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^{31}P- and ^{1}H-NMR investigations of the effect of *n*-alcohols on the hydrolysis by phospholipase A_2 of phospholipid vesicular membranes

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^{31}P- and ^{1}H-NMR spectroscopy of small, unilamellar egg yolk phosphatidylcholine (PC) vesicles in the presence of the lanthanide ion Dy^{3+} have been used to study the effect of various *n*-alcohols on the permeability induced by the action of the enzyme phospholipase A_2 (PLA_2). The method allows the monitoring of the number of PC and lysoPC molecules in the outer and inner monolayers. The results indicate that the initial rate of hydrolysis of PC by PLA_2 is increased by all the *n*-alcohols but in a chain-length dependent manner and that the maximum rate occurs at $n = 8$ (octan-1-ol). The subsequent rate is dependent upon the rate of transbilayer lipid exchange (flip-flop) of PC molecules from the inner to the outer monolayer. The vesicles only become permeable to the Dy^{3+} ions when lysoPC is mobilised in the flip-flop process of exchange of lipid molecules between the two monolayers. The *n*-alcohols affect both the time taken to initiate flip-flop of inner monolayer PC and the subsequent rate of permeability to Dy^{3+}. The *n*-alcohols are seen to affect all the above rates in an identical chain-length dependent manner, indicating a common cause for all observations which we identify as the degree of clustering of the *n*-alcohol molecules in the bilayer. The results are discussed in terms of the chain-length dependent mechanism of *n*-alcohol interactions with the membrane and the mechanism by which the vesicles become permeable to Dy^{3+} ions.

Introduction

Phospholipase A_2 (PLA_2) is a small ($M_r = 14000$), water-soluble enzyme which catalyses the specific hydrolysis of the *sn*-2 fatty acid ester of phosphatidylcholine to form lysophosphatidylcholine and fatty acid [1]. It is a calcium requiring enzyme present in the pancreas and in the venom of snakes and insects [2].

Even though extensive research work on the PLA_2 induced hydrolysis of phospholipids have been published [3–8], a detailed mechanism of the reaction at a molecular level is still not understood. The action of PLA_2 on phospholipids has been shown to be governed by the quality of the interface to which the enzyme is exposed [9] and it has been proposed that PLA_2 binding occurs at organisational defect sites in the interface [10]. These defects can be introduced into membrane bilayers by *n*-alcohols and several publications on the effect of various *n*-alcohols on PLA_2 induced hydrolysis of various lipid aggregates by PLA_2 have appeared [8,10,11].

Previous work has suggested that only limited hydrolysis occurs in small, unilamellar phospholipid vesicles where the action of the enzyme was seen to be restricted to the phospholipid molecules present in the outer monolayer only [12,13]. However, we have recently reported extensive hydrolysis of PC by PLA_2 with rapid transmembrane lipid exchange occurring, thus making inner monolayer PC available for hydrolysis [14,15]. We have also previously described the effect of the *n*-alcohols on various mechanism of vesicular permeability [16,17]. Here we describe the use of ^{31}P- and ^{1}H-NMR studies in conjunction with the lanthanide shift reagent Dy^{3+}, to deduce the quantitative extent of phospholipid hydrolysis and subsequent changes in vesicular structure in small, unilamellar egg phosphatidylcholine (PC) vesicles by monitoring the numbers of PC and lysoPC molecules present in each monolayer. The effect of various *n*-alcohols on PLA_2 was studied in an attempt to elucidate possible *n*-alcohol–enzyme interactions and the effect of the *n*-alcohols on the dy-

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namics of the flip-flop processes set up by the enzyme activity.

Materials and Methods

Egg phosphatidylcholine (egg PC), snake venom (*Naja naja*) phospholipase A_2 (PLA_2) and all the *n*-alcohols (spectroscopic grade where available) were purchased from Sigma, Poole, Dorset. Deuterium oxide (99.8% Gold Label) and dysprosium chloride (Gold Label) were purchased from Aldrich, Gillingham, Dorset. AnalaR calcium chloride was obtained from BDH Chemicals, Poole, Dorset. Stock solutions of alcohols where $n > 12$ were prepared in chloroform/methanol. The AnalaR chloroform used was purified by passing over alumina to remove ethanol and water, distilled and then stabilized by the addition of 1% v/v AnalaR methanol.

The small, unilamellar phospholipid vesicles were prepared by sonication of lipid in ^2H_2O as previously described [18] except that since egg PC contains unsaturated acyl chains, precautions were taken against oxidation by sonicating the egg PC under N_2 and at 4° C.

The PC dispersions used contained 30 mg lipid/ml D_2O for the ^{31}P-NMR experiments and 10 mg lipid/ml D_2O for the ^{1}H-NMR studies. ^{31}P- and ^{1}H-NMR spectra of the vesicles were obtained at 37° C using a Jeol FX90Q NMR spectrometer operating at 36.23 MHz for ^{31}P-NMR and 90 MHz for ^{1}H-NMR, respectively.

^{31}P-NMR spectra were accumulated using a total of 400 transients, employing a 20 μs, 45° pulse, an interpulse time of 2 s and a 9057 Hz sweep width with 8K data points to digitise the spectra. All ^{31}P-NMR spectra were accumulated in the presence of proton decoupling. For ^{1}H-NMR spectra, 40 pulse sequences were used (π-τ-$\pi/2$) with a pulse interval of 1 s to minimise the ^{2}HO-^{1}H signal.

The 10-mM NMR tubes contained 1 ml of vesicular dispersion confined by a vortex plug and capped. The desired concentrations of Dy^{3+} and Ca^{2+} in the extravesicular solution were obtained by adding known volumes from stock solutions of $DyCl_3$ and $CaCl_2$ in ^2H_2O to the vesicle dispersions in the NMR tube. A known volume of *n*-alcohol was introduced by pipetting into 1 ml of vesicle dispersion to give the appropriate concentration. Incorporation of alcohols which are solid at room temperature was achieved by making a stock solution in chloroform, pipetting the appropriate volume into the NMR tube and removing the solvent followed by the addition of 1 ml of vesicular dispersion. The required number of units of PLA_2 were introduced by pipetting small volumes of a stock solutions of the enzyme in ^2H_2O directly into the vesicle dispersions. In the experiments using ^{31}P-NMR, the vesicular dispersions contained 6 mM Ca^{2+}, 0.3 mM Dy^{3+} and 90 units PLA_2. For the ^{1}H-NMR studies, 2 mM Ca^{2+}, 0.15 mM Dy^{3+} and 30 units PLA_2 were used. The samples were incubated at 37° C for 30 min prior to the addition of the enzyme.

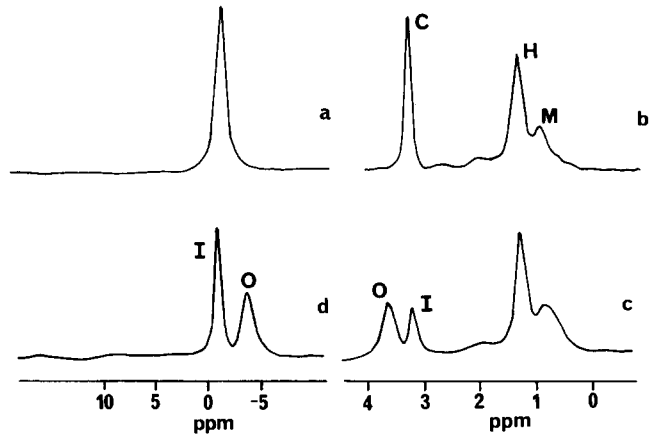

Fig. 1. NMR spectra of egg PC vesicles (30 mg lipid/ml) with extravesicular Ca^{2+} (6 mM) at 37° C. (a) ^{31}P-NMR spectrum. The peak arises from overlapping signals originating from the outer and inner monolayer phosphate moieties. (b) ^{1}H-NMR spectrum. Signals originate from the outer and inner choline headgroups (C), and methylenes (H) and terminal methyl groups (M) of the lipid acyl chains (c) and (b) but with the addition of extravesicular 0.3 mM Dy^{3+}. Signals originate from the outer (O) and inner (I) choline methyl groups. (d) and (a) but with the addition of extravesicular 0.3 mM Dy^{3+}. Signals originate from the phosphate moieties in the outer (O) and inner (I) monolayers. Shifts in the ^{31}P-NMR spectra are shown with respect to external phosphoric acid. Shifts in ^{1}H-NMR spectra are shown with respect to external TMS.

The ^{31}P-NMR spectrum of egg PC vesicles (30 mg lipid/ml) in the presence of 6 mM Ca^{2+} at 37° C shows a high resolution signal originating from the phosphate groups in the outer and inner monolayers as shown in Fig. 1(a). The corresponding ^{1}H-NMR spectrum from the same sample (Fig. 1(b)) includes high resolution signals from the terminal methyls (M) and methylenes (H) in the hydrocarbon chains and overlapping signals from the choline groups (C) in the outer and inner monolayers.

On adjusting the extravesicular concentration to 0.3 mM Dy^{3+}, the spectrum shown in Fig. 1(c) is obtained by ^{1}H-NMR and in Fig. 1(d) by ^{31}P-NMR. This shows separate signals originating from the extravesicular choline headgroups (O), and intravesicular choline headgroups (I). Such contact and pseudo-contact shifts are now well documented [19,20]. The mean ratio of the areas O : I = 1.7 obtained by integrating the two peaks in the ^{1}H-NMR spectrum indicates that vesicles of average diameter of 34 nm have been formed [21]. The O : I ratio of 1.72 obtained from the ^{31}P-NMR spectrum in Fig. 1(d) suggests that Nuclear Overhauser enhancement (NOE) has little effect on the ratio of the signal areas O and I.

When Dy^{3+} ions are transported across the lipid bilayer into the intravesicular solution the rise in in-

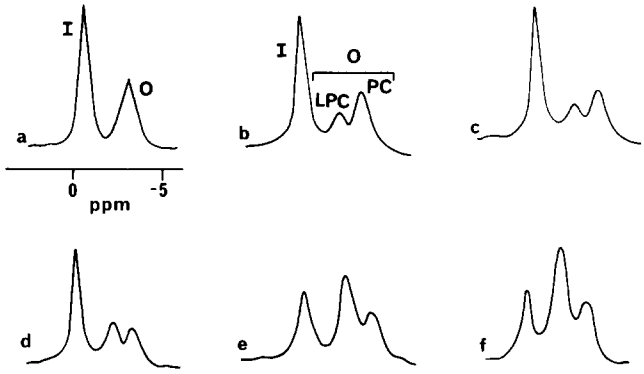

Fig. 2. ^{31}P-NMR spectra of egg PC vesicles (30 mg lipid/ml) at 37 °C in the presence of an extravesicular concentration of 6 mM Ca^{2+} and 0.3 mM Dy^{3+}. (a) Initial spectrum before addition of enzyme showing signals originating from outer (O) and inner (I) monolayer phosphate moieties. (b)–(f) Time-dependent changes in the ^{31}P-NMR signals after addition of 90 units PLA_2 showing the changes in intensity of the inside choline (I), outside PC (O) and outside lysoPC (LPC) signals. Spectra taken at (b) 110 min, (c) 205 min, (d) 390 min, (e) 2060 min and (f) 2800 min.

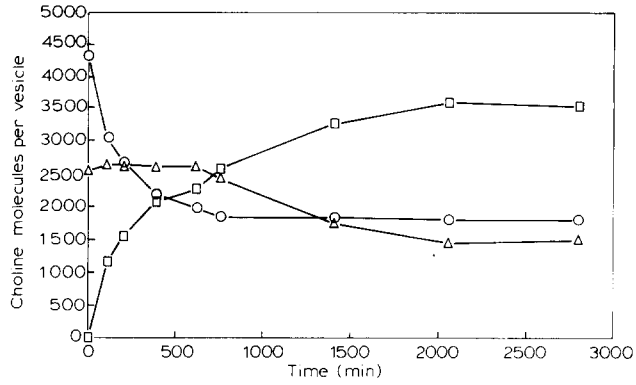

Fig. 3. Plot of the number of lysoPC (outside) (□), inside choline (△) and outside PC (○) molecules against time (min) for egg PC vesicles (30 mg lipid/ml) in the presence of 6 mM Ca^{2+}, 0.3 mM Dy^{3+} and 90 units PLA_2.

travesicular concentration of Dy^{3+} causes signal I to move towards signal O. By measuring the change in chemical shift (Hz) of signal I with time in the ^{1}H-NMR spectrum, the rate of transport of Dy^{3+} ions can be obtained. In order to convert experimentally shifts into an intravesicular concentration of Dy^{3+}, a calibration graph is necessary. This calibration graph (not shown) is obtained by sonicating known concentrations of Dy^{3+} into separate vesicle preparations and the adjusting the extravesicular concentration to 0.15 mM Dy^{3+}. At each different intravesicular concentration of Dy^{3+} ions the shift of signal I is measured [14,15].

Phospholipase A_2 has an absolute requirement for Ca^{2+} for enzymatic activity. The use of the relatively low concentrations of Dy^{3+} (0.15 and 0.3 mM) at the concentrations of lipid and enzyme used in these experiments does not result in significant competition for the Ca^{2+} binding sites on the enzyme [14,15].

Results

^{31}P-NMR studies

Figs. 2(a–f) show the time-dependent changes in the ^{31}P-NMR spectrum of the outside (O), inside (I) and lysoPC signals of egg PC vesicles (30 mg/ml) in the presence of 0.3 mM Dy^{3+}, 6 mM Ca^{2+} and 90 units of PLA_2. The observed lysoPC signal arises from lysoPC molecules on the outside monolayer only. Any lysoPC molecules on the inside monolayer will appear under the inside choline signal (since inside PC and inside lysoPC are unresolvable in the 36.23 MHz ^{31}P-NMR spectrum).

Fig. 2(a) shows the initial ^{31}P-NMR spectrum before the addition of the enzyme. Fig. 2(b) shows that 110 min after the addition of PLA_2, a signal due to the production of lysoPC can be observed 1.5 ppm downfield from the outside PC signal. Figs. 2(c–f) show further changes in the intensities of the inside choline, outside PC and lysoPC (outside) signals. The relative number of PC molecules present in the outer and inner monolayers of each vesicle can be readily calculated and by taking the ratio of lysoPC (outside) to outside PC and the ratio of inside choline to outside PC, the respective number of PC (outside), choline (inside) and lysoPC (outside) molecules in the vesicles can be calculated at various time intervals.

Fig. 3 is a plot of the number of outside PC, inside choline and lysoPC (outside) molecules present at various times calculated from ^{31}P-NMR spectra. Corresponding ^{1}H-NMR spectra were also taken to follow any changes in the O:I ratio. Table I presents the summarised data obtained as in Fig. 3 but for experiments done in the presence of the *n*-alcohols, $n = 3$, 6, 8, 10 and 14.

Fig. 4 is a collective plot of the number of lysoPC (O) molecules produced in the presence of various *n*-alcohols at a 5 mM concentration and this graph can be used to represent the rates of hydrolysis occurring at the various stages of enzymes activity and subsequent lipid exchange (as discussed below).

TABLE I

Effect of n-alcohols on (a) the rate of hydrolysis of outer monolayer PC, and (b) the rate of flip-flop of inner monolayer PC

	Control	*n*-Alcohols				
		3	6	8	10	14
(a) Hydrolysis (min) [a]	450	600	125	116	222	440
(b) Flip-flop (min) [b]	1167	1133	425	228	660	1250

[a] As measured by the time (min) for half conversion of outer monolayer PC into lysoPC.

[b] As measured by the time (min) required for the removal of 500 molecules (2500 to 2000) of PC from the inner monolayer.

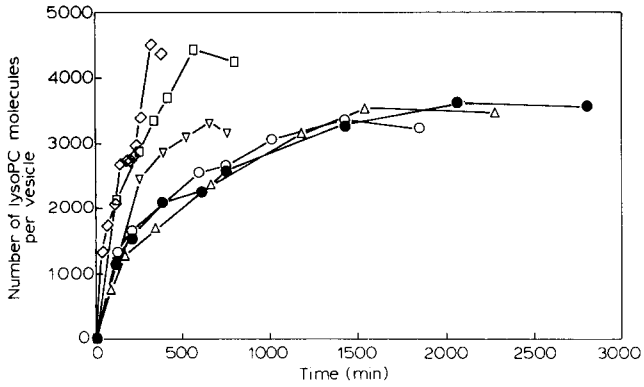

Fig. 4. Plot of the number of lysoPC(O) molecules produced for egg PC vesicles (30 mg lipid/ml) in the presence of 6 mM Ca^{2+}, 0.3 mM Dy^{3+}, 90 units of PLA_2 and various n-alcohols at a 5 mM concentration. (a) control (●), (b) propan-1-ol (△), (c) hexan-1-ol (□), (d) octan-1-ol (◇), (e) decan-1-ol (▽) and (f) tetradecan-1-ol (○).

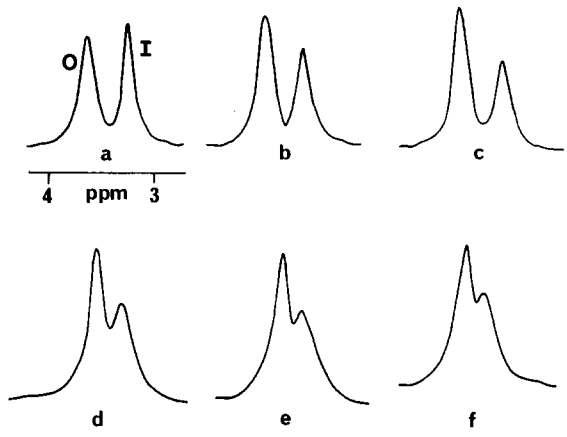

Fig. 5. (a–f) ^{1}H-NMR spectra of the headgroup region of egg PC vesicles (10 mg lipid/ml) run at 37 °C in the presence of 0.15 mM Dy^{3+}, 2 mM Ca^{2+} and 30 units PLA_2 taken at (a) 0 min, (b) 420 min, (c) 1660 min, (d) 2630 min, (e) 3150 min and (f) 3780 min. Spectra (a–c) show O : I ratio increases but no shift in the I signal. (d–f) show a downfield shift of signal I towards signal O resulting from the transport of the Dy^{3+} from the outer to the inner vesicle environment.

^{1}H-NMR studies

Figs. 5(a–f) show the time-dependent changes in the outer (O) and inner (I) headgroup region of the ^{1}H-NMR spectrum of egg PC vesicles (10 mg/ml) in the presence of 0.15 mM Dy^{3+}, 2 mM Ca^{2+} and 30 units of PLA_2. Figs. 5(a–c) show a gradual increase in the O : I ratio which continues until a ratio of 2.2 is obtained. There is an initial time period (approx. 400 min) where the O : I ratio remains constant. During the O : I ratio increase period, the I signal remains unshifted indicating that no Dy^{3+} ions have permeated into the intravesicular space. However, Figs. 5(d–f) show that after the O : I ratio has risen to 2.2 the *I* signal begins to shift downfield towards the O signal. This shift corresponds to increasing intravesicular concentrations of Dy^{3+} ions due to a permeability in the vesicular membrane.

The effect of a series of *n*-alcohols (C_3–C_{14}) at a 5 mM concentration on PLA_2 hydrolysis is shown in Figs. 6(a) and (b). The initial lack of transport corresponds to the period during which the O : I ratio is increasing. All the *n*-alcohols shows a chain-length dependent effect on (a) the rate of the O : I ratio increase and (b) the rate of Dy^{3+} permeability.

Discussion

From Fig. 3 it can be seen that the initial rate of lysoPC production is a mirror image of the rate of hydrolysis of outside PC. This explains the constant O : I choline ratio seen from the ^{1}H-NMR spectra in the initial part of the experiment. The number of inside PC molecules remain constant over this period. When a certain number of PC molecules remain in the outer

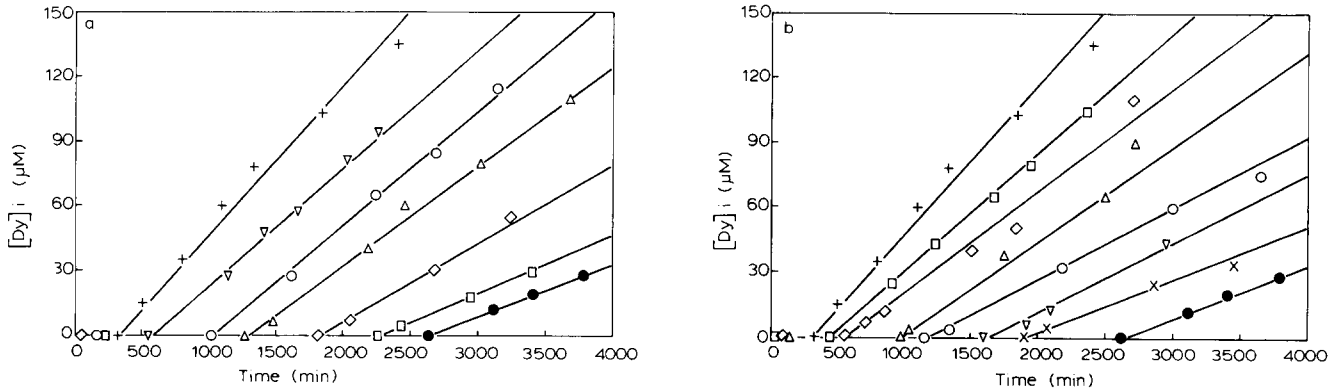

Fig. 6. Increase in the intravesicular concentration of Dy^{3+}, $[Dy]_i$, as a function of time in egg PC vesicles (10 mg lipid/ml) containing extravesicular 0.15 mM Dy^{3+}, 2 mM Ca^{2+}, 30 units PLA_2 and various *n*-alcohols at a 5 mM concentration. (a) *n*-alcohols from $n = 3$ to 8. Control (●), propan-1-ol (□), butan-1-ol (◇), pentan-1-ol (△), hexan-1-ol (○), heptan-1-ol (▽) and octan-1-ol (+). (b) *n*-Alcohols from $n = 9$ to 14. Control (●), octan-1-ol (+), nonan-1-ol (□), decan-1-ol (◇), undecan-1-ol (△), dodecan-1-ol (○), tridecan-1-ol (▽) and tetradecan-1-ol (×). The initial stages where no increase in $[Dy]_i$ is observed corresponds to the period where the O : I ratio is increasing.

monolayer (approx. 2000), a decrease in the number of inside PC molecules is observed and the rate of this decrease equals the rate of lysoPC production. This explains the observed increase in the O : I ratio in the ^{1}H-NMR spectra.

The ^{1}H-NMR observations in which the O : I ratio increases from 1.7 to 2.2 (Fig. 5) and the results illustrated in Fig. 3 show that there is a net transfer of choline-containing lipid from the inner to the outer monolayer. These results are in agreement with our previous studies of PLA_2 [14,15]. A mechanism for transmembrane lipid exchange has been proposed by Cullis and De Kruijff [22] who showed that the existence of inverted micelles within a bilayer structure allowed a rapid flip-flop movement across the membrane. The aqueous compartment within the inverted micelles provides a mechanism of transmembrane transport [23] but the permeability will depend upon the composition of the micelles and the size of the aqueous compartment [14]. When the number of PC molecules in the outer monolayer has decreased to approx. 2000, flip-flop of inside PC molecules with fatty acid from the outer monolayer to the inner occurs. These inverted micelles of fatty acid and PC may have too small an inner, aqueous compartment to allow the transport of Dy^{3+} ions across the bilayer. But when sufficient PC has been hydrolysed, flip-flop of lysoPC to the inner monolayer in substitution for PC, produces a larger inverted micelle capacity due to the bigger headgroups of the lysoPC/PC molecules and so allow ion transport which is seen in the ^{1}H- and ^{31}P-NMR spectra as a shift of the I signal towards the O signal.

Table I shows the effect of various *n*-alcohols at a 5-mM concentration on the processes that are occurring. All the alcohols effect the rate of lysoPC production. This can be attributed to the free space introduced into the bilayer by the presence of the various *n*-alcohols [10] and by the interaction energy that the *n*-alcohol molecules have with the lipid molecules in the bilayer [24].

It can be seen from Table I that the rate of lysoPC production is dependent upon the chain-length of the *n*-alcohols present. Fig. 4 is a collective plot of the number of lysoPC(O) molecules produced in each experiment and this graph represents the various rates of hydrolysis of egg PC vesicles by PLA_2 in the presence of a number of *n*-alcohols at a 5 mM concentration. The presence of propan-1-ol does not effect the rate of lysoPC production compared to the control (Table I). However, hexan-1-ol and octan-1-ol both greatly increase the rate of hydrolysis. Decan-1-ol increases the rate to a lesser extent and tetradecan-1-ol shows a similar effect to propan-1-ol (i.e., the rate is not altered compared to the control), i.e., maximal stimulation occurs at $n = 6$ to 8.

The idea that the quality of the bilayer surface influences the binding of PLA_2 has been widely published [10]. Brasseur and co-workers have calculated that for $n = 2$ to 4 the alcohols interact only with the lipid polar headgroups and therefore propan-1-ol causes little bilayer defects and the rat of hydrolysis is not greatly affected.. However, alcohol-alcohol clustering occurs when $n = 5$ to 8 [24]. The presence of octan-1-ol clusters in the membrane should lead to large structural defects in the bilayer and affect the quality of the interface. This will allow easier penetration of the PLA_2 and therefore result in an increase in the rate of hydrolysis [10]. As the alcohol chain-length increases, a more ordered lipid organization is obtained [24,25]. Penetration of PLA_2 into the bilayer will become less accessible as the chain-length of *n*-alcohol increases thereby resulting in a gradual decrease in the rate of hydrolysis.

The incorporation of *n*-alcohols in the phospholipid bilayers has been shown to accelerate the transmembrane lipid exchange of phospholipids and lysophospholipids [26]. Our results indicate that this effect is also very chain-length dependent. From Table I it can be seen that the rate of lysoPC production which is governed by the rate of transmembrane lipid exchange of inside PC, is not greatly affected by propan-1-ol compared to the control. However, hexan-1-ol and octan-1-ol greatly increase the rate of inside PC flip-flop and the hence the rate of hydrolysis. Decan-1-ol shows a lesser effect than both hexan-1-old and octan-1-ol. Tetradecan-1-ol does not significantly alter the rate of flip-flop compared to the control.

Following again the Brasseur model, the alcohols where $n = 2$ top 4 interact only with the polar headgroups of the lipid molecules [24] and therefore do not affect the packing of the bilayer structure. Transmembrane movement therefore, should not be significantly affected. However, as *n*-alcohol clustering occurs ($n = 5$ to 8), the packing the the bilayer will be greatly affected and the rate of flip-flop increased. As the chain-length of the alcohols further increase ($n > 9$), the order of the bilayer increases and results in a gradual decrease in the rate of flip-flop.

From Figs. 6 (a) and (b) it can be seen that the *n*-alcohols affect both (a) the rate of the O : I ratio increase to 2.2 and (b) the rate of Dy^{3+} transport. As the chain-length of the *n*-alcohols from propan-1-ol ($n = 3$) to octan-1-ol ($n = 8$) increases, the time taken for the O : I ratio to reach to 2.2 is shortened and the rate of Dy^{3+} permeability is increased (Fig. 6(a)). The *n*-alcohols from nonan-1-ol ($n = 9$) to tetradecan-1-ol ($n = 14$) exhibit reverse trends (Fig. 6(b)). These effects are seen, therefore, to be very dependent on the *n*-alcohol chain-length and both these (a) and (b) effects vary in exactly the same chain-length dependent order and for this to be the case there is very probably a single cause. This is again likely to be due to the chain-length tendency for the alcohols to cluster in the

bilayer. We have already discussed the effect of the n-alcohols on the rate of flip-flop of lipids. This determines the time before the onset of the O : I ratio change. The rate of Dy^{3+} permeability shown by the slopes of the lines in Figs. 6 (a) and (b) will depend on the rate of production of lysoPC-containing inverted micelles. This rate of formation of micelles will also depend on the effect of the *n*-alcohols on the degree of lipid–lipid interaction. Clearly this interaction is dependent on the extent to which the alcohols interact with the lipid which as discussed before is chain-length dependent and related to the tendency to cluster. When the *n*-alcohols strongly cluster ($n = 6$ to 8) they will have maximum disruptive effect on the packing of lipid so increasing the tendency to flip-flop and hence increase the permeability of Dy^{3+}.

It can be seen from the above discussion that this unitary hypothesis of the alcohol–alcohol and alcohol–lipid interactions adequately explains the effects that are seen on the rate of lysoPC production and on the rates of Dy^{3+} permeability observed. These interactions can also be used to explain other mechanisms of vesicular membrane permeability [16,17]. The combined use of ^{31}P- and ^{1}H-NMR studies therefore appears to have given an excellent insight into the mechanisms of vesicular permeability induced by PLA_2 and the effect of the *n*-alcohols on this enzymatic hydrolysis of small, unilamellar phospholipid vesicles.

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