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# A NMR STUDY OF THE IONIZATION OF FATTY ACIDS, FATTY AMINES AND *N*-ACYLAMINO ACIDS INCORPORATED IN PHOSPHATIDYLCHOLINE VESICLES

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## Summary

The ionization of fatty acids, fatty amines and *N*-acylamino acids incorporated in phosphatidylcholine single-walled vesicles has been measured. The guest molecules have been specifically enriched with $^{13}C$ and titrated by using NMR spectroscopy. The apparent p$K_a$ of fatty acids in phosphatidylcholine bilayers is 7.2—7.4 and those of fatty amines are approx. 9.5. These p$K_a$ values depend on many different parameters related to the structure of the lipid/solution interface, to the composition of the aqueous medium and to the localization of the ionizable groups. A special sensitivity to the ionic strength and to the surface charge has been found. A positive surface charge decreases the p$K_a$ value whereas a negative one increases it, the total range of variation being 2.5—3 units. In a qualitative macroscopic interpretation, it is proposed that p$K_a$ is essentially determined by the low polarity of the lipidic matrix.

## Introduction

In a phospholipid bilayer, the network of polar heads forms an interface separating the very hydrophobic chain region from the surrounding aqueous medium. This interface mediates the interactions between the bimolecular leaflet and water, ions, exogenous molecules and also, to a large extent, extrinsic proteins. In spite of different attempts to determine the conforma-

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tions of polar heads [1—4], their hydration [5,6] and their interactions with ions [7,8], the organization and the dynamics of the bilayer surface remain poorly understood. In the present work, we investigate the ionization of fatty acids, fatty amines and *N*-acylamino acids incorporated in phosphatidylcholine bilayers. As previously shown [9], such guest molecules can reveal local properties of the polar heads region in which their ionizable groups are anchored. Interfacial ionization phenomena have been previously investigated by different authors by using spin-labeled probes [10,11], $^{13}$C-labeled probes [9], fluorescent probes [12] or by determining the thermodynamical properties of pure phospholipid phases [13]. We have specially developed the use of $^{13}$C-labeled molecules which allows intrinsic nuclear magnetic resonance (NMR) titrations of these molecules embedded in single-walled vesicles.

Additional interest arises from the study of fatty acids since they are minor components of biological membranes. They are intermediates in phospholipid metabolism and it is thought they are involved in proton transport [14,15] and in fusion of lipid vesicles [16].

## Materials and Methods

Phosphatidylcholine was prepared from hen egg yolk (I.N.R.A., ref. PA12) by the method of Singleton [17] and stored under nitrogen at —20°C. Its purity was regularly checked by thin-layer chromatography. 90% enriched [1-$^{13}$C]stearic, and myristic acids were prepared by carbonation of the Grignard reagent of corresponding bromoalcanes (Ref. 9 and Bouloussa, O., unpublished account). Enriched stearic and palmitic acids were also purchased from Stohler Isotope Chemicals (Waltham, USA) and from CEN Saclay (France), respectively. Octadecylamine was approx. 45% $^{13}$C-enriched on the $\alpha$-$CH_2$ group by reduction of enriched octadecane nitrile with lithium aluminium hydride. *N*-Palmitoylglycine and *N*-palmitoyl L-alanine were prepared by condensing palmitoyl chloride and 90% enriched [1-$^{13}$C]glycine or L-[1-$^{13}$C]alanine (CEN Saclay products). *N*-Palmitoyl-DL-glutamic acid was prepared by condensing palmitoyl chloride with glutamic acid specifically labeled with $^{13}$C on the $\alpha$- or the $\delta$-COOH group (Bouloussa, O., unpublished account). Purity of all labeled compounds was checked, especially by mass spectrometry and $^{13}$C-NMR. A chloroform solution of lipid probes and phosphatidylcholine (1—16 mol% lipid in phosphatidylcholine according to experiments) was first evaporated to dryness. The film was then dispersed in a buffer solution and homogeneous solutions of mixed unilamellar vesicles were prepared by sonication of refrigerated samples and centrifugation. Mixed micelles were prepared by heating a 0.1 M detergent solution of sodium tetradecyl sulfate (STS), cetyltrimethylammonium bromide (CTAB) or of purified Triton X-100 containing $5 \cdot 10^{-3}$ M of myristic acid or of stearylamine, to above the dissolution temperature.

Proton decoupled $^{13}$C spectra were recorded at 22.63 MHz with a WH90 Bruker spectrometer operating in the pulse Fourier transform mode. Approx. 30 000 transients were accumulated in most experiments. Proton decoupled $^{31}$P-spectra were recorded at 36.43 MHz with the same equipment. In some $^{13}$C experiments, a partial precipitation occurs after some hours. That does not obscure the well resolved spectra of single-walled vesicles remaining in solution.

## Results

### *Ionization of fatty acids and fatty amines*

Intrinsic titrations of carboxylic groups and of amine groups were achieved by measuring the variations of $^{13}C$ chemical shifts of R-*COOH ($\Delta\delta \approx 5.2$ ppm) and R-*$CH_2$-$NH_2$ ($\Delta\delta \approx 0.65$ ppm) carbon nuclei with pH. An apparent $pK_a$ is then defined by the bulk pH value corresponding to half dissociation. For fatty acids incorporated in phosphatidylcholine vesicles, the $pK_a$ is 7.2–7.4 (0.1 M phosphate buffer, 310 K), i.e., approx. 2 units higher than the p$K$ of isolated molecules (approx. 5) (Fig. 1). For stearylamine, the $pK_a$, approx. 9.5, is more than one unit lower than the normal p$K$, approx. 10.7 (Fig. 1).

Considering the dissociation: R-COOH $\rightleftharpoons$ R-COO$^-$ + H$^+$ as fast on the NMR time scale, the measured chemical shift of the acidic group is:

$$\delta_{\text{mes}} = \delta_{\text{R—COO}^-} + [\delta_{\text{R—COOH}} - \delta_{\text{R—COO}^-}]\left[\frac{1}{1 + K/[\text{H}^+]}\right]$$

where $K$ is the equilibrium constant. The titration curves are conveniently represented by plotting $\alpha = f(\text{pH})$ where $\alpha$ is the dissociation coefficient:

$$\alpha = \frac{[\text{R—COO}^-]}{[\text{R—COO}^-] + [\text{R—COOH}]} = \frac{\delta_{\text{mes}} - \delta_{\text{R—COOH}}}{\delta_{\text{R—COO}^-} - \delta_{\text{R—COOH}}} = \frac{1}{1 + 10^{pK-\text{pH}}}\,.$$

These classical equations apply normally to simple Hasselbad acid-base equilibria in bulk media. If interfacial ionizations are described by such equilibria, the titration curves obey the previous relationship in which pH = bulk pH and p$K$ = $pK_a$. Small differences between calculated and experimental curves are observed only in the extreme parts of these curves (Figs. 1 and 3).

Interfacial acid-base equilibria should depend on many different parameters: temperature, concentration, ionic strength, permittivity, surface charge, electrical field, molecular interactions and dynamics of the interface. The effects of several of these parameters were tested and the results are summarized in Table I.

As previously described [9], the $pK_a$ of fatty acids decreases when the temperature increases: typically the $\Delta pK_a$ is approx. 0.15 unit for $\Delta T \approx 10$ K (stearic acid). Small effects of concentration were also observed for fatty acids: the $pK_a$ increase is approx. 0.2 unit when the fatty acid : lecithin molar ratio increases from 1% to 15%. A $pK_a \approx 7.45$ was found for stearic acid when $^2H_2O$ was used instead of $H_2O$ with 0.1 M sodium phosphate buffer (the pH was defined by meter readings). This value is somewhat lower than those given by Kantor and Prestegard [18]. (These authors reported only the change of the chemical shift ($\delta$ ppm) with pH and did not find any effect of ionic strength, a result which disagrees with the results reported here.) The $pK_a$ depends also on the buffer: $\Delta pK_a \approx 0.1$ unit was observed when sodium carbonate buffer was used instead of sodium phosphate buffer or when potassium phosphate replaced sodium phosphate. A more significant difference is obtained with a borax buffer in which the $pK_a$ is approx. 0.5 unit higher than in sodium phosphate buffer.

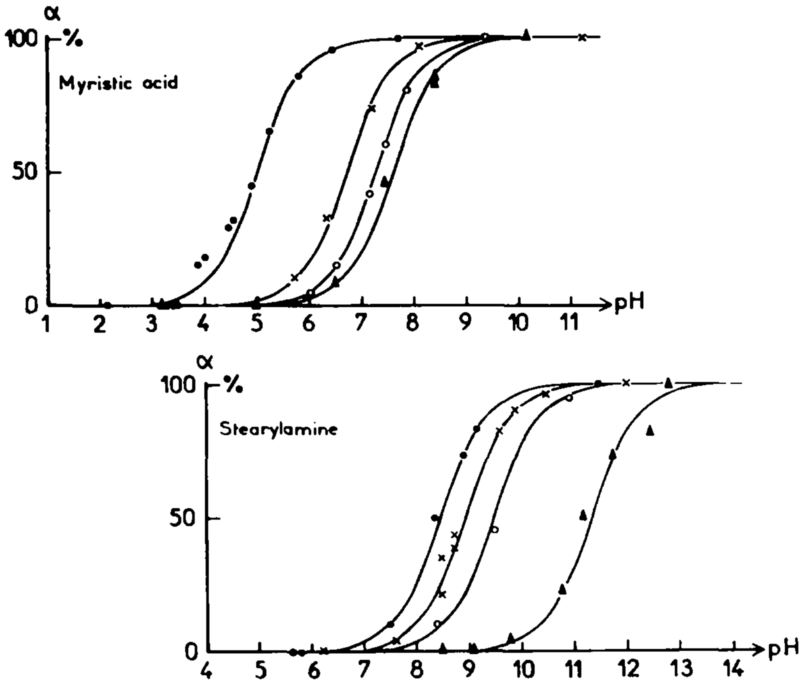


Fig. 1. Top, NMR titrations of myristic acid in single-walled phosphatidylcholine vesicles (○) and in detergent micelles. The dissociation coefficient α deduced from the variations of the chemical shift of the $^{13}C$-enriched carboxylic group is plotted versus the bulk pH. $T$ = 300 K, 0.1 M phosphate buffer in $H_2O$, 3.3 mol% fatty acid in phosphatidylcholine, 4 mol% fatty acid in detergents. Bottom, NMR titrations of stearylamine. $T$ = 300 K, 16 mol% fatty amine in phosphatidylcholine, 5 mol% fatty amine in detergents, 0.1 M phosphate buffer and 0.2 M carbonate buffer (pH > 9) in $H_2O$. CTAB, cetyl trimethyl ammonium bromide micelles (●); STS, sodium tetradecyl sulfate micelles (▲); PC, phosphatidylcholine vesicles (○); Triton X-100 micelles (X).

The effect of ionic strength is represented in Fig. 2. The $pK_a$ increases nearly linearly when the ionic strength decreases from $10^{-1}$ M to $10^{-3}$ M.

Dramatic changes in $pK_a$ values were induced by the surface charge. Nega-

TABLE I

STS, sodium tetradecyl sulfate; CTAB, cetyl trimethylammoniumbromide.

| Environment (*, 0.1 M buffer) | Myristic acid | | Stearic acid | | Stearylamine | |
|---|---|---|---|---|---|---|
| | $pK_a$ | $T$ (K) | $pK_a$ | $T$ (K) | $pK_a$ | $T$ (K) |
| $H_2O$ solution | ≈5.0 | | ≈5.0 | | ≈10.7 | |
| * CTAB micelles | 5.0 | 310 | 5.0 | 310 | 8.45 | 320 |
| * Triton X-100 micelles | 6.7 | 310 | | | 8.95 | 320 |
| * STS micelles | 7.65 | 310 | | | 11.25 | 320 |
| * Phosphatidylcholine vesicles | 7.25 | 300 | 7.4 | 300 | ≈9.5 | 302 |
| | | | 7.25 | 310 | | |
| * Phosphatidylcholine vesicles + 20% stearylamine | | | 6.1 | 300 | | |
| * Phosphatidylcholine vesicles + 10% phosphatidic acid | | | 7.6 | 300 | | |

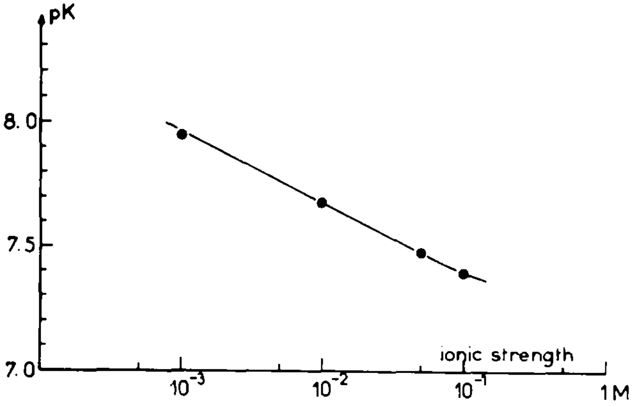


Fig. 2. Variations of $pK_a$ of 5 mol% stearic acid in single-walled phosphatidylcholine vesicles with ionic strength (phosphate buffer in $H_2O$).

tively charged vesicles were prepared by adding 10 mol% phosphatidic acid to neutral phosphatidylcholine. The $pK_a$ of stearic acid incorporated in such vesicles is increased approx. 0.2 unit. Unfortunately, the ionization of phosphatidic acid itself and a decreasing stability of vesicles for pH more than 8 make rather difficult a complete titration of the fatty acid probes. Positively charged vesicles were prepared by adding 20 mol% of stearylamine to phosphatidylcholine at pH less than 9. The $pK_a$ of fatty acids is then decreased approx. 1.3 unit. The surface charge effects were qualitatively confirmed by measuring the ionization of fatty acids and fatty amines incorporated in detergent micelles according to a procedure previously used by Fromherz [12]. The results reported in Table I and in Fig. 1 clearly show that a positively charged surface drives the $pK_a$ towards low values whereas a negatively charged surface drives it towards high values. The $\Delta pK_a$ is approx. 2.6 units for myristic acid and 2.8 units for stearylamine. For these two kinds of probe, the $pK_a$ is approx. 0.5 unit higher in neutral phosphatidylcholine vesicles than in Triton X-100 neutral micelles.

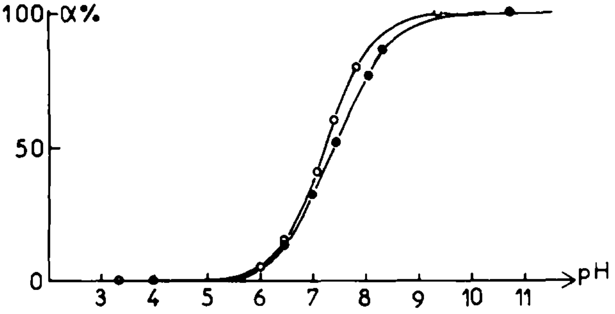


Fig. 3. NMR titrations of myristic (C 14 chain) acid (○) and stearic (C 18 chain) acid (●) in single-walled phosphatidylcholine vesicles. $T = 300$ K, 0.1 M phosphate buffer in $H_2O$. The full line follows the theoretical law: $\alpha = 1/1 + 10^{pK_a - pH}$ for myristic acid, but does not for stearic acid.

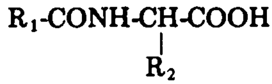
TABLE II

CTAB, cetyl trimethylammonium bromide.

| Environment | Pal-*N*-Gly | | Pal-*N*-L-Ala | | Pal-*N*-D,L-Glu | | | |
|---|---|---|---|---|---|---|---|---|
| | p$K_a$ | *T* (K) | p$K_a$ | *T* (K) | $^{\alpha}$COOH | | $^{\delta}$COOH | |
| | | | | | p$K_a$ | *T* (K) | p$K_a$ | *T* (K) |
| Dipeptide in $H_2O$ | Gly-Gly | | L-Ala-L-Ala | | L-Glu-L-Glu | | L-Glu-L-Glu | |
| | 3.1 | 298 | 3.35 | 298 | 3.14 | | 4.38 | |
| CTAB micelles (0.1 M citrate/ phosphate buffer) | | | 3.0 | 300 | | | | |
| Phosphatidylcholine vesicles (0.1 M citrate/phosphate buffer) | 5.2 | 300 | 5.25 | 300 | 4.65 | 300 | ≈6.3 | 300 |

As shown in Fig. 3, the position and the shape of the titration curves also depend on the length of the hydrocarbon chain of fatty acids.

*Ionization of N-acylamino acids*

The p$K_a$ of end carboxylic groups of  $R_1\text{-CONH-}\underset{\substack{|\\ R_2}}{\text{CH}}\text{-COOH}$ molecules ($R_1$ = $C_{15}H_{31}$. $R_2$ = H : Gly, $R_2$ = $CH_3$ : Ala) incorporated in neutral single-walled phosphatidylcholine vesicles is 5.2 (Table II). This value is higher than those found for dipeptides Gly-Gly or Ala-Ala, even if one takes into account the decreasing effect (<0.4 unit) due to end $NH_3^+$ group [19].

These two lipoamino acids act differently on the stability of single-walled vesicles solutions. *N*-Palmitoyl glycine induces partial precipitation below the p$K_a$ and two slightly different NMR signals are detected. On the other hand, there is no precipitation and no supplementary signal with *N*-palmitoyl-L-alanine.

The titration curves of the α and δ carboxylic groups of *N*-palmitoyl-DL-glutamic acid are presented in Fig. 4. The ionization of the δ group is very

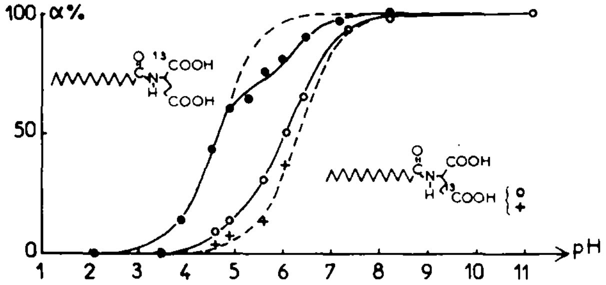


Fig. 4. NMR titration curves of 10 mol% *N*-palmitoyl-DL-glutamic acid in single-walled phosphatidylcholine vesicles. *T* = 300 K, 0.1 M citrate/phosphate buffer in $H_2O$.

clearly detected by the $\alpha$ group in a range of pH 5—7. As expected, the p$K_a$ values are higher than those obtained with a dipeptide in aqueous solution. Two slightly different resonances were often observed for the $\delta$ group. It must be noted that a remarkable stability is observed for solutions containing phosphatidylcholine vesicles in which some percent of *N*-palmitoyl-DL-glutamic acid were incorporated.

## Discussion

Schematically, the p$K_a$ of acids and amines inserted in phosphatidylcholine vesicle walls depends on: (a) the environment of ionizable groups; (b) local $H^+$ concentrations. The localization of guest molecules within the phospholipid bilayer must then be discussed first.

(1) The incorporation of spin-labeled fatty acids in vesicle walls was well demonstrated in many ESR experiments: (a) the ESR spectra are typically those of molecules oriented in a bilayer; (b) the $^{13}C$-resonances of surrounding phospholipid molecules are broadened through interaction with the paramagnetic nitroxide radical of fatty acid probes [20]. Our own experiments with spin-labeled fatty acids and also spin-labeled *N*-acylamino acids confirm the same result in the total range of pH, i.e., for ionized as well as for protonated molecules. Nevertheless, it must be mentioned that for palmitic acid (C 16 chain) a small ESR signal (less than 3% of the total intensity) attributed to the free form is observed at a very basic pH of approx. 11. Such partition was never observed for stearic acid (C 18 chain). This suggests that, for decreasing chain length, spin-labeled ionized fatty acids can partition between phosphatidylcholine vesicles and solution (even for low concentrations of 1—3 mol% of these fatty acids).

In NMR experiments, in which normal fatty acids are used, there is no evidence of partition between different environments. A single narrow line is always observed in $^{13}C$-NMR and the p$K_a$ values reflect well changes in the constitution of the lipidic matrix and in surface charge. Nevertheless, it must be mentioned that a fast equilibrium on the NMR time scale between incorporated and free forms would be undetectable. Furthermore, the absence of a polar nitroxide group should increase the hydrophobicity of the aliphatic moiety of ionizable probes.

Some restrictions may concern *N*-acylamino acids for which two NMR signals were detected in several experiments. The origin of these two signals was not elucidated. They could be due to an incomplete incorporation of lipoamino acids in vesicles or to heterogeneous structure of mixed vesicles.

(2) The possibility of segregation of fatty acids incorporated in phospholipid bilayers has been often discussed. The absence of segregation for spin-labeled fatty acids is well demonstrated by the absence of characteristic broadened ESR spectra due to spin-spin interactions. Segregation of normal fatty acids in their protonated form was recently proposed by Hauser et al. [21,22]. This assumption was essentially based on the incomplete ionization (in a range of pH 6—11) of fatty acids incorporated in multilamellar liposomes, this ionization being detected by electrophoretic mobility measurement and $Ca^{2+}$ binding. As incomplete ionization is also observed in the same range of pH for mono-

layers of pure fatty acids [23], these authors suggested clustering of fatty acids in phosphatidylcholine. ESR experiments [11] prove that the $pK_a$ of unaggregated spin-labeled fatty acids incorporated in phosphatidylcholine is strongly shifted: $pK_a \approx 6.15$ in multilamellar structures (approx. 35% water), $pK_a \approx 6.55$ in unilamellar vesicles.

The present study confirms the strong dependence of $pK_a$ on the environment created by the phospholipid matrix. More generally, the conditions of ionization are certainly different in pure fatty acid monolayers, mixed monolayers, multiwalled liposomes and single-walled vesicles. A high $pK_a$ cannot then be considered as a definitive proof of clustering. A perfect miscibility of fatty acids and phosphatidylcholine was found by Torosian and Lemberger [24] in monomolecular films spread on double-distilled water. In these conditions, the bulk pH should be approx. 5.5—6 and the fatty acids should be mainly protonated. From the phase diagram of dimyristic phosphatidylcholine-myristic acid multilamellar dispersions, Kantor and Prestegard found no evidence of segregation of fatty acids [18]. The phase diagram of the dipalmitoyl phosphatidylcholine-palmitic acid system established by Mabrey and Sturtevant [25] does not show the characteristic shape found for systems in which lateral phase separation occurs.

A clustering of fatty acids should involve an ionization mechanism more complex [23] than a simple dissociation: $AH \rightleftharpoons A^- + H^+$, i.e., very pronounced concentration effects and pronounced deviations of the experimental titration curves compared to calculated ones. The small effects observed here for single-walled vesicles do not support the formation of broad patches in which such complex mechanism should occur. Furthermore, the similarity in behaviour of fatty acids in phosphatidylcholine vesicles and in detergent micelles demonstrates that the $pK_a$ is mainly determined by the lipid or the detergent environment and not by a segregation phenomenon.

(3) The use of a shift reagent like $Pr^{3+}$ does not allow direct determination of the distribution of fatty acid molecules between the internal and external layers of vesicle walls. At acidic pH, there is a flip-flop of protonated fatty acids from one side to another (Sanson, A. et al., unpublished results) which prevents perturbation of carboxylic groups anchored on one side. Clearer conclusions are drawn by observing the shifts induced by $Pr^{3+}$ on $^{31}P$-resonance lines of phospholipid polar heads. The internal as well as the external $^{31}P$ signals are shifted differently when fatty acids are incorporated in vesicles, which suggests that these fatty acids are present on the two sides of the bilayer.

(4) Most of the attempts to interpret interfacial ionization in detergent micelles [12], lipid monolayers [23] and phospholipid bilayers [13] are based on a macroscopic description in which the electrical potential $\Phi_0$ and the activities of ions are the fundamental parameters.

Indeed, a microscopic description of the structure and dynamics of the interface is extremely difficult even for simple systems formed by homogeneous lipids [23]. Such a description is no longer possible for phosphatidylcholine interfaces. In multilamellar structures, the choline groups are thought to be tilted nearly parallel to the plane of the bilayer [2,3]. There is no evidence that such conformations are predominant in the curved bilayer forming a vesicle wall which requires, in addition, different packings inside and outside.

The hydration of choline groups and possibly of the glycerol backbone involves approx. 20 molecules of water [6]. The distribution of anions and cations in this region of the bilayer remains unknown. Furthermore, the exact location of ionizable guest groups, especially with regard to the phosphate group, is rather difficult to specify.

On the other hand, a simplified macroscopic model as proposed by Fernandez and Fromherz [12] can account for interfacial dissociations. Two acid-base equilibria are considered:

$$AH \rightleftharpoons A^- + H^+ \quad (1) \qquad BH^+ \rightleftharpoons B + H^+ \quad (2)\,.$$

By considering $A^-$ or B inserted in a neutral membraneous matrix and $H^+$ in the bulk phase, the difference between the $pK_i$ at the interface and the $pK_w$ in bulk water is written:

$$\Delta pK_i = pK_i - pK_w = \frac{1}{2.3RT}(\mu^{0m}_{A^-} - \mu^{0w}_{A^-}) - \frac{1}{2.3RT}(\mu^{0m}_{AH} - \mu^{0w}_{AH})\,.$$

$\mu^{0m}$ and $\mu^{0w}$ are the standard chemical potentials respectively referred to membrane and aqueous solution.

The above is consistent with a $\Delta pK_i > 0$ for fatty acids and a $\Delta pK_i < 0$ for fatty amines. A fundamental hypothesis is to consider the interfacial proton activity $a_i^{H^+}$ equal to the bulk proton activity $a_w^{H^+}$. When the dissociation occurs inside the matrix, a supplementary $\Delta pK$ is introduced by $(\mu^{0m}_{H^+} - \mu^{0w}_{H^+})$, i.e., by the energy difference of the proton in the membrane and in the aqueous phase. In detergent micelles, Fernandez and Fromherz concluded that the $pK_a$ was mainly determined by the local low polarity of the matrix and that there was no supplementary electrical potential. The difference of $pK_a$ determined in neutral (zwitterionic) phosphatidylcholine bilayers and in neutral (nonionic) Triton X-100 micelles (Fig. 1) could be due to such a potential induced by a special arrangement of polar glycerol backbone and choline groups.

When there is a surface charge, the potential $\Phi_0$ creates a $H^+$ concentration gradient: $[H^+]_i = [H^+]_b \exp(-e\Phi_0/kT)$, where $[H^+]_i$ = interfacial concentration, $[H^+]_b$ = bulk concentration. In spite of many simplifications, a Gouy-Chapman model accounts approximatively for ionizations in detergent micelles [12] and in non-zwitterionic phospholipid lamellar structures [13]. Such surface charge effects are reflected by the $pK_a$ of guest molecules in charged vesicles or micelles. However, an acceptable value of the $\Phi_0$ potential cannot be proposed if the exact position of the ionizable group with electrical field remain unknown.

The ionic strength effect observed in neutral phosphatidylcholine vesicles can be explained by changes in the chemical potential, $\mu^{0w}$, of the probes and possibly of $H^+$. Changes in the structure of the interface (polar head conformation, ion distribution) can also contribute to such effect.

(5) The internal volume of a vesicle diameter of approx. 200 Å is only = $9 \cdot 10^{-22}$ l. Such a small volume contains only some dozens of ions (0.1 M ionic strength) and one cannot define a pH in the usual sense. All ESR and NMR experiments show that there is no important difference between the degrees of ionization of internal and external guest molecules. Furthermore, $^{31}P$ NMR of an internal phosphate buffer demonstrates that its dissociation is equal to those

measured in the external bulk medium (Prigent, Y. and Gary-Bobo, C., personal communication). Formation of vesicles maintains the same conditions of dissociation for the buffer and surface probes in the internal microphase as that in the bulk medium, in spite of fundamental differences in their thermodynamical and kinetic properties. On the other hand, ion and $H^+$ transport through bilayers closing such small vesicles should involve very special mechanisms (Sanson et al., unpublished results).

## Conclusion

This qualitative approach of interfacial ionization in phospholipid bilayers displays the complexity of phenomena occurring at the interface formed by the choline polar heads. Changes in the constitution and in the charge of these polar heads should complicate more these phenomena. In a general way, the insertion of an ionizable molecule in a neutral bilayer induces appreciable shifts in its apparent $pK_a$ which is partly determined by the low polarity of its environment. This shift is modulated by many other factors which usually affect the acid-base equilibria or which more specifically modify the guest-bilayer interactions and the structure and the dynamics of the interface.

Positive and negative surface charges respectively decrease and increase the $pK_a$, the total shift is approx. 2.5—3 units for molecules investigated here.

It is very tempting to consider the biological relevance of such properties. The extreme sensitivity of interfacial ionization to the bilayer structure and to surface charge in a range of pH 6—9, suggests that pH and interactions with ions are factors which could modulate very effectively local interactions between phospholipids and ionizable molecules.

The present investigations are now carried on with special reference to pH gradient effects, proton transport by fatty acids and interactions between phosphatidylcholine vesicles induced by low concentrations of detergent, all phenomena involving interfacial ionization.

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