KINETICS OF PROTON EXCHANGE OF PHOSPHATIDYLETHANOLAMINE IN PHOSPHOLIPID VESICLES

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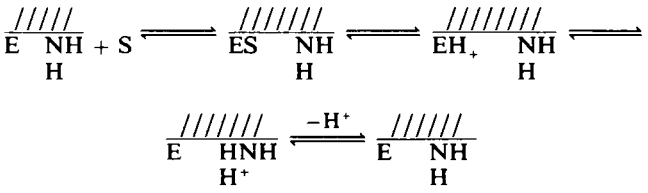
SUMMARY

The rate of proton exchange of the amino protons of phosphatidylethanolamine (PE) in sonicated mixed phospholipid vesicles has been determined by NMR spectroscopy. The rate of exchange increases with increasing pH and phosphate concentration. In the absence of buffer the dominant exchange process is an intrasurface reaction in which NH_2 groups react via water with NH_3^+ groups on the outer surface. Addition of cholesterol reduces the rate constant for intrasurface exchange. The experiments are evidence that such reactions could be dominant in proton transport in and to membrane surfaces.

The activity of an enzyme bound at or near a lipid-water interface may be very different from that of the soluble enzyme (Katchalski et al., 1971). Among the important factors for this modification are: local charge effects (Mauro, 1962), the hydrophobic character of the lipid bilayer, and the effect of localized concentration gradients on the diffusion of the substrates and products (Katchalski, 1970). In addition, it is possible to envisage a more direct role of the lipid in which the acid and base groups participate in the proteolysis reactions of the enzyme, such as in Scheme I

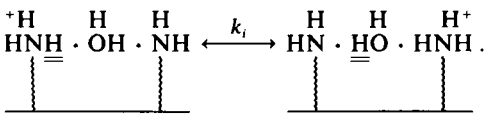


Scheme I

For simplicity only amino groups of the lipid bilayer are shown, whereas any acidic or basic group may be involved. Participation of such groups in the catalytic cycle could affect the pH optimum for the reaction and increase the reaction rate by providing a mechanism for two-dimensional proton diffusion in the plane of the lipid-water interface (Adam and Delbruck, 1968).

To learn more about the kinetics and mechanism of the proton transfer reactions of phospholipids, we have studied the rate of proton exchange of the amino protons of PE in sonicated mixed phospholipid vesicles using proton magnetic resonance. A previous study (Lange et al., 1975) has shown that the amino protons on the outside vesicle surface exchange rapidly with water protons at pH >5. The rates of exchange were determined more quantitatively in the present study by measuring the exchange broadening of the water resonance.

Because these methods may be unfamiliar, we first summarize the line of reasoning. The most important observation is an increase in the rate of transverse relaxation of the water protons in the presence of vesicles as the pH increases from 5 to 6. Well established NMR technology (primarily, study of the rate vs. radio frequency [RF] field) shows that this is a slow process, and almost certainly reflects the rate of transfer of protons between amino groups and water. This rate is too fast, by ~30 times, to be due to hydroxide catalyzed exchange. Instead, we invoke apparent transfer of a proton from an $-NH_3^+$ group to a neighboring $-NH_2$ group, via at least one bridging water molecule



Scheme II

Such a process not only transfers charge, but also exchanges at least one proton between solvent and the

amino proton pool, and is well established in small bifunctional molecules. The rate for this monomolecular process is easily estimated from the observed solvent relaxation rate weighted by the molarity of the neutral $-NH_2$ groups on the vesicle relative to the water molarity. The latter ratio is easily estimated from the known molarity of PE, the pH, and an assumed pKa of 9 for the amino groups. The rate constant k_i is thereby estimated from the data to be $>5 \times 10^6$ s^{-1}, as compared with a pseudo–first-order rate for protonation of an $-NH_2$ group of $\sim 10^3$ s^{-1} at neutral pH. Thus Scheme I describes a potent mechanism for charge transport on a PE-containing vesicle. However, we must caution that these results are fragmentary and require confirmation.

EXPERIMENTAL

Materials

Phosphatidylcholine (PC) and PE were obtained from fresh egg yolks as described by Litman, 1973. Cholesterol was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and further purified by recrystallization from ethanol. The purified lipids were shown by thin-layer chromatography each to give a single spot. PC and PE concentrations were determined, respectively, by inorganic phosphate determination (Gomori, 1942) and reaction with 2,4,6-trinitrobenzenesulfonic acid (Litman, 1973). Cholesterol concentration was determined by the method of Parekh and Jung, 1970. Vesicles were prepared by sonication, as previously described (Lange et al., 1975). PC-PE vesicles were at a final concentration of 30 mM of each lipid, and PC-PE-cholesterol vesicles were at a final concentration of 20 mM. The integrity of the PE and PC after sonication was checked by thin-layer chromatography.

4.55 atom % $H_2^{17}O$ from Miles-Yeda (Model 76326; Rehovoth, Israel) was distilled in a high vacuum system to remove nonvolatile impurities.

Measurements

Solutions were prepared by adding concentrated sodium hydroxide or phosphate buffer to the vesicle solutions by weight. The pH was determined before and after each measurement of the relaxation times. In solutions containing buffer the pH is accurate to ± 0.02, whereas in unbuffered solutions the accuracy is ± 0.07. The longitudinal relaxation time in the rotating frame ($T_{1\rho}$) and the spin lattice relaxation time (T_1) of the water resonance were measured with a magnet (model HR60; Varian Associates, Palo Alto, CA) and a pulse unit (Bruker Instruments Inc., Billerica, MA) at 56.4 MHz and a probe temperature of 25.0°C. $T_{1\rho}$ was determined by applying a 90° pulse followed by a 90° phase shift. The exponential decay was monitored by turning off the RF field for 5-ms intervals, during which the receiver voltage was sampled. The RF field strength, which varied between 505 and 5.0×10^4 rad s^{-1}, was calibrated from the time required for a 180° pulse. T_1 was measured from the recovery of the magnitization following a 180° pulse. Both measurements are accurate to ~1%.

RESULTS

The method of measurement is based upon the fact that the amino protons of PE exhibit a different chemical shift from the water protons with which they can exchange. The exchange process affects the transverse relaxation time (T_2) of the water protons (as well as the amino protons), causing exchange broadening. The use of the exchange broadening of the water resonance extends the upper limit at which specific rates may be determined to ~10,000 s^{-1} in the present study.

To measure rates of proton exchange there are advantages in using $T_{1\rho}$, the spin lattice relaxation time in the rotating frame rather than T_2. $T_{1\rho}$ is equal to T_2 when the RF field (ω_1) is zero and in the limit of large ω_1, $T_{1\rho}$ contains no contribution from NH—H_2O exchange broadening. By measuring the difference in $T_{1\rho}$ at high and low RF, the exchange broadening can be extracted. This difference is independent of the possible presence of long correlation times and of any small effect of static field inhomogeneity on the apparent $T_{1\rho}$. $1/T_{1\rho}$ of the water resonance contains contributions from several sources: broadening resulting from exchange of protons between amino sites and water, Δ_{NH}; broadening from the incomplete exchange averaged ^{17}O-H scalar coupling, Δ_O; and the intrinsic relaxation time in the absence of exchange, $T^\circ_{1\rho}$, as shown in Eq. 1.

$$1/T_{1\rho} = \Delta_{NH} + \Delta_O + 1/T^\circ_{1\rho}. \quad (1)$$

The first two terms in Eq. 1 depend upon the RF field strength (Meiboom, 1961), Eq. 2:

$$\Delta_{NH} = \frac{P_{NH}\delta^2_{NH}\tau_{NH}}{[1 + \tau^2_{NH}(\delta^2_{NH} + \omega_1^2)]}, \quad (2)$$

in which $P_{NH} = 3[NH_3]/(3[NH_3] + 2[H_2O])$, τ_{NH} is the mean lifetime of an NH proton in one cycle of exchange, δ_{NH} is the water to NH chemical shift, and ω_1 is the RF field strength, in units of radians per second (i.e., $\omega_1 = \gamma H_1$, where γ is the gyromagnetic ratio of protons, and H_1 is the radio frequency field). The value of Δ_O is proportional to the ^{17}O concentration, and is given by a similar equation with minor differences, the most important of which is that δ_{NH} is replaced by the proton-to-^{17}O spin-spin coupling constant in radians per second ~610 rad/s. The exact form does not matter because both Δ_O and $1/T_1{}^\circ_P$ could be estimated and subtracted from the measured data. This involved measuring the relaxation rate vs. ω and also vs. the concentration of added ^{17}O labeled water. Typical relaxation data of this type (Fig. 1) show strong RF field dependence, and the values for large ω_1 yield an estimate of $1/T^\circ_{1P}$ (after correction for Δ_O), while the ω_1 values for which the relaxation rate decreases give a confirmatory estimate of $1/\tau_{NH}$. The important point to note is that the definite ω_1 dependence is due to the contribution from Δ_{NH}.

To convert Δ_{NH} to a proton exchange rate it is necessary to determine which amino protons exchange with water. The exchange of water from inside to outside the vesicle is likely to be less than the NH—HOH exchange rate, and the signal from solvent inside the vesicles is negligible. Therefore we assume that the effective molarity of PE groups is 0.7 times the total molarity, equal to the approximate fraction of outside-facing PE head groups (Lange et al., 1975).

As the pH is increased in a solution containing 30 mM

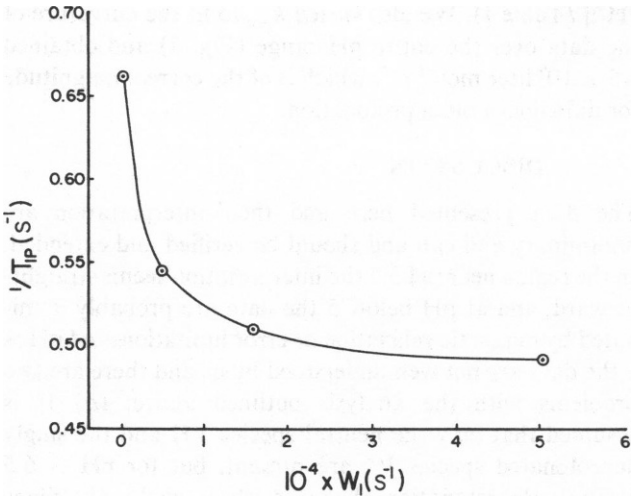


FIGURE 1 $1/T_{1P}$ vs. ω_1 for 30 mM PE, 30 mM PC at pH 5.31 at 25°.

PE and 30 mM PC, $\Delta_{NH}/(P_{NH})$ goes through a maximum as shown in Fig. 2. The maximum value with $\omega_1 = 505\ s^{-1}$ was used to calculate the NH-H_2O chemical shift, $\delta_{NH} = 1{,}040\ rad/s^{-1}$ at 56.4 MHz. This calculation is based on the simple fact that Δ_{NH}, Scheme I, is a maximum with respect to τ_{NH} when $\tau_{NH}^{-2} = \delta_{NH}^2 + \omega_1^2$, and is then $\Delta_{NH} = P_{NH}\ \delta^2/2\ (\delta_{NH}^2 + \omega_1^2)^{1/2}$. The value of δ_{NH} is in good agreement with an earlier, more accurate measurement (Lange et al., 1975) and supports the current analysis that assumes that only those amino groups on the outer surface contribute to Δ_{NH}. Using this value of δ_{NH}, we then estimate values for τ_{NH} as shown in Fig. 3. Note that the values obtained for $[H^+] < 10^{-6}$ in Fig. 3 are relatively model-independent since these correspond to $\tau_{NH} > (\delta_{NH}^2 + \omega_1^2)^{1/2}$, where $\Delta_{NH} \sim P_{NH}/\tau_{NH}$.

The specific rate of exchange increases rapidly above pH 5, and levels off near pH 7 at ~5,000 s^{-1} for vesicles both with and without cholesterol. We cannot set this limit very precisely. However, the pH dependence and magnitude of the rate is very different from that expected for a simple base catalyzed reaction. For example, a reaction of the

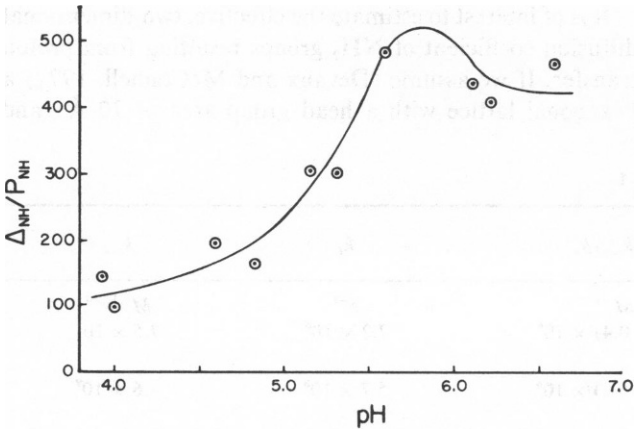


FIGURE 2 Δ_{NH}&P_{NH} vs. pH for 30 mM PE, 30 mM PC at 25°.

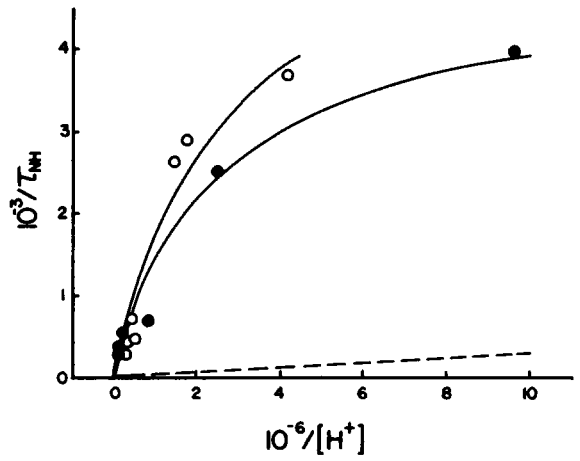


FIGURE 3 $1/\tau_{NH}$ vs. $1/[H^+]$; open circles, 30 mM PE, 30 mM PC; closed circles, 20 mM PE, 20 mM PC, and 20 mM cholesterol. Solid curves are calculated using the parameters in Table I. Dashed line is that calculated for reaction with hydroxide ion, Eq. 3.

NH_3 protonated vesicle, VH, with hydroxide ion (Scheme III) would yield a rate law (Eq. 3)

$$\underline{\text{VH}} + \text{OH}^-_{aq} \xrightarrow{k_{OH^-}} \text{V}^- + \underline{\text{HOH}}$$

Scheme III

$$1/\tau_{NH} = k_{OH}\text{-}[OH^-]/3 = k_{OH}\text{-}k_w/3[H^+], \qquad (3)$$

in which $1/\tau_{NH}$ is inversely proportional to the hydrogen ion concentration. Assuming that $k_{OH} = 1 \times 10^{10}\ M^{-1}\ s^{-1}$, $1/\tau_{NH}$ at pH 6 would be ~33 s^{-1}, while that observed is ~1,000 s^{-1} (Fig. 3). Thus, the observed rate is one and one-half orders of magnitude larger than can be explained by diffusion controlled hydroxide catalysis.

A mechanism that explains the kinetic data is as follows: we assume that a proton is removed from a vesicle by acid dissociation (Scheme IV):

$$\underline{\text{VH}} + \text{H}_2\text{O} \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} \text{V}^- + \underline{\text{HOH}_2}^+.$$

Scheme IV

Here, VH is a neutral vesicle and V^- is a vesicle containing a single neutral amino group. The species V^- can be viewed as set of many subspecies V_i^-, V_j^- differing only in the location of the neutral amino group on the surface. These subspecies interconvert by reacting rapidly with water by an intrasurface reaction as in Scheme V:

$$\underset{(V_i^-)}{\overset{+H}{\underline{\text{HNH}}}\cdot\text{OH}\cdot\text{NH}} \xleftrightarrow{k_i} \underset{(V_j^-)}{\text{HN}\cdot\overset{H}{\underline{\text{HO}}}\cdot\overset{H^+}{\text{HNH}}}.$$

Scheme V

Reaction V catalyzes NH-H_2O proton exchange if at least one water molecule participates, and here we will assume that only one does so. The number of protons exchanged in a single $VH \rightarrow V^- \rightarrow VH$ cycle depends upon the lifetime of V^- relative to the mean time between intermolecular transfers (Scheme V). This mechanism is an analogue of that found for the amino NH-H_2O exchange of hexaminoplatinum (IV) in aqueous solution (Grunwald and Fong, 1972). The rate law is

$$1/\tau_{NH} = k_a k_i/(k_i + 3nk_{-a}[H^+]), \quad (4)$$

in which the vesicle is viewed as a single species. n is the number of PE groups on the outer surface. The rate constant k_a is thus expected to be greater by a factor of n than the corresponding rate constant for deprotonation of a single amino group, whereas k_{-a} is the association rate of protons with a single neutral amino group that migrates about its surface (Scheme IV).

At low pH, for $[H^+] > k_i/3nk_{-a}$, this equation predicts a rate $\tau_{NH}^{-1} = k_a k_i/3nk_{-a}[H^+]$, which may be rearranged as k_i times the probability that any one of the three protons on a given amino group is missing. It therefore represents Scheme IV as a preequilibrium for the rate determining process of Scheme V. At much lower pH, not reached here, the rate of reprotonation (Scheme V) would exceed the apparent proton transfer rate (Eq. 4) and become rate limiting. At higher pH, for $[H^+] < k_i/3nk_{-a}$, Eq. 4 predicts $\tau_{NH}^{-1} = k_a$, and then the physical picture is that the lifetime of a deprotonated vesicle (species V^-) is so long that the neutral amino group diffuses over the entire vesicle before reprotonation occurs. Therefore, in this regime, each deprotonation results in the exchange of every proton. Multiple exchanges occurring during the lifetime of the species V^- do not produce much additional contribution to Δ_{NH} because they represent a relatively brief residence of a proton on an amino group compared with the lifetime of the neutral species VH.

The best fit of the rate law gives the values in Table I. To evaluate the rate constants we must make some assumptions about the equilibrium constant for Scheme IV. k_a/k_{-a} should be given by $10^{-9}\,n$, in which n is the number of amino groups on a vesicle and 10^{-9} is a reasonable assumption of their acid dissociation constant ($pK_a \sim 9$). This yields a direct value for k_i from the data around pH 5.5, where τ_{NH} is experimentally inversely proportional to $[H^+]$ (Table I). We also varied k_{-a} to fit the curvature of the data over the entire pH range (Fig. 3) and obtained $\sim 5 \times 10^9$ liter mol^{-1} s^{-1}, which is of the correct magnitude for diffusion-limited protonation.

DISCUSSION

The data presented here and their interpretation are preliminary and can and should be verified and extended. In the region near pH 5.5 the interpretation seems straightforward, and at pH below 5 the data are probably dominated by magnetic relaxation or error limitations. At pH > 6 the data are not well understood by us and there are two problems with the analysis outlined above: (*a*) It is assumed that only the neutral species VH and the singly deprotonated species V^- are present, but for pH > 6.5 multiple deprotonation of the vesicle is likely. (*b*) Since these experiments were done at low salt concentration, a possible net charge on the vesicle could yield misleading results. If there had been some hydrolysis of the ethanolamine side chain during preparation, the vesicle would be slightly negatively charged and near neutral pH a counterion charge layer of protons might neutralize this charge and give roughly pH-independent exchange.

Despite these problems, the evaluation of the effective proton transfer rate k_i inferred as described, mainly from data for pH ~5.5, seems relatively straightforward, is consistent with model studies, and is the best such estimate known to us. It is at least two orders of magnitude faster than is possible to explain by bimolecular reaction with hydroxide ion.

It is useful to compare the rate of apparent charge transfer $k_i = 7 \times 10^6$ s^{-1} with the reprotonation rate $k_{-a}[H^+]$, which is $\sim 7 \times 10^2$ at pH 7. Their ratio, 10^4, is the estimated average number of jumps that a neutral proton vacancy will make before being annihilated by a proton from solution. Therefore, a proton that disappears by being pumped actively through a membrane could be replenished by apparent transfer from neighboring NH_3 groups (Scheme V), and the group of 10^4 amino protons surrounding such a pump could act as an antenna to replenish the proton from solution.

It is of interest to estimate the effective, two-dimensional diffusion coefficient of NH_2 groups resulting from proton transfer. If we assume (Devaux and McConnell, 1972) a hexagonal lattice with a head group area of 70 $Å^2$, and

TABLE I

Vesicle composition	k_a	$3nk_{-a}/k_a$	k_i	k_{-a}
	s^{-1}	M^{-1}	s^{-1}	$M^{-1}s^{-1}$
PE, 30 mM PC, 30 mM	6240 ± 610	$(2.6 \pm 0.4) \times 10^6$	7.2×10^6	7.5×10^9
PE, 20 mM PC, 20 mM Cholesterol, 20 mM	4970 ± 260	$(2.6 \pm 0.3) \times 10^6$	5.7×10^6	6×10^9

that PE head groups are next nearest neighbors, we estimate $D = 5.8 \times 10^{-8}$ cm^{2}s^{-1} for PE/PC vesicles and 4.6×10^{-8} cm^{2}s^{-1} for PE/PC/cholesterol vesicles. Both estimates are somewhat larger than the diffusion coefficient of a spin label in di-dihydrosterculoylphosphatydlcholine $\sim 1.8 \times 10^{-8}$ cm^{2}s^{-1} (Devaux and McConnell, 1972). A possible explanation for such values of the diffusion coefficients is that the proton transfer may involve several water molecules so that the distance between nitrogen atoms may be as large as 9–10 Å during the proton transfer (Grunwald and Ralph, 1975).

Although the accuracy is not high, it is clear that the addition of cholesterol causes a small decrease in k_i (Fig. 3). Since the average density of amino groups on the surface of a vesicle is likely to decrease when the phospholipids are diluted with cholesterol, it would be expected that the rate of intramolecular proton transfer should be reduced when cholesterol is added.

The intrasurface proton transfer process provides a facile mechanism for the type of proton fluctuation postulated by Kirkwood and Shumaker, 1952. The rate constant is much larger than thought possible by some workers (Lumry and Yue, 1965; Scheider, 1965), who have considered only bimolecular reactions. Indeed, solvent water molecules are particularly adept at bridging acid and base centers, thereby catalyzing intramolecular proton exchange.

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