Enzyme–substrate interaction in lipid monolayers. I. Experimental conditions and fundamental kinetics

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Abstract A systematic study has shown the importance of the different factors which are concerned with the action of lipase on a substrate (1,3-didecanoylglycerol). These consist of *a*) the process of adsorption of lipase to the surface, *b*) the necessity of limited stirring to reach equilibrium, and *c*) the persistence during the reaction process of the enzyme molecules adsorbed on the monolayer. On the basis of this preliminary investigation, a technique was established to analyze the mechanism of lipase action with defined quantities of enzyme and lipid segregated in the monolayer. Thus, the process of the reaction itself is separated from the adsorption process, and it is demonstrated that the quantity of substrate hydrolyzed per minute depends only on the quantity of initially adsorbed lipase and not on the quantity of substrate or on the surface concentration of the enzyme. An appropriate new definition of the rate is consequently adopted.—**Dervichian, D. G., and J. P. Barque.** Enzyme–substrate interaction in lipid monolayers. I. Experimental conditions and fundamental kinetics. *J. Lipid Res.* 1979. **20:** 437–446.

Supplementary key words pancreatic lipase · monolayer · adsorption of lipase · 1,3-didecanoylglycerol

Before entering upon a new study of the mechanism of action of lipolytic enzymes on lipid monolayers, a preliminary and systematic search of the different concerned factors appeared to be needed. In fact, as will be seen in the present article, this preliminary investigation resulted in a better understanding and interpretation of previous results obtained either in this laboratory or by other authors (1–13). Consequently, the technique and procedure were revised.

It was demonstrated in this laboratory (14) that the lipolytic enzyme is strongly bound to the substrate monolayer. Thus, the adsorbed enzyme can be transferred, together with the lipid monolayer and under constant surface pressure, from one region of the surface, under which the water in the Langmuir trough contains the enzyme, to another region where the underlying water is devoid of enzyme. The monolayer is "washed" during the displacement to get rid of the underlying enzyme solution which may accompany it. Under these conditions, it was ascertained that the lipolytic reaction still proceeds.

This conclusion will be re-examined in the present report. What should be remembered here is that, although the enzyme is held by the substrate monolayer, its surface concentration follows an adsorption equilibrium with the underlying solution. But, as will be shown, the time required to reach equilibrium is very long, because diffusion of the enzyme molecules towards the surface is very slow. A *limited* agitation of the underlying solution, after the substrate layer has been spread, is necessary in order that convection makes up for the slowness of simple diffusion. The extreme slowness of the diffusion, far from being considered as a drawback, is an advantage, because, once the agitation is stopped, practically no new molecules of enzyme come to the surface, and only those enzyme molecules which are already adsorbed act in the lipolysis reaction.

These facts were ascertained in the present research and constitute the basis of a new well-defined experimental procedure. The essential point of this procedure is that, once the equilibrium quantity of enzyme is adsorbed and *the agitation stopped*, substrate and adsorbed enzyme form together a segregated system. On such a well-defined system, the experimental study of the reaction kinetics can be undertaken with the least a priori theoretical considerations or assumptions.

The same substrate was utilized as reported in the previous publications (7, 8, 14). The choice of 1,3-dicaprin (1,3-didecanoylglycerol) as substrate was dictated by the following conditions. First of all, the substrate monolayer must be insoluble even at high surface pressures and the reaction products must be very soluble in the underlying water in order to leave the surface immediately once they are produced. A triglyceride could not therefore be utilized, since the diglyceride produced would be either insoluble or

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DEFINITIONS AND NOTATIONS

Γ	Surface concentration of substrate in the monolayer, in moles per cm². (Order of magnitude: 10^{-10}.)
π	Surface pressure in the monolayer. π is a very sensitive function of Γ and is therefore used to control Γ by keeping π constant at a predetermined value during the lipolysis. (Order of magnitude: 0 to 30 dynes/cm.)
Σ	Area per mole of substrate in cm²/mole. $\Sigma = 1/\Gamma$. (Order of magnitude: 10^{10}.)
A	Molecular area of the substrate in Å². (Order of magnitude: 50 to 100 Å².)
S	Area of the monolayer in cm².
S_0	Initial area of the monolayer (magnitude 500 to 800 cm²).
m	Total quantity of substrate in the monolayer in moles. $m = S\Gamma = S/\Sigma$.
m_0	Initial quantity of substrate in the monolayer. $m_0 = S_0\Gamma = S_0/\Sigma$.
C	Concentration of enzyme in the underlying solution, in mg/l. (Order of magnitude 10^{-1} mg/l.)
z_t	Surface total *concentration* of enzyme.
Z_t	Total *quantity* of enzyme on the surface: $Z_t = z_t \cdot S$. (Initially $Z_t = z_e \cdot S_0$).
z_e	Equilibrium surface concentration of enzyme, i.e., initial surface concentration of enzyme adsorbed on the surface.
a	Specific enzymatic activity: moles product/min per mg enzyme.
$a \cdot Z_t$	Total enzymatic activity: moles product/min.

very slightly soluble, depending on the hydrocarbon chain length. Starting with a diglyceride, the monoglyceride and free fatty acid produced should both be soluble enough not to remain on the surface, even when incorporated in the remaining insoluble diglyceride. 1,3-Didecanoylglycerol fulfills all these conditions: its monolayer is stable up to the collapse pressure and the reaction products, namely decanoic acid (particularly at pH 8) and monodecanoylglycerol, cannot remain at the surface of water.

PRELIMINARY INVESTIGATIONS

Slowness of the spontaneous adsorption of the enzyme

Following the procedure of Hughes (1) as well as that of Schulman and Hughes (15) and Schulman and Rideal (16) for film penetration, Dervichian (7) and Olive and Dervichian (8) introduced a known quantity of enzyme under the lipid monolayer by injection of 30 or 50 ml of the enzyme solution with an appropriate pipette having a bent extremity. To get good mixing while injecting the solution, the end of the pipette was run along and under the region covered by the monolayer. Keeping the end of the pipette immersed, it was repeatedly filled by sucking the water in the trough and then was emptied while moving to and fro. In spite of this procedure, homogeneity was far from being fulfilled, so that to get comparable rates of lipolysis, 4–5 times more enzyme had to be used than with the procedure described in the present article.

In fact, by injecting colored solutions with the same procedure in the transparent trough and observing, either from above or from the side, it appeared that the distribution was very irregular: the dye remained concentrated in certain regions, often far from the surface, while other parts were colorless. This situation remains practically stationary, showing how slow the spontaneous diffusion is. Stirring with a sort of immersed rake lying with its teeth perpendicular to the bottom of the trough simply cuts the colored lumps, without producing a fine homogeneity. On the other hand, complete and rapid homogeneity is obtained by moving an immersed triangular prism, described below. The use of this prism allows adsorption equilibrium to be reached very rapidly.

The adsorption or fixation of the enzyme molecules to the substrate monolayer is one of the main factors to be taken into consideration. The concentrations of enzyme utilized in the case of monolayer lipolysis are much lower than those used when oil emulsions are subjected to lipolysis; they are of the order of 10^{-9} M. With such a dilution, the adsorption equilibrium takes considerable time to be reached by simple spontaneous diffusion. The slowness of the adsorption of proteins at the surface of their solutions is well known; in the absence of stirring, adsorption proceeds in an exponential manner. The adsorption equilibrium is evidently reached much more rapidly when mechanical convection by agitation replaces the slow spontaneous diffusion.

The following experiment illustrates this point. If the substrate monolayer is shifted from the surface of a region devoid of enzyme to the surface of a region containing the enzyme, the reaction takes a long time to start and reaches only a low rate. But, if, after the shift of the monolayer, the underlying solution is agitated, lipolysis starts immediately, reaching its maximum rate rapidly.

Laying the substrate directly on the surface of the

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enzyme solution by means of a volatile solvent leads to an immediate start of the reaction; this is how several investigators have operated. The maximum rate, however, is not reached (10), i.e., the adsorbed enzyme has not reached its equilibrium concentration. The immediate start of the reaction seems to be due to the local cooling produced by the evaporation of the solvent which produces convection currents in the underlying water. In fact, in the previous experiment, if, after the shift of the monolayer, drops of the *pure* solvent are deposited on the surface, the same behavior is observed.

Importance of the adsorbed enzyme

That the mere spreading of the substrate monolayer on a homogeneous solution of the enzyme is not enough to reach the equilibrium conditions between layer and solution is demonstrated by the experiments of Zografi, Verger, and de Haas (10). These authors discovered that, with a given substrate monolayer, the stirring of the underlying enzyme solution with a magnetic agitator increased 2 to 3-fold the rate of lipolysis. In fact, they were not considering an increase of the adsorption, but started from the idea that agitation would prevent the accumulation of the reaction products. Yet, the increase of the reaction rate persisted after the agitation was stopped, which showed that the effect was not due to an enhanced desorption of the reaction products.

These observations were difficult to interpret. But now, they can be understood since we know that *1*) agitation is necessary to bring to the surface the maximum quantity of enzyme corresponding to the adsorption equilibrium, and *2*) once a certain quantity of enzyme is adsorbed, the quantity of substrate hydrolyzed per minute depends only on this quantity of enzyme, and remains constant despite the reduction of the quantity of substrate in the course of time. These facts will be examined at length in the present report.

Necessity and realization of a rapid and limited stirring

Direct spreading on the enzyme solution also leads to an indeterminate homogeneity of the *adsorbed* enzyme. In fact, as the monolayer is deposited drop by drop, its surface pressure increases by steps and, as will be demonstrated in the second article of this series (17), the quantity of fixed enzyme depends on the surface pressure, particularly at relatively low pressures. On the contrary, if the monolayer is first spread on water and then shifted on the enzyme solution, there is time to bring the monolayer up to the chosen pressure and then to stir to start the reaction.

Thus, on one hand, stirring under the monolayer only when at its initial area fixes a definite amount of enzyme, and, on the other hand, the end of the stirring determines the zero time of the reaction process in a segregated system (substrate and enzyme in the monolayer). The technical problem was, therefore, to obtain in the shortest time possible the adsorption of the maximum quantity of enzyme corresponding to the equilibrium with the underlying enzyme solution. Considering the slowness of diffusion, it meant bringing rapidly the entire solution in contact with the surface. This was realized by the use of an immersed triangular prism made of Perspex. This prism extends all over the breadth of the trough and is placed on one of its lateral faces at the bottom (Fig. 2). The upper edge of this triangular prism is 2 to 3 mm below the surface of the water. A triangular section is chosen for the prism for evident hydrodynamic reasons. While the prism is moving, all the mass of the liquid slides on the advancing face of the prism and is forced to pass in a thin layer under the surface. The movement of the liquid is slow enough not to produce ripples. Nevertheless, it reaches the surface, as can be seen by sprinkling talcum powder on it; there is a slight flow on the surface which extends a few centimeters behind the moving prism. The travel takes less than 15 seconds, i.e., less than 30 seconds for one back and forth run. Magnetic agitators cannot be relied on for such a definite performance.

TECHNIQUE AND PROCEDURE

The technique and procedure used are entirely based on the preceding preliminary investigations and remarks.

The automatic apparatus for the measurement of the surface area and pressure was previously described (7). The trough (**Fig. 1**) had particularly large dimensions: 80 cm length, 23 cm width, and 3 cm depth. Such a large surface permits a correct spreading and a high precision in the area measurement, and also permits partitioning into different compartments.

In order to limit the water volume in which the enzyme is dissolved and to separate it from the region containing only the buffer solution, two barriers divide the trough into three compartments. These barriers, made of Perspex strips placed on edge at the bottom, extend over the whole width of the trough. Their height is such as to reach up to 2 mm below the surface of the water. Each barrier, made of two parallel strips separated by 5 cm, forms a baffle opposing diffusion (Fig. 1).

The enzyme is introduced in compartment II, while compartments I and III are isolated by the barriers.

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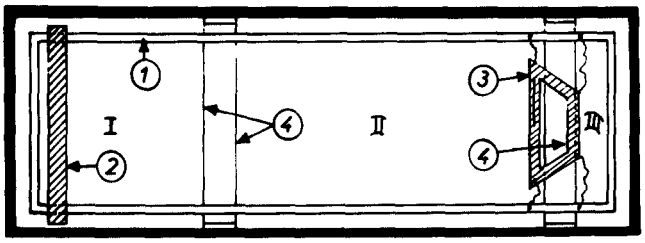

Fig. 1. The special Langmuir trough. (*1*) Floating frame limiting the surface area. (*2*) Piston barrier. (*3*) Float of the surface balance. (*4*) Immersed baffles preventing the diffusion of the enzyme from compartment II to the others. (II) Compartment containing the enzyme. (I) and (III) Compartments generally devoid of enzyme.

The triangular prisms that produce the agitation are previously placed in compartment II. To move them in one direction or the other, they are connected by a lateral arm to a carriage driven by a motor and running in a direction parallel to the length of the trough. The travel is limited in both directions to the barriers, agitation being produced only in compartment II. In order to reduce the time of agitation, a system with two joint triangular prisms was used (**Fig. 2**). This reduced the back and forth travel of the carriage.

To keep the surface pressure at a given constant value, a photoelectric cell device utilized by Benzonana (quoted in [11]) was adopted. This is fixed on the path of the light beam of the automatic apparatus, in combination with the spot follower recording the surface pressure. It stops or starts the movement of the piston-barrier which compresses the monolayer during the lipolysis.

A certain quantity of the freshly prepared mother solution of the enzyme is delivered uniformly at the bottom of compartment II with the bent pipette. Complete homogeneity is obtained by moving the triangular prisms to and fro.

After cleaning the surface, the substrate is deposited on compartment I. The quantity deposited, previously determined, is such that the initial surface pressure never exceeds 2 dynes/cm. (As will be demonstrated, at this pressure there is yet no enzyme fixation to the monolayer.) The monolayer is then compressed till the piston reaches the brink of the second compartment containing the enzyme. In these conditions, the initial area, S_o, is always the same, and the quantity of spread substrate is such that the surface pressure attains the desired value π. When needed, the pressure is adjusted by removing some of the monolayer by suction. With this procedure lipolysis does not start immediately and there is time to set in motion the agitating prisms. After the first traverse, hydrolysis starts and it is necessary to move the piston-barrier and to reduce slightly the surface in order to reset the pressure at its constant value π. Once the desired number of to and fro traverses of the agitating prism has been executed, the device that maintains the surface pressure constant by shifting the piston automatically is started. The time is marked every minute or 30 sec on the recorded diagram during the course of hydrolysis. All measurements were done at a temperature of T = 20 ± 1°C.

MATERIALS

Lipase

The porcine pancreatic lipase was kindly provided by Professor P. Desnuelle, and we thank him. The same enzyme preparation (stored below 0°C) was utilized throughout the experimental period of this work. The preparation and purification of the lipase has been published in detail (19). The sample as provided contained colipase. No surface denaturation was therefore encountered as is reported to occur with lipase completely separated from the colipase (18).

Substrate

The very pure synthetic diglyceride, 1,3-dicaprin (1,3-didecanoylglycerol) was kindly provided by the Astra-Calvé Society's research laboratories, and was used in all experiments. We express our thanks to Dr. R. F. Féron, technical manager of this Society.

Buffered solution

The water was doubly distilled and buffered at pH 8 with Tris acetate and HCl (Tris 0.005 M + NaCl

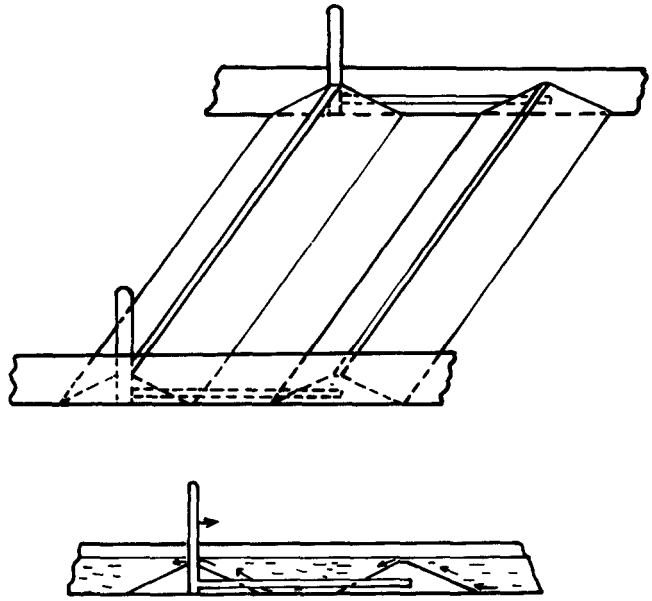

Fig. 2. Agitating device. Two joint triangular prisms immersed in the solution. They are connected to a carriage which moves them to and fro.

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0.15 M). Maximum activity of lipase was achieved by the addition of $CaCl_2$ to a final concentration of 4×10^{-4} M, according to Benzonana and Desnuelle (20).

RESULTS AND ANALYSIS

Quantity of adsorbed enzyme

Different enzyme concentrations produced different rates of lipolysis. With the lipase bulk concentration, C, of 50 μg/l, the dicaprin monolayer is completely hydrolyzed in about 15 min. Considering the depth of the trough beneath each cm² of the surface (i.e., 2.5 cm), we may calculate what the surface concentration z_t of the adsorbed enzyme would be if all the enzyme molecules had come to the surface. Taking 50,000 as the molecular weight of lipase, with 50 μg/l, z_t would be 15×10^{11} molecules of lipase per cm². This gives an average available area of ca. 6500 Å² for each molecule of enzyme. The diameter of a globular protein molecule with a molecular weight of 50,000 can be estimated to be about 50 Å and the largest cross section about 2000 Å². This is less than a third of the area available per molecule, *if all the enzyme* of the solution were adsorbed at the surface. That this is very far from being the case can be proved by the following experiment.

The substrate is spread, the solution agitated, and the onset of the reaction observed for several minutes. Then, while the compression barrier is simultaneously displaced to maintain the surface pressure constant, the monolayer is swept away together with the attached enzyme molecules and removed with the tapered end of a tube connected to a suction pump. If the quantity of enzyme bound to the monolayer is relatively important, repeating this procedure several times should impoverish the underlying enzyme solution and, consequently, the lipolysis rate should show a decrease. In fact, after repeating 20 times, no appreciable difference was found in the rate.

Another method is to take up the monolayer on a filter paper previously immersed under it and pulled out vertically through it by attached threads. This was inspired by the well-known Langmuir–Blodgett method for building up films. As the monolayer is progressively transferred to the wet paper with some of the underlying water, the compressing barrier has to be correspondingly displaced to maintain the surface pressure constant. A decrease of the reaction rate of hardly 10% was noticed after 40 repetitions of the operation.

Assuming that at the most one-hundredth of the total lipase is attached to each monolayer, there would be on the surface one lipase molecule on an area of at least 6500×100 Å, i.e., 6 or 7×10^5 Å². It should be kept in mind that this is with a protein bulk concentration of 50 μg/l and with the assumption that all the protein is pure lipase. The molecular area of dicaprin, under for example 15 dynes/cm, being 65 Å², the area of 6 to 7×10^5 Å² contains approximately 10^4 molecules of substrate. Thus, it can be stated that there are at least 10^4 molecules of dicaprin for each molecule of lipase and, therefore, if all is hydrolyzed in 15 min, the rate per molecule of lipase is at least 600 ester bonds per min, i.e., a catalytic constant of at least 600.

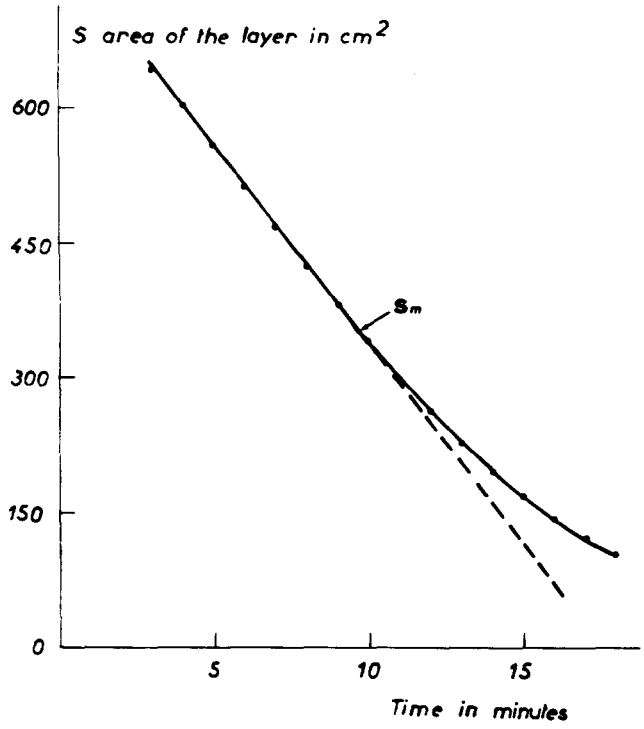

Fig. 3. Monolayer of dicaprin under a constant surface pressure of 10 dynes/cm. Variation of the surface area under the action of lipase. Concentration of lipase in the underlying solution, C = 30 μg/l.

With more than 10^4 molecules of substrate per molecule of enzyme, an average of more than 100 molecules of diglyceride stand linearly between neighboring lipase molecules. An interaction between the enzyme molecules has thus not been taken into account. Even when the area of the substrate is reduced, for example to a quarter, there are still at least 50 molecules of diglyceride separating linearly the nearest lipase molecules, the average distance between them being more than 400 Å.

The experimental kinetics of the surface reaction

When the monolayer area S in the course of the reaction (under constant surface pressure π) is plotted as a function of time, a graph, $S = f(t)$, is obtained with an important linear part (**Fig. 3**). This linear part starts

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when between 1/20th and 1/10th of the monolayer has disappeared and goes on to reductions of the surface reaching only a fourth of the starting area. If p is the constant slope of this linear part,

$$-dS/dt = p \qquad \text{Eq. 1}$$

At relatively high pressures π, the linear segment proceeds to still lower areas. Then the curve bends upwards and becomes definitely exponential, i.e., if the logarithms of the experimental points of Fig. 3, corresponding to the second curved part, are plotted versus time, a perfectly straight line is obtained. In other words, the experimental points obey an exponential of the form:

$$S = \alpha \cdot e^{-\beta t}$$

where α and β are constants. It follows that

$$-dS/dt = \beta \cdot S \qquad \text{Eq. 2}$$

The curve of Fig. 3 is relative to a constant π of 10 dynes/cm. It is one of the numerous curves obtained systematically at different constant π's that will be examined in the second article of this series (17). All these curves are alike, except that the minimum area S_m, where the linear part comes to an end and the exponential begins, depends on the value of π.

The consequences of these experimental facts are as follows. At any determined surface concentration Γ of the substrate (i.e., at any determined π), the area S of the monolayer is proportional to the quantity of substrate: $S = \Sigma \cdot m$ (Σ being the area occupied by 1 mole of substrate at the considered π, and m being expressed in moles). Consequently $dm = 1/\Sigma \cdot dS$. If therefore $S = f(t)$ is linear down to the point S_m and dS/dt is constant, it follows that dm/dt is also constant, i.e., the quantity of substrate disappearing per unit of time, $-dm/dt$, remains constant while the reaction proceeds. It should be stressed that the total quantity of substrate decreases.

In classical enzyme kinetics, the rate of the reaction expresses the variation of the *concentration* per unit of time, $-dc/dt$. As the volume v of the system is evidently constant, $v \cdot c = m$ is the total *quantity* of substrate and $v \cdot dc/dt = dm/dt$ is the *quantity* of substrate that disappears per unit of time in the whole volume of the solution. Since c decreases because of the reaction, m also decreases, and also $-dm/dt$. On the other hand, the total *quantity* E of enzyme does not change, and neither does its concentration $[E]$, since v does not change.

The conditions are different with the substrate monolayer under constant surface pressure π. Here, the surface concentration Γ of the substrate being kept constant (constant π), $d\Gamma/dt$ is zero. This evidently does not mean that the rate of the reaction is zero. To keep Γ constant, the area S must decrease, so that the total quantity of substrate $m = \Gamma \cdot S$ decreases with time and $dm/dt = \Gamma \cdot dS/dt$ is not zero; dm/dt is the decrease per unit of time of the total quantity of substrate which covers an area S at a given moment.

The question arises: what happens to the total quantity Z_t of enzyme adsorbed on the monolayer, and consequently to the surface concentration z_t when the area of the monolayer decreases because of the reaction? The answer follows very easily. As concerns the enzyme–substrate interactions, they cannot change since the substrate concentration Γ is invariable. On the other hand, the surface concentration of the enzyme, z_t, is so weak, as demonstrated above, that its specific activity a cannot be modified by the reduction of the area. Consequently, each molecule of enzyme keeps on hydrolyzing the same quantity of substrate per minute, whatever the total quantity of substrate may be, and

$$-dm/dt = a \cdot Z_t \qquad \text{Eq. 3}$$

Actually, it will be shown in the second article of this series (17) that the specific enzymic activity a is independent of the magnitude of Z_t (or z_t). This independence of a from Z_t leads to the conclusion that the rate of hydrolysis is strictly proportional to the quantity of adsorbed enzyme. Consequently, the rate of hydrolysis can be taken as a measure of the quantity of adsorbed enzyme.

The experimental fact is that, in the course of the linear process, dm/dt remains constant although m decreases considerably. Considering equation 3, if dm/dt is invariable, Z_t, the total quantity of enzyme, must not vary. But as S decreases and, as $z_t = Z_t/S$, the surface concentration z_t of the enzyme must increase during the linear process.

That dm/dt remains constant must not be surprising from the classical point of view. Indeed, in enzymic processes, when the substrate concentration is comparatively great, the rate no longer depends on the quantity of substrate, but rather on the quantity of enzyme. This is explained by saying that the enzyme is saturated by the substrate. Now, the enzyme could not be more saturated than it is in the monolayer, where the enzyme is embedded in the substrate itself and not dispersed in a common medium.

The exponential process which follows the linear process can be treated in the same rigorous way. Here, the experimental data show that the area decreases with time according to the relation $S = \alpha e^{-\beta t}$ and consequently

$$-dS/dt = \beta \cdot S \qquad \text{Eq. 4}$$

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As $dm/dt = 1/\Sigma \cdot dS/dt$, Eq. 4 gives:

$$-dm/dt = \beta/\Sigma \cdot S \qquad \text{Eq. 5}$$

Equation 3 is always valid since, when the exponential process commences, the enzyme molecules are still largely scattered and always embedded in the substrate itself. Combining equations 3 and 5, we get

$$Z_t = \beta/a\Sigma \cdot S \qquad \text{Eq. 6}$$

which means that Z_t decreases proportionally to S, so that

$$z_t = Z_t/S = \beta/a\Sigma = \text{constant} \qquad \text{Eq. 7}$$

Thus, *whatever the reason may be*, below S_m, while the area decreases, the surface concentration z_t of the enzyme remains constant.

The conclusions following mathematically from the experimental facts can now be summed up. During the linear reaction period and down to the area S_m, the total quantity Z_t of enzyme in the monolayer remains constant. Consequently, as S decreases, the surface concentration of the enzyme, which originally was $z_e = Z_t/S_o$, increases up to a maximum value $z_m = Z_t/S_m$ corresponding to the area S_m. From there on, the surface concentration remains constantly equal to z_m, and consequently the total quantity $Z_t = z_m \cdot S$ decreases progressively with the area S.

Expression of the rate

Only the initial linear period of the reaction will be considered here to define and measure the rate. The quantity of substrate disappearing per unit of time in the whole monolayer

$$-dm/dt = 1/\Sigma \cdot dS/dt \qquad \text{Eq. 8}$$

corresponds to the definition of the total enzymatic activity. Therefore, the constant a appearing in equation 3 is merely the classically defined *specific enzymatic activity*. It should be kept in mind that Z_t in equation 3 is the total quantity of enzyme segregated in the monolayer, which is the only quantity to take into account, excluding what is in the underlying solution.

Z_t is determined by the magnitude S_o of the initial area on which the enzyme is adsorbed. As in all adsorption or partition phenomena, the concentration C of the enzyme in the underlying solution has a role only in determining the equilibrium surface concentration z_e of the adsorbed enzyme. z_e depends also on the nature and the state of the surface, i.e., of the substrate monolayer. The total quantity Z_t of adsorbed enzyme is evidently proportional to the initial area S_o of the substrate:

$$Z_t = z_e \cdot S_o \qquad \text{Eq. 9}$$

Introducing equation 9 in equation 3, we get:

$$-dm/dt = a \cdot z_e \cdot S_o \qquad \text{Eq. 10}$$

For comparative measurements the same initial area S_o may be adopted. A better way is to introduce a normalized rate V which is independent of the initial size of the monolayer (i.e., independent of the initial quantity of substrate and, indeed, independent of the instrument used). This amounts to dividing both sides of equation 10 by S_o, and writing:

$$V = -1/S_o \cdot dm/dt = a \cdot z_e \qquad \text{Eq. 11}$$

Since a is the specific enzymatic activity, $V = a \cdot z_e$ *is the total enzymatic activity of the quantity* z_e *of enzyme which adsorbs (at equilibrium) on 1* cm^2 *of substrate monolayer*, for a given surface pressure π and a given bulk concentration C of the enzyme (i.e., the quantity of substrate, expressed in moles, hydrolyzed per minute by the enzyme molecules adsorbed on 1 cm^2 of the initial monolayer).

In fact, the experimental variable is not m but the area S of the monolayer, and dS/dt is given by the slope of the linear part of the $S = f(t)$ curve (Fig. 3). Using equation 8, we can write finally:

$$V = -1/\Sigma \cdot S_o \cdot dS/dt = a \cdot z_e \qquad \text{Eq. 12}$$

Practically, to get V at a given surface pressure π and for a given bulk concentration C of the enzyme, once the $S = f(t)$ curve is plotted from the recorded measurements, the slope $-dS/dt$ of the linear part has simply to be divided by the known initial area S_o and the known area Σ per mole of substrate.

This is not just an arbitrary definition of V. The third article of this series (21) reports the results of measurements at constant area, i.e., the area of the monolayer being fixed and the variation $d\Gamma/dt$ of the substrate surface concentration Γ with time being measured. This is equivalent to the classical measurement of the variation of $-dc/dt$ on a substrate dissolved in a fixed volume. It will then appear that the rate V, defined here by equations 11 or 12, is equivalent to $-d\Gamma/dt$ and therefore is equivalent to the $V = -dc/dt$ of classical enzymology.

DISCUSSION AND CONCLUSIONS

On a previous definition of the reaction rate

In two preceding articles (7, 8), a rate V_s was defined as the fraction of substrate hydrolyzed per minute under constant pressure:

$$V_s = -1/m \cdot dm/dt = -1/S \cdot dS/dt \qquad \text{Eq. 13}$$

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Here S is the decreasing area of the monolayer and should not be confused with the constant value S_o of the initial area which appears in equation 12. As a consequence, since equation 13 can also be written $-dS/S \cdot 1/dt$ or $-d\log S/dt$, $\log S$ was usually plotted against t.

The reasons for the introduction of V_s were the following. Because π is constant, it is not the surface *concentration*, Γ, which varies in the course of time, but the total *quantity*, m, of substrate. Therefore, it appeared that $-dm/dt$ must be proportional to the total quantity m present at each moment t, and consequently, dm/dt divided by m (equation 13) was considered as the interesting quantity. Like any physical property assessed per unit quantity of substance, this is a *specific* quantity and was termed "specific rate". This rate V_s was adopted by other authors; but it must be abandoned, because we know now that dm/dt (or dS/dt) depends on the quantity of adsorbed enzyme and is independent of m or S.

Using the technical procedure described in the present article, it appeared that V_s, deduced from the gradual decrease of S, increases progressively. It is only when the area is considerably reduced that V_s becomes constant. Plotting V_s against S gave systematically a perfect hyperbola obeying the relation $V_s = p/S$ (where p is a constant), which, considering equation 13, leads necessarily to the linear relation $S = S_o - pt$. This suggested plotting S directly versus time for all measurements, Fig. 3 being an example for one particular surface pressure.

The established facts

The experimental fact that, under constant surface pressure, the substrate decreases at first linearly down to a value S_m of the area and then exponentially has already been mathematically analyzed (see above). That the total enzymatic activity remains constant at the beginning is evidence that the involved enzyme does not change either in quantity or in specific activity.

The decrease of the total enzymatic activity after the point S_m can be due either to a decrease of the specific activity of the involved enzyme or to a decrease in its quantity. The experiments have shown quite consistently that the form of the decrease is absolutely independent of time. Indeed, as is shown in the second article (17), depending on the surface pressure at which the reaction proceeds, the rate varies considerably, so that the complete hydrolysis of the monolayer can last from less than 10 to more than 60 min. But, in all cases, during the whole period that follows the linear part, the area always decreases in the same exponential way. This means that, in all cases, the total enzymatic activity decreases proportionately to the area of the monolayer and shows that there is no relation with the length of time. Further experimental data obtained with measurements at constant area, given in the third paper (21), show that the beginning of the decrease in activity is bound exclusively to the reduction of the area at the same point whatever the length of time (4–25 min).

These converging facts make it unlikely that the decrease of activity is caused by some transformation of the enzyme which would necessarily depend on time, and particularly by denaturation. Rather, the conclusion which follows is that, beginning at the point S_m (Fig. 3), the total quantity of the involved enzyme (adsorbed enzyme) decreases proportionately to the area S, i.e., in such a way that its surface concentration $z_t = Z_t/S$ remains constant.

The reversible adsorption and desorption of the enzyme

A new inference can be drawn from the work (14) demonstrating the persistence of the enzyme in the monolayer after it is transferred on a region where the underlying water is devoid of enzyme. It was stated that the reaction rate did not significantly change after the displacement, $V_s = 6 \cdot 10^{-4} s^{-1}$ before and after with lipase, and $V_s = 9$ before and $8 \cdot 10^{-4} s^{-1}$ after with phospholipase A.

One would be tempted to deduce that the enzyme is permanently fixed to the monolayer since V_s remains the same. In fact, the rate considered at the time was V_s defined by equation 13. Because of the reaction process, the area at the end of the experiment was reduced to about a third of the original area. Consequently, if V_s was the same, it is because dS/dt (or dm/dt) was also reduced to one-third after the transference. As Z_t is proportional to dS/dt, it follows that only a third of the enzyme remained in the monolayer and ⅔ had desorbed when the monolayer was brought on the solution devoid of enzyme. This confirms that the adsorption of the enzyme is reversible.

The adsorption equilibrium can now be analyzed. In conformity with Gibb's classical equation, one can say that there is equilibrium between the surface phase and the bulk phase of the underlying solution when the chemical potentials of the enzyme are the same in both phases. Thus, when the concentration in the bulk is C, to have the same chemical potential μ in both phases, the surface concentration of the enzyme must be z_e. Suppose now C is reduced to zero, μ also becomes 0 in the bulk and, as the chemical potential corresponding to z_e in the surface is now higher, the enzyme will move from the surface towards the underlying solution, i.e., the enzyme desorbs. This is what happened when the monolayer was transferred from one

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region to the other (14). Desorption should also occur, if, keeping C the same in the bulk, the surface concentration z_t is increased by reduction of the area, thus producing an increase of μ in the surface phase.

How is it then that in the linear part of the reaction process, as Z_t is invariable, the surface concentration of the enzyme, $z_t = Z_t/S$, can increase well above the equilibrium value $z_e = Z_t/S_o$ which was set at the start, and why does desorption occur only after a certain reduction of the area corresponding to the point S_m?

Consider that, following the simple adsorption of the enzyme to the monolayer, association with the substrate occurs and, when a steady state is reached, only part of the enzyme molecules are free, while the others are held in the enzyme–substrate complex. Solely the surface concentration of the *free* enzyme molecules is to be taken into account in the equilibrium with the bulk concentration. The total surface concentration z_t can therefore proceed to increase in the segregated surface system as long as the concentration of the *free* molecules has not reached the limiting value z_e corresponding thermodynamically to the bulk concentration C in the underlying solution.

With regard to the experiment (14) where the monolayer is shifted on a region devoid of enzyme (see above), although the free enzyme molecules leave the surface quite rapidly, it is to be expected that the speed of desorption is governed by the rate at which those enzyme molecules which are bound in the intermediate enzyme-substrate complex are liberated before they can desorb. This explains the persistence for some time in the monolayer of part of the initially adsorbed enzyme.

Explanation of previous results

It is now possible to explain why, from the early stage of the reaction, an exponential variation of the area (i.e., a linear relation between log S and time) was found either in a preceding work from this laboratory or by other authors (10). The reasons are different.

In the work by Olive and Dervichian (8), the underlying solution was not homogeneous and no agitation was carried out. The fact that 4 to 5 times more enzyme was needed shows that under certain parts of the surface there was little or no enzyme at all, while under others the concentration was relatively high. But the principal reason lies in the absence of Ca^{2+} ions, as no $CaCl_2$ was added to the buffered solution. As shown in the next article (17), the linear part of the $S = f(t)$ curve is very short in the absence of Ca^{2+} ions, i.e., the desorption of the enzyme from the surface, and consequently the exponential process, begins very rapidly after a relatively small reduction of the area.

In cases similar to the experiments described by Zografi, Verger, and de Haas (10), the underlying solution was *continually agitated.* This keeps the surface concentration of *free* enzyme molecules permanently equal to z_e. Consequently, because of the equilibrium between associated and free enzyme molecules, the total surface *concentration* z_t is also maintained constant. The total *quantity* of enzyme in the monolayer, $Z_t = z_t \cdot S$, must therefore vary proportionally to S. On the other hand, as it was demonstrated in the present work, $-dm/dt = a \cdot Z_t$. It follows that $-dS/dt$ is proportional to S, and finally log S must decrease linearly with time.

In the case of the so-called "zero order" device introduced by Verger and de Haas (13), the area of the monolayer accessible to the enzyme remains constant, as new quantities of substrate are progressively added from a neighboring surface. Here also the underlying solution is continually agitated. Therefore both z_t and S are kept constant. Hence, $Z_t = z_t \cdot S$ is constant, and $-dm/dt = a \cdot Z_t$ must be constant. This explains why the authors found a simple linear decrease of the substrate with time. Thus, $-dm/dt$ being independent of m, the apparatus was named "zero-order trough".

Kinetics and order of the reaction

The linear decrease of the quantity of substrate, as found with the constant pressure method utilized in the present work, may tempt one to conclude that the reaction is of zero order. Care should be taken to avoid this identification, since in chemical kinetics, based on the law of mass action, thermodynamic activities, or at least *concentrations* (c), are always considered and certainly not *quantities* of reactants. Indeed, in classical kinetics, a reaction is said to be of zero order when dc/dt is constant while c varies. This occurs with high values of c, when it is said that the enzyme is saturated. As the volume v is evidently constant, and since $c = m/v$, if dc/dt is constant, dm/dt is necessarily also constant, while m varies as does c.

On the contrary, in the case of the surface reaction, the surface concentration Γ of the substrate (equivalent to c) is kept constant, and it is the area S (equivalent to v) which varies during the process. To say that the reaction is of zero order in the case of the bulk reaction means that dc/dt is independent of c, *which varies.* If here $d\Gamma/dt$ is constantly zero, because Γ *does not vary*, this means neither that the rate is zero nor that the reaction is of zero order. The total quantity m of substrate decreases necessarily, if not there would be no reaction, and, although $d\Gamma/dt = 0$, dm/dt has a constant value different from zero.

Nevertheless, the fact remains that in both cases the conditions are similar at the molecular level. In both,

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the enzyme is saturated by the substrate (excess of substrate), except that, in the case of the reaction in solution, as it proceeds, the substrate concentration gradually decreases and reaches a value at which the enzyme is no longer saturated, while with the surface reaction, the enzyme molecules are "dissolved" in the substrate itself and thus remain saturated up to the end.

In the monolayer, each molecule of enzyme keeps on hydrolyzing the same quantity of substrate per minute, although the enzyme concentration in the surface may increase to 4 or 5 times the initial concentration. This fact is easily explained since the environment in substrate of each enzyme molecule does not change (i.e., the substrate concentration is invariant).

Contrary to classical kinetics, instead of encounter and impact of enzyme and substrate molecules, what has to be considered here are the arrangement and coordination of the substrate molecules around each of the scattered enzyme molecules in the monolayer.

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