OBSERVATION OF THE PHOSPHATIDYL ETHANOLAMINE AMINO PROTON MAGNETIC RESONANCE IN PHOSPHOLIPID VESICLES: INSIDE/OUTSIDE RATIOS AND PROTON TRANSPORT*.

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Summary. The amino proton resonance of phosphatidyl ethanolamine in sonicated mixed phospholipid vesicles is observed 3.3 ppm downfield from H_2O. Above pH~ 5 it is broadened beyond detectability as a result of exchange with H_2O protons. In low salt, resonances of amino protons inside the vesicles appear to persist as the pH is raised, while those on the outside disappear. Solvent catalized proton conduction along the surface is proposed, with an effective $-NH_2$ to $-NH_3$ transfer rate of about 8×10^5 sec^{-1} at 25°C.

Several groups have used nuclear resonance to gain information about the fluidity and other properties of model phospholipid membranes (1,2,3). For proton magnetic resonance, samples have generally been prepared in D_2O buffer to avoid interference from the strong solvent proton signal. We have recently been able to obtain proton spectra of samples prepared in nearly 100% H_2O, and thereby to find the amino resonance of the phosphatidyl ethanolamine component of the membrane.

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MATERIALS AND METHODS. Phosphatidyl choline and phosphatidyl ethanolamine were obtained from fresh egg yolks as described by Litman (4). The purified phospholipids were shown by thin layer chromatography each to give a single spot. Phosphatidyl choline and phosphatidyl ethanolamine concentrations were determined respectively by inorganic phosphate determination (5) and reaction with 2, 4, 6-trinitrobenzenesulfonic acid (4). Stock solutions of the individual phospholipids in benzene were mixed in the appropriate volume ratio to obtain equimolar amounts of both phospholipids. The mixture was lyophilized, dispersed in distilled water containing 10% D_2O for field locking and sonicated for 50 minutes at 4°C under nitrogen. The dispersion was then centrifuged at 10,000 rpm for 10 minutes to remove undispersed lipid and titanium particles from the probe. The samples had a final concentration of approximately 60 μM/ml of lipid phosphorus.

Spectra were obtained at 90 mHz on a Bruker WH90 Fourier spectrometer, modified along the lines described previously (6) to suppress the H_2O signal. A special long, weak rf pulse is used which is designed to not affect the H_2O protons, while flipping resonances 100 or more Hz from the proton resonance. Further water suppression is obtained by audio filtering. Schemes of water resonance elimination based on destroying the water proton signal by prior radio frequency saturation would not work for observing the amino protons, which exchange rapidly with solvent.

RESULTS AND DISCUSSION. The sole resonance observed downfield of the water resonance is shown in Fig. 1, together with the upfield spectral region. It has the expected intensity and position for the amino resonance, and there are no other resonances expected or observed in this region.

This resonance is observable in samples prepared at pH 4 to 5. When the pH of such a sample is raised above 5 by adding sodium phosphate or sodium hydroxide, the resonance decreases in intensity, as shown in Fig. 1, remaining at a constant lower intensity as the pH is raised further, up to pH 6.6. We believe that the less intense resonance observed at higher pH is that of amino protons inside the vesicles, where the pH presumably remains constant at the value used in original preparation. This interpretation is supported by the observation that when the pH of a sample prepared at pH 6, for which no amino resonance was observed, was reduced to between 4 and 5, the resonance reappeared with the intensity expected if only the protons from the outside of the vesicle were contributing. This effect is seen only in samples prepared in low salt. In samples prepared in high salt, at pH 4 to 5, the resonance disappears entirely when the pH is raised, which shows that these samples transport hydrogen ions and their counterions.

The intensity of the residual resonance remaining after the pH is raised in the absence of salt can be used to assay the population of phosphatidyl ethanolamine inside relative to outside the membrane. Combined with similar use of

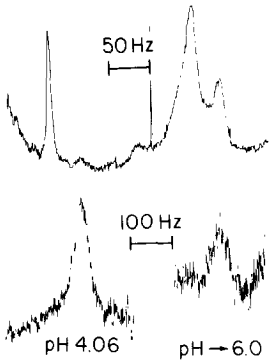
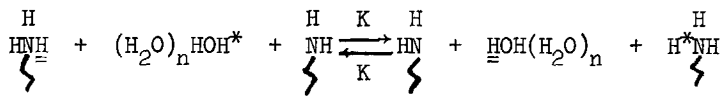


Fig. 1. Top, upfield spectrum of sonicated vesicles of equimolar phosphatidylcholine and ethanolamine, each 30 μM/ml, prepared in 90% H_2O, pH 4.06. The prominent peak furthest downfield is the choline methyl resonance and the sharp spike near the center is an artifact. Bottom, the sole resonance observed downfield of H_2O in this sample, 300 Hz from water (~3.3 ppm). The resonance on the left is observed in the sample as prepared while that on the right is that observed after the pH is raised to 6.6, and presumably arises from amino protons on the inside surface. It is plotted at twice the gain of that on the left, which is plotted at four times the gain of the top spectrum. All spectra were obtained in about two minutes of accumulation.

added magnetic ions to wash out choline resonances (2,3), it can be used to estimate the inside-outside ratio of a third component such as cholesterol.

The technique is applicable to only small vesicles in a limited pH range but has the advantage of relative gentleness and speed compared to chemical methods (7). Properties of vesicles prepared at higher pH could be studied by shifting the pH, if the property studied is stable enough.

As mentioned above, vesicles prepared at neutral pH in phosphate buffer show no amino resonance. The outside amino resonance reappears when the pH of the outside medium is reduced to pH 4. The disappearance is ascribed to broadening produced by exchange of the amino protons with solvent at a rate exceeding 10^3 sec^{-1}. The rate of exchange of amino protons on the outside surface was also investigated by means of transverse relaxation studies of the H_2O proton resonance in the presence of a strong radio frequency field (8). The results of this work are being analyzed, and our preliminary conclusion is that there is a rapid proton transfer between phosphatidyl ethanolamine groups in which water molecules participate as bridges:



The transfer rate, K, of a proton defect is estimated to be approximately 8×10^5 sec^{-1} at 25^o. It is highly probable that more than one water molecule participates because of the large distance between nitrogen atoms (>10A°). This process could be a physiologically significant source or sink of protons for membrane bound enzymes, or in invaginations of organelles.

In summary, the observed amino resonance has at least three applications: 1. Assay of inside/outside ratios or phosphatidyl ethanolamine, and thereby other components, in vesicles; 2. Study of proton transfer across vesicular membranes; 3. Study of proton transport along the surface of a vesicular membrane. The technique might be applicable to any membrane vesicle preparation on the 250 Å size range and stable at pH $\simeq$ 5.

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