Enzyme–substrate interaction in lipid monolayers. II. Binding and activity of lipase in relation to enzyme and substrate concentration and to other factors

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Abstract With the limited stirring procedure used in the present work, substrate and enzyme together form a segregated and well-defined system on the surface. The lipase molecules responsible for the lipolysis are only those that are adsorbed on the glyceride monolayer. After a study of the stirring procedure, two series of systematic experiments were done: *a*) the bulk concentration of the enzyme was varied with different constant surface concentrations of the substrate, and *b*) the surface concentration of the substrate was varied with different constant bulk concentrations of the enzyme. The influence of the surface concentration of substrate on *a*) the rate of lipolysis, V,; *b*) the enzyme activity, a,; and *c*) the enzyme adsorption, z_e, were each determined by a different procedure. The values obtained verify the enzymic activity equation ($a = V/z_e$). The roles of other factors (Ca^{2+} ions and pH) which govern the adsorption of the enzyme and its specific activity were also studied in preliminary experiments.—**Barque, J. P., and D. G. Dervichian.** Enzyme–substrate interaction in lipid monolayers. II. Binding and activity of lipase in relation to enzyme and substrate concentration and to other factors. *J. Lipid Res.* 1979. **20:** 447–455.

Supplementary key words pancreatic lipase · adsorption of lipase

In the preceding article (1), a systematic examination of the different factors involved in the mechanism of action of lipase on a lipid monolayer led to some modifications in the method, based on the following facts: *a*) spontaneous adsorption of the enzyme on the surface is extremely slow; *b*) an appropriate limited stirring of the underlying solution rapidly brings to the surface the maximum quantity of enzyme corresponding to the adsorption equilibrium; and *c*) at a given surface pressure, the rate of hydrolysis depends only on the initial quantity of adsorbed enzyme and remains constant (between certain limits) despite the reduction of the substrate monolayer in the course of time.

With the permanent stirring of the underlying solution, the surface reaction and the adsorption equilibrium of the enzyme are so intermingled that the different parts of the mechanism cannot be experimentally dissociated. Nor can the influence of the different factors be predicted on a classical basis, as the outcome of this investigation will show.

On the contrary, with a suitable and rapid stirring procedure, substrate and adsorbed enzyme form, on the surface, a segregated and well-defined system which can be used to study both the adsorption of the enzyme and the reaction itself, with the least number of assumptions.

With this procedure the different factors were systematically modified one by one, keeping all the others constant. The mere measurement of the rate of hydrolysis of the lipid in relation with either the bulk concentration of lipase in the underlying solution, or the surface concentration of the substrate, cannot by itself give thorough information on the different factors involved in the action of the enzyme. That the quantity of adsorbed enzyme should increase with the concentration in the underlying solution may be obvious; but the fact that it varies with the surface concentration of the substrate had to be demonstrated and the form of this variation determined. The mere measurement of the increase of the rate of hydrolysis with the increase of the surface concentration of the substrate does not show if this is due to an intensified adsorption, to an enhanced specific activity of the enzyme, or to both.

Under the proper conditions, the method enabled us to separate the adsorption process from the association and reaction processes which occur immediately after, and to evaluate each process. The variation of the rate of hydrolysis V was determined as a function of the bulk concentration C of the enzyme and as a function of the surface concentration Γ of the substrate

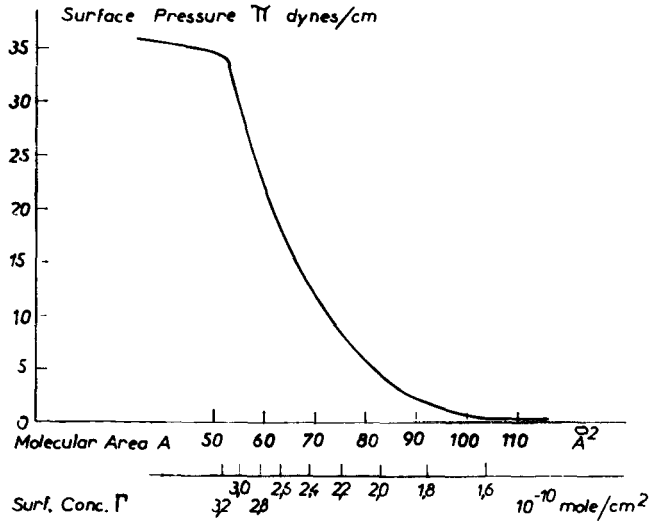

Fig. 1. Isotherm of 1,3-didecanoylglycerol monolayer at 20°C. Ordinates are relative to the surface pressure expressed in dynes per cm. Abscissae give both the molecular area in square Ångströms and the surface concentration in moles per cm².

(i.e., the surface pressure of the monolayer). In addition, the variation of the quantity of adsorbed enzyme z_e and the variation of the enzymic activity a as a function of Γ, were determined using three different means. The experimental verification of the relation $V = a \cdot z_e$ between these independently measured quantities enabled us to check the correctness of the measurements as well as the validity of the reasoning.[1]

TECHNIQUES AND PROCEDURE

The substrate (1,3-didecanoylglycerol), lipase associated with colipase, apparatus, double prism agitator, and the procedure were described in the first article (1). All experiments were done at the temperature of 20 ± 1°C.

RESULTS

Isotherm of the substrate monolayers

In the procedure utilized in the present study, the experiments were carried out at various constant surface concentrations Γ of the substrate. Practically, this amounts to maintaining the surface pressure π constant, since there is a precise relation between the mole area Σ in the monolayer and the surface pressure π; the latter can be easily controlled within 0.1 dyne/cm. The surface concentration Γ is the inverse of the mole area Σ. Therefore, it is necessary to establish accurately the mole area–surface pressure isotherm of the substrate. **Fig. 1** gives the $\Sigma-\pi$ isotherm of 1,3-didecanoylglycerol (dicaprin) at 20 ± 1°C. The layer collapses at a pressure of 34 dynes/cm at which it ceases to be entirely monomolecular. The molecular area A at this point is 52 Å² (i.e., $0.313 \cdot 10^{10}$ cm²/mole, corresponding to $\Gamma = 3.2 \cdot 10^{-10}$ mole/cm²). With the given sensitivity of the balance, 34 dynes/cm corresponds on the recorded graph to a displacement of the spot of 16.1 cm. Concerning the precision on the measurement of the area, it is enough to say that in the course of the recording of the entire isotherm, the piston barrier can readily move over 50 cm, measured within 1 mm.

A systematic study of stirring

The advantage of the double-prism agitator (1) is to reduce the number of agitations and to reach the adsorption equilibrium of the enzyme more rapidly. In order to follow in more detail the variation of the rate of hydrolysis as a function of the number of agitations (number of back and forth movements of the prism), the single prism was used. With the single prism the effect produced with each stroke is reduced.

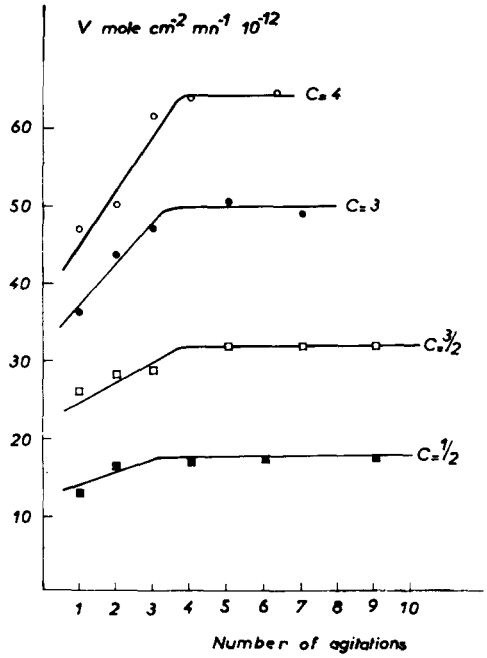

Fig. 2. Variation of the rate V as a function of the number of agitations of the enzyme solution in contact with the monolayer. Rates were measured under a constant pressure of 21 dynes/cm. The different curves correspond to different enzyme concentrations. $C½$ = 10 μg/l; $C¾$ = 30 μg/l; $C3$ = 60 μg/l; and $C4$ = 80 μg/l.

[1] A list of symbols and definitions is given in article I of this series (see reference 1).

To obtain the rate of hydrolysis corresponding to a given number of strokes the decrease of the area per minute ΔS was deduced from the slope of the initial linear part of the corresponding $S = f(t)$ curve. (This curve is similar to those of Fig. 4.) As explained previously (1), the rate is defined by the relation

$$V = -1/\Sigma S_0 \cdot dS/dt = a \cdot z_e. \qquad \text{Eq. 1}$$

Different enzyme concentrations were used. In order to have strictly comparative values for each concentration C, the successive measurements were done on the same enzyme solution. Furthermore, the substrate surface concentration Γ was always the same (21 dyn/cm) and the initial area S_0 of the monolayer was also the same.

In **Fig. 2** the values of V as a function of the number of agitations are plotted. Each of the curves is for a different concentration C of the enzyme. Similar curves were obtained for different constant surface concentrations, Γ.

For every enzyme concentration, V increases with the number of agitations, reaching a limiting value. In fact, V increases because z_e (i.e., the quantity of initially adsorbed enzyme per cm²) increases with the number of agitations and reaches the equilibrium value. It will be demonstrated below that V is strictly proportional to z_e. The fact that a limiting rate is reached after a certain number of agitations indicates that z_e itself reaches a limit.

Relation between the quantity of adsorbed enzyme and the enzyme concentration in the solution

It follows therefore that the limiting value of each of the curves of Fig. 2 should be taken as the characteristic rate for each *enzyme* concentration C in the underlying solution (the surface concentration Γ of *substrate* being definite and kept constant). These limiting values of V are plotted in **Fig. 3** as a function of C. The curve thus obtained necessarily passes through the origin, since it was verified that there was no spontaneous hydrolysis in the absence of enzyme.

Strictly speaking, this curve simply relates the rate of hydrolysis to the enzyme concentration C of the solution. But, since Γ is constant, the rate is proportional to the initial surface concentration z_e of adsorbed enzyme and the curve of Fig. 3 can be taken as the expression of the variation of z_e as a function of C in the underlying solution. It appears that, after a rapid increase at the beginning, the curve bends and then continues to rise linearly with increasing C. However, the plot of this curve could not be followed further, since the rate becomes so great that it is impossible to make reliable measurements.

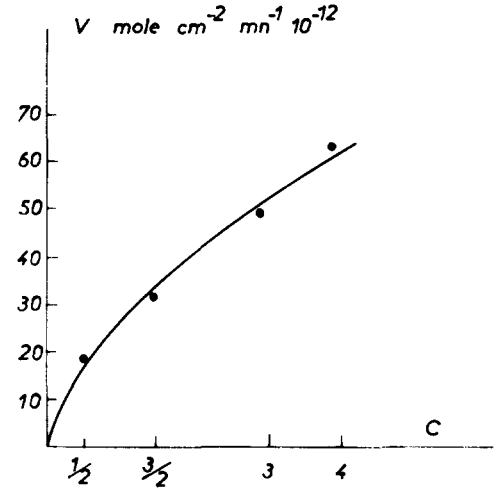

Fig. 3. Variation of the rate V with the enzyme concentration C for a determined number (five) of agitations and under a constant surface pressure of 21 dynes/cm.

Kinetics in relation to the bulk concentration of enzyme

In all the following experiments, a standard number of five agitations was considered as sufficient to reach the equilibrium value of the enzyme adsorption. Hydrolysis was followed over time, using a series of increasing concentrations of enzyme, but keeping always the same surface concentration Γ of substrate. Under the chosen conditions, the initial area S_0 was always $17 \times 40 = 680$ cm². **Fig. 4** shows the $S = f(t)$ curves with monolayers under a surface pressure of 10 dynes/cm (i.e., $\Gamma = 2.28 \cdot 10^{-10}$ mole/cm²) and lipase concentrations ranging from $C = 1 \times 30$ to 5×30 μg per liter. From the slopes dS/dt of the linear sections of these curves, the rates $V = -1/\Sigma S_0 \cdot dS/dt$ corresponding to the different bulk concentrations C were deduced and plotted on **Fig. 5** (lower curve). As in the case of Fig. 3, ($\pi = 21$ d/cm) the curve, starting from the origin, bends at first and then continues to rise linearly with increasing C.

Rate of hydrolysis as a function of the substrate surface concentration Γ

In all the preceding investigations concerning either the effect of the number of agitations or the bulk concentration of the enzyme, the determinations were carried out on a monolayer maintained under the same surface pressure (i.e., the same surface concentration Γ of substrate). In the following series of experiments, the whole process of complete hydrolysis with time was examined with monolayers held at different constant pressures π. In all cases, the bulk enzyme concentration C was the same (30 μg/l) and the

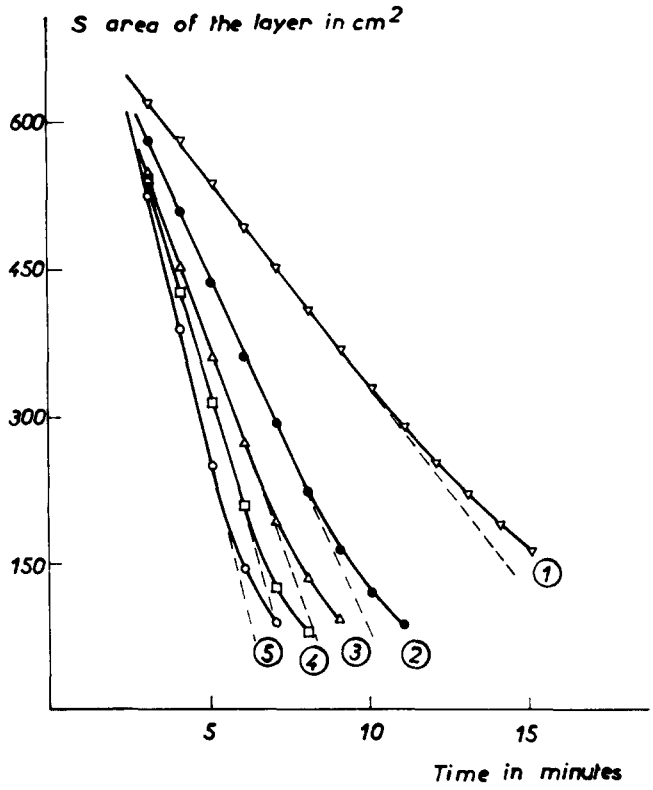

Fig. 4. Variation with time of the surface area of the substrate monolayer kept under a constant pressure of 10 dynes/cm ($\Gamma = 2.32 \cdot 10^{-10}$). Each curve is relative to a different bulk concentration C of enzyme. Curve (1) corresponds to C = 30 μg/l. Curves (2)–(5) correspond, respectively, to $2C$, $3C$, $4C$, and $5C$.

number of agitations also was the same (namely five), corresponding to the maximum adsorption of the enzyme to the monolayer. The different $S = f(t)$ curves are plotted in **Fig. 6.** As already stated, the hydrolysis rates V are deduced from the linear segments of these $S = f(t)$ curves. The values of V for different Γ (i.e., different π) have been calculated from the curves of Fig. 6 and plotted in **Fig. 7** (continuous curve).

Following the linear segment, the curves of Fig. 6 become exponential at a certain point, i.e., $-dS/dt$ decreases proportionally to S. As demonstrated in the preceding article (1), this corresponds to the beginning of the decrease of the total quantity Z_t of enzyme on the surface proportionally to S. It appears in Fig. 6 that the linear segment becomes shorter when the substrate surface concentration Γ is lower (i.e., π is lower). In other words, S_m/S_o (see below) increases with the surface concentration of the substrate.

The variation of V with C, while Γ is the same (preceding section and Fig. 3 or Fig. 5), shows that the quantity of adsorbed enzyme z_e increases when the bulk concentration C increases. But, the fact that V varies with Γ (Fig. 7, continuous curve), while C is the same, may result either from the increase of z_e or the increase of the enzymic specific activity a with Γ, or from the joint increase of both z_e and a. This problem can be solved by proceding in such a way that either z_e or a is kept the same while Γ is varied.

In the measurements represented in Fig. 6, from which are derived the values of V plotted in Fig. 7 (continuous curve), the initial adsorption of the enzyme and the measurement of the rate of hydrolysis are evidently made at the same surface pressure π (i.e., for the same Γ). On the contrary, taking advantage of the segregation of the adsorbed enzyme with the substrate in the surface, the procedure which will now be described permits us to modify independently z_e and a by changing the surface pressure between the two successive operations, one of adsorption of the enzyme, the other of rate measurement.

There are two possibilities. In the first, the adsorption is carried out in all cases at the same surface pressure, which necessarily gives the same quantity z_e at the start; but the rate is measured at different surface pressures at which the specific activity a of the enzyme may be different. In the second, the adsorption is carried out at different surface pressures, so that the quantity z_e may vary; but the rate is measured always at the same pressure, so a is necessarily the same. It is clear that this operating process is not a combination of the preceding measurements giving the values of V and that it leads to the direct determination of the variations of z_e and a with Γ. As a consequence, the experimental verification of the relation $V = a \cdot z_e$ with these results obtained by three different means, enables one to check the correctness of the measurements as well as the validity of the reasoning.

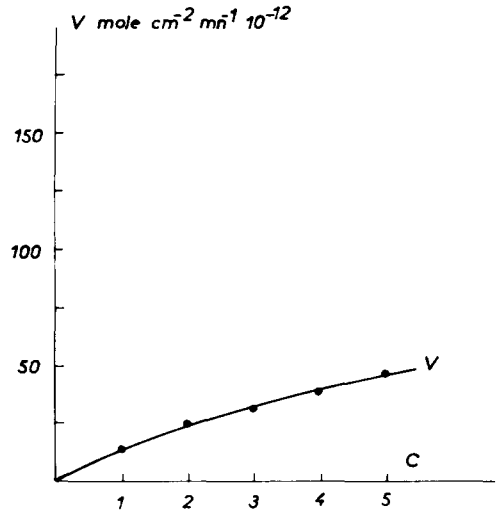

Fig. 5. Variation of the rate V with the concentration of the enzyme under a constant surface pressure of 10 dynes/cm.

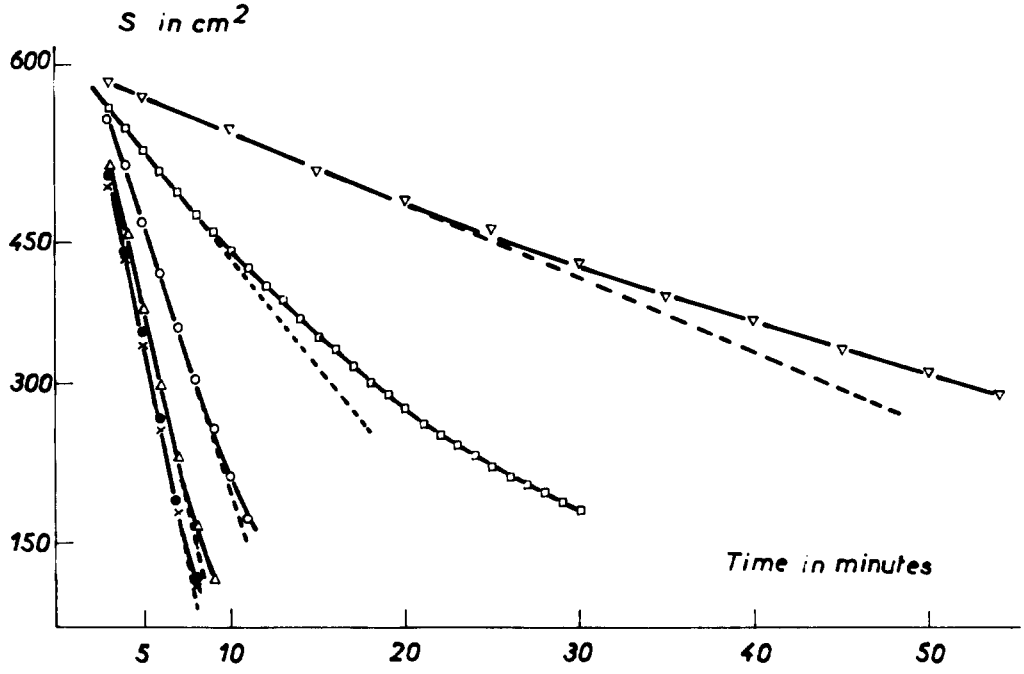

Fig. 6. Variation with time of the surface area of the monolayer with different surface concentrations, Γ, of the substrate and with a constant concentration of lipase, $C = 30$ μg/l. Values of Γ ($\times 10^{-10}$ mole/cm²) are as follows: ▽, 2.10; □, 2.20; ○, 2.32; △, 2.55; ●, 2.71; and ×, 2.85.

Variation of the specific activity *a* with the surface concentration of substrate

In practice, the automatic recording of the area S is cut every minute by a bar, so that the interval ΔS between two consecutive bars is in fact a recorded measure of $-dS/dt$. Therefore equation 1 can be written as well:

$$V = -1/\Sigma S_o \cdot \Delta S = a \cdot z_e \qquad \text{Eq. 1}$$

Hence

$$\Delta S = (\Sigma S_o) a \cdot z_e \qquad \text{Eq. 2}$$

where Σ and S_o are known quantities.

Now if, after the *same* arbitrary quantity z_e of enzyme per cm² is adsorbed on the surface, the hydrolysis is followed successively at two different surface pressures, π_1 and π_2, the corresponding values of ΔS must, according to equation 2 be proportional to the corresponding values of a:

$$\frac{\Delta S_2}{\Delta S_1} = \frac{a_2}{a_1}\left(\frac{\Sigma_2}{\Sigma_1}\right)$$

Inversely, from the measurement of ΔS_1 and ΔS_2 over a few intervals, the relative values of a_1 and a_2 can be estimated:

$$\frac{a_2}{a_1} = \frac{\Delta S_2}{\Delta S_1}\left(\frac{\Sigma_1}{\Sigma_2}\right) \qquad \text{Eq. 3}$$

(Σ_1 and Σ_2 are known from the established isotherm of the monolayer).

Several series of measurements of ΔS were carried out by bringing the substrate monolayer successively to two determined pressures, after the enzyme had been adsorbed to it at one of these pressures; e.g., measurement of ΔS at 15 and 24 dynes/cm, or 8 and 21 dynes/cm, the enzyme being adsorbed at either of

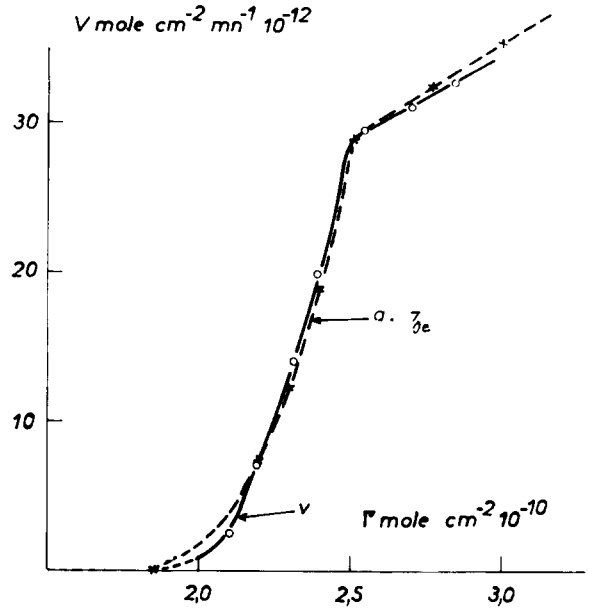

Fig. 7. Variation of the rate V with the surface concentration Γ of the substrate, the bulk concentration of the enzyme being 30 μg/l. The dotted curve corresponds to the product $a \cdot z_e$.

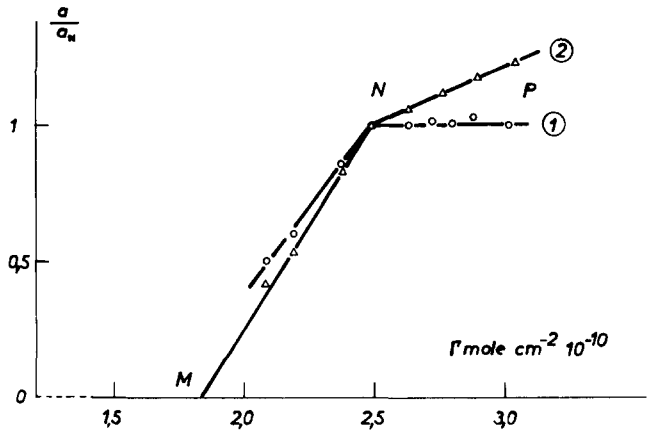

Fig. 8. Variation of the enzymatic specific activity a with the surface concentration of the substrate. The activity is expressed in relative values, a_N being the activity at the inflection point N. Curve (1) expresses the rate in area variation ΔS, while curve (2) expresses the rate in quantities, Δm, of substrate hydrolyzed per minute.

the two pressures of each pair. The results appear consistent if, instead of using absolute values of ΔS, one considers the ratio for each pair of measurements done on the same monolayer. Thus, with determinations made on 16 pairs of pressures ranging between 14 and 28 dynes/cm, the ratio $\Delta S_2/\Delta S_1$ of the two corresponding ΔS values fluctuated at random between 0.97 and 1.06. Only two pairs gave extreme values of 0.90 and 1.20. It can therefore be concluded that, beginning at 14 dynes and above, the hydrolysis rate ΔS, *expressed as an area variation per minute*, is independent of π. On the other hand, the comparison with pairs of pressures where one of the two is below 14 dynes/cm shows a decrease of the rate with the pressure; e.g., if the relatively constant value above 14 dynes/cm is normalized to unity, the rate of hydrolysis, expressed as ΔS, is 0.85 at 12 dynes/cm, 0.60 at 8 dynes/cm, and 0.50 at 6 dynes/cm. These relative values of the rate have been plotted in **Fig. 8,** curve *1*.

As stated above, these rates, ΔS, (provisionally used instead of V) simply express the area variation of the monolayer per minute and not the quantity of substrate hydrolyzed per minute and per cm², $-1/S_o \cdot dm/dt$. In fact, the quantity of substrate per cm² increases with the surface pressure. As shown on the isotherm of Fig. 1, the mole area Σ decreases when π increases. Therefore if $\pi_2 > \pi_1$, the factor Σ_1/Σ_2 appearing in equation 3 is greater than 1. Thus the constancy of ΔS above 14 dynes/cm corresponds in fact to an increasing variation of the quantity of substrate hydrolyzed per minute. To get the *relative* variation of the enzymic specific activity a, the values plotted on Fig. 8-*1* should be divided by the molecular area Σ corresponding to each surface pressure (refer to Fig. 1). Curve 2 of Fig. 8 is thus obtained, where the activity a at 14 dynes/cm is taken as unity. Here, the rate still increases very rapidly up to 14 dynes/cm and much slower thereafter.

In the next section, it will be shown that the quantity z_e of adsorbed enzyme varies with the surface pressure, at least in a certain region of pressure. Therefore the fact that, for a given pair of pressures, the ratio of the two rates remains the same whatever the pressure at which the enzyme was adsorbed (i.e., whatever the quantity z_e of adsorbed enzyme under the conditions used), proves, according to equation 2, that a, for a given surface pressure, is independent of z_e. If a is independent of z_e, it follows from equation 1 that V *is strictly proportional to* z_e.

Quantity of adsorbed enzyme in relation to the surface concentration of the substrate

The change of the rate of hydrolysis with the surface pressure of the monolayer, for a given quantity of adsorbed enzyme, was the point considered in the preceding section. Another point is the difference in the quantity of adsorbed enzyme according to the surface pressure. In other words, one should expect that the surface concentration z_e of the adsorbed enzyme depends on the surface concentration Γ of the substrate.

Equation 2 can be used to estimate the relative variation of z_e. However, since the specific activity a depends on the surface pressure of the monolayer, a comparative study of the adsorption requires that all the ΔS measurements should be carried out under identical conditions, i.e., at the same π, although arbitrarily chosen. Thus, as a, Σ, and S_o are the same for a pair of measurements, equation (2) leads to

$$\frac{(z_e)_2}{(z_e)_1} = \frac{\Delta S_2}{\Delta S_1} \qquad \text{Eq. 4}$$

Once the adsorption was performed at a given pressure, π was brought to the chosen standard value to measure ΔS. Actually, before bringing π to the standard value for measurements, a determination of ΔS was made at the pressure of adsorption. This measurement permitted a comparison of the rates at two different values of π for the same quantity of fixed enzyme, which was the object in the preceding section. Once the measurement was done at the reference pressure, the monolayer could be brought back to the initial pressure and, finding again the value of the initial rate, it was ascertained that the quantity of adsorbed enzyme had not changed. This proves, once more, the segregation of the active enzyme in the surface. It must be stressed that all these measurements have to be carried out while the process of hydrolysis is still linear.

Several series of determinations were made, not only by varying the pressure of adsorption of the enzyme from 6 to 28 dynes/cm (i.e., Γ varying from 2.09 to $2.96 \cdot 10^{-10}$ moles per cm^2) but also by choosing three different standard pressures to measure the rates, i.e., 14, 15, and 21 dynes/cm. The results obtained are quite consistent; on a total of 19 determinations, one single result, definitely inconsistent, was rejected. All rates were expressed as decrease, ΔS, of the area per minute, and considering ΔS as equal to 1.00 for $\pi = 15$ dynes/cm (i.e., $\Gamma = 2.50 \cdot 10^{-10}$ mole/cm^2), the values of ΔS found at 18, 21, 24, 25, and 28 dynes/cm vary at random between 1.00 and 1.03. Below $\pi = 15$ dynes/cm, ΔS decreases regularly: $\Delta S = 0.93$ for $\pi = 14$ ($\Gamma = 2.46 \cdot 10^{-10}$); $\Delta S = 0.70$ for $\pi = 12$ ($\Gamma = 2.37 \cdot 10^{-10}$); $\Delta S = 0.46$ for $\pi = 8$ ($\Gamma = 2.18 \cdot 10^{-10}$); and $\Delta S = 0.30$ for $\pi = 6$ dynes/cm ($\Gamma = 2.09 \cdot 10^{-10}$ mole/cm^2).

Now, according to equation 4, the quantities, z_e, of adsorbed enzyme are proportional to the rates, ΔS. It appears therefore that z_e increases rapidly with the surface pressure up to 15 dynes/cm, where the molecular area of the diglyceride is 66.4 Å^2 ($\Gamma = 2.54 \cdot 10^{-10}$ moles/cm^2) and above this pressure it remains constant. Taking the value of z_e at 15 dynes/cm for unity, the relative values of the enzyme adsorbed, z_e per cm^2, are plotted as a function of the surface concentration Γ of the substrate in **Fig. 9.**

Fig. 8 and Fig. 9 show that both the quantity of adsorbed enzyme z_e and the enzymatic specific activity a vanish below $\Gamma = 1.83 \cdot 10^{-10}$ moles/cm^2, which corresponds to $\pi = 2.3$ dynes/cm. It now becomes clear why, when spreading the substrate at the outset of each experiment, care was taken to keep the surface pressure below 2 dynes/cm. Thus, in the absence of any reaction, it becomes easy to adjust the instruments and start at the desired time.

A detail in the operation, which gives supplementary information, should be mentioned. It was specified that, to determine the change of enzymic activity with Γ, an initial measurement of the rate was made at a certain π and then, either by compressing or expanding the monolayer, a second measurement was made at a higher or lower surface pressure. In general, when the monolayer is brought back to the initial π, the same rate is found as at the first measurement. This reproducibility verifies that the quantity of adsorbed enzyme has not changed as a consequence of the compression or expansion. This is always true when the monolayer has been compressed. But, when it has been expanded, it is verified only when the expansion of the monolayer does not bring the pressure too low. In fact, if, after adsorbing the enzyme at a relatively high π and measuring the corresponding rate of hydrolysis, the monolayer is expanded at a π lower than ~8 dynes/cm and then brought back to the original pressure, a lower value of the rate is found. This is explained by a decrease of the quantity of adsorbed enzyme through desorption at a low π where the equilibrium quantity of adsorbed enzyme is in fact smaller. In view of this, for the comparative determination of the rates at low pressures, the operation was carried out by starting at the lower pressure and then compressing the monolayer to the higher pressure.

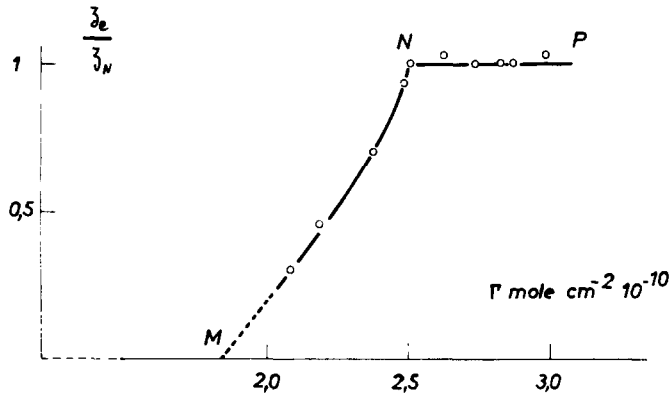

Fig. 9. Variation of the equilibrium surface concentration of the enzyme with the surface concentration of the substrate. The quantities of enzyme per cm^2 are expressed in relative values, z_N being the quantity at the inflection point N.

Consistency of the different elements of the kinetics

We know now that if V varies, it is because both the quantity of adsorbed enzyme z_e and its specific activity a have particular values for each Γ or π of the substrate monolayer.

Indeed, the two series of experiments which were described in the two preceding sections have led successfully to the dissociation of z_e and a and to the determination of their relative values by a way different from that of determining V directly. In the case of V, both the adsorption and the hydrolysis are carried out at the same surface pressure and no information can be derived on the corresponding values of z_e and a. But, as by definition $V = a \cdot z_e$ (see equation 1), by comparing the product of the measured values of a and z_e to the measured values of V, the consistency of the measurements and of the explanations can be tested.

The surface concentrations of the enzyme are known only in relative values z_e/z_N (Fig. 9); such is also the case for the enzymic activity a/a_N (Fig. 8-2). For the comparison, the product $a/a_N \cdot z_e/z_N$ should therefore be multiplied by the constant factor 29.2, which is the value of V at the inflection point for $\Gamma = 2.5$ on Fig. 7. (At the same inflection point in Figs. 8 and 9, a/a_N and z_e/z_N are equal to 1.) This gives the dotted curve in Fig.

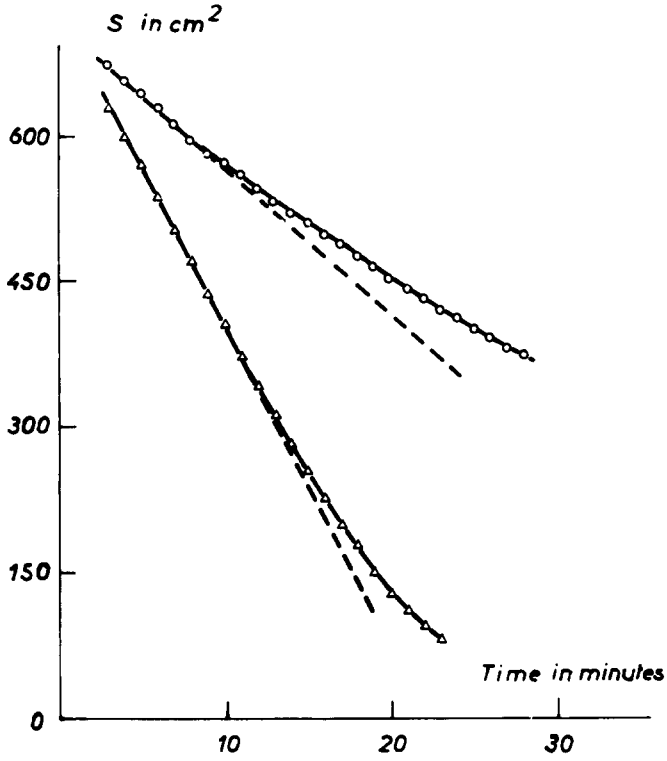

Fig. 10. Variation with time of the surface area of the monolayer in the absence of Ca^{2+} ions for two different surface concentrations of substrate. ○, $\Gamma = 2.32 \cdot 10^{-10}$ and △, $\Gamma = 2.55 \cdot 10^{-10}$ mole/cm². $C = 30$ μg/l. Compare with the curves of Fig. 6.

7, which tallies with the continuous curve representing V. Since V, a, and z_e were obtained by independent experimental means and since $V = a \cdot z_e$ derives merely from the definition of the enzymic specific activity, the coincidence of the two curves in Fig. 7 shows that the three series of measurements and the reasoning are correct.

Influence of the pH and of Ca^{2+} ions

As in ordinary bulk conditions, there is a maximum of lipase activity at pH 8 with monolayers of the substrate. Whether this is due to an increase of the adsorbed enzyme z_e or to an increase of the specific activity a can be ascertained through the following experiment. The trough was divided into two compartments, one where the pH was 5 and the other where it was 8. The enzyme was only in the pH 5 compartment. The monolayer was spread on the whole surface extending on top of both compartments and its pressure was brought to the desired value. After agitating the enzyme solution of the first compartment (pH 5), it was checked that there was no hydrolysis. The part of the monolayer covering this first compartment was then isolated with a barrier and the remnant of the monolayer was eliminated by suction. The whole of the isolated monolayer was then shifted to the compartment at pH 8 which included the surface pressure measuring device. The transfer was done without modifying the area of the monolayer so as to keep its pressure constant. Once at pH 8, the reaction took place; it was ascertained that the pressure fell gradually and that, to prevent it, it was necessary to reduce the area in the course of time. This experiment proves that the enzyme sticks to the monolayer at pH 5, but that the reaction does not take place. It also proves, once more, that the monolayer can be shifted, carrying with it the adsorbed enzyme. Finally, it proves that the pH affects the specific activity a. To determine the influence of pH on the quantity, z_e, of adsorbed enzyme, a systematic study should be made. However our results at two pH values agree with those of Sarda, Marchis-Mouren, and Desnuelle (3) on triglyceride suspensions. These authors showed, by centrifuging the triglyceride after it had been in contact with lipase, that the quantity of attached enzyme was maximum at pH 5 and that the rate of hydrolysis was maximum at pH 8 and zero at pH 5.

It is well known that calcium plays an activating rôle with lipase (2). Some experiments on the influence of Ca^{2+} ions were carried out particularly with a view to explaining the previous results of Olive and Dervichian (4) which were obtained without Ca^{2+} ions, as pointed out in the preceding article (1). The curves of **Fig. 10,** relative to measurements without Ca^{2+} ions, should be compared to the corresponding ones of Fig. 6, relative to measurements with Ca^{2+} ions ($4 \cdot 10^{-4}$ M of $CaCl_2$). In both cases, after the initial linear segment, the curves become perfectly exponential (i.e., $\log S$ versus time gives a straight line). But the reaction rates, deduced from the slope of the linear segment, differ considerably. If, for instance, the curves relative to $\Gamma = 2.32 \cdot 10^{-10}$ mole/cm² are compared, $-dS/dt = 48$ cm²/min with Ca^{2+} ions, and $-dS/dt = 14$ cm²/min without. On the other hand, the area reduction S_o/S_m after which the exponential process commences also changes considerably: $S_o/S_m = 2.4$ with Ca^{2+} ions, and $S_o/S_m = 1.15$ without.

DISCUSSION AND CONCLUSION

We have pointed out (1) that an apparent contradiction may be found in the facts concerning the persistence at the surface of the total quantity Z_t of adsorbed enzyme. If $z_e = Z_t/S_o$ is the surface concentration corresponding to the adsorption equilibrium, how can we explain that the enzyme can remain at the surface at higher concentrations, reaching a value $z_m = Z_t/S_m$ which may be five times greater, without

desorption occurring? We suggest that after their segregation in the surface monolayer, the total z_t of the enzyme molecules adsorbed per cm² become distributed at each moment into those, z^*, which are associated with the substrate and those, z, which remain free, so that $z_t = z^* + z$. Only the concentration z of the free molecules (which is necessarily lower than $z_t = Z_t/S$) needs to be considered in the thermodynamic equilibrium with the underlying solution. On the other hand, only those molecules that are substrate associated (z^*) govern the rate of hydrolysis.

If the stirring of the underlying solution was maintained permanently, the total surface *concentration*, z_t, of the enzyme would remain constant. Consequently, the total *quantity* of enzyme in the monolayer, $Z_t = z_t \cdot S$, would decrease proportionally to the area S (i.e., proportionally to the quantity m of substrate). Since $-dm/dt = a \cdot Z_t$ and $dS/dt = \Sigma \cdot dm/dt$, the area S must decrease exponentially with time, i.e., log S plotted versus time must give a straight line. This explains (1) the results of those investigators who have used a continuous magnetic stirring (5–6). In ordinary conditions of lipase titration, where oil emulsions are used, the mixture with the enzyme solution has to be shaken continuously. With the monolayer technique, the substrate is immobile on the surface, while the sheet of the enzyme solution, in contact with it, is continuously renewed by agitation. Practical information is thus obtained in both cases. But, in addition to the fact that the rate of hydrolysis increases with the bulk concentration C of the enzyme, the monolayer technique shows that it also varies with the surface concentration Γ of the substrate.

The limited stirring used in the present work enabled us to show that dm/dt depends on the total quantity of enzyme segregated on the surface and not on the quantity of substrate. However, the mere measurement of the rate of hydrolysis as a function of Γ or C was not enough if more information was desired on the mechanisms of lipase action. The present work amounts to the determination of the several different factors governing both the variation of the adsorption of the enzyme and the variation of enzymic specific activity. It was shown that, at a given surface pressure, the value of a is independent of the magnitude of z_e, as could be expected. This led to the conclusion that the rate of hydrolysis V is strictly proportional to the quantity of adsorbed enzyme z_e, and therefore that V can be taken as a measure of z_e.

That z_e depends on Γ may appear as self-evident, as it is to be expected that the affinity of the adsorbate (the enzyme) for the adsorbent (the substrate) must vary with the nature of the structure of the adsorbent. Much more important is the fact that a varies with Γ. Note that all determinations were carried out on a definite system of enzyme and substrate segregated on the surface. Then it should be stressed that a measures the activity only of those enzyme molecules that belong to the surface population. Finally, as these enzyme molecules are embedded in the substrate itself, it is to be expected that modifications in the conformation of the hydrocarbon chains, due to the change in the molecular area of the lipid, induce changes in the conformation of the enzyme molecules and consequently modify their reactivity. In kinetic terms, what is modified is the distribution equilibrium between substrate associated and nonassociated enzyme molecules of the surface population. In fact, any factor that can influence this equilibrium must modify both the slope of the linear portion of the $S = f(t)$ curve as well as its relative length, i.e., the value of S_o/S_m. This appears strikingly with the action of Ca^{2+} ions. The increase of the reaction rate by the Ca^{2+} ions can be due to an increase of the adsorbed enzyme, to an increase of the specific activity, or to both. But the simultaneous change of the length of the linear process shows that there is an influence on the equilibrium between the associated and nonassociated enzyme molecules in the monolayer.

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REFERENCES

1. Dervichian, D. G., and J. P. Barque. 1979. Enzyme–substrate interaction in lipid monolayers. I. Experimental conditions and fundamental kinetics. *J. Lipid Res.* **20:** 437–446.
2. Benzonana, G., and P. Desnuelle. 1968. Action of some effectors on the hydrolysis of long-chain triglycerides by pancreatic lipase. *Biochim. Biophys. Acta.* **164:** 47–58.
3. Sarda, L., G. Marchis-Mouren, and P. Desnuelle. 1957. Sur les interactions de la lipase pancréatique avec les triglycérides. *Biochim. Biophys. Acta:* **24:** 425–427.
4. Olive, J., and D. G. Dervichian. 1971. Cinétique de l'hydrolyse par une lipase d'un glycéride étalé en couche monomoléculaire. *Biochimie.* **53:** 207–213.
5. Zografi, G., R. Verger, and G. H. de Haas. 1971. Kinetic analysis of the hydrolysis of lecithin monolayers by phospholipase A. *Chem. Phys. Lipids.* **7:** 185–206.
6. Verger, R., M. C. E. Mieras, and G. H. de Haas. 1973. Action of phospholipase A at interfaces. *J. Biol. Chem.* **248:** 4023–4034.
7. Verger, R., and G. H. de Haas. 1973. A new technique to study enzyme reactions in monolayers. *Chem. Phys. Lipids.* **10:** 127–136.