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^{1}H-NMR of phosphatidylcholine liposomes at low p^{2}H in the presence of a paramagnetic shift reagent

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^{1}H-NMR spectra of egg phosphatidylcholine liposomes in ^2H_2O were obtained at several p^{2}H values in the presence of 10 mM $PrCl_3$ added after sonication of the phospholipid. It has been found that as the p^{2}H is lowered below 2, the two distinct signals corresponding to the outer and inner phospholipid trimethylammonium groups which arise by the shifting effect of the paramagnetic cation on the external surface of vesicles, tend to coalesce into a single, high-field peak, at the position corresponding to the internal, non-shifted $-N^+(CH_3)_3$ protons. These results can be interpreted to mean that the shifting effect of Pr^{3+} on phosphatidylcholine NMR spectra, is due to electrostatic interaction between the lanthanide and the ionized group of the lipid. At low p^{2}H, as the phosphodiester becomes protonated, the paramagnetic cation is no longer attracted by the liposome surface and its shifting effect on the phospholipid NMR signals disappears. The plot of the p^{2}H dependence of the chemical shift of the outer trimethylammonium resonance of phosphatidylcholine liposomes with praseodymium ions present only on the outside of vesicles, results in a sigmoidal titration curve with its midpoint at p^{2}H 1.5. In contrast, the inner signal is not affected by p^{2}H. If coalescence of signals is considered as indicative of complete protonation of the phosphate moiety, the value of 1.5 can be taken as the apparent pK for the ionization of that group under the experimental conditions employed, i.e., 10 mM $PrCl_3$ in ^2H_2O. That the low p^{2}H-induced merging of the signals is reversible, is shown by the reappearance of the two peaks when the p^{2}H of the phospholipid dispersion is raised from 1 to 5.7. Since the recovery of the trimethylammonium signal splitting indicates that Pr^{3+} has remained excluded from the liposome inner compartment, these experiments also demonstrate that the vesicles have not been disrupted by exposure to such an extreme acidic condition as p^{2}H 1.

Introduction

The initial report by Bystrov et al. [1] on the use of lanthanides as shift reagents in NMR spectroscopy of phospholipid vesicles, was followed by a number of studies dealing with the elucidation of the mechanism by which the shift of certain lipid resonances takes place [2–4] and with the application of this new methodological approach to the investigation of such topographical aspects as the different packing or the asymmetrical distribution of lipids in the external and internal monolayers of small vesicles [4–7]. In addition, as lanthanides are isomorphic replacements for calcium, the introduction of these cations as NMR shift reagents opened many interesting possibilities for investigations related to biomembrane

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properties [8]. From our laboratory (Fernández et al., 1973) came out the first report [9] on the use of an NMR shift reagent to detect ion transport across liposome bilayers mediated by ionophorous antibiotics. Such method has become increasingly popular [10,11]. Also, the application of lanthanides in NMR studies of the interaction of drugs with membranes, was proposed in 1973 by Fernández and Cerbón [12]. Using praseodymium ions added only on the outside of egg phosphatidylcholine liposomes, it was shown that the shift induced in the trimethylammonium proton resonance corresponding to the headgroups in the external surface of vesicles, can be reversed by the local anesthetic tetracaine. This result was interpreted as indicative of neutralization of charge of the lipid phosphate group by the cationic drug, which would lead to displacement of praseodymium ions from the vesicle surface.

The shifts induced by lanthanides in the NMR spectra of phospholipids, affect specifically the resonances of headgroup nuclei [1]. This implies that certain interaction must take place between the paramagnetic cations and the lipid polar moiety [2–4]. From spin-lattice relaxation-time measurements and line broadening in the presence of $GdCl_3$, Hauser et al. [3] concluded that the cations are bound to the phosphate group of phosphatidylcholine. Barsukov et al. have indicated that such interaction is probably electrostatic [13]. They found that the addition of negatively charged phospholipids to phosphatidylcholine liposomes, increases the shift induced by paramagnetic cations in the ^{1}H-NMR signal from phosphatidylcholine trimethylammonium groups.

An electrostatic interaction between lanthanides and the phosphate group of phospholipids should be extremely sensitive to pH changes in the range in which the ionization of the phosphodiester takes place. In the case of phosphatidylcholine, it is generally accepted that the phosphate group becomes protonated below pH 3 [14,15]. Although there have been some reports concerning the effect of pH on the interaction of polyvalent cations with phosphatidylcholine bilayers, none of them covered the ionization range of the phosphodiester [3,4]. What is known is that at 270 MHz no chemical shift changes can be detected in the pH range 0.3–10 for the trimethylammonium protons of egg phosphatidylcholine liposomes in the absence of lanthanides [3].

The purpose of this work is to investigate the effect of very low p^2H on the trimethylammonium proton magnetic resonance of egg phosphatidylcholine liposomes containing $PrCl_3$ only on the outside of vesicles. These experiments could give a conclusive proof of the electrostatic interaction between lanthanides and the phosphate group of phosphatidylcholine, if protonation of the phospholipid results in reversal of the paramagnetic shift induced by praseodymium. At the same time, the methodological approach presented here could provide a novel procedure for the estimation of the apparent pK characterizing the ionization of the phosphodiester group and for the ascertainment of the stability of liposomes at low p^2H.

Materials and Methods

Egg yolk phosphatidylcholine from Sigma Chemical Co. was purified by column chromatography on alumina [16]. The purity was checked by thin-layer chromatography. By determining the dry weight and phosphorus content [17], a mean molecular weight of 770 was assigned to the purified phospholipid. Praseodymium chloride (9.5%) was purchased from K&K Laboratories Inc.; ^{2}HCl and ^2H_2O (99.7%) were from Sigma Chemical Co. All other chemicals were reagent grade.

Depending on the required p^2H, the phospholipid was dispersed either in 25 mM sodium acetate/acetic acid in ^2H_2O (p^2H 4–6) or in the corresponding aqueous (^2H_2O) solution of ^{2}HCl (below p^2H 4). In all cases the solutions contained NaCl to bring the final electrolyte concentration to 0.13 M. Lipid vesicles were prepared as described previously [9,12]. A chloroform/methanol (9 : 1, v/v) solution containing the appropriate amount of phosphatidylcholine, was dried with nitrogen. Thereafter, to help remove the last traces of retained chloroform, benzene was added and evaporated with nitrogen. After addition of the required amount of the appropriate aqueous solution to give a final lipid concentration of 52 mM, the sample was sonicated for 30 min under nitrogen using a Branson sonifier (Model B 12) equipped with a titanium microtip, at a power output of 60 watts. During sonication, the sample

was kept in an ice-cooled vessel. Titanium powder and large, unsonicated vesicles, were eliminated by centrifugation at 5000 rpm for 10 min. At this point, $PrCl_3$ was added to give a final concentration of 10 mM. When necessary, a final adjustment of p^2H to the desired value, was performed by use of conc. ^2HCl or NaOH in ^2H_2O. The p^2H of the samples was measured before and after recording an NMR spectrum, with a Radiometer pH meter 22. Glass electrode pH readings were transformed to p^2H by means of the equation:

$$p^2H = pH + 0.4 \quad (1)$$

according to Glasoe and Long [18].

The 60 MHz ^1H-NMR spectra were recorded with a Varian A 60 spectrometer at 33°C [9,12]. Chemical shifts were assigned taking the $-(CH_2)_n$-signal of phosphatidylcholine as internal standard. All data presented are representative of at least three experiments.

Results

It is well known that at 60 MHz, the protons from the trimethylammonium groups of egg phosphatidylcholine liposomes in the absence of paramagnetic shift reagents exhibit a single resonance [1,9,12]. It has also been reported previously that at neutral pH the addition of $PrCl_3$ to the extravesicular space, resolves such single signal into two well differentiated components corresponding to the outer and inner phospholipid monolayers [9,12]. In the present paper, the pH dependence of such effect of Pr^{3+} is described.

Fig. 1 shows the influence of p^2H on the trimethylammonium ^1H-NMR signal of egg phosphatidylcholine liposomes with $PrCl_3$ present only on the outside of vesicles. All the spectra, except the one at very acidic p^2H (Fig. 1e), exhibit a signal split into two well defined components: one from the internal headgroups, which are not affected by the non-penetrating paramagnetic cation, and another from the external trimethylammonium protons, i.e., those exposed to the downfield shifting effect of praseodymium ions. Such an assignment is based on previous studies [9,12]. Lowering the p^2H from 5.7 (Fig. 1a) to 1.08 (Fig. 1e) does not modify the position of the highfield

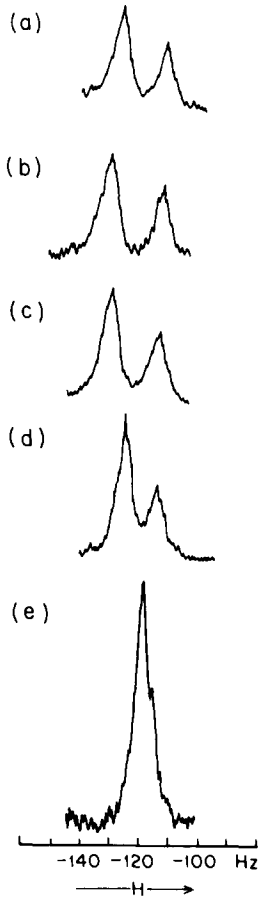

Fig. 1. The effect of p^2H on the splitting of the 60 MHz trimethylammonium ^1H-NMR signal of egg phosphatidylcholine liposomes induced by addition of 10 mM $PrCl_3$ following sonication of 52 mM phospholipid in ^2H_2O. The p^2H values are as follows: (a) 5.70, (b) 4.07, (c) 1.80, (d) 1.50, (e) 1.08. In each case, the lower-field signal results from the shifting effect of the externally added paramagnetic cation on the groups in the outside of the vesicles. The $-(CH_2)_n$-signal of phosphatidylcholine was taken as internal standard to assign chemical shifts.

resonance which remains constant at approx. −117 Hz. In contrast, the low-field peak (−133 Hz, Fig. 1a) only remains at an almost constant position at moderately acidic conditions (Fig. 1a, 1b, 1c) but as the p^2H is lowered below 2, it gradually shifts upfield (Fig. 1d) and finally coalesces with the resonance corresponding to the inner headgroups (Fig. 1e). This can be better appreciated in Fig. 2, where the chemical shifts of both, the external and internal trimethylammonium resonances as a function of p^2H, are

plotted. The data were taken from the spectra of Fig. 1. As can be observed, the titration curve obtained for the p^2H dependence of the chemical shift of the external groups in contact with Pr^{3+}, has its midpoint at p^2H 1.5.

In order to test the reversibility of the effect of low p^2H on the chemical shift induced by praseodymium ions, the following experiment was performed. Liposomes were prepared at low p^2H (1.08) and $PrCl_3$ was added to the phospholipid dispersion such that the shift reagent was present only on the outside of vesicles. After recording the ^1H-NMR spectrum shown in Fig. 3e, the sample was kept at room temperature for 1 h at such an extreme acidic p^2H. Subsequently, the p^2H was increased to 5.7 by addition of conc. NaOH and a new spectrum, shown in Fig. 3f, was recorded. It can be seen that the trimethylammonium signal is split again into two components at −130.0 and −117.0 Hz. Thus, despite of the exposure of liposomes to p^2H 1 for 1 h, the spectrum obtained is rather similar to the one in Fig. 1a, which was recorded for a sample prepared directly at p^2H 5.7. In this spectrum, the positions of the split trimethylammonium signals are −132.5 and −117.5 Hz. The small discrepancies found between the chemical shifts of the resonances in Figs. 1a and 3f, can be attributed to the dilution of the paramagnetic shift reagent resulting from the addition of conc. NaOH to the sample of Fig. 3f, that was necessary in order to bring its p^2H from 1 to 5.7. Nevertheless, since line positions are almost coincident, the reversibility of the effect of low p^2H can be considered as clearly demonstrated by this experiment.

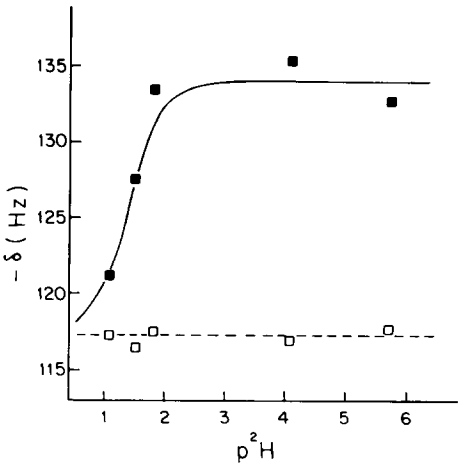

Fig. 2. The p^2H dependence of the chemical shifts of the outer (■———■) and inner (□-----□) trimethylammonium ^1H-NMR signals of egg phosphatidylcholine liposomes in ^2H_2O with 10 mM $PrCl_3$ only on the outside of vesicles. The 60 MHz spectra from which chemical shifts were determined, are the ones shown in Fig. 1. Line positions were determined with respect to the $-(CH_2)_n$-signal of phosphatidylcholine taken as internal standard.

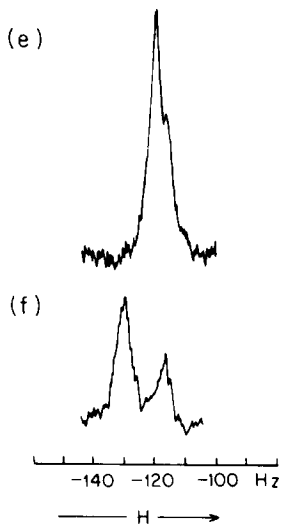

Fig. 3. Reversal of the effect of low p^2H on the praseodymium-induced splitting of the ^1H-NMR trimethylammonium signal of egg phosphatidylcholine liposomes. Upon sonication of 52 mM phospholipid in ^2H_2O, 10 mM $PrCl_3$ was added. (e) Coalescence of the inner and outer signals at p^2H 1.08. (f) Reversal of the low p^2H induced coalescence of the signals. After keeping sample (e) at p^2H 1.08 for 1 h, conc. NaOH was added to the lipid dispersion to increase the p^2H to 5.7; subsequently, the ^1H-NMR spectrum shown was obtained.

Discussion

The experiments presented in this paper were designed in order to investigate the influence of p^2H on the shifting effect of praseodymium ions in the trimethylammonium resonances of phosphatidylcholine vesicles. The p^2H range chosen was the one corresponding to ionization of the phosphodiester [14,15] to find out whether the presence of negative charge on the phosphate group, is indispensable for the interaction of the cation with the phospholipid, as judged by ^1H-NMR. It has been found that as liposomes are

acidified, the chemical shift induced by praseodymium diminishes in magnitude and finally disappears (Figs. 1 and 2).

The coalescence of signals at low p^2H shown in Fig. 1e, is consistent with the hypothesis that acidification of the liposomal dispersion would finally result in abolishment of the interaction of Pr^{3+} with phosphatidylcholine due to protonation of the binding site, i.e., the phosphate group. Although it is difficult to make a comparison between the results of experiments performed using different phosphatidylcholines which will not be packed to the same molecular area and surface charge density in the bilayers, it should be mentioned that studies of the pH dependence of the phase transition temperature of dipalmitoylphosphatidylcholine liposomes yield uncertain results concerning the possibility of achieving complete protonation of the phosphate group at pH 1 [19,20]. It can be speculated that the coalescence of trimethylammonium resonances reported in the present work might be due to a decrease of bound Pr^{3+} below the amount necessary to induce a shift and that despite of the merging of signals, the phosphate group could still bear some proportion of negative charge. An interpretation like that would imply the existence of a critical minimal concentration of lanthanide below which, the paramagnetic cation does not behave any longer as NMR shift reagent. That is unlikely since many previous experiments have shown that the dependence between the induced shift in phosphatidylcholine trimethylammonium resonance and lanthanide concentration can be described by a Langmuir-type adsorption isotherm [7,12,21–23]. Thus, either such hypothetical minimal praseodymium concentration does not exist or it is negligible. Further support to this conclusion is given by the fact that the shifting effect of lanthanides on phosphatidylcholine trimethylammonium resonances has been demonstrated to give an accurate estimation of the ratio of negatively charged lipid incorporated into the outer and inner monolayers of phosphatidylcholine vesicles [13]. Such estimation can be done because of the linear relationship existing between the induced shift and the content of negatively charged phospholipid in the bilayers. In summary, although the coalescence of signals found at low p^2H cannot be taken as an absolute proof of the complete protonation of the phosphate group, it does suggest strongly that such process have occurred. It appears that the effect of praseodymium on the ^1H-NMR spectrum of phosphatidylcholine, depends strictly on the negative charge of the phosphodiester such that protonation of this group results in complete reversal of the paramagnetic shift as if at low p^2H, the lanthanide were displaced from the liposome surface. Neutralization of the phosphate group by a cationic local anesthetic has a similar effect, as demonstrated in a previous article [12].

The plot of the chemical shift of the outer trimethylammonium signal of lecithin liposomes with praseodymium ions present only on the outside of vesicles, results in a sigmoidal titration curve with its midpoint at p^2H 1.5 (Fig. 2). In contrast, the inner signal remains at a constant position in the pH range studied, in agreement with previous reports showing that in the absence of shift reagents, there is no effect of pH on the position of the trimethylammonium resonance [3]. If, as discussed above, the coalescence of the trimethylammonium signals at low p^2H is considered to represent the fully protonated state of the phosphodiester, then the p^2H at the midpoint of the titration curve of Fig. 2, can be taken as an apparent pK for the ionization of phosphatidylcholine phosphate group. Such apparent pK should include the contribution of the electrostatic potential at the liposome surface, which will modify the concentration of protons at the interfacial region with respect to the bulk concentration [24–26]. In addition to considering the variation of surface charge with p^2H [26], the estimation of the intrinsic interfacial pK would require a previous determination of the binding constant of praseodymium ions to liposomes as a function of p^2H. Such a requirement is due to the fact that the paramagnetic cation does not seem to interact with phosphatidylcholine bilayers through a simple screening effect as assumed in the Gouy-Chapman [25,26] or Grahame [27] treatments, but through some form of specific mechanism [28].

It is worth mentioning that besides proving the reversibility of the effect of low p^2H on the shift induced by Pr^{3+} in the ^1H-NMR spectrum of phosphatidylcholine vesicles, the experiments of

Fig. 3 also show that liposomes resisted without being damaged, the exposure to p^2H 1 for 1 h. From the spectrum of Fig. 1e no clear conclusion concerning the intactness of liposomes could be drawn since, as demonstrated, at low p^2H the paramagnetic cation does not behave any longer as NMR shift reagent with respect to phosphatidylcholine. Thus, such spectrum could be due to an asymmetric system with praseodymium excluded from the inner compartment of intact liposomes, or to damaged liposomes which became freely permeable to praseodymium ions by the deleterious effect of the extremely acidic conditions employed. The experiments of Fig. 3 allows to discriminate between these two possibilities showing that even after being kept for 1 h at p^2H 1, liposomes remained impermeable to Pr^{3+}, as judged from the recovery of the splitting of the trimethylammonium signal, which is obtained by increasing the p^2H to 5.7.

The reversibility of the effect of low p^2H gives additional support to the use of the methodological approach presented here for the estimation of the apparent pK of the phosphate group of phosphatidylcholine, on one hand. On the other, it provides a new assay to determine the stability of liposomes in acidic solutions which could be of use in studies related to the use of lipid vesicles as carriers of drugs to be administered orally. Since such a route implies the exposure of liposomes to the low pH of the gastric juice, a knowledge of the effect of acidic media could be important.

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