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Effects of Cholesterol and Azelaic Acid in Lecithin Liposomes Exposed to ELF Fields: A Thermodynamic and Structural Study.

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Summary. — In the present work some thermodynamic and structural aspects of electric-, magnetic- and electromagnetic-field interaction at 50 Hz with lecithin liposomes mixed with azelaic acid or cholesterol have been investigated by using differential scanning calorimetry and X-ray diffraction. Calorimetric scans and X-ray diffraction patterns show that no significant modifications of the two mixtures occur, except a little increase of the freezing temperature of the free water after exposure to electromagnetic field.

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How extremely low frequency (ELF) fields can interact with biological systems has been the subject of a number of recent theoretical and experimental studies. The interest is due mainly to the possible dangerous effects of this type of fields for public health. With regard to the complexity of living beings, the more promising way to detect and understand these effects is to make *in vitro* experiments in order to analyse specific phenomena occurring at cellular level.

Because membranes are a likely site of interaction, it is a good choice to study membrane models, such as lipid liposomes, exposed to electric and/or magnetic fields. In fact microwaves can increase the permeability of lipid liposome [1,2]; planar phospholipid bilayers exposed to combined ELF a.c. and d.c. magnetic fields show a change of the d.c. current through them [3]. In this order of ideas, it is important to state which is the real meaning of the previously quoted effects, *i.e.* can ELF-fields exposure induce some permanent modifications onto pure or suitably contaminated liposomes? Are on the contrary these effects completely reversible, disappearing without memory when the ELF fields vanish?

Previous experiments [4] indicate that the thermodynamic and structural properties of DPPC pure liposomes are not affected by the exposure to ELF fields. Following the line of research previously outlined, we report in this paper a

thermodynamic study of the membrane transitions and a structural investigation on the effect of cholesterol (CHOL) and of the azelaic acid (AA) on *L*-α-dipalmitoyl-3*n*-phosphatidylcholine (DPPC) liposomes when the solutions are exposed to ELF fields.

Cholesterol, as a component of many plasma membranes, was chosen to render the samples more similar to cellular membranes. The azelaic acid is a 9 carbon saturated dycarboxylic acid having special biological properties [5]; it may be incorporated into a lipid matrix and localised in the neighbourhood of the phospholipid head group region, but not too far from hydrocarbon chains. It was chosen because the effects of such acid on lipid liposome have been deeply investigated in our laboratory.

L-α-dipalmitoyl-3*n*-phosphatidylcholine (DPPC: $C_{40}H_{80}NO_8P$, molecular weight: 734.0), azelaic acid and cholesterol were purchased from SIGMA chemical (St. Louis, MO, USA) and used without further purification. Weighed amounts of DPPC and azelaic acid to give the desired molar ratio $R = \text{mol}_{AA}/\text{mol}_{DPPC} = 0.3$, or DPPC and cholesterol to give the molar ratio $R = \text{mol}_{CHOL}/\text{mol}_{DPPC} = 0.5$, were dissolved in chloroform. The solutions were dried with a slow stream of nitrogen gas and then lyophilised in order to evaporate residual solvent. A buffer (solution of HEPES 10 mml with KCl 100 mml, *p*H: 7.4) was added in a weight ratio buffer $x = 3$. Liposomes were obtained by equilibrating the buffered solution for some hours at about 50 °C (above the chain melting temperature) with intermittent vortexing during this period. Finally, from each of the two solutions obtained, four homogenous samples were prepared to be irradiated separately in the electric, magnetic, electromagnetic unit and for the control unit.

The exposure apparatus [6] consists of four units: the sample (one for each unit) is exposed to a uniform magnetic *B*-field (M) in the first unit; a uniform electric *E*-field (E) in the second; a mutually orthogonal uniform *E*-field and *B*-field (EM) in the third or no field in the fourth for control. With reference to the earth's surface, the a.c. *B*-field and *E*-field were varying along the vertical and the horizontal, respectively. Faraday shields exclude electric noise in the units.

The *B*-field exposure system was designed to provide fields up to 0.2 mT. Its uniformity was kept within 5% of the mean value. Each *B*-field exposure system consists of three parallel copper plates (40×40 cm^2), the central one carrying a total current I, and the other two carrying currents $I/2$ in the opposite direction. The *B*-field between the plates is parallel to their surfaces and it is greatly attenuated outside in order to leave the relevant control unexposed [7]. The current ($I_{max} = 500$ A) to the plates is provided by a step-down transformer with three selectable ratios of 200:200, 220:36, or 220:18, whose primary winding is connected to 220 V, 50 Hz power source, followed by a variable autotransformer (Variac), which supplies a second step-down transformer (500:1 ratio) whose secondary winding is the set of copper plates. The *B*-field generating system was checked for exposure calibration, field uniformity and field level at the sham field location. The *B*-field generated by the test system and measured at the location of the sham system and of the *E*-field exposure system was < 1 μT.

The *E*-field generating system is of the parallel-plate type. The plates (10×12 cm^2) are energised by a step-up transformer (220:1000 ratio) whose primary winding is fed by a Variac connected to the power source. The maximum unperturbed *E*-field which can be obtained in the volume where the samples were placed is of about 20 kV/m (in air).

The samples were contained in four glass vessels placed in four Plexiglas

containers. All the samples were exposed at the peak value of electric (20 kV/m in air, reduced by all the dielectric media to a value of about 60 mV/m on liposomes) and/or magnetic field (0.2 mT) for up to 25 hours at 23 °C.

Differential scanning calorimetry (DSC) measurements were performed by using DSC 7 Perkin Elmer calorimeter connected to data processor unit. Sealed containers having 20 μl capacity were used as samples holders. The samples were heated or cooled at 5 K/min scan rate.

High- and low-angle X-ray diffraction profiles were performed by using a vertical powder diffractometer equipped with a RIGAKU DENKI RU300 rotating anode generator operating with Ni-filtered Cu-$K\alpha$ radiation ($\lambda = 0.154$ nm). Temperature was controlled by a HAAKE F3 control system.

For the DPPC-Cholesterol solution we have investigated by DSC heating scans in the temperature range 20–50 °C the phase transition temperatures $L_{\beta'} \rightarrow P_{\beta'}$, $P_{\beta'} \rightarrow$ $\rightarrow L_\alpha$ [8] of the unirradiated and irradiated samples. Then the samples were cooled from 50 °C to −60 °C in order to detect the freezing transition peaks of the free and trapped water.

For the DPPC-AA solution the heating scan was in the temperature range 20–50 °C, followed by a cooling scan in the range from 50 °C to −60 °C and finally by a second heating scan to 20 °C.

In table I the $L_{\beta'} \rightarrow P_{\beta'}$ transition is not reported because the presence in DPPC liposomes of cholesterol in molar ratio $R = 0.5$ [9] and of azelaic acid in molar ratio $R =$ $= 0.3$ induces the disappearance of this transition. Every reported value is averaged on the results obtained during six calorimetric heating and cooling scans.

It is evident that in the experimental uncertainty the temperature of chain melting and that of the freezing of the water trapped between the bilayers are not affected by the ELF-fields exposure; while the free water-ice transition temperature for samples exposed to EM field seems to be slightly different from that reported for the samples not exposed or separately exposed to magnetic field and electric field. In particular the samples with cholesterol show a free-water freezing temperature 2 °C higher than the corresponding one of all other samples, as already seen in the case of pure DPPC liposomes after ELF-fields exposure [4]. On the contrary the same transition, in the case of liposomes containing AA, occurs at an average temperature 1 °C lower than the others, but in this case the standard deviation is very large and renders every hypothesis on this difference devoid of meaning.

TABLE I. – *Transition temperatures.*

	$T_{L\alpha} \pm \sigma$(°C)	$T_{\text{free water}} \pm \sigma$(°C)	$T_{\text{trapped water}} \pm \sigma$(°C)
DPPC	42.5 ± 0.3	− 20.3 ± 0.5	− 43.1 ± 0.5
DPPC-CHOL	37.6 ± 1.0	− 20.5 ± 0.5	− 41.6 ± 0.4
DPPC-CHOL + EM	38.7 ± 1.2	− 18.1 ± 0.7	− 41.2 ± 0.2
DPPC-CHOL + M	37.1 ± 1.5	− 20.2 ± 1.9	− 41.6 ± 0.6
DPPC-CHOL + E	38.3 ± 0.8	− 20.0 ± 2.1	− 41.9 ± 0.7
DPPC-AA	38.5 ± 0.2	− 21.0 ± 2.1	− 44.5 ± 0.2
DPPC-AA + EM	38.9 ± 0.3	− 22.4 ± 1.2	− 44.7 ± 0.1
DPPC-AA + M	38.4 ± 0.1	− 20.7 ± 1.8	− 45.4 ± 0.2
DPPC-AA + E	38.8 ± 0.2	− 20.0 ± 1.5	− 43.4 ± 0.9

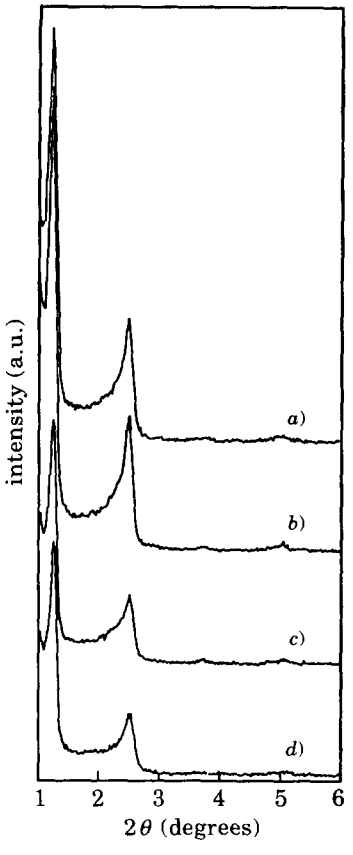

Fig. 1.

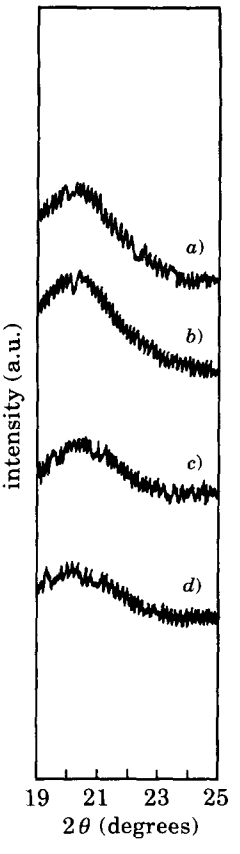

Fig. 2.

Fig. 1. – Low-angle diffraction patterns of DPPC liposomes with cholesterol at $R = \text{mol}_{\text{CHOL}}/\text{mol}_{\text{DPPC}} = 0.5$: *a*) control sample, *b*) exposed to EM, *c*) exposed to M, *d*) exposed to E. $T = 20$ °C.

Fig. 2. – High-angle diffraction patterns of DPPC liposomes with cholesterol at $R = \text{mol}_{\text{CHOL}}/\text{mol}_{\text{DPPC}} = 0.5$: *a*) control sample, *b*) exposed to EM, *c*) exposed to M, *d*) exposed to E. $T = 20$ °C.

The results obtained in the X-ray diffraction measurements do not show any difference in the structural properties of liposomes exposed to ELF fields. In fig. 1 we report the low-angle diffraction patterns of liposomes containing cholesterol: the peaks are typical of a lamellar structure and have very similar positions and intensities in all scans related to exposed and not exposed samples.

Figure 2 shows the high-angle diffraction patterns of the same samples reported in fig. 1. The rather large diffuse peaks indicate that the aliphatic chains are nearly melted but less disordered than the chains in L_α liquid crystalline phase: the low-high–angle profiles are typical of Γ phase which seems to be unaffected by ELF-fields exposure. Results very similar to those of the not exposed samples have been obtained in L_α phase at 50 °C and in all diffraction patterns obtained from liposomes containing AA.

In conclusion thermodynamic and structural properties of model membranes

containing cholesterol ($R = 0.5$) and azelaic acid ($R = 0.3$) seem to be not affected by ELF-fields exposure. The only doubt is regarding the case of DPPC-CHOL mixture, for which the freezing temperature of the free water increases after exposure to EM field; but this transition presents a supercooling effect also in not exposed samples and so only a great number of further measurements could give clearer information on this uncertain effect.

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