The Kinetic Study of Enzyme Action on Substrate Monolayers

PANCREATIC LIPASE REACTIONS*

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SUMMARY

The enzymatic reaction of porcine pancreatic lipase with insoluble monolayers of either trioctanoin or 1,2-dioctanoin can be followed quantitatively by monitoring the considerable change in surface pressure which occurs during the course of the reaction. The rate of each reaction is proportional to the surface concentration of the substrate. The calculated pseudo-first order rate constants are proportional to the enzyme concentration in the bulk solution.

At pH 7.6, the value of the second order rate constant for the hydrolysis of the primary ester function in trioctanoin was found to be $8.46 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$, and that for dioctanoin, $2.65 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$. The pH dependency of the rate constant for dioctanoin hydrolysis reveals an ionizable basic group at the active site of the enzyme with pK$\_a$ of 6.38. The results are consistent with fully hydrated enzyme molecules acting upon substrate molecules lying within the insoluble monolayer.

Many biological processes appear to be intimately associated with boundaries separating two phases. In particular, enzymatic modification of lipids occurs predominantly at the lipid-water interface of emulsions, micelles, membranes, and lipoproteins. A priori, the heterogeneity of these reactions could be a mere consequence of the water insolubility of the lipid substrate, or it could indicate that properties necessary for the optimization of the catalytic process are conferred by the interface to the enzyme, to the substrate, or to both. The possible contribution of the interface to heterogeneous enzymatic reactions could be assessed only by comparing the kinetic behavior of an enzymatic reaction in a homogenous system with that in aqueous solution. This necessitated the development of new methods for the determination of the kinetic parameters of enzymatic reactions in heterogeneous systems. Since the interaction of a lipid monolayer at the air-water interface with a hydrolytic enzyme solution is conceptually the simplest heterogeneous system, we undertook the kinetic study of an enzymatic reaction with a lipid monolayer.

The kinetic behavior of several nonenzymatic reactions involving monolayer molecules has already been successfully analyzed (1, 2). Furthermore, qualitative observations indicate that lipolytic enzymes can act upon lipid monolayers (3-5). For monitoring the time-dependent changes of surface concentration, the film pressure, $\pi$, i.e. the decrease in surface tension resulting from the spreading of a monolayer on the subphase, seems to be the most convenient and reliable physical parameter to measure. When both the substrate and the reaction products form a stable expanded monolayer (1), the surface pressure changes accompanying the reaction are, in general, rather small, since the number of hydrophobic chains in the monolayer does not change. If at least one of the products is water-soluble, pressure changes will be much larger, and hence the determination of the surface concentration will be more accurate. An additional potential advantage of soluble reaction products is the absence of possible interactions of the product with the enzyme or the remaining substrate monolayer. Because of the intimate relationship between the surface area and the surface pressure, one could monitor changes in surface concentration either by making measurements at constant surface area or at constant surface pressure. We chose the constant area (changing $\pi$) method because of its inherent experimental simplicity and because of the absence of such possible complications as barrier leakage, monolayer hysteresis, and viscous drag. This method also provides the possibility of determining in one experiment the reaction rates at a series of film pressures.

In order to explore the feasibility of this approach, we selected porcine pancreatic lipase (glycerol-ester hydrolase, EC 3.1.1.3) as the model enzyme because of its broad side chain specificity, and its ability to catalyze a heterogeneous reaction of an uncharged substrate. This relatively stable enzyme is easily purified to a homogenous form (6). As a monolayer substrate for pancreatic lipase, we chose glycerol trioctanate (trioctanoin). This triglyceride forms a stable monolayer, while the octanoate ion should easily dissolve in the subphase.

In this paper, we will show that pancreatic lipase does, indeed, catalyze the hydrolysis of trioctanoin monolayers. The progress of the reaction can be followed conveniently by the measurement of the change in the surface pressure and kinetic analysis of the data yields rate constants characterizing the enzymatic process. From these results interesting conclusions emerge concerning the behavior of the enzyme at the air-water interface.
**EXPERIMENTAL PROCEDURE**

**Materials and Methods**

Lipids—Trioctanoin (Eastman), distilled twice at 205° and 1.1 mm Hg, appeared as a single spot when chromatographed on Silica Gel G thin layer chromatography with a solvent system of hexane-ethyl ether (50:50). Spots were visualized by exposure to iodine vapor. This chromatographic system, adapted from Yurkowski and Brockerhoff (7), will separate tri-, 1,2-di-, 1,3-di-, and monoglycerides. The mass spectrometric data and monolayer force versus area results (ΔF = 95.4 A² per molecule) were in accord with pure trioctanoin. [carboxyl-14C]Trioctanoin (New England Nuclear Corp.) had a specific activity of 2.87 μCi per mg. When it was cochromatographed with a mixture of mono-, di-, and trioctanoin on analytical thin layer chromatography, 98.3% of the radioactivity was associated with the trioctanoin spot. Triolein (K & K Laboratories) and octanoic acid (Sigma Chemical Co.) were used without further purification.

1,2-Dioctanoin and monooctanoin were prepared from trioctanoin by partial digestion with pancreatic lipase. Worthington pancreatic lipase (200 units) was added to 110 ml of trioctanoin by partial digestion with pancreatic lipase. Worthington pancreatic lipase (Sigma Chemical Co.) were used without further purification. The monooctanoin, presumably a mixture of 1- and 2-monooctanoin, was obtained from Worthington Biochemical Corp. and had an activity of 78 Worthington units per mg (9).

Pancreatic lipase B, used in the monolayer experiments, was isolated essentially by the procedure of Verger et al. (6). For the final step, the original procedure calls for desalting by passage on a column of Sephadex G-25 followed by chromatography on CM-cellulose at pH 5.0. In our hands this step always led to a large irreversible loss of enzymatic activity. However, we were able to achieve good separation of lipases A and B after desalting by dialysis against 5 mM Tris-HCl buffer at pH 8.0 with 3 mM CaCl₂, followed by chromatography on DEAE-cellulose in the same buffer and by elution with a linear NaCl gradient from 0 to 0.24 mM (Fig. 1).

Samples of lipase B collected during the purification procedure were subjected to analytical electrophoresis according to the method of Ornstein and Davie (10, 11). The result of this electrophoresis, which separates readily the two lipases identified as A and B (6), together with the similarity of their ion exchange elution pattern to that of the original procedure indicate that the enzyme was produced 1 μmol of acid per min under these conditions.

**Monolayer Rate Assay**—In the enzyme purification process, pancreatic lipase concentrations were estimated with a simple and convenient monolayer rate assay. An aliquot of the fraction to be assayed (containing 1 to 5 μg of enzyme) was added with 50 ml of 0.1 M potassium phosphate buffer, pH 7.6, to the thermostated (20°) Teflon trough. The aqueous surface was swept free of contaminating materials, and the wire ring was set in the position of maximum pull. Trioctanoin in petroleum ether was added to the surface to form a monolayer having an initial film pressure of 9.0 ± 0.4 dynes per cm, and the decrease in film pressure due to the hydrolysis of the monolayer was recorded for several minutes. The pancreatic lipase activity, estimated from the slope of the film pressure versus time curve at 8.5 ± 0.16 dynes per cm, was converted to nanomoles hydrolyzed per cm² per min (1 dyne per cm per min change equals 4.41 × 10⁻³ nmol per cm² min).

Enzyme—Hog pancreatin lipase, used in the preparation of 1,2-dioctanoin and the mixture of 1- and 2-monooctanoin, was obtained from Worthington Biochemical Corp. and had an activity of 78 Worthington units per mg (9).

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![Fig. 1. DEAE-cellulose chromatography, pH 8, of the enzyme fraction prepared by elution from Sephadex G-100 according to the procedure of Verger et al. (6). The enzyme was eluted with 5 mM Tris-HCl buffer containing 3 mM CaCl₂ and a linear NaCl gradient as shown in the figure. Fractions of 9.1 ml were collected at a flow rate of 22 ml per hour. Open circles are absorption at 280 nm, closed circles are lipase activity, and the solid line is [NaCl].](http://www.jbc.org/)
our purified enzyme was lipase B and that it was free from contamination by lipase A or other proteins to the limits of detection of our method. In addition, the molecular weight of the purified enzyme (55,000), estimated by comparing its mobility during polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate to those of standard proteins by the method of Weber and Osborn (12), compared favorably to the value reported for lipase B by Verger et al. (6), based upon the same method. The identical electrophoretic mobilities observed in the presence and absence of mercaptoethanol indicated that lipase B was composed of a single polypeptide chain, again in agreement with the previous observations (6). While we were unable to measure the activity of the enzyme by the rate assay of the original procedure, the final steps in the purification showed the same increase in specific activity, when measured either by the emulsion assay1 or by the rate of the monolayer reaction.

Experimental Technique

Surface Tension Measurement—The aqueous subphase (50 ml) was added to a solid polytetrafluoroethylene (Teflon) trough (internal dimensions: 4 x 17 x 0.6 cm) in a copper vessel thermostated by water circulated from a constant temperature bath (see Figs. 2 and 3). The aqueous surface was cleaned by sweeping several times with Teflon barriers. The surface tension was determined from the maximum pull exerted on a du Noiuy ring of 9 mm diameter (0.1-mm Nichrome wire) attached to the “B” loop of a Cahn Electrobalance when the ring was in contact with and was raised above the aqueous surface. The force was recorded as a function of time with a Y recorder (Houston Omnigraphic Corp.). The ring constant, i.e. the conversion factor allowing the calculation of the surface tension from the measured force, was determined empirically with the use of pure liquids of known surface tension.

Lipid monolayers were spread from benzene or petroleum ether solutions delivered slowly to the aqueous surface with either an ultramicro “lambda” pipette or with a micrometer buret (Gilmont model RGI). The resultant change in force on the ring multiplied by the ring constant yielded the film pressure for a particular surface concentration. By the combination of the two addition techniques, a film pressure (τ) versus molecular area (A) curve was prepared for each lipid monolayer material. To calculate the kinetic parameters of the reaction, the experimental force values were first converted to film pressures. The subsequent conversion of the film pressure to surface concentrations of reacting molecules will be discussed under “Results.”

Monolayer Collection and Analysis—In order to correlate the observed surface tension changes with the chemical changes occurring in the pancreatic lipase-monolayer reaction, partially reacted films were collected and analyzed. At a given time during the reaction of a monolayer of [carboxy-14C]trioctanoin with pancreatic lipase, the subphase was removed in less than 30 s via a submerged glass tube attached to a vacuum line, until 2 to 3 ml remained. To this mixture were added 3.0 ml of a solution of mono-, 1,2-di-, 1,3-di-, and trioctanoin in 2,2,4-trimethylpentane. All material was removed from the trough. The organic phase was isolated, reduced by evaporation, and chromatographed on Adsorbosil-1 thin layer chromatography with a hexane-ethyl ether (50:50) solvent system. The spots, located by I2 vapor, were eluted with standard toluene scintillation fluid and counted in a Tri-Carb scintillation counter with an external standard. Control experiments showed that water-insoluble monolayer material could be collected in this way with greater than 95% yield.

1 Assays performed by Dr. N. D. Boyd.
RESULTS

Force-Area Curves

The collection and analysis of monolayers with film pressures between 0 and 10 dynes per cm revealed that [carboxy-\textsuperscript{14}C]trioctanoin added to the surface of an 0.1 M potassium phosphate buffer, pH 7.6, remained there quantitatively for at least several hours. In the 0.5 to 10 dynes per cm range, monolayers of trioctanoin and 1,2-dioctanoin yielded force-area curves typical of those of other glycerides (Fig. 4). In this film pressure range, the $\pi A$-plots (Fig. 5) for these two compounds were linear within experimental error, indicating that trioctanoin and 1,2-dioctanoin formed reasonably stable monolayer films of the liquid-expanded type obeying the relationship

$$\pi(A - A_0) = C$$

or

$$\pi A = \pi A_0 + C$$

(1)

where $\pi$ is the film pressure, $C$ is a constant, and $A$ and $A_0$ are the available and the limiting molecular areas. We found that at 25°, trioctanoin and 1,2-dioctanoin had a limiting area of 95.4 and 63.1 A$^2$ per molecule, respectively.

Monolayer Reactions with Pancreatic Lipase

Monolayers of [carboxy\textsuperscript{14}C]trioctanoin spread on buffer solutions in the absence of lipase B had constant film pressure, and when collected, all the radioactivity (95 ± 5%) was found in the monolayer as trioctanoin (Fig. 6a). Addition of pancreatic lipase B to the buffer subphase caused a time-dependent decrease in the film pressure (Fig. 7) which appeared to be biphasic, indicating the possibility of two consecutive reactions. The decrease in film pressure suggested loss of material from the monolayer. This was confirmed by the accumulation of radioactively labeled octanoic acid in the subphase during the reaction. The analysis of the monolayer after partial reaction yielded over 60% of the label as 1,2-dioctanoin (see Fig. 6b). In analogy with earlier studies (13) showing that emulsified triglycerides are successively hydrolyzed to 1,2-diglycerides and then to 2-monoglycerides, the conclusion was reached that the rapid initial portion of the monolayer reaction was the conversion of trioctanoin to 1,2-dioctanoin while the second slower phase was the hydrolysis of 1,2-dioctanoin to monoctanoin and octanoic acid. Since 1,2-dioctanoin formed a stable monolayer and accumulated during the trioctanoin monolayer reaction with lipase B, an investigation of the reaction of lipase B with 1,2-dioctanoin was then undertaken before the kinetic analysis of the trioctanoin reaction was attempted.

1,2-Dioctanoin Monolayer Reaction—A large decrease in film pressure was observed when 1,2-dioctanoin was spread as a

![Fig. 4. Force versus area curves for trioctanoin and 1,2-dioctanoin monolayers on 0.1 M potassium phosphate buffer, pH 7.0, at 20°. Open circles are trioctanoin and 1,2-dioctanoin force-area curves; closed circles are film pressures for mixed trioctanoin and 1,2-dioctanoin monolayers having the same average area per molecule. The percentage of trioctanoin is indicated next to the circle.](image)

![Fig. 5. $\pi A$ versus $\pi$ diagrams for trioctanoin and 1,2-dioctanoin monolayers on 0.1 M potassium phosphate buffer, pH 7.0, at 20°. The solid line represents linear approximation according to Equation 1 (see text).](image)

![Fig. 6. Time course of the surface pressure changes (left) and analysis (right) of monomolecular films of [carboxy\textsuperscript{14}C]trioctanoin on 0.1 M potassium phosphate buffer, pH 7.6, in the absence (a) and presence (b) of pancreatic lipase B. Symbols for right-hand diagram: $\text{-MG}$, 2-monooctanoin; $1,3$-DG, 1,3-diglycerides; $1,2$-DG, 1,2-diglycerides; $1,3$ diglyceride; OA, octanoic acid; TG, trioctanoin; ordinate, radioactivity in counts per min. Monolayers were collected after 12 min.](image)
FIG. 7. Recorder tracing of the film pressure changes for a trioctanoin monolayer at 20° on a subsolution of 0.1 m potassium phosphate buffer, pH 7.6, containing pancreatic lipase B. Abscissa, time in min; ordinate, $F$ is film pressure in dynes per cm. Sloping line at the top of the figure is the continuation of the record of hydrolysis without oscillation of the du Nouy ring.

FIG. 8. Recorder tracing of the film pressure changes for a 1,2-dioctanoin monolayer at 20° on a subsolution of 0.1 m potassium phosphate buffer, pH 7.6, containing pancreatic lipase B. Abscissa, time in min; ordinate, $F$ is film pressure in dynes per cm.

monolayer on a subsolution containing pancreatic lipase B (Fig. 8). This decrease was shown to result from the loss of monolayer material from the surface and by the accumulation of mono-, octanoin and octanoic acid in the subphase after the complete reaction of a monolayer of radioactive trioctanoin (see above). The rate of solution of the hydrolysis products was investigated. When octanoic acid or a mixture of mono-octanoin was added to the aqueous surface in quantities which would be sufficient to cover the surface five times with a moderately compressed monolayer, no changes in surface pressure were observed indicating that these compounds were too water-soluble to form stable monolayers. Therefore, we concluded that 1,2-dioctanoin was hydrolyzed to mono-octanoin and octanoic acid, both of which entered the aqueous subphase.

Since at any time the film pressure was due only to 1,2-dioctanoin, the surface concentration of unreacted molecules was easily determined from the force-area curves. While the uncertainty involved in the determination of film concentrations at low pressure ($\pi < 0.5$ dyne per cm) limited the extent to which the reaction could be followed accurately, plots of the logarithm of the number of film molecules versus time were linear up to 70% reaction indicating that the reaction was first order in substrate. The slope of the curve yielded the experimental rate constant ($k_{exp}$) for the reaction. The plot in Fig. 9 of ($k_{exp}$) as a function of the initial lipase B concentration ($E_0$) was linear and showed that the 1,2-dioctanoin monolayer reaction is also first order in enzyme. The slope of the curve yielded a second order rate constant ($k_{cat}/E_0$) of $2.65 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$.

Trioctanoin Monolayer Reaction—A typical reaction of a monolayer of trioctanoin with lipase B contained in the subphase is shown in Fig. 7. Monolayer collection and kinetic analysis in the second slower part ($t > 10$ min) showed that this final part of the trioctanoin monolayer reaction was the hydrolysis of 1,2-dioctanoin to mono-octanoin and octanoic acid. Therefore, the first rapid phase resulted from the hydrolysis of trioctanoin to 1,2-dioctanoin. Based on the observations (a) that monolayers of trioctanoin and 1,2-dioctanoin obeyed Equation 1, (b) that 1,2-dioctanoin reacted much more slowly than trioctanoin, and (c) that a mixed monolayer of these two materials was formed during the trioctanoin reaction, an equation could be derived to relate the observed film pressures to the mole fraction ($i$) of trioctanoin in trioctanoin-1,2-dioctanoin mixed monolayers with constant total number of film molecules.

Equation 1 can be rewritten as

$$\pi(nA - nA_0) = nC$$

(2)

where $A$ is the available area per mole of compound, $A_0$ is the limiting molar area of this compound, and $n$ is the total number of moles of the compound on the surface.

Assuming additivity of film pressures in mixed monolayers of dioctanoin and trioctanoin, then:

$$\pi_{exp} = \pi_1 + \pi_2$$

(3)

where $\pi_1$ and $\pi_2$ are the partial film pressures of trioctanoin and dioctanoin, respectively. The contribution to the total film
pressure of each component can be described by Equation 2, such that
\[ \pi_1(nA - n_1A_{\infty} - n_2A_{\infty}) = n_1C_1 \]  
and
\[ \pi_2(nA - n_1A_{\infty} - n_2A_{\infty}) = n_2C_2 \]
where \( n_1, n_2, A_\infty, \) and \( A_\infty \) are moles and limiting molar areas of trioctanoin and dioctanoin, respectively: \( n = n_1 + n_2 \); and A is the total surface area divided by n.

Inspection of Fig. 5 shows that \( C_1 = C_2 = C \), within our experimental error. Furthermore, since the hydrolysis of 1,2-dioctanoin is negligible during the trioctanoin reaction, we can assume that \( n \) remains essentially constant in the course of the first reaction. If we now define \( i \) as the mole fraction of trioctanoin at any time during the enzymatic reaction,
\[ i = \frac{n_1}{n} \]
then for a pure trioctanoin monolayer, \( \pi = \pi_2 \) and \( i = 1 \), while for a pure dioctanoin monolayer, \( \pi = \pi_\infty \) and \( i = 0 \). By using these relationships together with Equations 3, 4, and 5, one can show that
\[ i = \frac{1/\pi_2 - 1/\pi_\infty}{1/\pi_\infty - 1/\pi_\infty} \]
where \( \pi_\infty \) is the surface pressure of a mixed monolayer of mole fraction, \( i \).

In order to establish the validity of Equation 7 the film pressure of several trioctanoin-1,2-dioctanoin mixed monolayers of equal total number of molecules was determined (see intermediate points between \( \pi - A \) curves, Fig. 4) and \( i \) was plotted as a function of \( 1/\pi_\infty \) (Fig. 10). The linearity of this plot established the validity of Equation 7 for trioctanoin-1,2-dioctanoin mixed monolayers.

If the enzymatic hydrolysis of trioctanoin is first order with respect to the substrate, i.e. \( -d[S]/dt = k_{\text{exp}}[S] \), where \( [S] \) is the surface concentration of the substrate, then
\[ \ln \left( \frac{1/\pi_\infty - 1/\pi_\infty}{1/\pi_\infty - 1/\pi_\infty} \right) = k_{\text{exp}}t \]  
\( \pi_\infty \) was calculated by extrapolation from the final portion of the reaction. The plot of \( \ln (1/\pi_\infty - 1/\pi_i) \) versus t was linear (Fig. 11) indicating the reaction to be first order, and the slope of the plot yielded the experimental rate constant. Reactions studied at various lipase B concentrations indicated the trioctanoin reaction was also first order with respect to the enzyme concentration, i.e. \( -d[S]/dt = k_3[E_0][S] \) (Fig. 12), with \( k_{\text{exp}}/E_0 = 84.6 \times 10^4 \text{M}^{-1}\text{s}^{-1} \). When the second order rate constant was calculated from the initial rate of the trioctanoin monolayer reaction, a virtually identical value was obtained, within experimental error. This indicated the absence of product inhibition or a
perturbation of the surface pressure of glyceride mixtures in the presence of the enzyme.

**pH Dependency** —1,2-Dioctanoin was chosen as the substrate to study the pH dependency of the lipase B monolayer reaction because the kinetic parameters are less ambiguous and simpler to calculate than for trioctanoin. Reactions were followed on subphases ranging in pH from 5.5 to 8.0. The plot of the second order rate constant $k_s = \frac{k_{\text{lim}}}{1 + \frac{H^+}{K}}$ versus pH was sigmoid with a maximum in the alkaline region. The data fit a theoretical curve where the rate constant depends upon the ionization of a base of pK of 6.38 (Fig. 13).

**Control Experiments** —The rate of the enzymatic reaction was the same whether the substrate monolayer was spread immediately after the enzyme solution was poured into the trough and its surface cleaned, or after the surface of the enzyme solution aged for 30 min before monolayer spreading. This indicated that there was no slow accumulation of active enzyme at the surface.

The enzymatic reaction was carried out at several temperatures between 15 and 25°C, and appeared to be essentially independent of the temperature in this range.

In order to determine the rate of nonenzymatic hydrolysis of the glyceride monolayers, the time-dependent surface pressure changes for 1,2-dioctanoin and trioctanoin monolayers spread on 0.05 M NaOH were measured. After converting the data to surface concentration values, second order rate constants could be calculated. We found $k_{\text{OH}} = 9.6 \times 10^{-4} \text{ M}^{-1} \text{s}^{-1}$ for dioctanoin and $21 \times 10^{-3} \text{ M}^{-1} \text{s}^{-1}$ for trioctanoin. The reactivity of the primary esters toward hydroxide ion in the two compounds thus seems to be virtually identical.

**DISCUSSION**

The results reported here establish several features of the chemical interaction between a hydrolytic enzyme and lipid monolayers. First of all, there is a time-dependent change in the surface pressure of either trioctanoin or 1,2-dioctanoin monolayers when they are exposed to pure pancreatic lipase. By the use of radioactive trioctanoin, it was shown that the changes in surface pressure result from a chemical reaction, the initial products of which are 1,2-dioctanoin and octanoic acid in the case of trioctanoin. The diester remains on the surface while the acid rapidly enters the subphase. 1,2-Dioctanoin is more slowly converted to 2-monooctanoin and octanoic acid, both of which immediately enter the subphase.

The rate of either reaction is strictly proportional to the enzyme concentration in the bulk subphase. The specificity for the primary ester functions and the relative rates of tri and di-glyceride hydrolysis are those expected for catalysis by pancreatic lipase (13). This can be contrasted with the similar and much slower rates of hydroxide-catalyzed hydrolysis of tri- and dioctanoin monolayers.

Secondly, our results show that the substrate molecules which are subject to enzymatic hydrolysis are those in the monomolecular film. The monolayers are stable and show no tendency to dissolve in the absence of enzyme. The enzymatic hydrolysis is first order with respect to the surface concentration of glyceride substrate. Within the range we have studied, from about 10 dynes per cm down to 0.5 dyne per cm, the rate constant of the reaction is independent of the surface pressure. We have restricted our studies to this range because we have established that within it there are no phase transitions, and that only "liquid-expanded" state (1) is present. While it is possible to imagine that some molecules of glyceride extend below the water-glyceride interface and are extensively hydrated in the hydrocarbon chain region, we consider it unlikely that the enzymatic reaction involves only such molecules. We have shown earlier (14) that the second order rate constants for the lipase-catalyzed hydrolysis of monolayers of octanoate esters and of oleate esters differ by less than 2-fold. If molecules with extensively hydrated hydrocarbon chains were important in the reaction, one would expect considerable rate changes both with chain length differences and with surface pressure differences. Since these are not observed, we conclude that only molecules with the hydrocarbon chains lying within the monolayer undergo hydrolysis.

Thirdly, a corollary to this conclusion is that the enzyme molecules which participate in hydrolysis must lie close to the water-monolayer interface. We do not believe that an unhydrated enzyme monolayer has been formed, because such a process would be slow and diffusion-controlled (15, 16). If a film of active enzyme would form slowly, then one would expect to observe an increase in the enzymatic rate with time, which is not seen. That the enzyme is hydrated when it participates in the reaction is indicated by the pH dependency, which obeys a simple thermodynamic equilibrium when the hydrogen ion concentration of the bulk phase is altered, indicating that an ionizable group at the active site of the enzyme is in rapid equilibrium with the solution. On the other hand, it is perfectly reasonable that the enzyme could be present as a hydrated species in a soluble Gibbs monolayer (3) and in rapid equilibrium with enzyme in the bulk phase. The enzyme concentration in such a soluble monolayer could be different from that in the bulk phase, and at the moment we have no information as to the exact concentration of enzyme which participates in the reaction.

Sömörva et al. (17) have studied the pH dependency of the pancreatic lipase-catalyzed hydrolysis of tributyrin emulsions. The pH dependency of $V_{\text{max}}$ showed an inflection point at 5.8, when determined in 0.1 M NaCl. This value is not far from the inflection point which we report (6.4) for the dependency...
of the second order rate constant in the dioctanoin monolayer reaction, especially if one considers the much lower ionic strength used in the emulsion experiments. The similarity of these pH dependencies indicates that the mechanism of the enzyme-catalyzed reaction is the same with monolayers or emulsified substrates. Furthermore, from the data presented by Séméria et al. (17), a value for $k_{cat}/K_m$ of $3.35 \times 10^6$ M$^{-1}$ s$^{-1}$ can be calculated. This agrees remarkably well with our value of $8.46 \times 10^5$ M$^{-1}$ s$^{-1}$ for the trioctanoin monolayer reaction. It appears, therefore, that the catalytic efficiency of the enzyme is similar in each case. It is gratifying to see that monolayer-enzyme interaction reflects so closely the emulsion-enzyme behavior.

Independently from us Garner and Smith (18) described a similar approach to studying pancreatic lipase action on insoluble monolayers of octanoate esters. They also demonstrated the enzymatic reaction and indicated the usefulness of the technique. Recently, Olive and Dervichian (19) have also investigated the interaction of lipolytic enzymes with substrate monolayers. In the case of the reaction of Arrhizus lipase with a crude mixture of didecanoins (19) a slight surface pressure dependency of the enzymatic reaction might be present in the range from 2 to 12 dynes. Since we have observed no such pressure dependence for the pancreatic lipase reaction (see Fig. 11) further experimentation will be required to determine the nature of this discrepancy.

Our studies were undertaken in order to show the feasibility of quantitative kinetic analysis of enzymatic reactions on substrate monolayers. Although the experimental technique proved to be far from simple, it nevertheless yields readily interpretable kinetic results, provided due care is taken to establish: (a) the stoichiometry of the reaction, (b) the relationship between surface pressure and surface concentration, (c) the absence of phase transitions in the course of the reaction, and (d) the properties of mixed substrate-product monolayers. We feel that this technique will be useful not only for the study of the action of other lipolytic enzymes, but also in general investigation of lipid-protein interactions.

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**REFERENCES**

The Kinetic Study of Enzyme Action on Substrate Monolayers: PANCREATIC LIPASE REACTIONS
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