IONIZATION OF FATTY ACIDS AT THE LIPID–WATER INTERFACE

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1. Introduction

As demonstrated for phosphatidic acid [1] and for phosphatidyl serine [2], thermodynamic stability of lipid phases strongly depends on the ionization of the polar heads. Interfacial ionization phenomena must also be considered in interactions between ionizable extrinsic molecules and phospholipids as they could interfere in membrane stability and membrane function, especially in proton and ion transport. Fluorescent pH indicators have been used [3] to determine interfacial ionization. We use fatty acids as ionizable probes to investigate the interface in which the carboxylic group is anchored. For electron spin resonance (ESR) experiments, stearic acid was spin-labelled on the C5 position. For nuclear magnetic resonance (NMR) experiments, stearic and palmitic acids were selectively enriched with ^{13}C on the carboxylic group. These probes were incorporated in egg lecithin vesicles or multilayers, in which the zwitterionic phosphatidyl choline head groups define a neutral host system for the carboxylic group.

2. Experimental

Phosphatidyl choline was extracted from hen egg yolk (INRA ref. PA 12) as in [4] and stored under nitrogen at −20°C. Purity was checked by thin-layer chromatography. The 5-doxylstearic acid was purchased from Syva Co., Palo Alto, CA. ^{13}C-enrichment of the carboxylic group of stearic acid was achieved by carbonation of the Grignard reagent of bromoheptadecane [5]. The residue was dissolved in ligroin and the labelled fatty acid was purified by column chromatography on silica gel. ^{13}C NMR allows to control its purity and to determine the degree of enrichment (40–90%, depending on the enrichment of $BaCO_3$). 90% enriched [1-^{13}C]palmitic acid was purchased from C. E. N. Saclay, France. A chloroform solution of lecithin and of the fatty acid probes in the required amount (8 : 1000 molar ratio in ESR experiments, 1 to 16 : 100 molar ratio in NMR experiments) was first evaporated to dryness. Homogeneous vesicles 200–300 Å diameter were then prepared by sonication [6,7] using an ice bath and a nitrogen flux to avoid degradation. After chromatography on Sepharose 4B column, ESR samples were concentrated to 10–20 mg/ml. NMR samples (60–70 mg/ml) were only centrifugated at 130 000 × g for 30 min. The ESR spectra were recorded on a Varian V-4502 spectrometer. The ^{13}C NMR spectra (22.63 MHz) were recorded on a Bruker WH 90 spectrometer operating in the pulsed Fourier transform mode with complete proton noise decoupling. After several hours, partial precipitation of samples at alkaline pH occurs. The broad NMR lines of liposomes and precipitated particles do not obscure the narrow lines of small vesicles. In all experiments, the temperature was carefully controlled and the pH was measured before and after spectra recording.

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3. Results

3.1. *ESR experiments*

The existence of 2 different ESR spectra ascribed to the ionized and unionized forms of the spin-labelled stearic acid has been demonstrated for multilamellar samples of egg lecithin [8]. Two spectra were also detected with the same probe incorporated into sonicated vesicles, at sufficiently high temperature (fig.1). The carboxylic group was then indirectly titrated by measuring the relative magnitude of these 2 signals for different values of the bulk pH. The bulk pH value corresponding to 50% ionization defined an app. pK_a 6.55 which was more than one unit higher than the normal pK of stearic acid ($\simeq$ 4.9). For vesicles this pK_a was about 0.4 unit above that of multilayers (fig.3).

3.2. *NMR experiments*

NMR made it possible to titrate directly the carboxylic group by measuring the $^{13}\underline{C}$*OO(H) chemical shift. For multilamellar samples, line broadening prevented accurate measurements. On the other hand, a single narrow line was detected for stearic or palmitic acids incorporated into sonicated vesicles (fig.2). From the corresponding titration curve (fig.3) an app. pK_a 7.25 was deduced, which was higher than the pK_a measured by ESR. The NMR titration curve depended only slightly ($\simeq$ 0.2 pH unit) on the probe concentration in a broad range:

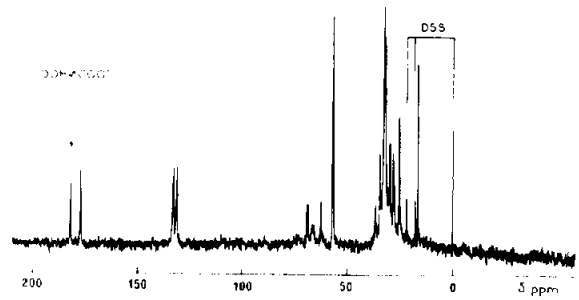

Fig.2. Typical ^{13}C NMR spectrum of 90% [1-^{13}C]stearic acid incorporated in sonicated vesicles of egg yolk lecithin. pH 7.2; T 27°C; stearic acid : lecithin, 1 : 100 mol; lecithin, 90 mg/ml; 40 000 transients. The main part of the spectrum is that of the [^{13}C]lecithin. The $^{13}\underline{C}$*OO(H) resonance and the extra peaks of the external reference DSS are assigned.

$1\% < C < 16\%$ (molar). The pK_a value decreased when the temperature was increased: $\Delta pK_a/°C \simeq 0.01$. It did not depend on the bulk ionic strength in a range: 0.1–1.1 M. No difference was detected between stearic and palmitic acids.

4. Discussion

4.1. *The intrinsic titration performed with ^{13}C NMR clearly confirms the ESR data:*

The pK of the fatty acids incorporated into lecithin bilayers is strongly shifted towards higher values, when

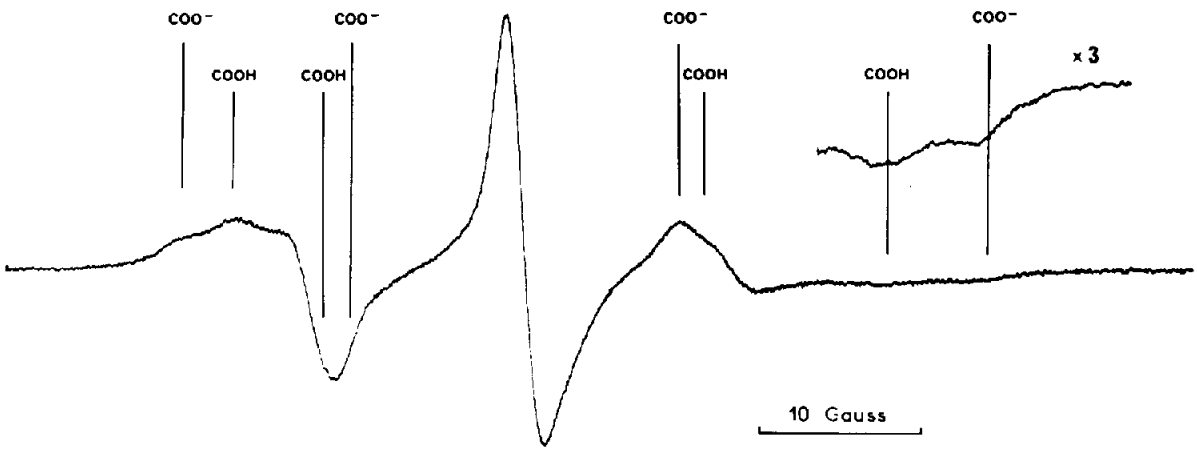

Fig.1. ESR spectrum of 5-doxylstearic acid incorporated in sonicated vesicles of egg yolk lecithin. pH 6.2; T 47°C; stearic acid : lecithin, 8 : 1000 mol; lecithin, 10 mg/ml. The spectrum is the sum of 2 spectra ascribed to the RCOOH and $RCOO^-$ forms.

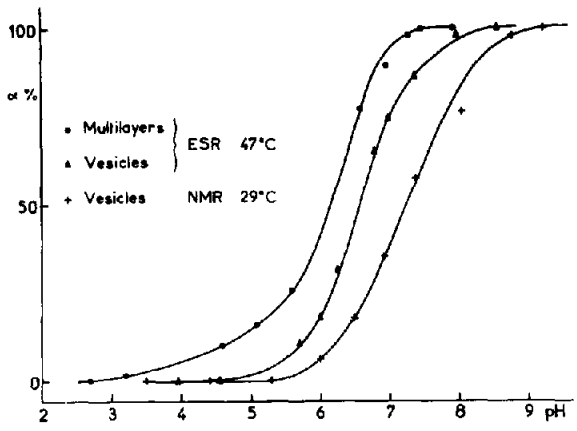

Fig.3. Titration curves of fatty acids incorporated in multilayers or in sonicated vesicles of egg yolk lecithin. In NMR experiments, the degree of ionization is deduced from the chemical shift of the carboxylic group. In ESR experiments, it is deduced from the relative intensity of the RCOOH and $RCOO^-$ spectra.

the degree of ionization is compared to the bulk pH. The ionization constant of a fatty acid is:

$$K = [H^+]\ [RCOO^-] / [RCOOH].$$

For carboxylic groups embedded in the lipid–water interface, the proton concentration is a local concentration $[H^+]_s$ which could be different from the bulk concentration $[H^+]_b$. Phosphatidyl choline heads of lecithin define a neutral host interface. The only surface charges are those of ionized fatty acid molecules. For negatively charged surfaces, a Boltzmann distribution of protons can be assumed:

$$[H^+]_s = [H^+]_b \exp(-e\phi_0/kT)$$

where ϕ_0 = electrical potential, e = absolute value of the electron charge, k = Boltzmann constant, T = temp. In a crude Gouy-Chapman theory, the relation between the bulk pH and the degree of dissociation is [10]:

$$pH = pK_i + \log_{10} \frac{\alpha}{1-\alpha} - 0.434\ e\phi_0/kT$$

where pK_i is the intrinsic pK and α the dissociation coefficient. Even for the highest concentration of fatty acids used here, one cannot account for a pK shift of about two pH units as observed in NMR experiments. Moreover, the absence of strong ionic strength effects clearly confirms that the displacement of the titration curve, when the ionization is referred to the bulk pH, is not related to the surface net charge. One must assume that environmental effects determine the ionization of the carboxylic groups. A relatively low dielectric constant is less favourable for the dissociation of 2 ions as recently proposed [11]. Other parameters such as water organization, local electrical field and molecular interactions should be also considered.

Moreover, ESR experiments reveal that the anchoring of the fatty acid molecules depends on their states of ionization [8]: the enchange frequency $RCOO^- \rightleftharpoons RCOOH$ is $< 10^7$ Hz. A single resonance in NMR experiments proves that it is $> 10^2$ Hz. The interfacial ionization is then a rather complex process involving local structural effects, local dynamical effects and surface effects (when the bilayer bears a net charge).

4.2. *For vesicles, there is a difference between the app. pK_a values provided by NMR and ESR (fig.3)*

Such a difference certainly reflects a disturbance due to nitroxide group of spin-labelled fatty acids. The bulky group positionned on the C5 carbon atom perturbs the organization of phospholipid molecules in the glycerol region [9], increasing the accessibility to ions and water molecules, therefore decreasing the difference between the bulk medium and the lipid–water interface. There is a difference between the 2 pK_a values measured by ESR for multilayers and sonicated vesicles. The lower value is obtained for multilayers which could correspond to a special equilibrium between the polar heads and the aqueous phase intercalated between 2 neighbouring layers.

4.3. *The partitioning of fatty acid molecules between the vesicle bilayers*

From the experiments described above, one could not decide on the partitioning of fatty acid molecules between the 2 sides of vesicle bilayers. NMR titrations by using shift reagents were unsuccessful because of precipitation and the possible formation of specific complexes between fatty acids and rare earth ions.

Preliminary ESR experiments on vesicles in which the outside bulk pH was progressively changed, suggested that fatty acids were effectively incorporated on both sides of the vesicle bilayers. Then, the observation that the $^{13}C^{*}OO(H)$ resonance of vesicles prepared at a given pH is a single line < 5 Hz width gives indication that the degrees of ionization of fatty acid molecules are nearly the same whether they are localized into the external or internal leaflet of the bilayer.

It must be observed that one cannot define a difference of pH between the inside and the outside in the usual sense. The inside of a vesicle 200–300 Å diameter is a microphase in which a macroscopic H^{+} concentration cannot be defined.

4.4. *Fatty acids as probes in ESR membrane studies*

Spin-labelled stearic acid is one of the most common probes used in membrane studies. In ESR experiments, fatty acids incorporated in egg lecithin vesicles are only 50% ionized for a bulk pH 6.5. Again, we recall the necessity to carefully control the degree of ionization before interpreting changes in ESR spectra.

Fatty acids are convenient probes to study the properties of lipid–water interfaces, inasmuch as the pK is extremely sensitive to the local conditions. For a low surface charge density, a discrete charge model would be certainly more adapted than the Gouy-Chapman theory to account for surface potential effects. In the experiments described above, such effects are very small since the net electrical charges are created by the extrinsic fatty acids. The ionization of these fatty acids depends primarily on the environment determined by the neutral interface constituted of phosphatidyl choline groups.

The activity of extrinsic ionizable molecules interacting with phospholipid bilayers depends on the pK determined by the lipid–water interface. A striking example is given by uncouplers of oxidative phosphorylation such as carbonyl cyanide *m*-chlorophenylhydrazone, activity of which is maximum for a bulk pH $\simeq 8$ when its normal pK is $\simeq 6.09$ [12]. Moreover small amounts of fatty acids could interfere in the stability and the proton permeability of lecithin bilayers.

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