Lung surface-active fraction as a model system for macromolecular ultrastructural studies with *Crotalus atrox* venom

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Abstract The dog lung surface-active fraction and phosphatidylcholine constituents were subjected to hydrolysis by *Crotalus atrox* phospholipase A_2. Relative rates of hydrolysis were: dipalmitoyl glycerophosphorylcholine > phosphatidylcholine isolated from the surface-active fraction > phosphatidylcholine as an integral component of the intact surface-active macromolecular structure. Cholesterol markedly inhibited, whereas tripalmitin increased, the rate of hydrolysis with both pure phosphatidylcholine substrates. The effect of temperature on the velocity indicated the enzyme was most active when the substrates were in the gel state. These kinetic results, in conjunction with surface chemistry studies, can be interpreted to indicate that the phosphatidylcholine in the intact surface-active macromolecular particle is liquid crystalline due to molecular interactions with other constituents. Gas–liquid chromatographic analysis of the 2-lysophosphatidylcholines and fatty acids produced from the enzymatic hydrolysis of the intact surface-active fraction indicated that palmitoyl residues were more accessible to the enzyme, perhaps because they occupied positions near the surface of the particle.

Supplementary key words dipalmitoyl glycerophosphorylcholine · phospholipase A_2 · molecular interactions · gel and liquid crystalline states

BOTH THEORETICAL CONSIDERATIONS and experimental results have established that the alveoli of mammalian lungs are lined with an acellular material containing a highly surface-active substance, designated pulmonary surfactant, whose presumptive function is the reduction of alveolar surface forces at low lung volumes during respiration, thereby preventing alveolar collapse (1–6). We have described the isolation and characterization of a surface-active fraction containing pulmonary surfactant from dog lung homogenates (7). Recently, we demonstrated the extra- and intracellular compartmentalization of this fraction (8). These studies have indicated that this surface-active fraction exhibits properties expected of a particulate lipoprotein macromolecular assembly. In our previous studies, we postulated that the ultrastructure of the alveolar lining might resemble a structured gel and we described how surface forces could arise in such a state without the necessity of a true liquid subphase in situ (7). No direct experimental evidence was given to support this postulate, rather it was based upon the known properties of the individual constituents of the surface-active fraction, principally the phospholipids (9).

The purpose of this communication is to present an approach to the study of biological ultrastructure with phospholipase A_2, utilizing the lung surface-active fraction and its constituents as model systems. Our results can be interpreted to support our previous conceptualization of the alveolar lining as a structured gel with liquid crystalline properties. It should be emphasized that phospholipase A_2 was used as a probe to investigate structure, organization, and interactions in the surface-active fraction and that the study of the properties of the enzyme itself was not the primary object of this report. The approach we have used should be applicable to the study of other particulate biological systems, especially membranes, with various phospholipases and other hydrolytic enzymes.

Abbreviations: IB, surface-active fraction isolated from a total lung homogenate; IB_I, intracellular IB, surface-active fraction isolated from a homogenate of residual lung after a prior saline lavage to remove material at the alveolar surface; IB_E, extracellular IB, surface-active fraction isolated from the saline lung lavage; IB-TL, total lipids from IB; IB-PC, phosphatidylcholine (diacyl glycerophosphorylcholine) from IB; C_{16}-PC, dipalmitoyl glycerophosphorylcholine; π, surface pressure; π_{max}, maximum surface pressure; C–M, volume-to-volume mixtures of chloroform and methanol; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

MATERIALS AND METHODS

Analytical methods and procedures

Procedures for the isolation of lipids, analytical methods including TLC and GLC, surface activity determinations, and scintillation counting have been described in our previous communications (7, 8, 10). Preparations of C_{16}-PC and IB-PC containing various mole fractions of cholesterol and tripalmitin for mixed monolayer studies were prepared from stock solutions of each lipid. Aliquots containing calculated amounts of each component were evaporated together under nitrogen and redissolved in the required amount of C–M 85:15 to give an overall concentration of 0.5–1.0 mg of lipid/ml. The molecular weights of the phospholipids were estimated from the phosphorus content. The areas/molecule for isotherms obtained in the mixed monolayer experiments were always expressed in terms of the calculated amount of phospholipid added to the surface of the subphase. Mixed monolayer studies were performed using a constant surface area, i.e., static method, while experiments designed to investigate the effect of phospholipase A_2 hydrolysis on intact IB involved cyclic compression–expansion of the surface area, i.e., dynamic method. These two techniques and the types of information obtained from each have been previously described in detail (7).

Isolation of the surface-active fraction from dog lung

The surface-active fraction, IB, was isolated by a modification of our previous procedure (7). Adult mongrel dogs of both sexes were anesthetized with pentobarbital, 26.4 mg/kg. A catheter, attached to a reservoir of 0.145 M NaCl at 37°C, was inserted into the jugular vein. The reservoir was approximately 3 ft above the level of the animal. The abdomen was opened and the vena cava was clamped with a hemostat. The abdominal aorta was then cut, and at least 2000 ml of NaCl solution was allowed to flow into the jugular vein. This usually took approximately 5 min, during which time heartbeat and respiration were spontaneous. The thorax was opened, and the lungs, which were white to very light pink in color, were removed and placed on ice. Routine phase microscopy of 1-μ Epon sections of lungs subjected to this procedure demonstrated a marked reduction in the number of erythrocytes present in the capillaries in relation to nonperfused lungs. The lungs were homogenized and the homogenate was filtered through nylon mesh and layered over 0.75 M sucrose. After centrifugation at 48,000 *g* (JA-20 rotor, J-21 centrifuge, Beckman Instruments, Palo Alto, Calif.), the washed interfacial material, I, from 10–15 g of lung was suspended in 10 ml of 0.65 M sucrose and placed in a single 50.1 rotor tube. The IC was precipitated by centrifugation (Beckman L2-65B ultracentrifuge) at 90,000 *g* for 60 min. The cloudy supernate was removed, diluted with homogenization medium to a sucrose concentration of 0.25 M as determined with a refractometer, and again centrifuged at 90,000 *g* for 60 min. This precipitated the surface-active fraction, which was suspended in 0.1 M Tris, pH 7.4, and then resedimented at 48,000 *g* for 20 min in the JA-20 rotor. This modification of our earlier procedure allows us easily to isolate the surface-active fraction from 120 g of dog lung in one day. When required, extra- and intracellular IB were isolated as before using the above modifications (8).

Diacyl glycerophosphorylcholines

IB-PC was isolated, recrystallized, and characterized as before (7, 10). C_{16}-PC was obtained from Calbiochem (La Jolla, Calif.) and purified (7).

Phospholipase A_2

The source of phospholipase A_2 was lyophilized *Crotalus atrox* venom purchased from Ross Allen Reptile Institute (Silver Springs, Fla.). During the course of this investigation, several different lots of venom were used. All were active at the glycerol C-2 position of PC, no C-1 activity was observed, but some variation in enzyme specific activity was seen in different lots. Consequently, the specific activity of each new lot was determined in the assay system described below using 1.0 μmole of C_{16}-PC at 37°C. This allowed us to standardize each venom so that equivalent hydrolysis rates could be obtained with the different enzyme lots. An arbitrary standard velocity of 67 nmoles of C_{16}-PC hydrolyzed/min under these conditions was chosen, and the amount of venom necessary was always between 10 and 30 μg/ml of reaction mixture.

Phospholipase A_2 assay

When IB-TL or one of the various PCs was the substrate preparation, an aliquot of the C–M 2:1 stock solution sufficient to prepare a 10% excess of the calculated amount was taken to dryness under nitrogen in a small beaker. After addition of 0.01 M Tris, pH 7.4 (unless otherwise indicated), the beaker was placed in an ice-water bath and sonicated for 1 min in a Branson Sonifier, model W185D (Heat Systems-Ultrasonics, Plainview, N.Y.) at a meter reading of 70 w. Clear suspensions, stable for several hours, were obtained. No effect upon hydrolysis rates was seen when the sonication period was varied up to 10 min. Other lipids were sonicated together with the appropriate PC in the calculated proportions. IB was dispersed in the buffer by vigorous mechanical agitation for 1 min on a Vortex-type mixer. The enzymatic reaction was carried out in 15-ml glass

stoppered centrifuge tubes. Each tube contained 1.0 ml of the dispersed substrate preparation. After temperature equilibration in a shaking water bath for 10 min, 10 μl of 0.5 M $CaCl_2$ in the Tris buffer was added and equilibration continued for 5 min. Phospholipase A_2 (10–30 μg of *C. atrox* venom) in 20 μl of Tris buffer was added at zero time. At the end of the incubation period, 5 ml of ice-cold C–M 2:1 was added with vigorous mixing to stop the reaction and extract the lipids (11). The resultant emulsion was separated by centrifugation at 2000 rpm and the upper aqueous methanol layer was removed by aspiration. The lower chloroform layer was washed with fresh upper phase solvents and then taken to dryness under nitrogen. The residue was taken up in 75 μl of C–M 2:1, and 2–25-μl aliquots were spotted on silica gel H plates for TLC. After development in chloroform–methanol–water 75:27.5:5, the plates were exposed to I_2 until the spots were lightly colored. Areas corresponding to unhydrolyzed PC and lyso-PC were scraped directly into digestion tubes for determination of phosphorus. The course of the reaction could, therefore, be followed by the disappearance of PC and the appearance of lyso-PC. Results were expressed as nanomoles PC hydrolyzed per minute. The hydrolysis of PC was linear for 15 min; generally, both 3- and 6-min incubation periods were used for a given initial velocity determination. Tubes containing no phospholipase A_2 and which were not incubated allowed the determination of initial substrate concentration in terms of PC. IB contains phospholipids other than PC, the major constituent (7), but only PC was hydrolyzed to an appreciable extent under these conditions. When the surface activity of reaction mixtures from the hydrolysis of IB was to be determined, enzymatic action was terminated by the addition of 1.0 ml of 2% EDTA.

In these experiments, the *C. atrox* venoms were not heat-treated to remove degradative enzymes other than phospholipase A_2. However, control experiments utilizing IB and C_{16}-PC as substrates were carried out to compare heat-treated venoms (12) with those prepared as above. Heat treatment did not affect the phospholipase A_2 kinetics, and the relationship between the two substrates shown in Fig. 4 was unaltered.

A schematic representation of the experimental design is given in Fig. 1. Although several different substrate preparations were used in these studies, a PC species was always the actual substrate of phospholipase A_2. For example, prior to lipid extraction, IB-PC was an integral constituent of the IB macromolecule and would be expected to participate in various interactions with the other molecular constituents. These interactions could conceivably affect susceptibility of the constituent IB-PC to hydrolysis by the enzyme. Since initial substrate concentrations and reaction velocities were expressed in terms of the actual amount of PC initially present and subsequently hydrolyzed at the given times in each particular substrate preparation, we could directly and quantitatively compare the kinetics of the enzyme reaction at the different stages outlined in Fig. 1. This approach was designed to yield information concerning the state of the PC as a constituent of IB and its participation in molecular interactions.

RESULTS

Kinetic studies

Under the conditions of our standardized assay, the pH optimum for the phospholipase A_2 hydrolysis was between 7.0 and 8.0 with IB as the substrate preparation (Fig. 2). The relationship between enzyme concentration and velocity with IB is shown in Fig. 3. The calcium concentration, 5×10^{-3} M, used in our assay system was found to be within the optimum range, and no reaction took place in its absence.

Results of the kinetic studies with the various substrate preparations are given as Lineweaver-Burk plots for ease of comparison (Figs. 4–6). The lines in Figs. 4–6 were drawn from least squares plots of experimental values obtained from a minimum of 10 different substrate concentrations throughout the range indicated in each figure. In no instance did any experimental point deviate more than 5% from the least squares line. In Fig. 4, a comparison of the IB and IB-TL lines demonstrates that removal of nonlipid components from IB to give IB-TL caused an increase in velocity throughout the range of initial substrate concentrations used, although V_{max} values for both substrates were similar. Results with IB_E and IB_I did not differ significantly from IB. When all non-PC components were removed

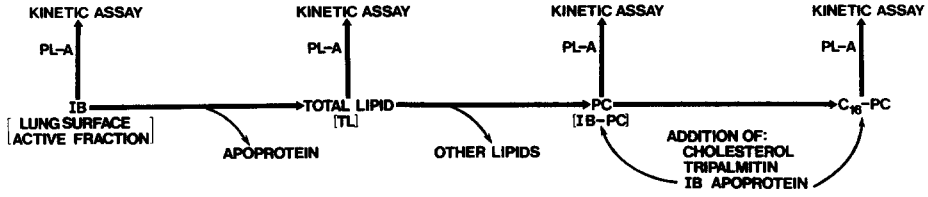

FIG. 1. Schematic outline of experimental design. Kinetics of phospholipase A_2 (PL-A) hydrolysis were determined at each successive stage after the sequential removal or readdition of IB constituents.

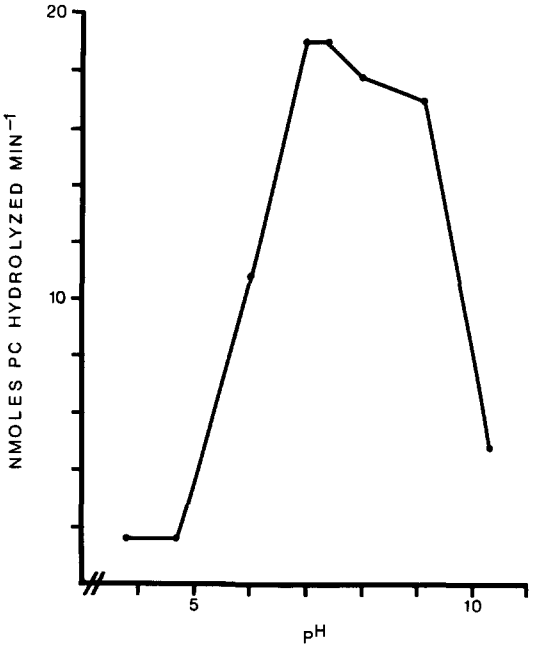

FIG. 2. Effect of pH on velocity of phospholipase A_2 hydrolysis with IB as the substrate preparation. Each tube contained 1.00 μmole of PC in the standard assay system except that the pH was as indicated.

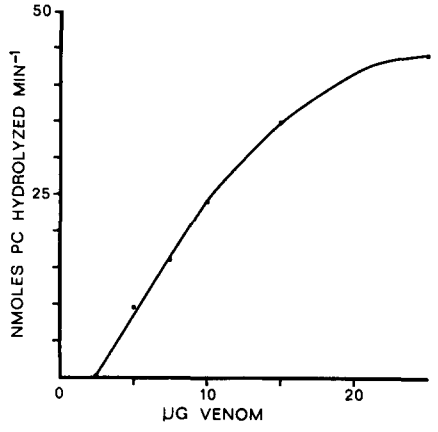

FIG. 3. Relationship between concentration of *C. atrox* venom (source of phospholipase A_2) and velocity of hydrolysis with IB as the substrate preparation. Each tube contained 1.45 μmoles of PC in the standard assay system except that the amount of venom was as indicated.

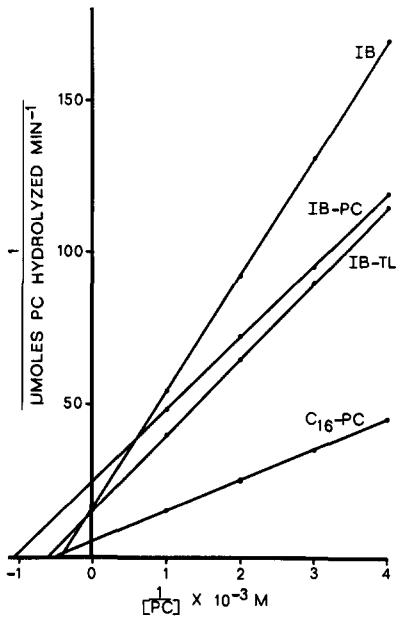

FIG. 4. Lineweaver-Burk plots of results obtained with various substrate preparations at the stages outlined in Fig. 1. Standard assay conditions as described in the text were used.

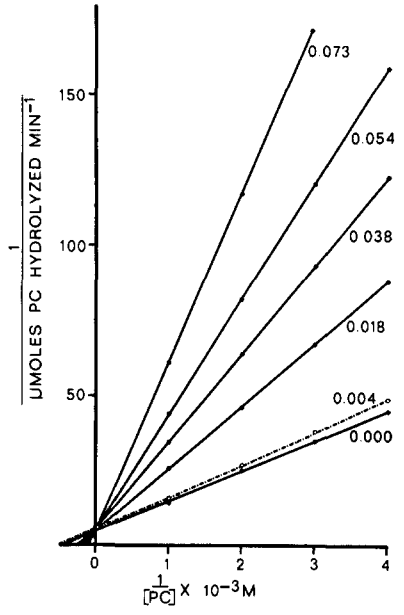

FIG. 5. Lineweaver-Burk plots showing effect of cholesterol on C_{16}-PC hydrolysis by phospholipase A_2. Numbers at each line indicate the mole fraction of cholesterol in the C_{16}-PC–cholesterol substrate.

from IB-TL to yield IB-PC, the velocity was still greater than that seen with the intact IB except at the highest initial substrate concentrations. V_{max} for IB-PC, however, was lower than with IB-TL or IB. We have previously shown that C_{16}-PC is the principal PC species present in IB (7). When C_{16}-PC was used as the substrate, V_{max} was greatest and a very marked increase in velocity was seen throughout the entire range of initial substrate concentrations.

After hydrolysis for various times, the free fatty acids and the 1-acyl-2-lyso-PCs produced from IB_E, IB_I, and IB-PC were isolated by TLC, and the fatty acid distribution pattern of each was ascertained by GLC (7, 10). This approach was designed to yield information concerning which molecular species of PC were hydrolyzed

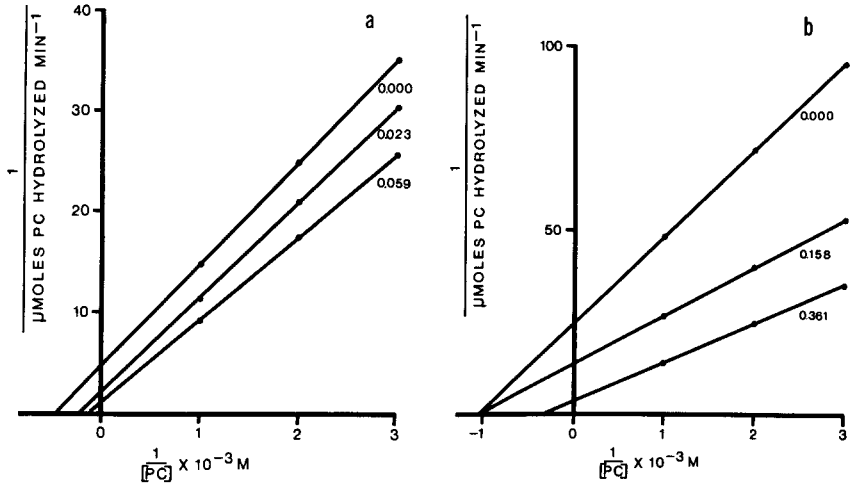

FIG. 6. Lineweaver-Burk plots showing the effect of tripalmitin on (*a*) C_{16}-PC and (*b*) IB-PC hydrolysis by phospholipase A_2. Numbers at each line indicate the mole fraction of tripalmitin in the tripalmitin–PC substrate.

through each time period in the substrate preparations. The data presented in Table 1 are rather involved, but several significant findings can be seen. The fatty acid distributions at the 1- and 2-positions at 0 time represent what would be expected at each time period if the hydrolysis took place nonselectively. If we first look at the percentages of total saturates for IB_E and IB_I, we see that the PCs actually hydrolyzed by the enzyme contain more saturated residues at each position than would be expected on the basis of the 0 time compositions, i.e., compare 0 with 3 min. The 1-position was, at least initially, considerably more saturated than the 2-position. In general, IB_E and IB_I gave similar results with respect to total saturates. However, the pattern seen with IB-PC differed significantly, especially in the initial phases of the hydrolysis. There does appear to have been selection for saturated residues at each position just as for IB_E and IB_I, but the 2-position for IB-PC contained a higher percentage of saturates than did the 1-position, in contrast to the findings with the IBs.

The predominant residue at each position was the 16:0. There was definitely a selection for PC species containing 16:0 at the 2-position in all of the substrate preparations. Again, IB-PC species actually hydrolyzed contained more 16:0 at the 2-position than at the 1-position while IB_E and IB_I had approximately equal percentages at both positions. The results with 18:0 for IB_E and IB_I indicate a pronounced selection for PC species with this residue at the 2-position, especially at the earliest hydrolysis periods. This selection was not as marked with IB-PC. At the 1-position, IB_E and IB_I exhibited some differences with 18:0. When the IBs were compared with

TABLE 1. Distribution of fatty acids at 1- and 2-positions of hydrolyzed PC as a function of time of hydrolysis[a]

Fatty acids	Minutes of Hydrolysis	Position 1			Position 2		
		IB_I	IB_E	IB-PC	IB_I	IB_E	IB-PC
		% of total fatty acids					
Total saturates	0[b]	82.3	84.8	83.6	65.2	65.5	65.4
	3	95.0	97.8	77.2	88.3	89.9	94.4
	6	97.4	97.2	83.5	75.0	85.9	97.3
16:0	0	66.7	73.2	70.0	51.2	54.2	52.7
	3	67.1	77.4	60.9	70.8	69.8	79.5
	6	72.8	75.4	67.2	60.8	71.5	82.6
18:0	0	12.0	7.8	9.9	1.6	1.7	1.6
	3	25.1	16.4	13.5	14.2	14.3	5.4
	6	21.2	18.4	14.1	8.5	7.7	4.8

[a] Standard assay conditions. For each substrate, each time point represents a single reaction tube. GLC analyses were done in duplicate as previously described (7). Initial PC concentration was 1.00 μmole/ml of reaction mixture. Although these results are from one experiment, they are entirely representative of other similar experiments.

[b] 0-time distribution at the 1- and 2-positions were determined on PCs extracted from IB_I, IB_E, and IB as previously described (7); the PCs were completely hydrolzed with *Naja naja* phospholipase A_2 in a diethyl ether reaction mixture. The 1-acyl-2-lyso-PC and 2-position fatty acids were isolated by TLC and subjected to GLC analysis.

IB-PC, the overall pattern was again different since 18:0 accounted for far fewer of the total residues at the 2-position of IB-PC in relation to the IBs.

The results presented in Fig. 4 and Table 1 could be interpreted to indicate that IB-PC and C_{16}-PC participate in lipid–protein and lipid–lipid interactions in the intact IB such that their susceptibility to hydrolysis by the enzyme is markedly affected. Cholesterol and triglyceride are also found in IB (7) and might reasonably be expected to participate in interactions with the constituent PC species of IB. As shown in Fig. 5, the rate of hydrolysis was inhibited with substrate dispersions prepared by sonicating increasing mole fractions of cholesterol in C_{16}-PC. The inhibition appears to be competitive but, when the enzyme was preincubated with a sonicated dispersion of cholesterol for 15 min prior to the addition of C_{16}-PC, no inhibition was observed. Results similar to those given in Fig. 5 were obtained with IB-PC and cholesterol.

Incorporation of tripalmitin into C_{16}-PC and IB-PC substrate preparations increased the rate of hydrolysis (Fig. 6). While the effect was seen with both PCs, no further increase in velocity with C_{16}-PC was found above a tripalmitin mole fraction of 0.059. On the other hand, velocity with IB-PC was increased up to a tripalmitin mole fraction of 0.361 (Fig. 6*b*). Again, preincubation of the enzyme with tripalmitin prior to the addition of IB-PC or C_{16}-PC gave no increase in the velocity over the control values shown in Fig. 6.

IB apoprotein obtained by our previous procedure (7) had no effect at all on the hydrolysis of C_{16}-PC or IB-PC.

The effect of temperature on the reaction velocity with various substrate preparations is seen in Fig. 7. The initial substrate concentration in terms of PC was the same for all preparations. An increase in velocity throughout the temperature range was observed with IB_E, IB_I, and IB-PC. There was a marked decrease in velocity seen with C_{16}-PC and C_{16}-PC + cholesterol between 40 and 45°C. The presence of cholesterol decreased the velocity of C_{16}-PC hydrolysis over the range of temperatures studied. In the presence of tripalmitin, C_{16}-PC was hydrolyzed much faster at all temperatures, and the point at which the profound decrease in velocity occurred was raised to 50°C.

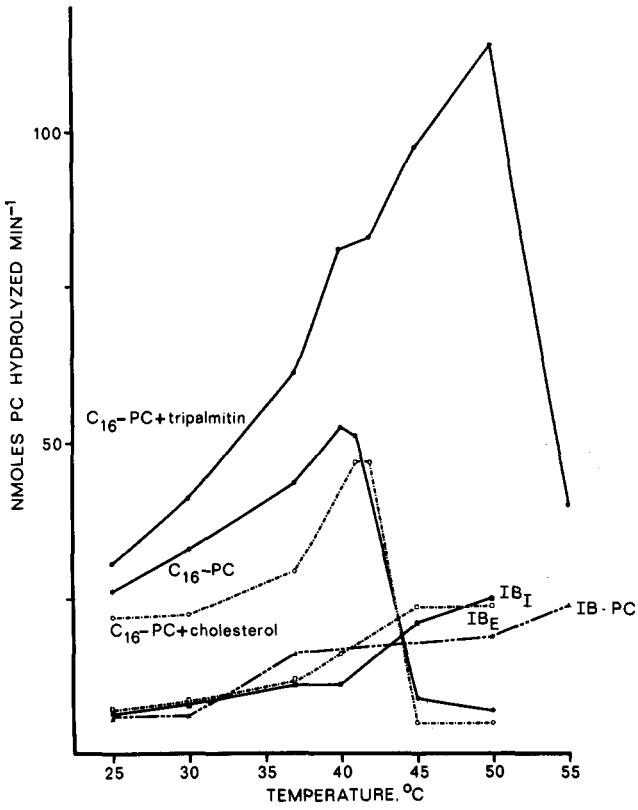

FIG. 7. Effect of temperature on phospholipase A_2 hydrolysis of PC in various substrate preparations. The initial PC concentration in all cases was 0.54 μmole/ml in the standard assay system. The mole fractions of tripalmitin and cholesterol in C_{16}-PC substrate preparations were 0.10 and 0.05, respectively.

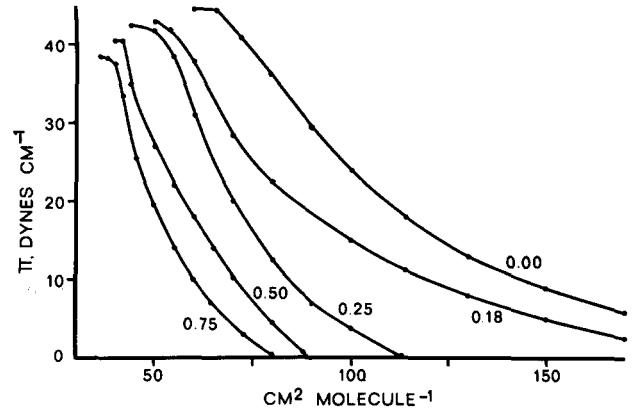

FIG. 8. Effect of cholesterol on surface isotherm of IB-PC. Numbers by each isotherm refer to the mole fraction of cholesterol. Isotherms were obtained with the static surface balance at constant surface area (7).

Surface chemistry studies

The above results emphasized the effect of substrate interactions on the nature of the enzymatic hydrolysis. Some of these possible substrate interactions and the effect of the hydrolysis on IB were investigated through the use of monolayer studies (7).

The isotherm for IB-PC was both less condensed and more compressible than that obtained with C_{16}-PC (Figs. 8 and 9 and Ref. 7). Increasing the mole fraction of cholesterol in IB-PC mixed monolayers gave isotherms which were more condensed and less compressible than the original IB-PC isotherm (Fig. 8). π_{max} was also decreased somewhat with the addition of cholesterol. Although Chapman et al. (13) have previously shown that cholesterol reduces the area occupied by each

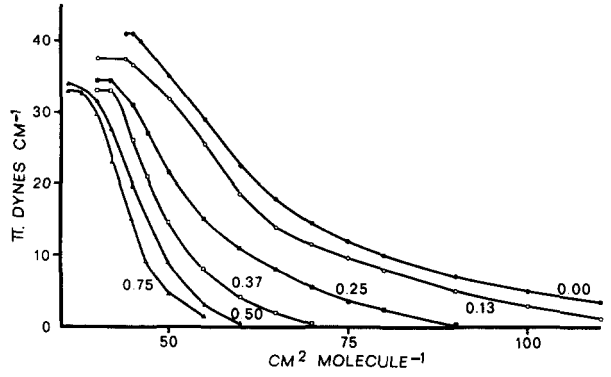

FIG. 9. Effect of cholesterol on surface isotherm of C_{16}-PC. Numbers by each isotherm refer to the mole fraction of cholesterol. Isotherms were obtained with the static surface balance at constant surface area (7).

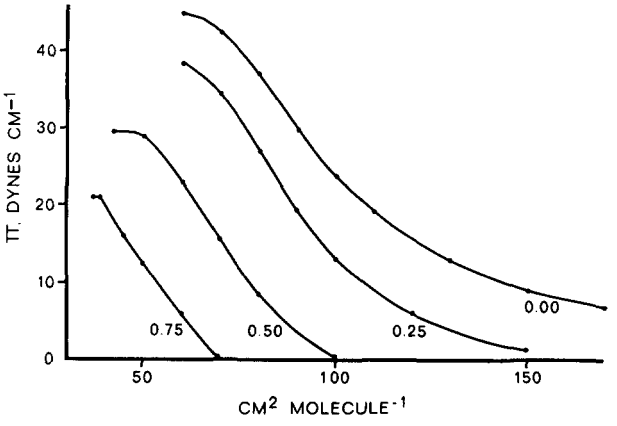

FIG. 10. Effect of tripalmitin on surface isotherm of IB-PC. Numbers by each isotherm refer to the mole fraction of tripalmitin. Isotherms were obtained with the static surface balance at constant surface area (7).

C_{16}-PC molecule, thereby causing a condensation of the C_{16}-PC isotherm, we have included our similar findings (Fig. 9) to provide a more direct comparison of our results with IB-PC and C_{16}-PC. It should be noted that the abscissas for Figs. 8 and 9 are drawn to different scales. π_{max} was reduced slightly with both phospholipids upon the addition of cholesterol. The condensation observed upon the addition of cholesterol to IB-PC was greater than with C_{16}-PC. This latter finding can be seen by comparing the percentage decrease in the area/molecule at a given π for the neat phospholipid and for a cholesterol mole fraction of 0.25. At a π of 10 dynes/cm, the decrease calculated in this manner for IB-PC was 58% and for C_{16}-PC was 41%. These changes were most obvious at the lower π values.

Increasing the mole fraction of tripalmitin in IB-PC mixed monolayers caused a large decrease in π_{max} and gave isotherms which were much more condensed than the original phospholipid (Fig. 10). The compressibility, as judged by the slopes of the isotherms, was not greatly affected by the addition of tripalmitin to IB-PC. When mixed monolayers of tripalmitin and C_{16}-PC were prepared, a condensation of the resultant isotherms and a reduction in π_{max} were again obtained, but increased compressibility was also seen with this phospholipid (Fig. 11).

Intact IB was subjected to hydrolysis for various times in the standard system at an initial substrate concentration of 1.56 μmoles of PC/ml. This corresponded to 500 μg of IB protein. The reaction was stopped by the addition of EDTA. When aliquots of the reaction mixtures were then applied to the surface of the subphase at constant surface area, very small changes in the isotherms were seen as the time of hydrolysis increased. Consequently, the dynamic surface balance was used for these studies since cyclic compression–expansion tends to magnify changes under these conditions. The results are given in Fig. 12 as plots of the maximum surface pressure

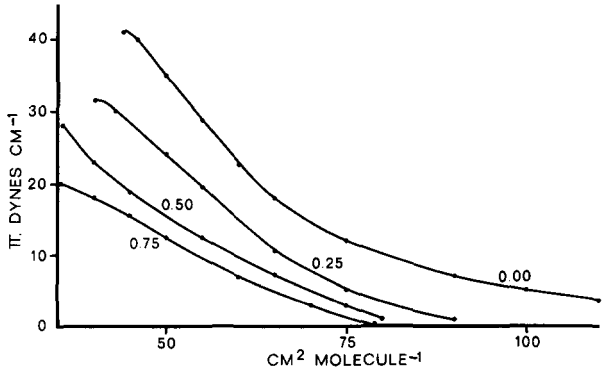

FIG. 11. Effect of tripalmitin on surface isotherm of C_{16}-PC. Numbers by each isotherm refer to the mole fraction of tripalmitin. Isotherms were obtained with the static surface balance at constant surface area (7).

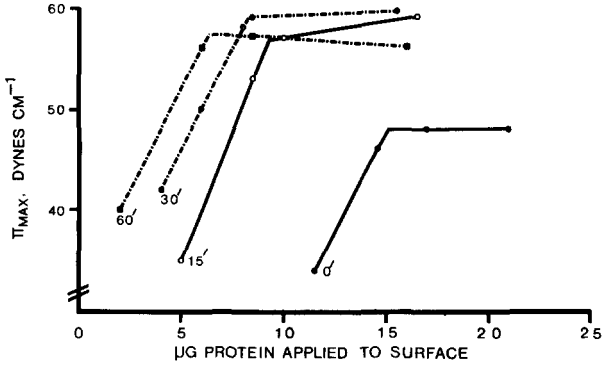

FIG. 12. Effect of phospholipase A_2 hydrolysis of IB on maximum surface pressure, π_{max}. IB, 1.56 μmoles of PC and 500 μg of protein, was subjected to hydrolysis in the standard assay system for the number of minutes indicated in the figure. The reaction was terminated by the addition of EDTA. Aliquots of the reaction mixtures were then placed on the surface of the subphase for the determination of the maximum surface pressure reached after cyclic compression–expansion of the surface area (7).

obtained at minimum surface area vs. the amount of original IB protein added to the surface. The π_{max} was usually reached after the fourth complete cycle. The results clearly show that, as the hydrolysis proceeded, the surface activity under these conditions actually increased. Not only were higher final π_{max} values reached with the hydrolyzed preparations, but less protein was required in order to reach any given π_{max}.

DISCUSSION

The study of fractions derived from the alveolar lining layer offers a unique opportunity to investigate molecular interactions and to correlate structure–activity relationships in a biological macromolecular assembly. The role generally proposed for pulmonary surfactant, a major functional component of this lining, is the reduction of alveolar surface tension at low lung volumes during respiration. This marked ability to reduce surface tension is associated with a propensity of alveolar constituents to orient at the air–liquid interface in vitro and presumably at the air–tissue interface in situ. In a previous report, assuming our total IB fraction was representative of pulmonary surfactant, we described in some detail how these surface forces could arise, and, if necessary, change in magnitude with lung volume (7). The phospholipid hydrocarbon residues were considered to be in the air phase of the interface, and a predominant role was ascribed to C_{16}-PC in the generation of surface forces. At that time, however, we pointed out that the surface properties of C_{16}-PC were modified by interaction with other constituents since surface isotherms for C_{16}-PC were not the same in the intact IB and the pure phospholipid.

The present studies with *C. atrox* venom as the source of phospholipase A_2 were designed to investigate some of the characteristics of those interactions which might take place in IB. Although these experiments were conducted with venom which had not been heat-treated and consequently contained enzymatic activities other than phospholipase A_2, the presence of these other enzymes apparently did not affect the hydrolysis of IB and C_{16}-PC. Implicit in these experiments is the assumption that susceptibility to and rate of hydrolysis are related to the accessibility of the enzyme to the substrate. Lack of access refers to substrate modification by interaction, i.e., noncovalent bonding, with various molecular species in such a way that the hydrocarbon chains of PC are rendered less available for attack by the enzyme.

If C_{16}-PC residues are preferentially located on the surface of the IB macromolecular particle, they could then be more accessible than other PC species to attack by the enzyme. That this may be so can be seen from the results given in Table 1. At the earliest times, using IB_E and IB_I as substrates, there was a preferential hydrolysis of C_{16}-PC as well as other disaturates. This is evident when the 0-time compositions are compared with, for instance, the 3-min compositions. The 0-time values indicate what would be expected if the hydrolysis were random with respect to PC species. Selectivity for saturation was also evident with IB-PC, especially at the 2-position. It would be difficult with these results to distinguish completely between selection by accessibility due to location and selection as the result of a "preferred" substrate molecular species giving a faster velocity (14). We can consider selection by substrate preference to be maximum in IB-PC because only PC–PC interactions would take place to modify availability of individual molecular species to the enzyme. Results with IB_E and IB_I differed considerably from IB-PC and, therefore, selection by locational accessibility as the result of multiple interactions might be important in these macromolecular substrate preparations.

From Fig. 4, velocities with C_{16}-PC were the highest found for any single substrate. IB-PC, except at the highest substrate concentrations, gave a greater velocity than did intact IB. We may then infer that molecular interactions in IB render the constituent PC, i.e., IB-PC as an integral component of the macromolecule, less accessible to the enzyme than with the "pure" phospholipid. A comparison of the IB-PC and IB-TL lines suggests that non-PC lipid components interact with PC in such a fashion that the PC as a component of IB-TL is somewhat more susceptible to hydrolysis than it is alone.

We cannot rigorously exclude the presence of some inhibitory substance in IB and its derivatives that acts upon the enzyme itself, especially if the effect of an inhibitor were temperature dependent, since the end result of substrate inaccessibility and inhibitor reaction with the enzyme would give similar results. The results shown in Fig. 4 could, at first glance, be interpreted as different types of inhibition in the normal connotation, with C_{16}-PC representing the substrate in the absence of inhibitor. Some observations, however, mitigate against the presence of an enzymatic inhibitor, viz., (*a*) IB apoprotein had no effect upon the hydrolysis of C_{16}-PC and IB-PC; (*b*) hydrolysis with IB-TL was faster than with IB-PC (Fig. 4); (*c*) cholesterol, which gave reduced rates when sonicated with IB-PC and C_{16}-PC (Fig. 5), had no effect when preincubated with the enzyme; and (*d*) the temperature studies shown in Fig. 7, along with the surface chemistry results (Figs. 8–11), indicate that the physical state of the substrate is the determinant criterion of hydrolysis.

The following considerations have led us to postulate that the PC hydrocarbon chains in intact IB have a high degree of liquid crystalline character.

(*a*) The results shown in Fig. 7 can be interpreted to indicate that phospholipase A_2 prefers substrates in the gel phase and is less active towards substrates in the liquid crystalline phase. PC species are lyotropic, thermotropic, mesomorphs exhibiting phase changes as a function of water content and temperature. The present studies were carried out in the presence of excess water, i.e., greater than 40% by weight, so that the temperature, Tc, at which the main endothermic transition takes place is at its minimum value (15, 16). At temperatures below Tc, a lamellar gel is formed in which the PC hydrocarbon chains are crystalline; above Tc, the PC–water system exists in a lamellar mesomorphic phase which is often termed the liquid crystalline phase. For C_{16}-PC in the presence of excess water, Tc is 38–41°C as determined from a variety of physical methods (15, 17–19). This is exactly the temperature range where the dramatic decrease in velocity takes place in the hydrolysis of C_{16}-PC. Snake venom phospholipases are very heat stable (20) and, because the velocity with IB-PC, IB_E, and IB_I continued to increase above this temperature, the fall in velocity with C_{16}-PC presumably was not due to thermal denaturation of the enzyme or to a specific temperature effect on an active (hydrophobic) site. The initial increase in velocity seen with C_{16}-PC up to 40°C can be attributed to increased kinetic energy in the system but, once the kinetic energy was sufficient to drive the system into the liquid crystalline phase, hydrolysis was inhibited by the change in phase structure.

(*b*) The PC gel–liquid crystalline transition is broadened in the presence of cholesterol (15). Cholesterol is found in IB; the calculated mole fraction of free cholesterol with respect only to PC is 0.20 (7). Below the Tc for the phospholipid, inclusion of cholesterol imparts a greater liquid crystalline character to the system, and above the Tc, cholesterol will act to give more gel character to the phospholipid. Another way of stating this is that, at any temperature between 25 and 40°C in Fig. 7, C_{16}-PC + cholesterol exhibits more liquid crystallinity than does C_{16}-PC alone. Thus, the decrease in velocity of hydrolysis in the presence of cholesterol can be attributed to an increase in liquid crystalline content.

(*c*) Increasing the unsaturation in PC hydrocarbon chains and mixing PCs with differing chain lengths also lowers and broadens the gel–liquid crystalline transition (21). The composition of IB-PC fatty acid residues exhibits significant unsaturation and variation in chain length.

(*d*) As a function of temperature, velocities with IB-PC, IB_E, and IB_I were similar (Fig. 7), especially in the absence of dramatic changes in the slopes of the curves. This indicates that Tc was broadened in these preparations.

(*e*) Phillips and Chapman (19) have pointed out that, since the temperature in the lung is lower than Tc for C_{16}-PC, a two-dimensional condensation of this phospholipid in the alveolar surface would be expected in the absence of constraining influences. The function of cholesterol and unsaturation in IB would then be analogous to their postulated role in other biological systems, i.e., increased fluidization.

The failure of IB apoprotein to modify the kinetics of the reaction may have been due to irreversible protein conformational changes induced by the method of preparation or to an inability to find the proper conditions to effect an interaction.

The increase in IB-PC and C_{16}-PC hydrolysis seen with tripalmitin (Figs. 6 and 7) would be the result of increased or retained lamellar gel character. The actual determination of this is beyond the scope of the present work. There was a sharp decrease in velocity seen with C_{16}-PC + tripalmitin which, however, occurred at a higher temperature than with C_{16}-PC alone. This could be interpreted as a phase change taking place above the normal Tc for C_{16}-PC.

Phillips and Chapman (19) have clearly delineated the similarities and differences between the monolayer and bulk systems in studies of phospholipid interactions and have shown how they may be compared. A PC monolayer is liquid-expanded (liquid crystalline) at lower surface pressures and larger areas/molecule. As the monolayer is compressed to higher surface pressures and smaller areas/molecule, it becomes condensed (gel). Our bulk phase studies suggest that phospholipase A_2 prefers substrates in the gel phase. This can be correlated with the results of Colacicco and Rapport (22) concerning the effect of initial surface pressure upon the hydrolysis of egg PC monolayers. At low surface pressures, hydrolysis proceeded slowly because the substrate was liquid crystalline. As the initial surface pressure was increased, the hydrocarbon chains were more gel-like and hydrolysis increased. The decrease in hydrolysis seen at even higher initial surface pressures could be the result of the even closer packing of the PC molecules, thereby presenting a barrier to attack by the enzyme. The monolayer at this point would be analogous to C_{16}-PC in our bulk phase system at the lower temperatures (Fig. 7). The hydrocarbon chains would be in the preferred gel state, but the kinetic energy was so low that the molecules were packed very tightly and hydrolysis was minimal.

Previous studies with mixed monolayers of C_{16}-PC and cholesterol (13, 23, 24), as well as our results presented in Figs. 8 and 9, have shown that cholesterol reduces the area occupied by the phospholipid molecules when they are in the liquid-expanded state. The exact mechanism leading to this condensation is discussed in the literature (13, 18, 24–28), but adding cholesterol to PC mono-

layers has been likened to lowering the temperature of the phospholipid, thereby decreasing the kinetic energy of the hydrocarbon chains (13). The result would be a closer packing of the phospholipid molecules as exemplified by the decrease in the area/molecule seen upon the addition of cholesterol. A more rigid structure would be obtained, and in this sense we would say that the degree of order was increased in the monolayer. This can also be seen from two other aspects of the isotherms shown in Figs. 8 and 9: (*a*) the decrease in compressibility indicates that the phospholipid molecules are brought closer together in the presence of cholesterol and (*b*) the π_{max} was not greatly decreased in the mixed monolayers. The π_{max} reached by a system may be considered a function of how close the components can be packed in order to "squeeze out" water from the film. The extrapolation of these monolayer considerations to our bulk phase results with phospholipase A_2 hydrolysis must, of course, be made with caution, but a simple explanation for the inhibition seen with cholesterol can also be offered on this basis. The closer proximity of the PC molecules in the presence of cholesterol presents a barrier to entry of the enzyme into the substrate micelles, thus effectively decreasing substrate accessibility.

The increase in velocity seen upon the inclusion of tripalmitin in IB-PC and C_{16}-PC substrate micelles (Fig. 6) may also be interpreted in terms of mixed monolayer studies (Figs. 10 and 11). Tripalmitin also caused a condensation of the PC isotherms, but the degree of order was presumably decreased. In the presence of tripalmitin, the π_{max} obtained was drastically lowered and, at least for C_{16}-PC, the compressibility was markedly increased. The resulting disorder of the PC hydrocarbon chains in the bulk phase micelles would not present an effective barrier to the enzyme.

The effect of phospholipase A_2 hydrolysis on the surface behavior of IB (Fig. 12) was rather surprising. Hydrolysis of one of the PC acyl linkages might be expected to decrease the surface activity of IB; yet, in terms of the π_{max} reached and the amount of IB necessary to attain a given π, hydrolysis seemingly increased IB surface activity. The following observations may offer a partial explanation for these findings. Phospholipase A_2 hydrolysis of monolayers of PC species similar to IB-PC does not greatly affect π under equilibrium conditions, i.e., the static surface balance (22, 29). Indeed, the changes are often very small and the course of the reaction can be followed only by surface potential measurements. A fatty acid and lyso-PC are the products of the hydrolysis. Both are surface-active to some extent. Lyso-PC will not reach as high a π_{max} as PC under static conditions, but at a π of 20 dynes/cm the areas/molecule are similar (30). Fatty acids are also surface-active. These molecules would presumably still be available as surfactants. Simpkins, Tay, and Panko (12), in a spin label study of the effect of phospholipase A_2 treatment on axonal and red blood cell membranes, found that immobility and order increased in the lipid regions and a condensation resulted in a more closely packed lipid array. If a similar process took place in our experiments with IB, the condensation of the lipid region might explain the increase in surface activity because the hydrocarbon chains could conceivably then "squeeze out" more water upon the over-compression which takes place on dynamic surface balances (7).

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