# Enzyme–substrate interaction in lipid monolayers. III. A study of the variation of the surface concentration with lipolysis

**J. P. Barque and D. G. Dervichian**

Department of Biophysics, Institut Pasteur, Paris 75015, and Department of Biophysics, University of Paris VI, Paris 75005, France

**Abstract** Monolayers of a diacylglycerol were submitted to the action of lipase, keeping the area constant. The variation of the surface concentration $\Gamma$ of the substrate with time was derived from the recorded reduction of the surface pressure $\pi$ (the isotherm of the monolayer being previously established). The rate $-d\Gamma/dt$ was determined both as a function of the surface concentration $\Gamma$ of the substrate and as a function of the bulk concentration $C$ of the enzyme in the underlying solution. The rate depends on the quantity of enzyme $z_e$ adsorbed on the monolayer and on the enzymatic specific activity $a$ of these adsorbed enzyme molecules. Both $z_e$ and $a$ vary with $\Gamma$. The two variations have been quantitatively dissociated. The curves of $z_e$ and of $a$ as functions of $\Gamma$ coincide with those previously established in the study of hydrolysis under constant surface pressure.—**Barque, J. P., and D. G. Dervichian.** Enzyme–substrate interaction in lipid monolayers. III. A study of the variation of the surface concentration with lipolysis. *J. Lipid Res.* 1979. **20:** 599–606.

**Supplementary key words** pancreatic lipase · adsorption of lipase · activity of lipase

In the work reported in preceding articles (1, 2), the monolayer of substrate (1,3-didecanoylglycerol), spread on the surface of water and submitted to the action of lipase, was maintained at constant surface concentration $\Gamma$ (i.e., at constant surface pressure $\pi$). Consequently, as the substrate gradually vanished because of the hydrolysis, the area $S$ of the monolayer had to be reduced with time in order to keep the surface concentration $\Gamma$ constant.

A suitable, rapid, and *limited* stirring was carried out at the outset, which results in the monolayer having the surface *concentration* $z_e$ of enzyme corresponding to the adsorption equilibrium with the underlying solution. With this procedure, once the *agitation is stopped*, substrate and adsorbed enzyme form together a segregated system on which the reaction kinetics can be studied experimentally with the fewest assumptions.

The *total quantity* $Z_t$ of enzyme present on the surface depends evidently on the initial area $S_o$ of the monolayer on which it was adsorbed, since $Z_t = z_e \times S_o$. As the quantity of substrate hydrolyzed per minute, $-dm/dt$, depends on this total quantity $Z_t$, and not on the surface concentration $z_t$ of enzyme at a given moment, a normalized definition of the rate $V$ of hydrolysis (i.e., independent of $S_o$) was given under the form:

$$V = -1/S_0 \times dm/dt \qquad \text{Eq. 1}$$

In the present investigation, contrary to the constant $\pi$ method, the area $S$ of the monolayer is kept constant. Therefore, because of the hydrolysis, the surface concentration $\Gamma$ (and consequently the surface pressure $\pi$) decreases gradually in the course of time.

When hydrolysis is measured under constant surface pressure, $d\Gamma/dt = 0$ since $\Gamma$ is kept constant. In the present procedure at constant area, $d\Gamma/dt$ varies and its value at every moment is derived from the measure of $d\pi/dt$, since the relation between $\pi$ and $\Gamma$ is known with precision through the established $\pi = f(\Gamma)$ isotherm of the substrate monolayer. As $\Gamma = m/S$ (where $m$ is the total quantity of substrate), $d\Gamma/dt = 1/S \times dm/dt$. Now, $S$ is kept constant and equal to the initial area $S_o$ on which is initially adsorbed the quantity $Z_t$ of enzyme.
Consequently, as $S = S_o$,

$$d\Gamma/dt = 1/S_o \times \mathrm{dm/dt} \qquad \text{Eq. 2}$$

Comparing equations 1 and 2, it appears that

$$-d\Gamma/dt = V \qquad \text{Eq. 3}$$

i.e., the rate $-d\Gamma/dt$, defined at constant area in the present investigation, is numerically equal to the normalized rate, defined when using the constant pressure method (1). This equality, Eq. 3, enables the direct comparison of the results of the two methods.

There is here a close analogy with enzyme reac-

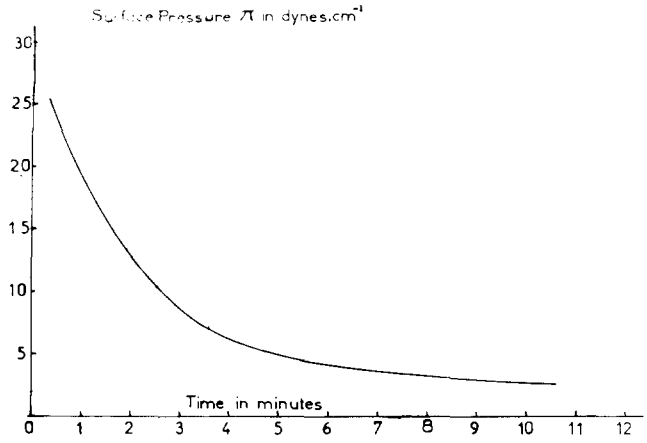


**Fig. 1.** Fall of the surface pressure $\pi$ with time, under constant area, due to hydrolysis of the monolayer. (Reduced reproduction of a continuous recorded curve.)

tions under classical conditions where the rate, expressed as $-dc/dt$, is determined as a function of the substrate concentration $c$ in a solution. In the case of a monolayer, the rate, expressed as $-d\Gamma/dt$, is determined as a function of the surface concentration $\Gamma$ of the substrate. In both cases, the quantity of involved enzyme is fixed. Yet, there is a fundamental difference: here, the reaction does not proceed in a common solvent (water), since the reacting enzyme is in, or adsorbed to, the monolayer, i.e., "dissolved" in the substrate itself.

Different authors have already made measurements at constant area, either with lipase acting on glycerides (3) or with phospholipase acting on lecithins (4, 5). Important in the present work appears to be the quantitative consistency of the results obtained with constant area and constant pressure methods. Using this latter procedure, it was demonstrated (2) that the enzymatic specific activity $a$ depends on the value of $\Gamma$. Thus, if the experiment is carried out at different surface concentrations $\Gamma$ of the substrate, not only $z_e$ and consequently $Z_t$ may be different, but also $a$ is different. With the constant area procedure utilized in this article, as $\Gamma$ varies in a continuous way, $a$ also varies continuously. It is noteworthy that the discrete values of $a$ found with the constant surface pressure method (2) coincide perfectly with the continuously determined variation of $a$ with $\Gamma$ obtained here. The same variation of $z_e$ with $C$ and $\Gamma$ was also obtained. Finally, desorption of the enzyme occurs here, not for a certain reduction of the area, since the area is kept constant, but when the surface concentration $\Gamma$ of the substrate falls below a certain value at which the free enzyme surface concentration $z$ exceeds the corresponding equilibrium concentration $z_e$. Again there is agreement between the two methods, as the beginning of desorption with the reaction at constant area is disclosed at the very point where it can be predicted from the results obtained with the constant pressure method.

## EXPERIMENTAL CONDITIONS AND PROCEDURE

The decrease of the surface concentration $\Gamma$ in the substrate is revealed by a decrease of the surface pressure $\pi$. The study of the hydrolysis of the monolayer at constant area thus imposes the recording of the variation of $\pi$ as a function of time. Now, the commonly used apparatus (1) is a special Langmuir trough recording the variation of $\pi$ as a function of the surface area. The recording device was therefore so modified that the recording paper moves at uniform speed with time instead of the area. In fact every minute corresponded to 1.4 cm on the paper.

Experiments can thus be performed, either by fixing at the start the enzyme on the substrate under definite different surface pressures and following the progress of lipolysis, or by fixing the enzyme always at the same surface pressure, then bringing the monolayer to definite, different initial surface pressures and following the reaction from there onward.

As the trough is divided into several compartments (see (1)), the substrate is deposited on the surface of the first compartment, which is devoid of enzyme, and is left to spread on the whole of the trough. In the course of this spreading, the surface pressure of the monolayer should never exceed 2 dynes/cm. This is because it was demonstrated in a preceding report (1), and will be confirmed here again, that at $\pi = 2$ dynes/cm there is yet no enzyme fixation to the monolayer. Thus in the absence of any reaction, it becomes easy to adjust the instruments and start at the desired time. The quantity of deposited substrate is approximately such that, by compressing the monolayer to the desired pressure, it exactly covers the compartment containing the enzyme. The final adjustment is rapidly made by taking away by suction any excess quantity. From this moment, the piston barrier compressing the monolayer is fixed in position so that the area is defined.

The underlying enzyme solution is then agitated; in all cases, five back and forth runs were carried out (1). As soon as the stirring comes to a stop, the recording paper is set in motion. According to the conditions, the automatic recording of the gradual decrease of $\pi$ lasts from 10 to 20 min. **Fig. 1** reproduces one of these recorded curves.

Using the $\pi = f(\Gamma)$ isotherm of the substrate monolayer (see (2)), the recorded curve can now easily undergo a change of the variable so as to represent the variation of $\Gamma$ as a function of time, $\Gamma = f(t)$. The determination of the slope on the $\Gamma = f(t)$ curve, at every point desired, gives directly the value of $d\Gamma/dt$ for each value of $\Gamma$. In practice, it is unnecessary to draw a new curve for $\Gamma = f(t)$. It is enough to translate $\pi$'s into $\Gamma$'s directly on the $\pi = f(t)$ curve for every minute or every 30 seconds and to deduce immediately the values of $\Delta\Gamma$.

## RESULTS

### Influence on the kinetics of the initial surface concentration of substrate

Questions arise when using the present method. First, does the initial surface concentration $\Gamma$ (or the initial $\pi$) have an effect on the subsequent process of the kinetics? Second, is there an influence of the surface pressure of the monolayer at which the underlying solution is agitated to fix the enzyme on the surface? Two types of experiments were carried out in order to answer to these questions.

*Adsorbing the enzyme at the same determined substrate surface concentration and starting the lipolysis process from different surface concentrations.* In a first series of experiments, after the enzyme had been adsorbed at a certain pressure, e.g., $\pi$ = 21 dynes/cm (i.e., $\Gamma = 2.69 \times 10^{-10}$ mol/cm$^2$ of substrate), the monolayer was compressed so as to bring it at a higher $\pi$ (i.e., a higher $\Gamma$), e.g., 24, 28, or 30 dynes/cm. The reaction was then followed starting from this new pressure. In each case, the rates of lipolysis were determined for the different values of $\Gamma$ through which the substrate monolayer gradually proceeds, using the method described above. In all cases, the rates (**Fig. 2**) at the same surface concentration $\Gamma$ were identical and equal to the rate found when hydrolysis was started at the very pressure under which the enzyme was adsorbed, namely 21 dynes/cm.

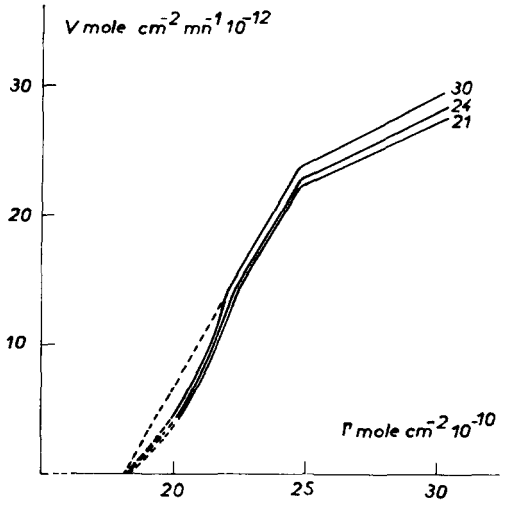


**Fig. 2.** Continuous variation of the rate $V$ with the continuous variation of $\Gamma$. The initial adsorption of the enzyme was accomplished in the three cases at the same surface pressure of 21 dynes/cm. The hydrolysis was started respectively at 21, 24, or 30 dynes/cm, after bringing the monolayer to these respective surface pressures. Bulk concentration of the enzyme in all cases: $C$ = 20 $\mu$g/l.

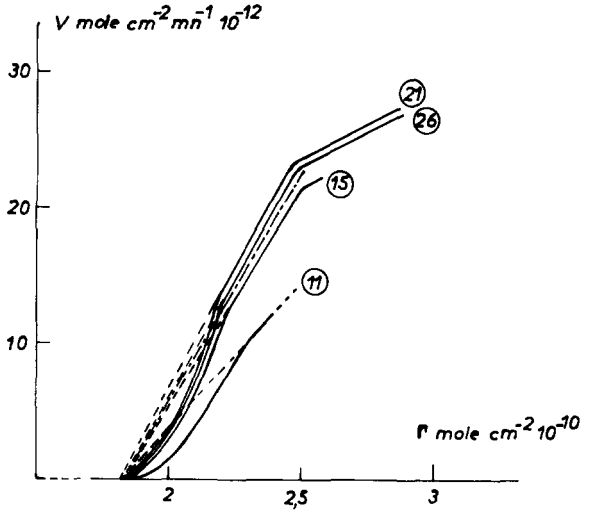


**Fig. 3.** Continuous variation of the rate $V$ with the continuous variation of $\Gamma$. Here, the pressure at which the enzyme was adsorbed and the pressure at which the hydrolysis was started were the same, namely 11, 15, 21, and 26 dynes/cm. ($C$ = 20 $\mu$g/l. in all cases).

Because of the compression necessary to bring the monolayer to a pressure above 21 dynes/cm, the area $S_o$ of the monolayer, which is kept constant in the course of hydrolysis, is slightly smaller. This affects the measure of $d\Gamma/dt$, since $d\Gamma/dt = 1/S_o \times dm/dt$. Yet, if the values found for $d\Gamma/dt$ are reduced to the same $S_o$ by correcting for the slight reduction of area, all the curves of Fig. 2 coincide. Thus the fact is confirmed that the rate $V$ depends only on the surface pressure (i.e., on the surface concentration $\Gamma$) of the monolayer at the time of adsorption. Consequently, for the reported experiments, $V$ is independent of the surface pressure at which hydrolysis commences.

*Influence of the adsorption of the enzyme at different substrate surface concentrations.* In a second series of experiments, the pressure at which the enzyme is adsorbed was varied and the change of the rate measured afterwards during the fall of $\Gamma$ produced by the hydrolysis. This is shown on **Fig. 3.**

When the adsorption was achieved at 11 dynes/cm, the rate found afterward at a certain surface pres-

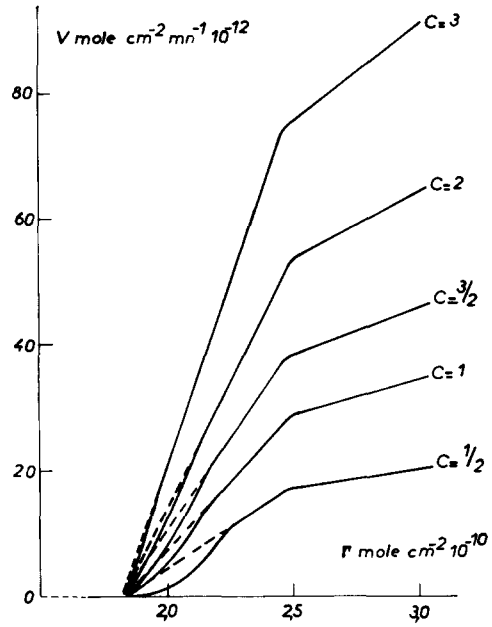


**Fig. 4.** Variation of $V$ with $\Gamma$ with different bulk concentrations $C$ of the enzyme. $C = 1$ corresponds to 30 $\mu$g/l. For all values of $C$, the initial adsorption of the enzyme and the start of hydrolysis were accomplished in identical conditions.

sure was lower than it was when the adsorption was achieved at, e.g., 21 dynes/cm. Following what was already said, one can conclude that the quantity of enzyme adsorbed at 11 dynes/cm is less than what is adsorbed at 21 dynes/cm. On the other hand it appears that the rates corresponding to the different pressures are nearly the same, within experimental errors, when the pressure of adsorption is equal to or greater than 15 dynes/cm. It is therefore concluded that the quantity of adsorbed enzyme is independent of $\pi$ when this is equal to 15 dynes/cm or more.

*Significance of the resulting curves.* These results corroborate the conclusions of the study of hydrolysis under constant surface pressure (1, 2). The agreement is not only qualitative, but also quantitative. Thus the determination of the variation of $z_e$ with $\pi$ by the previous method showed that at 11 dynes/cm the quantity of adsorbed enzyme is 0.66 of the amount adsorbed above 15 dynes/cm. In the present study the ratio between the slope of the linear part of the curve at 11 dynes (Fig. 3) to the average slope of the curves corresponding to $\pi = 15$ dynes/cm or more is 21.5/33.25 = 0.65.

The curves of Fig. 2 are similar to the one showing the variation of the enzymatic specific activity $a$ as a function of $\Gamma$ (see Fig. 8 in the second article of this series (2)). Similar to it, they cut the horizontal axis at about $\Gamma = 1.8 \times 10^{-10}$ mole/cm². Since $V = a \times z_t$, this similarity is consistent with the idea that $z_t$ is fixed once and for all at the beginning of the experiment and that it remains invariant, at least in a large range of variation of $\pi$, without either desorption of the enzyme or new adsorption occurring. A more direct proof will be given in the next section. But a desorption does in fact occur after a certain point. In the case of hydrolysis at constant $\pi$, this occurs when, because of the automatic reduction of the area, the surface concentration $z$ of the non-associated enzyme reaches the value $z_e$ of the equilibrium adsorption concentration. At constant $S$, we have to consider, on the one hand, the partition $z_t = z^* + z$ of the total adsorbed enzyme per cm² into enzyme associated to the substrate and free enzyme and, on the other hand, the variation of the equilibrium concentration $z_e$ of the adsorbed enzyme as a function of the surface concentration $\Gamma$ of the substrate: $z_e = f(\Gamma)$. Here, desorption begins when, because of the hydrolysis itself, the monolayer reaches a surface concentration $\Gamma$ at which the free enzyme surface concentration $z$ is equal to the equilibrium adsorption concentration corresponding to that $\Gamma$, i.e., when $z = z_e$. This must occur as $\Gamma$ falls below $2.3 \times 10^{-10}$ mol/cm², since the curves Figs. 2, 3, and 4 depart from linearity for about $\Gamma = 2.2 \times 10^{-10}$.

### Influence on the kinetics of the bulk concentration $C$ of the enzyme

In all the foregoing, the enzyme concentration $C$ in the underlying solution was always the same. In the following series of experiments the effect of the variation of $C$ was systematically examined (**Fig. 4**). The same surface pressure was adopted both for the fixation of the enzyme and for the starting point of hydrolysis.

Since $z_t$ increases with $C$ and since $V = a \times z_t$, each of the curves of Fig. 4 must derive from the same curve $a/a_N = f(\Gamma)$ (see Fig. 8 in (2)) multiplied by a different $z_t$. Consequently these different curves have a geometrical affinity. In fact, on one hand, their lower parts converge all to the same point $\Gamma = 1.83 \times 10^{-10}$ of the horizontal axis and, on the other hand, the ratio of the slopes of their two linear parts is the same for all of them, namely 3.8 ± 0.1. Besides, an important fact became apparent: the point where all the upper linear parts of these curves converge on the horizontal axis is $\Gamma = 0$. This means that, above $\Gamma = 2.50 \times 10^{-10}$, $V$ as well as $a$ are simply proportional to $\Gamma$. This fact had not been noticed when the $a/a_N = f(\Gamma)$ curve had been plotted in the second article of this series (2).

From the curves of Fig. 4, it is possible to calculate the variation of the surface concentration $z_e$ of the adsorbed enzyme as a function of the bulk concentration $C$. It is true that $z_e$ depends both on $C$ and

Γ. But it was demonstrated in the preceding article (2) and in the present work that, for a given $C$, $z_e$ remains the same for surface concentrations Γ of the substrate above $2.50 \times 10^{-10}$ (i.e., $\pi$ above 15 dynes/cm). Now, in all cases represented by the curves of Fig. 4, the adsorption was carried out well above 15 dynes, namely at $\pi = 30$ dynes/cm.

Some preliminary remarks have to be made. Each of the curves of Fig. 4 gives the variation of $V$ with $C$ for a quantity $z_e$ of enzyme adsorbed per cm² at the start. In fact, it is the quantity $z_e$ that is a function of $C$. Once adsorbed, it remains constant while the substrate concentration Γ varies. The adsorbed enzyme starts decreasing by desorption only when Γ falls below $2.3 \times 10^{-10}$. The extrapolated dotted straight line shows what the variation of $V$ would be if desorption did not occur. This extrapolated part is in fact born out by the study at constant $\pi$ (see (2)).

It was shown in (2) that the specific enzymatic activity $a$ depended only on Γ. As the curves of Fig. 4 have a geometrical affinity, this proves once more that $a$ depends exclusively on Γ and not on $C$. It is evident that whatever the relation $z_e = f(C)$ may be, the fact that $a$ is only dependent on Γ (and not on $C$) leads to geometrical affinity of the group of curves of Fig. 4. To find the relation $z_e = f(C)$, it is enough to examine, for a given value of Γ (that is on a vertical line cutting the different curves), how $V$ varies with $C$. In fact a fixed value of $a$ corresponds to a fixed value of Γ. It is thus ascertained that, for each value of Γ, $V$ varies proportionally to $C$ (**Fig. 5**). The conclusion is that $z_e$ is also proportional to $C$, as $V = a \times z_e$. Since the curves of Fig. 4 are made of two straight parts, the same conclusion can be deduced as well from the fact that the slopes of these straight lines increase proportionally to $C$.

In the preceding work (2), the values of $a$ could not be measured in an absolute way, because $z_t$ was unknown. The variation of $a$ could therefore be measured only in relative values $a/a_N$. Here, inversely, it is impossible to calculate the absolute values of $z_e$ when $C$ varies, because $a$ is known only in arbitrary units. Nevertheless, the linear form of the variation of $z_e$ with $C$ can be deduced.

### Comparison of the rates measured by the two methods and calculation of $z_e = f(\Gamma)$

The rate measured by the constant $\pi$ method will be designated by $V$ and the rate measured by the constant $S$ method by $V'$.

With the constant $\pi$ method, most of the systematic measurements were carried out with a bulk enzyme concentration of $C = 30$ μg/l. $V$ as a function of Γ is represented as curve II on **Fig. 6.** For the comparison to be valid, the curve giving the variation of $V'$ at constant area to be considered must also correspond to $C = 30$ μg/l. This is the curve marked $C = 1$ on Fig. 4 and has been redrawn as curve I on Fig. 6.

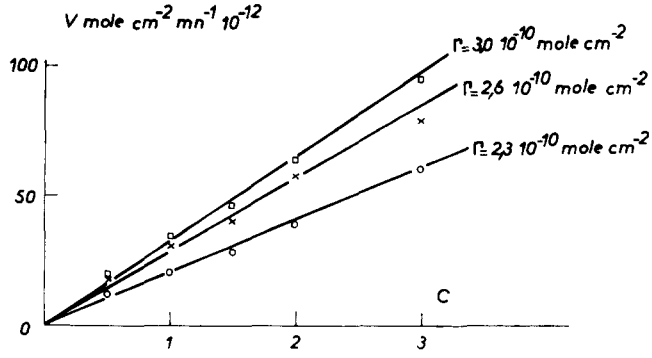


**Fig. 5.** Variation of the rate $V$ as a function of the bulk concentration of the enzyme for a given Γ. These curves are derived from the curves of Fig. 4 for values of Γ equal to 2.3, 2.6, and $3.0 \times 10^{-10}$.

It should be stressed that, with the first procedure, at the start of each measurement, the enzyme was adsorbed on the monolayer at the very surface concentration Γ (i.e., the same $\pi$) at which $V$ was determined afterwards. Consequently, the quantity of enzyme thus adsorbed per cm² varied with Γ (or $\pi$), at least for $\Gamma < 2.50 \times 10^{-10}$ (i.e., $\pi < 15$ dynes/cm). On the contrary, with the constant area method, the quantity of adsorbed enzyme $z_e$ is the same for all the values of the surface concentration Γ through which the substrate proceeds, since the adsorption is accomplished once and for all at the starting pressure.

It appears that curves I and II of Fig. 6 coincide

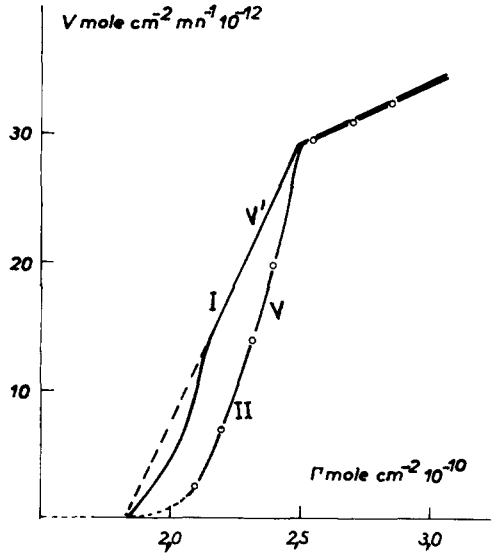


**Fig. 6.** Comparison of the rates measured (I) at constant area and (II) at constant pressure. (C = 30 μg/l).

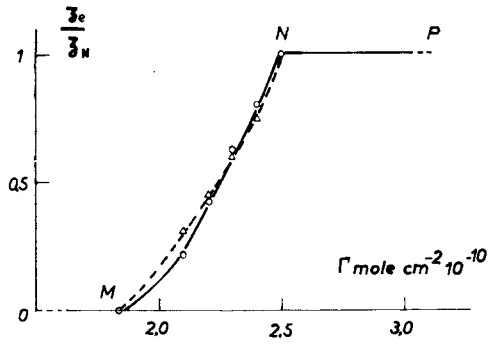


**Fig. 7.** Variation of the surface concentration $z_e$ of adsorbed enzyme as a function of Γ, expressed in relative values, the value at the point $N$, $z_N$, being taken as unity. ○, Constant area method; △, constant pressure method.

for values of Γ above $2.50 \times 10^{-10}$ (i.e., for $\pi > 15$ dynes/cm). This is easily explained, because, as shown in the present work and in the preceding one (2), above $\Gamma = 2.50 \times 10^{-10}$, the quantity of enzyme adsorbed, $z_e$ per cm², is the same whatever the value of Γ. Therefore, since in all cases $V = az_e$, the rate $V$ or $V'$ can only depend on the value of $a$ which depends only on Γ.

For values of Γ below $2.50 \times 10^{-10}$, the two curves I and II (Fig. 6) diverge. Use can be made of this divergence to calculate in a new way the relative variation of $z_e$ with Γ. Indeed the lower curve II shows the rate $V = a \times z_t = a \times z_e$ corresponding to the quantity of enzyme $z_t = z_e$ which is adsorbed at every surface concentration Γ of the substrate. Whereas, the upper curve I gives the rates $V' = a \times z_N$, corresponding to the quantity of enzyme $z_N$ which remains constant while Γ varies. This constant and maximum quantity $z_N$ is the surface concentration of enzyme which is adsorbed for values of Γ equal to $2.50 \times 10^{-10}$ (point $N$) or above it. Thus, as $a$ depends only on Γ, the ratio $V/V'$ gives immediately, for every value of Γ, the relative quantity $z_e/z_N$ of adsorbed enzyme. This relative quantity varies necessarily from 0 to 1 when Γ increases from 1.83 to $2.50 \times 10^{-10}$ and remains constant thereafter as shown in **Fig. 7.** The dotted curve on the same figure reproduces the values found by a quite different way described in the preceding article (2).

## DISCUSSION AND CONCLUSIONS

All of the results obtained by examining the hydrolysis of the substrate monolayer kept at constant area corroborate quantitatively those obtained by the constant surface pressure method.

It was confirmed that, for a given concentration $C$ of the enzyme solution, the quantity per cm² of adsorbed enzyme depends strongly on the surface concentration Γ of the substrate. Adsorption takes place only when Γ is higher than $1.83 \times 10^{-10}$ mol/cm². From $\Gamma = 1.83$ to $2.50 \times 10^{-10}$ mol/cm², the adsorbed quantity increases with Γ. Above 2.50 and up to the collapsing surface concentration of the monolayer, i.e., $\Gamma = 3.16 \times 10^{-10}$, the adsorbed quantity remains constant.

It was ascertained that all the curves showing the variation of the rate of hydrolysis as a function of Γ or $\pi$ are identical when the initial adsorption of the enzyme is accomplished at any value of $\pi$ above 15 dynes/cm (i.e., at any value of Γ above $2.50 \times 10^{-10}$ mol/cm²). On the other hand, when the adsorption is accomplished for instance at 11 dynes/cm ($\Gamma = 2.33 \times 10^{-10}$), the rates are distinctly lower. In this particular case, the rate measured, e.g., at 9 dynes/cm, is only $10 \times 10^{-12}$ instead of $15 \times 10^{-12}$. This difference is exactly the same as that found when using the constant $\pi$ method.

Both the variation of the adsorbed quantity of enzyme $z_e$ and the variation of the specific enzymic activity $a$ with Γ agree quantitatively when measured by the two different methods. With the constant $\pi$ method, the variation of $z_e$ and that of $a$ as a function of Γ had been determined, for $z_e$, by adsorbing the enzyme at different surface pressures and measuring its activity, always at the same standard pressure, and, for $a$, by adsorbing in all cases the same quantity of enzyme and measuring its activity at different surface pressures. With the constant area method, as the hydrolysis is left to proceed by starting from a certain high surface pressure at which the enzyme is adsorbed, the variation of the rate could be followed in a continuous way in terms of the surface pressure which decreases progressively. It was thus possible to establish directly the variation curve of $a$ in terms of Γ and to find out that it coincides with the curve found by the other method.

Measurements at constant area have led again to the conclusion that the quantity of enzyme that acts in the kinetics of the reaction is only the enzyme adsorbed at the start. We stress that the lipase is not only saturated by its substrate, but that, at the molecular scale, it is embedded in a very great excess of substrate. These are conditions quite different from those which prevail in ordinary enzymic reactions, even when the substrate concentration is such that the rate reaches its maximum value.

The hydrolysis of a monolayer corroborates the facts discovered by Benzonana and Desnuelle (6) with triglycerides dispersed as an emulsion in a lipase solution. They showed, not only that the enzyme is fixed at the surface of the substrate droplets, but

that it desorbs as soon as the enzyme concentration in the aqueous phase is decreased by dilution. Thus, adsorption increases with the enzyme concentration. But, when the reaction takes place on an emulsion, the surface concentration of the substrate molecules at the surface of the droplets is constant and cannot be modified. Since, with a monolayer, the surface concentration $\Gamma$ of the substrate can be controlled, it was possible to show that the adsorbed quantity of enzyme depends on $\Gamma$. Besides, the variation of the enzymatic specific activity $a$ in terms of $\Gamma$ could be distinguished from the variation of the quantity of adsorbed enzyme.

In the present study at constant area, it appeared again that it is not the total surface concentration $z_t$ of the enzyme which is in equilibrium with the bulk concentration $C$ in the water, but the surface concentration $z$ of the free enzyme only.

The constant area procedure confirms the fact that the enzyme is inactive below a definite surface concentration of the substrate, namely $\Gamma = 1.83 \times 10^{-10}$ mol/cm². To this corresponds a surface pressure of 2.3 dynes/cm. Discussing their own results and those of other authors, Esposito, Séméniva, and Desnuelle (7) had pointed out the absence of activity of lipase at low surface pressures. The new fact that now emerges is that, not only the specific activity is null below 2 dynes/cm, but also the adsorption of the enzyme does not take place. Some authors have tried to attribute this variation of the rate of hydrolysis with $\pi$ to some action of the surface energy. But, what we know of the properties of monolayers permits us to assert that the change in activity as well as the change in adsorption of the enzyme at the interface are in relation to the structure of lipid monolayers.

A last remark should be made on the matter of the reaction order in the hydrolysis of a monolayer. First of all, the definition of the rate itself should be considered. In the measurements at constant area carried out by Garner and Smith (3), the rate was empirically defined as the decrease $-d\pi/dt$ of the surface pressure per unit of time in the neighborhood of the collapsing pressure of the monolayer. This was based on the fact that, in that region, the surface concentration of the monolayer varies nearly linearly with $\pi$. They had ascertained that the rate (thus defined) was proportional to the bulk concentration $C$ of the enzyme in the underlying solution. In fact, we know now that the quantity of adsorbed enzyme $z_e$ is proportional to $C$ and that the enzymatic activity $a$ is invariable at a given surface pressure. Hence $V = a \times z_t = a \times z_e$ is indeed proportional to $C$ at every surface pressure.

If, instead of the simple variation of the surface concentration $\Gamma$, the variation of log $\Gamma$ is plotted as a function of time, starting for instance from $\pi = 10$ dynes/cm, a straight line may be obtained in certain conditions. This was obtained by Lagocki et al. (4) with lipase acting at constant area on di- and tri-octanoylglycerol when following the reaction below 6 dynes/cm. Since $d\log \Gamma/dt$ is constant, it follows that $d\Gamma/dt = k \times \Gamma$ and it could be concluded that the reaction is of the first order. This was the conclusion of Lagocki et al (4). In fact, we know now that, at relatively low surface pressures, the quantity of enzyme $Z_t$ involved in the reaction decreases by desorption, due to the decrease of $\Gamma$ itself. The bulk concentration $C$ of the enzyme in the aqueous solution is indeed constant, but the quantity $Z_t$ of enzyme "dissolved" in the monolayer varies, and it is $Z_t$ that is exclusively involved in the reaction equilibrium.

With regard to the order of the reaction, it is not the concentration $C$ of the enzyme in the underlying solution which has to be taken into consideration, but at most the surface concentration $z_t$ of the enzyme. But, as the proof was given, when the reaction proceeds at *constant surface pressure*, it is not $z_t$ which comes into play, but the total *quantity* $Z_t$ of adsorbed enzyme. When the reaction proceeds at *constant area*, the concentration $z_t$ remains constant, but this is so until the surface concentration $\Gamma$ of the substrate reaches a certain value. In fact, there are two processes in the kinetics, one below a substrate surface concentration of $2.50 \times 10^{-10}$ mol/cm², the other above it. For the higher values of $\Gamma$, $d\Gamma/dt$ varies proportionally to $\Gamma$. From this, one may conclude that the reaction is of the first order. But it should be pointed out that, according to classical enzymology, at high concentrations of the substrate (saturation) the reaction should be of zero order. All these remarks would tend to show that a discussion on the reaction order is trivial. In fact, the most important matter is the direct influence of the molecular structure of the substrate monolayer (in relation to $\Gamma$) on *1*) the substrate–enzyme interaction, and *2*) the specific activity of the enzyme. The two effects have now been dissociated quantitatively.

The authors gratefully acknowledge the technical assistance of G. Calmette.

*Manuscript received 23 May 1978; accepted 14 November 1978.*

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