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Osmotic Pressure Induced Pores in Phospholipid Vesicles†

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ABSTRACT: We report a comparative study of the leakage of hydrophilic molecules from vesicles of egg lecithin (EL) and of dipalmitoyllecithin (DPL). The effect of osmotic pressure differences on leakage is consistent with a model for a statistical pore nucleation process. The major difference in osmotic pressure induced leakage from DPL and EL is that the number of pore creation sites is much greater in DPL. We suggest that the difference in number of these sites also accounts for other differences in the properties of DPL and EL, namely for differences in vesicle fusion and apparent rate of "flip-flop".

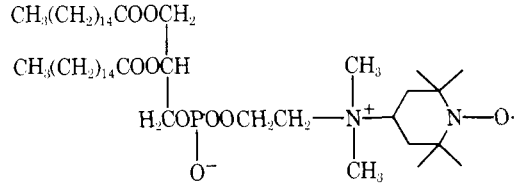
When dipalmitoyllecithin (DPL)[1] vesicles are preserved at a temperature above the "chain-melting point" (41°C) they undergo fusion. Evidence for this process based on the increase of internal volume, the transfer of phospholipids from the outer to the inner layer, and the mixing of the internal contents of the "fusing vesicles" has been presented by Taupin and McConnell (1972). Since then, different techniques have been used to confirm the existence of a fusion process in the case of vesicles made of saturated phospholipids (Papahadjopoulos et al., 1974; Prestegard and Fellmeth, 1974). On the other hand, egg lecithin (EL) vesicles do not fuse (Kornberg et al., 1972). Better knowledge of the bilayer properties is clearly required if we are to explain the different behavior of these two types of vesicles.

In this article, we present comparative measurements on DPL and EL vesicles which suggest the presence of a great number of defects in the DPL bilayer, these defects or pores being responsible for the observed leakage of hydrophilic solutes and an apparent increase in flip-flop rate. We propose a simple model for the pore nucleation process which leads to specific laws for leakage as a function of stress induced by an osmotic pressure difference across the vesicle wall. Experiments confirm this model.

Experimental Procedure

Dipalmitoyllecithin was purchased from Fluka. Egg lecithin was kindly provided by C. M. Gary Bobo (Laboratoire de Physiologie Cellulaire, Collège de France). The DPL (50 m*M* aqueous dispersion) was sonicated in buffered salt solutions at 50–55°C using titanium probes and then centrifuged to remove titanium debris. The conditions for egg lecithin were the same except that during sonication the tube was cooled in an ice bath. The buffer was 0.1 *M* phosphate (pH 6.9). The ionic strength of the buffer was varied by addition of sodium chloride for the tension-induced leakage measurements.

Several labels were used in the present work. Spin label I (dipalmitoylphosphatidyltempocholine) was synthesized by one of us (M.D.) following the general scheme of Kornberg and McConnell (1971) except for the production of phosphatidic acid which was prepared from (*S*)-(−)-1,2-dipalmitin (Fluka) following the process of Uhlenbroek and Verkade (1953).



I

The rotatory power of dipalmitoyl-L-α-glycerophosphoric acid thus obtained, $[\alpha]^{22}D$ +3.7° (*c* 7.9, dry $CHCl_3$), can be compared with the value of Baer (1951), $[\alpha]^{26}D$ +4.0° (*c* 9.6, $CHCl_3$). The condensation was performed by shaking the mixture of phosphatidic acid, tempocholine, and 2,4,6-triisopropylbenzenesulfonyl chloride in pyridine–chloroform for 4 hr in a closed flask containing glass beads. The spin label was purified by preparative silica thin-layer chromatography, in chloroform–methanol–NH_4OH (*d* = 0.90)–water (70:30:4:1, v/v). Anal. Calcd for $C_{48}H_{96}N_2O_{10}P\cdot 2H_2O$: C, 62.1; H, 10.85; N, 3.0; P, 3.3. Found: C, 62.0; H, 10.1; N, 3.0; P, 3.2.

The aqueous internal compartments of the vesicles were labeled using the water-soluble bilayer impermeable tempocholine (label II), prepared following the method of Kornberg and McConnell (1971). The label was dissolved

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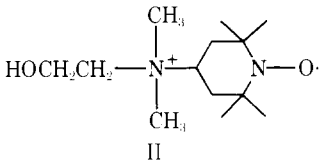
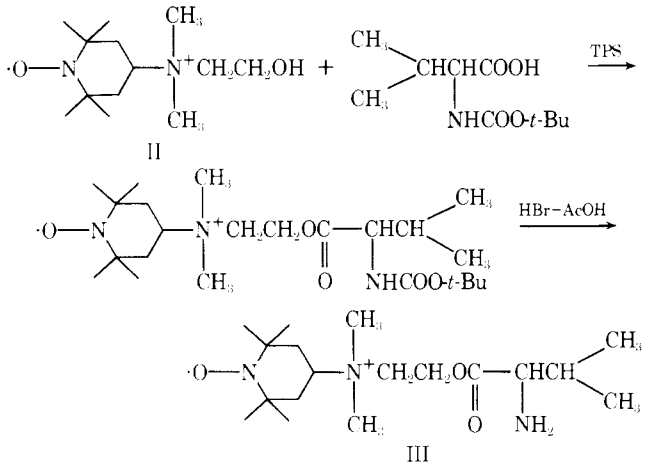
[1] Abbreviations used are: DPL, dipalmitoyllecithin; EL, egg lecithin.



II

in the buffer (5 mM) prior to the dispersion of the phospholipids.

We tried another hydrophilic label, the *O*-D,L-valyltempocholine (III), to detect the effect of size of the label on the leakage process; the label III was prepared as follows:



II

III

The butoxycarbonyl-D,L-valine (0.5 mmol), as described by Schwyzer et al. (1959), is shaken 15 hr with tempocholine II (0.5 mmol), 2,4,6-triisopropylbenzenesulfonyl chloride (1.5 mol), and glass beads in a mixture of 1 ml of dry chloroform and 0.5 ml of dry pyridine. After dilution with water and chloroform the orange organic layer is washed with 10% citric acid and water, dried over Na_2SO_4, and evaporated under reduced pressure ($T \leq 40°C$). To free the amino group the orange residue is treated at room temperature with a solution of HBr in acetic acid for 30 min. An orange syrup formed by addition of dry ether is separated and washed several times with chloroform to give 115 mg of crude III, after drying under reduced pressure. This product, being hygroscopic, was not submitted to composition analysis. The number of spins per mole was estimated to be about 0.1. We used this compound without further purification to indicate the qualitative dependence of our results on the size of the label.

From compact molecular models it is observed that for the fully extended conformations (all-trans links of the chain), the label III is about 1.5 times as long as II (~18 Å vs. 12 Å); if a coil conformation is considered (neglecting energy considerations) the more compact molecule can be contained in a sphere 1 Å larger in diameter than II (about 11.5 vs. 10.5 Å).

Different types of experiments were performed with the various spin labels. The labeled phospholipid I, which can be incorporated in the bilayer, was used to study the transfer of phospholipid molecules from the inner to the outer layer (flip-flop process) using sodium ascorbate at 0°C in the external solution as a reducing agent (Kornberg and McConnell, 1971). The hydrophilic label II was used for two distinct experiments.

(a) *Fusion Experiments.* It was previously shown (Taupin and McConnell, 1972) that during the fusion process, there is an increase of internal contents due to an increased volume to surface ratio of the larger vesicles arising from

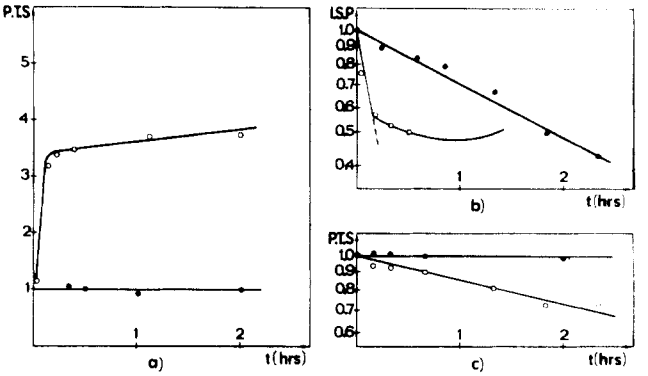


FIGURE 1: (●) EL vesicles preserved at 20°C; (○) DPL vesicles preserved at 46°C. (a) Typical evolution of the protected tempocholine signal (PTS) for 50 mM solutions of vesicles. PTS is expressed as a percentage of the signal before sodium ascorbate addition and is used as an index of the internal volume. (b) Time dependence of the internal surface paramagnetism (ISP) of sodium ascorbate treated vesicles (plotted as in Kornberg and McConnell, 1971). (c) Semi-log plot of the variation with time of the protected tempocholine signal (PTS) in vesicles passed over the Sephadex column.

fusion. We used this effect as an indicator of fusion. The vesicle solution was stored at 46°C. At various times, aliquots of the solution were treated at 0°C with sodium ascorbate to reduce the external label and the electron spin resonance (ESR) spectra were recorded. The ESR signal intensity was then proportional to the label protected within the vesicles and was called the protected tempocholine signal (PTS); it was used as an index of the internal volume. The increase of this volume as a function of time of preservation at 46°C reflects the fusion process.

(b) In order to measure the leakage of solutes through the vesicle wall, the vesicles were passed (after centrifugation) over a Sephadex column (Pharmacia, G25 fine) in order to remove both external sodium chloride and tempocholine. Consequently, an osmotic pressure difference was induced. The procedure was then identical with experiment a, the leakage of tempocholine being monitored by the decrease of the residual signal (after sodium ascorbate treatment) as a function of storage time at 46°C.

Results and Discussion

Comparative Measurements on DPL and EL Vesicles. Figures 1a, b, and c illustrate the behavior of EL and DPL vesicles for three types of experiments. (1) The protected tempocholine signal increases with time only in the case of DPL. It is to be noted on Figure 1a that the time during which fusion occurs is relatively short, about 10 min. No fusion occurs for EL vesicles.

(2) The flip-flop rate was measured by Kornberg's procedure (Figure 1b). The curve for EL exhibits (for time up to 2 hr) a single exponential decay of the internal paramagnetism, the slope of the curve being in agreement with the results of Kornberg and McConnell (1971). In contrast, the curve for DPL clearly exhibits two relaxation times, the longer one being the flip-flop time previously determined. Kornberg has shown that a spontaneous reoxidation masks the flip-flop process after a certain time. Unfortunately, as the DPL vesicles are maintained at a higher temperature than EL vesicles, this spontaneous reoxidation process is very efficient and it was not possible to observe the decrease of signal for a long time.

(3) Figure 1c shows that no leak occurs in EL vesicles. In contrast, a slow leak is present in the case of DPL vesicles

(half-time 4-5 hr). We can infer that DPL vesicles do not remain closed during the time of the experiment, unlike the EL vesicles.

We suggest that these differences can be explained by the existence of pores in DPL vesicles.

The Pore Model. Confirming the presence of pores in the vesicle wall calls for a study of the leakage from vesicles under different experimental conditions. Similar studies of leakage were made in the case of the hemolysis of red blood cells induced by a mechanical tension σ by Rand (1964). It is possible (de Gennes and Helfrich, 1974, unpublished) to reinterpret Rand's results in terms of an activation energy, E^*, for breakdown, E^* depending only on one phenomenological constant. Experimentally, E^* is found to be proportional to σ^{-1}. This behavior dependence law agrees with a model where pores or patches of a segregated phase can exist in the membrane, and become unstable under tension if their radius exceeds a certain limit, $R^*(\sigma)$.

We wish to present here a similar model developed by de Gennes (1974, private communication). In fact, two types of leakage may occur. In the first, the pores are unstable and their radii grow indefinitely leading to a complete emptying of the vesicle as in the case of Rand's experiments. The second type occurring at lower tensions corresponds to the alternate opening and closing of smaller pores and can be described by a permeability of the vesicle to the label.

Model. The energy E of a pore of radius R may be written:

$$E(R) = \gamma 2\pi R - \sigma\pi R^2$$

where γ is an "edge energy" (dimension dynes) and $\sigma\pi R^2$ represents the work of the tension σ. $E(R)$ reaches a maximum when $R = R^* = \gamma/\sigma$:

$$E(R^*) = E^* = \pi\gamma^2/\sigma$$

This means that when $R < R^*$ the pore tends to close again but when $R > R^*$ it tends to grow indefinitely leading to complete disruption of the vesicle.

Let the number of pores of energy, E, created per unit time and per vesicle be $1/\tau$:

$$1/\tau = \left(S/\Sigma_0\tau_0\right) \exp(-E/T)$$

T being the absolute temperature (expressed in units of energy), S being the surface of the vesicle, Σ_0 being a microscopic surface, and τ_0 being a microscopic time.

The expression for E shows that depending on γ and σ two cases may occur: (a) $E^*/T \ll 1$; unstable pores are thermally created ($R > R^*$) and they are the predominant process of leakage; (b) $E^*/T \gg 1$; only small pores are thermally created.

(a) *Unstable Pores.* The number of such unstable pores created per unit time and per vesicle is $1/\tau$:

$$1/\tau = \left(S/\Sigma_0\tau_0\right) \exp(-E^*/T)$$

For statistical nucleation in an interval of time $(t, t + dt)$ following the application of the tension σ, the probability of rupture of unbroken vesicles is: $dt/\tau \exp(-t/\tau)$ leading to a mean time $\bar{t}$ for the loss of internal content of $\bar{t} = \tau$. Thus, we are led to the relationship between $\bar{t}$ and σ:

$$\log \bar{t} = -\log \left(S/\Sigma_0\tau_0\right) + E^*(\sigma)/T$$

$$\log \bar{t} = A + (\pi\gamma^2/T)(1/\sigma)$$

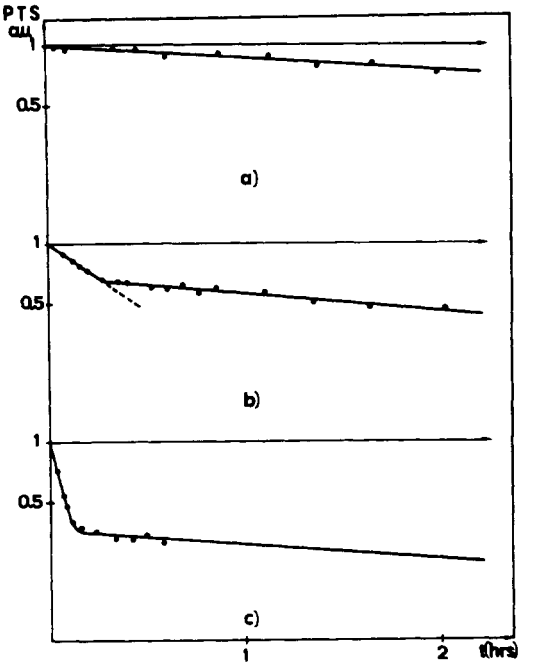


FIGURE 2: Evolution with time of the protected tempocholine signal (PTS) for various concentrations of NaCl (semi-log plot) in DPL vesicles passed over a Sephadex column: (a) without NaCl; (b) with 2 *M* NaCl; (c) with 5 *M* NaCl.

The tension, σ, is related to the concentration, c, of solute particles via the osmotic pressure, $\bar{\omega}$, by: $\sigma = \bar{\omega}(R_v/2)$; $\bar{\omega} = cT$, R_v being the radius of the vesicle. Finally:

$$\log \bar{t} = A + (\pi\gamma^2/T^2R_v)(2/c) \quad (1)$$

(b) *Pores without Rupture.* In this case, σ is too small to induce a significant number of unstable pores and one must take into account the small pores corresponding to $E < E^*$. The number of nucleated pores with radii in the range $(\rho, \rho + d\rho)$ per unit surface is proportional to $d\nu$:

$$d\nu = \exp[-(E(\rho)/T)]\, d\rho$$

In this case of low σ:

$$d\nu = (1 + \sigma\pi\rho^2/T) \exp\left(-\frac{2\pi\gamma\rho}{T}\right) d\rho$$

Neglecting the detailed procedure by which the pores permit leakage one can write a permeability, P, proportional to the total surface of the pores:

$$P = \text{const} \sum_\rho \rho^2 d\nu$$

$$P = \text{const} \int_0^{R^*} \rho^2 \left(1 + \frac{\sigma\pi\rho^2}{T}\right) \exp\left(-\frac{2\pi\gamma\rho}{T}\right) d\rho$$

the radii of the pores being smaller than the critical radius.

Letting

$$K = 2\pi\gamma/T:$$

$$P \sim \frac{2!}{K^3} + 4! \frac{\sigma\pi}{T} \times \frac{1}{K^5}$$

$$P = P_0[1 + 12(\sigma\pi/TK^2)]$$
$$= P_0[1 + (3/\pi)(T\sigma/\gamma^2)]$$

with $\sigma = \bar{\omega}R_v/2$ and $\bar{\omega} = cT$:

$$(P - P_0)/P_0 \sim (1/2)cR_v(T/\gamma)^2$$

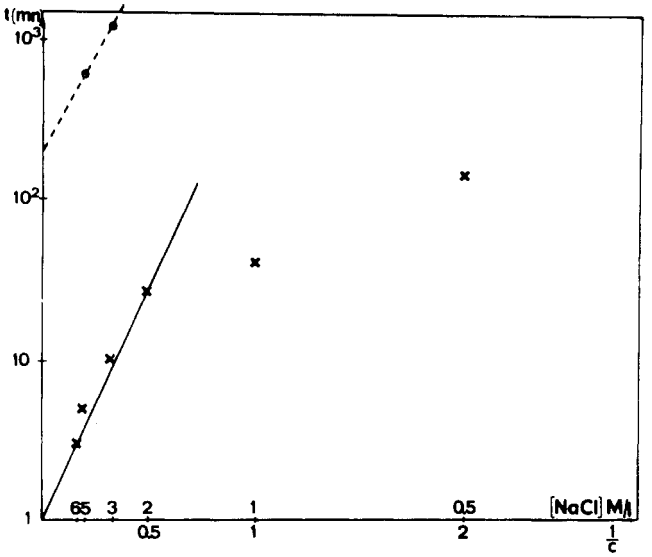


FIGURE 3: Semi-log plot of the half-time of leakage vs. the reciprocal concentration, $1/c$, of NaCl: (×) DPL; (⊙) EL.

Table I

	$\bar{t}$ (min) at [NaCl] (M)						
	0	0, 5	1	2	3	5	6
DPL	300 240 300	140	42	27	10	5	3
EL	∞				~20 hr	12 hr	

The time for rupture, $\bar{t}$, being inversely proportional to the permeability (see eq 2), $\bar{t}_0$ being the mean time in the absence of osmotic pressure.

This model thus leads to different laws for high and low surface tensions: (a) in the high tension case, eq 1 shows that log $\bar{t}$ is inversely proportional to the concentration c; (b) in the low tension case (eq 2) the ratio of the time of

$$\bar{t}_o/\bar{t} = [(1/2)R_v(T^2/\gamma^2)c + 1] \qquad (2)$$

leakage at zero tension to its value with tension is linear with the concentration c. The slopes of the curves depend only on R_v and γ.

Figure 2 shows typical evolution with time of the residual amount of tempocholine in DPL vesicles for various NaCl concentrations. A single decay rate is observed for low tensions (c up to 0.5 M NaCl) while a faster decay appears for higher c. The fast decay is due to the difference of osmotic pressure and clearly disappears when most of the tension has been released by the leakage process.

Table I gives the values of the time of half-leakage, t, for various NaCl concentrations both for DPL and egg lecithin. Figure 3 shows the (log $\bar{t}$ vs. $1/c$) plot and Figure 4 the $\bar{t}_0/\bar{t}$ vs. c plot for DPL. Clearly relation 1 fits for high tensions and 2 for low tensions. Comparison of the experimental slopes of Figures 3 and 4, respectively, with eq 1 and 2 gives, with R_v = 100 Å: (a) rupture process: $\gamma = 0.65 \times 10^{-6}$ dyn; (b) small pores: $0.55 \times 10^{-6} < \gamma < 0.80 \times 10^{-6}$ dyn.

The two results agree quite well with the values of the Frank elastic constants in liquid crystals (Frank, 1958): $\gamma \simeq 10^{-6}$ dyn.

With this value for γ, one can calculate the value of the

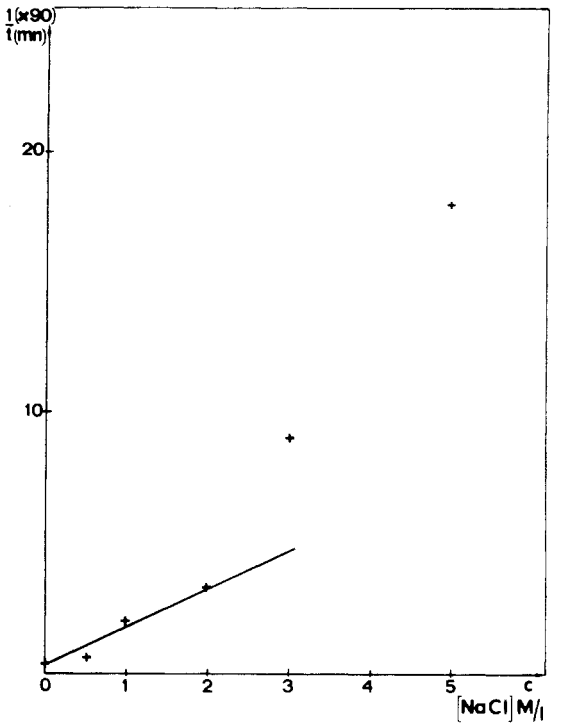


FIGURE 4: Plot of the reciprocal half-time of leakage vs. the concentration c of NaCl in moles per liter.

NaCl concentration c^*, which corresponds to the passage of rupture process to small pores process; it corresponds to $E^* \simeq T$ and gives $1 < c^* < 2$ M in agreement with the experiments. Furthermore, it should be noted that in case b (small pores without rupture) the size of the label is important since if it is too big no leak will occur. In contrast, type a leakage is not sensitive to the size of the label since there is complete opening of the vesicle. We observed that the leakage of label III is less than that of tempocholine only for concentrations of salt ≤0.2 M (in the b range).

The critical radius, R^*, of a pore is 2 Å for 1 M NaCl and 20 Å for 0.1 M. The fact that even a small osmotic pressure difference induces pores may have experimental consequences.[2] Similar measurements of tension induced leakage were made with EL vesicles at 20°C. The points plotted on Figure 3 indicate that: the slope of the line is similar, implying that γ is of the same order, which is not surprising, both phospholipids (DPL and EL) being in the liquid state; the intercept with the t axis is much higher for EL (200 times in t). This intercept is determined by the preexponential term in eq 1 and corresponds to the number of pore nucleation sites.

Thus, we are led to the conclusion that the main difference between DPL and EL resides in the number of pore creation sites, the number being much greater in DPL than in EL. During the experiments, DPL and EL are both in the fluid phase but DPL is only 5°C above its transition temperature.

[2] Some evaporation occurs during sonication, especially in the case of DPL, when the temperature is around 50°C. The resulting change in the buffer concentration leads, after the Sephadex column, to a small difference in osmotic pressure and consequently to the presence of pores. This causes an increase of the flip-flop rate, and an increase of permeability to substances such as sodium ascorbate. The transient increase of flip-flop rate observed in Figure 1b may be explained by these effects.

Conclusion

Several lines of evidence have been used to show the presence of pores in the DPL bilayer. The study of leakage as a function of osmotic pressure induced tension indicates a statistical nucleation process. The experimental value for the phenomenological constant γ is in agreement with values for other liquid crystals. The main difference between DPL and EL resides in the number of nucleation sites.

We suggest that these results are correlated with several observations on the coexistence of rigid and fluid phases in synthetically pure lecithin–water mixtures (Gottlieb and Eanes, 1974; Lee et al., 1974). This is analogous to cybotactic groups in liquid crystals.

The fusion and flip-flop results may be connected with the presence of pores. Fusion requires a breaking of the membrane, at least during the coalescence of the fusion vesicles. Moreover, the presence of pores in the membrane establishes a bridge between the two sides and, by means of fast lateral diffusion, permits an increase of exchange between inside and outside phospholipids, i.e. an apparently increased flip-flop rate.

It is also interesting to note that in the course of fusion of biological cells, Pasternak and Micklem (1973) observed temperature-dependent changes of the cell permeability. It is also evident that the well-known fusion inducing agent, lysolecithin, may operate by creating small defects in the bilayer.

Acknowledgments

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