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Phospholipases. II. Enzymatic Hydrolysis of Lecithin: Effects of Structure, Cholesterol Content, and Sonication

Mahendra Kumar Jain and E. H. Cordes

Department of Chemistry, Indiana University, Bloomington, Indiana 47401

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Summary. Hydrolysis of unsonicated liposomes of egg lecithin catalyzed by several phospholipases is markedly activated by addition of *n*-alkanols [Jain & Cordes, *J. Membrane Biol.* **14**:101 (1973)]. Further pursuit of these systems has established that several factors, including higher temperatures, increasing unsaturation of fatty acyl chains of the substrate, incorporation of cholesterol into the liposomes, and sonication, reduce the concentration of *n*-hexanol required to elicit maximal activation for enzymatic hydrolysis. Moreover, sonication or incorporation of cholesterol into lecithin liposomes reduces from C_8 to C_7 and C_6, respectively, the chain length of that alcohol eliciting maximal activation. These results are consistent with the hypothesis that sonication and increasing cholesterol content lead to liposomes which have a diminished thickness of the hydrocarbon region compared to that for unmodified liposomes derived from the same lecithin.

In the first paper of this series (Jain & Cordes, 1973), it was demonstrated that *n*-alkanols and other alcohols markedly modify the susceptibility of unsonicated liposomes of egg lecithin to hydrolysis promoted by several phospholipases. Details of that study strongly suggest that the observed behavior results from modification of the lipid/water interface as a consequence of incorporation of the alcohols into the liposomes. There are several additional variables known through which organization of lipid bilayers may be modified and, hence, through which the susceptibility of these structures to enzymatic attack might be altered. For example, sonication of phospholipid liposomes not only increases the lipid/water interfacial surface area, providing additional sites to which enzyme might bind, but also modifies the properties of the bilayer itself through accentuation of the curvature of the bilayer and related changes in the geometry of the system (Sheetz & Chan, 1972). Similarly, incorporation of cholesterol into phospholipid bilayers is known to modify the intermolecular interactions between the latter molecules (Ladbrooke, Williams & Chapman,

1968; Finer, Flook & Hauser, 1972*a*, *b*; Oldfield & Chapman, 1972; Jain, 1974[1]). As a continuation of the previous work, we have now explored the phospholipase-catalyzed hydrolysis of lecithins as a function of several such variables, including lecithin structure, sonication, cholesterol content, and temperature.

Materials and Methods

Dipalmitoyl-L-phosphatidylcholine (chromatographic grade) was obtained from the Sigma Chemical Co. and used without further purification. Soybean lecithin, obtained from the Sigma Chemical Co., and crude cabbage lecithin, isolated by the method of Wheeldon (1960) were purified through chromatography on neutral Woelm alumina according to the method of Singleton, Gray, Brown and White (1965). All lecithins employed in this study showed only a single spot on thin-layer chromatography on alumina plates developed with $CHCl_3$/MeOH/H_2O (90:10:4, lower phase) and on silica plates developed with $CHCl_3$/MeOH/acetic acid/H_2O (85:15:10:4). Sources of other chemicals and phospholipases, together with the assay procedures for the enzymes and the methodology employed to prepare unsonicated liposomes of lecithins are detailed in the preceding publication (Jain & Cordes, 1973). Liposomes, both sonicated and unsonicated, containing cholesterol were prepared as follows. Lecithins with appropriate concentration of cholesterol were mixed in chloroform solution. Following removal of the solvent, under reduced pressure at 35 to 45 °C or in a stream of nitrogen, the lipid film was swollen in an appropriate solution for 10 to 15 min and then shaken on a Vortex apparatus for 20 to 30 sec at the maximum setting (200 cycles/sec).

Sonicated liposomes were prepared from the unsonicated ones through sonication with a Bronson sonifier using a micro-tip probe at maximum setting (about 10 amp) at power level 5. Preparations showing only slight turbidity were obtained after sonication for 15 to 20 min for 5- to 10-ml samples containing 25 mM lecithin, with or without added cholesterol. Such preparations were centrifuged at 40,000 × *g* for 30 min and the clear supernatant solution was used in subsequent experiments after aging for 1 hr at appropriate dilutions of the original preparation. Thin-layer chromatography of lecithin extracted with diethyl ether from preparations of sonicated liposomes showed no evidence of deterioration of the sample. Rate measurements were carried out titrimetrically as previously described (Jain & Cordes, 1973). Unless otherwise specified, all measurements were made at 37 °C.

Results

Effect of Temperature on the Rate of Enzymatic Phospholipolysis

Zero-order rates in arbitrary units (*cf.* Jain & Cordes, 1973) for the bee venom phospholipase A-catalyzed hydrolysis of unsonicated liposomes of dipalmitoyl lecithin and egg lecithin are plotted as a function of the concentration of *n*-hexanol at several temperatures in Fig. 1. The bell-shaped profiles observed in each case are characteristic of rate-concentration profiles for related cases (Jain & Cordes, 1973). Note that the promotion of the

1 M. K. Jain, 1974. The role of cholesterol in membranes and related systems. *Curr. Top. Membranes Transport. (To be published.)*

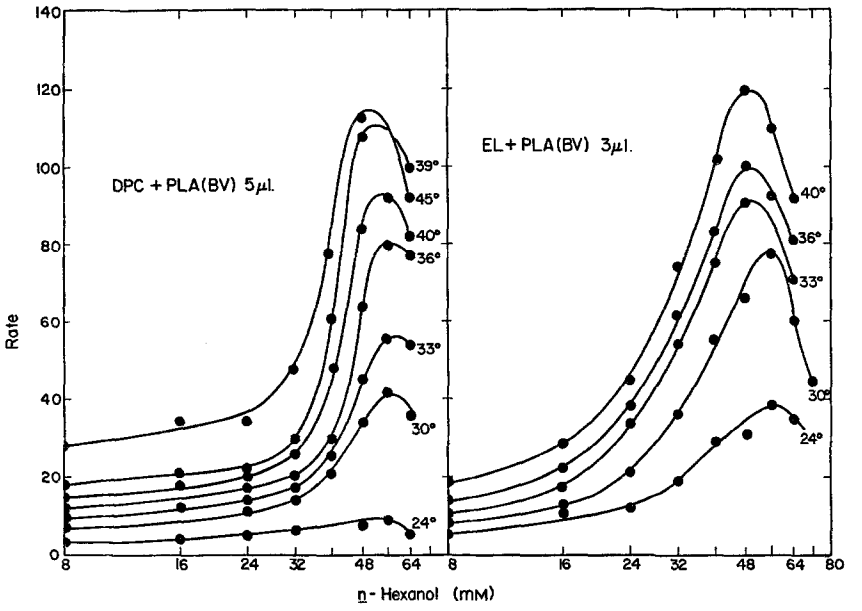

Fig. 1. The rate of bee venom phospholipase A-catalyzed hydrolysis of unsonicated liposomes of dipalmitoyl lecithin (DPC) and egg lecithin (EL) plotted as a function of the concentration of added *n*-hexanol at several temperatures. Maximal rates in the two parts of the figure are not directly comparable since different quantities of enzyme were employed, as noted

hydrolysis reaction by *n*-hexanol occurs over a much smaller concentration range of alcohol for dipalmitoyl as compared to egg lecithin. The concentration of *n*-hexanol required to elicit maximal activation of the enzymatic reaction appears to decrease slightly with increasing temperature. This point is shown graphically in Fig. 2 in which the concentration of *n*-hexanol at which maximal rates are obtained is plotted as a function of temperature for the hydrolysis of both lecithins. Note that this figure contains many data points in addition to those which can be extracted from Fig. 1. The dipalmitoyl lecithin liposomal system shows a sharp break in optimal *n*-hexanol concentration as a function of temperature; in contrast, the system derived from egg lecithin exhibits a smooth curve declining to a constant value at higher temperatures. Efforts were made to obtain corresponding data for sonicated egg lecithin liposomes and for both sonicated and unsonicated liposomes derived from mixtures of egg lecithin and cholesterol. However, the rate-concentration profiles for these systems are so broad that reliable values of the optimal concentration of *n*-hexanol cannot be readily measured.

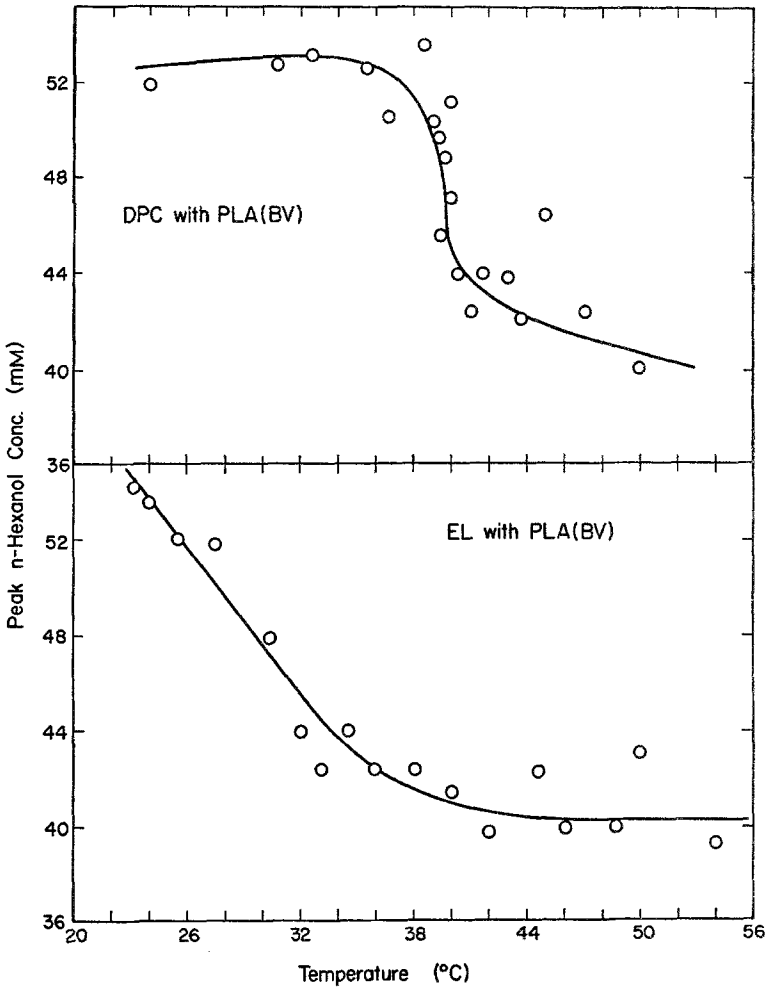

Fig. 2. Plots of the concentration of *n*-hexanol required to elicit maximal rates of bee venom phospholipase A-catalyzed hydrolysis of unsonicated liposomes of dipalmitoyl lecithin (DPC) and egg lecithin (EL) as a function of temperature. Optimal concentrations of *n*-hexanol were determined from rate-concentration profiles of the type shown in Fig. 1

Effect of Degree of Unsaturation in Lecithins on n-Hexanol Activation of Phospholipolysis

Lecithins containing various degrees of unsaturation in their fatty acyl moieties form liposomes which are suitable substrates for a variety of phospholipases. In Fig. 3, zero-order rates for hydrolysis of unsonicated liposomes derived from several lecithins (degrees of unsaturation are indicated in Table 1) are plotted as function of the concentration of added *n*-hexanol for hydrolysis catalyzed by phospholipases A from bee venom and snake

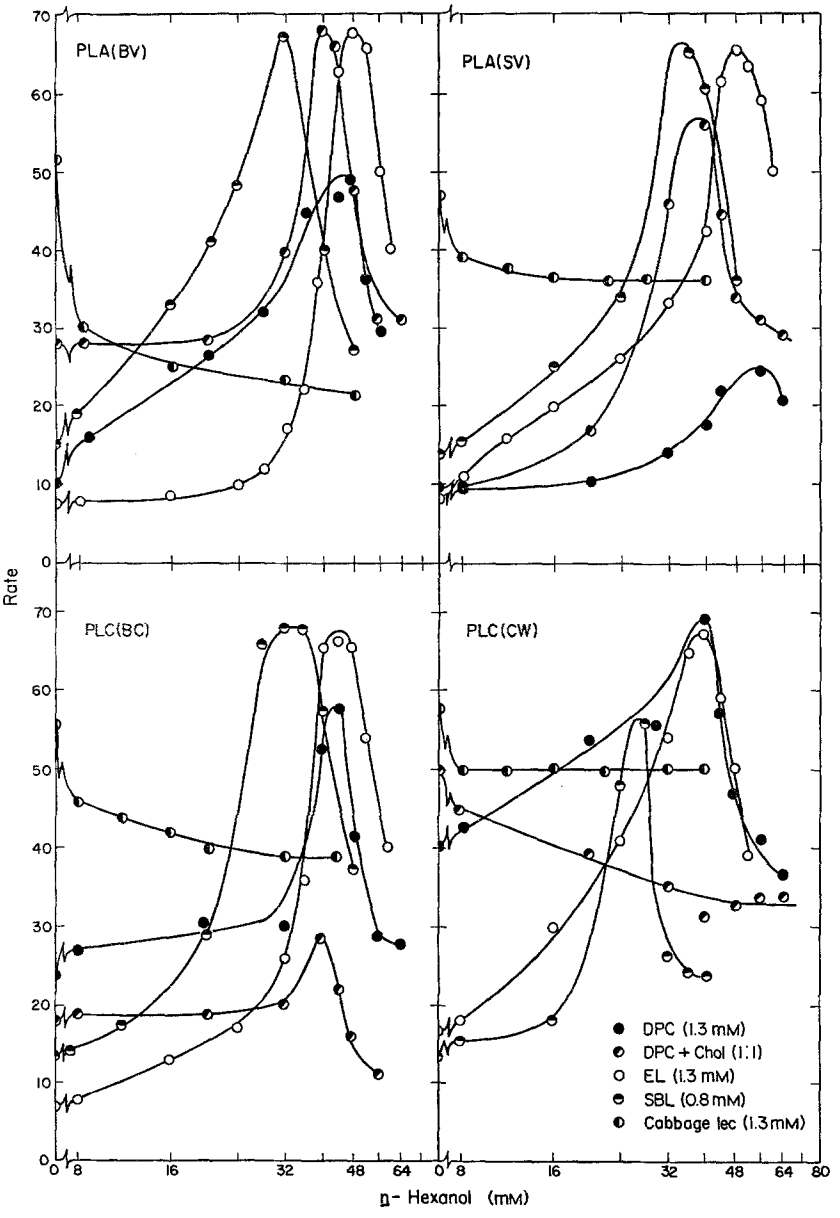

Fig. 3. Rates of phospholipase-catalyzed hydrolysis of unsonicated liposomes of several lecithins plotted as a function of the concentration of *n*-hexanol. Enzymes employed include phospholipases A from bee venom and snake venom and phospholipases C from *B. cereus* and *C. welchii*. DPC, dipalmitoyl lecithin; EL, egg lecithin; SBL, soybean lecithin; chol, cholesterol

venom and for phospholipases C from *Bacillus cereus* and *Clostridium welchii*. In most instances, typical bell-shaped profiles are obtained. Note that the peak rates in this figure are not comparable. Individual profiles

Table 1. Values of V_{max} and K_m for enzymatic hydrolysis of a series of unsonicated liposomes derived from several lecithins in the presence of *n*-hexanol

Lecithin	No. of double bonds/ lecithin	Hexanol, optimal conc. (mM)	K_m (mM)	V_{max} (arbitrary units)
	Phospholipase A (bee venom)			
Dipalmitoyl lecithin	0	50	0.364	60
DPC + cholesterol	0	42	0.73	75
Egg lecithin	1.8	40	0.286	87
Soybean lecithin	2.8	32	0.6	80
Cabbage lecithin	4.2	0	2.6	40
	Phospholipase A (snake venom)			
Dipalmitoyl lecithin	0	51	0.78	80
DPC + cholesterol	0	43	0.442	50
Egg lecithin	1.8	42	0.36	110
Soybean lecithin	2.8	33	0.91	240
Cabbage lecithin	4.2	0	13	2000
	Phospholipase C (*Bacillus cereus*)			
Dipalmitoyl lecithin	0	46	<0.05	40
DPC + cholesterol	0	41	0.52	110
Egg lecithin	1.8	40	0.962	130
Soybean lecithin	2.8	34	0.55	150
Cabbage lecithin	4.2	0	0.52	150
	Phospholipase C (*Clostridium welchii*)			
Dipalmitoyl lecithin	0	46	0.13	38
DPC + cholesterol	0	40	0.91	70
Egg lecithin	1.8	38	0.21	110
Soybean lecithin	2.8	28	0.092	51
Cabbage lecithin	4.2	0	0.47	80

differ substantially in two respects: (*i*) The concentration of *n*-hexanol required to obtain a maximal rate of hydrolysis decreases with increasing unsaturation of the lecithin, although there is little or no difference between that for dipalmitoyl lecithin and egg lecithin. The extreme example is provided by cabbage lecithin which shows, for each enzyme, a maximal rate of hydrolysis in the absence of added alcohol. (*ii*) The rate-concentration profile tends to become sharper with decreasing unsaturation.

Values of V_{max} and K_m have been calculated from the usual double reciprocal plots for the phospholipase-catalyzed hydrolysis of several lecithins in the presence of optimal concentrations of *n*-hexanol: results are collected in Table 1. The enzymes show distinctive patterns of behavior. Both phospholipases A exhibit generally increasing values of K_m with increasing degree of unsaturation; only that for snake venom shows increasing

values of V_{max} as a function of this variable. For the phospholipases C, values of K_m vary randomly with degree of unsaturation and values of V_{max} tend to be rather intensive to this variable under the conditions of these measurements.

Cholesterol Content and Kinetic Constants for Phospholipase-catalyzed Hydrolysis of Lecithin Liposomes

Liposomes containing cholesterol were prepared from various lecithins as described in Materials and Methods. These were employed as substrates for enzyme-catalyzed reactions. Rate-concentration profiles for phospholipase-catalyzed hydrolysis of liposomes of egg and dipalmitoyl lecithins containing equimolar concentrations of cholesterol in the presence of several *n*-alkanols generated typical bell-shaped curves (Fig. 3, Jain & Cordes, 1973). The presence of cholesterol has little influence on the shape of the rate-concentration profiles for egg lecithin but broadens those for dipalmitoyl lecithin. Plots (not shown) of the logarithms of the optimal concentration of activating alcohol against the logarithm of the *n*-octanol/water partition coefficient (Hansch plot; Hansch & Dunn, 1972) for the bee venom phospholipase A-catalyzed hydrolysis of egg lecithin yield satisfactory straight lines (however, *see below*). The slopes and intercepts of the plots for liposomes with and without added cholesterol are collected in Table 2. Note that the values obtained are not significantly altered by the addition of cholesterol.

Values of V_{max} and K_m for phospholipase-catalyzed hydrolysis of unsonicated liposomes of egg lecithin in the presence of optimal concentrations of *n*-hexanol were determined as a function of the mole fraction of cholesterol: the results are summarized in Fig. 4. The general pattern of behavior observed is one of parallel changes in V_{max} and K_m, even though the actual

Table 2. Slopes and intercepts of Hansch plots for bee venom phospholipase A-catalyzed hydrolysis of lecithin in the presence of several *n*-alkanols

Liposomes	Slope	Intercept
Unsonicated EL[a]	1.10±0.1	0.60±0.05
Unsonicated EL plus Chl[b]	1.0 ±0.1	0.55±0.10
Sonicated EL	0.9 ±0.15	0.40±0.10
Sonicated EL plus Chl[b]	0.85±0.25	0.30±0.1
Unsonicated SBL[c]	1.2 ±0.1	0.50±0.05

[a] EL, egg lecithin.
[b] Chl, cholesterol (equimolar with respect to lecithin).
[c] SBL, soybean lecithin.

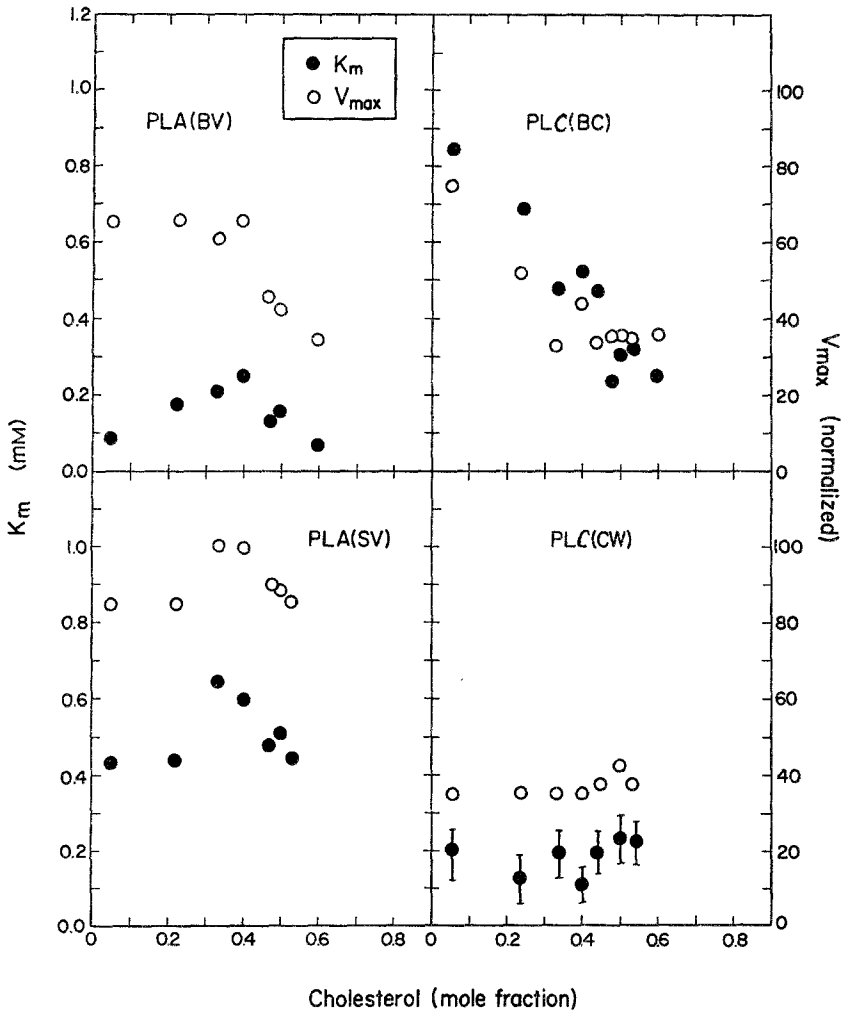

Fig. 4. Plots of K_m and V_{max} for the phospholipolysis of unsonicated liposomes of egg lecithin as a function of the mole fraction of cholesterol. Enzymes employed are the same as those indicated in Fig. 3 and Table 1

nature of the changes observed is strongly dependent on the enzyme studied. Some specific values of V_{max} and K_m for each enzyme studied have been included in Table 1.

The Effect of Sonication on the Rate of Phospholipase-catalyzed Hydrolysis of Egg Lecithin Liposomes

Plots of zero-order rates against the concentration of added *n*-hexanol for phospholipase-catalyzed hydrolysis of sonicated and unsonicated lipo-

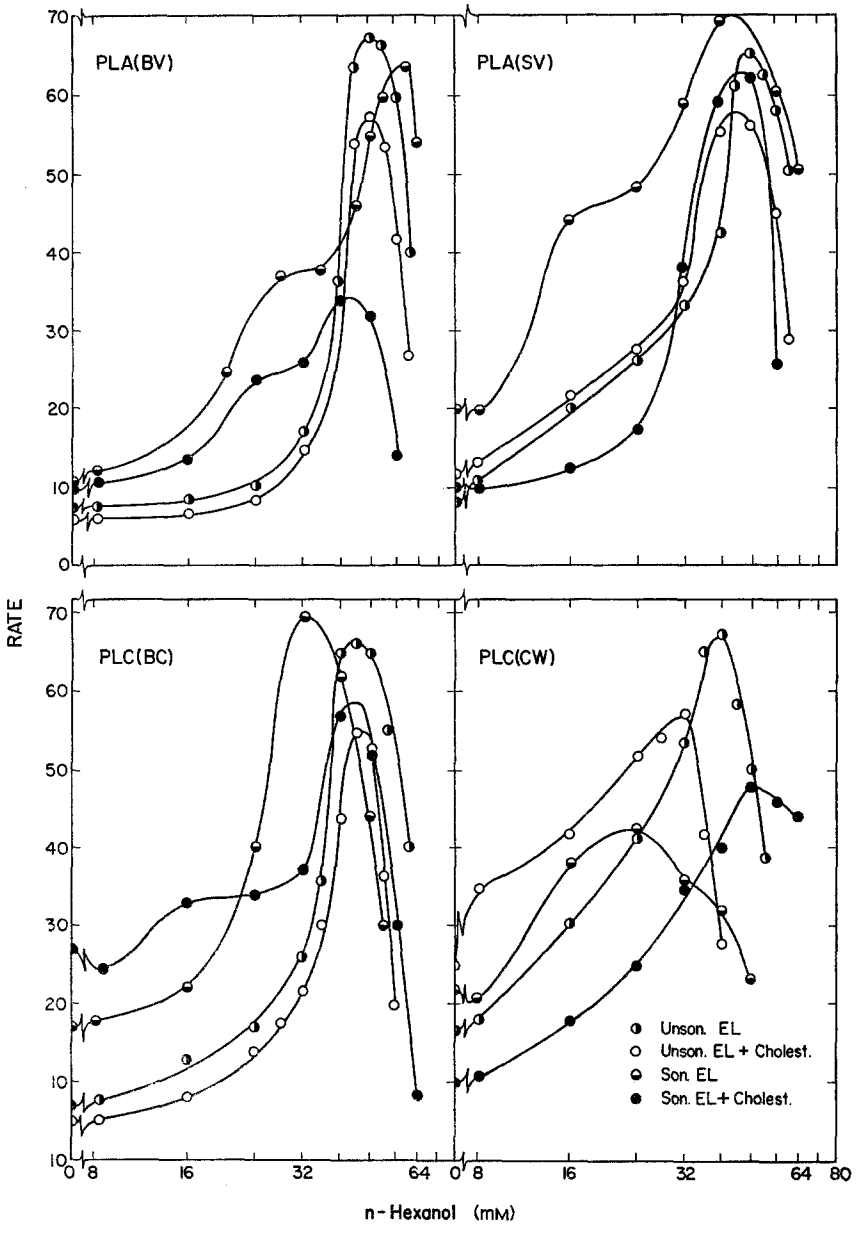

Fig. 5. Rates of phospholipase-catalyzed hydrolysis of egg lecithin (EL) in the form of unsonicated liposomes, sonicated liposomes, and the corresponding structures into which 0.5 mole fraction of cholesterol has been incorporated plotted as a function of the concentration of *n*-hexanol. Enzymes employed are the same as those specified in Fig. 3 and Table 1

somes of egg lecithin, alone and with 0.5 mole fraction of cholesterol, are provided in Fig. 5. For each of the phospholipases studied, the concentra-

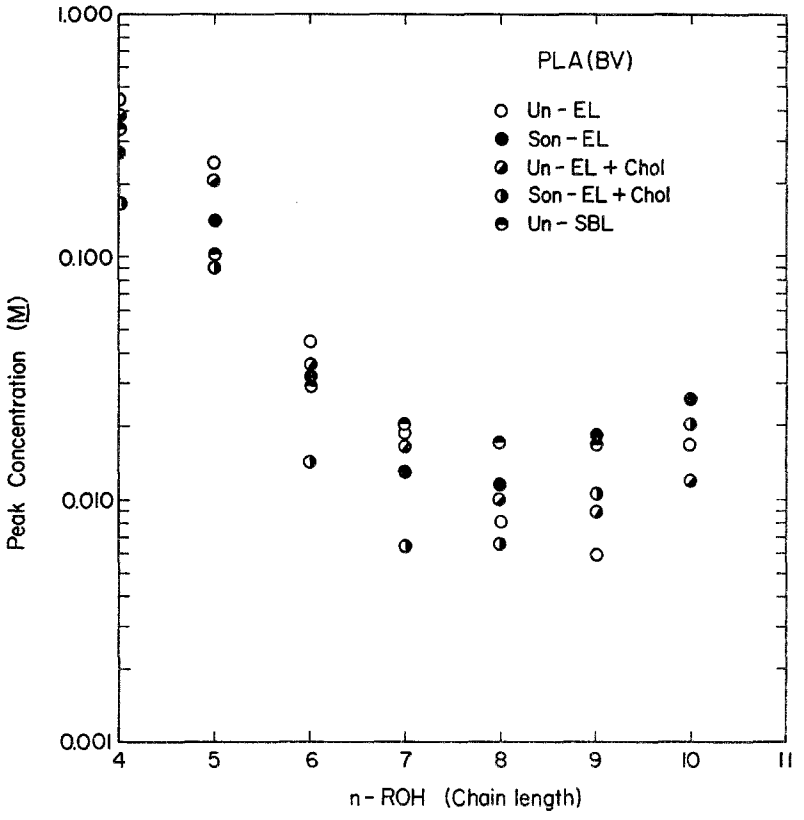

Fig. 6. Concentrations of *n*-alkanols required to elicit maximal susceptibility of sonicated and unsonicated liposomes of egg lecithin (EL), with and without 0.5 mole fraction cholesterol, to bee venom phospholipase A-catalyzed hydrolysis plotted as a function of alkanol chain length. Data for unsonicated liposomes of soybean lecithin (SBL) are included for the sake of comparison

tion of *n*-hexanol required to elicit optimal activation is only modestly affected by sonication of the substrate, with or without added cholesterol. Note that sonication has the effect of inducing a shoulder in these plots at concentrations of *n*-hexanol lower than the optimal ones. Both sonication and addition of cholesterol have the effect of broadening the rate-concentration profiles. Studies of the type shown in Fig. 5 have been extended to include other *n*-alkanols. In contrast to the behavior found for unsonicated liposomes, data for the sonicated ones do not yield satisfactory Hansch plots. That is, for the sonicated liposomes, the kinetic behavior of the system cannot be accurately predicted from *n*-octanol/water partition coefficients for the activating alcohols (however, *see below*).

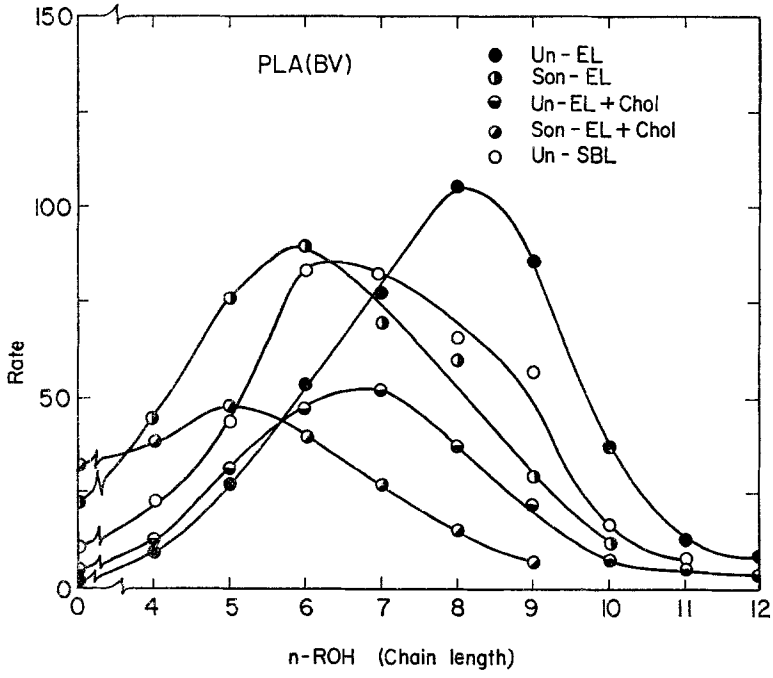

Fig. 7. Rates of bee venom phospholipase A-catalyzed hydrolysis of sonicated and unsonicated liposomes of egg lecithin (EL), with and without 0.5 mole fraction of cholesterol, at the optimal concentration of activating alcohols plotted against the chain length of those alcohols. Data for soybean lecithin (SBL) is included for the sake of comparison

The Effect of Various Alcohols on the Rate of Phospholipase A (bee venom)-catalyzed Hydrolysis of Various Lecithins

Activation rate profiles similar to those shown in Fig. 5 were obtained for phospholipase A (bee venom)-catalyzed and *n*-alkanol-activated hydrolysis of unsonicated and sonicated egg lecithin with and without cholesterol, and for unsonicated egg lecithin. The peak-activating concentrations of alcohols and the maximal rates obtained from these profiles are plotted in Figs. 6 and 7, respectively, as a function of the chain length of the activating alcohol. The results show that for a given liposomal system greater activation is observed as the chain length of the activating alcohol increases. However, this trend reverses beyond a given chain length dependent on the physical and chemical state of the lecithin. Thus, the peak activating concentration for *n*-nonanol is lower than that for either the lower or higher congeners. However, the lowest activating concentrations (*cf.* Fig. 6) for sonicated egg lecithin, unsonicated egg lecithin + cholesterol, sonicated egg

lecithin + cholesterol, and unsonicated soybean lecithin are observed for C_8, C_7-C_8, C_7-C_8, C_7-C_9 alcohols, respectively. The maximal rates observed for alkanol-activated hydrolysis (Fig. 7) show much larger variation. Thus, the maximal rates for hydrolysis of unsonicated egg lecithin, sonicated egg lecithin, unsonicated egg lecithin + cholesterol, sonicated egg lecithin + cholesterol, and unsonicated soybean lecithin are observed for C_8, C_6, C_7, C_5, and C_6-C_7 alkanols, respectively. It may be noted that the trends of effects shown in Figs. 6 and 7 are qualitatively similar. Some quantitative differences observed are probably within the experimental uncertainty.

Discussion

Recent studies of the effect of temperature (Ladbrooke *et al.*, 1968; Träuble, 1971; Sackmann & Trauble, 1972), cholesterol content (Oldfield & Chapman, 1972; Jain, 1974, *see* footnote 1), and sonication (Sheetz & Chan, 1972) indicate that these factors significantly affect the geometry of phospholipid vesicles, including their bilayer thickness, and the molecular spacings of the aliphatic chains. In a preceding study we demonstrated that the interaction of phospholipases with bilayer substrates is dependent on details of phospholipid organization in bilayers (Jain & Cordes, 1973). It follows that temperature, cholesterol content, and sonication should affect the susceptibility of phospholipid liposomes toward enzyme-catalyzed hydrolysis.

Certain pure long-chain paraffins undergo a thermotropic phase change in which, like the lecithin hydrocarbon chains, they go from a state of hexagonal symmetry to a melted state of lower symmetry. This transition in a phospholipid-water system is well characterized for several phospholipids: it occurs at 40.5 to 41.5 °C for dipalmitoyl lecithin. The changes in molecular organization that accompany the transition from gel to liquid-crystalline phase may be detected by changes in ANS fluorescence and bromothymol blue absorbance (Träuble, 1971), increase in molal volume (Träuble & Haynes, 1971; Melchoir & Morowitz, 1972; Sheetz & Chan, 1972), capacity to form stable BLM (Jain, 1972), and changes in low angle X-ray diffraction bands (Chapman, DeGier & Ladbrooke, 1967; Engelman, 1971).

The gross features of plots of rate against *n*-hexanol concentration for the bee venom phospholipase A-catalyzed hydrolysis of unsonicated liposomes of dipalmitoyl lecithin do not change dramatically as one passes

through the phase-transition temperature for this substrate (Fig. 1). There is, however, a detectable and quite sharp change in the concentration of *n*-hexanol required to achieve maximal stimulation of the enzymatic reaction which occurs at a temperature near that for the phase transition (Fig. 2). Hydrolysis of egg lecithin, which has a phase transition temperature below 20 °C, shows only a gradual increase in optimal alcohol concentration with decreasing temperature (Fig. 2). Overall, the data can be rationalized by assuming that increasing temperature causes a change in membrane organization in the polar head group region which facilitates those changes resulting from incorporation of *n*-hexanol.

It has been established that incorporation of cholesterol into dipalmitoyl lecithin bilayers lowers the phase transition temperature: there is no detectable phase transition at a cholesterol mole fraction of 0.5. As noted in Table 1, the peak activating concentration of *n*-hexanol for phospholipase-catalyzed hydrolysis of dipalmitoyl lecithin + cholesterol liposomes is comparable to the values obtained for egg lecithin at 37 °C or for dipalmitoyl lecithin at 44 to 45 °C. Incorporation of cholesterol into egg lecithin liposomes also alters the kinetic constants for hydrolysis by phospholipases A; however, the differences are not significant for phospholipases C. Clearly, different phospholipases respond differently to the same change of bilayer organization.

In several respects, the effect of sonication on liposomes is similar to that observed following incorporation of cholesterol into unsonicated liposomes as monitored by changes in susceptibility to phospholipase-catalyzed hydrolysis. Although K_m and V_{max} (*cf.* Table 1) are not significantly affected following sonication of liposomes, definite differences are observed for peak activating concentration of various alcohols (Fig. 6). Even more dramatic differences are observed for maximal rate activation produced by various alcohols (*cf.* Fig. 7). These observations suggest that the hydrocarbon region of sonicated liposomes can optimally incorporate alcohols with only 6 to 7 methylene residues instead of 8 to 9 residues which are optimally incorporated into liposomes which are unsonicated. Sonicated liposomes containing equimolar amounts of lecithin + cholesterol are optimally activated with alcohols containing only 5 to 6 methylene residues (*cf.* Fig. 6). Thus, incorporation of cholesterol into liposomes accentuates the effect of liposomes. This would imply that sonication and incorporation of cholesterol affect the hydrophobic region of the bilayer such that its capacity to incorporate *n*-alkanols of various chain lengths changes significantly. A shift of approximately one methylene residue to lower homologue is observed following either sonication or incorporation of cholesterol. Incorpora-

tion of cholesterol and sonication shift the peak activation to a lower homologous alkanol by 2 to 3 methylene residues.

These observations are consistent with the hypothesis that the bilayer of sonicated liposomes and/or cholesterol-containing liposomes may have dynamic fluctuations of the free volume in the vicinity of the oxygens of glycerol residues of lecithin in the bilayer. Furthermore, in sonicated and cholesterol-containing liposomes the Chapman transition appears at a lower temperature and over a wider range (Sheetz & Chan, 1972). Indeed, for geometrical reasons the glycerol backbone region of phospholipids in sonicated liposomes has a lower packing density in the outer monolayer. Similar changes in packing of the glycerol backbone region may be postulated for cholesterol-containing liposomes. Thus, a molecular organization which may permit a limited penetration of water into the hydrocarbon region, perhaps for the first two or three methylene residues, may occur in sonicated or cholesterol-containing liposomes. Such a reduction of dielectric thickness is consistent with high electrical capacity observed following incorporation of cholesterol (Ohki, 1969) or sonication (Redwood, Takashima, Schwan & Thompson, 1972), or in specially prepared BLM (Montal & Mueller, 1972). A concomitant lower activation energy of water permeation could also be expected and has been observed for sonicated liposomes (*cf.* Sheetz & Chan, 1972).

In this and the preceding paper (Jain & Cordes, 1973) we have shown that the rate activation profiles for various alcohols with various phospholipases are all typically bell-shaped and possess several characteristics which are of interest. Thus, for example, at lower concentrations of alcohols there is hardly any noticeable activation of phospholipase activity. Following this initial lag in activity on concentration scale there is a steep rise in rate. If the steep rising phase is extrapolated to the abscissa, one finds that the intercept on the abscissa is characteristic not only of each alcohol but also for each lecithin type. The maximal alcohol concentration not eliciting increased susceptibility to phospholipolysis may reflect the difference between the average volume per lecithin molecule and the limiting volume per lecithin molecule beyond which lecithin molecules cannot be compressed, i.e., membrane void volume.

Following incorporation of an alcohol into the void volume of the bilayer, any further increase in alcohol concentration in the membrane would result in membrane expansion (*cf.* Seeman, 1972); i.e., intermolecular distances in the membrane planes or overall area per molecule would increase. If the interaction of phospholipases with their substrates depends

upon an optimum intermolecular distance between the substrates, one would anticipate the bell-shaped curves of rate versus alcohol concentration repeatedly observed. Other explanations based on altered fluidity and micelle-bilayer equilibria in the membrane could also account for the observed bell-shaped curves.

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