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ACTION OF PHOSPHOLIPASE A_2 ON BILAYERS CONTAINING LYSOPHOSPHATIDYLCHOLINE ANALOGS AND THE EFFECT OF INHIBITORS

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Abstract—The effects of several lysophospholipid analogs on the phase properties of codispersions with diacylphosphatidylcholine with or without fatty acids were examined. These ternary codispersions were readily hydrolyzed by phospholipase A_2, and they underwent a rapid change in turbidity. Nonideal mixing or phase separation in the ternary codispersions is postulated to be responsible for their enhanced susceptibility to pig pancreatic phospholipase A_2, as well as for their tendency to undergo spontaneous change in turbidity, presumably due to spontaneous fusion of the vesicles. Both of these processes were inhibited by a variety of structurally unrelated solutes like *n*-hexanol and mepacrine. These and other observations are interpreted to suggest that structural defects in bilayers of ternary codispersions are a common locus for the binding of phospholipase A_2 and are responsible for the process underlying the change in turbidity. The experiments described here suggest that many of the common inhibitors of phospholipase A_2 owe their effects to their ability to modify the quality of the substrate interface, rather than to a direct interaction with the enzyme.

An aqueous dispersion of either lysophospholipid or fatty acid alone forms micelles under most conditions ([1]; see, however, Refs. 2 and 3), whereas an aqueous dispersion of these two types of compounds together forms a bilayer, as evidenced by a variety of physical studies [4–6]. The ability of a ternary codispersion containing diacylphosphatidylcholine, lysophosphatidylcholine, and fatty acid to interact with pig pancreatic phospholipase A_2 [7, 8] is particularly striking because this enzyme does not interact with bilayers formed from any one or two of these three components [7, 8]. To account for these observations, we have postulated that in binary codispersions lysophosphatidylcholine and fatty acid molecules interact to form a functional dimer [4, 5], and that in ternary codispersions phospholipase A_2 binds to the defect sites created by phase separation of the components [7, 8]. In this paper we further examine the consequences of these two postulates.

Phase separation in codispersions implies different degrees of interaction between the components [9, 10]. To understand such interactions, we have studied the properties of codispersions of a variety of lysophosphatidylcholine analogs with fatty acids and/or diacylphosphatidylcholine. Thus, we have examined the thermotropic transition properties, the ability to interact with phospholipase A_2, the ability to undergo an increase in turbidity (presumably due to fusion and aggregation), and the inhibitory effect of a variety of solutes on these processes. The results show that, while the specific interaction between a lysophospholipid and a fatty acid is not dominant in regulating the thermotropic properties of the binary codispersion, the properties related to phase separation in the ternary codispersions may depend upon such interactions. Moreover, it is shown that similar features of bilayer organization regulate the change in turbidity of vesicles as well as the interaction of phospholipase A_2 with the vesicles.

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MATERIALS AND METHODS

All compounds used in this study were analytical grade or better than 99% pure as judged by thin-layer chromatography. Phospholipids and inhibitors were purchased from Calbiochem (La Jolla, CA), Sigma (St. Louis, MD), Medmark (Munich), or Senn Chemicals (Basel), or prepared by appropriate methods described in the literature [11].

Unless stated otherwise, all codispersions were prepared by mixing the lipid components in chloroform + methanol (1:1) solution, removing the organic solvent and drying the film in vacuum, and then suspending the lipid film in an aqueous buffer at 50–70° [7, 8]. Typically, for measuring the turbidity change and for the phospholipase A_2 interaction, the dispersions were sonicated in a bath-type sonicator for 5–15 min until they became clear. In all cases, whether sonicated or not, the dispersions were stored for several hours above their phase transition temperature. Under most conditions, ternary codispersions undergo a rapid increase in turbidity at room temperature; therefore, they were routinely stored at 55°. For calorimetric studies, unsonicated dispersions (15 or 25 μl) containing 1–2 μmoles of lipids in 100 mM KCl and 200 mM morpholino-propane sulfonic acid (MOPS) or Tris at pH 8.0 were used in sealed aluminum pans. These samples were usually scanned between a 10 and 70° range on a Perkin–Elmer DSC-1B or Mettler TA2000B calorimeter at 2.5° or 1°/min at appropriate sensitivity

[12]. For kinetic measurement of phospholipase A_2-catalyzed hydrolysis by titration on a pH-Stat titrator (Radiometer) at pH 8.0, the sonicated lipid dispersions were made in 100 mM KCl and 10 mM $CaCl_2$ [8]. The reaction was initiated by adding 0.5 μg phospholipase in 2 μl buffer to 5 ml reaction mixture containing 0.5 mM substrate. The rates of hydrolysis are given as micromoles per minute per milligram of protein. Binding of pig pancreatic phospholipase A_2 to the lipid dispersions according to the criteria described elsewhere [7] was monitored by measuring the increase in intrinsic fluorescence intensity of tryptophan at 328 nm on a Perkin–Elmer MPF-45 or SLM-4800 spectrofluorometer [7]. The kinetics of the change in turbidity was monitored at 360 nm in stirred and thermostated curvettes on a Beckman Acta III spectrophotometer. The sonicated dispersions for turbidimetric measurements were prepared in 100 mM KCl, 200 mM Tris or 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) at pH 8.0.

Specific experimental conditions are given in the figure legends and in the footnotes of the tables. To improve the clarity in some of the figures, the data points are not given.

RESULTS

Phase transition properties of aqueous codispersions of lysophospholipids with palmitic acid or n-*hexadecanol.* Elsewhere we have shown that equimolar codispersions of 1-acylphosphatidylcholine with various fatty acids exhibit a thermotropic gel to liquid crystalline phase transition that depends on the acyl chain length of the two components as well as on the state of ionization of the fatty acid [4, 5]. It was, therefore, of interest to examine the effect of changing the structure of the glycerophosphorylcholine moiety on the thermotropic phase transition properties of the binary codispersions of the analogs of lysophosphatidylcholine with palmitic acid or with 1-hexadecanol. All the codispersions containing lysophospholipid analogs that we examined exhibited an endothermic transition that was also observed on repeated scans and at a somewhat lower temperature (about 5° lower) in the cooling scan. None of these codispersions exhibited a pre- or a subtransition even after storage for several weeks at 4°. As summarized in Table 1, the mid-points of the transitions for the palmitic-acid-containing binary codispersions of lysophospholipids were in the 42–45° range, and their enthalpies were between 6 and 8 kcal/mole (data not given). The data summarized in Table 1 demonstrate that the phase transition temperatures for the binary codispersions did not depend upon the minor structural changes in the glycerophosphorylcholine moiety. The ether and the hydrocarbon analogs of the ester moiety in diacylphosphatidylcholines exhibit similar behavior [13]. The codispersions containing fatty acids had lower transition temperatures and enthalpies than the hexanol-containing codispersions. There was little effect of changing the oxygen functions in the glycerol backbone. In fact, codispersions of *n*-hexadecylphosphocholine and 1-palmitoyl-lysophosphocholine with fatty acid or *n*-hexadecanol have essentially similar phase transition characteristics [5]. Particularly interesting is the codispersion of 1-palmitoylglycerol and hexadecylphosphocholine which showed a broad transition at 39°. A 5° range in the transition temperatures of the various binary codispersions could have been due to entropic factors arising from differences in hydration or to differences in the interfacial pK_a of

Table 1. Phase transition temperatures (°) of codispersions containing lysophospholipids with palmitic acid or *n*-hexadecane-1-ol*

	Lysophospholipid†	Temperature	
		+ Palmitic acid	+*n*-Hexadecanol
I	1-Palmitoylglycol PC	43°	55°
II	*n*-Hexadecylphosphocholine	45°	55°
III	1-Palmitoyl-2-deoxy-lyso-PC	44°	57°
IV	1-Palmitoyl-lyso-PC	43°	57°
V	1-Palmitoyl-lyso-β-PC	42°	53°
VI	1-Hexadecyl-lyso-PC	44°	57°
VII	1-Hexadecyl-2-methyl-lyso-PC	44°	58°
VIII	1-Hexadecyl-2-acetyl-lyso-PC	40.5°	48°
IX	II + 1-Palmitoylglycerol	39°	
X	1-Palmitoyl-lyso-PE	58°	63°
XI	1-Palmitoyl-lyso-PG	43°	58°
XII	1-Elaedoyl-lyso-PC	43°	57°
XIII	1-Stearoyl-lyso-PC	49°	59°
XIV	1-Myristoyl-lyso-PC	42°	53°
	IV + Myristic acid	36°	
	IV + Stearic acid	54°	

* All dispersions were made by premixing equimolar amounts of components and then dispersing the dried film in 100 mM KCl + 200 mM MOPS or Tris at pH 8.0. Fifteen microliters of the codispersions containing 1–2 μmoles of lipids was scanned at 2.5°/min on a Perkin–Elmer DSC1B. Transition temperatures given here are the midpoints of the profile in the heating rum.

† Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; and PG, phosphatidylglycerol.

the fatty acid. Similarly, 10–12° higher transition temperatures for the *n*-hexadecanol-containing codispersions were probably due to an increase in the effective acyl chain length by at least one methylene residue. While changes in the glycerophosphocholine moiety had little effect on the transition temperature, the codispersions of lysophosphatidylethanolamine exhibited the transition at 58° for palmitic acid and at 63° for the *n*-hexadecanol-containing codispersions, compared to a transition temperature of 65° for the dispersions of dipalmitoylphosphatidylethanolamine. Thus, the transition temperatures of the binary dispersions were generally very similar to the transition of the corresponding diacylphospholipid. Such a similarity between the transition temperatures of the binary codispersion and the corresponding diacylphospholipids suggests that specific interactions in the head group region are not required for the packing of the acyl chains in the gel phase. The gross orientation of the components in a bilayer is predominantly due to their amphiphilic character, and the acyl chains in the gel phase are hexagonally packed. Therefore, rotational freedom along the long axis of the acyl chains does not change significantly during the gel to liquid crystalline phase transition in these binary dispersions, compared to their covalent analog diacylphospholipids.

Phase transition properties of the ternary codispersions of diacylphosphatidylcholine, palmitic acid, and analogs of lysophosphatidylcholine. The ternary codispersions of diacylphosphatidylcholine, lysophosphatidylcholine and fatty acid exhibit several peaks in their broad phase transition profiles [5]. Similar behavior was observed with the ternary codispersions containing the various lysophosphatidylcholine analogs. A typical differential thermal analysis profile and the temperature dependence of the steady-state fluorescence anisotropy of diphenylhexatriene in the ternary codispersions are shown in Fig. 1. In this and all the other ternary codispersions we have tested, the transition range was broad, and often separate transitions approximately corresponding to those of diacylphosphatidylcholine and those corresponding to the binary codispersions of lysophospholipid and fatty acid were discernible, even though the ternary codispersions were prepared from the components premixed and dissolved in organic solvents. Such profiles with multiple transitions strongly suggest that nonideal mixing or phase separation occurs in the ternary codispersions containing the various lysophospholipids. For most studies in this paper we have used the ternary codispersions containing dimyristoylphosphatidylcholine, palmitic acid, and palmitoyllysophospholipids. In such dispersions, the transitions corresponding to the two or more phases occurred between 23 and 45°, and they were discernible by calorimetry and by the fluorescence anisotropy measurements (Fig. 1). The ternary codispersions containing identical chains in all the three components exhibited phase separation over a much narrower temperature range.

Interaction of phospholipase A_2 with ternary codis-

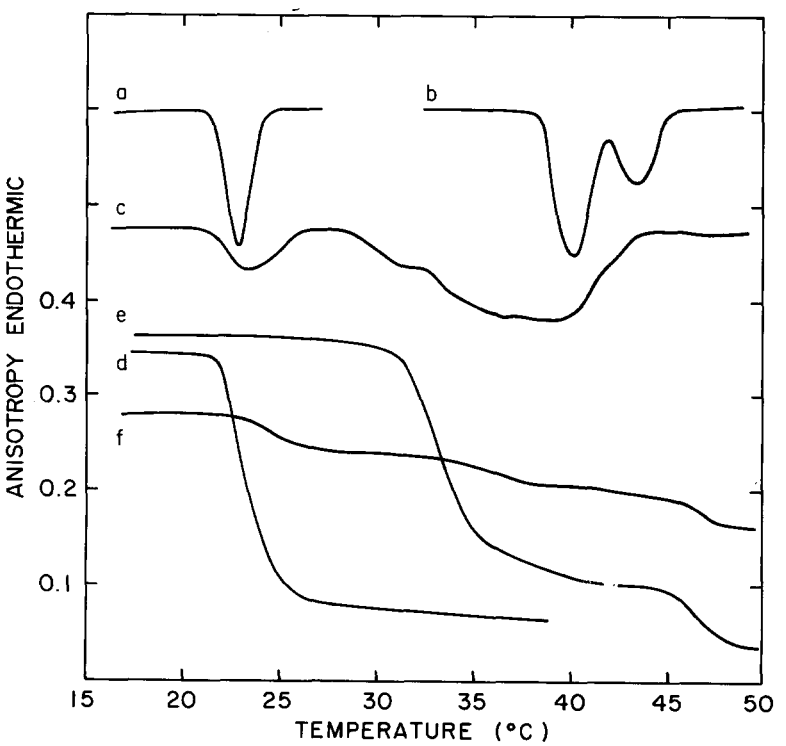

Fig. 1. Phase transition profiles by differential scanning calorimetry (top, a–c) and anisotropy of fluorescence polarization of diphenylhexatriene (bottom, d–f). Profiles a and d are for dimyristoylphosphatidylcholine; b and e for 1-palmitoyllysophosphatidylcholine and palmitic acid (1:1 mole ratio); and c and f for the ternary codispersions containing dimyristoylphosphatidylcholine, 1-palmitoyllysophosphatidylcholine and palmitic acid (2:1:1 mole ratio). All samples were buffered at pH 8.0.

persions. Elsewhere we have shown that an enhanced action of pig pancreatic phospholipase A_2 with the ternary codispersions is due to an increased binding of the enzyme [7, 8]. Now we have extended this observation. As summarized in Table 2, the ternary codispersions containing several lysophospholipid analogs and fatty acid homologs also facilitated binding and catalytic action of the enzyme. The dispersions containing lysophospholipid analogs exhibited essentially the same affinity for the enzyme, whereas the ternary mixtures containing homologous free fatty acids or homologous lysophosphatidylcholines appeared to show some specificity for the phospholipase interaction. This is mainly because, as shown in Fig. 2 (curves a and b), both the binding and the catalytic action of the pancreatic phospholipase A_2 on the ternary codispersions were seen only over their phase transition range. For the ternary codispersions containing a shorter or a longer chain component, the phase transition range was shifted below or above the experimental temperature of 30°. As expected, interaction of phospholipase A_2 with the ternary codispersions with lower and higher transition temperatures was demonstrated over the temperature range of the phase transition (Fig. 2).

Table 2. Interaction of phospholipase A_2 with the ternary codispersion containing dimyristoylphosphatidylcholine + fatty acid + lysophospholipid (4:1:1 mole ratio)*

Lipid mixture (DMPC +)	Initial rate (arbitrary)	ΔF (%)
+ I + FA_{16}	80	40
+ II + FA_{16}	70	55
+ III + FA_{16}	70	58
+ IV +FA_{16}	80	62
+ VII + FA_{16}	70	60
+ VIII + FA_{16}	70	65
+ V + FA_{16}	12	15
+ XII + FA_{16}	9	16
+ XIII + FA_{16}	35	60
+ XIV + FA_{16}	45	55
+ XV + FA_{16}	62	50
+ IV + 16-ol	60	30
+ IV + FA_{10}	0 ($\tau > 15$ min)	<5
+ IV + FA_{12}	0 ($\tau \sim 5$ min)	<5
+ IV + FA_{14}	30	40
+ IV + FA_{18}	60	50
DSPC + DMPC	0 ($\tau > 10$ min)	<5
DPPC + DMPC	0 ($\tau > 10$ min)	<5
DLPC + DPPC	0 ($\tau \sim 5$ min)	<5
DMPC	0 ($\tau \sim 5$ min)	<5
DMPC + FA_{10-18}	0 ($\tau > 5$ min)	<5

* All measurements were done at 30° in solutions containing 100 mM KCl, 10 mM $CaCl_2$ at pH 8.0. The medium for the binding runs was buffered with 20 mM Tris. The initial rates of hydrolysis were measured by starting the reaction with the addition of 2.5 μg pig pancreatic phospholipase A_2 to 5 ml reaction mixture containing 600 μM dimyristoylphosphatidylcholine, 150 μM fatty acid and 150 μM lysophospholipids (for details see Ref. 14). For binding studies, the mixture contained 600 μM ditetradecylphosphatidylcholine + 150 μM fatty acid, and 150 μM lysophospholipid and 5 μM pig pancreatic phospholipase. In some binding experiments, appropriate homologous ether or the D-analog was used. Abbreviations: FA_n fatty acid of chain length n; 16-ol, *n*-hexadecanol; DRPC, diacylphosphatidylcholines. R = S, stearoyl; P, palmitoyl; M, myristoyl; and L, lauroyl. The lipid components with roman numerals refer to the lysophospholipids given in Table 1.

It may be noted that the interaction of phospholipase A_2 was not seen with dispersions of single phospholipids or with several binary mixtures at their phase transition temperatures. For example, the mixtures of dimyristoyl- and distearoylphosphatidylcholines or didecanoyl- and dipalmitoylphosphatidylcholines did not interact with phospholipase A_2, even though these dispersions are known to exhibit nonideal mixing [9, 10]. The negative charge on the interface did not appear to be absolutely necessary for the interaction of phospholipase A_2 with the ternary codispersions because the palmitic acid could be replaced by *n*-hexadecanol with only a slight reduction in binding. On the other hand, the binary codispersions containing fatty acids with lysophospholipids, or fatty acids and diacylphospholipids, or lyso- and diacylphospholipids do not interact with phospholipase A_2 ([7, 8]; see, however, Ref. 14). These observations essentially rule out the possibility that binding of monomer or micellar lysophospholipid is one of the first steps involved in the interaction of phospholipase A_2 with diacylphospholipids. Also, the "fluidity" of the bilayer was not an important factor for the binding because the bilayers in the liquid crystalline phase, as in didecanoyl- or 1-palmitoyl-2-oleoylphosphatidylcholine liposomes at 25–30°, did not interact with phospholipase A_2. These observations suggest that the interactions of pig pancreatic phospholipase A_2 with the phospholipid dispersions were not simply regulated by "fluidity" or by hydrophobic partitioning.

The phospholipase activating effect of lysophospholipids and fatty acids in dimyristoylphosphatidylcholine vesicles depended upon their mole fractions. As shown in Fig. 3, the rate of hydrolysis and the extent of binding of phospholipase A_2 to the ternary codispersions increased non-linearly with the increasing mole fraction of the products in the ternary codispersions.

Inhibition of phospholipase A_2 action on the ternary codispersions. A variety of lipid soluble agents are known to modulate the phase properties of bilayers [10, 12, 15] and thus influence the action of phospholipase A_2. We have examined the effects of several additives. The effect of *n*-hexanol was particularly interesting because elsewhere we have shown that *n*-hexanol facilitates the action of phospholipase on bilayers of diacylphosphatidylcholines [16]. As shown in Fig. 4, *n*-hexanol inhibited the action of pig pancreatic phospholipase A_2 on the ternary codispersions. The kinetics of hydrolysis is rather complex. Hexanol inhibits not only the initial rate but also the extent of hydrolysis. Such features of the kinetics are under investigation; however, for the present we have taken the amount of product formed in 2 min as a measure of the initial rate of hydrolysis of the ternary codispersions. As shown in Fig. 5, the rate of hydrolysis decreased with increasing concentration of *n*-hexanol, and 50% inhibition of the phospholipase A_2 activity was attained at about 2.5 mM hexanol concentration ($= I_p$). As shown in Fig. 5, the inhibitory effect of *n*-hexanol was apparently due to perturbation of the binding of

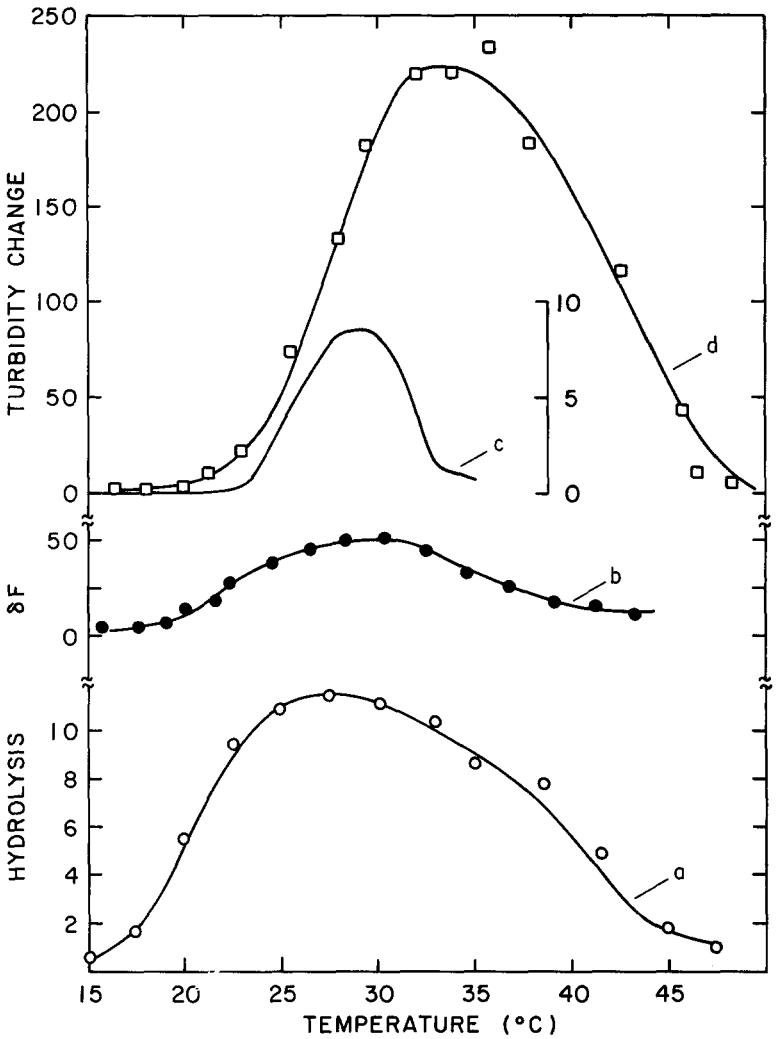

Fig. 2. Temperature dependence of phospholipase A_2 catalyzed rate of hydrolysis (bottom), fluorescence change on binding of phospholipase A_2 (middle), and the rate of change in turbidity (top). Curves a, b, and d were obtained with the ternary codispersions of ditetradecyl- (for b) or dimyristoylphosphatidylcholine (for a and d), 1-palmitoyllysophosphatidylcholine, and palmitic acid (3:1:1). Ordinate for curve c is expanded 10-fold (scale on the right; data points not shown) and the rate of turbidity change was obtained by adding 1-palmitoyllysophosphatidylcholine to vesicles of dimyristoylphosphatidylcholine.

the enzyme, as indicated by quenching of tryptophan fluorescence enhancement of the bound enzyme. This change in binding of the enzyme was most likely due to a change in the phase transition characteristics of the ternary codispersions in the presence of *n*-hexanol. As shown in Fig. 6, the phase transition profile of the ternary codispersions containing *n*-hexanol did not exhibit the characteristic multiple phase transitions seen with the ternary codispersions alone (cf. Fig. 2).

The inhibitory effects of several other solutes including butacaine, dibucaine, and mepacrine were examined, their I_p values are given in Table 3. Indeed, some of the most effective inhibitors of phospholipase action reported in the literature [15], such as mepacrine, were among the most effective inhibitors in this substrate system. There does not appear to be any obvious correlation of inhibitory potency with the hydrophobicity or positive charge of these drugs, even though like *n*-hexanol they apparently interfered with the binding of the enzyme to the substrate codispersions. We have found similar inhibitory effects with several uncharged polyethers which are known to exhibit anti-tuberculosis activity [17].

Effect of lysophospholipid analogs on the turbidity change in the ternary codispersions. Quite early in our work with the ternary codispersions we noted that during storage at room temperature the sonicated ternary codispersions became turbid, even though these dispersions remained clear indefinitely when stored above or below their phase transition temperature. Time dependence of the increase in turbidity of the ternary codispersions quenched from 55° to 30° is shown in Fig. 7. We have not yet established the process underlying the change in

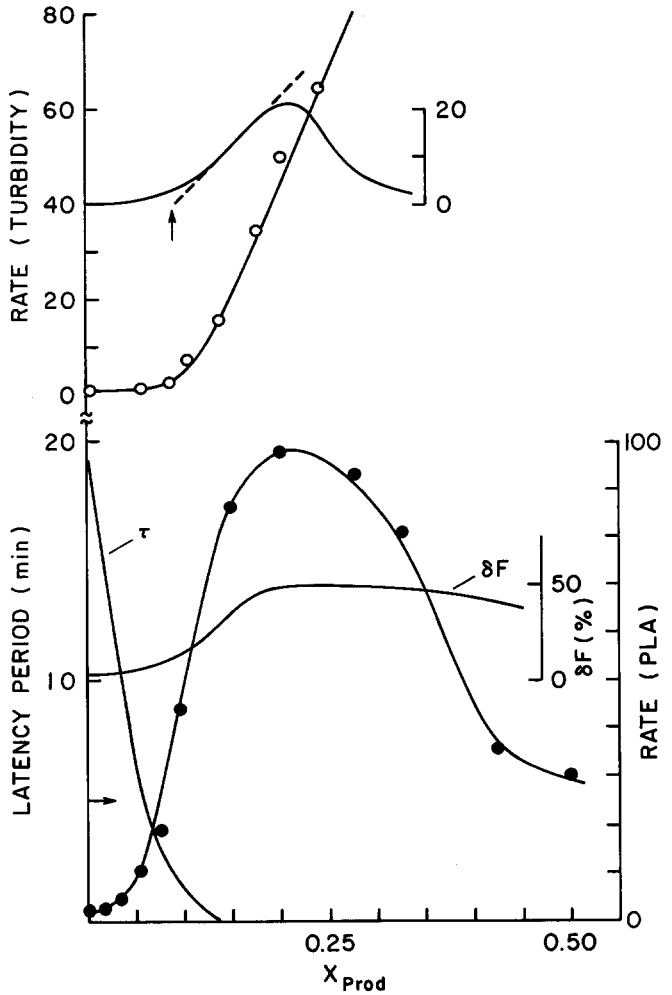

Fig. 3. Initial rate of phospholipase A_2 catalyzed hydrolysis of dimyristoylphosphatidylcholine and its latency period (bottom) and the rate of turbidity change (top) as a function of the mole fraction of 1-palmitoyllysophosphatidylcholine without (smooth curve given without data points, top) or with palmitic acid (curve with data points). Fluorescence change indicative of binding of phospholipase A_2 to the ternary codispersions containing ditetradecylphosphatidylcholine is also shown in the lower part of the figure. All measurements were done at pH 8.0 and 30°. It may be pointed out that elsewhere [14] we also reported on the dependence of the latency period on the initial rate of phospholipase A_2 catalyzed hydrolysis of the ternary codispersion prepared by adding the binary codispersions of the products to the preformed dimyristoylphosphatidylcholine vesicles. For the data presented here, the ternary codispersions were prepared by dispersing the premixed lipid components. This difference in the method of preparation of the sample accounts for the discrepancy in the results.

turbidity; however, the following observations suggest that the change in turbidity was substantially, if not exclusively, due to fusion of the vesicles in the ternary codispersion.

(a) Dilution of the dispersions at the end of the increase in turbidity did not result in a decrease in turbidity beyond what was expected on the basis of the dilution factor.

(b) The rate of change in turbidity showed a second order dependence upon the concentration of the ternary vesicles, indicating that collision of two vesicles was rate limiting. A similar concentration dependence has been noticed in the fusion process [18].

(c) The rate of change in turbidity showed a strong dependence upon temperature. As shown in Fig. 2, the maximum rate was observed within the temperature range of the phase transition of the ternary vesicles.

(d) Substitution of palmitic acid with *n*-hexadecanol in the ternary vesicles did not change significantly the rate of change in turbidity. Similarly, the rate of change in turbidity did not depend significantly upon the monovalent ion concentration between 5 and 500 mM KCl or NaCl. However, the rate of change in turbidity of the fatty-acid-containing ternary codispersions increased several-fold in the presence of calcium ions. Such an effect of calcium ions was not observed with *n*-hexadecanol-containing ternary dispersions.

(e) Dispersions prepared from premixed dimyristoylphosphatidylcholine with palmitic acid or

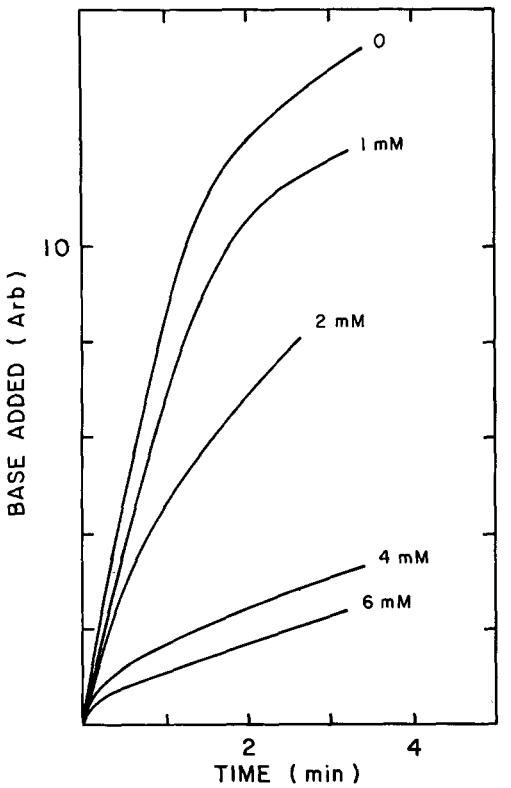

Fig. 4. Reaction progress curves for the hydrolysis of the ternary codispersions (10:2:2) by phospholipase A_2 in the presence of *n*-hexanol at the concentrations indicated.

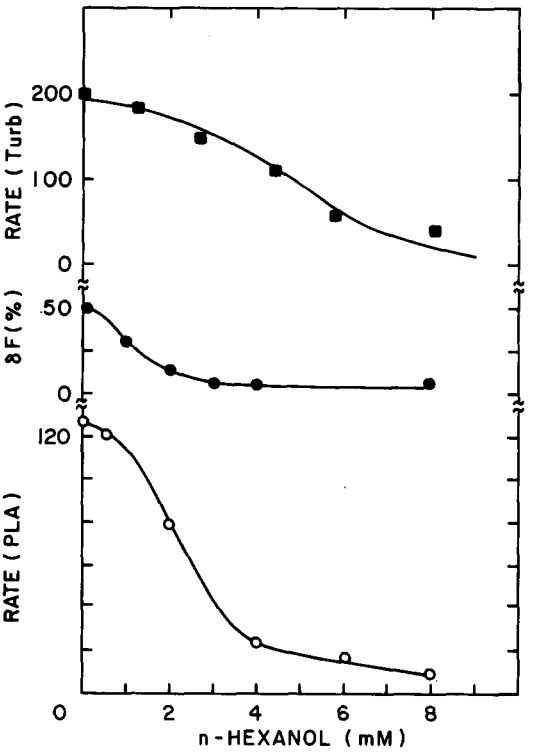

Fig. 5. Dependence of the rate of hydrolysis by phospholipase (bottom) and of the fluorescence change on the binding of phospholipase (middle) to the ternary codispersions and the rate of change of turbidity of the ternary codispersions on the concentration of *n*-hexanol.

with lysophosphatidylcholine exhibited only a slow (× 0.01) change in turbidity even at their phase transition temperatures (Fig. 2). However, a rapid change in the turbidity at the phase transition temperature of the diacylphospholipid vesicles can be induced by adding a solution of lysophosphatidylcholine [18] or with premixed fatty acids [19].

Taken together these observations strongly suggest that the time-dependent change in turbidity shown in Fig. 7 was due predominantly to fusion or a related process. The contribution of vesicles aggregation was probably insignificant. Unfortunately, due to a spontaneous and rapid rate of the underlying process, we could not use the experimental criteria developed for monitoring the induced fusion in bilayers [20, 21]. We are still investigating other possibilities to identify the process underlying the change in turbidity. However, for the main theme of this paper, a knowledge of the exact mechanism of the change in turbidity is not crucial. In the rest of this paper we show that the factors which govern the change in turbidity also regulate the interaction of pig pancreatic phospholipase with the ternary codispersions.

Effect of inhibitors on the change in turbidity. As shown earlier, several compounds inhibited the action of pig pancreatic phospholipase A_2 on the ternary codispersions. Indeed, these compounds also inhibited the rate of change in turbidity of the ternary vesicles. Concentrations of several compounds required for 50% inhibition (I_t) of the rate of turbidity change are given in Table 3. All the inhibitors that blocked the change in turbidity also inhibited the phospholipase action. There is a significant correlation between the two sets of data summarized in Table 3 (the correlation coefficient is better than

Table 3. Concentrations of inhibitors for phospholipase A_2 action (I_p) and for the change in turbidity (I_t) of the ternary codispersions*

Inhibitor	I_p (μM)	I_t (μM)
n-Hexanol	2340	4340
EMD 21657†	348	232
Mepacrine	58	55
Chloroquin	273	390
Chlorpromazine	42	78
Butacaine	106	183
Amitryptyline	100	140
Dibucaine	50	158
Tetracaine	124	432
AC 3574	37	63

* I_p is the inhibitor concentration that reduced the rate of pig pancreatic phospholipase A_2 catalyzed hydrolysis by 50%. I_t is the concentration that inhibited the rate of change in turbidity by 50%. Conditions were as given in the legends to Figs. 5 and 7. The ternary codispersions contained dimyristoylphosphatidylcholine, 1-palmitoyllysophosphatidylcholine and palmitic acid (4:1:1 mole ratio).

† Compound was obtained from E. Merck A.G., Darmstadt, West Germany.

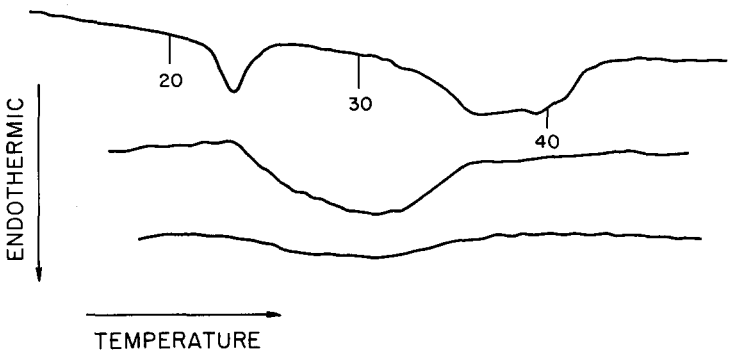

Fig. 6. Phase transition profiles of the ternary codispersions (top) in the presence of *n*-hexanol (middle) and dibucaine (bottom).

0.90). Thus far we have examined over 100 compounds reported to be inhibitors of phospholipase A_2. In all these cases we found a strong correlation between the inhibitory effect on phospholipase action and on the turbidity change (to be published). Such a correlation suggests that the primary effect of these inhibitors is on the characteristics of the bilayer that facilitate binding of the enzyme.

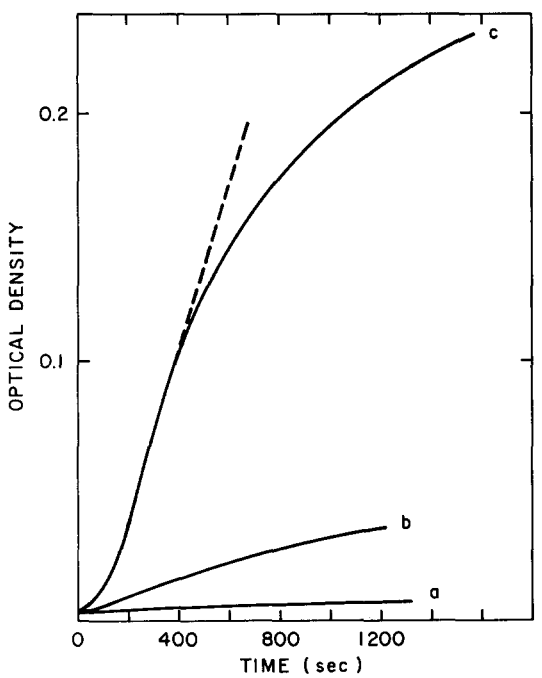

Fig. 7. Time dependence of the change in turbidity of the sonicated codispersions of dimyristoylphosphatidylcholine premixed with lysophosphatidylcholine (a), with freshly added lysophosphatidylcholine (b), and with premixed lysophosphatidylcholine and fatty acid (1:1) (c). The rate of change in turbidity is taken as the slope of the dotted line. All such measurements were made in 100 mM KCl, 200 mM Tris at pH 8.0 and 30° unless stated otherwise. Typically dimyristoylphosphatidylcholine was 2.0 mM, and the reaction was initiated by quenching 75 μl of the stock lipid dispersions preincubated at 55°. For curve (b), a stock solution of lysophospholipid was added to the preformed vesicles.

Although inhibition of the phospholipase A_2 binding dependent fluorescence change could be shown by nonchromophoric compounds like *n*-hexanol (Fig. 5), methods have as yet to be developed to demonstrate similar changes for the chromophoric inhibitors. Some of the qualitative trends are also interesting: several lipid soluble agents like indomethacin, halothane, and A_2C (the "membrane mobility agent") did not have any inhibitory effect; not all inhibitors were cationic; several uncharged solutes like *n*-hexanol also inhibited both the processes. These observations suggest that, at least qualitatively, the inhibitory effects of these compounds are not due simply to their lipid solubility alone or to their positive surface charge.

Effect of freshly added lysophosphatidylcholine analogs on the rate of turbidity and phospholipase A_2 action on dimyristoylphosphatidylcholine vesicles. Elmaharani and Blume [18] have shown that addition of aqueous lysophosphatidylcholine solution to diacylphosphatidylcholine vesicles at their phase transition temperature induces a change in turbidity presumably due to fusion of the vesicles. Similar changes in turbidity can be induced by other lysophospholipids. As shown in Fig. 3, the rate of change in turbidity increased with the mole fraction of freshly added aqueous solution of the lysophospholipid. At low mole fractions the rate changed slowly, but at higher mole fractions the change was faster. From such data we obtained the mole fraction of lysophospholipid (X_t) at which the steeper slope just began. These X_t values for several lysophospholipids are summarized in Table 4.

The hydrolytic action of pig pancreatic phospholipase A_2 on diacylphosphatidylcholine vesicles is accompanied by a latency phase, the duration of which depends upon the presence of additives in the substrate vesicles [8]. Elsewhere we showed that the latency period during the action of phospholipase A_2 on dimyristoylphosphatidylcholine vesicles is decreased by freshly added lysophospholipids [14]. Direct binding experiments with the vesicles of ditetradecylphosphatidylcholine show that this decrease in latency period with freshly added lysophospholipids is due to an increase in the fraction of the phospholipase A_2 bound to the vesicles. Thus, a change in the latency period is a direct measure of the enzyme bound to the vesicles. As shown in Fig.

Table 4. Mole fractions of freshly added lysophospholipids required to increase the rate of change of turbidity (X_t) and that required to lower the latency phase (X_p) in the reaction progress curve for hydrolysis by pig pancreatic phospholipase A_2*

	Lysophospholipid	X_t	X_p
I	1-Palmitoylglycol PC	0.095	0.13
II	*n*-Hexadecylphosphorylcholine	0.091	0.056
	Tetradecylphosphorylcholine	0.057	0.091
	Octadecylphosphorylcholine	0.074	0.21
III	1-Palmitoyl-2-deoxy-lyso-PC	0.09	0.082
IV	1-Palmitoyl-lyso-PC	0.011	0.09
XV	1-Lauroyl-lyso-PC	0.16	0.16
XIV	1-Myristoyl-lyso-PC	0.070	0.074
XIII	1-Stearoyl-lyso-PC	0.085	0.010
XII	1-Elaedoyl-lyso-PC	0.08	0.15
VI	1-Hecadecyl-lyso-PC	0.070	0.055
VII	1-Hexadecyl-2-methyl-lyso-PC	0.055	0.057
VIII	1-Hexadecyl-2-acetyl-lyso-PC	0.074	0.07
X	1-Palmitoyl-lyso-PE	0.22	0.27
V	1-Palmitoyl-lyso-β-PC	0.25	0.30

* The values of X_t and X_p were obtained as shown in Fig. 9. The abbreviations for lysophospholipids are given in the † footnote of Table 1. All studies were done at pH 8.0 and 30° on dimyristoylphosphatidylcholine vesicles to which an aqueous solution of lysophospholipid was freshly added.

8, the latency period decreased with increasing mole fractions of lysophospholipid freshly added to the vesicles of dimyristoylphosphatidylcholine. The steady-state rate of hydrolysis at the end of the latency phase did not change at low mole fractions of lysophospholipids. From such data we have obtained the mole fraction of lysophospholipid (X_p) required to decrease the latency period to 5 min. The values of X_p for the various lysophospholipids are summarized in Table 4. It appears that the inhibitors somehow modulate the effect of freshly added lysophosphatidylcholine. As shown in Fig. 9, preincubation of dimyristoylphosphatidylcholine vesicles with butacaine increased the latency period, whereas the steady-state rate was inhibited only at higher butacaine concentrations. The effect of butacaine on the latency period in this system is somewhat intriguing, because in the ternary system the effect of the inhibitors was on the initial rate and the extent of hydrolysis. This is probably because the mole fractions of the products in the ternary codispersions used in these studies were about 20%, whereas the mole fraction of lysophosphatidylcholine added to the preformed dimyristoylphosphatidylcholine was about 7%.

The X_p and X_t values are defined arbitrarily; however, they provide a measure of the change in dimyristoylphosphatidylcholine vesicles induced by freshly added lysophospholipids. Although the values of X_p and of X_t for the various lysophospholipids are not identical, there is a significant correlation between the X_p and X_t values. This would imply that similar features of bilayer organization induced by lysophospholipids regulate the change in turbidity, as well as promote binding of phospholipase A_2. It should be noted that the X_p and X_t values fall in narrow range, and the resulting concentrations do not bear any obvious correlation to the critical micelle concentrations of these phospholipids. The activating effect of lysophospholipids on the turbidity change or on phospholipase A_2 interaction was seen only when added to preformed diacylphospholipid vesicles. In this regard, 1-alkyl-2-acetyl-phosphatidylcholine (PAF or platelet activating factor) and its analog 1-alkyl-

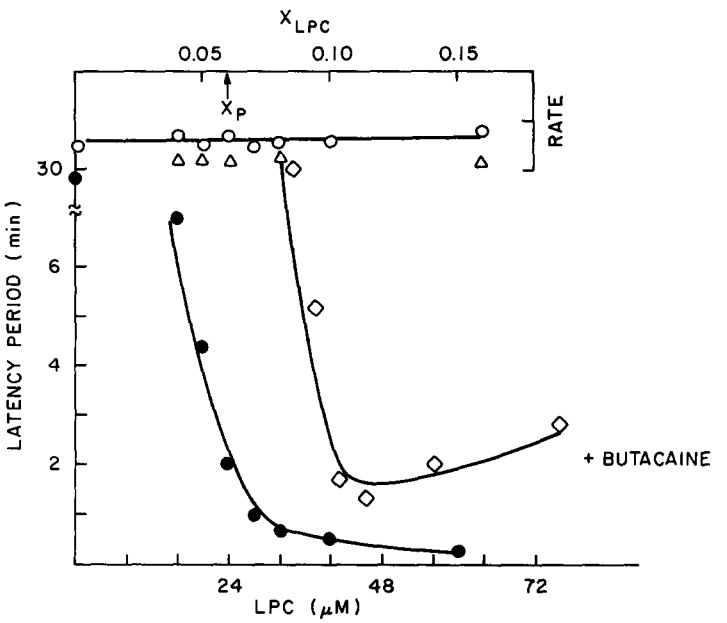

Fig. 8. Dependence of the latency period for the action of pig pancreatic phospholipase A_2 on deoxylysophosphatidylcholine concentration added to the vesicles of dimyristoylphosphatidylcholine concentration (400 μM) in the presence (◇) and absence (●) of butacaine (160 μM). The steady-state rates of hydrolysis in the presence (△) and absence (○) of butacaine did not change significantly.

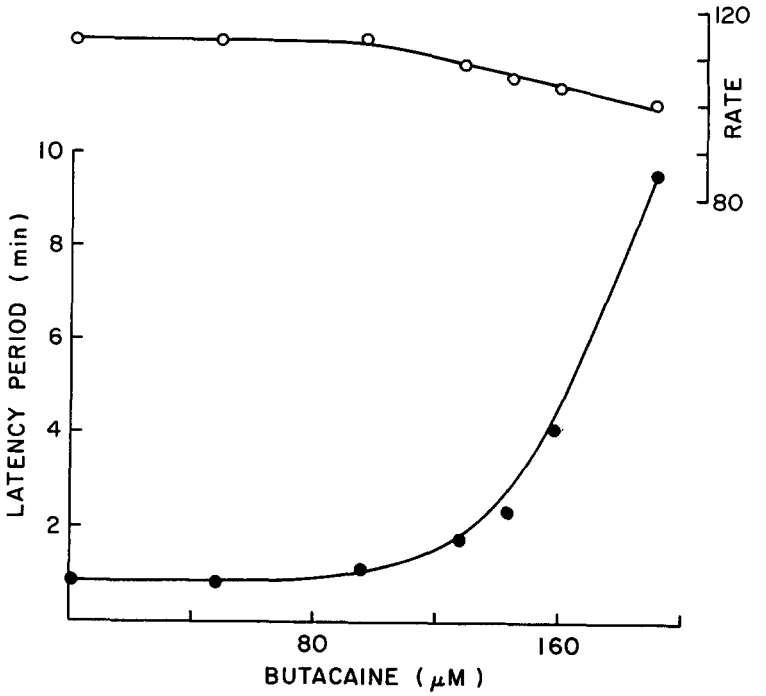

Fig. 9. Dependence of the latency period (●) and the steady-state rate of hydrolysis (○) on the butacaine concentrations for the hydrolysis of dimyristoylphosphatidylcholine vesicles (400 μM) activated by freshly added lysophosphatidylcholine (20 μM).

2-methyl-phosphatidylcholine are among the most effective lysophospholipids we have studied. Physiologically, an asymmetric distribution of these compounds could be accomplished by secretion or by activation of intracellular lysophospholipase.

DISCUSSION

In this paper we have shown that binary codispersions of a variety of monoacylphospholipids and lysophospholipids with fatty acids exhibited a thermotropic gel-to-liquid crystalline phase change in bilayers. The ternary codispersions containing diacylphosphatidylcholine along with the above lipids exhibited multiple transitions over a broad temperature range. In this temperature range, the ternary codispersions were readily hydrolyzed by pig pancreatic phospholipase A_2 and they underwent a spontaneous change in turbidity.

Both the rate of change in turbidity and the rate of phospholipase A_2 catalyzed hydrolysis were inhibited by solutes such as phenothiazines, local anesthetics, and *n*-hexanol. These and other experiments suggest that similar features of bilayer organization in the ternary codispersions, as well as in dimyristoylphosphatidylcholine vesicles containing freshly added lysophospholipids, are a common locus for the action of phospholipase A_2 and for the change in turbidity.

The observations reported here have a bearing on several features of the action of phospholipase A_2 on bilayers. The results show that the activating effect of freshly added lysophospholipids or of premixed lysophospholipid and fatty acid codispersions was primarily due to a modification of the bilayer organization. Some of the specificity exhibited by lysophospholipids could be due to differences in their abilities to modify the phase properties of the bilayer. Similarly, the inhibitory effects of certain solutes also appear to be due to their abilities to modify the phase properties of the bilayers. Furthermore, the effects of the inhibitors were probably not due to modulation of the surface charge but to their effects on the phase properties of the codispersions.

Obviously we have not explored all the possible combinations of fatty acids, lysophospholipids, diacylphospholipids, and inhibitors. Based on the data presented here alone one can make a strong argument for the primary importance of the phase properties of the substrate bilayer in regulating the action of phospholipase A_2 and the change in turbidity. This is in general agreement with some of the suggestions made elsewhere [7, 8, 16]. The data presented here and elsewhere [7, 8, 16] can be used to elaborate further on this theme. For example, it can be essentially ruled out that the "fluidization" of the bilayer or coexistence of gel and liquid crystalline phases in the substrate bilayer alone leads to significant interaction with phospholipase A_2. Thus, for example, phospholipase A_2 binds only weakly to bilayers of single or mixed dialkylphospholipids, such as ditetradecylphosphatidylcholine, 1-palmitoyl-2-octadec-9-enyl-phosphatidylcholine, D-didecanoyl- and D-dipalmitoylphosphatidylcholine alone or in mixed bilayers, even at their phase transition temperatures. This is further confirmed by the fact that the reaction progress curves for these substrates exhibit a measurable latency phase. In contrast,

freshly added lysophospholipids or premixed lysophospholipid and fatty acid led to a dramatic increase in the binding of the enzyme, and no discernible latency phase was observed when the additive concentration reached above a critical mole fraction. The mechanisms underlying this behavior are not known; however, our working hypothesis is that in bilayers the binding sites for pig pancreatic phospholipase A_2 are the organizational defects in the phase-separated domains [7]. At this stage we are not even sure whether the phase-separated system represents coexisting laterally phase separated domains in the bilayer or whether in the ternary codispersions there are separate aggregates whose components are able to exchange. It is certain, however, that there is some organized aggregated species in the ternary codispersions that contains all three components and acts as a substrate for phospholipase A_2. An intuitive explanation for poor affinity of pure diacyl- or dialkylphosphatidylcholine bilayers for the phospholipase may be that the life-time of the defects in these bilayers is much shorter because the probability is the same that defects will appear anywhere in the plane of a single component of bilayer [10]. In contrast, in the mixed lipid bilayers, where there is a greater tendency for certain components to interact with some but not with others, the defects will be more localized and their life-times will be limited by the rate of lateral diffusion and mixing of components. A great deal of work remains to be done to prove the validity of this speculation.

Equally provocative questions are raised by the observed correlation between the factors governing the change in turbidity of the codispersions and their interaction with phospholipase. If we postulate that the binding sites for phospholipase A_2 are also the sites that determine the rate of change in turbidity of the codispersions, then understanding the process underlying the change in turbidity needs to be explored further. Two obvious possibilities are aggregation and fusion. We have little direct evidence but, based on the evidence presented in the Results, we tend to discount the possibility that aggregation contributes significantly to the change in turbidity. If fusion or some related process is responsible for the change in turbidity, the organizational changes in the bilayer leading to fusion should be of interest. We are investigating this further. Meanwhile, the significance of the data presented here is that activation of the substrate interface for phospholipase binding can be independently characterized by turbidity changes, and the modulators of the turbidity change also modulate the action of phospholipase A_2 on the bilayers.

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REFERENCES

1. H. U. Weitzien, *Biochim. biophys. Acta* **559**, 259 (1977).
2. C. J. A. Van Echteld, B. DeKruijffe, J. G. Mandersloot and J. DeGier, *Biochim. biophys. Acta* **649**, 211 (1981).
3. W. Wu, C. Huang, T. G. Conley, R. B. Martin and I. W. Levin, *Biochemistry* **21**, 5957 (1982).
4. M. K. Jain, C. J. A. Van Echteld, F. Ramirez, J. DeGier, G. H. DeHaas and L. L. M. Van Deenen, *Nature, Lond.* **284**, 486 (1980)
5. M. K. Jain and G. H. DeHaas, *Biochim. biophys. Acta* **642**, 203 (1981).
6. P. R. Allegrini, G. van Scharrenburg, G. H. DeHaas and J. Seelig, *Biochim. biophys. Acta* **731**, 448 (1983).
7. M. K. Jain, M. R. Egmond, H. M. Verheij, R. J. Apitz, R. Dijkman and G. H. DeHaas, *Biochim. biophys. Acta* **688**, 341 (1982).
8. R. J. Apitz, M. K. Jain and G. H. DeHaas, *Biochim. biophys. Acta* **688**, 349 (1982).
9. M. K. Jain and H. B. White, *Adv. Lipid Res.* **15**, 1 (1977).
10. M. K. Jain, in *Membrane Fluidity in Biology* (Ed. R. Aloia), Vol. 1, pp. 1–37. Academic Press, New York (1983).
11. A. J. Slotboom, H. M. Verheij and G. H. DeHaas, *Chem. Phys. Lipids* **11**, 295 (1973).
12. M. K. Jain and N. M. Wu, *J. membr. Biol.* **34**, 157 (1977).
13. R. Bittman, S. Clejan, M. K. Jain, P. W. Deroo and A. F. Rosenthal, *Biochemistry* **20**, 2790 (1981).
14. M. K. Jain and G. H. DeHaas, *Biochim. biophys. Acta* **736**, 157 (1983).
15. M. K. Jain, *Handbook of Inhibitors*. John Wiley, New York (1982).
16. G. C. Upreti, S. Rainier and M. K. Jain, *J. membr. Biol.* **55**, 97 (1980).
17. P. D. Hart and S. N. Payne, *Br. J. Pharmac.* **43**, 190 (1971).
18. K. Elmaharani and A. Blume, *Biochemistry* **21**, 521 (1982).
19. H. L. Kantor, S. Mabrey, J. H. Prestegard and J. M. Sturtevant, *Biochim. biophys. Acta* **466**, 402 (1977).
20. J. Wilschut, N. Duzgunes, R. Fraley and D. Papahadjopoulos, *Biochemistry* **19**, 6011 (1980).
21. J. N. Weinstein, S. Yoshikami, P. Henkert, R. Blumenthal and W. A. Hagins, *Science* **195**, 489 (1977).