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Enzymic Hydrolysis of Various Lecithin Monolayers Employing Surface Pressure and Potential Technique

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Enzymic hydrolysis of dipalmitoyl, egg, soybean, and dioleoyl lecithin monolayers was studied by measuring the changes in surface pressure (π) and surface potential (ΔV) upon injection of snake venom (*Naja Naja*) into the subsolution. The presence of Ca^{++}, which could not be substituted by Mg^{++}, Sr^{++}, or Ba^{++}, was essential for hydrolysis. Thin layer chromatography of monolayers removed after injection of venom into the subsolution showed that the snake venom indeed hydrolyzed lecithin monolayers into lysolecithin and free fatty acid. The rates of hydrolysis of different lecithins were in the order: dioleoyl lecithin > soybean lecithin > egg lecithin > dipalmitoyl lecithin, which is also the order of their molecular areas and hence their intermolecular spacings in monolayers. The solubility of lysolecithin molecules in the subsolution depends upon the chain length and unsaturation of the fatty acyl chains, which affects the changes in ΔV and π upon hydrolysis.

The presence of citrate buffer or chelating agents with excess of Ca^{++} in the subsolution increased the rate of hydrolysis by decreasing the binding of Ca^{++} to lecithin monolayers. The hydrolysis of egg lecithin monolayers on different buffers was in the order, tris > veronal > collidine; which is the reverse of that found in the bulk reaction. Curves of π-area and ΔV-area of egg lecithin on these buffers indicated penetration of veronal and collidine ions into monolayers; this prevents the formation of the enzyme-substrate complex at the surface. In bulk reactions, these buffer ions act as lipid dispersants increasing the lipid/water interfacial area and consequently increase the rate of hydrolysis.

Anionic, cationic, and neutral spacer molecules (15 mole %) were introduced in egg lecithin monolayers. The rate of hydrolysis increased in the presence of eicosanyl trimethylammonium, decreased in the presence of diceteyl phosphate or dipalmitin, and remained unchanged in the presence of cholesterol, indicating the importance of surface charge and state of the monolayer.

INTRODUCTION

Phospholipase A (EC.3.1.1.4) is known to split the ester linkage at the β-position of the lecithin molecule (1, 2). Hughes (3) showed that snake venoms produced a fall in the surface potentials of lecithin monolayers. It was assumed that the fall in surface potential was due to hydrolysis of lecithin, since the products, lysolecithin and free fatty acid, exhibit lower surface potentials. This assumption has been verified in the present work by thin layer chromatography (TLC) of the monolayers. The influence of metal ions, chelating agents, film pressure, and buffers on hydrolysis of lecithin monolayers is also described. The surface pressure and potential of egg lecithin monolayers were measured on various buffers to deter-

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[2] The untimely death of Dr. Jack H. Schulman, a truly brilliant and creative scientist is reported with profound regret. Those who worked with him share a deep sorrow in this tragic event.

TABLE I

FATTY ACID COMPOSITIONS OF LECITHINS

Fatty acid[a]	Egg lecithin	Soybean lecithin
14:0	tr.	—
16:0	34.34	13.8
16:1	tr.	—
18:0	13.01	3.8
18:1	32.85	13.3
18:2	16.17	62.5
18:3	—	6.3
20:3	tr.	tr.
20:4	3.6	—

[a] Number of carbon atoms: number of double bonds.

mine the interaction of buffer ions with lecithin monolayers. In addition, the effect of intermolecular spacing, which is related to unsaturation of fatty acyl chains, on the hydrolysis of lecithin monolayers has been investigated by using dioleoyl, soybean, egg, and dipalmitoyl lecithins. Anionic, cationic, and neutral spacer molecules were introduced in egg lecithin monolayers in order to study the effect of surface charge and state of monolayers on hydrolysis.

EXPERIMENTAL

Materials

Lipids. Chromatographically pure dipalmitoyl lecithin was purchased from Mann Research Laboratories (New York). Egg lecithin was supplied by Sylvana Chemical Company, (Millburn, New Jersey). Cholesterol and soybean lecithin were supplied by Applied Science Laboratories (State College, Pennsylvania). Table I shows the fatty acid composition of egg and soybean lecithins analyzed by gas-liquid chromatography through the courtesy of the laboratory of Dr. E. H. Ahrens, Jr. (Rockefeller University, New York). The dioleoyl lecithin was a gift from Dr. L. L. M. Van Deenen. Dicetyl phosphate and eicosanyl trimethylammonium bromide were purchased from K & K Laboratories (Plainview, New York). The purity of all lipids was checked by thin layer chromatography.

Venoms. Snake venom *Naja naja* was obtained from Ross Allen Reptile Institute (Silver Springs, Florida) and stored at −10°C.

Buffers. Tris, veronal, collidine, and citrate buffer solutions (0.05 *M*) were prepared (4) and adjusted to pH 7.2. To these buffer solutions NaCl and $CaCl_2$ were added such that the resulting solution contained buffer (0.05 *M*) + NaCl (0.02 *M*) + $CaCl_2$ (0.01 *M*). Inorganic chemicals of reagent grade and twice distilled water were used in all experiments.

Preparation of Phospholipase A Solution

The venom (1 mg) was dissolved in a 100 ml. solution of 0.02 *M* NaCl + 10^{-3} *M* EDTA-Na_2, pH 4.5. The venom solution was heated to 90°C in a water bath for 10 minutes and was subsequently cooled to room temperature. Venom solution stored in cold at 4°C for 15 days showed no change in enzymic activity.

TLC of Phospholipid Monolayers

A Lucite trough (19 × 74 cm.^{2}) of 2200 ml. capacity was used for spreading monolayers. The trough was filled with the subsolution consisting of Tris buffer (0.05 *M*) + NaCl (0.02 *M*) + $CaCl_2$ (0.01 *M*) at pH 7.2. The lecithin solution was spread by means of a microsyringe, and the venom solution was injected under the monolayer. The surface of the trough (i.e., monolayer and a small amount of subsolution) was sucked through a glass nozzle into a 50-ml. conical flask connected to an aspirator. This was quickly transferred to a red actinic volumetric flask (250 ml.) containing 20 ml. of chloroform. After the flask was shaken gently, the lipids were extracted in the chloroform and the aqueous phase was discarded. Similarly 12 additional monolayers were transferred to the flask in order to collect a sufficient amount of lipids in the chloroform. The chloroform was evaporated and the lipids were analyzed by TLC with the solvent system chloroform-methanol-water (85:35:5 v/v/v). Spots on the TLC plate were made visible by spraying the plate with a 1% solution of iodine in methanol. As a control, lecithin monolayers were removed in the absence of venom in the subsolution and were analyzed by TLC.

Apparatus and Procedure

The method of measuring surface pressure by a modified Wilhelmy plate and surface potential by a radioactive electrode has been described previously (5). A stirring device was introduced into the apparatus for this work. Two Teflon-coated cylindrical magnets were placed in the trough (11.5 ×22 cm.²; 400 ml. capacity) and were moved to and fro by two magnets on a motorized shaft placed below the trough. This type of stirring was found necessary to mix small quantities of venom (20 μg.) efficiently in the subsolution. The temperature of the subsolution was kept constant at 25°C. The lecithin solution (0.025 ml.) was spread on a clean surface of subsolution. Five minutes after spreading, the monolayer was compressed to a desired surface pressure. The subsolution was stirred by magnets for 3 minutes to check the constancy of surface pressure and potential. About 2 ml. of venom solution containing 20 μg. of venom was injected into the subsolution and surface pressure and potential were recorded simultaneously at intervals of 1 or 2 minutes.

FIG. 1. TLC of egg lecithin monolayers. (*1*) Egg lecithin stock solution, (*2*) egg lecithin monolayers in the absence of snake venom in the subsolution, (*3*) egg lecithin monolayers in the presence of snake venom in the subsolution. *O*, origin; *LL*, lysolecithin; *L*, lecithin; *FA*, fatty acid; *OP*, oxidation products.

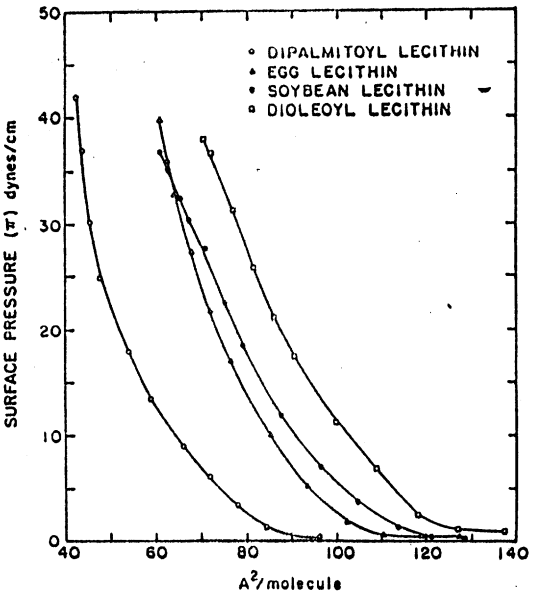

FIG. 2. Surface pressure-area curves of dipalmitoyl, egg, soybean, and dioleoyl lecithins on 0.02 *M* NaCl subsolution, pH 5.6, at 25°C.

RESULTS AND DISCUSSION

TLC of Egg Lecithin Monolayers

Figure 1 shows a TLC plate which presents direct evidence to show that snake venom indeed hydrolyzes lecithin monolayers into lysolecithin and free fatty acid. Since lysolecithin and fatty acid monolayers are known to exhibit lower surface potentials than that of lecithin (6, 7), this hydrolysis results in a decrease of surface potential of lecithin monolayers. It is evident that egg lecithin is oxidized to a small extent in the process of removing monolayers from the interface. The oxidation products move with the solvent front on the TLC plate. The control study without snake venom showed only two spots, one corresponding to oxidation products and the other to lecithin.

Surface Pressure-Area and Surface Potential-Area Curves of Dipalmitoyl, Egg, Soybean, and Dioleoyl Lecithins

Figure 2 shows the surface pressure-area curves of dipalmitoyl, egg, soybean, and

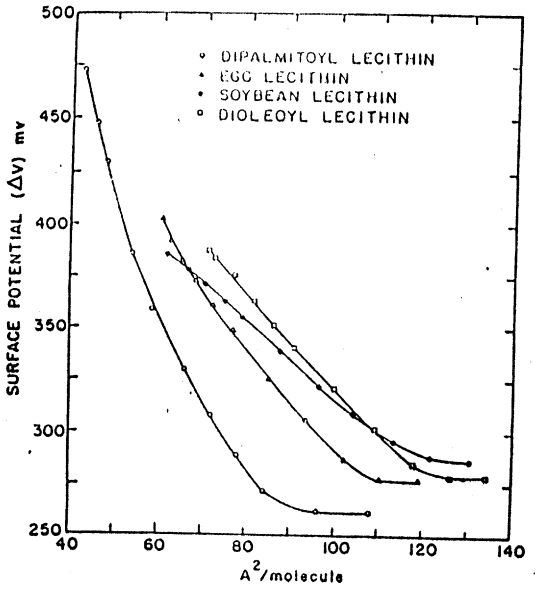

FIG. 3. Surface potential-area curves of dipalmitoyl, egg, soybean, and dioleoyl lecithins on 0.02 *M* NaCl subsolution, pH 5.6, at 25°C.

dioleoyl lecithins on subsolutions of 0.02 *M* NaCl. The fatty acid composition of egg and soybean lecithins (Table I) shows that most egg lecithin molecules consist of a saturated and an unsaturated fatty acyl chain whereas in most soybean lecithin molecules the fatty acyl chains are polyunsaturated. The area/molecule of these lecithins follows the order: dipalmitoyl < egg < soybean < dioleoyl lecithin. The deviation which occurs in the π-A curve of soybean lecithin above 32 dynes/cm. is presumably due to an alteration in the structure of the soybean lecithin monolayer permitted by the lesser cohesive force between polyunsaturated chains. Figure 3 shows the ΔV-area curves of these lecithins on 0.02 *M* NaCl subsolutions.

The intermolecular spacing (i.e., average distance between two adjacent phosphate groups) in lecithin monolayers can be calculated approximately by assuming the "limiting area" as the area of a circle with a radius r; then the intermolecular spacing is $2r$. If we consider molecular areas of 42.5 A², 61 A², and 72 A² for dipalmitoyl, egg, and dioleoyl lecithins, respectively, the corresponding intermolecular spacings are 7.36 A, 8.8 A, and 9.58 A. We have shown that this change of 1–1.5 A in the intermolecular spacing strikingly influences the interaction of metal ions (5) and phospholipase A with lecithin monolayers.

Enzyme Activity of Snake Venom Solution

It has been shown (8) that the addition of EDTA to venom solution increases the enzyme activity. In the present work, when EDTA was not added to the venom solution, the enzyme activity was reduced to 50% on the following day, whereas in the presence of EDTA the enzyme activity did not change for 15 days. This is due to the fact that EDTA inhibits the proteolytic enzymes present in snake venoms (9). Since phospholipase A is heat-stable (10), the venom solution was heated to denature other proteins present in the venom without influencing the phospholipase A activity.

Influence of Unsaturation of Fatty Acyl Chains on Hydrolysis of Lecithin Monolayer

Figures 4 and 5 show the changes in surface pressure and potential of dipalmitoyl

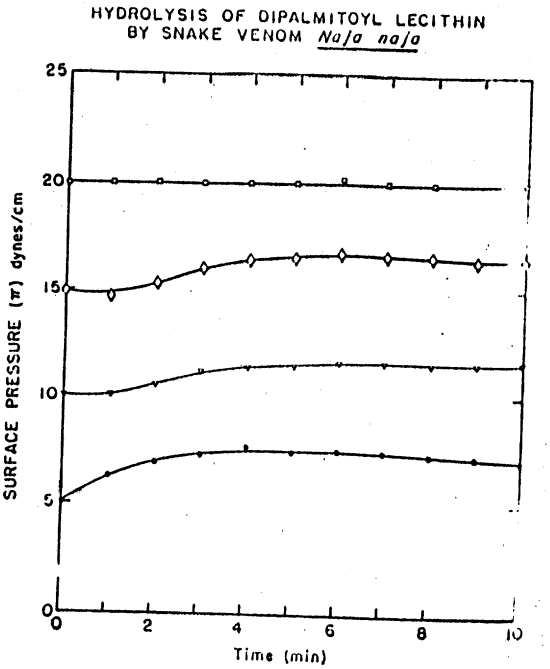

FIG. 4. The changes in the surface pressure of dipalmitoyl lecithin monolayers upon hydrolysis by snake venom *Naja naja* (20 μg.). The subsolution consists of tris (0.05 *M*) + NaCl (0.02 *M*) + $CaCl_2$ (0.01 *M*), pH 7.2, at 25°C.

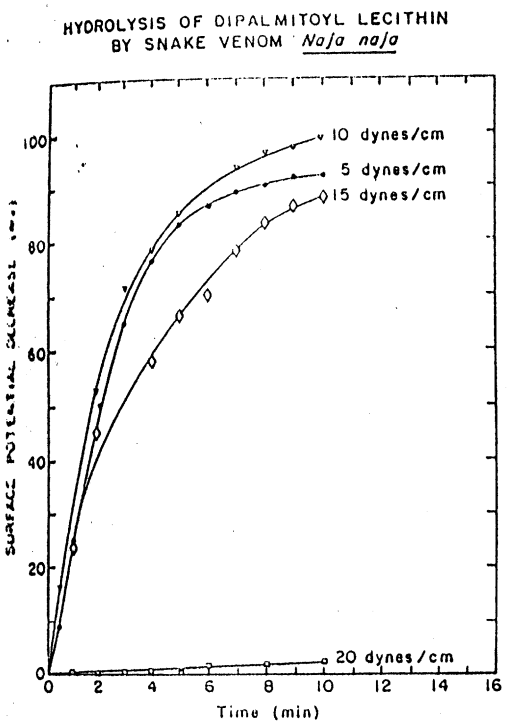

FIG. 5. The decrease in the surface potential of dipalmitoyl lecithin monolayers at various surface pressures upon hydrolysis by snake venom *Naja naja* (20 μg.). The subsolution consists of tris (0.05 *M*) + NaCl (0.02 *M*) + $CaCl_2$ ((0.01 *M*), pH 7.2, at 25°C.

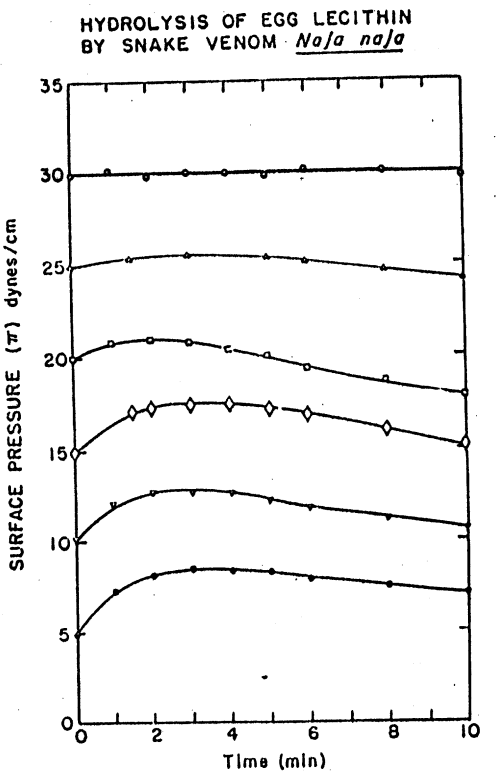

FIG. 6. The changes in the surface pressure of egg lecithin monolayers upon hydrolysis by snake venom *Naja naja* (20 μg.). The subsolution consists of tris (0.05 *M*) + NaCl (0.02 *M*) + $CaCl_2$ (0.01 *M*), pH 7.2, at 25°C.

lecithin monolayers which occur upon injection of 20 μg. of snake venom *Naja naja* into the subsolution. Surface pressure showed an increase of 2-3 dynes/cm., which can be explained as follows. It has been shown that lysolecithin occupies approximately the same area as lecithin (6); therefore, the hydrolysis products lysolecithin plus fatty acid must occupy a larger area than unhydrolyzed lecithin. Since the hydrolysis is studied at constant film area, this results in an increase of surface pressure. It also indicates that palmitoyl lysolecithin remains in the monolayer in contrast to palmitoleoyl lysolecithin, which dissolves in the subsolution resulting in a fall of surface pressure upon hydrolysis of yeast lecithin.[3]

It is also evident from Fig. 5 that dipalmitoyl lecithin monolayers are not hydrolyzed above a surface pressure of 20 dynes/cm. This suggests that the intermolecular spacing in dipalmitoyl lecithin monolayers at this surface pressure does not permit the active site of the enzyme to penetrate into the lecithin monolayers.

Figures 6 and 7 show the changes which occur in surface pressure and surface potential of egg lecithin monolayers upon hydrolysis. It is clear that as in the case of dipalmitoyl lecithin monolayers, lysolecithin derived from egg lecithin does not dissolve in

[3] Recent work (unpublished) done in our laboratory indicates that the hydrolysis of monolayers of yeast lecithin, which consists mostly of palmitoleic (16:1) acid, by snake venom, results in a fall of surface pressure and surface potential at all surface pressures. This indicates that lysolecithin derived from yeast lecithin dissolves in the subsolution. A comparison of the hydrolysis of dipalmitoyl and yeast lecithin monolayers suggests that unsaturation of the fatty acyl chain substantially increases the solubility of lysolecithin in the subsolution.

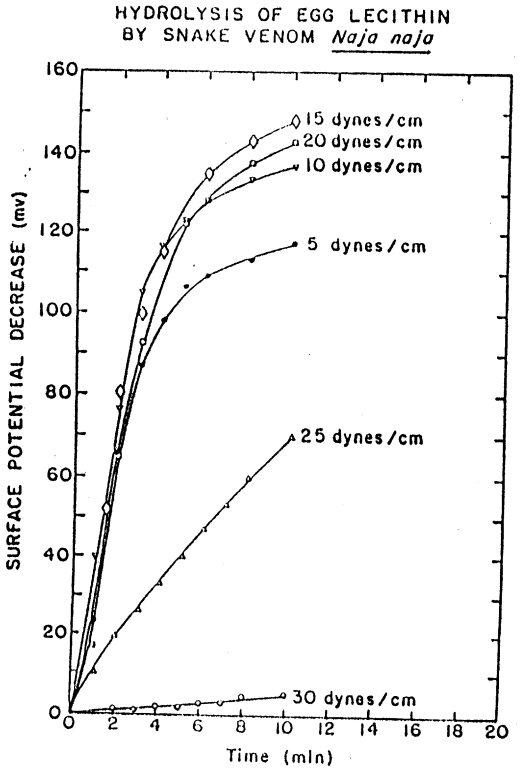

FIG. 7. The decrease in the surface potential of egg lecithin monolayers at various surface pressures upon hydrolysis by snake venom *Naja naja* (20 μg.). The subsolution consists of tris (0.05 *M*) + NaCl (0.02 *M*) + $CaCl_2$ (0.01 *M*), pH 7.2, at 25°C.

the subsolution, since there is no significant decrease in surface pressure. Moreover, the intermolecular spacing above a surface pressure of 30 dynes/cm. does not permit the enzyme to penetrate into egg lecithin monolayers (Fig. 7).

Figures 8 and 9 show the changes which occur in surface pressure and surface potential of soybean lecithin monolayers upon hydrolysis. In contrast to lysolecithin derived from dipalmitoyl and egg lecithins, lysolecithin from soybean lecithin contains polyunsaturated fatty acyl chains (Table I) and dissolves in the subsolution, resulting in a fall of surface pressure (Fig. 8). It is also evident from Fig. 8 that the solubility of lysolecithin increases with the surface pressure. Hydrolysis does not occur at a surface pressure of 37 dynes/cm. (Figs. 8 and 9).

Figures 10 and 11 show the changes which occur in surface pressure and surface potential of dioleoyl lecithin monolayers upon hydrolysis. The surface pressure does not decrease significantly up to 20 dynes/cm. this indicates that oleoyl (18:1) lysolecithin remains in the monolayer in contrast to palmitoleoyl (16:1) lysolecithin.[1] Above a surface pressure of 25 dynes/cm., hydrolysis is accompanied by a decrease in surface pressure; this indicates that lysolecithin is squeezed out of the monolayer at high surface pressures, presumably owing to its strong hydrophilic group. It is known from penetration experiments that several water soluble surfactants, or those with strong hydrophilic groups, are squeezed out of monolayers at high surface pressures (11,12). Thus

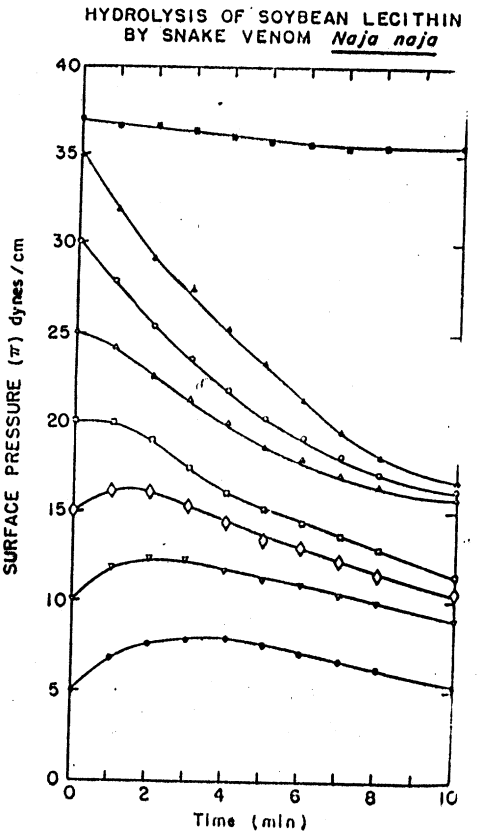

FIG. 8. The changes in the surface pressure of soybean lecithin monolayers upon hydrolysis by snake venom *Naja naja* (20 μg.). The subsolution consists of tris (0.05 *M*) + NaCl (0.02 *M*) + $CaCl_2$ (0.01 *M*), pH 7.2, at 25°C.

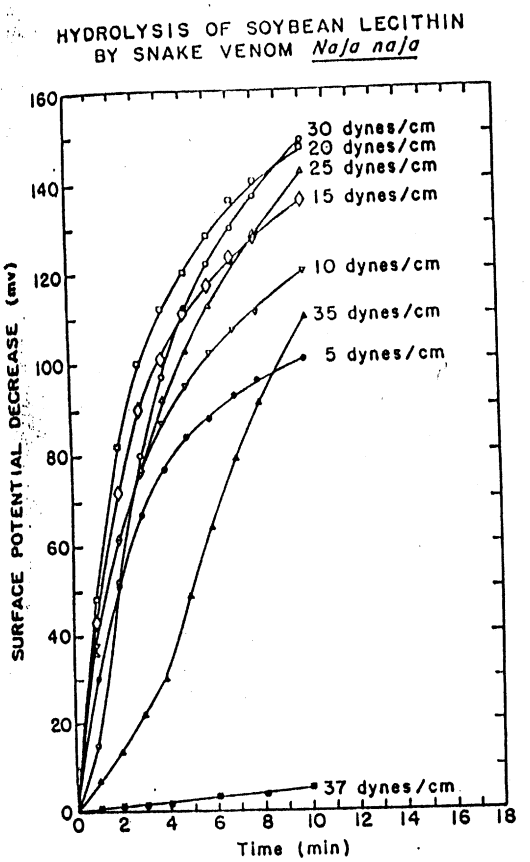

FIG. 9. The decrease in the surface potential of soybean lecithin monolayers at various surface pressures upon hydrolysis by snake venom *Naja naja* (20 μg.). The subsolution consists of tris (0.05 *M*) + NaCl (0.02 *M*) + $CaCl_2$ (0.01 *M*), pH 7.2, at 25°C.

chain length and the degree of unsaturation of fatty acyl chains together with the state of compression of monolayers determine the solubility of lysolecithin in the subsolution. The changes in surface potential indicate that considerable hydrolysis occurs in dioleoyl lecithin monolayers even up to a surface pressure of 40 dynes/cm. (Fig. 11).

The compression of a monolayer results in an increase of surface concentration of molecules and simultaneously a decrease in the intermolecular spacing in the monolayer. The former increases the rate of hydrolysis by increasing the frequency of collision between enzyme and substrate molecules, whereas the latter decreases the rate by preventing the penetration of the enzyme molecule into the monolayer. These counterbalancing factors, which directly influence the rate of hydrolysis, determine the optimum surface pressure for hydrolysis of lecithin monolayers. Figure 12 shows the initial decrease in surface potential in 2 minutes (which is considerered to be proportional to the initial rate of reaction) plotted against surface pressure. It is evident that the optimum surface pressures for hydrolysis are 10, 15, 20, and 25 dynes/cm. for dipalmitoyl, egg, soybean, and dioleoyl lecithins, respectively. At optimum surface pressures, their initial rate of reaction is in the order: dioleoyl lecithin > soybean lecithin > egg lecithin > dipalmitoyl lecithin. This is also the

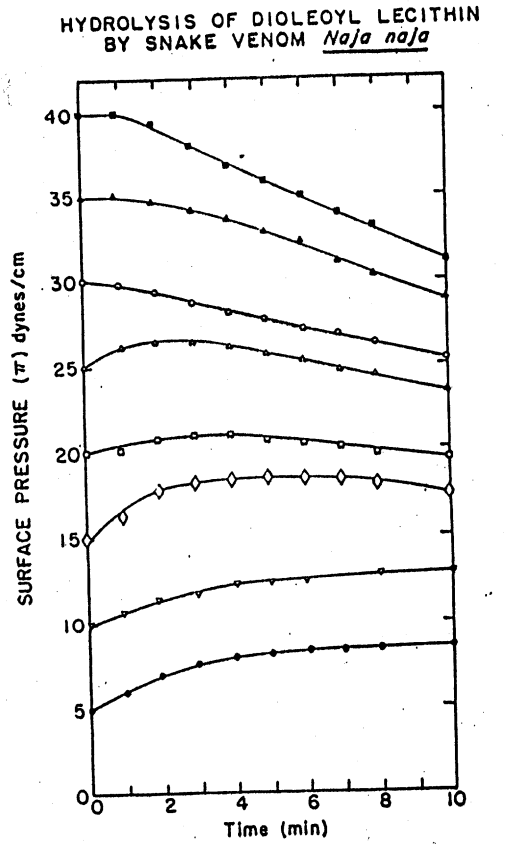

FIG. 10. The changes in the surface pressure of dioleoyl lecithin monolayers upon hydrolysis by snake venom *Naja naja* (20 μg.). The subsolution consists of tris (0.05 *M*) + NaCl (0.02 *M*) + $CaCl_2$ (0.01 *M*), pH 7.2, at 25°C.

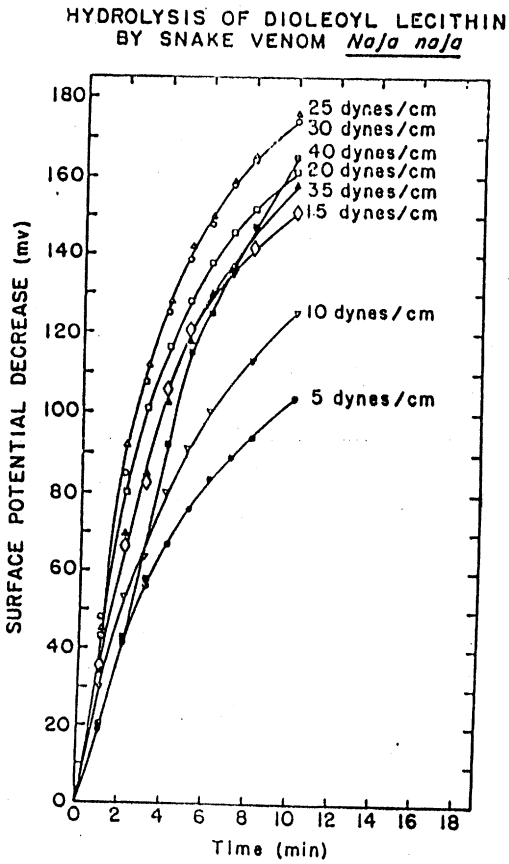

FIG. 11. The decrease in the surface potential of dioleoyl lecithin monolayers at various surfaces pressures upon hydrolysis by snake venom *Naja naja* (20 μg.). The subsolution consists of tris (0.05 *M*) + NaCl (0.02 *M*) + $CaCl_2$ (0.01 *M*), pH 7.2, at 25°C.

order for the final surface pressures at which hydrolysis does not proceed. Moreover, the area per molecule and hence the intermolecular spacing in lecithin monolayers follow the same order (Fig. 2). These results suggest that *the rate of hydrolysis of lecithin monolayers is determined by the unsaturation of fatty acyl chains and hence the intermolecular spacing in monolayers.* Figure 13 shows schematically the effect of unsaturation of fatty acyl chains on intermolecular spacing in lecithin monolayers.

Recently Moore and Williams (13) have shown that the rate of hydrolysis of different species of lecithin in the bulk reaction follow a similar order; this was attributed to the specificity of phospholipase A. The results presented here indicate that different rates of hydrolysis of lecithins are related to the intermolecular spacing of lecithin molecules rather than to the enzyme. Since the ester bond at the β-position of lecithin is involved in this process, a larger intermolecular spacing should increase the probability of formation of an enzyme-substrate complex and hence increase the rate of hydrolysis. Colacicco and Rapport (14) reported 12 dynes/cm. to be the optimum surface pressure for hydrolysis of egg lecithin monolayers. The difference of 3 dynes/cm. which we find is presumably due to a difference in techniques employed.

Influence of Metal Ions and Chelating Agents on Hydrolysis of Lecithin Monolayers

The presence of Ca^{++} is essential for hydrolysis of lecithin monolayers by snake venom *Naja naja*. Other divalent cations

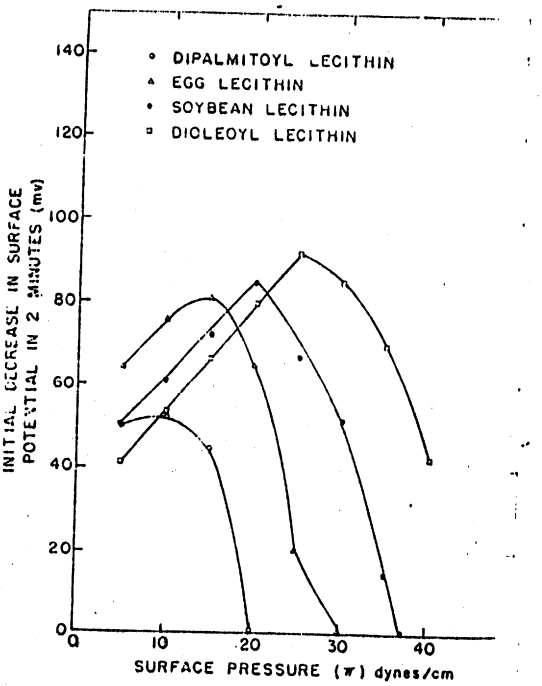

FIG. 12. The effect of surface pressure on initial rate of hydrolysis, as measured by the initial decrease of surface potential in the first 2 minutes, for various lecithin monolayers. The subsolution consists of tris (0.05 *M*) + NaCl (0.02 *M*) + $CaCl_2$ (0.01 *M*), pH 7.2, at 25°C.

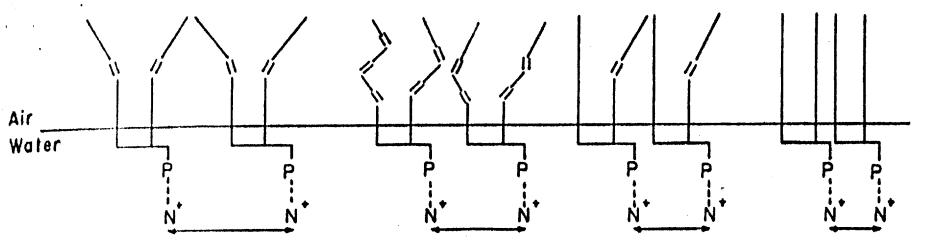

FIG. 13. A schematic representation of the influence of unsaturation on the intermolecular spacing in lecithin monolayers. The lecithin molecules are shown in the order from left to right: dioleoyl lecithin, soybean lecithin, egg lecithin, dipalmitoyl lecithin.

such as Mg^{++}, Sr^{++}, and Ba^{++} are unable to replace Ca^{++} for hydrolysis (Fig. 14). However, the presence of an equivalent amount of Mg^{++} in addition to Ca^{++} causes significant reduction in the rate of hydrolysis. Dipalmitoyl lecithin monolayers are employed in this study since they exhibit maximal interaction with metal ions (5).

EFFECT OF METAL IONS AND CHELATING AGENTS ON HYDROLYSIS OF DIPALMITOYL LECITHIN

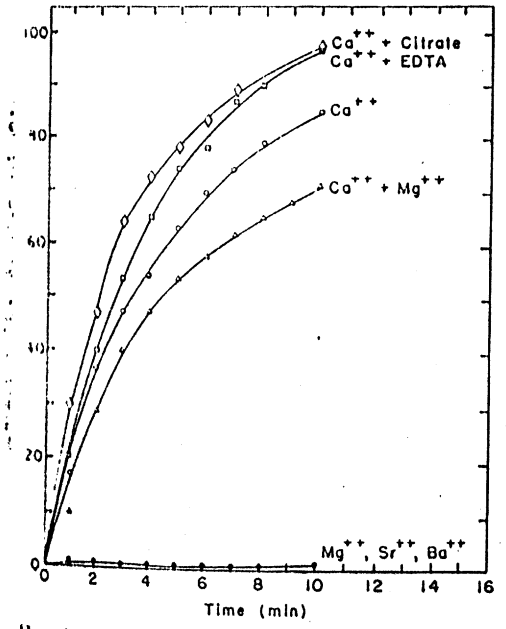

FIG. 14. The effect of metal ions and chelating agents on hydrolysis of dipalmitoyl lecithin monolayers, at a surface pressure of 15 dynes/cm., by snake venom *Naja naja* (20 μg.) on subsolutions of Tris (0.05 *M*) + NaCl (0.02 *M*) + additives, pH 8.2, at 25°C. The additives are as follows: ●— 0.01 *M* $MgCl_2$, $SrCl_2$, $BaCl_2$, or 0.01 *M* $CaCl_2$ + 0.01 *M* EDTA-Na_4 (or Na_3 citrate); ○— 0.01 *M* $CaCl_2$; ▽— 0.01 *M* $CaCl_2$ + 0.01 *M* $MgCl_2$; □— 0.01 *M* $CaCl_2$ + 0.005 *M* EDTA-Na_4; ◇— 0.01 *M* $CaCl_2$ + 0.005 *M* Na_3 citrate.

In relation to the role of Ca^{++} in hydrolysis of lecithin by phospholipase A, it should be emphasized that dioleoyl lecithin, which interacts least with Ca^{++} (5, 15), shows the highest rate of hydrolysis. This suggests that the specificity and requirement of Ca^{++} in the hydrolysis reaction are due to the enzyme phospholipase A and not due to charge requirements of the substrate (lecithin molecules). When equivalent amounts of Ca^{++} and a chelating agent (Na_3 citrate or EDTA-Na_4) are present in the subsolution, hydrolysis does not occur. However, when the chelating agent is present with excess of Ca^{++}, hydrolysis occurs at a higher rate than in the presence of Ca^{++} alone (Fig. 14). This agrees with the results reported by Dawson using bulk reaction kinetics (16). As previously mentioned, a comparison of the rate of hydrolysis and the interaction of Ca^{++} with different lecithins suggests that the binding of Ca^{++} to lecithin monolayers reduces the rate of hydrolysis. The presence of chelating agents in the subsolution reduces the binding of Ca^{++} to lecithin monolayers and thereby increases the rate of hydrolysis. The excess Ca^{++} is required for the activation of phospholipase A. This conclusion is further supported by the rate of hydrolysis of lecithin monolayers on citrate buffer, which also prevents the binding of Ca^{++} to lecithin monolayers (see Fig. 15).

Influence of Buffer Solutions on Hydrolysis of Egg Lecithin Monolayers

It has been shown (17) from the bulk reaction kinetics that the rate of hydrolysis of egg lecithin by phospholipase A varies in different buffers at the same pH. Figure 15

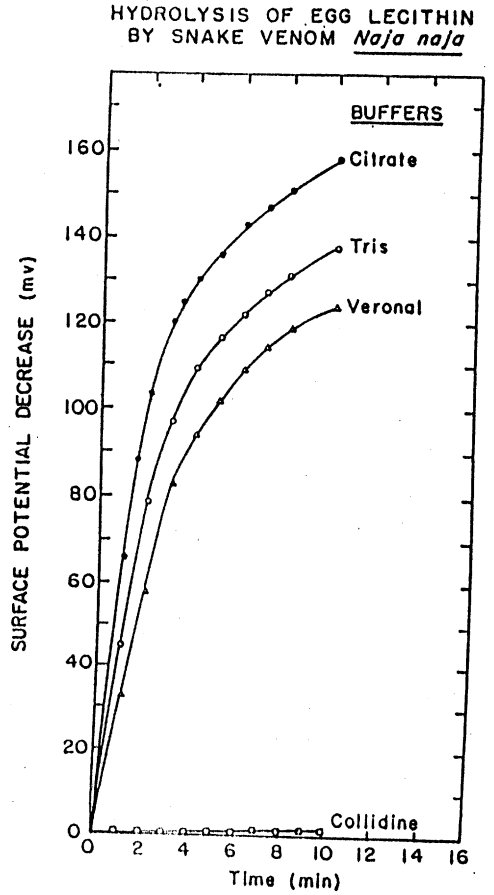

FIG. 15. The effect of various buffers on hydrolysis of egg lecithin monolayers, at a surface pressure of 15 dynes/cm., by snake venom *Naja naja* (20 μg.) on subsolutions of buffer (0.05 *M*) + $CaCl_2$ (0.01 *M*), pH 7.2, at 25°C.

shows the changes in surface potential upon hydrolysis of egg lecithin monolayers at a surface pressure of 15 dynes/cm. The changes in surface pressure upon hydrolysis were the same as shown in Fig. 6 at the same surface pressure. The rate of hydrolysis is highest on citrate buffer owing to inability of Ca^{++} to interact with lecithin monolayers in the presence of citrate ions. This conclusion is supported by surface potential measurements (see Fig. 17). The rates for the other three buffers are in the order: tris > veronal > collidine. Collidine buffer is widely used in bulk studies and is known to yield complete hydrolysis of lecithin (17), whereas in the surface reactions reported here it completely inhibits hydrolysis (Fig. 15). It is interesting to note that the order for the rate of hydrolysis found in the surface reaction is the reverse of the order found in the bulk reaction (i.e., in bulk, collidine > veronal > tris) (17). To investigate the possibility of interaction of buffer ions with lecithin, surface pressure and potential of egg lecithin monolayers were measured on the same buffer subsolutions.

The Surface Pressure-Area and Surface Potential-Area Curves of Egg Lecithin Monolayers on Various Buffer Solutions

Surface tension measurements of buffers showed that tris and citrate buffers were not surface active (i.e., their surface tension was close to that of water), whereas veronal and collidine were surface active and showed surface tensions of 66 and 57 dynes/cm., respectively. Figure 16 shows the surface pressure-area curves of egg lecithin monolayers on various buffer (0.05 *M*) solutions containing 0.01 *M* $CaCl_2$ at pH 7.2 and 25°C. On veronal and collidine buffers the lecithin monolayers

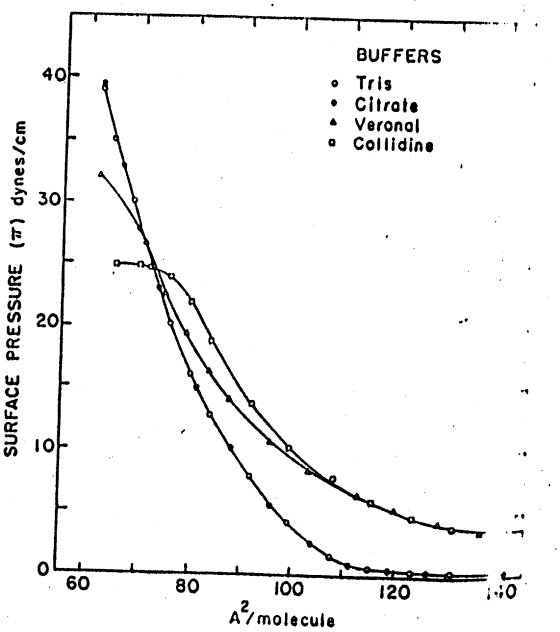

FIG. 16. Surface pressure-area curves of egg lecithin monolayers on subsolutions of buffer (0.05 *M*) + $CaCl_2$ (0.01 *M*), pH 7.2, at 25°C.

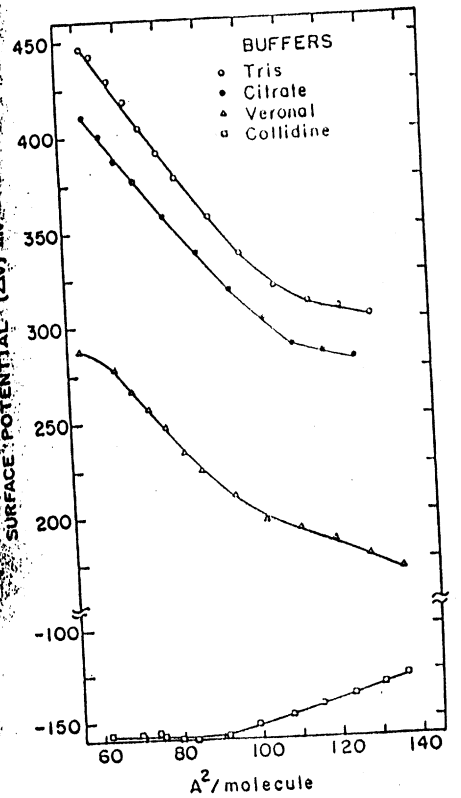

FIG. 17. Surface potential-area curves of egg lecithin monolayers on subsolutions of buffer (0.05 *M*) + $CaCl_2$ (0.01 *M*), pH 7.2, at 25°C. The filled circles (●) also indicate the surface potential of egg lecithin on subsolutions of citrate or tris buffers without $CaCl_2$ in the subsolution, pH 7.2, at 25°C.

showed a larger area per molecule, indicating penetration of buffer ions (veronal and collidine) into the monolayers. This results in reduced stability of the monolayers, which is shown by the lower collapse pressures of lecithin monolayers on veronal and collidine buffer solutions. On the other hand, surface pressure-area curves on tris and citrate buffer solutions are identical to that obtained on subsolutions of 0.02 *M* NaCl (Fig. 2).

The interaction of buffer ions with lecithin monolayers is strikingly shown by surface potential-area curves on these buffer solutions (Fig. 17). Surface potentials on veronal and collidine buffers are 175 mv. and 600 mv. lower than on tris buffer solution, indicating strong interaction of veronal and collidine buffer ions with lecithin monolayers.

These results enable us to explain the reversal of the rate of hydrolysis in surface reaction as compared to bulk reaction. In hydrolysis of monolayers, the adsorption and penetration of buffer ions (veronal and collidine) prevent the formation of an enzyme-substrate complex, which results in the lower rate of hydrolysis. On the other hand, in bulk reaction, the surface-active veronal and collidine buffers act as lipid dispersing agents (or lipid dispersants) increasing the area of the lipid/water interface and consequently increase the rate of hydrolysis.

Although the π-area curves of egg lecithin on tris and citrate buffers are identical (Fig. 16), the surface potential on tris is higher than that on citrate buffer, both con-

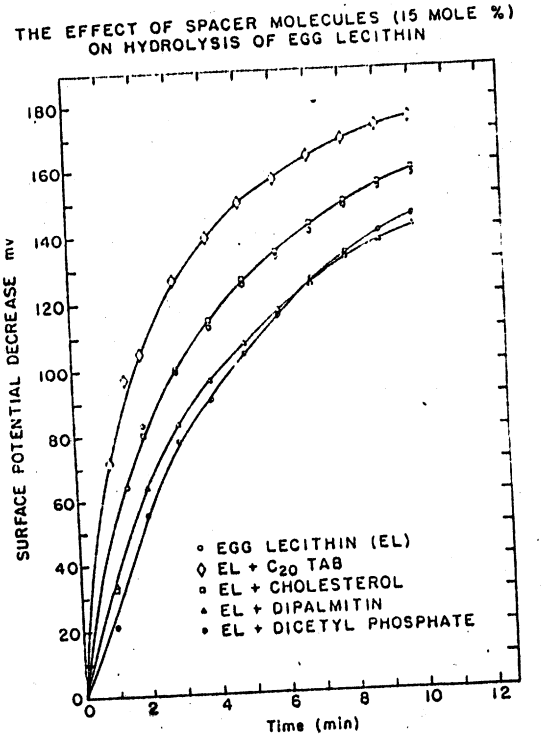

FIG. 18. The effect of the presence of various spacer molecules (15 mole %) in egg lecithin monolayers, at a surface pressure of 15 dynes/cm., on hydrolysis by snake venom *Naja naja* (20 μg.) on subsolutions of tris (0.05 *M*) + NaCl (0.02 *M*) + $CaCl_2$ (0.01 *M*), pH 7.2 and 25°C. Eicosanyl trimethylammonium bromide is denoted by C_{20}TAB.

taining 0.01 *M* $CaCl_2$ (Fig. 17). This indicates that Ca^{++} added to tris buffer interacts with egg lecithin but when added to citrate it does not. This is supported by the following observations: (*a*) surface potentials were identical on citrate buffer in the presence or absence of $CaCl_2$ and (*b*) the surface potential on tris buffer without added $CaCl_2$ was identical to that on citrate buffer. Thus, citrate buffer prevents the binding of Ca^{++} to lecithin monolayers and consequently increases the rate of hydrolysis.

Influence of Spacer Molecules on Hydrolysis of Egg Lecithin Monolayers

It is known that the presence of small amounts of charged lipids significantly influences the hydrolysis of lecithin monolayers (18,19). The added lipid molecules which influence the spacing between lecithin molecules are termed *spacer molecules*. In the present study, 15 mole % of four different types of spacer molecules, namely, anionic, cationic, and nonionic (liquefying and solidifying) were separately added to egg lecithin to study the effect of surface charge and state of monolayers on hydrolysis. It has been shown (20) that cholesterol tends to liquefy and dipalmitin tends to solidify lecithin monolayers, suggesting that cholesterol is a liquid spacer and dipalmitin a solid spacer molecule.

Recently Dawson reported (21) that addition of 15% dicetyl phosphoric acid (but not stearylamine) to yeast lecithin monolayers increased the loss of P^{32} from high-pressure films due to hydrolysis by snake venom. In contrast to this observation, none of the spacer molecules used in the present study were able to initiate hydrolysis of egg lecithin monolayers at a surface pressure of 30 dynes/cm., where phospholipase A is unable to hydrolyze egg lecithin monolayers (Fig. 7). Hydrolysis at this surface pressure does not occur even upon increasing the amount of spacer molecules to 50 mole % in egg lecithin monolayers. The discrepancy between our results and those of Dawson (21) is presumably due to the difference in fatty acid composition of egg and yeast lecithins (5).

At a low surface pressure (15 dynes/cm.), the presence of spacer molecules in egg lecithin monolayers produced significant differences in hydrolysis. Figure 18 shows changes in surface potentials of egg lecithin monolayers in the presence of different spacer molecules (15 mole %) upon injection of 20 μg. of venom into the subsolution. The presence of cholesterol, which is a nonionic liquid spacer does not influence the hydrolysis of egg lecithin monolayers. On the other hand, dipalmitin, a nonionic solid spacer, reduces the rate of hydrolysis. This suggests that solidification (i.e., higher surface viscosity) of lecithin monolayers reduces hydrolysis. The presence of dicetyl phosphate in egg lecithin monolayers reduces the rate of hydrolysis. This agrees with the results reported by Dawson (16) on hydrolysis of lecithin particles in the presence of diethyl ether. The negative charges of dicetyl phosphate presumably act in a similar way as the negative charges of liberated free fatty acids. The excess of negative charge in the monolayer would tend to increase the binding of Ca^{++} to the monolayer, which in turn would reduce the rate of hydrolysis. The presence of eicosanyl trimethylammonium bromide (C_{20}TAB) in egg lecithin monolayers strikingly increased the rate of hydrolysis. This is caused by a combination of factors, namely, repulsion of Ca^{++} from the vicinity of the monolayer due to the positively charged trimethylammonium groups of C_{20}TAB, and neutralization of negative charges of liberated fatty acids by the cationic C_{20}TAB. It is known from the mixed monolayers of anionic and cationic surfactants that the opposite charges neutralize each other, forming ionic salt linkages in mixed monolayers (22). Therefore, the presence of C_{20}TAB in lecithin monolayers does not allow initial accumulation of negative charges, which accumulate only after all C_{20}TAB is neutralized by free fatty acids. However, it is interesting to note that in bulk reactions the presence of C_{16}TAB along with lecithin particles does not significantly influence hydrolysis (16). The difference is due to the fact that C_{16}TAB is soluble and C_{20}TAB insoluble in water; this could influence the structural arrangement of these spacer molecules in relation to the polar group of lecithin. The

results presented here suggest that both the surface charge and the state of monolayers are of considerable importance in hydrolysis of lecithin.

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