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Intrinsic Differences in the Perturbing Ability of Alkanols in Bilayer: Action of Phospholipase A_2 on the Alkanol-Modified Phospholipid Bilayer

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Summary. The kinetic parameters for the steady-state rate of hydrolysis of egg phosphatidylcholine in multilamellar vesicles by bee venom phospholipase A_2 are measured in the presence of 27 alkanols and several organic solvents. In general, small nonpolar solutes like enflurane, tetrahydrofuran, benzene, chloroform and diethylether do not promote the hydrolysis of multilamellar vesicles. The rate of hydrolysis shows a biphasic dependence upon the alkanol concentration for all higher (C_5–C_9) alcohols examined, i.e., an optimal rate of hydrolysis is observed at a characteristic concentration for each alcohol. The alkanol to lipid mole ratio (D/L ratio) in the bilayer at the peak activating concentration of an alkanol was computed from its bilayer/water partition coefficient. The branched chain alcohols induce peak activation of hydrolysis at lower D/L ratios in the bilayer than the corresponding straight chain analogs. Similarly, the longer chain *n*-alkanols at peak activating concentration have a lower D/L ratio than the corresponding lower alcohols. Both the K_m and V_m for phosphatidylcholine increase as a function of the chain length of the activating alcohol. These kinetic parameters also depend upon the position of the substituents on the activating alcohols. Both the D/L ratio and V_m for an alcohol are found to change with the cross-sectional area of the activating alcohol across its long axis: alcohols with a more asymmetric cross-section exhibit higher V_m and a lower D/L ratio. Such correlations of V_m and D/L ratio with the molecular parameters of the alkanols are interpreted to suggest that the accessibility of the substrate molecule in the bilayer to the phospholipase is modulated by the free space introduced by the alkanols in the bilayer.

Effect of tetradecane derivatives and A_2C (a membrane fluidizing agent) on the phase transition characteristics of DPPC bilayers, and their susceptibility to phospholipase A_2 from bee venom and pig pancreas is also reported. These solutes cause a broadening of the transition profile and reduce the size of the cooperative unit and the enthalpy of transition. These effects depend upon the mole fraction of a solute in the bilayer; however, equal concentrations of these solutes do not induce equal response. Susceptibility of the modified bilayers to phospholipase A_2 depends not only upon the structure of the solute but also upon the source of the enzyme. The data show that the activity of the membrane-bound enzyme is modulated to different extents by different solutes, and the bilayer perturbing ability of these solutes may be related to the asymmetry of their cross-sectional area and to the free space introduced by the alkanols in a bilayer.

The action of phospholipases on phospholipids is governed by the "quality" of the interface to which the enzyme is exposed to during its course of action [24]. The action of phospholipases on a phospholipid bilayer, for example, is facilitated by long chain alcohols [6]. In previous publications we reported: that the effect of these alcohols depends upon their concentrations [6] and chain length [7]; that the activating effect of hexanol is due to a modification of the bilayer rather than the solubilization of the substrate [23]; that the optimal activating effect of hexanol is observed when the hexanol to phospholipid mole ratio in the bilayer is ~ 1.3 [23]; that the ability of alcohols to perturb the gel phase in dipalmitoylphosphatidylcholine bilayer correlates well with their ability to activate the phospholipase A_2 catalyzed hydrolysis of egg phosphatidylcholine bilayer [8]; and the behavior of the alkanol modified bilayer is compar-

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able to the behavior of phospholipid monolayer as the substrate for the bee venom and pig pancreatic phospholipase A_2 [5]. In this paper we describe additional experiments on the kinetics of the action of phospholipase A_2 on egg phosphatidylcholine bilayer modified by isomers and homologs of octanol and tetradecanol. These experiments are designed to evaluate the structural features of the alcohols that govern their activating effect on the action of phospholipase A_2 on phospholipid bilayer. The results demonstrate that the activating effect of an alcohol depends both upon its bilayer/water partition coefficient and upon its structure, i.e., equal concentrations of different alcohols in membrane do not necessarily induce equal activation. Indeed, the activation profiles depend both upon the nature of the alcohol and the source of the enzyme.

Materials and Methods

The bee venom and pig pancreatic phospholipase A_2 were obtained from Sigma. These preparations were judged to be better than 95% pure on the basis of their specific activity [23]. They migrate as single bands on polyacrylamide gel at pH 7.8 in Tris-glycine buffer. Egg phosphatidylcholine was isolated from hen yolk and chromatographed on silicic acid or neutral alumina. Its purity was tested by thin-layer chromatography where a single spot was observed in several solvent systems. A_2C was purchased from Makor Chemicals (Jerusalem). *Δ9-cis*-tetradecenol was purchased from Sigma and *Δ9-trans*-tetradecenol was from Nu-Check Prep. All other alkanols used in this study were purchased from Aldrich Chemical Co. Most of these were found to be >95% pure by gas chromatography; some alkanols were, however, purified by column chromatography or by vacuum distillation to give samples of >95% purity. Handshaken liposomes (multilammelar vesicles) were used for most of the kinetic studies described in this paper. The unilamellar vesicles were prepared by sonication of 25 mM multilamellar vesicles in a bath type sonicator (Sonicor Instrument Corporation, New York) for 60–120 min under nitrogen. The sonicated mixture was centrifuged at 30,000× *g* for 20 min to remove larger particles. Usually, the amount of the larger particles thus removed is less than 10% of the total lipid that is sonicated. The titration of the fatty acid released by the action of phospholipase A_2 with AMPD (2-amino-2-methyl-1,3-propane-diol) was performed at pH 7.4 in 5 ml reaction volume in an atmosphere of nitrogen at 37 °C. The response time of the instrument under all the conditions described here is less than 5 sec. The enzyme concentration was maintained such that the rate of hydrolysis was 20–90 nmol proton released min^{-1}. Typically this required 50 ng bee venom enzyme in 5 ml reaction mixture at optimal alkanol concentration. All the rates presented in this paper are normalized for 50 ng enzyme/5 ml of the reaction mixture. The liposomes prepared in 150 mM KCl and 6 mM $CaCl_2$ where subjected to an osmotic shock for phospholipase assay by diluting in a 6 mM $CaCl_2$ solution. Under these conditions about 70% of the total substrate in liposomes becomes available for phospholipase action under zero order rate conditions [23]. Details of the preparation of egg lecithin, preparation of liposomes, dilution and storage of the enzyme, protocol for following the hydrolysis of phosphatidylcholine by phospholipase A_2 are described in detail elsewhere [6, 7, 23].

Partition coefficient is defined as the ratio of equilibrium concentrations of a solute in two immiscible phases. In lipid bilayers, the equilibrium concentration of a solute cannot be determined directly. Operationally, the partition coefficient can be defined as the ratio of the number of moles of solute per gram of lipid in the bilayer to the number of moles of solute per gram of the aqueous phase. The number of moles of solute in the membrane phase is defined as the difference in the number of moles of solute in the aqueous phase in the absence and in the presence of a known amount of lipid. The partition coefficients in egg phosphatidylcholine (P_{EL}) liposomes at 24 °C were measured by determining the total (C_T) and the residual (C_W) aqueous phase concentrations of alkanols by gas chromatography. Handshaken liposomes (multilamellar vesicles) were osmotically shocked in the aqueous solution (at 40–50% saturation) of alkanol and allowed to stand for more than 30 min. The mixture was centrifuged at 12,000× *g* for 2×2 min and the concentration of the alkanol in the supernatant was defined as C_W. Independent measurements showed that all (>99.8%) the lipid in these tubes settled in the pellet [23]. The concentration of alcohols in the control tubes containing all the components except liposomes was defined as C_T. Partition coefficient P was calculated as $(C_T - C_W)/(C_W \cdot L)$, where L is the weight of lipid in grams per grams of water in the tube. The values obtained by the procedure are expected to have a standard deviation of up to ±10%. Similarly, the mole fraction of a solute in the bilayer is expressed as $X_S = [S]/[S]+[L]$, where $[S]$ is the concentration of solute and $[L]$ is the concentration of phospholipid.

Preparation of D-α-*dipalmitoylphosphatidylcholine*

DL-DPPC (2 g) dissolved in 500 ml diethylether+water (98:2) was mixed with *naja naja* phospholipase A_2 (2 mg) and incubated for 2 days at 30°. The residue left after evaporation of ether was chromatographed on 100 g of neutral alumina (Woelm). D-DPPC (0.92 g) was eluted with chloroform/methanol (7:3). It was judged to be pure by thin-layer chromatography. Liposomes prepared from D-DPPC were not hydrolyzed by *naja naja* phospholipase A_2, indicating that the L-isomer was completely removed by the first phospholipase treatment of DL-DPPC.

Differential Scanning Calorimetry

The DSC scans of the liposome dispersions were carried out on a Perkin-Elmer DSC-1B [10] as described elsewhere. The sample (15 µl) was placed in sealed aluminium pans and scanned from lower to higher temperatures at 1.25 of 2.5 °K/min at maximum sensitivity. Several parameters characteristic for a DSC profile were obtained from such scans. These are T_m, the temperature at which the DSC profile exhibits its maximum; T_c and T_e, the temperatures at which the transition begins and ends; ΔC_p, excess heat capacity is given by the height of the transition profile; ΔH, calorimetric enthalpy of transition given by the area under the transition profile; HHW, half-height width of the transition; and HHW', the shift in the transition profile of modified liposomes at half height width compared to that of the unmodified liposomes. From these measurements one could obtain a phase diagram (a plot of T_c and T_e as a function of X_s, the mole fraction of the solute) and calculate the average size of the cooperative unit undergoing the phase transition: $n = 4RT_m^2 \Delta C_p / \Delta H^2$, where ΔC_p is excess specific heat. The values of the various phase transition parameters reported here are within ±0.5 °C for T_c and T_e, within ±0.2 °C for T_m, within 3% for ΔC_p, HHW, and HHW', and within ±10% for ΔH.

Iodination of Phospholipase A_2 with ^{125}I

Both the bee venom and pig pancreatic enzymes were iodinated by the following procedure using the iodination kit supplied by Biorad (USA). Phospholipase A_2 (100 μg), β-D-glucose (250 μg), enzymobead reagent (500 μg; a sepharose bound lactose peroxidase and glucose oxidase preparation supplied by Biorad), and NaI^{125} (Amersham) were mixed in a total of 200 μl phosphate buffer (0.2 M, pH 7.2). The reaction was allowed to proceed at room temperature (22 °C) for 25 min. The mixture was then quenched by filtering it through the filter assembly provided for in the iodination kit. The filtrate was passed through a precalibrated Sephadex G-25 column (bed volume 10 ml) preequilibrated with fetal calf serum (Microbiological Associates) in 0.5 M Tris buffer at pH 7.4. One ml fractions were collected and counted directly by a ^{125}I-gamma counter. The fractions corresponding to iodinated phospholipase A_2 were pooled and dialyzed (4 × 500 ml, 24 h) against Tris buffer (0.5 M, pH 7.4) to which 50 mM KCl was added. Specific radioactivity of the final preparation was estimated to be more than 2.5 μCi/μg protein. This corresponds to an iodination efficiency of better than 40%. It has been demonstrated elsewhere that the properties of the free and iodinated pig pancreatic phospholipase A_2 are quite similar [14]. Our own experiments (not reported here) with iodinated (cold) bee venom enzyme showed that its kinetic characteristics are indistinguishable from those of the native enzyme.

Binding of Radiolabeled Phospholipases to DPPC Liposomes Containing the Tetradecane Derivatives

D-DPPC (1.2 mg) was mixed in polypropylene tubes with tetradecane derivative in chloroform to give a mixture of an appropriate mole fraction of the solute. After removing the organic solvents in a stream of nitrogen at 45°, the tubes were dried in vacuum for 3–5 hr. The lipid+solute film was dispersed in 400 μl of the aqueous phase for 1 h at 50 °C. At the end of this period the tubes are sonicated for 3–5 sec in a bath-type sonicator and then mixed thoroughly on a Vortex shaker for 2 min at maximum setting. The tubes were then incubated at 37° before centrifugation. The composition of the dispersions at pH 7.4 was 4 mM D-DPPC, 0–6 mM solute (X_s=0 to 0.66), 50 mM Tris, 50 mM KCl, 6 mM $CaCl_2$, 10 μg BSA, and 400 μg I^{125}-labeled phospholipase A_2 containing ~25,000 cpm. Appropriate control tubes lacked either lipid or $CaCl_2$. The tubes incubated at 37° were centrifuged at 12,000 × *g*, 37 °C, for 2 min, and once again for 2 min. 370 μl of the supernatant is withdrawn: first 100 μl is discarded, next 200 μl is placed in two scintillation vials (100 μl each), and last 70 μl is discarded. The same pipet tip is used for all the four withdrawals. To the 30 μl pellet 90 μl water was added, and the suspension (40 μl each) was transferred to two scintillation vials. The scintillation fluid (12 ml/vial) was Amersham PCS. The tubes were counted on a Beckman L-100 scintillation counter with 70% efficiency.

The binding data is expressed as the ratio of the number of counts in the total pellet (lipid phase) to the number of counts in 400 μl of the supernatant (R_B). The number of counts in the lipid phase is obtained by subtracting the number of counts in the 30 μl of the aqueous supernatant from the total number of counts in the dispersed pellet. Significant difficulties were encountered due to adsorption of counts on the tubes and pipet tips. In the protocol outlined above ~5% of the total counts were reproducibly adsorbed on the tube in the presence of phospholipid. This introduces some uncertainty in our estimate of the ratio of labeled phospholipase in the bilayer and the aqueous phase. However, the counts adsorbed on the tube are quite reproducible, and corrections for these counts with appropriate controls were made in the data presented in this paper. The number of adsorbed counts on the tube was significantly larger or irreproducible when the tubes were coated with fetal calf serum, bovine serum, albumin, or cold phospholipase. Use of siliconized glass tubes, detergent,

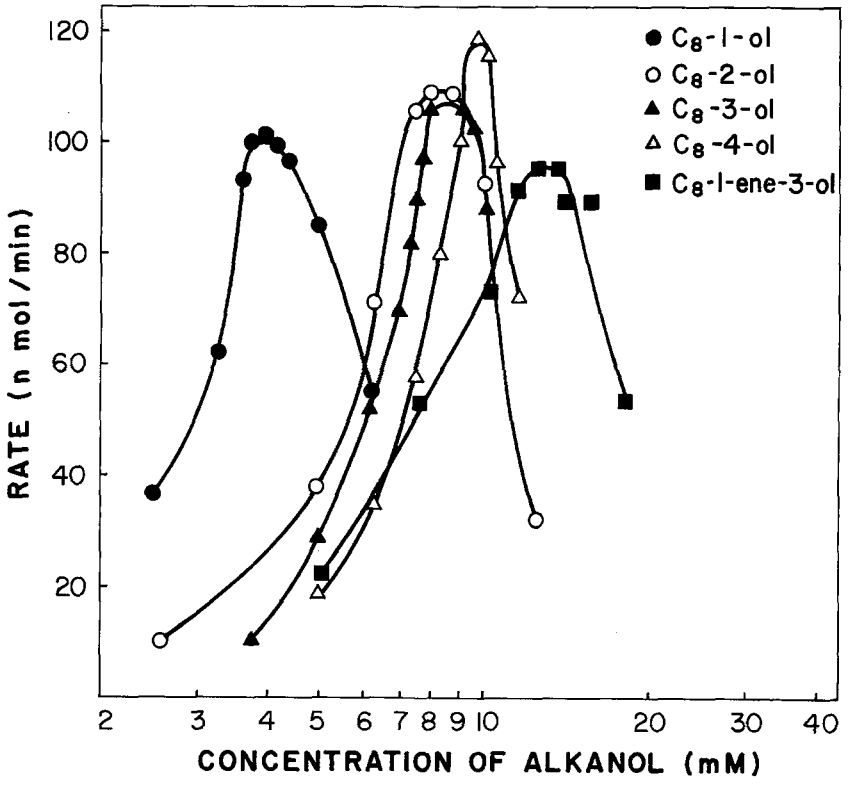

Fig. 1. Initial rate *vs.* *n*-alkanol concentration profiles for the hydrolysis of osmotically shocked egg phosphatidylcholine multilamellar vesicles. The profiles are presented for *n*-octan-1-ol (●), *n*-octan-2-ol (○), *n*-octan-3-ol (▲), *n*-octan-4-ol (△), and *n*-oct-1-en-3-ol (■). The reaction mixture (5 ml) contained 500 μM phospholipid, 50 ng bee venom phospholipase A_2, 6 mM $CaCl_2$, 1 mM KCL at pH 7.4 and 37 °C. All the alcohols examined showed similar biphasic profiles. The only difference between different alcohols is the height of the peak (peak rate) and the position of the peak (peak concentration).

Table 1. Partition coefficient and kinetic constants for phospholipase A_2

I.	II.	III.	IV.	V.	VI.	VII.	VIII.	IX.	X.	XI.	XII.	XIII.	XIV.
	Alkanol	P_{EL}	Peak rate (nmol/min)	Phospholipase A_2 on egg-PC				Molecular parameters					
				Peak conc. (*m*M)	K_m (μM)	V_m (nmol/min)	D/L Ratio	a	b	A_r ($=a\times b$)	A_e ($=a\times a$)	A_v ($=A_e-A_r$)	a/b
1.	*n*-Butan-1-ol	~5	4.5	491	–	–	1.84	5.8	5.8	33.64	33.64	0	1.00
2.	*n*-Pentan-1-ol	18	29.5	126.7	25.6±3.2	40.6±1.8	1.71	6.3	5.8	36.54	39.7	3.16	1.09
3.	*n*-Hexan-1-ol	50	59	48.0	27.5±4.4	61.3±2.3	1.52	6.6	5.8	38.28	43.56	5.28	1.14
4.	*n*-Heptan-1-ol	170	73	11.0	43.8±4.3	80.2±1.8	1.32	7.0	5.8	40.6	49	8.4	1.21
5.	*n*-Octan-1-ol	378	99	4.19	39.0±5.6	108 ±3.3	1.04	8.2	5.8	51.04	67.24	16.24	1.41
6.	*n*-Nonan-1-ol	1,400	112	1.8	67 ±7.9	137 ±4.8							
7.	*n*-Decan-1-ol	4,500	110	1.2	72.9	130.8							
8.	*n*-Undecan-1-ol	–	90	4.04	–	–							
9.	*n*-Dodecan-1-ol	–	102	11.5	–	–							
10.	*n*-Octan-2-ol	159.3	107	8.08	26.6±3.1	109.2±2.9	0.911	7.2	5.8	41.8	52	10.04	1.24
11.	*n*-Octan-3-ol	97.4	105	8.53	34.8±5.0	112 ±3.7	0.601	8.5	6.0	51	72.2	21.3	1.42
12.	*n*-Octan-4-ol	97.7	117	10.04	41.8±5.1	124.7±7.3	0.709	9.5	5.8	48.72	70.6	21.8	1.45
13.	*n*-Oct-1-en-3-ol	71	97	12.8	19.5±3.9	92 ±3.7	0.84	8.35	6.0	50.1	70	19.9	1.39
14.	2-Ethyl-hexan-1-ol	145.2	102	6.4	23.8±2.9	102.9±2.6	0.661	8.75	6.70	58.6	76.6	18.0	1.31
15.	2-Methyl-hexan-2-ol	16.5	64	64.2	28.6±5.1	63 ±2.5	0.79	7.0	5.8	40.6	49	8.4	1.21
16.	6-Methyl-heptan-2-ol	90	90	9.8	29.4±2.5	92.1±1.87	0.605	7.5	5.7	42.8	56.3	13.5	1.32
17.	3-Methyl-heptan-2-ol	66.0	99	9.9	24.1±3.0	94.8±2.6	0.482	8.0	5.7	46.6	64	18.4	1.37
18.	Heptan-2-ol	41.0	79	24.05	31.7±4.6	78.7±3.03	0.727	7.2	5.8	41.8	52	10.3	1.24
19.	Heptan-3-ol	18.2	87	31.13	45.3±9.2	73 ±5	0.422	7.9	5.8	45.6	63	17.4	1.36
20.	6-Methyl-5-hepten-2-ol	60.6	45	23	37.5±6	51 ±2.3	1.005	7.0	5.7	39.9	49	9.1	1.22

Partition coefficient of alkanols and kinetic constants for phospholipase A_2 action on bilayers. The partition coefficient of alcohols column *II*) egg phosphatidylcholine bilayer/water (column *III*), and the kinetic constants (column *IV–VII*) were determined as described in the text. The alkanol to lipid mole ratio (D/L ratio) in the bilayer is at the peak alkanol concentration (column *V*). The molecular parameters (column *IX–XIV*) of the alkanols were determined on the CPK space-filling models. These are presented in arbitrary units where 1.5 cm = 1 Å on the molecular scale. P_{EL}, partition coefficient; K_m, Michaelis-Menten constant; V_m, maximum velocity; D/L, alkanol to lipid mole ratio at the peak alkanol concentration; a and b are the long- and the short axes of the cross-sectional area of the alkanol across its longest axis.

higher salt, or BSA concentration to release the adsorbed counts did not give reproducible results.

Results

Phosphatidylcholine dispersed in water is a poor substrate of bee venom phospholipase A_2. The rate of hydrolysis of both multilamellar and unilamellar vesicles is increased more than 200-fold in the presence of hexanol, and >95% of the total substrate can be hydrolyzed under these conditions [23]. We have characterized the kinetic parameters of bilayers modified by alkanols by measuring the rate of hydrolysis of phosphatidylcholine as a function of alkanol concentration, and by measuring the rate of hydrolysis as a function of substrate concentration at optimal alkanol concentration.

Action of Phospholipase A_2 on the Egg-Phosphatidylcholine Liposomes Modified by Alkanols

Several homologs and analogs of *n*-octanol enhance the rate of hydrolysis of egg phosphatidylcholine in multilamellar vesicles. As shown in Fig. 1, their effect is biphasic, i.e., a maximal activation is observed at a characteristic concentration for each alkanol. While they all show biphasic activation profiles, several differences in the effect of these alcohols may be noted. As the position of hydroxyl groups in the chain changes, the peak activating concentration of the alcohol as well as the peak rates (maximum rates of hydrolysis) change.

Several other analogs and homologs of octanol also activate phospholipase A_2 catalyzed hydrolysis. As presented in Table 1, both the optimal activating concentration of the alcohols (column *V*) and the

Table 2. Effect of organic solutes on the hydrolysis of MLV by bee venom phospholipase A_2

Solute	Concentration range (mM)	Peak rate[a] (nmol/min)	Peak conc.[a] (mM)	
Chloroform	28.6 –114.4	18	85.8	
Carbon tetrachloride	5.16– 10.32	10.32	7.8	A biphasic profile was not obtained
Enflurane	Up to 549	–	–	Not effective[b]
Halothane	11.05–66.30	60.0	33.15	–
Tetrahydrofuran	87 –3,480	7.6; 8.8	1,740	–
Benzene	Up to 28	–	–	Not effective[b]
Diethyl ether (24 °C)	Up to 958	–	–	Not effective[b]
Fluroxene	45–450	10.8	112.5	
Methanol	Up to 5 M	–	–	No effect[b]
Ethanol	Up to 3.4 M	–	–	No effect[b]
Propanol	334 –1,672	30.8	1,003	
Butanol	136.6– 956.2	4.5	491	

[a] The conditions for the determinations of rate-concentration profiles are described in the legend to Fig. 1.
[b] No effect on the hexanol activation profile was observed.

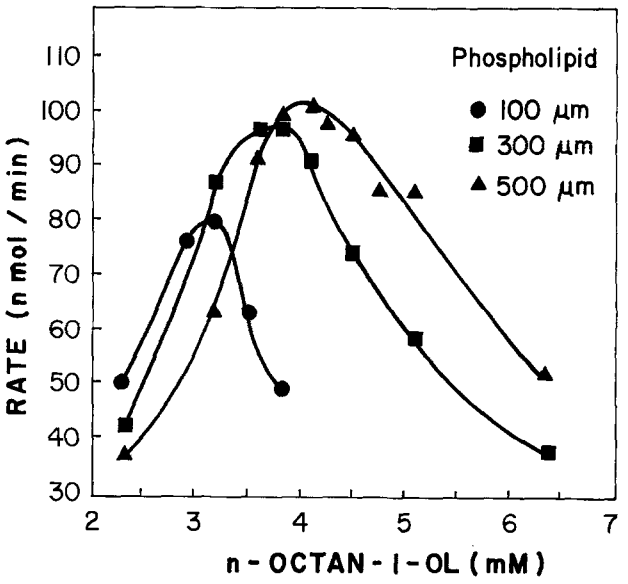

Fig. 2. Initial rate-*n* alkanol concentration profiles at various phospholipid concentrations for a *n*-octan-1-ol. The peak alcohol concentration changes with the phospholipid concentration in the medium it the partition coefficient is high (> 200). The experimental conditions are the same as in the legend of Fig. 1; however, the phospholipid concentration for the various profiles is as indicated with the profiles: for Fig. 2 – 100 μM (●), 300 μM (■), 500 μM (▲).

peak-rates (column *IV*) for the various alcohols are quite different. It may be noted that both of these characteristics change as a function of the chain length, the position of the hydroxyl group, and the position of branched alkyl group in the activating alkanol. This would imply that the "affinity" and the "efficacy" of these alcohols for their activating effect are different. The effect of several nonpolar organic solutes on the rate of hydrolysis was also studied. The data presented in Table 2 show that most of these solutes do not promote the rate of hydrolysis over a wide concentration range. Halothane and propanol are the only solutes that show a significant activation of hydrolysis. Such a lack of effect of lipid soluble solutes demonstrates that the activating effect of higher alkanols is not simply due to their incorporation ($P_{EL} \sim 20$) into the bilayer. In the following experiments, we have tried to establish the factors that govern the activating effect of the higher ($> C_5$) alcohols.

Kinetic Parameters for the Phospholipase A_2 Catalyzed Hydrolysis of Egg-Phosphatidylcholine Bilayer Modified by the Various Alkanols

To understand whether a difference in the effects of the various alcohols on the peak rate of hydrolysis is due to a difference in the affinity of the enzyme for the substrate or due to altered catalytic parameters, we studied the effect of varying the substrate concentrations on the peak rate of hydrolysis. Since the alcohols are partitioned into a bilayer, their concentration in the membrane phase and the aqueous phase would change as the liposome concentration is altered at a constant total alkanol concentration in the medium. This change would be quite significant if the bilayer/water partition coefficient of an alkanol is large or if the lipid concentration is large. Therefore, the peak activating concentrations of alcohols with partition coefficients greater than 100 were determined at several lipid concentrations. Typically, the peak activating concentration of alcohols were determined at 100, 300, and 500 μM substrate (Fig. 2). From a plot of lipid concentration *vs.* optimal alcohol concentration (data not shown), the optimum amount of alcohol for a corresponding lipid concentration was obtained. For lower alkanols ($< C_7$) such corrections were not necessary since their aqueous phase concentration is not significantly affected by less than 500 μM lipsomes in the medium.

The initial rates of hydrolysis under zero order steady-state conditions were determined at several (generally at 10, 15, 20, 30, 50, 100, 150, 200, 300, and 500 μM) phospholipid concentrations and the cor-

responding peak activating concentrations of an alcohol. This procedure assures that the lipid to alkanol mole ratio in the bilayer at the various lipid concentrations is optimal and the same. The liposomes were osmotically shocked [23]. Under these conditions about 70% of the total substrate in liposomes is exposed to the enzyme. The initial rates of hydrolysis as a function of phospholipid concentration at optimal alcohol concentration were curve fitted to the Michaelis-Menten hyperbola by a nonlinear regression algorithm on a PDP 11-10 minicomputer. The K_m and V_m values thus obtained are presented in columns *VI* and *VII* of Table 1. The data shows certain trends: the V_m values are very similar to but slightly larger than the peak rates for all the alkanols, suggesting that the peak rates were determined at the saturating substrate concentrations; both the K_m and V_m values increase with the chain length for the *n*-alkan-1-ol series; among the isomeric octanols, *n*-oct-1-en-3-ol and 2-ethyl-1-hexanol modified bilayers have a lower K_m and higher V_m; among the *n*-alkan-1-ols an even-odd alteration is observed, that is the difference between the K_m and V_m values induced by an even chain-length alcohol are closer to those for the lower odd-chain alcohol than those of the higher odd-chain alcohol.

The effect of alkanols on the kinetics of phospholipid action could be due to their effect on either the enzyme or on the lipid bilayer or both. A direct effect of alcohol on the enzyme is expected to show up in a change in the affinity (K_m) of the enzyme. However, the K_m values for multilamellar vesicles have a contribution from the total available substrate, thus masking the true affinity of the enzyme for the substrate. In unilamellar vesicles such effects are not expected to arise since 65% of the substrate is present in the outer monolayer [23, 19]. The K_m values for the substrate in unilamellar vesicles thus provides a better measure of the relative affinity of the enzyme for the substrate in the presence of the various alkanols. The kinetic data for the action of phospholipase A_2 on unilamellar vesicles modified with three alkanols is presented in Table 3. The K_m values for unilamellar vesicles modified by three alcohols are the same within the experimental error. Since the K_m values for multilamellar vesicles modified by the various alkanols is within a twofold range, we feel that the K_m values for multilamellar vesicles are approximately the same in the presence of the various alcohols. It is possible that the alkanols also interact with the hydrophobic site on the enzyme (G.H. de-Haas, *personal communication*) where they would compete for the binding of the enzyme with the bilayer.

Table 3. Kinetic parameters for the hydrolysis of unilamellar vesicles by bee venom phospholipase A_2 in the presence of alkanols

Alkanols	Conc. (mM)	Peak rate[a] (nmol/min)	K_m[a] (μM)	V_m[a] (nmol/min)
None	–	<0.02	>800	>0.02
n-Hexan-1-ol	20	22	21.7±3	20±2.4
n-Octan-1-ol	3.0	86	22.5±4.2	82±3
n-Oct-1-en-1-ol	12.8	74.9	17.2±2	73±2

[a] The kinetic parameters were determined as described in Materials and Methods.

Partition Coefficient of Alkanols in Lipid Bilayer/Water

The partition coefficient of *n*-hexanol in 50 mM egg phosphatidylcholine (P_{EL}) was determined [9, 23] at several hexanol concentrations (10 to 40 mM). The P_{EL} values ranged from 47–51 at these hexanol concentrations. Thus the P_{EL} values do not change significantly as a function of *n*-hexanol concentration in the aqueous phase. This shows that the concentration of alkanols in the bilayer changes linearly with its aqueous phase concentration. The values of the partition coefficients of the various alkanols in egg phosphatidylcholine bilayer are presented in column *III* of Table 1. These values were determined at approximately the same concentration as a peak activating concentrations (column *V*, Table 1). The data shows that the partition coefficient of *n*-alkan-1-ols increases with the chain length. An average increase in the free energy for the transfer of a methylene group is -660 ± 60 cal/mol. The partition coefficients for the branched chain alcohols are consistently smaller than those for the isomeric straight chain 1-alkanols. This probably reflects anisotropy of the organization of the bilayer.

From the partition coefficient values we calculated the concentration of alkanols in the bilayer at which these alkanols exhibit the peak rates (*cf.* Fig. 1). The alkanol to lipid molar ratio (D/L) in the bilayer at the peak activating concentrations is defined as:

$$\frac{D}{L}=\frac{PL}{PL+1}\cdot\frac{C}{M}$$

where P is the partition coefficient of the alcohol, L is the weight of lipid in grams per grams of the aqueous phase, M is the molarity of lipid in the aqueous phase, and C is the concentration of the alcohol in mols/liter. The D/L values corresponding to the peak alcohol concentrations (column *V*, Table 1) are presented in column *VIII* (Table 1). If an equal number of alkanol molecules in the bilayer

induced an equal response, one would expect the D/L ratio to be constant for the various alkanols at the concentrations at which they induce an optimal (peak) activation of the phospholipase A_2 catalyzed hydrolysis. The D/L values presented in Table 1 vary over a fourfold range, i.e., for example, on the average 0.48 molecules of 3-methyl-2-heptanol, or 1.70 molecules of *n*-pentanol per lipid molecule in the bilayer cause an equal perturbation of the bilayer to facilitate an optimal action of the phospholipase. If one assumes that the effect of alkanols is only on the lipid bilayer, a difference in the D/L ratio at equipotency concentration would imply that the intrinsic perturbing ability of the alkanols in a bilayer is different.

Correlation of the D/L Ratios and the V_m Values With the Molecular Parameters of the Activating Alcohols

Since the incorporation of alkanols in a bilayer increases the susceptibility of phospholipids in the bilayer to phospholipase A_2, it is likely that molecular parameters of alkanols related to their geometry may correlate well with the D/L ratio or the V_m values or both. This is based on the assumption that the presence of alkanols in the bilayer increases the amount of the enzyme in the bilayer. Alkanols are expected to be incorporated into a bilayer such that the hydroxyl group is placed near the polar groups and the alkyl chain is aligned parallel to the acyl chains of the phospholipid molecule [10]. The phase diagram for the lower alkanols ($<C_{10}$) suggests that the alkanols are ideally mixed with phospholipids in the bilayer. If the alkanol molecules are assumed to be uniformly distributed in the bilayer, the bilayer perturbing ability of alkanols would depend upon their cross-sectional area across their long axis. As shown in Fig. 3, the cross-sectional asymmetry or the ratio of the long, *a*, and the short, *b*, axes will depend upon the type and the position of the substituents on the polymethylene chain. Since very little direct information is available on the conformation of the alkanols used in this study, we obtained the values of the long, *a*, and short, *b*, cross-sectional axes as presented in columns *IX* and *X*, from the space filling CPK models. The values are given in cm such that 1.5 cm = 1 Å. The following simplifying assumptions were made. All C–C bonds are assumed to be in *trans*-conformation except that the dihedral angle at the C_4–C_5 bond is assumed to be in a gauche conformation. A rationale for this is presented in the discussion section. Thus, *n*-butan-l-ol has a symmetrical cross section ($a=b$), whereas for the higher alkanols $a>b$. For the C_9 and higher alcohols we assumed

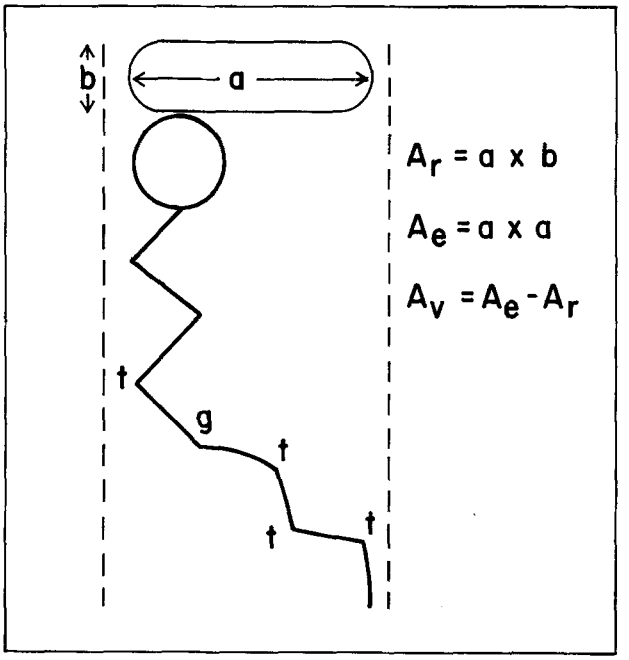

Fig. 3. A schematic for the conformation of *n*-alkanol containing a gauche, *g*, and otherwise all-*trans*, *t*, conformation. A gauche conformation in position 4 would swing the rest of the chain to the side thus making the cross section of the molecule more asymmetrical. Assuming the cross-sectional long axis = *a*, and width = *b*, we have calculated the actual cross-sectional area ($A_r = a \times b$), effective area ($A_e = a^2$), and the void area ($A_v = a^2 - ab$).

on the average two gauche conformers in a chain. This may give rise to a 2gl kink, thus effectively reducing the a/b ratio.

From the values of *a* and *b* we calculated the values of the "real" cross-sectional area ($A_r = a \times b$), the "effective" cross-sectional area ($A_e = a^2$), and the void area ($A_v = A_e - A_r$) contributed by an alkanol. Correlations of D/L ratio, V_m and K_m with A_r, A_e, and A_v are presented in Table 4. Such correlations with K_m are poor ($r<0.4$); however, significant correlations were observed between other parameters. In general, V_m increases with A_r, A_e, and A_v, whereas D/L decreases with these areas. As shown in Fig. 4, best correlations are observed between the parameters related to void space (A_v, $A_v^{1/2}$) introduced by alkanols and the corresponding V_m and the D/L ratios. This implies that the activating effect of alcohols may be related to their ability to introduce a free space in the bilayer; that the various alcohols introduce the free space in proportion to the D/L ratios at optimal alcohol concentration; and that the V_m values increase with the free space in the bilayer. Significant but not as strong correlations are observed with A_e and A_r values, presumably because these parameters also contain contribution of the free space introduced by an alkanol.

Table 4. Correlation of V_m and D/L ratio with the various parameters

Plot		Slope		Y-Intercept		Corr. coeff.
X-axis	Y-axis					
D/L ratio	a/b	−0.24	±0.047	1.5	±0.48	−0.82
D/L ratio	A_v	−0.57	±0.15	1.7	±0.15	−0.72
D/L ratio	$(A_v)^{1/2}$	−2.1	±0.37	5.4	±0.38	−0.85
D/L ratio	A_e	−22.4	±5.0	78.4	±5.3	−0.77
D/L ratio	$(A_e)^{1/2}$	−1.56	±0.34	9.0	±0.36	−0.79
D/L ratio	A_r	−9.7	±3	53.6	±3	−0.66
D/L ratio	$(A_r)^{1/2}$	−0.79	±0.2	7.4	±0.22	−0.71
V_m	a/b	0.0039	±0.0006	0.96	±0.05	0.86
V_m	A_v	0.19	±0.029	−2.43	±2.6	0.855
V_m	$(A_v)^{1/2}$	0.039	±0.00049	0.17	±0.44	0.897
V_m	A_e	0.37	±0.07	27.6	±6.4	0.80
V_m	$(A_e)^{1/2}$	0.028	±0.0049	5.3	±0.43	0.83
V_m	A_r	0.176	±0.044	30.7	±3.8	0.72
V_m	$(A_r)^{1/2}$	0.013	±0.0029	5.6	±0.255	0.75
K_m	$(A_v)^{1/2}$	0.014	±0.014	3.0	0.57	0.25

The data from Table 1, for 20 alkanols was correlated by a standard linear regression analysis on PDP-11 computer.

Effect of Tetradecane Derivatives

Since lower alkanols have relatively small partition coefficients, we studied the effect of a series of long-chain compounds on the susceptibility of bilayers to phospholipase A_2 and on the thermotrophic phase transition of liposomes. The compounds used for the modulation of the bilayer organization are:

I. *n*-Tetradecan-1-ol
II. Δ^9-*cis*-*n*-Tetradecen-1-ol
III. Δ^9-*trans*-*n*-Tetradecen-1-ol
IV. *n*-Tetradecane
V. *n*-Tetradecan-1-oic acid (myristic acid)
VI. *n*-Tetradecane trimethylammonium bromide
VII. A_2C [2-(2-methoxyethoxy)ethyl 8-(*cis*-2-*n*-octylcyclopropyl)octanoate]

These solutes were mixed with phospholipids before the preparation of bilayers. Due to their partition coefficients, the aqueous phase concentration of these compounds is expected to be negligibly small in the presence of liposomes. Since the activating effect of

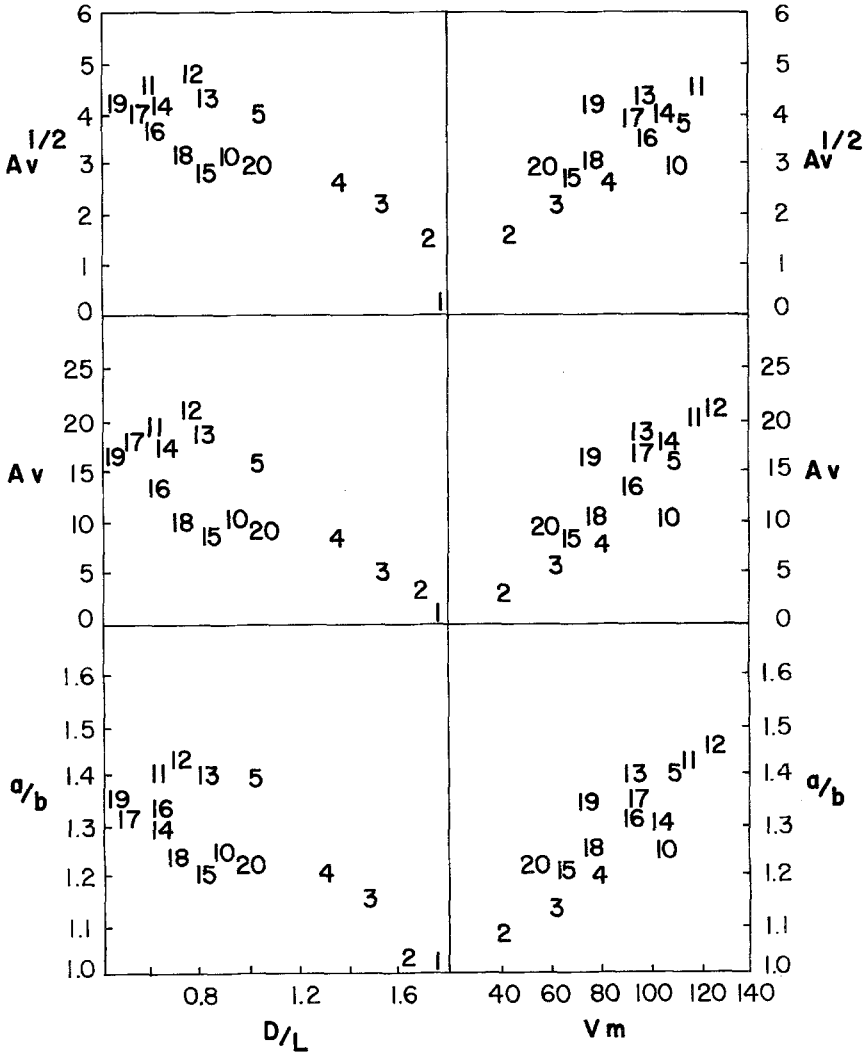

Fig. 4. Plots of alkanols to lipid mole ratio in the bilayer (D/L ratio) at the peak activating concentrations (column *V*, Table 1) and V_m (column *VII*, Table 1) as a function of the cross-sectional parameters as derived in Fig. 3. Other correlations of this type are summarized in Table 4.

lower alkanols is predominantly on V_m, for tetradecane derivatives we studied their effect only on the initial rate of hydrolysis at high liposome concentration.

Effect of Tetradecane Derivatives on the Rate of Hydrolysis of DPPC Bilayers

Data presented in Fig. 5 shows that the rate of hydrolysis by both the bee venom and the pancreatic phospholipase A_2 is stimulated by the various tetradecane derivatives; however, the peak rates are considerably smaller than those observed with octanol analogs. The rate activation profiles as a function of X_s for these solutes are significantly different. For bee venom the rate activation profiles for solutes, I, III, V, and VI are biphasic, i.e., the rate of hydrolysis increases and then decreases as a function of alcohol concentration (X_s). Both A_2C and tetradecane do not cause any increase in the rate of hydrolysis. The activation profile for tetradecanol is biphasic, similar to that observed with hexanol [5]. In contrast, the activation profiles for *cis*- and *trans*-tetradecanols are monotonic, and the rate of hydrolysis increases rather abruptly at $X_s \sim 0.4$. Interestingly the behavior of the bee venom and the pig pancreatic enzyme is similar with the *cis*-tetradecanol modified bilayer, whereas their effects on bilayers modified by *trans*-tetradecanol are significantly different. These observations demonstrate that phospholipase A_2 from two different sources respond differently to bilayers modified by the isomeric tetradecenols. Not only two different types of effects, monotonic and biphasic, are observed with isomeric tetradecanols, but the rate activation profiles for these two enzymes with a given solute (II and V) are also different. These observations demonstrate that microenvironments in a solute modified bilayer experienced by two closely related proteins are significantly different. Moreover, A_2C and tetradecane do not have any activating effect on phospholipases, even though their effects on the phase transition characteristics represent almost two extremes (*see* below).

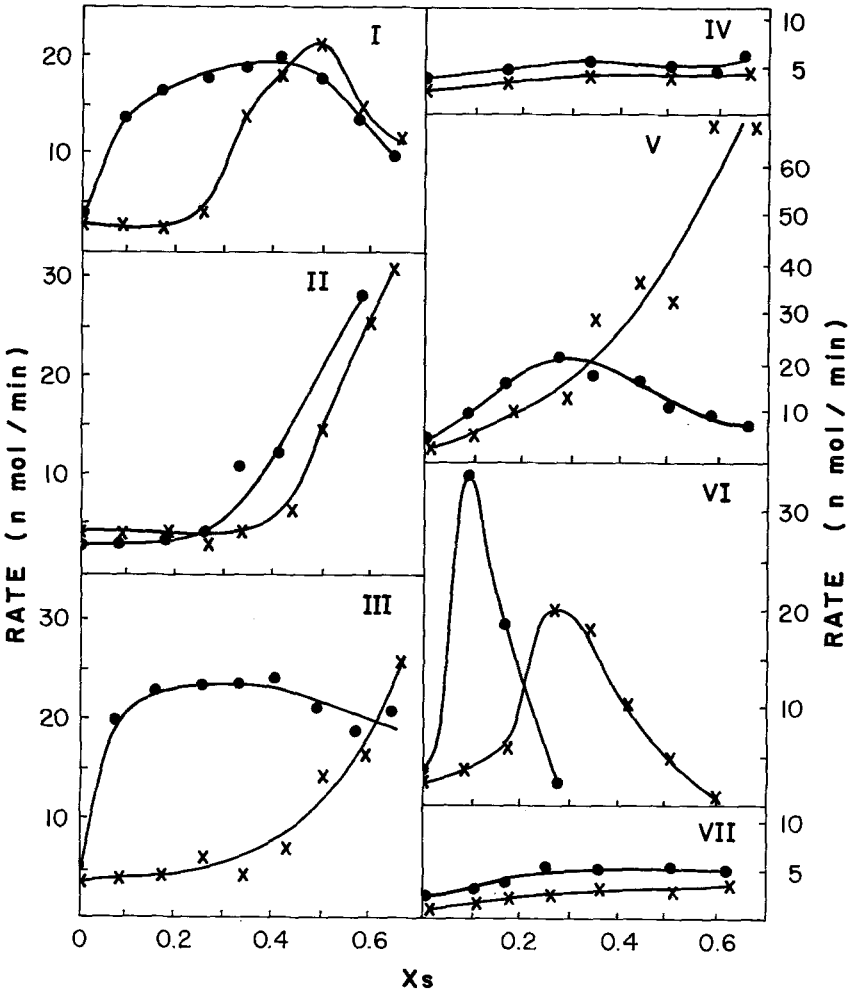

Fig. 5. Initial rates of hydrolysis of DPPC liposomes containing varying mole fractions (X_s) of solutes I–VII by bee venom (–●–) and pig pancreatic (–×–) phospholipase A_2 at 37°, pH 7.4. Phospholipid concentration in each sample was 2 mM; enzyme concentration was 40 ng/5 ml for the bee venom and 100 ng/5 ml for the pancreatic enzyme.

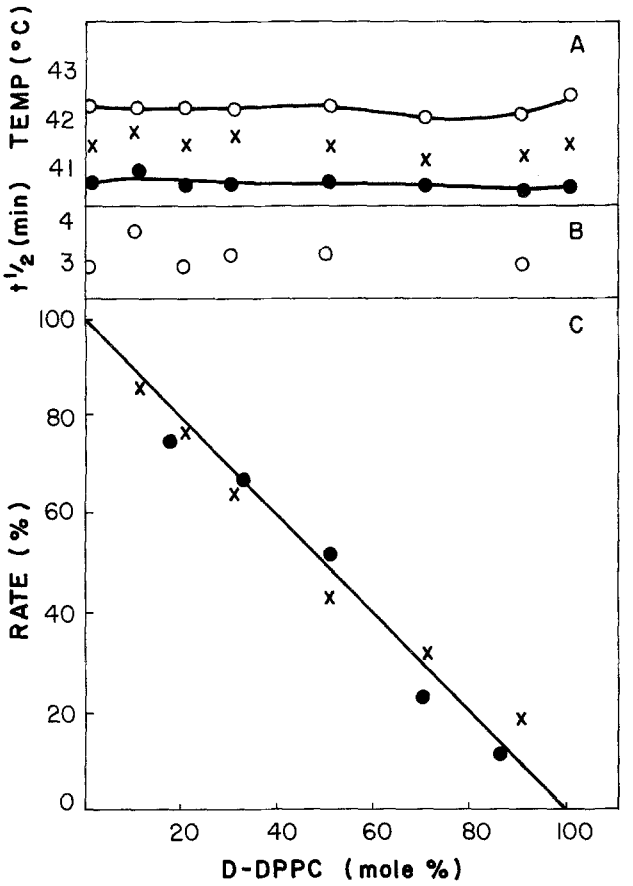

Fig. 6. Effect of varying mole % of D-DPPC in L-DPPC upon (*A*) the phase diagram (*see* legend Fig. 8), (*B*) $t_{1/2}$ for the lag phase [5] of pig pancreatic phospholipase with hexanol modified egg phosphatidyl choline liposomes, and (*C*) the relative rate of hydrolysis of hexanol modified egg phosphatidyl choline liposomes by the bee venom (●) and pig pancreatic phospholipase A_2 (– × –).

Effect of D-DPPC on the Hydrolysis of L-DPPC by Phospholipase A_2

The rate of hydrolysis of L-DPPC by bee venom and pig pancreatic phospholipase A_2 as a function of the mole fraction of D-DPPC in liposomes is shown in Fig. 6. These liposomes were prepared by premixing the two stereoisomers in appropriate amounts, and the mixed lipid film was dispersed in the salt solution. The rate of hydrolysis of L-DPPC by both the enzymes decreases linearly with the mole fraction of D-DPPC in the liposomes. The results show that for both the phospholipases D-DPPC is not a substrate; that D-DPPC inhibits hydrolysis of L-DPPC; that equal concentrations of D- and L-DPPC inhibition is 50%. Moreover, the latency period [5] for the pig pancreatic enzyme does not change with the mole proportion of D-DPPC, which suggests that the latency period is not a function of the surface dilution of the solute. This suggests that the affinity of both the bee venom and pig pancreatic phospholipase A_2 for D- and L-DPPC is the same. This confirms the observation that K_m of pig pancreatic phospholipase A_2 for micelles of the L-form of the substrate is the same as its K_i for the D-isomer [1]. The fact that K_i and K_D are equal implies that either the D- and L-isomers bind equally well to the two enzymes [1] or their binding to the bilayer is a rate limiting step, in which case the inhibitory effect of D- is due to surface dilution [2].

Binding of Radiolabeled Phospholipase to the Alkanol Modified Bilayer

One of the possible explanations for the dependence of the rate of hydrolysis on X_s is that alkanols regulate the amount of the enzyme in the bilayer phase, and a difference in the behavior of the two enzymes could be due to a difference in their ability to partition into the bilayer. To test this possibility we studied the direct binding of ^{125}I-labeled phospholipase A_2 with phospholipid bilayer in liposomes. Binding experiments require incubation of the enzyme with the substrate, and under these conditions the products of hydrolysis perturb the bilayer. To overcome this problem the binding experiments were done with bilayers prepared from D-DPPC, which is not hydrolyzed by bee venom or pig pancreatic phospholipase A_2. The fact that K_i and K_m for D- and L-DPPC are identical, makes D-DPPC bilayers an ideal system for the enzyme-binding studies. The fraction of bee venom and pig pancreatic ^{125}I-labeled phospholipase A_2 bound to D-DPPC bilayers is shown in Fig. 7. The results show that the ratio of membrane bound enzyme to the enzyme in the aqueous phase (R_B) increases with X_s for all the solutes examined. The fraction of the bilayer bound enzyme in the presence or the absence of the modifying alcohol remains the same in the presence or absence of Ca^{2+} (data are not presented here). Several features of this binding data (Fig. 7) may be noted: more phospholipase is partitioned into the bilayer as a function of X_s; more of the bee venom enzyme is incorporated into the bilayer modified by I–IV, and VI; the partitioning of the two enzymes in the bilayer modified by V is almost the same. There is a parallel between the fraction of the bound enzyme and the rate of hydrolysis (*compare* Figs. 5 and 7), i.e., the rate of hydrolysis increases when more enzyme is bound. Such correlations are particularly evident at $X_s < 0.4$. However, significant departures from this trend may be noted: the binding and the rate curves do not exactly overlap for a given system; at high X_s the rate decreases,

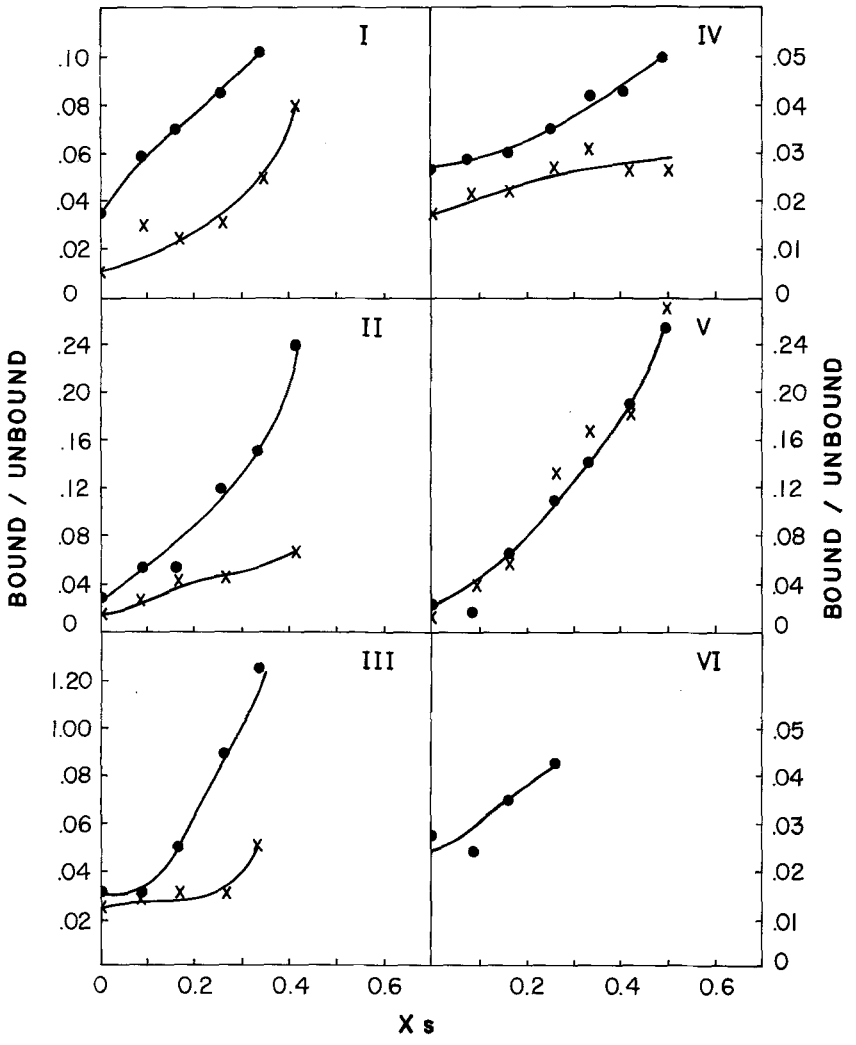

Fig. 7. Ratio of the bound/unbound radiolabeled bee venom (-●-) and pig pancreatic (-×-) phospholipase A_2 to D-DPPC bilayers containing varying mole fractions (X_s) of solutes I–VI. The mixture for binding studies contained 0.5 M Tris (pH 7.4), 50 mM KCl, 4 mM D-DPPC, 0–6 mM solute, 400 μg labeled enzyme, 10 μg BSA in a total volume of 400 μl at 37 °C.

whereas the fraction of the bound enzyme increases. Although from our data we cannot calculate the absolute amount of the bound enzyme, the data demonstrate that the amount of the bound enzyme is only one of the many factors that determines the rate of hydrolysis. Other factors involved in the turnover cycle of the enzyme must contribute, especially at high X_s.

Effect of Tetradecane Derivatives on the Thermotropic Phase Transition Characteristics of DPPC Liposomes

Thermotropic phase transition characteristics of the mixed bilayers containing various mole fractions of tetradecane derivatives are similar to those observed for the lower alkanols [10], i.e., the phase transition profiles are broadened. Some differences may be noted: I and V shift the transition profiles towards higher temperature; II, VI and VII shift the profiles towards a lower temperature; and the DSC profile for the liposomes containing III and IV is shifted only slightly. At higher concentration, VI disrupts the bilayer to give mixed micelles as indicated by the formation of clear solutions. Although these compounds modulate the phase properties of bilayers, some differences in the magnitude of the shift in T_m, the temperature range of transition, and possible phase separation (coexistence of two or more phases undergoing the transition at different T_m) were also noted.

The phase diagrams for the tetradecane derivatives I–VII constructed from the phase transition profiles are shown in Fig. 8. Complete phase diagrams (up to $X_s = 1$) for these solutes could not be obtained since at high X_s the dispersions were either unstable or they comicellized. The bilayer/water partition coefficient of tetradecane derivatives is expected to be more than 10^4 [9]; therefore, for the construction of the phase diagrams it is assumed that all the tetradecane derivative added during the preparation of liposomes is present in the bilayer. The shapes of

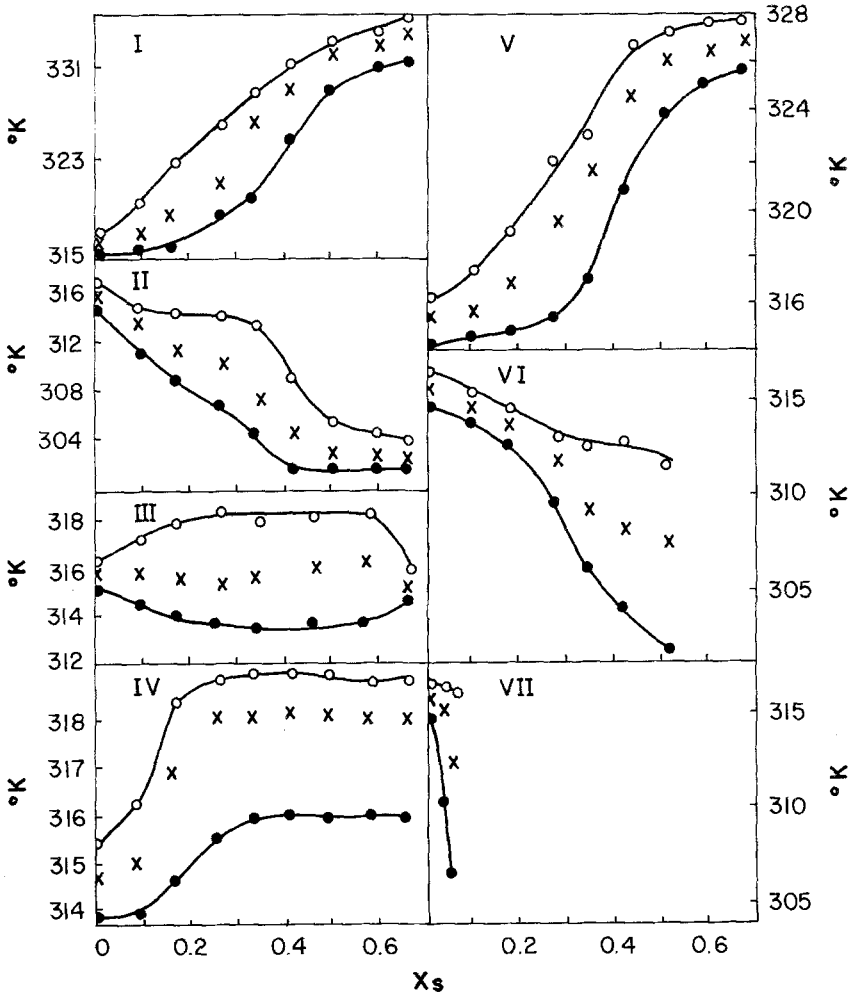

Fig. 8. Phase diagram for DPPC liposomes containing solutes I–VII. The onset (-●-), middle (-×-), and the end (-○-) of transition profile were obtained by differential scanning calorimetry. *See* Materials and Methods section for details. No transition profiles could be observed for liposomes containing 5 mole % A_2C.

the phase diagrams suggest that I, III, V and VII mix with DPPC gel phase, although calculations similar to those presented by Mabrey and Sturtevant [13] suggest that the mixing is not ideal over the whole range of X_s. Solutes II, IV, and VI exhibit significant departure from ideal mixing over a wider range of X_s. Such a nonideal mixing is also indicated by asymmetric and split phase transition profiles from which the phase diagrams are constructed. The effect of these solutes on the phase diagram is a manifestation of their interaction with the gel phase in the bilayer. A difference in their effect suggests that the mode of interaction and/or the effect of the various tetradecane derivatives with DPPC in the gel phase is different. It is quite likely that some of these solutes (like tetradecanoic acid and tetradecanols) form stoichiometric complexes with distinct phase transition characteristics, however, the miscibility of such complexes with pure DPPC gel phase appears to depend strongly upon the nature of the solute. In general, chains with shape and size closer to palmitic acid appear to mix ideally with dipalmitoyl phosphatidylcholine in bilayers, provided a polar group is attached to these chains to orient them parallel to phospholipid molecules in the bilayer. The nature of the polar group on the solute is also important since the phase diagrams for I, V, and VI are quite different. This could be due to a difference in the size of the polar groups. Indeed, this is best reflected in the behavior of A_2C (VII) modified bilayers, where the phase transition profile disappears completely at $X_s \geqq 0.1$.

Plots of ΔH, n, and HHW' as a function of X_s are shown in Fig. 9. Such a dependence of the various phase transition parameters upon the mole fraction of solutes I–VII leads to several interesting conclusions. ΔH is altered only slightly at low mole fractions of solutes. Excess specific heat (ΔC_p), which is related to the size of the cooperative unit, is quite sensitive to low concentrations of the various solutes in the bilayer. However, this parameter is not sensitive to the nature of the solute. Also at higher mole proportions of solutes, ΔC_p cannot be readily interpreted

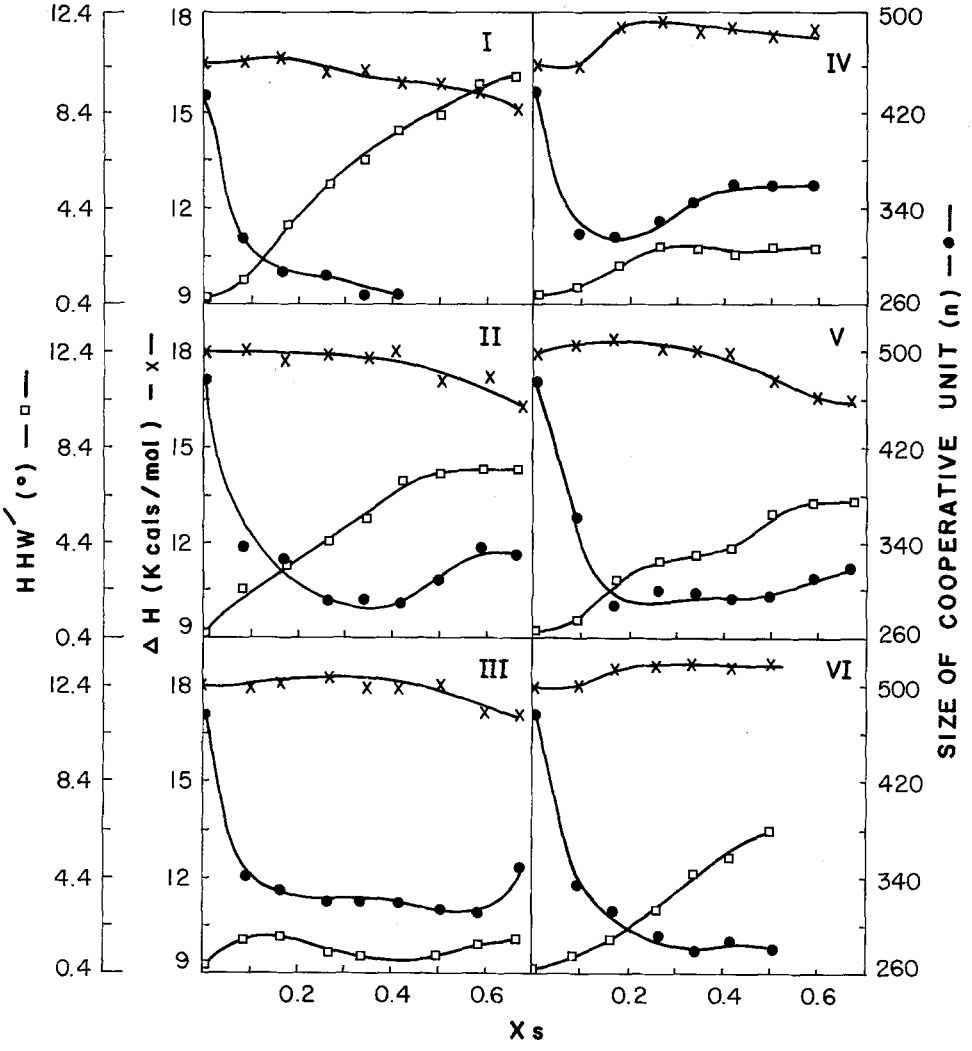

Fig. 9. Effect of varying mole fraction (X_s) of solutes I–VI on the phase transition parameters of DPPC bilayers: enthalpy of transition, ΔH (×), size of cooperative unit, n (–●– for the main peak), shift in half height width, HHW' (–□–).

because relative contributions of the various phases present in the bilayer are difficult to discern. A change in T_m as a function of X_s would be expected if the solute modified bilayer exhibited an ideal colligative behavior. Although T_m changes as a function of X_s, both the direction and magnitude of such a change is different for different solutes; for solutes like Δ^9-*trans*-tetradecanol (III) T_m changes very little as a function of X_s. HHW' is a composite parameter whose magnitude and direction changes as a function of X_s. All the solutes examined in this paper show a change in HHW'. Unfortunately, it is difficult to attribute a molecular significance for this parameter.

Discussion

The observations presented in this paper show that the hydrolysis of phosphatidylcholine in a bilayer is facilitated by the presence of linear and branch chain alkanols in the bilayer. Although it is possible that the alcohols also intereact with the enzyme, the experiments presented here are designed to understand the role of alkanols in bilayer during the action of bee venom phospholipase A_2. The effect of *n*-alkanols on the various properties of phospholipid bilayer [3, 9, 20, 23 and references therein] suggest that: they are incorporated into lipid bilayer; their partition coefficient increases with the chain length; they modify the packing of phospholipid in bilayer such that the lipid molecules have greater degrees of freedom; their partition coefficient does not change significantly as a function of alkanol concentration. The sedimentation behavior, X-ray diffraction pattern, thermotropic phase transition, and permeability of water suggest that the MLV modified by alkanols retain the multilamellar bilayer structure intact, and that the phospholipid from the bilayer is not "solubilized" into the alkanol containing aqueous phase. Such observations rule out the possibility that the

higher alkanols ($\geqq C_5$) increase the surface area of the substrate available to the enzyme in the aqueous phase. This is further substantiated by the fact that the rates comparable to those of MLV modified by alkanols cannot be obtained even if the unmodified MLV concentration is raised several hundredfold. These and other observations are best interpreted to suggest that the alkanols modify the packing of phospholipids (fluidity or mobility) in bilayer. A difference in the effect of different alkanols is generally accounted for by the assumptions that the partition coefficient of alkanols increases with chain length and that an equal number of solute molecules in the bilayer induce an equal response. In this paper, we have further tested these assumptions. The data presented in Table 1 shows that the alcohol to lipid mole (D/L) ratios at the peak activating concentrations are different for the various alcohols. This means that different alcohols perturb the bilayer to different extents or in qualitatively different modes. Since the behavior of all the alcohols ($< C_{10}$) examined here is qualitatively similar, we have assumed that the activating effect of alcohols on the phospholipase A_2 catalyzed hydrolysis is due to the free space they introduce in the bilayer.

If the penetration of the enzyme in the bilayer is a limiting step in its turnover [21, 24], the factors related to the lateral compressibility and the free space in the bilayer should play an important role in determining the amount of the enzyme bound to the bilayer. For example, the free space introduced by an alkanol in the bilayer could make more sites available for the incorporation of phospholipase A_2. Thus, the D/L ratio is an indirect measure of the free space introduced by the alkanol: a larger D/L ratio implies a smaller free space introduced by an alkanol. Similarly, an increase in V_m with the free space would mean a larger turnover of the enzyme or a greater proportion of the enzyme in the bilayer. If one assumes that optimal penetration of the enzyme in the bilayer is required for optimal catalytic activity, a biphasic dependence of rate upon the alkanol concentration would be predicted. Lower rates would arise from an inadequate penetration or sub-optimal residence times of the enzyme in the bilayer at low alkanol concentration. An increased penetration of the enzyme in the bilayer containing a greater than peak activating concentration of the alcohol could result in an increased residence time for the enzyme in the bilayer which would effectively reduce the turnover number and, therefore, the rate of hydrolysis.

When considering the rotation about C–C bonds in alkyl chains, it is necessary to take into account the steric interactions between the adjacent chains. Calculations show that the steric hindrance to a rotation about C–C bonds will vary throughout the length of an alkyl chain [18]. Probability of an out-of-*trans* or a gauche conformation and the amplitude of oscillation away from the *trans* position is expected to increase with the chain length and temperature. This means that the cylinder of rotation of medium length alkyl chain would have larger cross-sectional area than that of a small chain. For still larger chains, the steric interactions can be reduced by correlating the motion of either the neighboring chains or that within a single chain. The later possibility would give rise to a correlation of two gauche conformations to give a kink [22] which would not drastically disrupt the regular packing of the neighboring chains. This model would predict that the bilayer perturbing ability would increase with the chain length till the kink formation would fold back the oscillation of the alkyl chain away from the close-packed *trans*-conformation. The free energy difference between *trans*- and gauche conformations is about 500 cal/mol [15], i.e., at 37 °C the ratio of *trans*- to gauche methylene conformers is about 4:1. Thus on the average, C_5–C_9 alkanols would have at least one gauche conformation. By assuming that the gauche conformation occurs at the same methylene residue one can calculate the cross-sectional area and the asymmetry of an alkyl chain as a function of its length. For the calculation of molecular cross-sections, we have localized the gauche conformation on the C_4–C_5 bond of the alkanols. Statistically, this must be the average position. A gauche conformation away or towards the hydroxyl group would lead to a large or smaller cross-sectional area which would average out to a value corresponding to the localization of the bend near the center of the chain. With these assumptions, using molecular models we calculated the cross-sectional areas and the axial ratios (a/b). The best fitting data was obtained by placing the off-*trans* conformation at the C_4–C_5 bond in an alkanol. Thus *n*-butanol has little or not out-of-*trans*-rotation; therefore, its ability to perturb the bilayer would be minimum. Indeed, the peak rates (directly proportional to V_m) at optimal *n*-butanol concentrations are 4.5 nmol/min compared to 29.5 nmol/min for pentanol. Similarly, the D/L ratio for *n*-butanol is highest. Moreover, a bent chain arising from a gauche conformation would exhibit a significant even-odd alteration; that is each additional odd methylene imparts a greater increase in the cross-section than an even methylene residue as the chain-length is increased. The V_m values presented in Table 1 do suggest such a trend.

As the chain length increases the probability of two gauche conformers on the same alkyl chain also increases. Two such off-*trans* conformations, when correlated on the neighboring carbon atoms would

form a kink, thus effectively reducing both the cross-sectional area and the asymmetry of the alkanol. This means that the bilayer perturbing ability of alkanols would show a "cut-off" when the probability of correlating two off-*trans* conformations into a kink is finite. Typically, this would be observed at nonanol or decanol. This is indeed the case. The peak activating concentration of decanol is much higher than that predicted on the basis of its partition coefficient, that is, D/L ratio is higher than that for octanol. Not only for the bilayer systems described here but also for several other unrelated bilayer systems such a cut-off at C_{10}–C_{12} has been observed [11]. It must be emphasized that the exact value of the free energy difference between *trans*-gauche conformations and the correlation of the off-*trans* conformation to form a 2gl kink may depend upon the mobility of the neighboring alkyl chains. In any event, such a cut-off effect would not be very sharp, since the asymmetry of the molecule would depend upon the distance between the two gauche conformers.

The effect of tetradecane derivatives is particularly interesting. While all of these solutes (I–VII) perturb the bilayer, their bilayer perturbing ability is more or less related to the asymmetry of their cross section. These results are more or less parallel to those reported in literature for these solutes [13, 16]. The results presented here demonstrate that the rate of phospholipase catalyzed hydrolysis at DPPC in liposomes is indeed activated in the presence of the compounds containing a polar group attached to the tetradecane chain. However, the shape of the activation profile depends upon both the nature of the activating alcohol and the source of the enzyme. This observation suggests that not only the bilayer perturbing ability of the various compounds is different, but the perturbation caused by a given agent may be experienced in a qualitatively different fashion by the two different enzymes.

The factors governing the action of phospholipase A_2 on phospholipid bilayer interface are not known. To a first approximation, most of the solutes that increase the rate of hydrolysis indeed increase the partition coefficient of the enzyme in the bilayer. However, other factors governing the catalytic efficiency of the bilayer bound enzyme must be invoked to account for the differences in the behavior of the two enzymes. The data presented in this paper suggests that there are no obvious quantitative relationships between phospholipase activity and the phase transition parameters, such as the relative proportion of the various phases, the size of the cooperative unit, T_m, and possibly the regions of mismatch between the gel and the liquid crystalline phases. Verger and DeHaas [24] have suggested that the pig pancreatic phospholipase A_2 is incorporated in the bilayer, and the membrane bound enzyme "scoots" from one substrate to the other in the plane of the bilayer. It appears that the bee venom phospholipase A_2 which does not show a lag phase, "hops" from the aqueous phase to the membrane phase and back to the aqueous phase during its turnover cycle analogous to the Path 1 proposed by Tinker and Wei [21]. If the constraints of molecular motion and bilayer organization for the scooting and hopping are different, these two proteins will behave differently. Even though we do not yet understand these constraints, the bilayer-solute-enzyme system could serve a useful model to characterize such constraints. For example, Pringle and Miller [16] have reported that II and III are equally effective anesthetics, whereas their ability to perturb the bilayer is quite different. The existing theories of anesthesia [17] would predict that since II is a better perturbing agent for bilayer it should be a more effective anesthetic. The behavior of the two proteins reported in this paper suggests that the effect of solute on the function of a membrane protein may be quite different than its effect on other proteins, and the effect would depend upon constraints that are unique to a given protein. By analogy, it may be implicated that the sodium channel, which is interrupted by anesthetics from opening, may involve lateral movement of the component subunits. Indeed, Hodgkin and Huxley [4] did suggest that movement of charged particles is responsible for opening and closing of the sodium channel.

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