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STUDY OF WATER PERMEABILITY THROUGH PHOSPHOLIPID VESICLE MEMBRANES BY ^{17}O NMR

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SUMMARY

Vesicle suspensions of up to 5 % egg lecithin and 2.5 % cholesterol have been found to have no effect on the NMR relaxation times of ^{17}O from water. Addition of 1–5 mM Mn^{2+} to an equimolar vesicle suspension of egg lecithin and cholesterol permitted resolution of the free induction decay into two exponential components, a fast one arising from the external water and a slow one arising from the intravesicular fluid. From the rates of relaxation the mean life time of the water molecules within the vesicles was calculated to be 1 ± 0.1 ms at 22 °C. The size of the vesicle was estimated from electron micrographs to be about 500 Å in diameter. These data yield an equilibrium water permeability, P_w, of about 8 μs^{-1} for the vesicle membranes. From the temperature dependence of P_w an activation energy of 12 ± 2 kcal/mol was obtained. The longitudinal relaxation time (T_1) of water within vesicles remained the same as in pure water.

INTRODUCTION

Phospholipid vesicles, generated by ultrasonic irradiation of aqueous suspension, have been shown to consist of single spherical shells of bilayers some 50 Å thick [1]. Each shell separates an intravesicular water phase a few hundred angstrom in diameter from the external bathing medium. It is by now well established that the structural core of biological cellular membranes consists of a similar phospholipid bilayer [2].

Since the bilayer consists of a hydrophobic phase, it exhibits a very low ionic permeability, which can be followed by conventional isotopic labeling techniques. Surprisingly the water flow through biological membranes is relatively fast and therefore the measurements of the water permeability are much more demanding technically [3].

With regard to water transfer, one has to distinguish between hydraulic conductivity (L_p) characterizing the net flux of water driven by an external driving force, and the permeability (P_w) characterizing the transfer of labeled water under equilibrium conditions, where exchange of water between the two media occurs in the absence of net water transport [3]. In cellular systems like red blood cells, L_p was measured by light scattering and P_w by isotopic labeling [4]. More recently, NMR

techniques have been used to measure P_w through the membranes of red blood cells [5–7]. Light scattering techniques have also been used to measure the L_p in phospholipid vesicles [8–10]. The small dimensions of the phospholipid vesicles do not permit the application of isotopic labeling for measurements of P_w. Very recently, proton NMR has been used to monitor the P_w of vesicular membranes [11].

The impermeability of the vesicles to paramagnetic ions has been used very elegantly to differentiate the internal from the external membrane surface by proton NMR [12]. This distinction can be made either by inducing a chemical shift of the surface protons (for which the rare earth ions are most suitable), or by enhancing the rates of NMR relaxation (for which Mn^{2+} is most effective) [12]. The fast exchange of water molecules prevents the proton NMR techniques from distinguishing between water in the intravesicular and bathing media [12]. Andresko and Forsen have prepared vesicles of synthetic dipalmitoyl phosphatidylcholine containing intravesicular concentrations of Mn^{2+} in excess of 45 mM [11]. In that preparation the proton relaxation times could be resolved into two components reflecting the intravesicular and extravesicular media, from which the water permeability through the membrane was calculated.

In this present study we measured the equilibrium water permeability P_w through phospholipid vesicle membranes by means of ^{17}O NMR. The effect of Mn^{2+} on the transverse relaxation times of ^{17}O is about ten times greater than on protons [13]. Therefore we were able to differentiate between intravesicular and extravesicular water using concentrations of only a few mM Mn^{2+} in the external medium.

MATERIALS AND METHODS

Egg lecithin grade I dissolved in methanol/chloroform mixture within sealed ampoules was purchased from Lipid Products. Thin-layer chromatography showed a single spot, so that the material was used with no further purification. The cholesterol (CH-Ln) was purchased from Sigma Chemical and also used without further purification. Weighted amounts of cholesterol were added to the original solutions of egg lecithin. A dry film of the lipid was produced by evaporating the solvent by a stream of argon and subsequent vacuum pumping. The lipid was then suspended in 1–5 ml water enriched with up to 20 % with ^{17}O. Vesicles were produced by ultrasonic radiation with a Braunsonic 300S, using a metal tip immersed in the suspension. Sonication was carried out for 1–2 h to optical clarity under argon in glass tubes within an iced-water bath. The sonicated suspension was centrifuged at $20\,000 \times g$ for 30 min at 4 °C. Usually three phases could be distinguished after centrifugation, a middle bulk phase and two smaller surrounding phases. The vesicles of only the bulk middle phase were studied in our experiments. The appropriate amounts of $MnCl_2$ or $PrCl_3$ solutions were added to the suspension before the NMR experiments.

The electron micrographs were obtained by negative staining according to the following procedure. The suspension was diluted down to 1–5 mg/ml of lipid, a drop was applied to a 400 mesh copper grid coated with parlodion and sprayed with a thin film of carbon. After 30 s most of the liquid was removed with a blotter and a drop of phosphotungstic acid was added. 30 s later the grid was blotted to remove excess liquid. The experiments were performed on a Philips 300 electron microscope operating at 60 kV.

The ^{17}O NMR experiments were conducted on a Brucker BKR 322S pulsed NMR spectrometer operating at a frequency of 8.1 MHz combined with a Varian 12-inch high resolution model V-4012 A-HR electromagnet. Sample volumes of about 1 ml were examined in thin-walled glass tubes (10 mm external diameter). T_2 was obtained directly from the free induction decay pattern. The contribution of field inhomogeneity was shown to be negligible by comparing results obtained by the Carr Purcell sequence with those obtained from the free induction decay for ^{17}O in pure water.

Values for T_1 were obtained by the conventional technique of applying paired pulse of 180° and 90°.

In order to enhance the signal : noise ratio the free induction decay was time averaged in all experiments. Proton spectra were obtained at 60 MHz on a Varian T60 spectrometer.

RESULTS AND INTERPRETATION

The longitudinal (T_1) and transverse (T_2) relaxation times of the ^{17}O from $H_2{}^{17}O$ in equimolar suspension of about 5 % egg lecithin and 2.5 % cholesterol at 22 °C were 6.5±0.5 ms, the same as in pure water. After exposing the suspension to ultrasonic treatment to form vesicles, the T_1 values remained unchanged while the T_2 values were usually slightly reduced to about 5 ms. We attribute the small change in T_2 to contamination introduced by the metal probe during sonication.

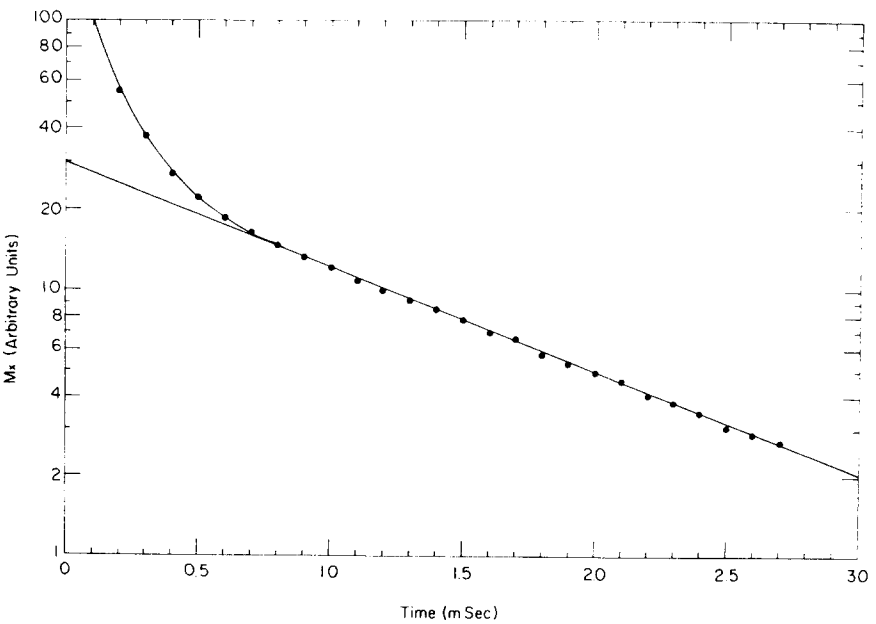

Fig. 1. The free induction decay of ^{17}O from $H_2{}^{17}O$ in a suspension of vesicles containing 5 % egg lecithin and 2.5 % cholesterol in the presence of 5 mM $MnCl_2$. Two exponential components can be distinguished, a fast one of the external water and a slow one of the intravesicular water, from which the mean life time of water molecules in the vesicles can be deduced.

In order to differentiate the intravesicular water from bulk medium, up to 5 mM Mn^{2+} was added to the vesicle suspension. ^{17}O is affected by this paramagnetic ion mainly via the direct contact interaction between the unpaired electrons of the metal with the nuclear magnetic moment of ^{17}O [13]. This mechanism leads to a very significant shortening of T_2 while T_1 is practically unaffected by the concentration of Mn^{2+} used in our experiments. In such suspensions the free induction decay reflecting the transverse relaxation rate of the ^{17}O signal from water was resolved into two exponential components, a fast component of the external water relaxed by the Mn^{2+}, and a slow component of the intravesicular water significantly affected by the limited dwelling time of the water molecules in the intervesicular medium. Fig. 1 demonstrates such an experiment. Since Mn^{2+} at concentrations used in our experiment affects very little the chemical shift of ^{17}O in water, the following equation can be used to calculate the mean life time (τ) of the water molecules within the vesicles [14].

$$1/\tau = \frac{(1/T_{2o} - 1/T_2)(1/T_2 - 1/T_{2i})}{(P_o/T_{2o} + P_i/T_{2i} - 1/T_2)} P_o \qquad (1)$$

where $1/T_2$ is the relaxation rate obtained from the longest component, $1/T_{2o}$ is the relaxation rate of water in the presence of Mn^{2+}, $1/T_{2i}$ is the relaxation rate of pure water, P_i is the fraction of water within the vesicles and P_o is the fraction of water in the extravesicular medium.

In our preparation $P_i \ll P_o$ and $1/T_{2i} \ll 1/T_{2o}$, so that a much simpler expression can be used.

$$1/\tau = 1/T_2 - 1/T_{2i} \qquad (2)$$

The size of the vesicles has been estimated from electron micrographs obtained by negative staining. A typical picture is shown in Fig. 2. The average diameter estimated from such pictures for vesicles containing 1 : 1 mol ratio cholesterol to phosphatidylcholine is about 500 Å.

In Fig. 3 proton spectra of similar preparation with and without $PrCl_3$ are shown. The Pr^{3+} is used here as a shift reagent that splits the signal of the choline group to a signal arising from the external surface, which is shifted upfield, and the signal from the internal surface, which is unaffected. The intensities of the two peaks are expected to reflect the ratio of the external and internal surface areas [12]. Owing to differences in linewidth between the two peaks the accuracy of such an estimate is rather limited. However, the greater intensity of the outer shifted peak in our preparations indicates that the vesicles are composed mainly of single bilayered shells.

In Fig. 4 the longest component of the ^{17}O transverse relaxation rate of the vesicle suspension in the presence of Mn^{2+} is plotted as a function of the reciprocal of absolute temperature. In the same figure are also plotted the relaxation rates for the vesicle suspension prior to the addition of $MnCl_2$. The values for $1/\tau$ calculated from the data in Fig. 4 are shown in Fig. 5. The data over the temperature range 4–30 °C yield an activation energy of 12 ± 2 kcal/mol for the diffusion of water through the membranes. Experiments have also been conducted beyond the freezing point down to −10 °C, probably in supercooled solutions. At temperatures close to the freezing point a significant deviation from the Arrhenius temperature dependence

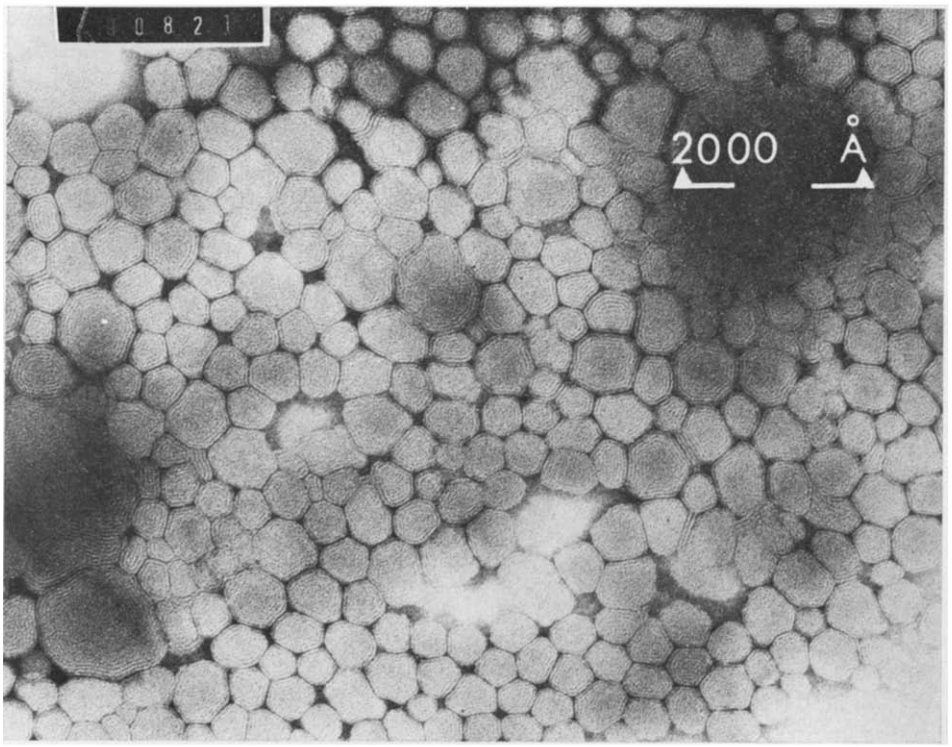

Fig. 2. A typical micrograph picture of vesicles containing 1 : 1 molar ratio of egg lecithin to cholesterol, obtained by negative staining with phosphotungstic acid.

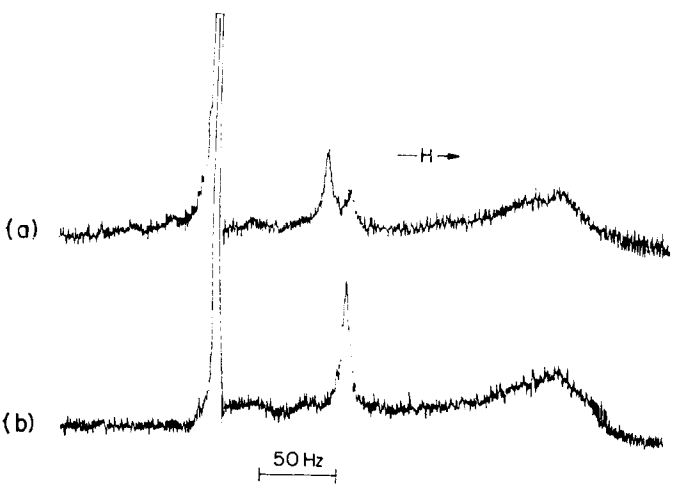

Fig. 3. 60 MHz proton NMR spectra with (a) and without (b) $PrCl_3$, of vesicles in a ^2H_2O suspension containing 5 % egg lecithin and 2.5 % cholesterol. In spectrum (a) the choline peak is split due to a downfield shift of the outer surface choline protons induced by Pr^{3+}.

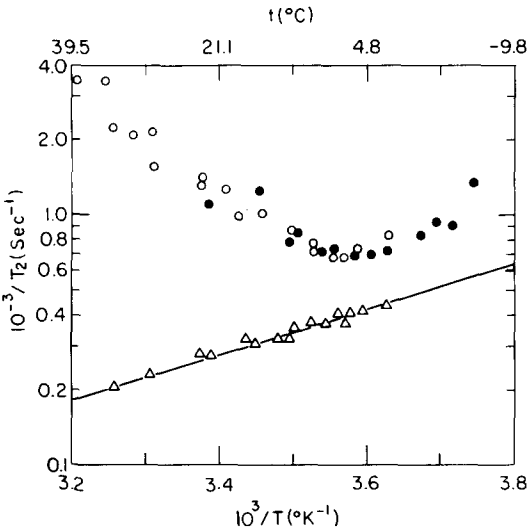

Fig. 4. Temperature dependence of the longest ^{17}O T_2 component of ^{17}O from $H_2{}^{17}O$ in a vesicular suspension containing 5 % egg lecithin and 2.5 % cholesterol in the presence of $MnCl_2$. The open and closed circles represent data from two different bottles. The triangles show the temperature dependence of the single T_2 value obtained in the same suspensions before adding the $MnCl_2$.

is observed. At this low temperature the water permeability seems to be further hindered owing to changes of the membrane properties.

In a forthcoming publication we show that within a few hours after mixing egg lecithin vesicles containing cholesterol and vesicles without cholesterol, the

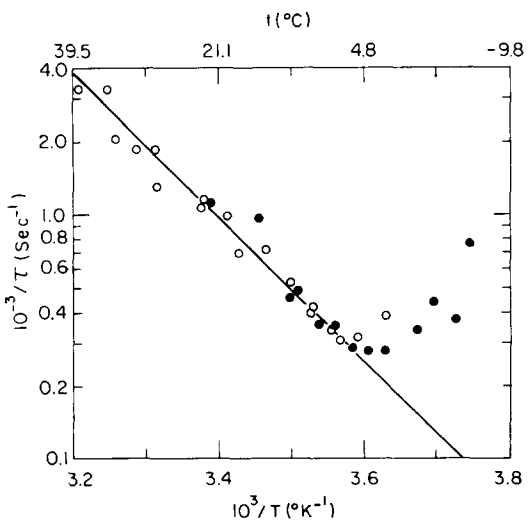

Fig. 5. Temperature dependence of the mean lifetime of water molecules in the vesicles calculated by Eqn. 2 from data in Fig. 4.

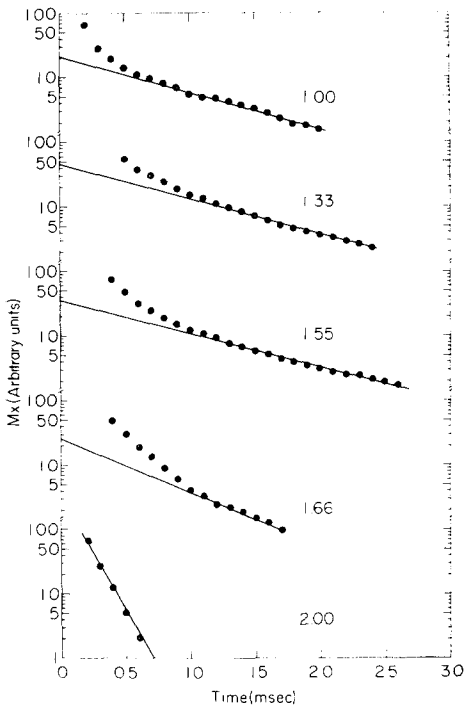

Fig. 6. ^{17}O free induction decay patterns from $H_2{}^{17}O$ in suspensions in the presence of 5 mM $MnCl_2$ of vesicles containing different molar ratios of egg lecithin to cholesterol (as indicated in the figure). A significant modification can be observed from 1 : 1 to about 1 : 0.5 molar ratio.

cholesterol is redistributed among the vesicles, ending up with a homogeneous average cholesterol content. We also show that the internal media of the vesicles are not mixed, thus indicating that fusion does not occur. This phenomenon was used in the present study in order to compare membrane permeability of vesicles with different cholesterol content. Vesicles containing a 1 : 1 molar ratio of phosphatidylcholine to cholesterol were mixed with various amounts of vesicles without cholesterol, incubated for about 4 h, and then Mn^{2+} was added to the bathing medium. In Fig. 6 the ^{17}O free induction decay patterns of vesicles with different cholesterol contents are compared. As can be appreciated from this figure, the longest component of the transverse relaxation rate is significantly enhanced only when the cholesterol/phosphatidylcholine ratio is reduced to 0.5 : 1, while it is almost unaffected by smaller gradual reductions of the cholesterol content.

This experiment indicates that the water permeability does not change smoothly with the cholesterol content at molar ratios between 1 : 1 and 0.5 : 1. Only when the molar cholesterol content is brought to about half the molar phosphatidylcholine amount, does a change induced in the membrane structure increase significantly water permeability through the membrane.

As mentioned before, the Mn^{2+} of the concentrations used in our experiments does not affect the longitudinal relaxation of ^{17}O in water. This relaxation rate is determined by the nuclear quadrupolar interaction and reflects the rate of rotational

tumbling of the water molecules. The T_1 of the intravesicular water was measured in the presence of 5 mM Mn^{2+} and found at 22 °C to be 6.5±1 ms, which is similar to the value found in pure water.

DISCUSSION

The mean lifetimes of water molecules within vesicles evaluated from the NMR data combined with the estimated size of vesicles from the electron micrograph pictures permit the calculation of the equilibrium water permeability (P_w) through the membrane.

$$1/\tau = P_w S/V \tag{3}$$

where S is the membrane surface area and V is the volume of the vesicle. For spherical vesicles, the following expression can be obtained:

$$P_w = r/3\tau \tag{4}$$

where r is the vesicle radius.

$1/\tau$ at 22 °C has been calculated to be 10^3 s^{-1} and r estimated from the electron micrograph about 250 Å. From these data one can calculate $P_w = 8 \cdot 10^{-4}$ $cm \cdot s^{-1}$, which is an order of magnitude higher than the value measured in the black lipid membrane preparation by isotopic labeling [15]. The higher rate of water flow in our measurements is not surprising if we consider the differences in membrane composition in the two preparations. Furthermore, the experiments with the black lipid membranes are susceptible to systematic errors due to unstirred layer phenomena, which reduce the measured permeability [3].

More striking is the similarity of the temperature dependence of the permeability in the different types of membrane preparation. 12±2 kcal/mol is the activation energy from our experiment, which is in the same range as those obtained for the different preparations of black lipid membranes [16], and also in a recent study by proton NMR of vesicles prepared from synthetic dipalmitoyl phosphatidylcholine [11]. It was suggested [17] that water diffusion via the membrane might occur through large pores containing water with bulk properties. This model predicts an activation energy of about 5 kcal/mol for the water diffusion. Our results for the activation energy are significantly higher than this figure and this model may therefore be rejected. The similar values in the activation energies in the different membrane preparations suggest that they really reflect an activation energy for the water permeability through the membrane rather than variation of membrane structure or composition with temperature. A variation in the membrane properties probably occurs in our preparation in the vicinity of 0 °C, where the temperature dependence deviates from the simple Arrhenius relationship.

It is also interesting to note that the values of T_1 indicated that the intravesicular water maintains the same molecular mobility as bulk water; thus the microviscosity of the small core of water enclosed by the lipid bilayer does not differ from a normal phase of water.

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