The interaction between dipalmitoylphosphatidylcholine large unilamellar vesicles and porcine pancreatic phospholipase A$_2$ has been studied under a variety of conditions. It was found that the presence of large unilamellar vesicles inhibits the hydrolysis of small unilamellar vesicles at room temperature, and reaction calorimetric experiments showed that protein-lipid interactions in the absence of Ca$^{2+}$ occur in the gel state with a stoichiometry of about 40 phospholipid molecules/protein-binding site. However, hydrolysis can be induced in the gel state under conditions of osmotic shock. On the other hand, hydrolysis is usually observed within the lipid transition temperature range, but then it occurs only after a latency phase during which the hydrolysis is very slow. The duration of this latency phase reaches a minimum near the phase transition temperature. However, if the enzyme-substrate mixture is heated from low temperatures (continuously or by a temperature jump) to a temperature within the phase transition range, hydrolysis occurs instantaneously. These results are in accordance with the conclusions of the preceding paper (Menashe, M., Romero, G., Biltonen, R. L., and Lichtenberg, D. (1986) J. Biol. Chem. 261, 5328-5333) that effective binding of the enzyme to lipid vesicles occurs relatively rapidly in the gel state and that activation of the enzyme-substrate complex requires the existence of structural irregularities in the lipid bilayer.

Although hydrolysis products may have a pronounced effect on the time course of the reaction in the transition range, instantaneous hydrolysis can be induced in the phase transition region in the absence of reaction products by appropriate manipulation of the experimental conditions during which no reaction products are produced. Thus reaction products are not essential for activation of porcine pancreatic phospholipase A$_2$. Furthermore, it is shown that the fraction of lipid hydrolyzed during the latency period is a function of the initial substrate concentration in a manner inconsistent with the proposition that the accumulation of a constant critical fraction of reaction products is the basis for activation. Comparison of the results of this study with those of the preceding paper strongly support the previously proposed reaction scheme.

The hydrolysis of dipalmitoylphosphatidylcholine (DPPC$^1$) small unilamellar vesicles (SUV) by the porcine pancreatic phospholipase A$_2$ (PLA$_2$) was described in the preceding paper (1). Those studies led to the conclusion that hydrolysis is preceded by at least two distinctly different steps. First, the enzyme binds to the lipid bilayer in a Ca$^{2+}$-independent step which occurs best in the gel phase lipid. This step is followed, prior to hydrolysis, by activation of the initially formed enzyme-substrate complex. Although the exact nature of the latter step is unknown, it is clear that it requires Ca$^{2+}$ and that in SUV it can only occur in the phase transition range or in the gel phase region. On the basis of these findings we hypothesized that activation of the bound enzyme occurs only when the membrane exists in a state in which the lipid is undergoing highly cooperative structural fluctuations. Such a situation prevails in the phase transition region of vesicles of any size due to the existence of gel and liquid crystalline regions. In SUV, a similar situation apparently exists in the gel state due to the irregular (faceted) packing of the lipid (2, 3) resulting from the high curvature of these bilayers. Large unilamellar vesicles (LUV) do not possess packing irregularities in their gel state (3), and indeed the present study shows that PLA$_2$ is not activated under such conditions.

The use of LUV for studying PLA$_2$ activity has two additional advantages. First these vesicles are more stable than the SUV, which tend to fuse below the phase transition temperature (4-7). This makes acquisition of precise data on the hydrolysis of LUV and their quantitative interpretation easier than for SUV. Second, results obtained with LUV are probably more relevant to biological membranes, which are usually not as curved as the SUV. Results of PLA$_2$ hydrolysis of LUV reported in this communication support the previously proposed reaction scheme and its implications (1).

**MATERIALS AND METHODS**

The sources of the various chemicals and the kinetic procedures used in the study of PLA$_2$-induced hydrolysis were described in the preceding paper (1). LUV were made through spontaneous fusion of the SUV (4), which were made as described in the preceding paper (1). This fusion process was allowed to proceed for 3-4 weeks before use of the LUV.

Calcium was added to all the dispersions only after the large vesicles were formed because the fusion of small vesicles is slower in

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1 The abbreviations used are: DPPC, dipalmitoylphosphatidylcholine; SUV, small unilamellar vesicles; PLA$_2$, porcine pancreatic phospholipase A$_2$; LUV, large unilamellar vesicles; $\tau$, latency period, time at which the rate of hydrolysis abruptly increases; $P_\tau$, the cumulative amount of lipid hydrolyzed at time $\tau$. 

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the presence of Ca$^{2+}$ and results in much less reproducible preparations than those formed by fusion in the absence of Ca$^{2+}$. The fused vesicles were dialyzed for at least 4 h at the phase transition temperature $T_m$ against a solution of 35 mM KCl and 10 mM CaCl$_2$ to ensure equilibration of the added Ca$^{2+}$ between the inner and outer compartments of the vesicles.

The fused vesicles were characterized using a high sensitivity differential scanning calorimeter (5). LUV have a $T_m$ of 41 °C compared to a $T_m$ of 37 °C for SUV (4). Vesicles fused as described above and equilibrated with Ca$^{2+}$ exhibited a transition at 41 °C (Fig. 1A). No evidence for a transition that could be associated with SUV was found. Both SUV and LUV exhibit a pretransition in the 30–35 °C temperature range (6).

Mixed vesicles of lysophosphatidylcholine and palmitic acid were prepared by dissolving equimolar amounts of the two components in CHCl$_3$-CH$_2$OH (3:2, v/v), evaporating the solution to dryness and dispersing the residue in 50 mM KCl at 60 °C. Subsequently, the dispersion was incubated for 2–3 h at 50 °C and sonicated at this temperature to clarity before being added to a 100 mM dispersion of pure DPPC LUV. Calorimetric scans obtained shortly after mixing and equilibrated with Ca$^{2+}$ exhibited a transition at 41 °C (Fig. 1C). Additional incubation of the mixture for 40 h did not produce any further changes (not shown). These results indicate that lipid reorganization occurs and leads to a stable dispersion after 35 h. Since this mixture contained a 20-fold excess of DPPC LUV over mixed aggregates (presumably SUV) of the reaction products, we believe that the mixed aggregates formed in this dispersion are LUV containing reaction products. PLA$_2$ was added to aliquots of these mixtures 50–70 h after mixing and the hydrolysis monitored.

Binding of the phospholipase to DPPC LUV was studied in the absence of Ca$^{2+}$ by isothermal reaction calorimetry using a LKB 10700 batch microcalorimeter. The reactant solutions were placed in two separate compartments of the calorimeter cell. The reaction was initiated by mixing the reactants, and the heat evolved was measured using a Keithley digital nanovoltmeter interfaced to a PDP 11/23 minicomputer. The data were analyzed as described previously (8). The reaction medium was 50 mM KCl, pH 8, containing 1 mM EDTA. The final lipid concentration used in these experiments was 2.9 mM. The concentration of phospholipase PLA$_2$ was varied from 0.6 to 2.5 mg/ml.

RESULTS AND DISCUSSION

The pancreatic phospholipase A$_2$-catalyzed hydrolysis of DPPC liposomes is a complex reaction, the details of which are strongly influenced by the existence of a variety of structural and thermodynamic states accessible to the lipid substrate under different experimental conditions. We have presented in the preceding paper some of the salient features of the hydrolysis of DPPC SUV by pancreatic PLA$_2$ (1). It was shown that rapid activation of the enzyme and hydrolysis of the SUV occurred only at temperatures where the lipid exists exclusively in the gel state. At temperatures near or above the thermotropic phase transition of the substrate, the enzyme activation reaction became very slow and the hydrolysis profile was characterized by a long latency period whose duration could be altered by manipulation of the temperature, the enzyme and substrate concentrations, and other treatments (1).

However, DPPC SUV are unstable below $T_m$ (4, 7) and are far from being an ideal choice of substrate for a detailed analysis of the reaction mechanism of PLA$_2$. It has been shown that incubation at 4 °C induces fusion of the sonicated liposomes into vesicles of an average diameter of 950 Å (4). The rate of this reaction depends on the lipid concentration. Wong et al. (4) have shown using gel chromatography techniques that not more than a fourth of the lipid was still contained in vesicles of the original size after 4 h of incubation of a 120 mM dispersion of sonicated DPPC liposomes at 4 °C. One day later, less than 15% of the DPPC was present as SUV. After 1 week, the average diameter of the vesicles was found to be about 700 Å, with no evidence for the existence of SUV. Further fusion over an additional period of 2 weeks resulted in the formation of larger bilayers of 950 Å diameter (4). No difference between the thermotropic behavior of vesicles fused for 1 week or 2 was observed, in accordance with previous differential scanning calorimetric studies (9).

The fusion process is reflected in the kinetic profile of the PLA$_2$-catalyzed hydrolysis of DPPC liposomes. After sonication at 50 °C, a 100 mM dispersion of DPPC was incubated at 4 °C. At various times after incubation, aliquots were diluted to 2 mM, and their hydrolysis by PLA$_2$ (0.5 mg/ml) was monitored at room temperature using a pH stat apparatus. As shown in Fig. 2, a 2-h incubation of the SUV dispersion at 4 °C resulted in a significant decrease in the rate of hydrolysis of the lipid by PLA$_2$ (compare Fig. 2, B and A). After 21 h of incubation, the rate of hydrolysis of DPPC at room temperature was further reduced (Fig. 2C). Further incubation had only a small

![Fig. 1. Curve A is the apparent heat capacity of pure DPPC LUV (prepared by 4 weeks of incubation of SUV at 4 °C and dialyzed against a medium containing 10 mM CaCl$_2$ and 35 mM KCl) as a function of temperature. DPPC concentration in all the dispersions is 10 mM. B and C are heat capacity curves obtained 1 and 35 h after the addition of 6 mol % reaction products, respectively (see text for details).](image1)

![Fig. 2. Time course of hydrolysis by PLA$_2$ at 23 °C of DPPC vesicles prepared by ultrasonic irradiation. A dispersion of 100 mM DPPC was sonicated and then incubated at 4 °C for 4 weeks. At various times after incubation, aliquots of this dispersion were diluted to provide 2 mM DPPC dispersions. PLA$_2$ (0.5 µg/ml) was added to the diluted aliquots at room temperature, and phospholipid hydrolysis was monitored by the pH stat. Curve A is the course of hydrolysis of SUV immediately after preparation (after adjustment to 23 °C and pH 8.0). Curves B, C, and D are the time courses of the reaction obtained after 2 h, 21 h, and 14 days of incubation of the 100 mM dispersion at 4 °C, respectively.](image2)
additional effect on the rate of hydrolysis; the kinetics of hydrolysis after 45 h (not shown) was indistinguishable from that obtained after 21 h. Two additional weeks of incubation, which according to Wong et al. (4) lead to a size transformation of the vesicles from an average diameter of 700 Å to an average diameter of 950 Å, had very little effect on the initial rate of hydrolysis (Fig. 2D). These results indicate that the activity of pancreatic PLA₂ toward gel state DPPC vesicles becomes independent of the radius of curvature of the liposomes after they reach a diameter of 700 Å. It thus appears that the activation of the enzyme necessary for rapid hydrolysis does not occur in the gel phase of vesicles of diameter 700 Å or more. We must, therefore, conclude that packing irregularities in the gel phase, which exist in SUV but not in the larger fused vesicles, are responsible for activation of SUV in the gel phase.

The rate of hydrolysis of a mixture of SUV and LUV is shown in Fig. 3. It is shown that the presence of LUV (broken line) reduces the rate of hydrolysis of the mixture as compared to pure SUV under identical conditions (solid line). This phenomenon is observed at all temperatures below the phase transition of LUV. It thus appears that PLA₂ binds to the LUV in the gel phase and acts as an inhibitor of hydrolysis in SUV in the gel phase.

We have previously suggested that dramatic structural fluctuations or disruption of the membrane play a major role in PLA₂ activation (1, 9). The lack of such events for gel phase LUV (3) is likely responsible for the low PLA₂ activity observed. However, such events might be expected if the LUV are osmotically "shocked." The observed time courses of hydrolysis of LUV at room temperature in the presence of externally added glucose are consistent with this hypothesis. While little hydrolysis is observed in the absence of glucose (Fig. 4A) addition of 15% (w/v) glucose to a pre-equilibrated Ca²⁺-enzyme-LUV mixture results in an immediate increase in the rate of hydrolysis (Fig. 4B). This phenomenon presumably occurs because the osmotic gradient formed causes shrinkage of the vesicles (3) resulting in structural changes of the membrane. Dissipation of the osmotic gradient by allowing the glucose to equilibrate on both sides of the bilayer during 15 h of incubation at 41 °C results in a preparation which is hydrolyzed at a much slower rate as shown in Fig. 4C. Similar behavior has been observed using different agents to produce the initial osmotic imbalance. These observations lend further support to the conclusion that enzyme binding occurs in the gel phase and that structural irregularities in the bilayer and their attendant fluctuations are necessary for activation of the enzyme-substrate complex.

Addition of PLA₂ to LUV at temperatures below or above the transition range results in little or no hydrolysis. As with MLV (10), hydrolysis is observed only in the transition range. In this temperature range the time course of hydrolysis is characterized by a latency period (r) whose duration strongly depends on the exact temperature as shown in Fig. 5. The maximal rate of hydrolysis is comparable to that observed for the SUV at 37 °C (1). The exact length of this latency period at a given temperature varies somewhat from preparation to preparation. Nonetheless, the trend in the temperature dependence of the latency period is always maintained and is a function of the concentrations of enzyme and substrate. In all cases, the minimum in r occurs in the phase transition region at temperatures slightly below or equal to Tₘ. The number of gel-liquid crystalline interfacial regions and the number of fluctuating clusters are maximal near this temperature (11). The temperature dependence of r, therefore, constitutes strong suggestive evidence that membrane structural fluctuations play a dominant role in PLA₂ activation on the membrane surface.

In order to gain more insight into the details of the mechanism of activation of PLA₂, a variety of temperature jump and temperature-scanning hydrolysis experiments were performed. When we temperature jumped a mixture of enzyme and LUV in the presence of Ca²⁺ from room temperature to 45 °C no hydrolysis of the lipid was observed. This result is in contrast to the rapid hydrolysis obtained under similar conditions with SUV. However, when a mixture of enzyme (25 μg/ml) and LUV (1 mM) is preincubated for 15 s at room temperature and then temperature jumped into a medium at 41.2 °C, the substrate is completely hydrolyzed in less than 5 s (not shown). This result is identical to those obtained with

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G. Romero, K. Thompson, and R. Biltonen, unpublished results.
SUV. This is in contrast to the hydrolysis profile obtained when the enzyme is not preincubated with the substrate and where significant hydrolysis was observed only after a latency phase (Fig. 5). These experiments indicate that the enzyme can effectively bind to the substrate at room temperature, and this binding is sufficient to result in an immediate activation and hydrolytic reaction after a temperature jump into the phase transition region, where, apparently, the irregular packing of the phospholipids provides the right conditions for activation.

The enzyme-substrate reaction in the gel phase is complex. In contrast with the results obtained with high concentrations of enzyme (25 μg/ml), a 20-s preincubation at room temperature using a lower enzyme concentration (2.5 μg/ml) was insufficient to induce instantaneous hydrolysis upon temperature jumping the mixture to 41.2 °C. Longer preincubation times (about 5 min) at room temperature were required to observe rapid initial hydrolysis. It thus appears that the enzyme concentration in the preincubation mixture and the time of preincubation at low temperature are important variables in determining whether or not instantaneous hydrolysis can be induced by temperature jumping to the transition range. This result suggests that the rate of effective binding below Tm or other relatively slow enzyme concentration-dependent events occurring on the membrane surface in the gel phase play a significant role in enzyme activation.

As shown in Fig. 6A, continuous heating (at approximately 5 degrees/min) of a mixture of PL2 and LUV in a Ca2+-containing medium from room temperature to 45 °C results in rapid and complete hydrolysis of the lipid when the transition region is approached. However, if the same mixture is prepared at 48 °C and then continuously cooled through the transition region, little hydrolysis is observed (Fig. 6B). Reheating this sample from 36 °C does not result in any significant hydrolysis in the phase transition region, whereas reheating it from 30 °C yields rapid hydrolysis of all the lipid when the transition range is entered (Fig. 6C). These results support the view that while effective binding can occur in the gel state, it does not occur in the liquid crystalline state.

Additional support for the proposed model comes from the temperature jump experiments shown in Fig. 7. In these experiments concentrated mixtures of PLAl and LUV were either incubated for 3 min at 41 °C and then 3 min at room temperature or for 3 min at room temperature and then for 3 min at 41 °C. Following these two steps of preincubation the mixtures were temperature jumped to 49.5 °C and the hydrolysis monitored. Little hydrolysis was observed if the preincubation at 41 °C preceded the one at room temperature (Fig. 7A), whereas when the room temperature preincubation preceded the one at 41 °C rapid hydrolysis was obtained (Fig. 7B). It is difficult to assess exactly how much of the lipid in the experiment described in Fig. 7B was hydrolyzed during the second preincubation at 41 °C. However, the shape of the observed hydrolysis profile (Fig. 7B) indicates that more than a third of the lipid was involved. In any event, it is clear that DPPC LUV in the liquid crystalline phase can be instantaneously hydrolyzed at 49.5 °C provided that the “right sequence of events” which leads to activation of the enzyme-substrate complex occurs. This sequence of events is first effective binding of PLAl in the gel phase and then activation in the phase transition region. Such a sequence is also likely to occur if cooling of an enzyme-substrate mixture from 48 °C to the transition region is preceded by a temperature jump of the mixture from the gel phase to 48 °C. In fact, the temperature jump of an enzyme-substrate mixture from room temperature to 48 °C results in no significant hydrolysis, but upon subsequent cooling of the mixture rapid hydrolysis is observed as the transition range is approached (Fig. 8A).

Our previous studies using SUV as substrate suggested that Ca2+ was not required for the initial binding step but that it

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**Fig. 6.** Time, temperature course of hydrolysis of DPPC LUV (1 mM) by PL2 (25 μg/ml) in temperature-scanning experiments. In A, the enzyme was added to the LUV dispersion at 28 °C, whereas in B and C the two components were mixed at 48 °C. In all three experiments no hydrolysis occurred over an initial period of 5 min. Sample A was heated by an external water bath kept at 48 °C. Samples B and C were cooled by removing the heating source. Sample B was reheated when the temperature in the reaction vessel reached 36 °C. In C, reheating was initiated when the cooled reaction reached 29.5 °C.

**Fig. 7.** Temperature jump experiments on the hydrolysis of DPPC LUV at 49.5 °C by PL2. Mixtures of concentrated solutions of enzyme and substrate were premixed for 3-min periods at 41 °C and then at 23 °C (A) or at 33 °C and then at 41 °C (B). Following these preincubations the mixtures were added to a reaction medium pre-equilibrated to pH 8.0 at 49.5 °C, and the hydrolysis monitored by the pH stat. The final enzyme and substrate concentrations were 2.5 μg/ml and 1 mM, respectively.
was required for activation of PLA2 (1). Our results with LUV confirm these conclusions. When a mixture of a high concentration of PLA2 (25 μg/ml) and LUV (1 mM) is incubated at room temperature for 30 s in the absence of Ca²⁺ and then diluted 30-fold into a Ca²⁺-containing medium at 41 °C rapid and complete hydrolysis is observed. This result supports our conclusion that effective binding of PLA2 to the gel state lipid does not require Ca²⁺. On the other hand, if the enzyme-substrate mixture is diluted in a Ca²⁺-free medium and the reaction mixture is then heated to 48 °C prior to the addition of Ca²⁺, no hydrolysis is observed (not shown), indicating that activation requires Ca²⁺.

A conclusive demonstration of the Ca²⁺-independent binding of PLA2 to LUV in the gel state was obtained by reaction calorimetry experiments. Increasing concentrations of enzyme were mixed with 8.8 μmol of DPPC LUV (2.9 mM final concentration) in a batch microcalorimeter at 25 °C in the presence of 1 mM EDTA. The binding reaction was followed measuring the heat released upon mixing. The results obtained are shown in Fig. 9 and unequivocally demonstrate interaction of the enzyme with the lipid under the conditions of the experiment. These experiments, however, were carried out at rather high lipid concentrations, and the apparent binding constant of the protein to the vesicles could not be estimated with sufficient precision from the data. However, an apparent stoichiometry of about 40 phospholipid molecules/enzyme-binding site was calculated from the data, a figure in excellent agreement with previous measurements carried out by others using substrate analogs, either in pure form (12) or in ternary codispersions containing reaction products (13). The ΔH° of interaction between the enzyme and the lipid bilayer was estimated as approximately −8 kcal/mol of enzyme.

Previous reports have suggested that reaction products increase the binding of PLA2 to certain vesicle surfaces (13) and that the rate of hydrolysis may be greatly enhanced after a critical fraction of the lipid has been hydrolyzed (14). Thus it has been suggested that reaction products play a critical or essential role in the slow process(es) leading to activation of PLA2 in the transition range (13–15). On the other hand, our observation that substrate preincubated with PLA2 at room temperature in a Ca²⁺-deficient medium (in which no hydrolysis occurs) is instantaneously hydrolyzed upon temperature jumping the enzyme-substrate mixture to the transition range into a Ca²⁺-containing medium clearly demonstrates that reaction products are not essential for activation of the enzyme. Furthermore, our calorimetric experiments clearly demonstrate that pancreatic PLA2 binds phospholipid membranes in the absence of Ca²⁺ and reaction products with a binding constant larger than 10⁸ M⁻¹ expressed in moles of lipid and with a stoichiometry that is in accord with that observed in the presence of reaction products. These lower estimates of the binding parameters are only somewhat smaller than those obtained by Jain and co-workers (14, 15) using ternary codispersions of dialkyllecithins and reaction products. Furthermore, the affinity of PLA2 for LUV appears to be comparable to that for SUV as shown by the inhibition experiment depicted in Fig. 3 and by comparison of the LUV-binding parameters to the SUV kinetic constants calculated in the preceding paper (1). Finally, cobra venom enzymes, which readily bind to dialkyllecithin vesicles (14), have also been shown to exhibit latency periods in the presence of DPPC LUV and multilamellar vesicles (16). Thus, it must be concluded that reaction products are not essential for enzyme binding and that poor binding of PLA2 to the surface of the vesicles cannot be responsible for the lack of activity observed in the presence of gel state DPPC LUV.

Nevertheless, in order to obtain additional information on the possible role that reaction products might play in the hydrolytic reaction catalyzed by porcine pancreatic PLA2, we have carried out a number of experiments with mixed systems...
containing vesicles made of DPPC and its hydrolysis products. As a first approach, we prepared LUV and then mixed them with a cosonicated dispersion of equimolar concentrations of 1,2-

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palmitoylphosphatidylcholine and palmitic acid. Although the system thus formed is not well characterized, calorimetric data indicate that mixing of lipids in this fashion yield LUV which contain reaction products (see “Materials and Methods”). These mixed systems behave similarly to pure LUV if PLAs is added to them at room temperature or 48 °C or when a mixture of PLAs and substrate vesicles is heated from room temperature to the phase transition region (compare Fig. 10A to Fig. 6A). However, in the transition region, the latency periods in the mixed systems are either eliminated or reduced in comparison to those observed with pure DPPC LUV (not shown). Furthermore, when PLAs is added to such a mixed system at 48 °C and the mixture then cooled, rapid hydrolysis is immediately obtained in the transition region (Fig. 10B). The latter result is in contradistinction to that observed using pure DPPC LUV as substrate (Fig. 6B). These results demonstrate that reaction products can accelerate the PLAs-catalyzed hydrolysis of DPPC under some, but not all, experimental conditions. The exact basis of this effect is as yet unclear.

Another finding which may be the result of the presence of reaction products is described in Fig. 8B. In this experiment, a mixture of PLAs and pure DPPC LUV prepared at 48 °C was cooled to 34.5 °C. This procedure resulted in no hydrolysis. Six subsequent cycles of heating to 45 °C and cooling to 35 °C each resulted in some hydrolysis when the temperature of the mixture approached the transition region upon heating (Fig. 8B). In the course of these cyclical steps approximately 5–10% of the total lipid was hydrolyzed. When the mixture was then reheated, a rapid and complete hydrolysis occurred upon approaching the transition region. This result could be due to activation induced by reaction products or to the fact that the enzyme-substrate mixture spent sufficient time in the phase transition region during the cycles of heating and cooling for activation to occur.

While the formation of a critical fraction of reaction products and the observed abrupt onset of enzymatic activity in the vicinity of the gel to liquid crystalline phase transition of the lipid may be qualitatively correlated, it is important to ask whether this correlation is causal or casual. The fact that product formation is not essential for activity in the phase transition region suggests only a casual relationship. The simplest quantitative statement of the “critical fraction” hypothesis is that after a constant precise fraction of the lipid is hydrolyzed an abrupt enhancement of the catalytic activity occurs. However, the fraction of substrate hydrolyzed at time \( \tau \), \( p \), is neither a constant nor a simple function of \( [S] \), as shown in Fig. 11. Thus, this simple model for a critical fraction of hydrolysis to explain the apparent abrupt onset of activity of PLAs is not consistent with our data, and the correlation between \( \tau \) and product formation under these conditions appears to be only a casual one.

**CONCLUDING REMARKS**

The results reported here are consistent with the following conclusions regarding the mechanism of PLAs hydrolysis of DPPC unilamellar vesicles: 1) The initial step of the reaction scheme is binding of the enzyme to the lipid surface. This step does not require Ca\(^{2+}\) and the optimal conditions for its occurrence are when the lipid is in the gel phase. 2) Subsequent to binding, an activation process occurs on the membrane surface. This step is Ca\(^{2+}\) dependent and requires the existence of structural fluctuations within the membrane. 3) While one or both of these steps are influenced by the presence of reaction products, reaction products are neither essential nor sufficient to induce enzyme activity by themselves. The exact nature of the product-induced acceleration of the rate of hydrolysis is not yet clear, but is probably related to the effect of products on the character of the phase transition. 4) The results of the preceding paper indicate that enzyme aggregation (possibly dimerization) plays an important role in enzyme activation. Since we have no data concerning the Ca\(^{2+}\) dependence of this event, we cannot conclude whether enzyme aggregation is a part of the initial binding or the subsequent activation step(s).

We would finally like to note that when hydrolysis of DPPC occurs at any temperature within or above the transition range in both SUV and LUV, it is complete. On the other hand, at temperatures below the transition region only 60–65% of the lipid in SUV and 45–55% in LUV is hydrolyzed in the time regime of our experiments. This finding suggests that the lipid of the inner monolayers of the vesicles is hydrolyzed below \( T_n \), much slower than that of the outer monolayer. At higher temperatures the lipid of the inner

![Fig. 10. Time, temperature course of the hydrolysis of DPPC LUV containing 5 mol % of reaction products by PLAs. The enzyme was added to the LUV dispersion at 23 °C (A) or 48 °C (B). The final enzyme and substrate concentrations were 25 μg/ml and 1.5 mM, respectively. Subsequently, the temperature was varied as indicated.](image1)

![Fig. 11. The substrate concentration dependence of the fractional degree of hydrolysis at the end of the latency period (P). The latency period was calculated as described in the preceding paper (1), and the fractional degree of hydrolysis at \( \tau \) was estimated from the pH stat data. The experiments shown were carried out at 38 °C in the presence of 10 μg/ml pancreatic phospholipase A\(_2\).](image2)
monolayer of the vesicles can become accessible to the enzyme through increase in the rate of "flip flop" (17), increased permeability of the vesicles to PLAs, fusion of the partially hydrolyzed vesicles, or more than one of these factors. In some experiments, the time course of the reaction appears to indicate that following activation and partial hydrolysis, the reaction rate decreases and only after an apparent "second latency" period another abrupt increase in activity occurs which results in hydrolysis of the remaining (35-55%) phospholipid.

Thus, while we believe that this and the preceding paper constitute an important step toward understanding of the mechanism responsible for the activation of PLA2, many fundamental questions remain unresolved. Present and future effort will be devoted by our laboratories to gain more insight into these important but very complex and poorly understood enzymatic reactions. Presently, we are in the process of attempting to quantitatively analyze phospholipid hydrolysis by PLA2 in terms of a general model which assumes that enzyme-enzyme interaction on the substrate surface is fundamental to the activation process.

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