Short communication

# Changes in Vesicular Membrane ESR Spin Label Parameters Upon Isotope Solvent Substitution

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Isotope solvent substitution, most frequently $D_2O$ for $H_2O$, has widely been used to investigate mechanisms of water interaction with biological objects, and to reveal the role of water in a number of biochemical and physiological processes. Numerous works have suggested changes occurring in biological membranes and their properties upon isotope exchange, involving passive and active transport of ions through biological membranes (Kaminer and Kimura 1972; Gillespie 1975). Kremlev and Lobyshev (1978) have shown that the substitution of heavy for normal water resulted in a substantial increase in the stability of artificial bilayer membranes with a decrease of the kinetic rate of incorporation of polyaminoacids which modify membrane electrical conductance. This can be explained by the formation of a much firmer net of deuterium hydrogen bonds, including water molecules localized in the phospholipid polar groups region. This should finally result in changes in dynamic characteristics of the bilayer. The properties of a bilayer are directly related to the properties of protein structures in the membranes which form channels of various types. Prompted by the lack of reports of experimental results concerning this problem, we started studying dynamic characteristics of cardiolipin and cholesterol-supplemented egg lecithin vesicular membranes in normal and heavy water, using the method of ESR spin probes.

Vesicles were prepared according to Vladimirov and Dobretsov (1980) from egg lecithin (Kharkov Chemical Works, USSR) in a concentration of 2.1 mg/ml in 0.1 mol/l NaCl and $10^{-3}$ mol/l EDTA. Solutions were prepared with redistilled water, or with commercially available $D_2O$ (Izotop, USSR) with a deuterium concentration of 99.8 %. The pH values of the solutions with $H_2O$ and $D_2O$ were identical (approx 7.0). No correction of pH-meter indicated values (using glass electrodes) was performed for $D_2O$ solutions; this granted an equivalence of the electrostatic status of macromolecules in both types of solution (Kalinichenko and Lobyshev 1976). Experiments were performed at

20 °C. The spin probes used were hydrophobic I(12,3), I(1,14) as well as spin-labelled cholesterol (cholestane) (Syva, USA). Liposomes were also prepared with the addition of cardiolipin (Kharkov Chemical Works, USSR) and cholesterol (Fluka). Chemically pure saccharose and glycerol (Reachim, USSR) were used. The concentrations of the probes added to the liposome solutions in electrolyte were $2 \times 10^{-4}$ mol/l. The probe cholestane was added to the lipid solution in a concentration of $2 \times 10^{-3}$ mol/l shortly before vesicle preparation. ESR were recorded at 9.18 GHz using a Varian E-4 ESR spectrometer (USA) with following settings: for $I_{12,3}$ — microwave power 10 mW, modulation amplitude 0.4 mT, scan rate 2.5 mT/min; for $I_{1,14}$ — microwave power 10 mW, modulation amplitude 0.1 mT, scan rate 1 mT/min; for cholestane — microwave power 50 mW, modulation amplitude 0.4 mT, scan rate 2.5 mT/min. Typical patterns of spectra, and parameters measured are shown in Fig. 1. The other parameter $S$, and correlation time $\tau_c$ were computed using the relationship (Kuznetsov 1976; Lichtenstein 1974):

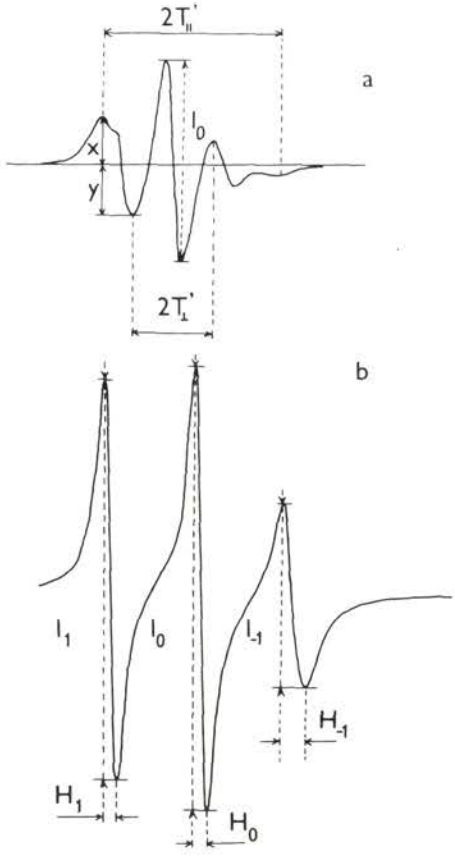


**Fig. 1.** *a*) A typical pattern of spectra of probe $I_{12,3}$ and cholestane in membranes. *b*) A typical pattern of probe $I_{1,14}$ spectrum in membrane. Parameters of spectra used for calculations are shown.

$$S = \frac{2T' - 2T'_{\perp}}{2[T_{zz} - (T_{xx} + T_{yy})/2]} \cdot \frac{T_{xx} + T_{yy} + T_{zz}}{T' + 2T'_{\perp}},$$

where $T_{xx} = T_{yy} = 0.58\,\mathrm{mT}$, $T_{zz} = 3.1\,\mathrm{mT}$

$$\tau_c(0, -1) = 6.73 \times 10^{-10} H_0 [(I_0/I_{-1})^{1/2} - 1]$$

$$\tau_c(-1, 1) = 6.65 \times 10^{-10} H_1 [(I_1/I_{-1})^{1/2} - 1]$$

Since the long axis of the cholestane molecule does not coincide with the $2p\pi$ orbital of nitrogen atom in contrast to fatty acids-based probes, the value of the molecule long axis order parameter (Table 2) was computed by $S_{mol} = S[(3\cos^2\varphi - 1)/2]^{-1}$. A value of 90° was taken for the angle $\varphi$.

Table 1 shows correlation times of rotational diffusion of probe $I_{1,14}$, corresponding to the fast isotropic movement model ($\tau_c < 10^{-9}$ s), along with the respective correlation time ratios $\tau_c(0, -1)/\tau_c(-1, 1)$, the latter characterizing the degree of rotational anisotropy. Isotropic rotation corresponds to the ratio of 1. The above condition is not met in cholesterol-containing membranes, and computed values of $\tau_c$ reach $1.5 \times 10^{-9}$ s. The relationship used however, exceeds the actual values of correlation times at $\tau_c = 5 \times 10^{-9}$ s by 20 % only. Around $\tau_c = 2 \times 10^{-9}$ s, the actual correlation times is almost identical with the computed value (Lichtenstein 1974). Owing to this, all the computations are correct.

Results shown in Table 1 indicate that substitution of $D_2O$ for $H_2O$ is associated with a prolongation of the correlation times in lecithin membranes and in cardiolipin-containing membranes, with an increasing anisotropy of rotational diffusion. The effect of $D_2O$ is more pronounced in lecithin membranes, while being weaker in cardiolipin-containig membranes.

Heavy water has by about 20 % higher viscosity than does normal water. To check the possible effect of viscosity, experiments were performed with glycerol and saccharose solutions having viscosities identical with that of $D_2O$. The higher viscosity of saccharose solution did not produce changes in dynamic characteristics of the probe. Glycerol induced, however, changes in correlation times; this may have resulted from glycerol being incorporated into the lipid bilayer. Owing to this, glycerol cannot be considered under our experimental conditions, as a substance having alterated merely the viscosity of the solution. The addition of cholesterol was followed by a prolongation of the spin correlation times and an increase in rotational diffusion anisotropy , thus confirming earlier results (Lichtenstein 1974). The substitution of $D_2O$ for $H_2O$ in membranes containing 20 mol % cholesterol resulted in shortening of relaxation time and in anisotropy of the probe movement.

Table 2 presents values of the order parameter and of amplitude of first derivation of zero adsorption maximum $I$ for probe $I_{12,3}$ and cholestane in egg

**Table 1.** Correlation times for probe $I_{1,14}$ in membranes

| Lipid composition | Solvent | $\tau_c(0,-1) \times 10^{-10}$ s | $\tau_c(-1,1) \times 10^{-10}$ s | $\frac{\tau_c(0,-1)}{\tau_c(-1,1)}$ |
|---|---|---|---|---|
| lecithin | $H_2O$ | 8.5 | 7.7 | 1.1 |
| lecithin | $D_2O$ | 10.2 | 7.9 | 1.3 |
| lecithin + cardiolipin | $H_2O$ | 8.8 | 7.6 | 1.2 |
| lecithin + cardiolipin | $D_2O$ | 9.8 | 7.8 | 1.3 |
| lecithin | 8.5 % glycerol | 9.8 | 6.5 | 1.5 |
| lecithin | 7.0 % saccharose | 8.5 | 7.6 | 1.1 |
| lecithin + cholesterol 11.2 mol % | $H_2O$ | 12.1 | 8.9 | 1.4 |
| lecithin + cholesterol 20 mol % | $H_2O$ | 15.1 | 10.4 | 1.5 |
| lecithin + cholesterol 11.2 mol % | $D_2O$ | 12.2 | 9.9 | 1.2 |
| lecithin + cholesterol 20 mol % | $D_2O$ | 12.8 | 10.3 | 1.2 |

**Table 2.** Parameters of ESR spectra of probe $I_{12,3}$ and cholestane in membranes

| Lipid composition | Solvent | X/Y | $I_0$, rel. units | $S$ |
|---|---|---|---|---|
| lecithin | $H_2O$ | 0.67 | 279 | 0.52 |
| lecithin | $D_2O$ | 0.68 | 250 | 0.54 |
| lecithin + cardiolipin | $H_2O$ | 0.72 | 228 | 0.55 |
| lecithin + cardiolipin | $D_2O$ | 0.71 | 213 | 0.55 |
| lecithin + cholesterol 11.2 mol % | $H_2O$ | 0.82 | 168 | 0.56 |
| lecithin + cholesterol 20 mol % | $H_2O$ | 0.74 | 156 | 0.57 |
| lecithin + cholesterol 11.2 mol % | $D_2O$ | 0.65 | 206 | 0.58 |
| | | Probe — cholestane | | |
| lecithin | $H_2O$ | 1.5 | 26 | 0.63 |
| lecithin | $D_2O$ | 1.3 | 23 | 0.66 |

lecithin membranes supplemented with cardiolipin (50 : 3.3, w/w), and for cholesterol supplemented lecithin membranes. In homogenous membranes, $D_2O$ induced an increase in the order parameter of the spin probe long axis. In other words, the probe movement in vesicular membranes in $H_2O$ is more chaotic, i.e. in the latter case, membrane lipids appear more fluid. This effect does not occur in heterogeneous membranes. Measurements of parameter $I$, characterizing probe concentration in the membrane, gave surprising results. Owing to a lower solubility of the probe in $D_2O$, an increase in hydrophobic probe concentration in the lipid phase would be expected (Lobyshev and Kali-

nichenko 1978). However, the parameter $I$ decreased for both probes by approximately 10 % in lecithin membranes. A decrease of parameter $I$ by up to 5 % and an increase in probe concentration by about 15 % were observed in cardiolipin-containing membranes and cholesterol-containing membranes, respectively.

Hianik et al. (1986) have defined relationships between micro- and macroscopical characteristics of bilayer membranes. We could show that the pattern of changes of parameter $I$ is very similar to that of the reciprocal value of modulus of membrane elasticity in direction perpendicular to its surface, $1/E_{\perp}$, as a result of varying cholesterol concentration in lecithin membranes. This means that the decrease of parameter $I$ observed in our experiments would correspond to an increase in modulus of elasticity by about 10 % for lecithin membranes. It thus can be concluded that the water phase/membrane distribution coefficient for the probe represents a function of mechanical properties of the lipid bilayer.

The results obtained have confirmed our original hypothesis concerning the stabilization of the bilayer through a net of hydrogen bonds with the participation of water molecules. The addition of cardiolipin and/or cholesterol likely disturbs the regular structure of bound water on the bilayer surface, thus reducing the component of the additional stabilization through a regular net of hydrogen bonds. The growth of energy of the O ... D-O bond as compared to O ... H-O results in a decrease of lateral lipid diffusion, as shown by a prolongation of relaxation times and an increase in degree of order of spin probes following the substitution of $D_2O$ for $H_2O$. These changes spread from the bilayer surface into the depth of the membrane. This was suggested by the results obtained with probe $I_{1,14}$ which has a free radical distant from bilayer surface. Changes in dynamic characteristics of the bilayer may induce corresponding changes in kinetic characteristics of macromolecular structures forming channels. This aspect offers a ready explanation for earlier results suggesting a growth of kinetic constants of ionic channels by approximately 15 %. These observations could not have been satisfactorily explained as yet (Schauf and Bullock 1982).

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