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# Phospholipases. I. Effect of *n*-Alkanols on the Rate of Enzymatic Hydrolysis of Egg Phosphatidylcholine

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*Summary.* The rate of hydrolysis of unsonicated liposomes of egg lecithin by phospholipase A (from bee venom and Russell viper venom) and phospholipase C (from *Bacillus cereus* and *Clostridium welchii*) is markedly dependent on the nature and concentration of a variety of added alcohols. Typical plots of rate against alcohol concentration are bell-shaped. The maximum rate and the alcohol concentration at which it is achieved are alcohol-specific. In a homologous series of *n*-alkanols, the maximal rates increase and the optimal concentrations decrease as the chain length is increased from $C_4$ to $C_8$. For longer alcohols ($C_9$ to $C_{12}$), progressively higher concentrations are required to elicit maximal activation. The optimal activating concentrations $C$ for $C_4$ to $C_8$ *n*-alkanols obey the relationship $pC = a \log P_{\text{octanol}} + \text{constant}$ [*cf.* Hansch & Dunn, *J. Pharm. Sci.* **61**:1 (1972)], suggesting that the alcohol-activating effect is a consequence of their incorporation into the liposomes with resultant modification of liposomal structure.

Phospholipases are an important group of enzymes which exhibit a complex pattern of substrate specificity (Ansell & Hawthorne, 1964) associated with the function of these enzymes in the *in vivo* catabolism of several classes of phospholipids (Thompson, 1970). Phospholipids rarely form simple molecular solutions in water but exist in one or more of a variety of organized structures, the nature of which depends on the structure of the phospholipid, the composition of the aqueous phase, temperature, and other environmental parameters (Jain, 1972). Such aggregates of phospholipids or detergent-dispersed phospholipids are the usual substrates employed for the *in vitro* study of phospholipases: biological phospholipids or their analogues which form simple solutions in water are not generally suitable substrates. This fact emphasizes the probable importance of the state of aggregation and mode of organization of the substrate for regulation of enzyme-substrate interactions in such systems (Entressangles & Desnuelle, 1968). Indeed, previous observations strongly suggest that the fine structure

of the lipid-water interface is a highly important parameter for phospholipase action (DeHass *et al.*, 1971; Bonsen *et al.*, 1972). Results of studies employing detergent-dispersed or sonically dispersed phospholipids as substrate (Roholt & Schlamowitz, 1961; Van Deenen & De Haas, 1963; Seppala *et al.*, 1971; Uthe & Magee, 1971*a*, *b*; Scherphof *et al.*, 1972; Smith *et al.*, 1972; Wells, 1972) are difficult to interpret in detail since the nature of phospholipid-detergent interactions and the resultant structures are not well understood.

In addition to those structures formed by isolated phospholipids, phospholipases catalyze the hydrolysis of membrane-bound phospholipids and, as a consequence, modify the structure and properties of biological membranes in a number of ways (Jain, 1973). At this level of structural complexity, the nature of lipid-protein interactions and their consequences for the surface properties of the membrane become important for an understanding of the specificity of phospholipases.

The above considerations establish the importance of thorough investigations of phospholipase activity in terms of substrate structure at the molecular level and in terms of the molecular parameters which govern the interfacial properties of complex structures, including the lamellar bilayer, which are formed by phospholipids. As a possible entree into the latter matter, the observation that *n*-alkanols are known to affect lipid organization in membranes (Cherry *et al.*, 1970; Seeman *et al.*, 1971*b*) suggests that these compounds may also affect enzyme-substrate interactions by modifying the state of organization of the aggregates of the substrate. Since physicochemical methods are capable of yielding considerable insight into the organization of phospholipids in biological membranes and model systems (Jain, 1972), it appeared of interest to investigate the effect of *n*-alkanols on phospholipase activity toward model substrates, with the long-range goal of relating the observed effects to changes in surface properties of these substrates. This manuscript describes results obtained in the first phase of these studies.

## Materials and Methods

Lecithin was isolated from commercial hens' eggs by acetone precipitation and chromatography on neutral Woelm alumina (Singleton *et al.*, 1965), and was stored in chloroform solution at $-25$ °C. Samples employed in kinetic studies showed a single spot on thin-layer chromatography in several solvent systems. Alcohols used in this study were either analytical grade or were distilled under reduced pressure; center cuts boiling over a range $<1$ °C were collected. All other chemicals were analytical grade. Phospholipase A (Bee venom and Russell viper) and phospholipase C (*Clostridium welchii*) were purchased from Sigma Chemical Co., St. Louis, Missouri. Phospholipase C (*Bacillus*

*cereus*) was obtained from General Biochemicals, Chagrin Falls, Ohio. These enzymes were used without further purification. The products of action of each phospholipase employed on egg lecithin were assayed by thin-layer chromatography on alumina plates developed with $CHCl_3/CH_3OH/H_2O = 90:10:4$. In each case, only those products expected on the basis of the known specifity of the enzyme were observed, indicating that the commercial products are essentially free of extraneous phospholipase activities. Each phospholipase was also examined for protease activity against bovine serum albumin employing a titrimetric assay; in no case was detectable protease activity observed.

The course of enzymatic hydrolysis of egg lecithin was measured by continuous titration of the liberated fatty acid or choline phosphate with a Radiometer pH-Stat, consisting of a Titrator TTT-1, Titrigraph SBR-2 and burette SBU-1. All measurements were performed at pH 7.4 and 37 °C unless stated otherwise. A known amount of phospholipase is added to a standardized (pH 7.4) suspension of liposomes in 10 ml of a solution containing 50 mM KCl, 230 mM mannitol, and 6 mM $CaCl_2$. Protons liberated during the enzymatic reactions were neutralized by titration with a solution of $5.0 \times 10^{-3}$ M AMPD (2-amino-2-methyl-1,3-propanediol) with the proportional band set at 0.1. The speed of the strip chart was regulated so that the slope of the titration curve was between 20 and 65 °. In those cases in which quantitative values of the extent of phospholipid hydrolysis were calculated from titrimetric data, each mole of fatty acid liberated was considered to yield one mole of protons; each mole of choline phosphate liberated was considered to yield 0.9 moles of protons (corresponding to an estimate of the $pK_a$ of the second ionization of this substance of 6.0).

Unsonicated liposomes were prepared by evaporation to dryness of chloroform solutions of lecithin in a round bottom flask containing 3 to 4 glass beads (1 to 2 mm radius) either under a stream of nitrogen or at reduced pressure. Appropriate amounts of the KCl-mannitol-$CaCl_2$ solution (*see above*) were added to the flask which was maintained at 40 to 45 °C under nitrogen. At the end of a 10- to 30-min period, the mixture is gently shaken until all the lipid is loosened from the wall of the flask. The resulting cloudy suspension is dispersed by agitation for 30 sec on a Vortex apparatus set at full speed. The liposome dispersion was then permitted to age for at least 2 hr prior to use in kinetic measurements.

## Results

Egg lecithin in the form of unsonicated liposomes prepared as described above was observed to be only very slightly subject to phospholipase-catalyzed hydrolysis (see Fig. 1 *a*, for example). With each enzyme employed, the rate of hydrolysis of these preparations under standard conditions (*see* Materials and Methods) is too slow to make accurate kinetic measurements possible with the titrimetric method used. This conclusion was not changed by several variations in experimental conditions including change of pH in the range 6 to 8, variation in temperature from 18 to 48 °C, and increase in the time of dispersal of lecithin in liposome formation from 30 sec to as much as 15 min. We conclude that, for the various sets of conditions employed at least, unsonicated liposomes of egg lecithin are poor substrates for those enzymes employed in this study, in confirmation of the work of others (*see* Jain, 1972).

### *Effect of n-Alkanols on the Rate of Phospholipase-catalyzed Lecithin Hydrolysis*

In the presence of appropriate concentrations of certain *n*-alkanols, as developed below in detail, unsonicated liposomes prepared from egg lecithin are converted into suitable substrates for a variety of phospholipases. All reactions were initiated by the addition of enzyme. Under the conditions described above, plots of base consumption against time were linear for at least 8 to 10 min; this point was verified several times in the course of these studies under a variety of conditions. Zero-order rates were evaluated from the slope of such plots over time periods ranging from 1 to 5 min; a typical set of examples is shown in Fig. 1 *a*.

The effect of concentration of *n*-alkanols on the zero-order rate of hydrolysis of egg lecithin liposomes was determined in two ways. First, rates were measured for 8 to 10 min for several different preparations of liposomes to which had been added varying amounts of alcohol 5 min prior to initiation of reaction, maintaining the enzyme concentration constant. Second, rates were measured on a single preparation of liposomes to which successive aliquots of alcohol were added (Fig. 1 *a*). Mixing of the suspension in the reaction vessel was adequate so that a new zero-order rate was established within 10 to 15 sec of the addition of each quantity of alcohol. As shown in Fig. 1 *b*, for the case of bee venom phospholipase A, the two methods yield identical results within experimental error. Since the latter method permits rapid determination of rate-concentration profiles for alcohols with a minimum consumption of lecithin and enzyme, it was routinely employed. From time to time, results obtained in this way were checked employing the former method. In all cases, satisfactory agreement was obtained.

The zero-order rate of hydrolysis of unsonicated egg lecithin liposomes is plotted as a function of the concentration of several alcohols for snake venom and bee venom phospholipase A and *B. cereus* and *Cl. welchii* phospholipases C in Fig. 2. Note that the addition of each of the various alcohols increases the susceptibility of the unsonicated liposomes to attack by the phospholipases. Careful examination of the data contained in Fig. 2 will reveal the following generalities concerning alcohol activation of phospholipase action.

a) In most cases, the rate-concentration profiles are bell-shaped; rates increase with increasing alcohol concentration until an optimal concentration is reached and further increases of concentration elicit rate decreases.

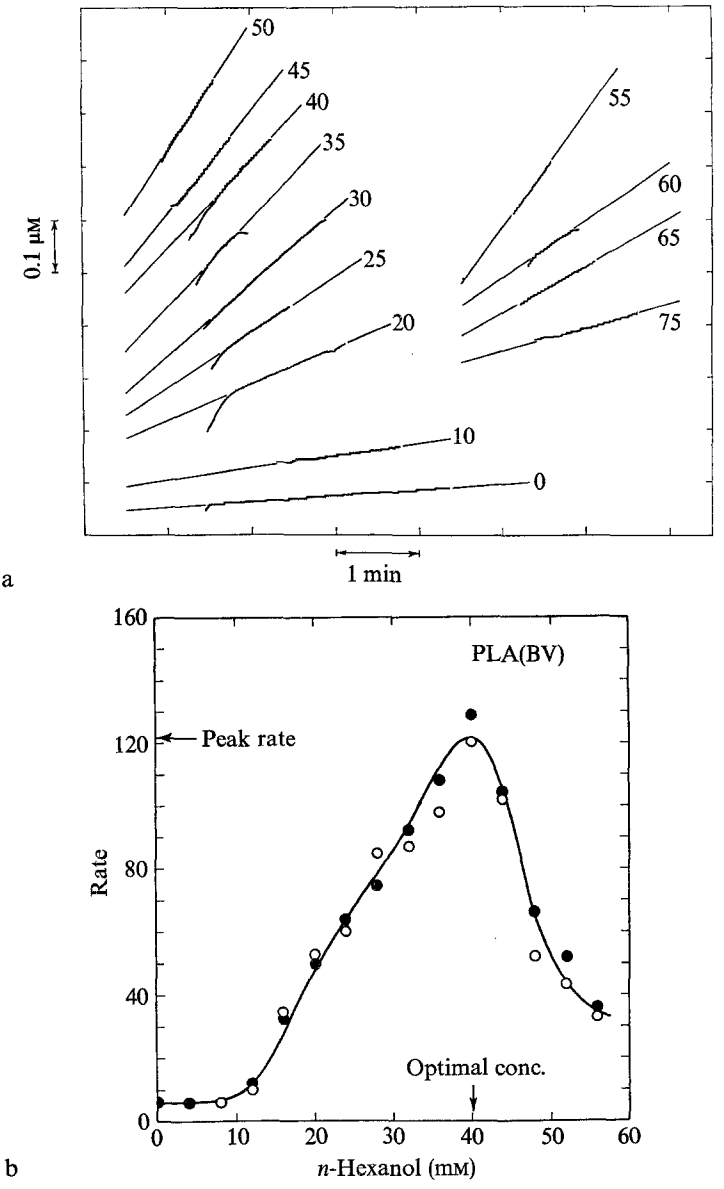



Fig. 1. (*a*) Titrimetric records of the course of hydrolysis of unsonicated liposomes of 1.3 mM egg lecithin in the presence of 0.1 μg of bee venom phospholipase A in the presence of varying concentrations of *n*-hexanol; total reaction volume is 10 ml. The ordinate indicates the number of micromoles of base added to neutralize the acid liberated during the hydrolytic reaction. The numbers associated with each trace indicate the number of microliters of *n*-hexanol added to the reaction mixture; note that 10 μliters of *n*-hexanol correspond to a concentration of 8.1 mM. (*b*) Zero-order rate of hydrolysis of unsonicated liposomes of 1.3 mM egg lecithin in the presence of 0.1 μg/10 ml of bee venom phospholipase A plotted as a function of the concentration of *n*-hexanol. Open circles refer to measurements made after addition of alcohol to fresh samples of lecithin; closed circles refer to measurements made by successive addition of aliquots of *n*-hexanol to the same sample of lecithin (Fig. 1 *a*; *see text* for details). Rates in this and subsequent figures are given in arbitrary units in which 100 units corresponds to the hydrolysis of 0.2 μmoles of lecithin per min

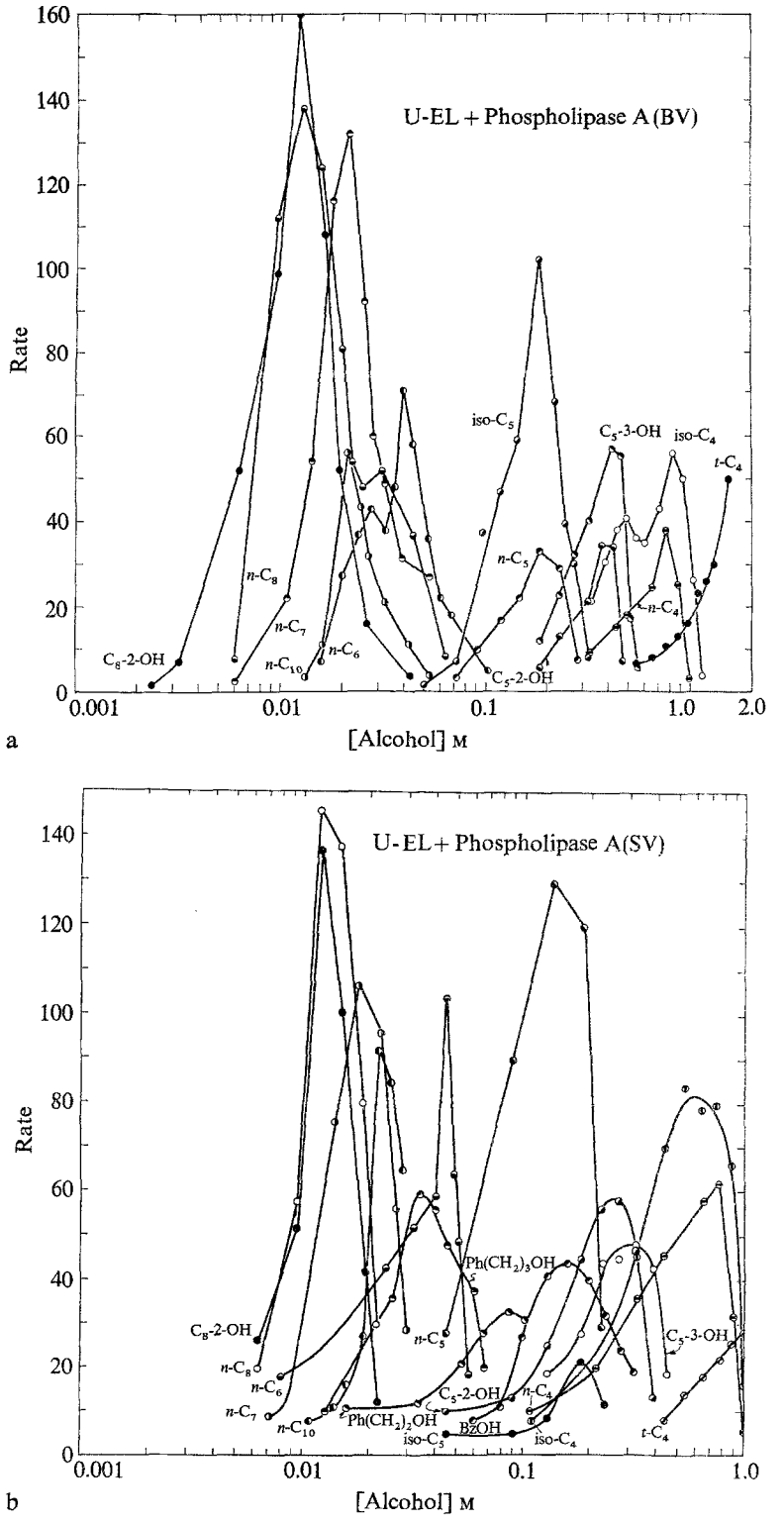
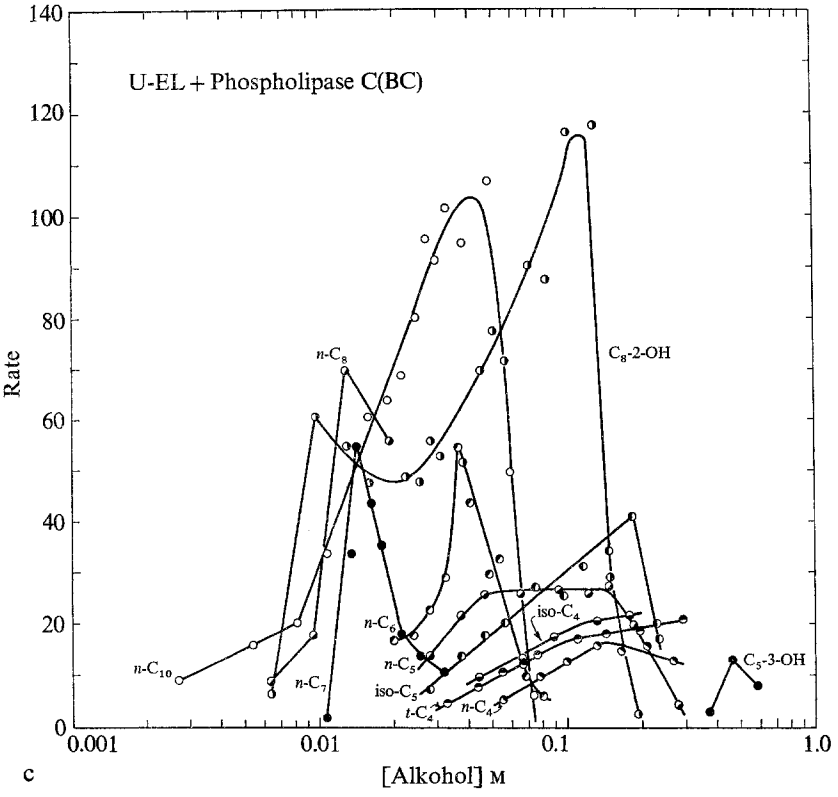
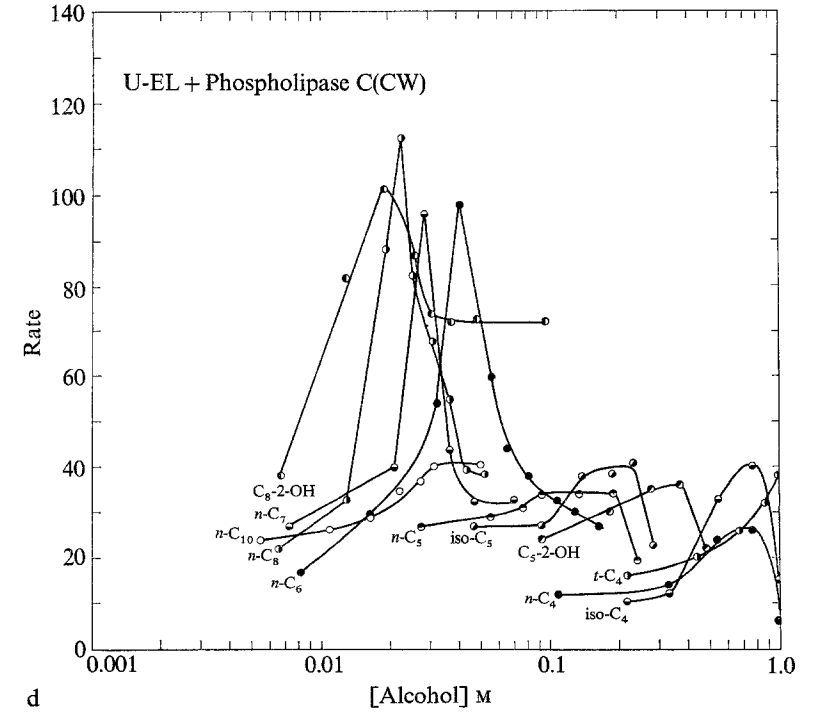



Fig. 2. Rate-concentration profiles for hydrolysis of unsonicated liposomes of 1.3 mM egg lecithin by (*a*) bee venom phospholipase A, (b) snake venom phospholipase A, (*c*) *Bacillus cereus* phospholipase C, and (*d*) *Clostridium welchii* phospholipase C in the presence of several alcohols. *n*-$C_4$, *n*-butyl; *t*-$C_4$, tertiary butyl; iso-$C_4$, isobutyl alcohol;




c




d

$n$-$C_4$-$C_{10}$ refer to the corresponding $n$-alkanols; BzOH, benzyl alcohol; $Ph(CH_2)_2OH$, phenylethyl alcohol; $Ph(CH_2)_3OH$, phenylpropyl alcohol; $C_5$-2-OH, 2-pentanol; $C_5$-3-OH, 3-pentanol; $C_8$-2-OH, 2-octanol. The same amount of phospholipase and lecithin were used for all plots in a given set. The amount of phospholipases was chosen arbitrarily

Table 1. Specific activity (μmoles/min/mg protein) of several phospholipases assayed in various systems

| Enzyme | Present method [a] | Manufacturers' assay [b] |
|---|---|---|
| Phospholipase A (Bee venom) | 1,600 | 1,600–2,400 |
| Phospholipase A (Russell viper) | 10 | 9–10 |
| Phospholipase C (*Bacillus cereus*) | 9.3 | 10–11 |
| Phospholipase C (*Clostridium welchii*) | 0.7 | 0.5–2 |

[a] Substrate is unsonicated liposomes of egg lecithin in the presence of optimal concentrations of *n*-hexanol at 37 °C, pH 7.5.
[b] Substrate is egg lecithin dispersed through sonication in presence of deoxycholate at 37 °C, pH 8.2.

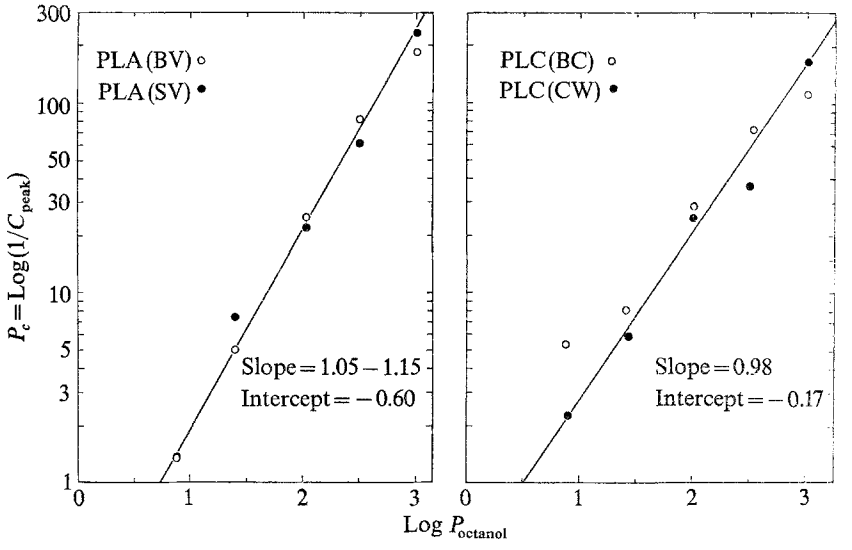



Fig. 3. Plots of the logarithm of the reciprocal of the optimal activating concentration and concentration at which half-optimal activation occurs as a function of the logarithm of the *n*-octanol/water partition coefficients, $P_{octanol}$, for several *n*-alkanols, which function as activators for hydrolysis of unsonicated liposomes of egg lecithin for the indicated phospholipases

b) In a homologous series of alcohols, for example from *n*-butanol to *n*-octanol, the concentration of alcohol required to elicit maximal activation decreases with increasing chain length and the maximal rate achieved increases with increasing chain length. However, with longer chain alcohols more complex behavior is observed. Thus, *n*-alkanols possessing from 9 to 14 carbon atoms are less effective activators than is *n*-octanol. Data for *n*-octanol are included in Fig. 2 to illustrate this point.

c) As detailed in Table 1, optimal concentrations of *n*-hexanol yield peak rates for hydrolysis of unsonicated egg lecithin liposomes which are comparable to those obtained employing conventional means of phospholipase assay.

d) Plots of the logarithm of the optimal concentration of activating alcohol or the logarithm of the concentration required to yield half the maximal activation against the logarithm of the *n*-octanol/water partition coefficient for the alcohols (Hansch plot; Hansch & Dunn, 1972) yield straight lines with slopes in the range 1.0 to 1.2 (Fig. 3). That is, the *n*-octanol/water partition coefficients are directly related to activating capacities for these alcohols, suggesting that their effects may result from incorporation into the liposomes. The behavior of aromatic alcohols and branched chain alcohols is, in contrast, more complicated (*see* Fig. 2; *also* Jain, *unpublished observations*). This may reflect the substantial perturbation of structure which results from incorporation of such alcohols into liposomes (Colley & Metcalfe, 1972).

*Enzyme Concentration and n-Hexanol Activation of Lecithin Hydrolysis*

In Fig. 4*a*, the zero-order rate of hydrolysis of unsonicated liposomes of egg lecithin is plotted as a function of the concentration of added *n*-hexanol at several concentrations of *B. cereus* phospholipase C. Note that the concentration of alcohol required to elicit maximal activation is independent of enzyme concentration. As shown in Fig. 4*b*, the maximal rates observed vary in a linear way with enzyme concentration. Similar behavior was observed for the phospholipase C from *Cl. welchii* as well as for phospholipases A from bee venom and Russell viper snake venom (plots not shown).

*Substrate Concentration and n-Hexanol Activation of Lecithin Hydrolysis*

In Fig. 5*a*, the zero-order rate of hydrolysis of unsonicated liposomes of egg lecithin in the presence of a fixed concentration of *B. cereus* phospholipase C is plotted as a function of the concentration of added *n*-hexanol at several concentrations of lecithin. That concentration of *n*-hexanol required to elicit maximal activation of the enzymatic reaction is independent of lecithin concentration. Data of this type may be employed to construct double reciprocal plots of rate versus substrate concentration in the presence of optimal concentrations of *n*-hexanol; a typical example is provided in Fig. 5*b*. Note that satisfactory straight line behavior is obtained. Such plots permit calculation of values of $V_{max}$ and $K_m$ in the usual manner.

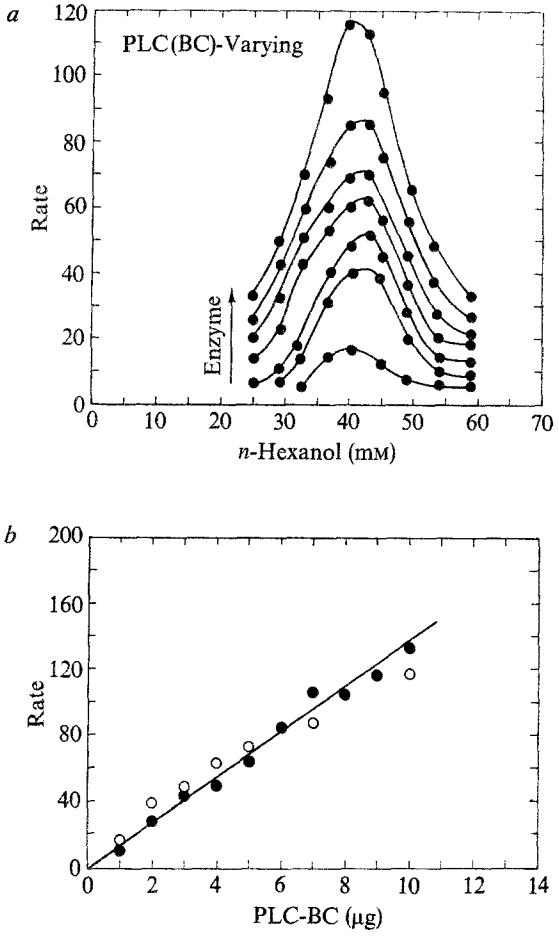



Fig. 4. (*a*) Plots of zero-order rates for *Bacillus cereus* phospholipase C catalyzed hydrolysis of unsonicated liposomes of 1.3 mM egg lecithin as a function of the concentration of added *n*-hexanol for several enzyme concentrations. (*b*) Plots of the zero-order rate obtained at optimal concentrations of *n*-hexanol against the concentration of *B. cereus* phospholipase C. The open circles refer to data taken from rate-concentration profiles of the type shown in (*a*); the closed circles are independent measurements made at that concentration of *n*-hexanol judged to be optimal. Rates are given in units in which 100 units correspond to hydrolysis of 0.2 μmole of lecithin per min

### *Kinetic Parameters for Phospholipase-catalyzed Hydrolysis of Lecithin as a Function of n-Hexanol Concentration*

Employing the methodology described just above (*see* Fig. 5*b*), values of $K_m$ and $V_{max}$ for enzyme-catalyzed hydrolysis of unsonicated liposomes of egg lecithin were measured as a function of the concentration of added *n*-hexanol. Data for all four of the phospholipases studied is collected in

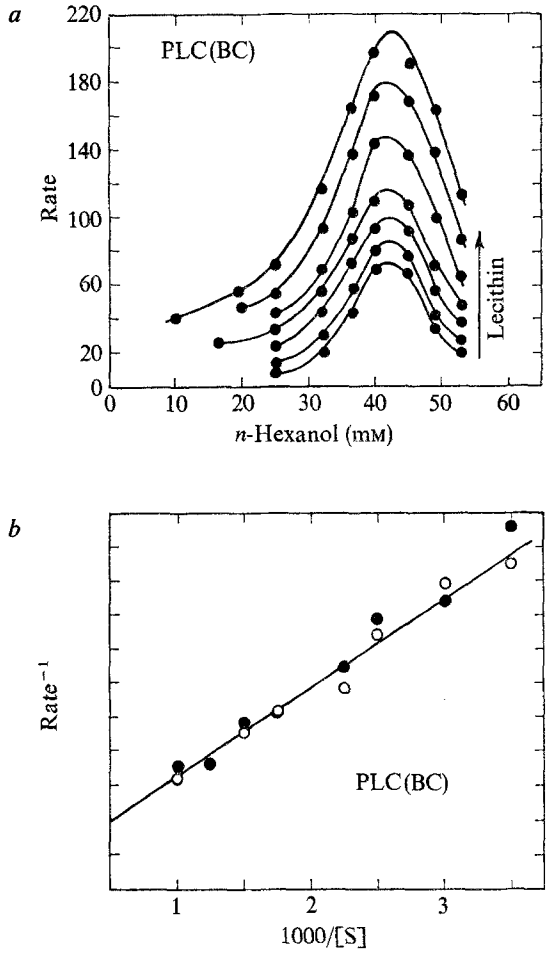



Fig. 5. (*a*) Plot of zero-order rates for *Bacillus cereus* phospholipase C-catalyzed hydrolysis of unsonicated liposomes of egg lecithin as a function of the concentration of added *n*-hexanol for several concentrations of lecithin at a fixed enzyme concentration. (*b*) A double reciprocal plot of zero-order rate versus substrate concentration for *B. cereus* phospholipase C-catalyzed hydrolysis of egg lecithin in the presence of the optimal concentration of *n*-hexanol. Open circles are data points taken from the optima in the rate-concentration profiles shown in (*a*); closed circles are independent measurements made at that fixed concentration of *n*-hexanol judged to elicit maximum activation. Rates are given in units in which 100 units correspond to hydrolysis of 0.2 μmole of lecithin per min

Fig. 6. For the sake of comparison, zero-order rates at a constant concentration of lecithin as a function of alcohol concentration are also included in this figure. Both the values of $V_{max}$ and $K_m$ exhibit a complex pattern of dependence on alcohol concentration. Thus, values of $V_{max}$ for phospho-

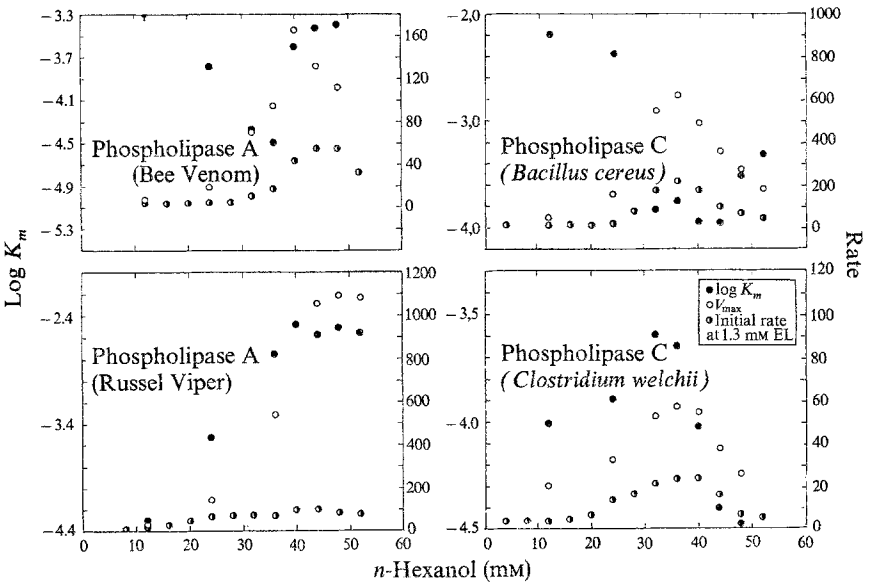



Fig. 6. Plots of logarithms of Michaelis constants (closed circles), maximal velocities (open circles) and initial rates at 1.3 mM egg lecithin (half-open circles) against the concentration of *n*-hexanol for catalysis of the hydrolysis of unsonicated liposomes of egg lecithin by the indicated phospholipases

lipase C-dependent reactions show a bell-shaped dependence on hexanol concentration but those for phospholipase A-dependent ones do not. Values of $K_m$ show a similarity diversity of behavior from one enzyme to another. In consequence, in some cases the hexanol-activation of enzyme-catalyzed hydrolysis reflects positive contributions from both $V_{max}$ and $K_m$ and in others the quantitative difference between two parameters varying in opposite fashions.

### *Kinetic Parameters for Phospholipase-catalyzed Hydrolysis of Lecithin as a Function of the Chain Length of n-Alkanols*

Values of $K_m$ and $V_{max}$ were measured for the enzyme-catalyzed hydrolysis of unsonicated liposomes of egg lecithin at the optimal activating concentrations of *n*-butanol, *n*-pentanol, *n*-hexanol, *n*-heptanol, and *n*-octanol. Results for all four phospholipases studied are collected in Table 2. A complicated pattern is observed with each enzyme responding in its own unique way. In three of the four cases, values of $V_{max}$ pass through optimal values as the length of the alkyl chain of the activating alcohol is increased. In the fourth, a monotonic increase in $V_{max}$ with chain length is observed.

Table 2. Kinetic constants characterizing hydrolysis of egg lecithin by various phospholipases in the presence of peak-activating concentrations of several *n*-alkanols [a]

| Enzyme | $C_4$ | $C_5$ | $C_6$ | $C_7$ | $C_8$ |
|---|---|---|---|---|---|
| Phospholipase A (Bee venom) | | | | | |
| $K_m$ (mM) | 0.49 | 0.118 | 0.086 | 0.043 | 0.078 |
| $V_{max}$ | 5 | 63 | 86 | 117 | 54 |
| Phospholipase A (Snake Venom) | | | | | |
| $K_m$ (mM) | 0.26 | 0.6 | 1.01 | 3.25 | 5.2 |
| $V_{max}$ | 165 | 260 | 810 | 950 | 1,330 |
| Phospholipase C (*Bacillus cereus*) | | | | | |
| $K_m$ (mM) | 2.6 | 2.1 | 1.52 | 1.3 | 1.4 |
| $V_{max}$ | 650 | 650 | 650 | 650 | 650 |
| Phospholipase C (*Clostridium welchii*) | | | | | |
| $K_m$ (mM) | 0.91 | 0.62 | 0.55 | 0.26 | 0.32 |
| $V_{max}$ | 40 | 65 | 80 | 50 | 60 |

[a] The peak-activating concentrations of *n*-alkanols are the same as those given in Fig. 2*a–d*. The kinetic constants for a given phospholipase were determined on a single preparation of liposomes. The values of $V_{max}$ are given in arbitrary units.

Similarly, in three of the four cases, values of $K_m$ pass through minima as the length of the activating alcohol is increased. In the fourth, $K_m$ increases regularly with increasing chain length.

## Discussion

The findings just described establish that the rate of hydrolysis of unsonicated liposomes of egg lecithin by two phospholipases A and two phospholipases C is markedly sensitive to the nature and concentration of alcohols in the medium. At optimal concentrations of activating alcohols, the lecithin liposomes behave as excellent substrates for all enzymes tested and such systems may find utility for the assay of these enzymes.

Alcohol activation for enzymatic hydrolysis of egg lecithin liposomes may be the consequence of any or all of the following factors: (*i*) an increased area of the phospholipid-water interface caused by disorganization or dispersal of the liposomes in the presence of alcohol; (*ii*) modification of the catalytic properties of the enzymes induced by interaction with the alcohols; and (*iii*) modification of the microenvironment of individual phospholipid molecules within the liposomal aggregate, reflecting alcohol-induced alterations in liposomal organization.

A number of lines of evidence strongly suggest that the first of these possibilities cannot account for the observed behavior of these systems. First, most of the rate-concentration profiles are bell-shaped (Fig. 2). Dispersal of liposomes to yield increased phospholipid-water interfacial area can account for the ascending portion of these curves but does not provide a rational explanation for the descending leg. Second, it has been established in an independent study (Jain, Toussaint & Cordes, 1973) that unsonicated liposomes of egg lecithin treated with those alcohols employed in the present study remain intact and impermeable to KCl and sucrose. Third, sonication of egg lecithin liposomes does not result in an increased rate of enzymatic hydrolysis which is proportional to the increase in interfacial area (Jain & Cordes, 1973).

There exists no concrete evidence to firmly establish the role of added alcohols in modifying the inherent catalytic capacities of the phospholipases employed in this study. There is, however, substantial circumstantial evidence strongly suggesting that this is not the underlying cause of the bulk of the effects observed. Specifically, all four phospholipases employed in this study behave in a qualitative and quantitatively similar manner as revealed by shapes of rate-concentration profiles and similarities in concentrations of various alcohols required to elicit maximal activation (Fig. 2). Were the enzymes themselves the primary site of alcohol action, one might have expected that more divergent patterns of behavior would have been observed. The marked similarities suggest that unmodified enzymes are responding in distinct but related ways to a modified substrate. This is, however, no means of ruling out a small contribution, which may vary from enzyme-to-enzyme, to the effects observed resulting from changes in enzyme organization resulting from the binding of one or more molecules of alcohol.

The bulk of the data suggest that the modification of susceptibility of lecithin liposomes to enzymatic hydrolysis induced by the alcohols is a direct result of changes in liposomal properties resulting from incorporation of the alcohols into them. Indeed, there is abundant evidence indicating that alcohols modify a wide variety of membrane-associated phenomena (cf. Hansch & Dunn, 1972). Moreover, the concentrations of alcohols required to elicit maximal activation of the enzymatic reactions are closely related to those required to cause a maximal rate of swelling of unsonicated egg lecithin liposomes (Jain *et al.*, 1973) and to those required to elicit a maximal change in the absorption properties of bromothymol blue adsorbed on such liposomes (Jain & Toussaint, *unpublished observations*). These observations, taken together, strongly suggest that perturbations in the liposomal membrane organization induced by alcohols affect the susceptibility

of the liposomes to enzymatic hydrolysis, the rate of swelling under osmotic shock, and the change in bromothymol blue absorption in a quantitatively similar manner; that is, one effect underlies all three phenomena.

Given that the effects of alcohols on the rate of enzymatic hydrolysis of lecithin liposomes principally reflect incorporation of the alcohols into the membrane microphase, it is possible to place a more nearly quantitative interpretation on the data for the *n*-alkanols containing four to eight carbon atoms. This treatment is based on the considerations of Hansch and Dunn (1972) who have suggested that *n*-octanol/water partition coefficients, $P_{\text{octanol}}$, are useful quantities in accounting for membrane-associated behavior on the basis that *n*-octanol mimics the apolar character of the interior of membranes while simultaneously providing opportunity for hydrogen bond formation and polar interactions that might characterize the membrane surface. According to this treatment, the concentration of a series of congeners, in this case the *n*-alkanols in the aqueous medium, in equilibrium with the liposomal microphase is given by:

$$\log(1/C_{H_2O}) = pC = a \log P_{\text{octanol}} + b$$

in which $a$ and $b$ are constants which provide information concerning the microenvironment of the sites at which the molecules of interest are localized within the membrane microphase. The slope $a$ is a measure of the sensitivity of the system to perturbations reflecting the hydrophobic character of the congeners. Values of 0.85 or greater are found for systems highly sensitive to hydrophobicity of the perturbants. The values observed for activation of phospholipase-catalyzed lecithin hydrolysis by various *n*-alkanols are in the range 1.05 to 1.15. This strongly suggests that the sites of interaction of alcohols with the lipid-phospholipase system are highly hydrophobic.

The value of intercept $b$ is a function of the sensitivity of the system to occupation of those sites occupied by the perturbants and the intrinsic activity of a given set of these molecules. Thus, at those concentrations of congeneric alcohols required to elicit peak hydrolytic activity of various phospholipases toward lecithin, the same intermolecular organization of phospholipid molecules in the liposome may or may not obtain. Thus, interpretation of values of $b$ requires that one consider the *demands placed upon the system*; in the present situation, this refers to the packing characteristics of lipids and alkanols in the membrane phase treated with alkanols.

It is now well established that the organization of phospholipid molecules in liposomes corresponds to that of concentric spheres bounded by lamellar lipid layers. Results presented in this work suggest that such structures

formed from egg lecithin either do not permit ready access of the phospholipase to individual phospholipid molecules or that the liposomes bind these enzymes so tightly so that they cannot rapidly complete their catalytic process. The activating effect of alcohols can be visualized as resulting from modifications of the intermolecular associations among lipid molecules in the bilayer. This might result in significant reorientation of the lipid molecule so as to accommodate phospholipases at the interface in such a way as to permit their productive access to substrate molecules. This may be reflected as altered values of $K_m$ for enzyme-substrate interaction (Fig. 6 and Table 2). That such effects are important is also consistent with the observation that a pronounced optimum for hydrolytic activity of phospholipase A (Colacicco & Rapport, 1966; Shah & Schulman, 1967; Colacicco, 1971; Zografi *et al.*, 1972), B (Bangham & Dawson, 1960), C (Miller & Ruysschaert, 1971), and D (Quarles & Dawson, 1969) toward phospholipid monolayers is observed at 10 to 30 dynes $cm^{-1}$ surface pressure. Considerable independent evidence exists suggesting that alkanols alter the organization of phospholipid molecules in lipid bilayers (Metcalfe *et al.*, 1968; Johnson & Bangham, 1969; Cherry *et al.*, 1970; Seeman *et al.*, 1971*a*; Colley & Metcalfe, 1972; Paterson *et al.*, 1972). However, the specific details of the consequences of these intermolecular interactions are yet to be worked out. There are a number of such consequences possible including changes in the spacing, packing, orientation or mobility of lipid molecules in the bilayers, in the charge profile of the electrical double layer at the surface, or in the organization of interfacial water. Such changes following incorporation of *n*-alkanols, may alter the properties of associated components such as membrane-bound enzymes. Consequently, alteration of catalytic properties of the phospholipases as well as their access to substrate may underlie the alcohol-induced effect. Such changes might alter other properties of the interface as well; for example, it has been noted by Seeman *et al.* (1971*b*) that the binding of $Ca^{2+}$ to the red cell membrane is increased by as much as a factor of 2.5 by $C_2 - C_5$ alcohols. However, the rate activations observed in the present study are far too large to be accounted for on the basis of an alcohol-induced increase in the interfacial concentration of $Ca^{2+}$.

The results of the present study indicate that the action of phospholipases on phospholipid aggregates is extremely sensitive to the organization of the lipid/water interface. The factors determining the probability of productive interaction of the enzyme with the substrate seem to reside in details of the specific orientation of the substrate at the interface. However, these results cannot yet distinguish between the possibilities of whether the interaction of enzyme with interface or the formation of enzyme-substrate complex,

or the dissociation of enzyme from the interface is the rate-limiting step in the reaction cycle. Studies are in progress to sort out the various possibilities.

## References


Ansell, G. B., Hawthorne, J. N. 1964. Phospholipids: Chemistry, Metabolism, and Function. Elsevier Publishing Co., Amsterdam

Bangham, A. D., Dawson, R. M. C. 1960. The physicochemical requirements for the action of *Penicillium notatum* phospholipase B on unimolecular films of lecithin. *Biochem. J.* **75**:133

Bonsen, P. P. M., DeHaas, G. H., Pieterson, W. A., Van Deenen L. L. M. 1972. Studies on phospholipase A and its zymogen from porcine pancrease. IV. The influence of chemical modification of the lecithin structure on substrate properties. *Biochim. Biophys. Acta* **270**:364

Cherry, R. J., Dodd, G. H., Chapman, D. 1970. Small molecule-lipid membrane interactions and the puncturine theory of olfaction. *Biochim. Biophys. Acta* **211**:409

Colacicco, G. 1971. Significance of surface potential measurements on lipid monolayers during action of phospholipase A on lecithins. *Nature* **233**:202

Colacicco, G., Rapport, M. M. 1966. Lipid monolayers: Action of phospholipase A of *Crotalus atrox* and *Naja Naja* venoms on phosphatidylcholine and phosphatidal choline. *J. Lipid. Res.* **7**:258

Colley, C. M., Metcalfe, J. C. 1972. The localization of small molecules in lipid bilayers. *FEBS* **24**:241

De Haas, G. H., Bonsen, P. P. M., Pieterson, W. A., Van Deenen, L. L. M. 1971. Studies on phospholipase A and its zymogen from porcine pancrease. III. Action of the enzyme on short-chain lecithins. *Biochim. Biophys. Acta* **239**:252

Entressangles, B., Desnuelle, P. 1968. Action of pancreatic lipase on aggregated glyceride molecules in an isotropic system. *Biochim. Biophys. Acta* **159**:285

Hansch, C., Dunn, W. J. 1972. Linear relationships between lipophilic character and biological activity of drugs. *J. Pharm. Sci.* **61**:1

Jain, M. K. 1972. The Bimolecular Lipid Membrane: A System. Van Nostrand Reinhold Co., New York

Jain, M. K. 1973. Effect of hydrolytic enzymes on biomembranes and related systems. *In:* Current Topics in Membranes and Transport. Vol. 4. (*In press*)

Jain, M. K., Cordes, E. H. 1973. Phospholipases. II. Enzymatic hydrolysis of lecithin: Effects of structure, cholesterol content, and sonication. *J. Membrane Biol.* **14**:119

Jain, M. K., Toussaint, D. G., Cordes, E. H. 1973. Kinetics of water penetration into unsonicated liposomes. Effects of *n*-alkanols and cholesterol. *J. Membrane Biol.* **14**:1

Johnson, S. M., Bangham, A. D. 1969. The action of anaesthetics on phospholipid membranes. *Biochim. Biophys. Acta* **193**:92

Metcalfe, J., Seeman, P., Burgen, A. S. V. 1968. The proton relaxation of benzyl alcohol in erythrocyte membranes. *Mol. Pharmacol.* **4**:87

Miller, I. R., Ruysschaert, J. M. 1971. Enzymic activity and surface inactivation of phospholipase C at the water/air interface. *J. Colloid Interface Sci.* **35**:340

Paterson, S. J., Butler, K. W., Huang, P., Labelle, J., Smith, I. C. P., Schneider, H. 1972. The effect of alcohols on lipid bilayers: A spin label study. *Biochim. Biophys. Acta* **266**:597

Quarles, R. H., Dawson, R. M. C. 1969. The hydrolysis of monolayers of phosphatidyl [Me-$^{14}$C]choline by phospholipase D. *Biochem. J.* **113**:697

Roholt, O. A., Schlamowitz, M. 1961. Studies of the use of dihexanoyllecithin and other lecithins as substrates for phospholipase A with addendum on aspects of micelle properties of dihexanoyllecithin. *Arch. Biochem. Biophys.* **94**:364

Scherphof, G., Scarpa, A., von Toorenengergen,A. 1972. The effect of local anesthetics on the hydrolysis of free and membrane bound phospholipids catalyzed by various phospholipases. *Biochim. Biophys. Acta* **270**:226

Seeman, P., Chau, M., Goldberg, M., Sanks, T., Sax, L. 1971*b*. The binding of Ca to the cell membrane increased by volatile anesthetics (alcohols, acetone, ether) which induce sensitization of nerve or muscle. *Biochim. Biophys. Acta* **225**:185

Seeman, P., Roth, S., Schneider, J. 1971*a*. The membrane concentrations of alcohol anesthetics. *Biochim. Biophys. Acta* **225**:171

Seppala, A. J., Saris, N.-E. L., Gauffin, M. L. 1971. Inhibition of phospholipase A-induced swelling of mitochondria by local anesthetics and related agents. *Biochem. Pharmacol.* **20**:305

Shah, D. O., Schulman, J. H. 1967. Enzymic hydrolysis of various lecithin monolayers employing surface pressure and potential technique. *J. Colloid Interface Sci.* **25**:107

Singleton, W. S., Gray, M. S., Brown, M. L., White, J. L. 1965. Chromatographically homogeneous lecithin from egg phospholipids. *J. Amer. Oil Chem. Soc.* **42**:53

Smith, A. D., Gul, S., Thompson, R. H. S. 1972. The effect of fatty acids and of albumin on the action of a purified phospholipase $A_2$ from cobra venom on synthetic lecithins. *Biochim. Biophys. Acta* **289**:147

Thompson, G. A. 1970. Phospholipid metabolism. *Comprehensive Biochem.* **18**:157

Uthe, J. F., Magee, W. L. 1971*a*. Phospholipase $A_2$: Action of purified phospholipids as affected by deoxycholate and divalent cations. *Canad. J. Biochem.* **49**:776

Uthe, J. F., Magee, W. L. 1971*b*. Phospholipase $A_2$: Comparative action of three different enzyme preparations on selected lipoprotein systems. *Canad. J. Biochem.* **49**:785

Van Deenen, L. L. M., De Haas, G. H. 1963. The substrate specificity of phospholipase A. *Biochim. Biophys. Acta* **70**:538

Wells, M. A. 1972. A kinetic study of the phospholipase $A_2$ (*Crotalus adamanteus*) catalyzed hydrolysis of 1,2-dibutyryl-*sn*-glycero-3-phosphorylcholine. *Biochemistry* **11**:1030

Zografi, G., Verger, R., De Haas, G. H. 1972. Kinetic analysis of the hydrolysis of lecithin monolayers by phospholipase A. *Chem. Phys. Lipids* **7**:185