Effects of Alcohol-Induced Lipid Interdigitation on Proton Permeability in L-α-Dipalmitoylphosphatidylcholine Vesicles

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ABSTRACT 6-Carboxyfluorescein was employed to examine the effect of alcohol-induced lipid interdigitation on proton permeability in L-α-dipalmitoylphosphatidylcholine (DPPC) large unilamellar vesicles. Proton permeability was measured by monitoring the decrease of 6-carboxyfluorescein fluorescence after a pH gradient from 3.5 (outside the vesicle) to 8.0 (inside the vesicle) was established. At 20°C and below 1.2 M ethanol, the fluorescence decrease is best described by a single exponential function. Above 1.2 M ethanol, the intensity decrease is better described by a two-exponential decay law. Using the fitted rate constants and the vesicle radii determined from light-scattering measurements, the proton permeability coefficient, *P*, in DPPC vesicles was calculated as a function of ethanol concentration. At 20°C, *P* increases monotonically with increasing ethanol content up to 1.0 M, followed by an abrupt increase at 1.2 M. The vesicle size also exhibits a sudden increase at around 1.2 M ethanol, which has been shown to result from vesicle aggregation rather than vesicle fusion. The abrupt increases in *P* and in vesicle size occur at the concentration region close to the critical ethanol concentration for the formation of the fully interdigitated gel state of DPPC. At 14°C, the abrupt change in *P* shifts to 1.9–2.0 M ethanol, completely in accordance with the ethanol-temperature phase diagram of interdigitated DPPC. Effects of methanol and benzyl alcohol on lipid interdigitation have also been examined. At 20°C, DPPC large unilamellar vesicles exhibit a dramatic change in *P* at 3 M methanol and at 40 mM benzyl alcohol. These concentrations come close to the critical methanol and benzyl alcohol concentrations for the formation of fully interdigitated DPPC structures determined previously by others. It can be concluded that proton permeability increases dramatically as DPPC is transformed from the noninterdigitated gel to the fully interdigitated gel state by high concentrations of alcohol. This marked increase in proton permeability can be attributed to the combined effect of the changes in membrane thickness and surface charge density, due to the ethanol-induced lipid interdigitation. The possible effects of the increased proton permeability caused by ingested ethanol on gastric mucosal membranes are discussed.

INTRODUCTION

The alcohol-induced phase transition from the noninterdigitated to the fully interdigitated gel state in lipid vesicles is well documented (Fig. 1) (reviewed by Slater and Huang, 1988). X-ray diffraction data have demonstrated that L-α-dipalmitoylphosphatidylcholine (DPPC) vesicles are converted to a fully interdigitated structure when the ethanol concentration is elevated to 0.8–1.2 M at 20°C (Simon and McIntosh, 1984). Ethanol-induced lipid interdigitation has also been studied by differential scanning calorimetry (DSC) and fluorescence spectroscopy (Rowe, 1985; Veiro et al., 1987; Nambi et al., 1988; Zeng and Chong, 1991; Yamazaki et al., 1992). The formation of the fully interdigitated phase depends on lipid acyl chain length (Rowe, 1983), temperature (Nambi et al., 1988), and pressure (Zeng and Chong, 1991) and is hindered by the addition of cholesterol (Komatsu and Rowe, 1991). A temperature/ethanol phase diagram for DPPC has been determined (Nambi et al., 1988; Ohki et al., 1990).

Previously, Simon et al. (1986) have discussed the possible correlation between anesthetic potency and lipid interdigitation in the membrane. However, in a recent study, we have demonstrated that pressure facilitates, rather than antagonizes, the effects of ethanol on the formation of the fully interdigitated gel phase of DPPC (Zeng and Chong, 1991). Thus, the well-known antagonism effect between pressure and ethanol-induced anesthesia must not originate from lipid interdigitation. It was then concluded that lipid interdigitation is unlikely to be the main cause of ethanol-induced anesthesia (Zeng and Chong, 1991).

Compared to noninterdigitated lipid bilayers, fully interdigitated structures have a shorter membrane thickness (Simon and McIntosh, 1984), a reduced membrane surface charge density, and a higher molecular order in lipid acyl chains (Wu et al., 1982; Simon and McIntosh, 1984; O'Leary and Levin, 1984; Hui and Huang, 1986; Boggs et al., 1989). Thus, although ethanol-induced anesthesia does not seem to be related to ethanol-induced lipid interdigitation, the structural changes in the bilayer due to lipid interdigitation are substantial and ought to affect membrane properties. Indeed, it has been shown that the ethanol-induced lipid interdigitation causes disappearance of pretransition (Veiro et al.,

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Abbreviations used: DPPC, L-α-dipalmitoylphosphatidylcholine; DSC, differential scanning calorimetry; Prodan, 6-propionyl-2-(dimethylamino)-naphthalene; 6CF, 6-carboxyfluorescein; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; POPC, 1-palmitoyl-2-oleoyl-L-α-phosphatidylcholine; C_r, critical alcohol concentration for the formation of the fully interdigitated gel phase of DPPC.

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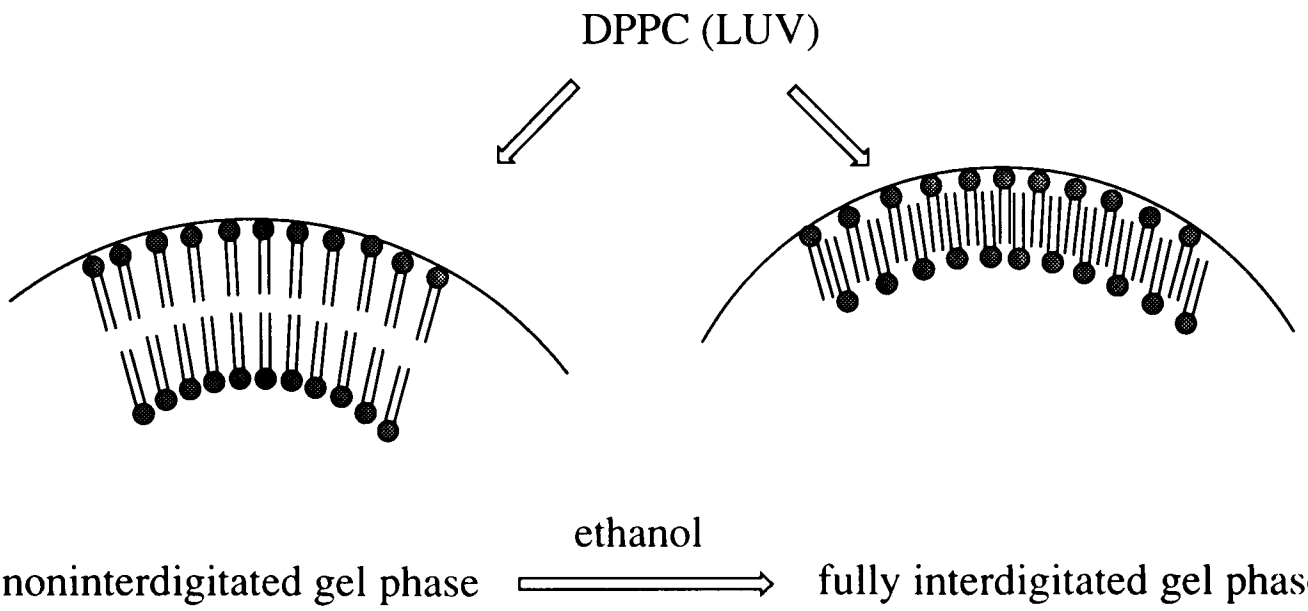

FIGURE 1 Schematic diagram showing the conversion of DPPC from the noninterdigitated gel phase to the fully interdigitated gel phase at [ethanol] $> C_r$. C_r is the critical alcohol concentration for the formation of the fully interdigitated gel phase of DPPC.

1987), an increased mobility in the head group of DPPC (Herold et al., 1987), and a decrease in the membrane solubilities for water (McDaniel et al., 1983) and 6-propionyl-2-(dimethylamino)naphthalene (Prodan) (Zeng and Chong, 1991).

In the present study, we have examined the effect of ethanol-induced lipid interdigitation on proton permeability in DPPC vesicles. Proton flux across the membrane bilayer is a vital part of most bioenergetic systems (Nicholls, 1982). The typical value for the permeability coefficient of protons ranges from 10^{-4} to 10^{-8} cm/s, depending on the degree of saturation in the lipids, the size of the vesicle, and the size of the pH gradient imposed across the membrane (Perkins and Cafiso, 1987). It has been reported that the dielectric constant on the membrane surface is enhanced by ethanol (Rottenberg, 1992) and that an increase in membrane dielectric constant raises ion permeability of the membrane (Hauser et al., 1973; Perkins and Cafiso, 1987). However, these studies were limited to low ethanol concentrations. Little has been done with regard to the proton permeability at ethanol concentrations where lipid membranes are fully interdigitated.

In this study, we have used a pH-sensitive fluorescent probe, 6-carboxyfluorescein (6CF), trapped inside DPPC large unilamellar vesicles (LUVs), to monitor the effect of ethanol-induced lipid interdigitation on proton permeability. This probe and its derivatives have previously been used for the measurement of pH changes inside lipid vesicles and cell membranes (Thomas et al., 1979; Barchfeld and Deamer, 1985; Hays and Alpern, 1990; Cybulska et al., 1992; Graber et al., 1992). The fluorescence intensity of 6CF in its unquenched state is pH dependent, decreasing about 20% per pH unit, over the pH range of 8–5, as the proportion of the ionized species of the 6CF molecules decreases (Szoka et al., 1979). Using this property, we have measured the proton permeability in terms of the pH change inside the DPPC vesicle in the ethanol concentration range of 0–1.8 M. Our data have demonstrated a marked increase in proton permeability through the ethanol-induced phase transition from the noninterdigitated to the fully interdigitated gel state. The possible factors contributing to this phenomenon and its physiological implications have been discussed.

MATERIALS AND METHODS

Materials

DPPC was purchased from Avanti Polar Lipids (Alabaster, AL). 6CF and Prodan were obtained from Molecular Probes (Eugene, OR). Valinomycin was purchased from Sigma (St. Louis, MO). High-performance liquid chromatography-grade organic solvents were used in all experiments.

Liposome preparations and size determination

DPPC dissolved in chloroform was dried under vacuum overnight and then suspended in a buffer solution containing 0.1 M KCl, 0.01 M Tris, and 5 mM 6CF or 2.34 mM Prodan at pH 8.0 to form multilamellar vesicles (MLVs). The dispersions were heated to 65°C for 20 min and then cooled to 4°C for 20 min. This heating/cooling cycle was repeated four more times to enhance the 6CF trapping efficiency (Hope et al., 1986). Large unilamellar vesicles (LUVs) were prepared by using a lipid extruder (Lipex Biomembranes, Vancouver, Canada) according to the method of Hope et al. (1985). Typically, 1.5 ml of 10 mM lipid dispersions were extruded 10 times through two stacked polycarbonate filters with a pore size of 100 nm under nitrogen (200–300 psi) at 60°C. The free 6CF molecules were removed by passing the sample through a 1.4 × 28 cm Sephadex G-50 column equilibrated with a 10 mM Tris buffer at pH 8.0. The column was maintained at 23°C and the flow rate was 0.8 ml/min. The fractions containing DPPC (LUVs) plus the trapped 6CF were collected. The size of the extruded liposomes after gel filtration was determined to be 100 ± 20 nm in diameter by a quasielastic light-scattering methodology using a Coulter N4 MD particle size analyzer (Coulter Electronics, Hialeah, FL). The light source of the

analyzer was a He-Ne laser. Detection was at an angle of 90° with respect to the excitation. Phospholipid concentration was determined as inorganic phosphate by the method of Bartlett (1959).

Measurements of 6CF fluorescence

Aliquots of diluted alcohols (<2.5 M) were added to the 6CF-trapped DPPC vesicles. The mixture was incubated at the desired temperature for 1 h before establishing a pH gradient across the bilayer. Diluted alcohols were used to minimize the disruption of liposomes due to the exposure to high local alcohol concentrations. A pH gradient from 8 (interior) to 3.5 (exterior) was established by adding 0.1 N HCl. The fluorescence intensity of 6CF was monitored as a function of time on a SLM DMX-1000 fluorometer (Urbana, IL). For the measurement of 6CF intensity in the liquid crystalline phase of 1-palmitoyl-2-oleoyl-L-phosphatidylcholine (POPC), a smaller pH gradient (8.0 to 5.5) was used to avoid the problem of the sharp intensity drop. The fluorescence intensity was measured at 525 nm with an excitation at 489 nm. The lipid concentration was 0.8 mM for all of the samples examined.

Measurements of Prodan fluorescence

Emission spectra of Prodan fluorescence were measured on a SLM DMX-1000 fluorometer (Urbana, IL) at 20°C and were corrected for instrument response. The excitation wavelength was 359 nm, and the bandpass was 4 nm for both excitation and emission monochromators.

DSC measurements

Calorimetric measurements were made with a Hart Scientific differential scanning calorimeter (Provo, UT). The samples were loaded at room temperature and held at room temperature for 30 min before a heating scan was initiated. The scan rate was 15°C/h.

RESULTS

Control experiments

Ethanol-induced lipid interdigitation is well documented for DPPC (MLVs) but not for DPPC (LUVs). Since LUVs were used in the present study, it is necessary to demonstrate that ethanol can induce lipid interdigitation in DPPC (LUVs). In a previous study, we have shown that an abrupt decrease in the ratio of Prodan fluorescence intensity at 435 nm to that at 510 nm, F435/F510, indicates the formation of ethanol-induced, fully interdigitated structure (Zeng and Chong, 1991). Fig. 2 *A* shows that such an abrupt decrease in F435/F510 appears at 0.8–1.2 M ethanol for both DPPC (MLVs) and DPPC (LUVs). This concentration region has been shown previously by x-ray diffraction to be the critical ethanol concentration for the formation of the fully interdigitated DPPC structure at 20°C (Simon and McIntosh, 1984). Thus, the data in Fig. 2 *A* can be taken to indicate that ethanol-induced lipid interdigitation not only takes place in large unilamellar vesicles but also occurs at essentially the same ethanol concentration as that for multilamellar vesicles.

The above notion is further supported by the DSC results (Fig. 2 *B*). Using DSC, Rowe (1983) previously showed that ethanol has a biphasic effect on the main phase transition temperature T_m of DPPC (MLVs). The biphasic effect was attributed to the ethanol-induced phase transition from the noninterdigitated gel to the fully interdigitated gel state (Simon and McIntosh, 1984). As shown in Fig. 2 *B*, this biphasic effect is observed over the same ethanol concentration for both DPPC (LUVs) and DPPC (MLVs).

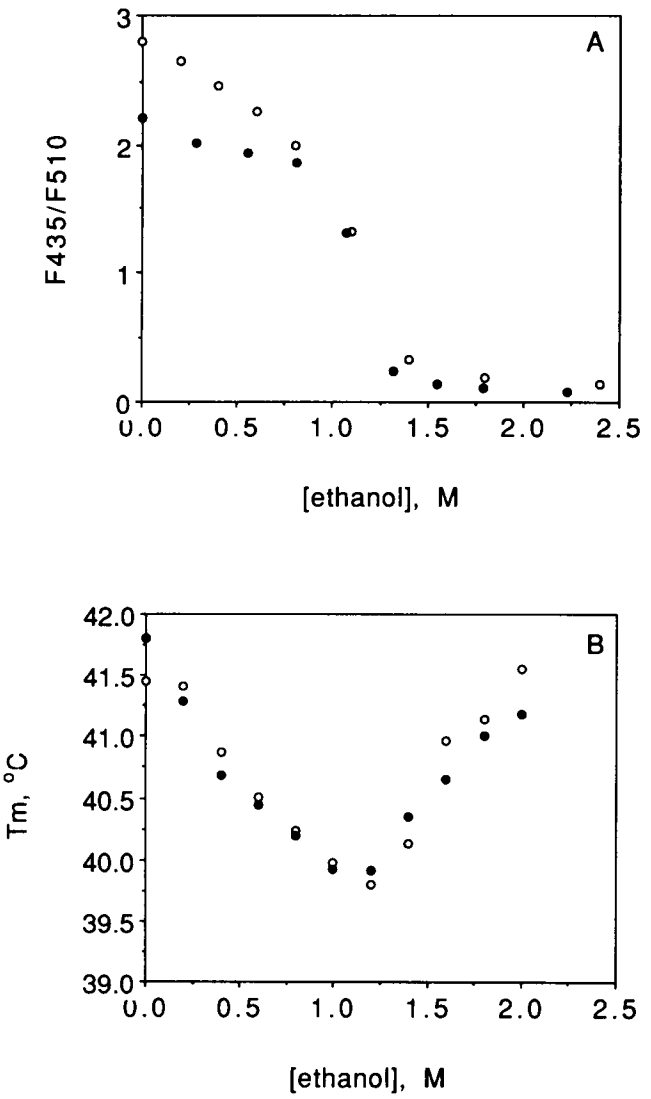

FIGURE 2 (*A*) Ethanol dependence of F435/F510 derived from Prodan fluorescence at 20°C; (*B*) ethanol dependence of T_m determined by DSC for DPPC (LUVs) (○) and DPPC (MLVs) (●).

Fig. 3 *A* shows that the emission spectrum of 6CF varies little with ethanol concentration. Fig. 3 *B* shows that the fluorescence intensity measured at 525 nm for 6CF trapped in DPPC (LUVs) decreases only 5% or less as the ethanol concentration increases from 0 to 2.0 M. In contrast, the intensity decreases appreciably when the pH is lowered from 8.0 to 3.0. Similar results were obtained when methanol or benzyl alcohol was used instead of ethanol (data not shown). These results form the basis for interpreting the data obtained from proton permeability measurements (Fig. 5).

Although the half-time of 6CF leakage in DPPC small unilamellar vesicles at 5°C in the absence of ethanol is known to be on the order of weeks (Weinstein et al., 1977),

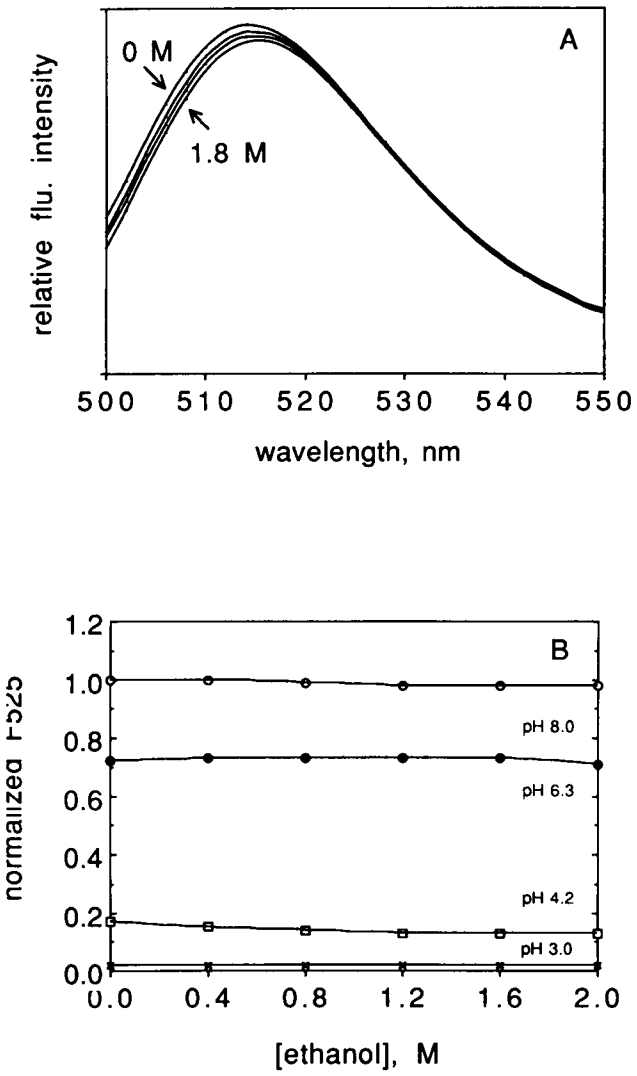

FIGURE 3 (*A*) Emission spectra of 6CF trapped in DPPC (LUVs) as a function of ethanol concentration. The excitation wavelength was 489 nm. (*B*) Effects of pH and ethanol content on the normalized F525 for 6CF trapped in DPPC (LUVs). F525 stands for the fluorescence intensity measured at 525 nm. Temperature = 20°C.

it is not clear whether this stability persists in DPPC large unilamellar vesicles at 20°C in the presence of alcohol. In order to address this question, we have prepared 6CF-trapped DPPC (LUVs) in the presence of 100 mM 6CF using the method described in Materials and Methods. At this concentration, 6CF self-quenches (Weistein et al., 1977; Lichtenberg et al., 1981). In this case, any 6CF leakage would lead to an increase in fluorescence intensity due to the release of self-quenching. It is found that the fluorescence intensity changes only 3% or less over the period of 1000 s in the concentration ranges of 0–2.0 M ethanol, 0–4.5 M methanol, and 0–60 mM benzyl alcohol (data not shown). The results suggest that these alcohols cause negligible leakage of 6CF in DPPC (LUVs) under the experimental conditions.

It has been shown that encapsulated 6CF will leak out as DPPC goes through its main phase transition from gel to liquid crystalline state (Lichtenberg et al., 1982). To determine whether leakage would occur through the phase transition from the noninterdigitated gel to the interdigitated gel phase of DPPC (LUVs), we have measured the effect of temperature on the fluorescence intensity of 6CF trapped inside DPPC (LUVs) in the absence and presence of 1.4 M ethanol (Fig. 4). According to the temperature-ethanol phase diagram (Nambi et al., 1988), at 1.4 M ethanol, DPPC should exhibit a phase transition from the noninterdigitated to the fully interdigitated gel state at 18–19°C and a main transition from the liquid-crystalline to the noninterdigitated gel state at about 41°C. If the trapped 6CF leaks out of the vesicles through these two phase transitions, the fluorescence intensity would increase abruptly both at 18–19°C and at 41°C. Fig. 4 shows that fluorescence intensity increases abruptly only at 41°C, not at 18–19°C, suggesting that the phase transition from the noninterdigitated gel to the interdigitated gel state does not lead to any appreciable leak of 6CF.

Effects of ethanol on vesicle size

The vesicle size has been determined as a function of ethanol concentration by the light-scattering methodology. The measurements were made on a Coulter N4 MD particle size analyzer (Coulter Electronics). The measurements were made 30 min after the addition of ethanol. The results are presented in Table 1. The original size of the DPPC vesicle was about 100 nm in diameter. This size remains virtually unchanged up to 1.0 M ethanol. Above 1.0 M, large vesicles with a mean diameter of about 1300–2300 nm were detected and accounted for about 25–53% of the total vesicle. The percentage of the vesicles retaining the original size decreases abruptly at 1.0–1.2 M ethanol. This concentration region is

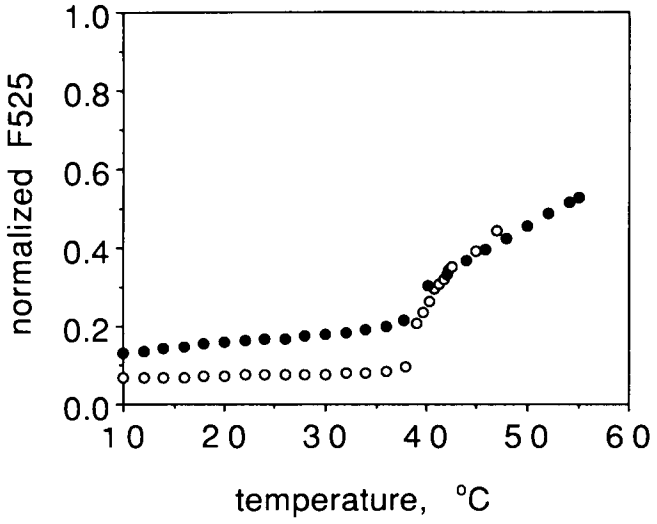

FIGURE 4 Effects of temperature on the normalized fluorescence intensity of 6CF trapped in DPPC (LUVs) at 0 M ethanol (○) and at 1.4 M ethanol (●). F525 was set to unity when the vesicles were disrupted by the addition of Triton X-100.

TABLE 1 Effect of ethanol on the size and the size distribution of DPPC (LUVs) at 20°C

[Ethanol] (M)	Population 1 Diameter (nm)	Population 1 Percentage	Population 2 Diameter (nm)	Population 2 Percentage
0.0	97 ± 17	100	—	0
0.2	101 ± 25	100	—	0
0.4	98 ± 15	100	—	0
0.6	101 ± 15	100	—	0
0.8	107 ± 30	100	—	0
1.0	108 ± 29	100	—	0
1.2	106 ± 24	75	1285 ± 369	25
1.4	110 ± 31	50	1376 ± 460	50
1.6	141 ± 47	58	2280 ± 750	42
1.8	149 ± 46	55	2430 ± 760	45
2.0	140 ± 47	47	2360 ± 770	53

close to the critical values shown in Fig. 2. Additional tests, as outlined in Table 2, further indicate that a large mean diameter (e.g., 1350 nm) appears only in the fully interdigitated gel state of lipid vesicles. It thus appears that the ethanol-induced lipid interdigitation results in an increase in vesicle size.

In order to determine whether the increase in vesicle size is due to vesicle aggregation or vesicle fusion, the following tests have been performed (Table 3). In the absence of ethanol, the mean diameter of the DPPC (LUVs) is about 100 ± 20 nm. At 1.6 M ethanol, the mean diameter is increased to 1350 ± 520 nm, presumably as a result of ethanol-induced lipid interdigitation. Aggregated vesicles can be dissociated into individual vesicles by low-power bath sonication and by raising temperature (Wong and Thompson, 1982), whereas fused vesicles cannot. As clearly shown in Table 3, after the DPPC samples containing 1.6 M ethanol are sonicated for 30 min or after the temperature is increased from 20°C to 50°C, the mean diameter of the vesicle returns to its original size (about 100 nm). These tests strongly suggest that the increase in vesicle size at high ethanol concentrations is due to vesicle aggregation, rather than vesicle fusion.

Effects of ethanol on proton permeability in DPPC (LUV)

Fig. 5 shows that the fluorescence intensity of 6CF trapped in DPPC (LUVs) decreases with time after a pH gradient from 3.5 (exterior) to 8.0 (interior) is established (indicated by the arrow in Fig. 5). The initial rapid drop in fluorescence intensity (about 10% of the total intensity) is probably due to a trace of external probes not removed by gel filtration

TABLE 2 Effects of 1.6 M ethanol on the mean diameter of vesicles in different lipid physical states

System	T (°C)	Physical state	Mean diameter (nm)
DPPC	20	Fully interdigitated gel state	1350
DPPC	10	Noninterdigitated gel state	175
DPPC	55	Liquid crystalline state	77
DMPC	20	Noninterdigitated gel state	124

TABLE 3 Treatments used to distinguish membrane aggregation from membrane fusion in DPPC (LUVs) at 20°C

	Mean diameter (nm)
Before treatments	
0 M ethanol	100 ± 20
1.6 M ethanol	1350 ± 520
After treating the sample containing 1.6 M ethanol by	
Bath sonification for 30 min	123 ± 26
Raising temperature from 20°C to 55°C	105 ± 19

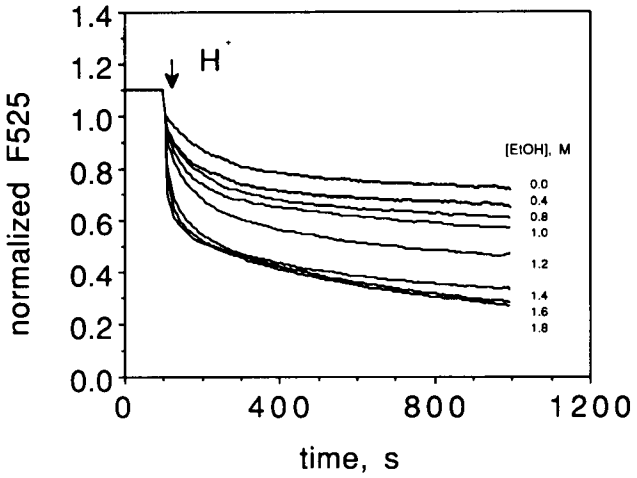

FIGURE 5 Time course of the change in the normalized F525 for 6CF trapped in DPPC (LUVs) at ethanol concentrations of 0 to 1.8 M. A pH gradient from pH 3.5 (exterior) to pH 8.0 (interior) was established by adding 0.1 N HCl to the external buffer at the time indicated by the arrow. Temperature = 20°C.

(Andersen, 1989); consequently, this portion will not be considered in the determination of proton permeability. The subsequent slow decay of fluorescence intensity is believed to result from the decrease of pH inside the vesicle due to proton influx driven by the pH gradient (Szoka et al., 1979). The beginning intensity of this slow decay is normalized to unity (normalized F525 = 1).

Fluorescence intensity changes were computer fit to single and double exponential decay functions using a simplex error minimization procedure (Nelder and Mead, 1965). The goodness of fit for different functions was compared both visually and by comparing the values of

$$\chi^2 = \sum[F_i - f(t_i,p)]^2/(n_d - n_p), \quad (1)$$

where F_i is the fluorescence intensity at time t_i, $f(t_i,p)$ is the value of the function at time t_i for parameter set p, n_d is the number of data points and n_p is the number of parameters used to define the function. The simplex was iterated and recalculated until the difference in χ^2 between successive iterations was less than 0.001%. To avoid local minima in χ^2, each data set was fit to each function several times using different starting parameters.

As shown in Table 4, at 20°C and below 1.2 M ethanol, the fluorescence change can be satisfactorily described by a

TABLE 4 Data analysis of the decay of fluorescence intensity of 6CF entrapped in DPPC (LUVs) after a pH gradient from 3.5 (exterior) to 8.0 (interior) is established (temperature = 20°C)

[Ethanol] (M)	Single exponential fit (see Eq. 2)				Double exponential fit (see Eq. 3)			
	A	$k \times 10^3$	C	$\chi^2 \times 10^4$	A	$k_1 \times 10^3$	$k_2 \times 10^4$	$\chi^2 \times 10^4$
0.0	0.242	7.8920	0.757	0.046	0.214	9.0260	0.891	0.040
0.4	0.255	10.332	0.704	0.563	0.194	25.760	3.984	0.420
0.8	0.299	10.193	0.665	0.417	0.230	20.800	4.217	0.337
1.0	0.306	12.625	0.646	0.720	0.260	26.110	4.245	0.478
1.2	0.369	10.737	0.552	1.550	0.287	31.956	7.737	1.165
1.4	0.410	14.141	0.433	6.250	0.388	61.930	1.181	2.236
1.6	0.387	15.917	0.428	9.350	0.408	90.392	1.160	1.914
1.8	0.348	13.536	0.412	12.80	0.409	162.90	1.253	1.645

single exponential plus a finite value

$$F(t) = A \exp(-kt) + C, \quad (2)$$

where A is the pre-exponential factor, k is the rate constant, and C is a constant. Above 1.2 M, it is apparent in the χ^2 values (Table 4) that the double exponential decay function

$$F(t) = A \exp(-k_1 t) + (1 - A) \exp(-k_2 t) \quad (3)$$

provides a better approximation of the data than the single exponential decay. Here k_1 and k_2 are the rate constants. The second (slow) exponential decay component detected in the change of 6CF fluorescence at ethanol concentrations higher than 1.2 M (Table 4) may result from the vesicle aggregation mentioned earlier.

Using the fitted rate constants (Table 4) and the vesicle radii determined by the light-scattering methodology (Table 1), the proton permeability coefficient P can be calculated from the following equation (Elamrani and Blume, 1983):

$$P = kR/3 = kV/S, \quad (4)$$

where R, V, and S are the radius, volume, and surface area of the vesicle, respectively. For the samples at ethanol concentrations above 1.2 M, the average rate constant $\langle k \rangle$ ($= Ak_1 + (1 - A)k_2$) was used to calculate the proton permeability coefficient. The same pattern of increase in P above 1.2 M ethanol was seen when rate constants were calculated using the same equation, either single- or double-exponential decay, for all ethanol concentrations. However, the χ^2 values suggest that a double-exponential decay is a better description of the data than a single-exponential decay at high ethanol concentrations.

Fig. 6 *A* shows the effects of ethanol on the proton permeability coefficient in DPPC (LUVs) (filled circles) and in POPC (LUVs) (open circles). In DPPC (LUVs), P initially increases monotonically with increasing ethanol content up to 1.0 M, followed by an abrupt increase at 1.2 M ethanol. As mentioned earlier, this concentration is within the ethanol concentration region (0.8–1.2 M) for the formation of the fully interdigitated DPPC structure previously reported by x-ray diffraction (Simon and McIntosh, 1984). Thus the data in Fig. 6 *A* (filled circles) suggest that the ethanol-induced lipid interdigitation has greatly enhanced the proton permeability. To ensure that the abrupt increase in P at 1.2 M ethanol is the result of lipid interdigitation, a control experiment using POPC (LUVs) has been conducted at 20°C. At this temperature, POPC is in the liquid crystalline state and is

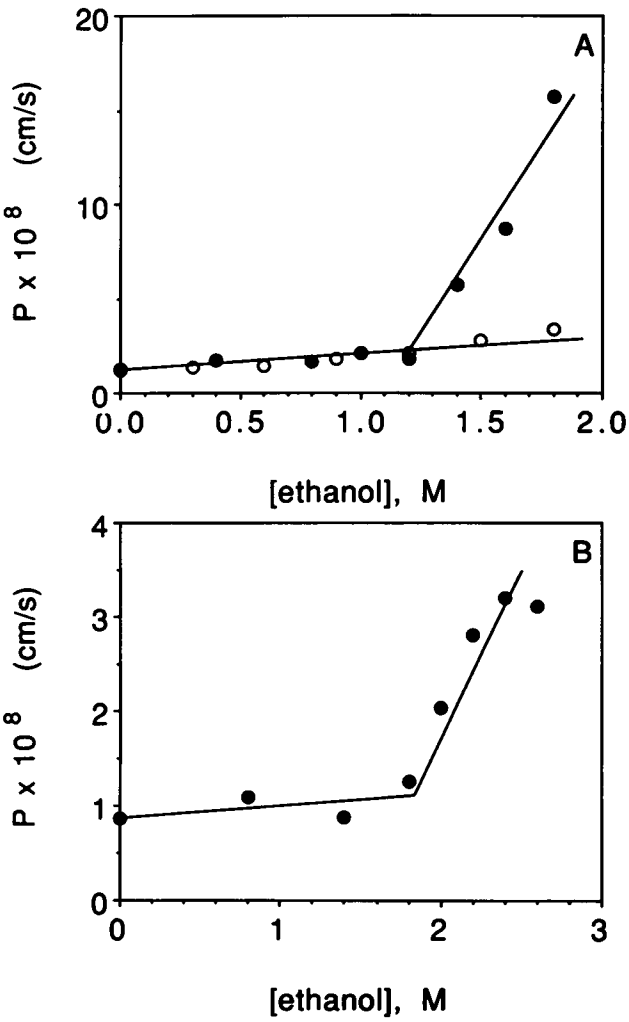

FIGURE 6 (*A*) Variation of proton permeability coefficient, P, as a function of ethanol concentration at 20°C for DPPC (LUVs) (●) and for POPC (LUVs) (○). (*B*) Plot of P versus ethanol concentration for DPPC (LUVs) at 14°C.

known not to be converted to the interdigitated state over the ethanol concentrations examined. Thus, no abrupt changes in proton permeability are expected. Fig. 6 *A* (open circles) shows that this is indeed the case.

Moreover, according to the temperature-ethanol phase diagram (Nambi et al., 1988), the critical ethanol concentration for the formation of interdigitated DPPC vesicles is about 2.0 M at 14°C. Fig. 6 *B* shows that at 14°C the proton permeability coefficient displays an abrupt change at 1.9–2.0 M ethanol, completely in accordance with the phase diagram. This once again demonstrates that an abrupt increase in P occurs when lipid vesicles are converted from the noninterdigitated to the fully interdigitated structure.

The proton permeability coefficients in noninterdigitated lipid vesicles range from 10^{-9} to 10^{-4} cm/s (Deamer and Nichols, 1983). Elamrani and Blume (1983) reported that the proton permeability coefficient is about 10^{-8} cm/s in the noninterdigitated gel phase of dimyristoylphosphatidic acid large unilamellar vesicles. These values are comparable to the P values shown in Fig. 6.

Effects of other short-chain alcohols on proton permeability in DPPC (LUVs)

X-ray diffraction studies have demonstrated that, in addition to ethanol, short-chain alcohols such as methanol and benzyl alcohol can induce the fully interdigitated phase of DPPC (MLVs) (McDaniel et al., 1983; McIntosh et al., 1983). Thus, it is of interest to examine the effects of methanol and benzyl alcohol on proton permeability in DPPC (LUVs). At 20°C, an abrupt change in P is observed at 3 M methanol (Fig. 7 *A*) and at 40 mM benzyl alcohol (Fig. 7 *B*). These critical concentrations, along with the critical alcohol concentrations (C_r) for the formation of fully interdigitated DPPC determined from other physical parameters (e.g., T_m determined from DSC, and the F435/F510 parameter derived from Prodan fluorescence), are summarized in Table 5. Clearly, there is a good agreement on C_r determined from various methods.

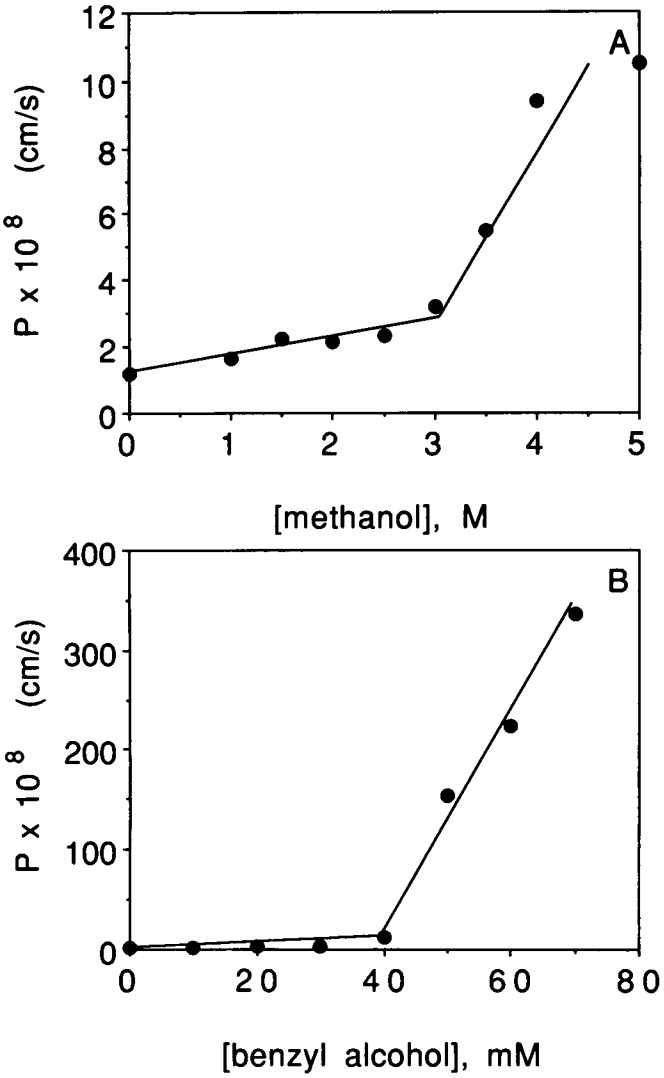

FIGURE 7 Plots of P versus the concentration of methanol (*A*) and benzyl alcohol (*B*) in 6CF-containing DPPC (LUVs) at 20°C.

DISCUSSION

At 20°C, DPPC (LUV) is in the noninterdigitated gel state at ethanol concentrations below 1.0 M. In this low ethanol concentration region, the proton permeability coefficient P increases monotonically with increasing ethanol content (Fig. 6 *A, filled circles*). This result can be due to the ethanol-induced increase in dielectric constant of the membrane (Parsegian, 1969; Orme et al., 1988) or to the disordering effect on lipid packing caused by the partitioning of ethanol into the bilayer (Hubbell and McConnell, 1969; Jain and Wu, 1977; Harris and Schroeder, 1981; Rowe, 1982; Ives and Verkman, 1985; Barchfeld and Deamer, 1985, 1988).

The dramatic increase in proton permeability at 1.2 M ethanol at 20°C (Fig. 6, *filled circles*) can be interpreted as being due to the formation of the fully interdigitated DPPC phase. Two pieces of evidence support this assertion. First, the ethanol concentration, at which the abrupt increase in P is observed (Fig. 6 *A*), comes close to the critical ethanol concentration C_r for the formation of the fully interdigitated gel phase in DPPC determined from x-ray diffraction (0.8–1.2 M at 20°C) (Simon and McIntosh, 1984), Prodan fluorescence (1.1 M at 20°C) (Zeng and Chong, 1991), and DSC (0.87–1.1 M) (Rowe, 1985). Second, the temperature dependence of the abrupt increase in P (Fig. 6) is completely in accordance with the ethanol-temperature phase diagram of interdigitated DPPC (Nambi et al., 1988). Their phase diagram indicates that C_r is 1.1 M at 20°C and 2.0 M at 14°C. If the dramatic increase in the plot of P versus ethanol concentration (Fig. 6 *A, filled circles*) is due to lipid interdigitation, then the breakpoint should be shifted from 1.1 M ethanol at 20°C to 2.0 M ethanol at 14°C. As shown in Fig. 6 *B*, this is almost the case.

It has been suggested that passive proton transport across lipid bilayers is mediated by the hydrogen-bonded chains of water (Nagle and Nagle, 1983; Deamer and Nichols, 1989).

TABLE 5 Comparison of the critical alcohol concentrations, C_r (M), for the formation of the fully interdigitated DPPC gel state, determined from various methods (temperature = 20°C)

Methods	Membrane systems	C_r (M)			References
		Methanol	Ethanol	Benzyl alcohol	
Proton permeability	DPPC (LUVs)	3.0	1.2	0.040	This study
F435/F510 of Prodan fluorescence	DPPC (LUVs)	3.0	1.2	0.035	This study; also Zeng and Chong, unpublished results
T_m of DSC	DPPC (LUVs)	2.8	1.2	0.050	This study
T_m of DSC	DPPC (MLVs)	2.5	1.1	N/D	Rowe, 1985; also this study
X-ray	DPPC (MLVs)	N/D	1.3*	0.075*	Simon and McIntosh, 1984; McIntosh et al., 1983

* Lowest alcohol concentrations at which fully interdigitated structures were demonstrated.

However, McDaniel et al. (1983) reported that the solubility of water in lipid bilayers is decreased in the fully interdigitated state, as compared to the noninterdigitated state. Thus it is unlikely that the significant increase in proton permeability in the interdigitated state, as shown in Fig. 6, is due to an increase of the hydrogen-bonded water chain. On the other hand, ethanol has been proposed to reside both in the hydrocarbon core and at the membrane surface of the noninterdigitated DPPC vesicles (Rowe, 1983; Kreishman et al., 1985; Sarasua et al., 1989). One may thus propose that passive proton transport across lipid bilayers is mediated by the hydrogen-bonded chains of ethanol. But, when the lipid is converted from the noninterdigitated to the fully interdigitated structure, ethanol tends to move from the hydrocarbon region toward the membrane surface (Rowe, 1983; Sarasua et al., 1989) or toward the bulk solution (Zeng and Chong, unpublished results). This argues against the idea that the dramatic increase in P at high ethanol concentrations (Fig. 6) is due to an increase of the hydrogen-bonded ethanol chain.

The dramatic increase in proton permeability from the noninterdigiated state to the fully interdigitated gel state (Fig. 6) can be explained in terms of the alteration in membrane thickness. X-ray data have indicated a bilayer thickness of 42 Å for the noninterdigitated gel phase in DPPC and a thickness of 30 Å for the fully interdigitated gel state (Simon and McIntosh, 1984). Using the Born-Parsegian expression (Parsegian, 1969), Orme et al. (1988) have calculated that decreasing membrane hydrocarbon thickness from 50 to 30 Å will raise the ionic permeability by a factor of almost 10.

The proton permeability discussed in this study is inferred from the rate of 6CF fluorescence intensity decrease, which is a measure of the rate of pH change inside the vesicle, $\Delta pH/\Delta t$ (where t is the time). The parameter $\Delta pH/\Delta t$ is proportional to proton flux J and to the total membrane surface area (Barchfeld and Deamer, 1985). Assume that both the surface area of each lipid molecule and the total number of lipids in each vesicle remain unchanged through the ethanol-induced phase transition. The total surface area of the vesicle will be doubled, and R will be increased by a factor of 1.4, according to Eq. 4, when the vesicle is converted from the noninterdigitated to the fully interdigitated gel phase. Interestingly, our data in Table 1 show that the diameter of population 1 is increased by exactly a factor of 1.4 at high ethanol concentrations. This increase in the radius should bring about a 1.4-fold increase in $\Delta pH/\Delta t$.

For an ion to cross the bilayer, it must (*a*) enter the membrane by overcoming any interfacial free energy barrier, (*b*) diffuse across the bilayer, and (*c*) exit the membrane on the opposite side (Gennis, 1989). The positive charge residing at the choline group of DPPC would impose an energy barrier to a proton entering the interior of the vesicle. However, the charge density at the surface of fully interdigitated DPPC vesicles is less than that in noninterdigitated vesicles, due to the fact that the nonpolar terminal methyl groups of DPPC are relocated to the membrane surface when DPPC becomes fully interdigitated. A decrease in charge density should facilitate step (*a*) and step (*c*), thus enhancing proton permeability.

Perhaps it is the combined effect of the changes in membrane thickness and surface charge density that brings about the marked increase in proton permeability through the ethanol-induced phase transition from the noninterdigitated to the fully interdigitated DPPC.

This study demonstrates that lipid interdigitation promotes vesicle aggregation. DPPC vesicles can undergo spontaneous aggregation (Wong and Thompson, 1982). This process, however, is slow (on the order of hours to days), whereas the vesicle aggregation caused by alcohol-induced lipid interdigitation occurs within 30 min (Table 1). Using dehydrating agents such as ethylene glycol, dextran, glycerol, and sucrose, some investigators concluded that membrane-bound water in the membrane surface is the main barrier to the close contact of two membranes (Rand et al., 1988; Ohki and Arnold, 1990). McDaniel et al. (1983) proposed that the fully interdigitated membrane decreases the membrane solubility for water. Therefore the effect of lipid interdigitation on vesicle aggregation shown in Table 1 may result from the dehydration of lipids. On the other hand, the aggregation can be understood from an energetic viewpoint without the involvement of dehydration. In contrast to the noninterdigitated vesicles, the membrane surface of the fully interdigitated vesicle is covered by both the polar headgroup and the nonpolar terminal methyl group (Fig. 1). The nonpolar regions in the surface create van der Waals contact points between vesicles that favor vesicle aggregation. It should be

mentioned that an increase in membrane permeability has been previously suggested to couple with an enhancement in vesicle aggregation or fusion (Smaal et al., 1987). Our present data suggest that the formation of the fully interdigitated lipid structure leads to both an increase in proton permeability and the enhancement of vesicle aggregation.

The pH gradient used in this study is 4.5 units. Large pH gradients have been shown to produce proton diffusion potential in the liquid crystalline phase of lipid vesicles (Deamer and Nichols, 1983). But this situation is uncertain in the gel state of lipid vesicles. Boheim et al. (1980) suggested that valinomycin (a potassium ionophore) may not function in the gel state of hexadecyltetradecylphosphocholine because valinomycin is frozen out of the membrane. However, Hsu and Chan (1973) reported that valinomycin molecules are associated with DPPC bilayers below and above the phase transition temperature. In the present study, whether the measured proton permeability in DPPC is limited by counter-ion diffusion has been tested by using valinomycin. Valinomycin was incorporated into 6CF-trapped DPPC (LUVs) at 55°C, with the ratio of valinomycin to DPPC equal to 1/2300. The proton permeability experiment (Fig. 5) was then repeated at 20°C in the presence of valinomycin. Our results (not shown) indicate that valinomycin has virtually no effect on the decay of F525. This negative result would suggest that proton diffusion across the gel phase DPPC is not limited by the diffusion of potassium counter-ion, provided that valinomycin is still active in the gel phase of DPPC. Since this assumption may not be correct (Boheim et al., 1980), we cannot rule out the possibility that the measured P is limited by counter-ion diffusion. However, the existence of counter-ion diffusion will at most affect the P values, not the qualitative conclusion on the ethanol dependence of the P value.

In order to assess the physiological consequence of the sharp increase in proton permeability caused by ethanol-induced lipid interdigitation, two questions should be addressed. First, will cells ever be exposed to ethanol concentrations greater than the critical ethanol concentration, C_r, for the formation of fully interdigitated lipids under pathophysiological conditions? Second, can such exposure actually lead to lipid interdigitation in biological membranes?

Persons who have a blood ethanol concentration of 100 mg/dl (or 22 mM) or higher are considered to be intoxicated. This level of ethanol is much lower than the C_r (0.8–1.2 M at 20°C) (Rowe, 1983; Simon and McIntosh, 1984) for the formation of the fully interdigitated DPPC vesicles. This may give the impression that model membrane studies, such as the one presented here, bear little relevance to real alcoholic problems. It is important, however, to point out that the argument based on the blood alcohol concentration is misleading; it ignores the fact that about 25% of the ingested alcohol is absorbed in the stomach and 75% is absorbed in the small intestines (Marinetti, 1990). When 1 ounce (29.6 ml) of 100 proof bourbon (equivalent to 15 ml of absolute ethanol at 17 M) is consumed as several doses over a short period of time (e.g., <1 h), cells in the stomach and in the small intestines experience, at least momentarily, an ethanol concentration of 1.5 M or higher. These concentrations certainly exceed the C_r for DPPC (0.8–1.2 M at 20°C) (Rowe, 1983; Simon and McIntosh, 1984). This calculation has taken into account the dilution of the ingested ethanol by gastric juice, which is less than 30 ml in fasting and about 100–150 ml during eating. At body temperature, this situation becomes even worse, since higher temperatures favor lipid interdigitation (Nambi et al., 1988; Zeng and Chong, 1991). Note that the main phase transition temperature of DPPC is about 41°C, which is above body temperature, and that DPPC can become interdigitated by ethanol at temperatures below the main phase transition temperature. Thus, during heavy drinking, gastrointestinal mucosal membranes are likely to be exposed to an ethanol content higher than the critical ethanol concentration C_r for the formation of the fully interdigitated DPPC.

Direct evidence for lipid interdigitation in a biological membrane is unattainable at the present time due to the complexity of the system. However, it is still feasible to estimate the possibility of the ethanol-induced lipid interdigitation in gastrointestinal mucosal membranes. Ethanol-induced lipid interdigitation has been found only in saturated diacylphosphatidylcholine model membranes (Rowe, 1983, 1985, 1987). Wassef et al. (1979) reported that, in rat gastric mucosa, DPPC makes up about 31% of the total phosphatidylcholine, which comprises 46% of the total phospholipid. They also pointed out that rat gastric mucosa is similar to lung in that both contain elevated amounts of DPPC. It was suggested that DPPC forms the main active component in the mucosal barrier (Butler et al., 1983) to protect the stomach from acid-induced damage (Lichtenberger et al., 1983). On the other hand, Schmitz and Renooij (1990) reported that, in rat, human, and canine gastric mucosa, saturated diacylphosphatidylcholine comprises only 2–6% of the total phosphatidylcholine. The reason for the discrepancy between their results and those obtained by Wassef et al. (1979) is not known. However, Schmitz and Renooij (1990) indicated that although saturated diacylphosphatidylcholine is a minor component, its biosynthesis may be specifically triggered for gastric mucosal renewal and repair processes. It thus appears that a significant amount of disaturated phosphatidylcholine does exist in human, rat, and canine gastric mucosa, and it may play a critical role in protecting the gastric mucosal barrier. These saturated diacylphosphatidylcholine molecules can, in principle, form fully interdigitated structures at high ethanol concentrations, resulting in an abrupt increase in proton permeability. This possibility becomes even more significant when considering the fact that a high content of ethanol (100 mg/ml) can induce lateral phase separation in phosphatidylcholine/phosphatidylethanolamine mixtures, giving rise to the coexistence of the noninterdigitated gel and the fully interdigitated gel phase (Rowe, 1987).

If the above notions are correct, we should observe an abrupt increase in proton permeability in gastric mucosal membranes at certain characteristic alcohol concentrations. In fact, an alcohol-induced increase in proton permeability

was observed in gastric and duodenal mucosal epithelial membranes isolated from rabbit (Wilkes et al., 1989). It is interesting to compare their results with our present results. Wilkes et al. (1989) found that, at 20°C, the proton permeability in gastric and duodenal membranes increases sharply at about 0.8–1.0 M ethanol and at about 70–90 mM benzyl alcohol. These alcohol concentrations come close to the critical alcohol concentrations for the abrupt increase in proton permeability determined at 20°C from our present DPPC study (Figs. 6 *A* and 7 *B*) and close to the critical alcohol concentrations for the formation of the fully interdigitated DPPC determined by other methods (Rowe, 1983; Simon and McIntosh, 1984; summarized in Table 5). This correlation suggests that the observed increase in proton permeability in rabbit gastrointestinal mucosal membranes (Wilkes et al., 1989) may result from the alcohol-induced interdigitation of saturated diacylphosphatidylcholines.

Alcohol-induced lipid interdigitation and the accompanying increase in proton permeability may be related to alcohol-induced gastrointestinal mucosal bleeding (reviewed in MacMath, 1990). The pH of normal gastric juice is about 0.8, and it is near 7 inside the gastric luminal epithelia. The ability of the stomach to maintain such a high proton gradient is mediated by the gastric mucosal barrier. Disaturated phosphatidylcholine (e.g., DPPC) has been suggested to be the main active phospholipid component in the mucosal barrier (Butler et al., 1983). The initial event of the ethanol-induced gastric bleeding is the damage of the gastric mucosal barrier, evidenced by the increased proton permeability, which causes great damage to the mucosa and its microvasculature (Davenport, 1976; Bailey et al., 1986, 1987; Wilkes et al., 1987; Lacy, 1987). Our present data show that the alcohol-induced lipid interdigitation in DPPC greatly increases proton permeability. Thus, it is likely that alcohol-induced lipid interdigitation may have an etiological role in the alcohol-related damage of the gastric mucosal barrier.

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