

KINETIC ANALYSIS OF THE HYDROLYSIS OF LECITHIN MONOLAYERS BY PHOSPHOLIPASE A

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Enzymic hydrolysis by pancreatic phospholipase A (E.C. 3.1.1.4) of L-dioctanoyl-, L-didecanoyl- and L-didodecanoyllecithin monolayers was studied under constant surface pressure by measuring the amount of substrate which disappears per unit area per unit time. The reaction is first-order with respect to the total number of substrate molecules allowing the determination of a rate constant. Apparent limitations of the monolayer techniques are often caused by diffusion problems. Experimental conditions are discussed to detect and control these difficulties.

pH dependence and calcium requirements of the enzymatic reaction are similar under monolayer and bulk conditions. For all three substrates plots of velocity vs. surface pressure show bell-shaped curves with a similar maximum rate at a surface pressure of about 8 dynes/cm. This result is discussed in relation to conformational changes in the lecithin molecules. With the monolayer techniques one can determine only a minimal specific activity, because of the unknown amount of enzyme involved in the catalysis. This minimal specific activity is compared with the value obtained with lecithin micelles as substrate in similar bulk conditions.

Inhibition of the enzyme by competitive inhibitors present in mixed films cannot be studied by the monolayer technique. Comparison of the monolayer and bulk methods showed that both techniques are complementary.

I. Introduction

Lipids which are hydrolyzed by lipolytic enzymes generally are dispersed in water as interfacial structures such as micelles, lyotropic mesophases, liposomes or oil-in-water emulsions. Common to all of these structures is the apparent orientation of lipid molecules at the interface with their non-polar portions directed away from the aqueous environment and polar portions directed towards it. Therefore, in addition to the usual parameters influencing

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enzymatic reactions, factors influencing the state of the lipid-water interface must be considered when studying lipolytic enzyme activity¹⁾).

Phospholipase A, obtained from various sources, catalyzes the hydrolysis of fatty acid ester bonds at the 2-position of 1,2-diacyl-*sn*-phosphoglycerides²⁾. Porcine pancreatic phospholipase A, purified and characterized as to amino acid sequence^{3, 4)}, has been used to study the hydrolysis of various synthetic phosphatidylcholine derivatives⁵⁾. Compounds having acyl chains short enough to produce significant water solubility yet long enough to give micellar structures (1,2-dihexanoyl-*sn*-glycero-3-phosphorylcholine* to 1,2-dioctanoyl-*sn*-glycero-3-phosphorylcholine) are hydrolyzed significantly once their critical micelle concentrations are exceeded. Shorter-chain compounds, existing as monomers, show no tendency to be hydrolyzed, nor do longer chain derivatives which exist in water as insoluble dispersions (liposomes).

From these studies it was concluded that affinity constants between enzyme and similar substrates cannot be compared with each other because of their differing lipid-water interfaces. Likewise, an understanding of the observed differences in maximal velocity for different substrates requires that the interfacial parameters be defined in addition to any chemical differences which might exist.

In order to define better the influence of the interfacial state on this reaction, one should use a technique where surface density (number of molecules per unit area) can be altered systematically. Spreading pure insoluble substrates on the surface of water as monomolecular films, compressing them to various surface densities and then measuring enzymatic activity would seem to meet this criterion. Indeed a number of studies with lipolytic enzymes have been carried out using this technique⁶⁻¹⁴⁾. Most of these studies have utilized long-chain glycerides or phospholipids with relatively crude enzyme preparations. The use of long-chain substrates has led to difficulties in interpretation of kinetic data because of product accumulation in the surface film which produces an ill-defined change in the composition of the monolayer**. To quantitate these reactions, therefore, it would be better to use substrates which form monomolecular films but whose products are readily water soluble and leave the surface rapidly. Olive and Dervichian reported for the first time such an approach utilizing a lipase preparation from *Arrhizus rhizopus* and a mixture of 1,2 and 1,3 dicaprins as the substrate^{13, 14)}.

* For the sake of simplicity, these compounds will be abbreviated to L-dihexanoyllecithin, etc.

** In most cases surface pressure has not been maintained constant during the reaction, although the rate of the reaction has been shown to depend on this value^{6, 8, 9)}.

The present work was designed to study the hydrolysis of some synthetic lecithins spread as insoluble monomolecular films, using homologues yielding water soluble products. The use of pure substrates and pure enzyme was seen as a decided advantage in drawing conclusions as to possible reaction mechanisms. Our first goal was to study enzymatic hydrolysis as a function of interfacial parameters such as surface density and surface pressure. We hoped to be able to gain some insight into the possible effects of the "quality of the interface", as determined by molecular orientation, charge density, water structure, etc. A second goal was to attempt to compare enzymatic activity in such systems with that obtained using micellar solutions. For this purpose we tried to determine a minimal value for specific activity of phospholipase A during the hydrolyses of the lecithin monolayers.

II. Materials and methods

A. Enzyme

Porcine pancreatic phospholipase A was obtained by the activation of the pure zymogen with trypsin, as described previously¹⁵). Stock solutions of enzyme of about 1 mg/ml were prepared each week and stored in a refrigerator. Less than 15% loss in activity was noted during this time. Each day from this solution a 0.1 mg/ml solution was prepared for the kinetic studies. These were kept in an ice-bath and showed no loss in activity over a 12 hr period. The exact enzyme concentration in solution was determined spectrophotometrically at a wavelength of 280 nM. A value of 14.0 for E 1%/1 cm was used.

B. Lipids

The short-chain L-diacyllecithins with two identical acyl chains (C_8 , C_{10} and C_{12}) were prepared as described by Cubero Robles et al.^{16,17}) while the D-dioctanoyllecithin and *rac*-1-octanoyl-2-deoxy-2-octanamido-glycero-3-phosphorylcholine were prepared by the method of Bonsen et al.¹⁸). All phospholipids were rapidly percolated on an alumina column as a final step and were checked for purity using thin-layer chromatography. Fatty acids used were the same as those used to prepare the various phospholipids and 1-dodecanoyl-*sn*-glycero-3-phosphorylcholine (dodecanoyllysolecithin) was prepared by treatment of L-didodecanoyllecithin with phospholipase A. All concentrations of phospholipid in solution were determined by phosphorus analysis according to the method of Fiske and Subbarow¹⁹).

C. Film balance and accessories

The film balance consisted of a Teflon trough having the dimension of 32 cm

length, 17.3 cm width and 2 cm depth. A glass coil connected to a constant temperature bath was used to maintain a temperature of $25^{\circ} \pm 0.1^{\circ}$. The unit was housed in a metallic box also containing coils with the same circulating water.

Surface pressure π was measured by the Wilhelmy plate method, using a thin platinum plate (perimeter 3.94 cm) attached to a RIIC electro-microbalance model LM 500. This was attached to a Hitachi model OPD 53 recorder so that mass changes could be recorded continuously. To minimize the contribution of buoyancy to the mass recorded, the plate was lowered carefully until it just touched the surface. The minimum vertical movement of the electrobalance during the rapid measurement of surface tension change due to a rapid nulling and the fact that surface tension or surface pressure was maintained constant during the reaction, minimized any possibility of contact angle change at the plate surface during measurement of a reaction rate. Thus, any serious error in the surface tension measurement was limited by avoiding measurements of decrease in surface pressure.

D. Procedure for following reaction rates

All materials were spread from a benzene solution. Comparison with chloroform as the spreading solvent indicated no differences due to the choice of solvent. Pure solvent added in great excess gave no surface pressure change when the surface was compressed rapidly. Solutions were added using an Agla Microliter Syringe Unit (Burroughs-Wellcome).

The water used was twice distilled first from alkaline permanganate and subsequently in an all-glass distillation apparatus. Absolute surface tension values and the absence of surface tension changes upon compression in the absence of films indicated that no surface active impurities were present after cleaning of the surface by suction into a glass pipette.

The general procedure used to bring the enzyme and substrate together was to spread the substrate on a solution of the enzyme. In this way possible problems caused by non-homogeneous solutions after injection of the enzyme were avoided. In most cases stirring was accomplished by means of two magnetic stirrers placed in the trough (200 rpm).

As soon as the enzyme and substrate were together the surface pressure was set by compression to a constant value and maintained by manual adjustment. At fixed time intervals, usually every 15 sec, the position of the barrier, L , was noted, measurement generally being taken until the original area was reduced to about 50%. Since a first-order process does not require that one knows the exact zero time for the reaction, time zero and its corresponding L_0 were taken arbitrarily after reaching a constant value of π , usually about 1 min after initial contact between enzyme and substrate.

Fig. 1 shows a tracing taken from the recorder during a typical run.

E. Kinetic treatment of data

Pure insoluble films compressed to a given surface density, N_s (number of molecules per unit area) will produce a corresponding reduction in the surface tension of the pure subphase solution. The change in surface tension is called the surface pressure, π , and has the units of dynes per cm.

When N molecules of a pure substrate are spread on S cm² of an aqueous subphase and phospholipase A is added to the subphase, substrate molecules are hydrolyzed. The experimentally obtained linear relationship between $\log N$ and time (cf. fig. 2) allows us to write:

$$-\frac{dN}{dt} = kN, \quad (1)$$

where k is a first-order rate constant expressed in units of reciprocal time*. Reactions involving phospholipase A at interfaces are also dependent on π or on N_s , thus it is best to carry out such reactions at constant π . Since the number of molecules in the surface is reduced during the reaction, if the products leave the surface rapidly, it will be necessary to reduce S in order to maintain N_s and π constant. Since N is directly proportional to S at constant N_s :

$$N = N_s \cdot S. \quad (2)$$

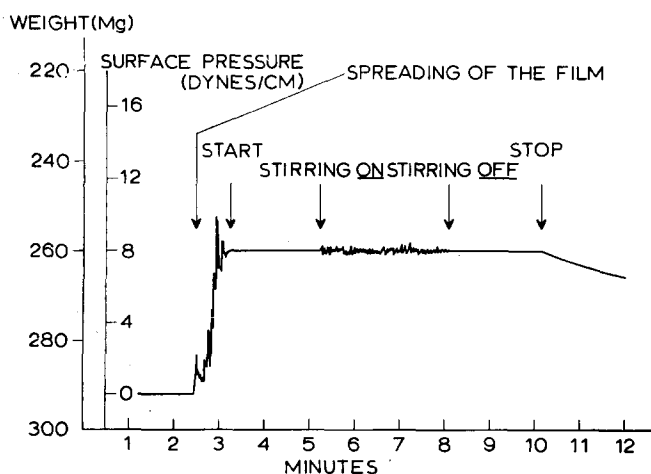


Fig. 1. Recorder tracing of the surface pressure-time relationship during hydrolysis of L-diocanoyllecithin by phospholipase A.

* This rate constant k , being surface pressure dependent, should not be confused with the classical first order rate constant used in bulk kinetics which is concentration independent.

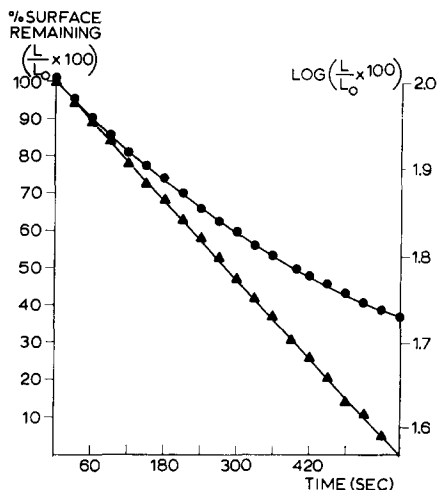


Fig. 2. Surface area-time relationship during the hydrolysis of L-dioctanoyllecithin by phospholipase A at a constant surface pressure of 5 dynes/cm. The upper curve shows the variation in the percent surface remaining with time (left scale). The lower curve shows the linear relationship between the logarithm of the percent surface remaining and time (right scale).

The change in S with time, dS/dt , can be used directly to estimate k in eq. (1). Solving for the first-order equation leads to:

$$\log \frac{S}{S_0} = \frac{-kt}{2.303}, \quad (3)$$

where S_0 equals the area of the trough at time zero. Since the width of the trough is constant, one can use the fraction of trough length remaining at time t , (L/L_0) and thus:

$$\log \frac{L}{L_0} = \frac{-kt}{2.303}. \quad (4)$$

Hence a plot of $\log L/L_0$ vs. t should give a linear plot from which the value of k can be obtained. Fig. 2 demonstrates the first-order behavior typical of all data collected.

It has been suggested that for such a system it would be more useful to express the rate of the reaction as the number of molecules hydrolyzed per unit area per time¹³). Since:

$$N = N_s \cdot S \quad (5)$$

then,

$$-\frac{dN}{dt} = kN = kN_s S \quad (6)$$

thus,

$$-\frac{1}{S} \frac{dN}{dt} = kN_s, \quad (7)$$

where the term on the left represents the number of molecules reacting per unit area per unit time. Thus, after estimating k at constant π , multiplication by N_s at that π gives the velocity term desired. Eq. (7) was used in this study to compare the rates of hydrolysis of different substrates.

III. Results

A. Properties of phospholipid films

In order to interpret kinetic data carried out at constant π , it is essential to know the relationship between π and the surface density N_s , the number of molecules present per unit area. Fig. 3 presents plots of π vs. N_s for L-dioc-tanoyl-, L-didecanoyl- and L-didodecanoyllecithin. All three curves are in agreement with previous studies which utilized the Langmuir horizontal film balance^{20, 21}). The plots for the D isomer of di-octanoyllecithin and for *rac*-1-octanoyl-2-deoxy-2-octanamidoglycero-3-phosphorylcholine were identical to that obtained for the L isomer of dioctanoyllecithin.

Since these films are maintained at constant π during the reaction it is

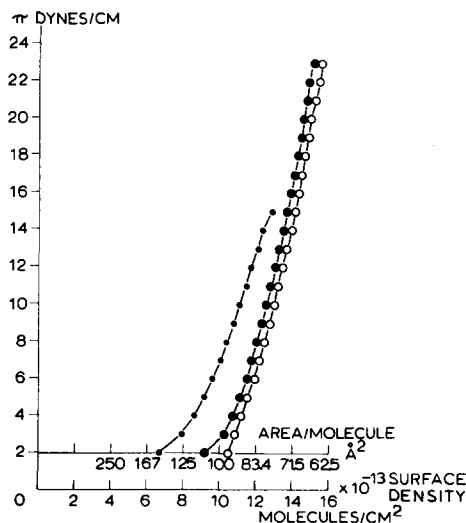


Fig. 3. Surface pressure-area per molecule curves of monomolecular films of L-dioc-tanoyllecithin (●—●), L-didecanoyllecithin (●—●) and L-didodecanoyllecithin (○—○). The lower abscissa represents surface density and is expressed as number of molecules per cm². For experimental conditions, see text.

important to know whether or not π changes in the absence of enzyme at constant area. Under all conditions, including stirring, no loss in π was noted for the L-didecanoyl- and L-didodecanoyllecithins, indicating no problems due to solubility or leakage. Some correction was required for the various dioctanoyl derivatives above 8 dynes/cm surface pressure due to slow solubilization into the subphase. Thus, all kinetic results concerning L-dioctanoyllecithins are corrected and the values are presented later with the corresponding corrections. It was generally seen that the surface pressure versus surface density relationships of these zwitterionic species were not influenced by the concentration of NaCl up to 2 M, calcium concentrations up to 33.5 mM and pH in the range 4.3 to 9.3. The presence of a Tris-Acetate buffer had no effect either. Using the D isomer of dioctanoyllecithin, which is not hydrolyzed by phospholipase A and which is a competitive inhibitor²²) we did not observe any surface pressure change due to enzyme "penetration" of the monomolecular film in the concentration range of enzyme used for kinetic studies (1×10^{-6} – 1×10^{-5} mg/ml). No apparent "penetration" of enzyme was seen with the L isomer of dioctanoyllecithin when Ca^{2+} was deleted so that no hydrolysis could take place.

B. Possible problems of product accumulation

Since the products of the hydrolysis of lecithins themselves may be surface active, the possibility that desorption of products could be rate limiting had to be considered. In order to investigate this possibility we have measured the rate of desorption into the subphase solution of pure fatty acid films and a 1:1 molar mixture of dodecanoyllysolecithin and dodecanoic acid under the conditions of our enzyme studies. The influence of surface pressure, pH and stirring was considered. We have used the kinetic treatment of Ter Minassian-Saraga²³) to evaluate our data and have found reasonably good agreement with her results. The more recent results of Gershfeld were also found to be in good agreement with ours²⁴).

To evaluate desorption kinetics the following equations have been used²³). First:

$$\phi(t) = - \frac{1}{S} \frac{dN}{dt}, \quad (8)$$

where $\phi(t)$ represents the superficial flow rate expressed as the number of molecules dN desorbed by unit time dt through the surface S . If it is assumed that in a steady-state condition the process of desorption is diffusion controlled across an "unstirred layer", the superficial flow rate may be expressed as a function of the diffusion constant D , the thickness of the "unstirred layer" ϵ and the concentration of the dissolved product in equilibrium with

the film C_0 . Thus:

$$\phi(t) = \frac{C_0 D}{\varepsilon}. \quad (9)$$

From this equation one first can see qualitatively how such parameters as pH, surface pressure and stirring will affect desorption rate. Stirring will diminish the thickness of the "unstirred layer" while increases in pH and surface pressure will increase concentration C_0 (pH only in the case of a fatty acid).

Results with the octanoic, decanoic and dodecanoic fatty acids and the 1:1 mixture of dodecanoyllysolecithin and the dodecanoic acid indicate the following. At pH 9.1 where the acid should be completely ionized we have not been able to develop any insoluble surface films, even at highest speed of compression possible (about 60–75 cm per minute). At pH 6.2 only the dodecanoic acid and the 1:1 mixture give measurable rates of desorption as indicated in table 1. Note the increase in desorption rate with increasing π and with stirring as compared to non-stirring. In all such cases we noted also that cessation of stirring leads to an immediate and significant decrease in the rate of desorption.

We have also noticed, as did Ter Minassian-Saraga²³) that a presteady-state condition gives an initial region of non-first-order behavior during fatty acid desorption, followed by good first-order data. As will be shown

TABLE 1
Rate of desorption of dodecanoic acid and dodecanoyllysolecithin
from monomolecular films

Film (on a pH 6.2 subphase)	Surface pressure	$k^* \times 10^3 \text{ sec}^{-1}$	
		no stirring	stirring
dodecanoic acid	5 dynes/cm	2.56**	14.6
	12 dynes/cm	9.05	too fast
dodecanoic acid + dodecanoyllysolecithin (1:1 molar ratio)	5 dynes/cm	1.99	11
	12 dynes/cm	2.62	13.3

* k is the velocity constant of the rate of desorption after reaching the steady-state conditions. The presteady-state is characterized by higher and variable rates. One effect of stirring is to establish rapidly the steady state conditions.

** Literature value²³): 2.5.

later, in those cases where product accumulation seems to be limiting the apparent rate of enzyme hydrolysis, the first-order plots also show an initial period of apparent non-steady state. Likewise no decrease in velocity is seen when stirring is stopped, except in those same cases suspected of product accumulation.

Based upon these experiments we are able to conclude that the problem of product accumulation is not serious, except for dodecanoic acid. However, even in this case, if we choose conditions of low enzymatic activity where the speed of formation of products is low compared with the rate of desorption, it is possible to collect meaningful kinetic data. Of course, this precaution is valid only if we assume that the fatty acid and lysoderivative desorb from the mixed film with lecithin during reaction in the same way as they do from a pure film. The exact reaction conditions of product formation in the film, followed by desorption are difficult to simulate.

C. Establishing solution conditions for studying the reaction

Initial studies were conducted to establish solution conditions giving maximal activity and to determine that indeed we were studying parameters which directly influence the reaction rate. As seen in fig. 2, linear first-order plots were obtained over a wide extent of the reaction. Only at pH 6.2 and lower with L-didodecanoyllecithin did we observe an initial non linear portion which we attribute to product desorption. If the reaction was studied while

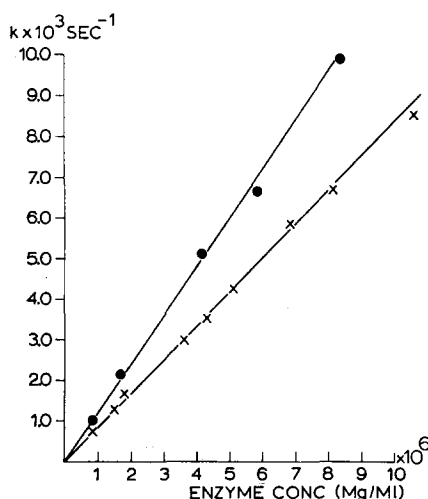


Fig. 4. Variation of the velocity constant with enzyme concentration. Hydrolysis of L-dioctanoyllecithin (surface pressure 8 dynes/cm) by phospholipase A was carried out at pH 6.2 (●—●) and pH 8.0 (×—×) under standard conditions.

the subphase was stirred, and then stirring stopped, no change in slope was seen for L-dioctanoyllecithin under all conditions. The L-didecanoyllecithin exhibited a 15% reduction in rate at pH 6.2 and 9.1, while for L-didodecanoyllecithin the reduction rate was 50% at pH 6.2 and 9.1. The similarity of the results at each pH would be expected from (9), if the unstirred layer thickness, reduced by stirring, was the only changing factor.

First-order rate constants obtained were found to be independent of the initial number of molecules placed on the surface, as long as the reaction was studied at constant surface pressure. The values of k , in general, were reproducible within 10%. It was also found that respreading more substrate over the same enzyme solution, even after a number of previous reaction runs, gave reproducible values of k . This assured us that no change in the enzyme activity was occurring due to depletion or inactivation.

Enzyme proportionality: One criterion used for studying these reactions was to check for proportionality between rate constants and enzyme concentration*. Enzyme proportionality was observed for L-dioctanoyllecithin under all conditions and for L-didecanoyllecithin down to pH 6.2. One plot for L-dioctanoyllecithin is given in fig. 4 to indicate the enzyme concentration range used and the upper limit of reaction rate which could be studied by our techniques. For L-didodecanoyllecithin no proportionality was observed at pH 6.2 but proportionality was attained at pH 9.1 and at low rates of hydrolysis.

Ca^{2+} : The requirement for calcium ion is well established and this was observed also in the present study. Table 2 expresses the dependence of reaction rate constants on calcium ion concentration in terms of percent of enzyme activity or maximum calcium ion effect for one set of conditions. A value of 6.7 mM Ca^{+2} was chosen for use throughout the study since higher Ca^{2+} did not increase the reaction rate.

pH: In the present study we were interested in choosing a pH value for study which gave optimum reaction rates. Fig. 5 shows a plot of reaction rate constant vs. pH for L-dioctanoyllecithin under one set of conditions. Since no product accumulation occurs with L-dioctanoyllecithin even at low pH values and since pH did not affect the lecithin monolayers, we assume this

* A simple calculation shows that in our conditions, if all enzyme of the bulk phase was adsorbed, only a few percent of the total film surface would be saturated with enzyme. We think that the plateau in activity at "high" enzyme concentration observed in the case of lipase¹⁴⁾ cannot be attributed to a saturation at the interface. It is more probable that some rate limiting factor due to diffusion is the reason for such an apparent saturation.

TABLE 2
Influence of calcium concentration on the hydrolysis rate of
L-dioctanoyllecithin films by phospholipase A

Ca ²⁺ concentration (mM)	Relative velocity*
0.10	15.6
0.20	28.3
0.34	34.0
0.67	56.5
1.70	79.0
6.70	100
16.8	100
33.5	100

* The runs are performed without stirring in a 2 cm depth trough at a surface pressure of 8 dynes/cm, pH 6.25 and NaCl to give a total ionic strength of 0.1.

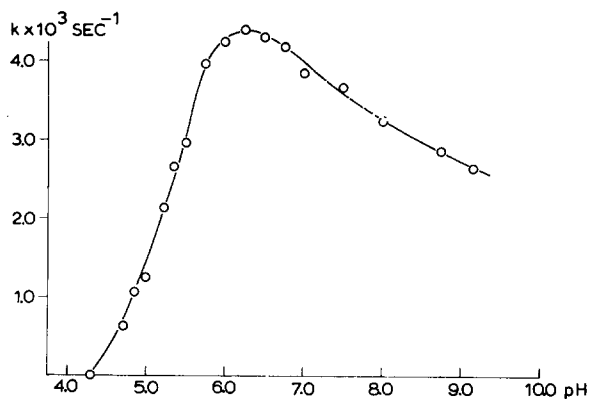


Fig. 5. Variation of the velocity constant with pH of the bulk phase. Hydrolysis of L-dioctanoyllecithin (surface pressure 5 dynes/cm) by phospholipase A was performed under standard conditions. Enzyme concentration: 7.4×10^{-6} mg/ml.

behavior reflects a variety of possible effects on the enzyme*. For most subsequent experiments pH 6.25 was chosen for study, except when comparing L-dioctanoyl-, L-didecanoyl- and L-didodecanoyllecithins where a higher pH value of 9.1 was used.

NaCl: Sodium chloride was introduced in the subphase solution to maintain a constant ionic strength. Comparison of reaction rates indicate no

* No attempt was made to study this further at the present time. However, it is interesting to note that the maximum velocity measured in bulk presents a similar pH dependence with an optimum also at pH 6.2.

ionic strength effect up to 0.3 M NaCl. In all subsequent studies ionic strength was adjusted to 0.1 with NaCl.

Buffer: The Tris-Acetate buffer was shown to have no effect on the reaction up to 0.01 M concentration, and in most studies a 0.001 M solution was sufficient to maintain pH constant.

D. Pressure profile

Having established the optimum solution conditions of this reaction, we next attempted to determine the influence of monolayer compression to various surface pressures and surface densities. We chose first to work with L-dioctanoyllecithin because problems of product diffusion do not exist at pH 6.2.

The first factor to consider was the role of stirring at various pressures during the course of the reaction. We noticed that at all surface pressures the speed of the reaction was 2–3 times faster when the stirrer was turned on in the middle of a run as compared with the rate obtained before stirring. This increase is not due to acceleration of the desorption rate of product because, as mentioned earlier, when stirring is stopped for L-dioctanoyllecithin the reaction continues at the higher speed caused by stirring. Likewise this effect is not accounted for by any significant increased instability of L-dioctanoyllecithin monolayers as this was checked in a blank run without enzyme. It is interesting to note also that in both stirring and non stirring cases, enzyme proportionality is maintained. Our explanation for the increase in reaction rate with stirring is that the number of enzyme molecules acting at the surface is increased by stirring in the presence of the spread film, presumably due to an increase in the speed of enzyme diffusion to the surface. Perhaps only until one stirs in the presence of a monolayer does one approach a more true equilibrium between the bulk phase and the surface. This effect is not very sensitive to speed of stirring or the time of stirring since after 1 min of stirring at 200 RPM the reaction velocity is no longer increased. This type of behavior is best seen from fig. 6 where 2 types of surface pressure vs. activity plots are presented. First, we spread on an enzyme solution at a given pressure and without stirring allow the reaction to proceed to an extent of about 30%. The surface pressure profile obtained (lower curve) has a maximum value around 8–9 dynes/cm. If we then switch on the stirrer and measure at the same surface pressure we obtain the increased velocity just discussed as seen in the upper curve with a maximum still at 8–9 dynes/cm surface pressure. Exactly the same type of behavior is seen with L-didecanoyl- and didodecanoyllecithin.

For comparing the hydrolysis rates of L-dioctanoyl, L-didecanoyl- and

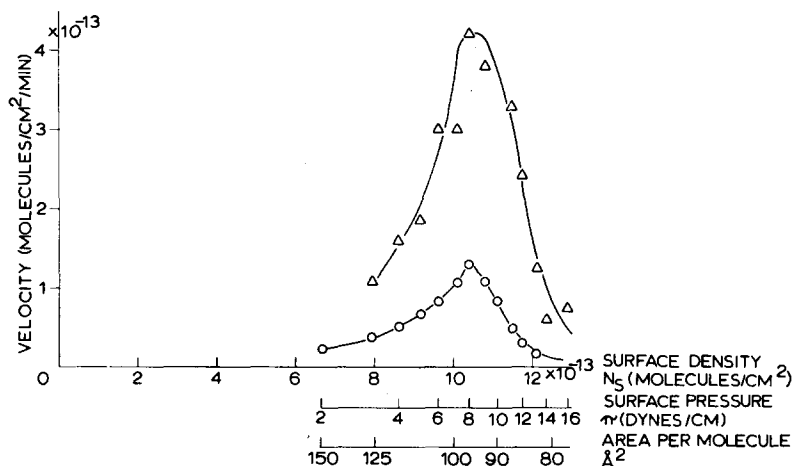


Fig. 6. Influence of the compression state of a short-chain lecithin monolayer on the rate of hydrolysis by phospholipase A (standard conditions). Enzyme concentration 5.7×10^{-6} mg/ml. ($\circ - \circ$) without stirring; ($\triangle - \triangle$) with stirring of the subphase.

L-didodecanoyllecithins the method of spreading to a certain surface pressure and running the reaction with stirring was used since equilibrium conditions are better approached by stirring. We also chose a comparison at pH 9.1 since product accumulation can be considered minimum for all compounds. Enzyme proportionality was checked and found to exist for all compounds under these conditions. It was most interesting to note that under these conditions L-dioctanoyl-, L-didecanoyl and L-didodecanoyllecithins gave the same surface pressure optimum and about the same absolute reaction rates at the optimum (8–9 dynes/cm). This is in sharp contrast with bulk studies⁵⁾ and will be discussed later.

E. Molecular activity of phospholipase A

Once an activity is measured it would be of interest to express it as a specific activity, as is done for enzyme reactions studied in bulk solution. In classic enzymology, e.g., one can calculate the number of enzyme molecules responsible for the observed reaction by determining the Michaelis Menten constants V_{\max} and K_m . In the present case, however, the total amount of substrate does not influence the velocity because, at a fixed surface pressure the ratio of enzyme to substrate remains constant. One of the major problems in using the monolayer technique for studying enzyme kinetics, is the unknown amount of enzyme which is really involved in the catalysis at the interface. However, a minimal value for the specific activity can be estimated for phospholipase action on monolayers by assuming that all the enzyme of

the bulk phase is adsorbed at the interface. We refer to this as minimal specific activity because in all likelihood this assumption is not correct and fewer molecules are actually involved in the reaction. The first indication of this possibility is seen from the reproducibility of results when measuring activity on the same subphase 3 or 4 times after cleaning the surface and spreading a new monolayer each time. A more direct proof of this fact is provided by the use of several troughs of decreasing depth down to 1 mm. At the same bulk concentration of enzyme each decrease in depth leads to a proportional decrease in total number of enzyme molecules. Table 3 gives the relative values of rate constant found for one set of conditions (conditions given in table 3). It can be seen that the extent of reduction in activity is much less than the reduction in amount of enzyme. This means that the number of enzyme molecules forming the active complex is negligible as compared with the total amount of enzyme used.

Below we give the calculation of minimal specific activity for L-dioctanoyllecithin at optimum conditions of study and stirring during the reaction*. The trough used was 0.1 cm deep. For the monolayer system choosing the following values for velocity (7×10^{-5} μmol per min per cm^2) and enzyme concentration (5.7×10^{-6} mg per ml), we can calculate a minimal specific activity in the following manner:

$$SA = \frac{7 \times 10^{-5}}{0.1 \times 5.7 \times 10^{-6}} = 122 \mu\text{Mole/min/mg/protein}.$$

To have some idea of how the interfacial state of the substrate influences the rate of the reaction, we calculated what should be the specific activity of the enzyme if the lipid film were dispersed in the bulk as a micellar solution. Since enzymatic hydrolysis of micellar solutions have been shown to follow Michaelis-Menten kinetics⁵) we can calculate at each substrate concentration

TABLE 3
Influence of the depth of the trough on the hydrolysis rate
of L-dioctanoyllecithin films by phospholipase A

Depth of trough (mm)	Relative velocity*
23.5	100
3.7	91
2.8	93
1.7	81
1.0	80

* In all experiments the enzyme concentration in the bulk phase is identical (10^{-5} mg/ml). The runs are performed without stirring at a surface pressure of 5 dynes/cm at pH 6.25, Ca^{2+} 6.7 mM and NaCl 0.1 M.

the corresponding velocity. Using the experimentally determined values* of V_{\max} and K_m (1750 μ moles per Min per mg protein and 2.52 mM respectively), one finds for a substrate concentration of 2.10×10^{-3} mM

$$v = \frac{V_{\max} \cdot S}{K_m + S} = \frac{1750 \cdot 2 \times 10^{-3}}{2.52 + 2 \times 10^{-3}} = 1.39 \mu\text{Moles/min/mg protein.}$$

Thus comparing this value of the specific activity with the one found for the monolayer film (min. specific activity = 122), we can conclude that the orientation of the phospholipid molecules at the interface has caused an 88 times increase in velocity as compared with the activity in the micellar state.

This result was rather unexpected, but does not necessarily mean that the turn-over number of phospholipase A towards a monolayer is 88 fold higher than for a micelle! With the monolayer technique we cannot measure maximal velocities; therefore one possible explanation is that the affinity of the enzyme for a film of substrate in the ideal conformation is higher than for a micelle of the same substrate. Nevertheless, it is striking to note that a strong increase in activity is found when comparing in bulk studies the maximal velocities on L-diheptanoyllecithin micelles in solutions of low and high ionic strength⁵). From an enzymatic point of view, can the effect of ionic strength in micellar solutions be compared with the effect of the compression on a monolayer film?

F. Mixed films

Since the monolayer technique offers the possibility of altering the composition of the surface in a known way, it was interesting to investigate the influence of different competitive inhibitors included to form mixed films with the lecithins. For this purpose we used 3 mixtures of L-dioctanoyllecithin and its optical isomer in various proportions. This last compound is absolutely insensitive to the action of phospholipase A, but behaves as a pure competitive inhibitor with an affinity constant identical to the normal L derivative²³). The surface pressure-area curves of the two isomers are identical as are all curves obtained with mixtures. In fig. 7 we have plotted the variation with time of total surface for the different mixtures during the course of the reaction.

Because the ratio of L to D isomers is continuously changing with time it is necessary to know the exact amount of lipid added on the surface and the zero time when the reaction starts. To make the comparison clearer, purposely we choose to express the kinetic data as surface remaining relative to the same initial amount of L isomer (100 in arbitrary units). E.g., in this type

* Ca^{2+} 6.7 mM; NaCl 0.1 M; Tris-Acetate 1 mM; pH 6.25; 25°C; $\mu = 8$ dynes/cm.

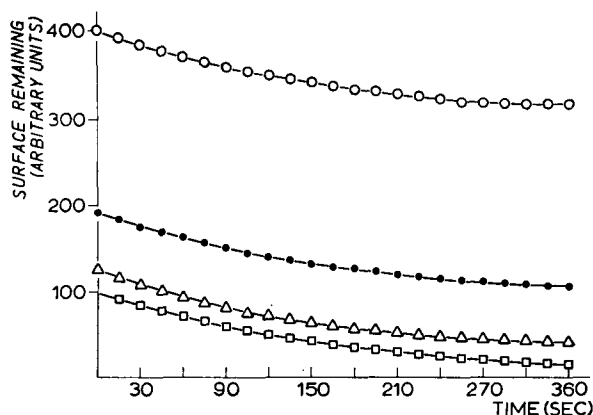


Fig. 7. Surface area-time relation during the hydrolysis by phospholipase A of mixed films of L- and D-dioctanoyllecithin. Surface pressure: 8 dynes/cm; enzyme concentration: 4.7×10^{-6} mg/ml. (○—○) 23.8% L-isomer, (●—●) 48.4% L-isomer, (△—△) 73.8% L-isomer, (□—□) 100% L-isomer. For further details, see text.

of presentation a 1:1 molar ratio of L and D lecithin will be represented at zero time by the number 200 and after an infinite time by the number 100. Under these conditions the absolute decrease in surface with time, dS/dt is equal to the decrease in the total number of substrate molecules with time, dN/dt . If one takes the initial slopes dS/dt of the 4 different curves of fig. 7, one finds that they are identical. Because in all experiments the total initial number of substrate molecules, N , is identical, we can say that the rate constant of all 4 reactions studied is the same.

This result suggests that a film of L lecithin is enzymatically hydrolyzed with the same speed whether we use a pure film or any mixture of the L and D antipodes. This lack of inhibition seems to be in contrast with bulk studies where the D isomer is found to be a pure competitive inhibitor²²). In fact, this contrast is only apparent because in the monolayer technique an extremely small amount of the total bulk enzyme is really involved in the reaction (see section: molecular activity of phospholipase). We can consider, therefore, that the bulk phase is a reservoir with a constant enzyme concentration, unaffected by the presence or absence of a lipid film. On this basis we would assume that even a powerful inhibitor with high affinity for the enzyme probably cannot deplete all the enzyme from the bulk phase and consequently it will not affect the equilibrium of the enzyme with the substrate, leading to the active complex. We attempted to confirm this assumption by using mixed films of L-dioctanoyllecithin and an analogue with an amide bond instead of an ester bond at the 2-position of the glycerol backbone. This "amide" derivative is, up to now, the most potent competitive inhibitor for

phospholipase A²²). Despite this fact, we found no major influence of this compound on the enzymic hydrolysis of L-dioctanoyllecithin using a 23.5 mm or a 1 mm depth trough. Thus we conclude that inhibition of the enzyme by competitive inhibitors present in mixed surface films cannot be studied by the monolayer technique.

IV. Discussion

Since the introduction by Hughes⁶) in 1935 of the monolayer technique to study enzymatic reactions, several authors have used this method to follow lipolytic activities against triglycerides and phospholipids. However, the general use of long-chain substrates, which upon enzymatic hydrolysis produce insoluble reaction products, has given rise to difficulties in interpreting changes in surface pressure. Surface potential changes have been measured, but this method is indirect and liable to errors in interpretation. Recently Garner and Smith¹⁰) as well as Lagocki et al.¹¹) used insoluble short-chain glycerides (dioctanoin, trioctanoin) as substrates for pancreatic lipase. In this way all reaction products are soluble and diffuse away in the subphase. The former group performed their kinetic runs at constant area, which forced them to determine initial reaction rates in short time intervals where the decrease in film pressure is still roughly linear with respect to time. The latter authors, working at constant area as well, state: "in the surface pressure range investigated the reaction is independent of the compression." In that case, the experimentally found first-order relationship between total number of substrate molecules and time allows the determination of the rate constant. Although such an independence of reaction rate from film pressure may be found in a limited region of surface pressures, the results published by Olive and Derivchian¹⁴) and those described in the present paper (fig. 6) demonstrated that this is certainly not a general phenomenon. In fact, the very pronounced maximum in the hydrolysis rate as a function of surface pressure found for such different substrates as diglycerides and lecithins strongly favors the constant-pressure technique as applied first by the French workers.

How can we understand such a maximum in the enzyme activity/surface pressure relationship as shown in fig. 6? Shah and Schulman⁹), who found a similar optimum surface pressure, explain this behaviour by two counterbalancing factors: "the compression of a monolayer results in an increase of surface concentration of molecules and simultaneously in a decrease in the intramolecular spacing in the monolayer. The former increases the rate of hydrolysis by increasing the collision-frequency between enzyme and substrate molecules whereas the latter decreases the rate by preventing the

penetration of the enzyme molecule into the monolayer." Although with long-chain substrates the descending part of the curve might be explained by assuming a more difficult penetration by the enzyme of a more closely packed monolayer, we do not agree as regards the interpretation of the ascending part. Assuming the formation of an enzyme-substrate complex along the Midhaelis-Menten model, we have

$$v = \frac{V_{\max} \times S}{K_m + S}.$$

There are two extremes; when $S \gg K_m$, then $v = V_{\max}$, or when $S \ll K_m$, then

$$v = \frac{V_{\max} \times S}{K_m} = \text{constant} \times S.$$

According to fig. 6, between 3 and 8 dynes, the surface density N_s increases with a factor of about 1.3. This means that according to Michaelis-Menten kinetics the increase in velocity between 3 and 8 dynes surface pressure can never exceed a factor of 1.3 unless the Michaelis-Menten model is not applicable. It is clear, however, that the increase in velocity between 3 and 8 dynes surface pressure is at least a factor of 4! Therefore, we prefer to consider the strong increase in activity upon compression followed by the rapid decrease as a consequence of the continuously changing orientation or conformation of substrate molecules during compression. Possibly around 8 dynes/cm surface pressure, the substrate molecules obtain an orientation and/or conformation which is optimal for interaction with the enzyme. At the same time, it cannot be excluded that at a certain free energy of the lipid/water interface (which is related to the surface pressure π), the enzyme molecules also possess a conformation which is optimally adapted for binding to or degradation of the substrate. Such a dependence of enzyme conformation on the physical state of the substrate has been suggested earlier by Desnuelle et al.²⁵.

Because of the fact that under the experimental conditions used, the substrate can never adsorb all enzyme available, it is impossible to separate K_m and V_{\max} contributions, as is usually done under normal kinetics in bulk conditions. In other words, we do not know if the apparent surface pressure optimum is related to optimal binding between enzyme and substrate and/or to a maximum value of the rate-limiting constant in the hydrolysis reaction. One may even wonder if the surface pressure π is really an important parameter determining the rate of hydrolysis. Although for all three lecithins, L-dioctanoyl-, L-didecanoyl- and L-didodecanoyllecithin maximum hydroly-

ysis occurs around a surface pressure of 8 dynes/cm, this substrate range is too limited to draw more general conclusions*.

On the other hand, our previous assumption⁵⁾ that enzyme activity might be related to the mean area per molecule of substrate in the interface, is certainly not true either. At the optimal surface pressure of 8 dynes/cm, the three lecithins being hydrolyzed at a similar rate possess different areas per molecule (fig. 3).

L-dioctanoyllecithin	96	Å ² /molecule
L-didecanoyllecithin	83	Å ² /molecule
L-didodecanoyllecithin	80.5	Å ² /molecule

If the area per molecule or intermolecular spacing is the critical factor governing the enzymic breakdown of lecithins, one would expect, e.g., at 96 Å²/molecule, for L-dioctanoyl-, L-didecanoyl- and L-didodecanoyllecithin an optimal action at 8 dynes/cm, 3.3 dynes/cm and 2 dynes/cm, respectively. Obviously this is not the case in the present study. We must conclude that the measurable characteristics of a monolayer such as surface pressure and mean area per molecule are probably parameters too rough to be related directly at the molecular level with the efficiency of the enzymatic action.

In conclusion, classical kinetic analysis of the hydrolysis by lipolytic enzymes of their substrates is still difficult to perform. E.g., pancreatic phospholipase A is unable to attack its natural, long-chain substrate such as lecithin, unless the organized liposomal structures are converted into smaller mixed micelles by addition of certain detergents. Even in the case of shorter-chain homologues which produce water-clear micellar dispersions and which are rapidly hydrolyzed in bulk by phospholipase A without any addition of detergent, there is a large influence of still unknown parameters of the interface on the hydrolysis rate. How to explain, e.g., the highly efficient breakdown by pancreatic phospholipase A of dioctanoyllecithin in bulk (specific activity about 1000 μmoles/min/mg protein) compared with the total inertness of higher homologues such as dinonanoyl- and didecanoyllecithins? The difficulties in obtaining rapid enzymatic hydrolysis of the higher phospholipid homologues in pure water are undoubtedly related to the fixed orientation and/or conformation of the substrate molecules under bulk conditions. The monolayer technique allowing a continuously changing orien-

* Shah and Schulman⁸⁾, who investigated phospholipase A hydrolysis of several long-chain lecithins, differing in degree of unsaturation found considerable differences in the surface pressure values where optimal breakdown occurs. They suggest that the rate of hydrolysis of lecithin monolayers is determined by the unsaturation of the fatty acyl chains and hence by the intermolecular spacing in the monolayer.

tation c.q. conformation of substrate molecules at the air/water interface upon compression, clearly shows that these higher homologues are hydrolyzed as expected at about the same rate as the shorterchain compounds. On the other hand, the monolayer technique also has its drawbacks as compared with bulk methods. The unknown amount of enzyme which is adsorbed to the monolayer and which catalyzes the observed reaction makes it impossible to measure maximal velocities and to determine the Michaelis constant. The two techniques, monolayer and micelle, are therefore complementary.

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References

- 1) L. Sarda and P. Desnuelle, *Biochim. Biophys. Acta* **30** (1958) 513
- 2) L. L. M. van Deenen and G. H. de Haas, *Biochim. Biophys. Acta* **70** (1963) 538
- 3) G. H. de Haas, A. J. Slotboom, P. P. M. Bonsen, L. L. M. van Deenen, S. Maroux, A. Puigserver and P. Desnuelle, *Biochim. Biophys. Acta* **221** (1970) 31
- 4) G. H. de Haas, A. J. Slotboom, P. P. M. Bonsen, W. Niewenhuizen, L. L. M. van Deenen, S. Maroux, V. Dlouha and P. Desnuelle, *Biochim. Biophys. Acta* **221** (1970) 54
- 5) G. H. de Haas, P. P. M. Bonsen, W. A. Pieterse and L. L. M. van Deenen, *Biochim. Biophys. Acta* **239** (1971) 252
- 6) A. Hughes, *Biochem. J.* **29** (1935) 437
- 7) R. M. C. Dawson and A. D. Bangham, *Biochem. J.* **72** (1959) 493
- 8) G. Colacicco and M. M. Rapport, *J. Lipid Res.* **7** (1966) 258
- 9) D. O. Shah and J. H. Schulman, *J. Colloid Interface Sci.* **25** (1967) 107
- 10) C. W. Garner and L. C. Smith, *Biochem. Biophys. Res. Comm.* **39** (1970) 672
- 11) J. W. Lagocki, N. D. Boyd, J. H. Law and F. J. Kézdy, *J. Am. Chem. Soc.* **92** (1970) 2923
- 12) I. R. Miller and J. M. Ruyschaert, *J. Colloid Interface Sci.* **35** (1971) 340
- 13) D. G. Dervichian, *Biochimie* **53** (1971) 25
- 14) J. Olive and D. G. Dervichian, *Biochimie* **53** (1971) 207 and J. Olive, Thesis, University of Paris (1969).
- 15) G. H. de Haas, N. M. Postema, W. Niewenhuizen and L. L. M. van Deenen, *Biochim. Biophys. Acta* **159** (1968) 118

- 16) E. Cubero Robles and H. de Jongh, *Rec. Trav. [Chim.]* **86** (1967) 762
- 17) E. Cubero Robles and D. van den Berg, *Biochim. Biophys. Acta* **187** (1969) 520
- 18) P. P. M. Bensen, G. J. Burbach-Westerhuis, H. H. de Haas and L. L. M. van Deenen, *Chem. Phys. Lipids*, accepted for publication.
- 19) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.* **66** (1925) 375
- 20) M. C. Phillips and D. Chapman, *Biochim. Biophys. Acta* **163** (1968) 301
- 21) R. Joos and R. A. Demel, *Biochim. Biophys. Acta* **183** (1969) 447
- 22) P. P. M. Bensen, G. H. de Haas, W. A. Pieterse and L. L. M. van Deenen, submitted for publication.
- 23) L. Ter Minassian-Saraga, *J. Chem. Phys.* **52** (1955) 181
- 24) N. L. Gershfeld, *Adv. in Chem. Series* **84** (1968) 115
- 25) P. Desnuelle, L. Sarda and G. Ailhaud, *Biochim. Biophys. Acta* **37** (1960) 571