Isobaric titration of reacting monolayers: kinetics of hydrolysis of glycerides by pancreatic lipase B

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Abstract The rate of lipolytic enzyme-catalyzed reactions yielding water-soluble products can be measured by isobaric titration. The method is based upon the measurement of the amount of substrate that must be added to a monolayer to maintain constant surface pressure during the course of the enzymatic reaction. The rate constants determined for the hydrolysis of trioctanoin and 1,2-dioctanoin by pancreatic lipase were identical with those determined by the variable surface pressure method and by a radioactive substrate technique. This direct titrimetric method has a wider dynamic range and more versatility for following surface reactions than previously described systems.

Supplementary key words constant surface pressure · constant surface area · negative feedback · pressure dependence · trioctanoin · 1,2-dioctanoin

The current interest in enzymatic reactions at membranes and other lipid–water interfaces has prompted research into methods for studying the details of these processes, in particular the kinetics of the action of enzymes upon substrate monolayers spread on aqueous subphases. Methods currently employed for this purpose depend upon the continuous measurement of some physical property of a monolayer in a closed system, i.e., all the substrate is present in the monolayer and all the enzyme is present in the subphase at time zero. The physical property monitored is usually the surface potential, the area occupied by the monolayer, or the surface pressure. Systems have been devised to measure, as a function of time, the change in surface pressure at constant area (1) or the change in surface area at constant surface pressure (2–6). For the latter system, several variants of the method have been used, but all of them entail displacement of a preformed monolayer by a moving barrier. With all of these physical methods, precise knowledge of the surface properties of the substrate is necessary for converting the experimental data into the kinetically meaningful rate expression of moles of substrate hydrolyzed per second. In the case of constant surface pressure systems, one further needs to ascertain that the physical properties of the monolayer do not change upon compression, dislocation, or aging.

In order to reduce the number of variable parameters, we felt it necessary to develop a new technique by which the number of moles reacting could be measured volumetrically while maintaining both the surface pressure and the surface area constant. To accomplish this and still retain the advantage of continuous monitoring, we abandoned the idea of a closed system and turned our attention to an open system in which molecules disappearing from the monolayer could be replaced continuously. We studied the feasibility of determining reaction velocities by spreading the substrate from a volatile organic phase at a rate necessary to maintain constant surface pressure. The theoretical aspects of such steady-state reactions are well known, and the necessary feedback systems are commercially available. A priori, any surface property could have been employed to monitor the steady state, but we chose the surface pressure because it can be measured, both accurately and reproducibly, using a Cahn Electrobalance equipped with a du Noüy ring (1).

In this paper, we hope to show that, using this method, rate measurements can be made directly and rate constants can be determined even without precise knowledge of the surface properties of the monolayer. As an example, we measured, with isobaric titration, the hydrolysis of glycerides by pancreatic lipase B at the air–water interface.

MATERIALS AND METHODS

Reagents

Lipase B. Porcine pancreatic lipase B was purified by the method of Verger et al. (7) as modified by Lagocki, Law, and Kézdy (1). The purified enzyme was homoge-

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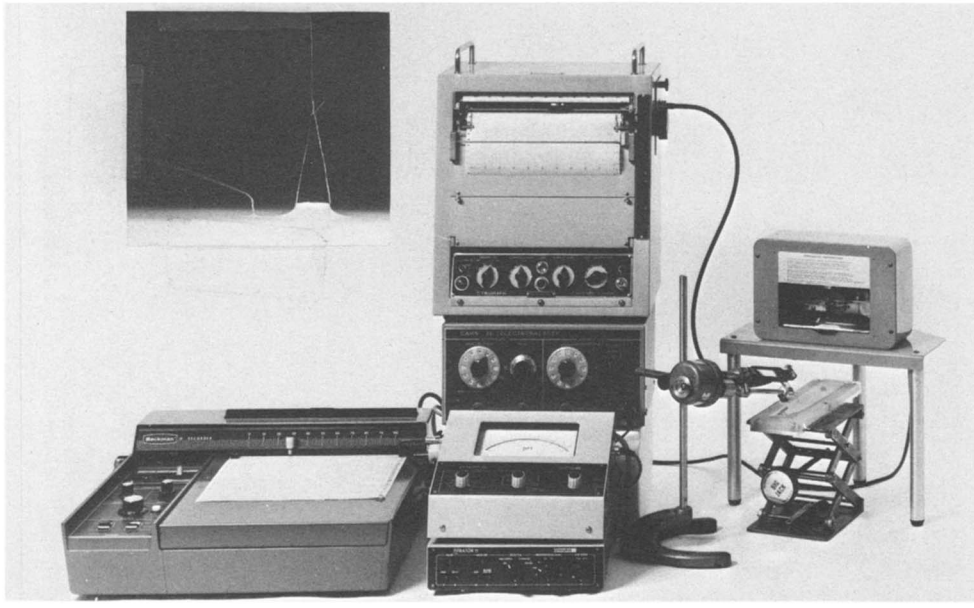

Fig. 1. Isobaric titrator, showing arrangement of the components. See text and Fig. 2 for details. Inset shows du Noüy ring and syringe needle tip at air–water interface.

neous as judged by polyacrylamide gel electrophoresis at pH 8.9. Enzyme molarity was calculated from UV absorbance measurements using $E^{1\%}_{280} = 13.3$ (8) and mol wt = 50,000 (7). The specific activity of the purified protein was identical with that previously described (9). The pure enzyme was stored as a lyophilized powder at −12°C, and solutions were prepared in 0.1 M potassium phosphate buffer, pH 7.6, as needed.

Lipids. Trioctanoin (glycerol trioctanoate, Eastman) was redistilled, bp 197–199°C (0.5 mm), lit. bp 233–233.5°C (1 mm) (10). 1,2-Dioctanoin was prepared as described by Lagocki et al. (1). [*carboxy*-^{14}C]Trioctanoin (New England Nuclear Corp.) had a specific activity of 5.67 Ci/mole and was shown to contain more than 99.8% triglyceride by chromatography on silica gel G with a solvent system of hexane–ethyl ether 50:50. This system resolves triglycerides, 1,2-diglycerides, 1,3-diglycerides, monoglycerides, and fatty acids (1).

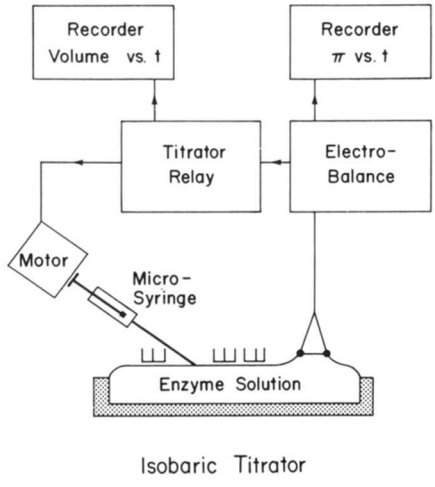

Fig. 2. Block diagram of components of isobaric titrator. Upon a decrease in the surface pressure, π, the Electrobalance generates a DC signal, which is detected by a Radiometer titrator. This, in turn, activates the titrigraph, which adds glyceride (⊔⊔) from a syringe until the initial surface pressure is restored.

Buffer. Water was purified by passing distilled water through a mixed-bed deionizer. After preparation of 0.1 M phosphate buffer, trace surface impurities were removed by foaming. The lack of surface-active contaminants in foamed buffer was indicated by the absence of time-dependent increases in the film pressure of a monolayer of trioctanoin spread on this buffer to 2 dynes/cm.

Other chemicals. All organic solvents and chemicals were reagent grade and were used without further purification.

Experimental technique

Surface tension was continuously monitored using a recording Electrobalance (model RG, Cahn Instruments) equipped with a 9-mm du Noüy ring.

Constant surface pressure measurements. During the course of a reaction, constant surface pressure was maintained by adding substrate molecules to the air–solution interface from a syringe driven by a Radiometer pH stat. The apparatus is shown photographically and schematically in **Figs. 1** and **2.** At first, the substrate in petroleum ether was spread manually onto the cleaned surface of an enzyme solution until the desired pressure was reached. At this point, the output of the Electrobalance was nulled using a compensating voltage. Any small change in the pressure then generated a DC voltage (10 mV/mg) pro-

portional to the size of the change. This signal was fed into a titration system consisting of the following Radiometer components: pH meter, PHM28; titrator, TTT11; titrigraph, SBR2C; and syringe burette, SBU1A. When the signal exceeded the deadband (0.5 μg) of the titrator, the syringe burette was activated, causing addition of titrant solution from a gas-tight syringe (Hamilton, 50 or 100 μl) onto the surface. The amount, and hence moles, of titrant added as a function of time was recorded by the titrigraph. Simultaneously, the surface pressure was measured as a function of time using a Beckman model 1005 potentiometric recorder. With this instrumentation, the surface pressure could be maintained with $\pm$ 5 $\times$ 10^{-3} dynes/cm, but values of $\pm$0.02 dynes/cm optimized the signal-to-noise ratio.

Determination of force–area curve. With the instrumentation described above and the titrator in titration curve mode, continuous force–area curves of lipids could be determined. For this purpose, the Electrobalance output was initially nulled in the absence of lipid. Titrant was then automatically added to the surface to keep the Electrobalance output voltage equal in magnitude, but opposite in sign, to a reference voltage that was changed linearly with time by the titration mode circuit of the pH stat. This resulted in a curve relating the volume of titrant added to the surface pressure.

Monolayer collection and analysis. Monolayers containing [^{14}C]trioctanoin and reaction products were collected using 2,2,4-trimethylpentane and nonradioactive carriers as previously described (1). After collection of the monolayer, an aliquot of the organic phase was chromatographed on silica gel (Eastman Chromagram, 5 $\times$ 20 cm) as described above, and the spots were visualized with iodine vapor. The chromatogram was then cut into 1-cm strips, and the radioactivity in each strip was measured in a Nuclear-Chicago Isocap/300 scintillation counter. The scintillation fluid was a mixture of 3.92 g of 2,5-diphenyloxazole (PPO), 0.08 g of *p*-bis(*o*-methylstyryl)benzene (bis-MSB) dissolved in 667 ml of toluene, and 333 ml of Triton X-100 (Packard).

Instrumental details. For monitoring surface reactions, the Electrobalance was operated at a sensitivity at which the amplification was strictly linear only when the output signal was not more than 0.2 mV/mg. However, even at an output setting of 2 mV/mg, approximate linearity was obtained in the 0–100 mV range (ca. 0–10 dynes/cm). For reactions where only the constancy of the pressure is monitored, higher, nonlinear amplification can be employed.

In practice, the noise level is influenced by the concentration of the titrant, the spreading properties of the titration solvent, the rate of addition of titrant, and the location of the syringe needle relative to the du Noüy ring. The lowest noise levels were obtained when a dilute substrate solution in a solvent having a low spreading coefficient was being added to the surface at a relatively slow rate with large spacing between needle and ring.

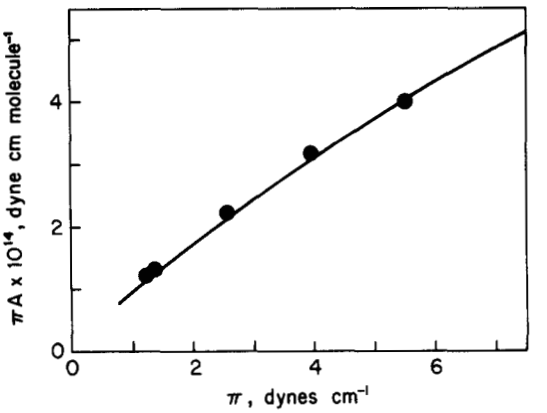

Fig. 3. Plot of πA vs. π for 1,2-dioctanoin monolayers. Subphase was 0.1 M potassium phosphate buffer, pH 7.6, at 24°C. The solid line shows data obtained using the isobaric titrator. Trough area, 124 cm^2; titrant solution, 2.70 $\times$ 10^{-4} M 1,2-dioctanoin in petroleum ether (30–60°C). Initial aliquot of titrant contained 1.85 $\times$ 10^{-8} moles. *Closed circles* are data taken from Ref. 1 and were obtained by spreading aliquots of substrate.

The syringe most often used was a Hamilton 1710LT equipped with a KF-730 needle, although larger and smaller needles and syringes were successfully employed. To allow only the end of the needle to touch the surface of the buffer, the needle was bent approximately 80° at a point 1 cm from the tip.

The rectangular troughs routinely used were made of Kel-F (26.7 $\times$ 5 $\times$ 0.5 cm) or Teflon (26 $\times$ 5 $\times$ 0.5 cm) and had measured surface areas of 127 and 124 cm^2, respectively.

RESULTS

Force–area curve. We will first discuss the use of this method for the measurement of force–area curves. Surface properties of 1,2-dioctanoin were measured as described in Materials and Methods. A large amount of lipid is required to produce a monolayer with a surface pressure of 1 dyne/cm compared with that required to increase the pressure from 1 to 8 dynes/cm. Therefore, the sensitivity for measurement in the liquid expanded phase can be increased by spreading, prior to the titration, an aliquot of lipid solution sufficient to give a monolayer of 1 dyne/cm. The results of such a titration are shown as the solid line in **Fig. 3,** where πA, the product of the surface pressure, π, and A, the area per molecule, is plotted against π. Comparison of these data with those obtained by the technique of discontinuous spreading (1) (Fig. 3, *closed circles*) demonstrates satisfactory agreement between the two methods. As previously noted (1), the experimental curve is reasonably well described by Eq. 1

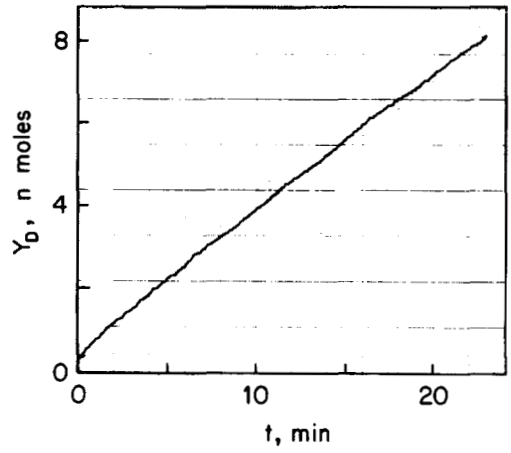

Fig. 4. Titrigraph recording of the hydrolysis of a 1,2-dioctanoin monolayer by pancreatic lipase B. π, 2.5 dynes/cm; $[E_o]$. 1.90×10^{-9} M; trough area, 124 cm^2; potassium phosphate buffer, 0.1 M, pH 7.6, at 24°C. Titrant was 5.23×10^{-4} M 1,2-dioctanoin in petroleum ether (30–60°C).

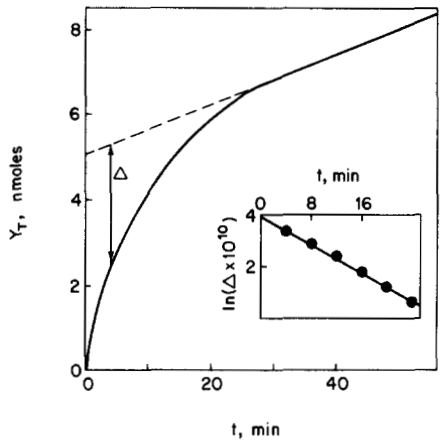

Fig. 5. Titration of the hydrolysis of trioctanoin by a trioctanoin solution. For definition of Y_T and Δ and the determination of the rate constant (*insert*), see Appendix. π, 4 dynes/cm; $[E_o]$, 3.76×10^{-9} M; trough area, 124 cm^2; 0.1 M potassium phosphate buffer, pH 7.6, at 24°C. Titrant was 1.24×10^{-4} M trioctanoin in petroleum ether (30–60°C).

(see Appendix for equations and their derivations). Agreement of the two methods proves that spreading of the lipid was instantaneous and quantitative even at rates of addition far in excess of those used in subsequent enzymatic rate measurements. Thus, in our kinetic experiments, neither the spreading of the lipid nor the evaporation of the solvent is rate limiting. In summary, these experiments demonstrate that the surface properties of 1,2-dioctanoin are independent of the manner or rate of monolayer formation.

Kinetic experiments. Initially, we studied the hydrolysis of monolayers of 1,2-dioctanoin by pancreatic lipase B using the substrate as the titrant. Earlier studies have demonstrated that medium-chain glycerides yielding instantaneously water-soluble products are suitable for studying the hydrolysis of substrate monolayers by pancreatic lipase (1, 11, 12). After an initial adjustment of the surface pressure to the desired value, the measured rate remained constant for the duration of the experiment. **Fig. 4** shows a typical titrigraph recording of moles of substrate, Y_D, delivered to the surface as a function of time. In all experiments, linearity was observed even when the extent of the reaction exceeded one full turnover of the substrate molecules on the surface. At high surface pressures, a small nonenzymatic rate was observed, apparently due to the dissolution of the substrate. In no case did this rate exceed 3% of the measured enzymatic rate.

Whereas the hydrolysis of 1,2-dioctanoin is a simple, one-step process, the hydrolysis of trioctanoin monolayers by pancreatic lipase occurs in two consecutive steps: trioctanoin is rapidly hydrolyzed to 1,2-dioctanoin and octanoic acid followed by the much slower hydrolysis of 1,2-dioctanoin (1). Since water-soluble products are generated in each step, the overall process still should be amenable to isobaric titration. **Fig. 5** shows the number of moles of trioctanoin added to the surface as a function of time for the isobaric, enzymatic hydrolysis of a trioctanoin monolayer. The curve is composed of a rapid burst followed by a slower, linear rate of addition of titrant. At the end of the burst, the monolayer is composed of essentially pure 1,2-dioctanoin molecules, as was shown later by experiments with radioactively labeled trioctanoin. Thus, the burst must correspond to the hydrolysis of trioctanoin whereas the linear portion represents the steady-state hydrolysis of 1,2-dioctanoin. On the basis of these observations and the properties of the mixed monolayers (1), Eq. 9 can be derived for the kinetic analysis of this curve. The intercept, I, was determined by graphical extrapolation of the steady-state portion of the curve, and the slope of the steady-state portion yielded V_∞ (defined by Eq. 11 in Appendix). The apparent first-order rate constant, b, was determined by plotting the logarithm of the difference between the extrapolated line and the reaction curve, Δ, as a function of time (*inset,* Fig. 5). In all experiments, such plots were linear over more than 90% of the reaction. From the three experimental parameters, I, V_∞, and b, the rate constants k_T and k_D can be calculated from Eq. 13 as described in the Appendix. **Tables 1** and **2** show the calculated values of k_D and k_T from a large number of experiments performed at three different pressures. Although the k_D values show an apparent pressure dependency, the first-order behavior observed in the pre-steady state (*inset,* Fig. 5) indicates that at any given pressure the reaction is first order with respect to the concentration of substrate in the monolayer. Thus, values of k_D can also be calculated from the experimental rates of dioctanoin hydrolysis where dioctanoin is the titrant (e.g., Fig. 4). At any pressure, the rate constant is calculated as the rate divided by the enzyme concentration and by the surface

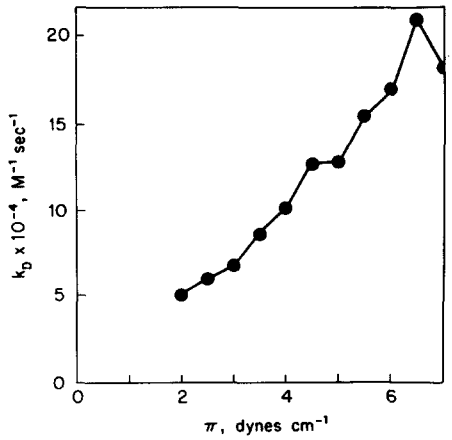

Fig. 6. Pressure dependency of k_D. Values of k_D were calculated using Eq. 5. Potassium phosphate buffer, at 24°C, 0.1 M, pH 7.6; $[E_o]$, 1.9×10^{-9} M; trough area, 124 cm^2. Titrant was 5.23×10^{-4} M 1,2-dioctanoin in petroleum ether (30–60°C).

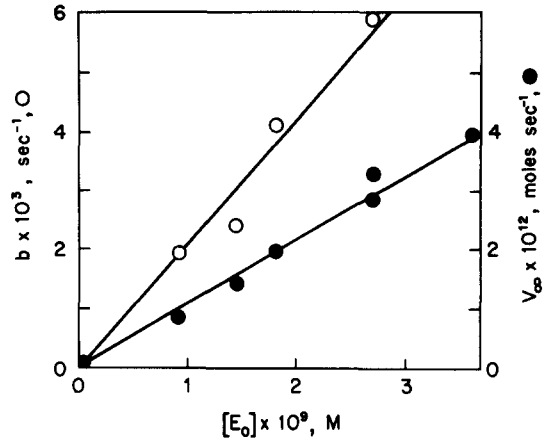

Fig. 7. The dependence of b and V_∞ on $[E_o]$. π, 3 dynes/cm; 0.1 M potassium phosphate buffer at 24°C, pH 7.6; trough area, 124 cm^2. Titrant was 2.16×10^{-4} M trioctanoin in petroleum ether (30–60°C).

concentration of substrate (moles/cm^2). These constants are shown in Table 1 as well as in **Fig. 6.** The dependency of V_∞ and b on the enzyme concentration (see Eqs. 11 and 12) is shown in **Fig. 7.** The linearity of each of these plots shows that both reactions are first order with respect to the enzyme concentration. Similar rate constants were also determined using the variable pressure technique (1) with 1,2-dioctanoin as the substrate. From the surface concentration vs. time curves, instantaneous rates were estimated at a series of pressures. Again, dividing these rates by the enzyme concentration and the substrate concentration gives the values of k_D shown in Table 1.

To validate our kinetic data obtained by the physical method of surface pressure determinations, we measured rates of trioctanoin hydrolysis under constant pressure by a chemical method using radioactively labeled substrate. In these experiments, labeled trioctanoin monolayers were spread and nonisotopic trioctanoin was used as the titrant. The reaction was stopped before completion for analysis of the products as described in Materials and Methods. From the data obtained, k_T values were calculated using Eq. 15 (see Table 2). Because the reactions were not continued into the steady state, Eq. 13 could not be used to calculate k_T from the same experiment; therefore, approximate k_T values were calculated from the initial rate of trioctanoin addition using Eq. 14 (see Table 2).

A nonreactive compound, oleic acid, was also used to titrate the hydrolysis of a trioctanoin monolayer. The rate

TABLE 1. Second-order rate constants for the hydrolysis of monolayers of 1,2-dioctanoin by pancreatic lipase B

π dynes cm^{-1}	$k_D \times 10^{-4}$ M^{-1} sec^{-1} Variable Pressure	Constant Pressure	Constant Pressure from Trioctanoin Steady State
3.0	6.9 ± 1.6 (17)	6.8	5.9 ± 2.5 (13)
5.0	13 ± 1.9 (16)	13	9.6 ± 4.8 (10)
7.0	24 ± 4.2 (15)	18	15 ± 3.5 (11)

The subphase was 0.1 M potassium phosphate buffer, pH 7, 24°C. Parentheses enclose the number of determinations from which standard deviations were calculated. From data obtained by the variable surface pressure method of Lagocki et al. (11), values of k_D were calculated using Eq. 5. Trough area was 127 cm^2 and $[E_o]$ ranged from 1.1×10^{-9} to 7.0×10^{-9} M. At constant surface pressure with 1,2-dioctanoin as both substrate and titrant, values of k_D were calculated using Eq. 5. See Fig. 6 for experimental details. From the hydrolysis of trioctanoin monolayers with trioctanoin as the titrant, k_D values were calculated using Eq. 13. Trough area was 124 cm^2 and $[E_o]$ ranged from 0.4×10^{-9} to 1.9×10^{-9} M.

TABLE 2. Second-order rate constants for the hydrolysis of monolayers of trioctanoin by pancreatic lipase B at constant surface pressure

π dyne cm^{-1}	$k_T \times 10^{-6}$ M^{-1} sec^{-1} From Pre-steady State	From Initial Velocity	From Surface Radioactivity	From Inert Titrant
3.0	3.1 ± 0.8 (13)	2.8 ± 0.9	2.4 ± 0.6 (3)	2.9
5.0	4.2 ± 1.2 (10)			
7.0	4.7 ± 1.2 (11)	4.6 ± 0.7	4.4 ± 0.3 (4)	

The subphase was 0.1 M potassium phosphate buffer, pH 7.6, 24°C. Parentheses enclose the number of determinations from which standard deviations were calculated. The values of k_T calculated with Eq. 13 were obtained from complete reactions with trioctanoin as the titrant. Trough area was 124 cm^2 and $[E_o]$ ranged from 0.38×10^{-9} to 1.9×10^{-9} M. The values obtained from initial rate measurements with trioctanoin as the titrant were calculated using Eq. 14. Trough area was 124 cm^2 and $[E_o]$ ranged from 1.9×10^{-10} to 7.6×10^{-10} M. From analysis of radioactively labeled reaction products, k_T values were also calculated from these initial rate experiments using Eq. 15. The value of k_T from an initial rate measurement with oleic acid as the titrant was calculated using Eq. 16. Trough area was 124 cm^2 and $[E_o]$ was 1.9×10^{-10} M.

constant k_T at $\pi = 3$ dynes/cm was calculated from an initial rate using Eq. 16 (see Table 2).

DISCUSSION

The results presented here show that true enzymatic hydrolysis rates can be measured by the method of isobaric titration. In the case of the enzymatic hydrolysis of dioctanoin monolayers, where the titrant is the substrate itself, our method provides a direct measurement of the number of molecules disappearing from the surface because all the added molecules spread rapidly on the subphase. In the kinetically more complicated case of trioctanoin hydrolysis, direct measurement of product formation using ^{14}C-labeled substrate also yielded rate constants identical, within experimental error, with those obtained from titration data. Furthermore, analysis of the steady-state portion of the reaction of trioctanoin, where trioctanoin is the titrant, yielded rate constants for the hydrolysis of 1,2-dioctanoin that are in reasonable agreement with those obtained by the direct method. In all of our experiments, strict first-order dependency upon the enzyme concentration was observed. Finally, initial velocity measurements for trioctanoin hydrolysis, using trioctanoin or oleic acid as the titrant, were consistent with direct radioactive product analysis. The consistency of all of these rate constants determined by four different methods shows that the assumptions used in the derivation of the kinetic equations are approximately valid for the system. Also, the good agreement between the constant and the variable pressure results shows that the kinetic behavior of the system is independent of the previous history of the monolayer.

A gradient of composition must exist in the monolayer in the case of the isobaric titration of trioctanoin hydrolysis because the surface concentration of the titrant must be higher at the point of addition than at the periphery of the trough. The kinetic equations have been derived in terms of total number of molecules instead of surface concentrations. Since these equations describe the kinetic phenomenon correctly, the surface heterogeneity does not influence the kinetic behavior of the whole system, i.e., the reactivity of the surface molecules is not influenced by the chemical nature of their nearest neighbors. Such a behavior is not unexpected when the monolayer is in the liquid expanded state.

In apparent variance with earlier studies utilizing constant area (1) or constant pressure (5) techniques within the liquid expanded phase, we found a slight pressure dependency for the trioctanoin reaction and a somewhat more pronounced pressure dependency for the dioctanoin reaction. The enzyme preparation used in our recent work (9) and in the present study is identical in chromatographic and electrophoretic properties to earlier preparations (1, 7). However, it exhibited substantially higher specific activity than previously reported (1) when measured by the variable pressure assay using 1,2-dioctanoin as the substrate. This raises the possibility that the heterogeneity of lipase species is not limited to the A and B forms (7). Such a heterogeneity has been shown for pancreatic phospholipase, which can be isolated in several related forms (13, 14) differing in their selectivity toward the state of substrate dispersion (14). In their action on phospholipid monolayers, these forms show either pressure-dependent or pressure-independent kinetics, depending upon the species. Thus, apparent pressure dependency observed with some lipase preparations must reflect an intrinsic property (e.g., tendency to denature at interfaces) of the enzyme rather than of the substrate or the catalytic process.

Isobaric titration has a wider dynamic range than other techniques because the titrant concentration can be varied between extremely wide limits. Because it is based upon stoichiometric and volumetric methods, it has additional advantages over other methods based upon indirect physical methods. Although its conceptual simplicity is confined to reactions in which all products are instantaneously soluble in the subphase, the method is applicable to any surface reaction that is accompanied by a decrease in limiting molecular area. In the latter case, as with other methods, determination of the rate will depend upon the particular surface properties of substrate and product. Because of the shape of the force–area curve, the sensitivity of the method decreases rapidly below 1 dyne/cm. At high pressures, the monolayer heterogeneity from the point of substrate addition to the end of the trough may lead to inaccuracies because of molecular interactions in the regions of high substrate concentrations. Provided that appropriate control experiments exclude these possible artifacts, the method is also suitable for the study of a wide variety of other surface phenomena such as dissolution of monolayers, complex formation, enzyme denaturation, and surface reactions in the presence of inert substances.

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APPENDIX

In the following deviations, all monolayer constituent concentrations will be expressed in units of moles per square centimeter, and the enzyme concentrations in moles per liter.

In a liquid expanded monolayer, the relationship between the surface pressure, π, and the area occupied by a mole of the substrate, A, can be described with good approximation (1) as an equation of the form:

$$\pi(A - A_o) = C \qquad \text{Eq. 1}$$

where C and A_o are constants, A_o is usually termed the limiting molar area. For n moles of substrate and a total available area of S cm^2 (i.e., $S = nA$) Eq. 1 becomes

$$\pi(S - nA_o) = nC \qquad \text{Eq. 2}$$

For an ideal mixture of m species in the liquid expanded state, the partial pressure, π_i, of the ith species is expressed by

$$\pi_i\left(S - \sum_{i=1}^{m} n_i A_{oi}\right) = n_i C_i \qquad \text{Eq. 3}$$

and the total surface pressure becomes

$$\pi = \sum_{i=1}^{m} \pi_i \qquad \text{Eq. 4}$$

A. One-step reaction yielding only soluble products; hydrolysis of 1,2-dioctanoin

In a closed system, if the reaction is first order with respect to the enzyme concentration, $[E_o]$, and to the surface concentration of substrate, D/S, the total rate of formation of product, P, for an area of S cm^2 would be

$$\frac{dP}{dt} = k_D[E_o]D$$

where D is the number of moles of substrate on the surface, and k_D is the second-order rate constant in M^{-1} sec^{-1}. In an open system under steady-state conditions (i.e., $dD/dt = 0$), when the titrant used is the substrate itself, the maintenance of constant surface pressure requires the addition of 1 mole of substrate to the surface for each mole hydrolyzed. Designating the number of moles of titrant in the syringe as M_D, it follows that

$$\frac{dP}{dt} = \frac{-dM_D}{dt} = k_D[E_o]D_o$$

where D_o is the number of moles of substrate that yields the constant pressure, π, on a surface area of S. Since the titrigraph measures the difference, Y_D $(=M_{SD} - M_D)$, between the moles of substrate present in the syringe at $t = 0$, M_{SD}, and t, M_D, then

$$\frac{dY_D}{dt} = k_D[E_o]D_o = \text{a constant} \qquad \text{Eq. 5}$$

i.e., k_D can be calculated by dividing the slope of the straight line tracing of the titrigraph by $[E_o]$ and D_o.

B. Two-step reaction yielding an insoluble intermediate and soluble final products

(1) Hydrolysis of trioctanoin. For a two-step enzymatic surface reaction in which the substrate and the intermediate coexist on the surface, the maintenance of constant pressure, π, imposes a relationship between the number of moles of substrate, T, and intermediate, D, on the surface. The equation relating these two variables can be derived from the properties of the mixed monolayer. For a system in which Eqs. 1–3 are valid, the restriction imposed by constant pressure conditions is that D and T obey the relation:

$$T = T_o(1 - D/D_o) \qquad \text{Eq. 6}$$

where T_o and D_o are the number of moles of substrate and intermediate that alone would produce π when spread on a surface area of S.

If both substrate and the intermediate react with the enzyme in a first-order fashion, and if M_T is the number of moles of substrate in the syringe, the following system of differential equations can be written:

$$\frac{dD}{dt} = k_T[E_o]T - k_D[E_o]D \qquad \text{Eq. 7}$$

$$\frac{dT}{dt} = \frac{-dM_T}{dt} - k_T[E_o]T \qquad \text{Eq. 8}$$

Eq. 7 can be transformed, using Eq. 6, to express T in terms of T_o and D, and then solved, giving an equation for D as a function of time $[D(t)]$. Eq. 6 is differentiated and $D(t)$ is differentiated, both with respect to time, and dD/dt is substituted in the differentiated Eq. 6. The expression for dT/dt obtained in this way is now set equal to dT/dt in Eq. 8, and the resulting equation is solved (9). Using the conditions that at $t = 0$, $M_T = M_{ST}$, $D = 0$, and $T = T_o$, we obtain

$$Y_T = M_{ST} - M_T = I + V_\infty t - Ie^{-bt} \qquad \text{Eq. 9}$$

where

$$I = T_o\left[\frac{k_T[E_o]}{b} - 1\right]\left[1 - \frac{k_D[E_o]}{b}\right] \qquad \text{Eq. 10}$$

$$V_\infty = \frac{k_T k_D[E_o]D_o}{k_T + k_D(D_o/T_o)} \qquad \text{Eq. 11}$$

and

$$b = k_T[E_o](T_o/D_o) + k_D[E_o] \qquad \text{Eq. 12}$$

Eq. 9 is of the classical form of "burst" kinetics, i.e., a fast exponential reaction followed by a linear, zero-order portion. The slope of the line determines V_∞, and graphical extrapolation of the latter portion yields I as the intercept on the ordinate axis. The parameter b is determined from the slope of a logarithmic plot using as a variable the difference, Δ, between the extrapolated line and the actual curve. Solving Eqs. 10–12 for k_T yields the quadratic expression:

$$[k_T[E_o]]^2 - \left[\frac{bI}{T_o} + b + \frac{V_\infty}{T_o}\right]k_T[E_o] + \frac{bV_\infty}{T_o} = 0 \qquad \text{Eq. 13}$$

Thus, k_T, and subsequently k_D, can be calculated from this equation using the experimental values of I, V_∞, and b. From Eq. 9, the initial rate of the reaction is

$$V_{OT} = \left(\frac{dY_T}{dt}\right)_{t=0} = V_\infty + bI$$

If $k_D << k_T$, this becomes

$$V_{OT} = k_T[E_o]T_o(1 - T_o/D_o) \qquad \text{Eq. 14}$$

(2) Hydrolysis of labeled trioctanoin under constant pressure. Because the reactivity of the radioactively labeled trioctanoin initially present on the surface is not influenced by the addition of unlabeled trioctanoin for maintenance of constant pressure, the hydrolysis of this compound into 1,2-dioctanoin followed by

the hydrolysis of the latter can be treated as a series of two consecutive first-order reactions (1).

If the trioctanoin is labeled in the carboxyl carbon atoms, the specific activity of the 1,2-dioctanoin generated will be two-thirds of that for trioctanoin, and for the case where $k_D << k_T$, it can be shown that

$$\ln\left[\frac{C_T}{3/2C_D + C_T}\right] = -k_T[E_o]t \qquad \text{Eq. 15}$$

where C_D and C are the measured radioactivities associated with 1,2-dioctanoin and trioctanoin in the isolated reaction product mixture.

(3) Hydrolysis of trioctanoin using a nonreactive titrant. If the titrant used is not a substrate for the enzyme but forms a stable monolayer on the surface, the hydrolysis of the trioctanoin initially present can again be treated as a series of two consecutive first-order reactions. If under these conditions the titrant on the surface obeys Eq. 1, and if $k_D << k_T$, it can be shown that

$$\left(\frac{dY_x}{dt}\right)_{t=0} = -k_T[E_o]X_o[1 - T_o/D_o] \qquad \text{Eq. 16}$$

where Y_x is the moles of titrant added from the syringe and X_o is the moles of titrant that yield the surface pressure π on a surface of area S. Values of X_o were calculated from data in Ref. 15.

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