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Direct determination by ^{2}H-NMR of the ionization state of phospholipids and of a local anaesthetic at the membrane surface

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Protonation and deprotonation of the primary amino acid group of phosphatidylethanolamine, and of the lipid phosphate groups of phosphatidylethanolamine with phosphatidylcholine, have been observed directly and isothermally in equimolar mixed fluid bilayers of the two phospholipids. In addition, the acid-base titration of the secondary amino group of the local anaesthetic, tetracaine, whilst partitioned into the bilayers, has also been determined. Here we show how studies by deuterium nuclear magnetic resonance of non-perturbing deuterons, specifically placed at the membrane polar-apolar interface, can give direct information about the electrostatics at a membrane surface.

Cellular stability is determined at the plasma membrane surface by steric and electrostatic interactions, the latter being responsible for peripheral protein binding [1], specific integral protein interactions with lipids [2], cellular adhesion [3] and the physical state of the hydrophobic core of a membrane [4,5]. In addition, nerve impulse transmission and lateral communication are thought to occur along membrane surfaces through electrostatic mechanisms [6]. The surface of biological membranes are, however, very heterogeneous and contain a range of chemical groups which can exist in different states of ionization determined either by counter ions in the bulk extracellular phase or by direct molecular associations, for example between a basic amino acid side-chain and an anionic phospholipid [7]. Thus, the red cell has a rather high average surface charge density of 6.3 e/nm^2 at pH 7.4 which because of the three-dimensional structure of the cell surface, is considerable higher than the zeta potential of 0.09 e/nm^2 determined by electrophoretic mobility measurements [8].

Considerable effort, therefore, has been devoted to understanding membrane electrostatics [9]. The practical information required for such discussions has usually come from studies on single, one-component phospholipid model membranes whose thermally induced ordered-disordered phase transitions depend upon their surface charge [3–5,10]. Here we demonstrate the potential for studying isothermally the ionization state of membrane components in heterogeneous fluid bilayer membranes by observing the nuclear magnetic resonance spectra from non-perturbing deuterons incorporated into specific positions in phospholipids such that they probe the membrane bilayer surface directly.

Abbreviations: DMPE, 1,2-dimyristoylphosphatidylethanolamine; DMPC, 1,2-dimyristoylphosphatidylcholine; TTC, tetracaine; NMR, nuclear magnetic resonance; $\Delta\nu_Q$, quadrupole splitting; pK_a, apparent dissociation constant; K_p, partition coefficient.

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The bilayer forming phospholipid, 1,2-dimyristoylphosphatidyl ethanolamine (DMPE), has been specifically deuterated in the α-CH_2 and β-CH_2 groups of the polar head group (Fig. 1). In mixed bilayers with 1,2-dimyristoylphosphatidylcholine (DMPC), only the DMPE was deuterated and in this way the effect of one lipid on the other can be observed [11,12]. Fig. 1 shows typical ^{2}H-NMR spectra in which the distance between the spectral maxima is the quadrupole splitting, $\Delta\nu_Q$, which is determined by both the amplitude and conformation of the C^2H-bound in the polar lipid head group [11–13]. The measured values of $\Delta\nu_Q$ are rather small, being less than about 10% of the possible maximum of 127 kHz for methylene groups [14], but the lines are narrow, well defined and small changes can be measured relatively accurately to within $\pm$ 50 Hz.

In Figs. 2 and 3a, are shown the values of $\Delta\nu_Q$ for the α- and β-deuterons in fluid DMPE-d_4/DMPC bilayers in which DMPE and DMPC are ideally mixed in an equimolar ratio. Values of $\Delta\nu_Q$ for the α-C^2H_2-group do not vary significantly above pH 4.0, which is in contrast to the values for the β-C^2H_2-group of the same phospholipid type. The changes in the quadrupole splittings for the β-C^2H_2-group of DMPE-d_4 to the acid-base dissociation of its primary amino group reflect an apparent dissociation constant, pK_a, of about 9.6, which is lower than the value of 11.0 for pure DMPE bilayers measured from phase transition determinations using fluorescent probes [5]. This difference may be due to the presence of DMPC or alternatively to inherent differences in the experimental methods. Similarly under acidic conditions, although the values of $\Delta\nu_Q$ for the β-C^2H_2-group are unchanged (Fig. 3), those measured for the α-C^2H_2-group (Fig. 2) decrease significantly as the lipid phosphate groups of both lipids are protonated giving an average pK_a of somewhat less than 3.5 A complete titration for the phosphate group was not achieved since some morphological change in the sample from its appearance was seen to take place. Since each of the sets of values of $\Delta\nu_Q$ for the two groups vary independently of each other, such changes are clearly due to the bulk pH and were not caused by motional averaging of the quadrupole splittings by small vesicle rotation [15]. The ^{31}P-NMR spectra for all

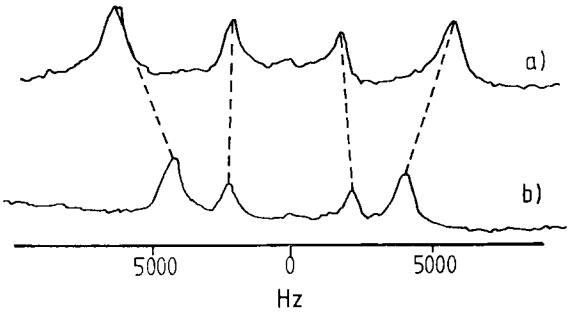
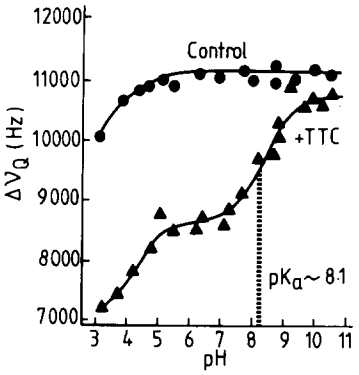


Fig. 1. ^{2}H-NMR spectra at 46.02 MHz, 57°C and pH 7.8 of fully hydrated mixed bilayers of 1,2-dimyristoylphosphatidylethanolamine (DMPE-d_4) and 1,2-dimyristoylphosphatidylcholine (DMPC) in an equimolar ratio in which the ethanolamine polar head group contains deuterons: -O-$P(O_2)^-$-O-$\overset{\alpha}{C^2H_2}$-$\overset{\beta}{C^2H_2}$-$\overset{+}{N}H_3$ in the absence (a) and presence (b) of 25 mol% tetracaine with respect to phospholipid showing how the quadrupole splitting, $\Delta\nu_Q$, is determined for the α-C^2H_2 (larger splitting) and β-C^2H_2-group of the ethanolamine group. DMPE-d_4 was synthesized by phosphorylating 1,2-dimyristoyl-*sn*-glycerol using phosphorus oxychloride in the presence of per-deuterated ethanolamine [11,12] (Merck, Canada). DMPC (Fluka) and DMPE-d_4 were pure by thin-layer chromatography and less than 5% degradation of the lipids was observed after any NMR experiment, which was for a maximum of 12 h with any one dispersion. Tetracaine (TTC) was obtained from Sigma. The lipid mixtures were produced by dissolving DMPC and DMPE-d_4 in chloroform/methanol (2:1, v/v) before removing the solvent with O_2-free N_2 followed by placing the sample under vacuum for at least 12 h. The dry lipid was dispersed to homogeneity in 0.4 ml of buffer, 0.1 M NaCl, 0.01 M NaH_2PO_4, 0.01 M EDTA made with deuterium-depleted water (Aldrich), at a temperature of 60°C, to give a final lipid concentration of 0.7 M. To alter the pH of a dispersion, 1 M NaOH or 1 M HCl (in deuterium-depleted water) was added in small amounts and the pH measured electrometrically for each sample. For samples containing tetracaine, 10 mol% of the solid anaesthetic was added to the phospholipid dispersion which was then re-dispersed. Spectra were recorded using a Bruker WH-300 spectrometer using a 90° pulse of 29 μs with a post-pulse delay of 45 μs, relaxation delay of 120 ms and sweep width of 30 kHz. Each spectrum required 20000 scans.

samples (not shown) were characteristic for phospholipid bilayers with a chemical shift anisotropy of about 6 kHz, which is inbetween the quadrupole splittings measured for the two deuterated groups.

Such highly specific sensitivity shown in the changes in $\Delta\nu_Q$ for the adjacent methylenes to the phosphate groups of DMPC and DMPE, and the amino group of DMPE as they are being titrated



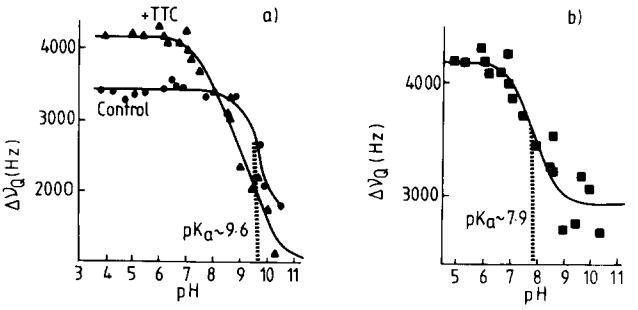


Fig. 2 (left). Variation of the quadrupole splittings for the α-C^2H_2-group of DMPE-d_4 for mixed DMPC/DMPE-d_4 bilayers from spectra similar to those shown in Fig. 1 as a function of bulk pH in the absence for the control dispersion, and presence of 10 mol% tetracaine (+TTC). For the +TTC values of $\Delta\nu_Q$ above pH 5, curve fitting was performed to the general formula

$$\Delta\nu_Q(\text{observed}) = \left(\Delta\nu_Q(\text{acid}) + \Delta\nu_Q(\text{base}) \times 10^{(\text{pH}-\text{p}K_a)}\right)/(1 + 10^{(\text{pH}-\text{p}K_a)})$$

using a VAX 11/780 computer, and the best fit was determined by the method of least squares to determine the apparent dissociation constant, pK_a, of the protonation-deprotonation of the aliphatic secondary amino group of tetracaine. A complete titration of the phosphate groups of DMPE/DMPC bilayers or primary amino group of DMPE was not carried out.

Fig. 3 (right). Variation of the quadrupole splittings with bulk pH for the β-C^2H_2-group of DMPE-d_4 in equimolar mixed bilayers with DMPC from spectra similar to those shown in Fig. 1 in the absence and presence of 10 mol% tetracaine (TTC) (a). The control sample in (a) shows the titration of the proton on the primary amino group of ethanolamine adjacent to the β-C^2H_2 group to give a pK_a of about 9.6 whilst in the presence of tetracaine a second titration is observed. By subtracting the control titration curve in (a) from the individual values of $\Delta\nu_Q$ for the sample with tetracaine, the difference values were plotted in (b) and curve fitted as described in Fig. 2 to obtain a pK_a for the protonation-deprotonation of tetracaine of approximately 7.9.

separately, is surprising. The local sensitivity of the deuterium spectra from labelled methylenes to titration of adjacent ionizable groups is not caused by the chemical shielding effects as detected by ^{1}H-NMR, these being small (typically less than a 100 Hz change in the Zeeman energy) and would affect the spectral density symmetrically. (Deuterium has a nuclear spin, $m_I = 1$, and two spectral lines are observed symmetrically about the chemical shift line position determined by the magnitude of Zeeman energy with the applied magnetic field [14,17].) Therefore chemical shift changes do not alter the measured quadrupole splitting itself. The quadrupole splitting changes are most probably caused by orientational or motional changes of the methylene groups independently of each other. Any modification of hydrogen bonding caused by an alteration in the ionization of the lipid head group might be expected to alter the orientation or amplitude of the motion of the whole group and therefore changes would be detected simultaneously in the spectra from both labelled groups. We have, however, suggested earlier that the ethanolamine group of PE is relatively restrained at the membrane surface [11], a view supported by X-ray crystal data for phospholipids [16].

Having determined directly the pK_a of the amino group of DMPE and phosphate groups of DMPE and DMPC in mixed bilayers, the acid-base titration of the secondary amino group of the local anaesthetic tetracaine was then investigated. The partition coefficient, K_p, for tetracaine into phospholipid bilayers varies depending upon the lipid type, temperature, bulk pH and ionic strength. Values for K_p between 660 (for egg PC bilayers at 30°C, pH 9.5 and in 0.1 M NaCl) and 4.1 (for egg PE bilayers at room temperature, pH 9.5 and in low salt) have been reported [18]. To determine the pK_a of tetracaine in phospholipid bilayers, there may be variations in the absolute changes in $\Delta\nu_Q$ due to changes in K_p as the bulk pH is

varied; these changes may alter the measured pK_a of TTC.

A linear change in $\Delta\nu_Q$ was observed for the quadrupole splittings of each C^2H_2-group up to total concentrations of tetracaine of 25 mol% of the phospholipid at pH 7.0 in buffered saline of 0.1 M NaCl. A gradient of -36 Hz/mol% and $+24$ Hz/mol% was measured for the α- and β-C^2H_2-groups, respectively, in mixed DMPE/DMPC bilayers at 62°C. For the pH titration experiments a tetracaine concentration of 10 mol% with respect to the total phospholipid concentration was used which was 2.5% of the total volume of the dispersion and close to the concentrations of around 0.5–1.0% used clinically [19]. In Fig. 2 is shown the change in the values of $\Delta\nu_Q$ for the α-C^2H_2 methylene when tetracaine was present in the bilayer giving a pK_a of about 8.1 for the titration of the aliphatic amine, whereas in the control experiment in the absence of tetracaine, no change in $\Delta\nu_Q$ for this group was measured over this same pH range.

In Fig. 3 a similar pK_a of 7.9 is determined for the titration of the secondary amino group of tetracaine was determined from measurements of $\Delta\nu_Q$ for the β-C^2H_2-group of DMPE, which also senses the deprotonation-protonation of its own primary amino group. The small difference of the two independently determined values of pK_a for tetracaine are within experimental error and may not be significant. Also the titration of the aromatic amino group, with a pK_a of 2.1 [20] is not approached in these present experiments. Both values of pK_a for the aliphatic amino group of tetracaine determined in this study are in the range (7.5–8.47) determined in aqueous buffer [20–22] and in the presence of detergent micelles (8.26 [20]) and bilayers of phosphatidylcholine (between pH 5.5 and 9.0 [18] and at pH 7.5 [21] from NMR studies of the anaesthetic itself). It is possible, however, that the different pK_a values measured here in Figs. 2 and 3, are due to repositioning of tetracaine in the bilayer during its titration [20,23] or to reorientations of the lipid dipoles within the membrane double layer. Any repositioning, if it happens, must take place quickly (faster than 10^3 times per second) since single, well resolved NMR spectra are recorded with no evidence for lateral phase separation of tetracaine-lipid complexes. The results suggest that tetracaine can perturb the conformation/amplitude of the entire polar head group of membrane lipids with the secondary amino group being close to both methylene segments of the DMPE head group. From studies with other deuterated lipid types (data not shown) there appears to be no specific association of tetracaine with any of the major classes of bilayer forming phospholipids.

From the work described here, it may well now be possible to investigate the ionization state of chemical groups in a variety of large biological macromolecules which normally give unresolved spectra by ^{1}H-NMR, this being a common technique for determining such information. Deuterons have been successfully incorporated into globular and membrane proteins, the sugars of glycoproteins, membrane soluble lipophiles as well as phospholipids, and studied by NMR and neutron diffraction to give structural and dynamic information about these components. Now ^{2}H-NMR may be additionally useful for such labelled molecules in giving directly electrostatic information, and for membrane associated macromolecules, when they are actually embedded in the membrane itself.

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References

1 Boggs, J.M. (1983) in Membrane Fluidity in Biology (Aloia, R.C., ed.), Vol. 2, pp. 80–130, Academic Press, New York
2 Marsh, D. and Watts, A. (1982) in Lipid-Protein Interactions (Jost, P.C. and Griffith, O.H., eds.), Vol. 2, Ch. 2, Wiley Interscience, New York
3 Rand, R.P. (1981) Annu. Rev. Biophys. Bioeng. 10, 277–314
4 Cevc, G., Watts, A. and Marsh, D. (1981) Biochemistry, 20, 4955–4965
5 Träuble, H. and Eibl, H. (1974) Proc. Natl. Acad. Sci. USA 71, 214–219
6 Kuffler, S.W. and Nicholls, J.G. (1976) From Neurone to Brain, Sinauer, MS
7 Sixl, F., Brophy, P.J. and Watts, A. (1984) Biochemistry, 23, 2032–2039
8 Gunze, M. and Heuck, C.C. (1986) Biochim. Biophys. Acta 856, 41–44
9 Parsegian, V.A., Rau, D.C., Evans, E.A. and Rand, R.P. (1985) Stud. Biophys. 110, 71–76

10 Watts, A., Harlos, K. and Marsh, D. (1981) Biochim. Biophys. Acta 645, 91–96
11 Sixl, F. and Watts, A. (1983) Proc. Natl. Acad. Sci. USA 80, 1613–1615
12 Sixl, F. and Watts, A. (1982) Biochemistry 21, 6446–6452
13 Seelig, J., Seelig, A. and Tamm, L. (1982) in Lipid-Protein Interactions (Jost, P.C. and Griffith, O.H., eds.), Vol. 2, Ch. 3, Wiley Interscience, New York
14 Seelig, J. and Seelig, A. (1980) Q. Rev. Biophys. 13, 19–61
15 Burnell, E.E., Cullis, P.R. and De Kruijff, B. (1980) Biochim. Biophys. Acta 603, 63–69
16 Hauser, H., Pascher, I., Pearson, R.H. and Sundrell, S. (1981) Biochim. Biophys. Acta 650, 21–51
17 Seelig, J. (1977) Q. Rev. Biophys. 10, 353–418
18 Boulanger, Y., Schreier, S. and Smith, I.C.P. (1981) Biochemistry 20, 6824–6830
19 De Jong, R.H. (1977) in Local Anesthetics (Thomas, C.C., ed.), Springfield
20 Garcia-Soto, J. and Fernàndez, M.S. (1983) Biochim. Biophys. Acta 731, 275–281
21 Boulanger, Y., Schreier, S., Leitch, L.C. and Smith, I.C.P. (1981) Can. J. Biochem. 58, 986–995
22 Skou, J.C. (1954) Acta Pharm. 276, 1–17
23 Kelusky, E.C., Boulanger, Y., Schreier, S. and Smith, I.C.P. (1986) Biochim. Biophys. Acta 856, 85–90