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Interaction of isopropylthioxanthone with phospholipid liposomes

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Abstract

Isopropylthioxanthone (ITX) is a highly lipophilic molecule which can be released in foods and beverages from the packages, where it is present as photoinitiator of inks in printing processes. Recently it was found in babies milk, and its toxicity cannot be excluded. The structure of the molecule suggests a possible strong interaction with the lipid moiety of biological membranes, and this is the first study of its effects on phospholipid organization, using differential scanning calorimetry (DSC) and spin labelling techniques. The data obtained with multilamellar liposomes of saturated phospholipids of different length, with and without cholesterol, point out that the molecule changes the lipid structure; in particular, in the gel state, behaving like a disordering agent it increases the mobility of the bilayer, while, in the fluid state, tends to rigidify the membrane, in a cholesterol like way. This behavior supports the hypothesis that ITX experiences a relocation process when the lipid matrix passes from the gel to the fluid state.

Keywords: Isopropylthioxanthone (ITX); Phospholipid membranes

1. Introduction

Isopropylthioxanthone (ITX), whose structure is represented in Fig. 1, is a molecule commonly used as photoinitiator of inks for food packaging. The interest on its possible biological effects and toxicity became topical since traces of ITX ranging from tens to hundreds μg/l were recently found in a number of beverages, especially milk.

A detailed report was carried out by the European Food Safety Authority [1] concluding that a possible genotoxic effect of ITX can be excluded on the basis of in vivo existing studies, but no other toxicity data are available.

At the same time the US Environmental Protection Agency considers ITX a potential hazard for human health and environment at lower concentrations than those found in packaged milks and other drinks [2], while episodes of reddening occurred to workers with the substance after sunlight exposure [3].

This paper is a first DSC and spin labelling study of the interactions of ITX with model membranes with the aim of getting information on the basic modifications induced by the substance on the lipid organization, starting from the idea that ITX is virtually insoluble in water but highly lipophilic, and the eventuality of accumulation phenomena, in particular at the level of cell membranes, should be accounted for.

The experiments were performed on multilamellar liposomes of saturated L-a phosphatidylcholine, dimyristoyl (DMPC; C14:0), L-a phosphatidylcholine, dipalmitoyl (DPPC; C16:0), L-a phosphatidylcholine, distearoyl (DSPC; C18:0) phospholipids, with and without cholesterol, and demonstrated that the molecule behaves like a disordering agent in the gel state, while tends to rigidify the membrane, in a cholesterol like way, over the transition temperature. As it will be discussed, these interactions support the hypothesis that ITX experiences a relocation process when passing from the gel to the fluid state.

Interestingly, a similar behavior was observed for chlorpromazine and phenothiazine derivatives [4–6] which share with ITX a near identical structure of rigid rings, so the results could be extended to, or confirm, the properties of a more general class of molecules of biological and pharmacological interest.

2. Materials and methods

2.1. Chemicals

All chemicals, of the highest available quality, were obtained from Sigma Chemical Co. (St. Louis, USA), while the solutions were prepared with quality milliQ water.

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2.2. Liposome preparation

Multilamellar vesicles were prepared following the method of Kusumi et al. [7]. Phospholipids, ITX and cholesterol were dissolved in a 2:1 chloroform methanol mixture then dried with a stream of nitrogen gas and kept under vacuum for at least 14 h. The dried mixtures were suspended, in a HEPES 0.1 M buffer, pH 7.2. The lipid dispersion, with a 101 mM final lipid concentration, was warmed at about 330 K, mixed repeatedly with a vortex for 30 s and used, just as obtained, for DSC measurements. A solution of TEMPO, 1 mM final concentration, was added to the phospholipid dispersion for the determination of the partition to TEMPO.

When required, spin labels (SASL) were added to the chloroform methanol mixture (final concentration of SASL = 1.8 mM).

2.3. DSC measurements

Calorimetric measurements were performed on a Setaram DSC 92.

About 50 mg of phospholipid dispersion was placed in an aluminium crucible. An identical crucible was filled with an equivalent weight of HEPES solution and placed in the reference cell.

The temperature scanning rate was 0.5 K min^{-1}. The transition temperature T_c from the L_β to L_α phase of liposomes was taken at the peak of the DSC profiles.

2.4. ESR measurements

ESR measurements were performed on a Bruker ESP300E, 9 GHz spectrometer at 10 mW microwave power. Samples were placed in a gas permeable TPX tube 1 mm i.d. (Wilmad, N.J. USA) and centered in the resonant cavity, then deoxygenated under nitrogen flow above T_c for 10 min.

ESR spectra of *n*-doxylstearic acid spin labels (*n*-SASL, with $n = 5$, 10 or 16) incorporated into DPPC liposomes were collected both over (317 K) and below (305 K) the transition temperature T_c of DPPC (310.5 K) and analyzed in terms of order parameter S, correlation time τ and maximum hyperfine splitting A_{max}.

In particular, above T_c, the order parameter S was used to monitor the lipid order and motion of 5 and 10-SASL according to [8], while the spectra of 16-SASL, due to the intense motional narrowing, are better described by the effective correlation time τ, according to [9,10].

The following formula holds:

$$S = 0.5407 \times [3(A_{max} - A_{min})/(A_{max} + 2A_{min})]$$

$$\tau = 6.5 \times 10^{-10} w_0 [(h_0/h_{-1})^{1/2} - 1]$$

where A_{max} and A_{min} are suitable peak positions, and w_0, h_0 and h_{-1} are the midfield line width and mid- and high-field line amplitudes of the ESR spectrum.

Below T_c, in the conditions of very slow motion, A_{max}, the separation between the outer hyperfine lines, is the most appropriate parameter for monitoring the effects of dopants on the ordered organization of the alkyl chains [8].

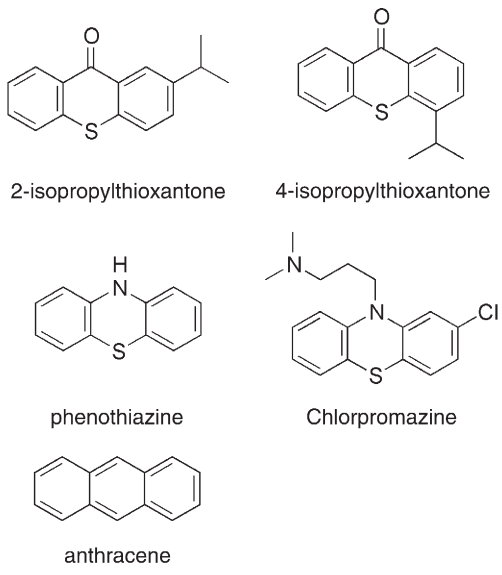

Fig. 1. Molecular structure of Isopropyl-9*H*-thioxanthen-9-one (ITX), mixture of 2- and 4-isomers, and analogue tricyclic compounds.

3. Results and discussion

3.1. Differential scanning calorimetry

The calorimetric profiles of the main gel to fluid state transition of 100 mM DMPC, DPPC and DSPC multilamellar liposomes at different concentrations of ITX are plotted in Fig. 2. The dopant demonstrated to be a rather strong perturbing agent which conflicts with the interactions between the phospholipids acyl chains. The effect is particularly relevant in the case of phospholipids of shortest tails (DMPC): it involves the lowering of T_c and a strong reduction of the total enthalpy which makes the transition hardly detectable at ITX 10 mM. In the case of DPPC and DSPC we observe a concentration dependent decrease of T_c and a marked, non-symmetric line broadening which could indicate an inhomogeneous distribution of the drug in the bilayer, originated from the formation of domains with different drug concentrations. Anyway, the sharper profile of the high temperature side of the peaks suggests another interpretation, which is consistent with the ESR data. In fact, as it will be discussed in the following, the position of ITX inside the bilayer depends on whether the membrane is in the gel or fluid state. In the gel state, ITX may be tentatively located in the interfacial region between the polar and the hydrophobic sides, a place determinant for introducing defects in the lipid matrix and perturbing the cooperativity. This could explain the broader left side of the transition, while the steepest second half could reflect the repositioning of the drug in a less critical position and a consequent recovery of cooperativity.

The contributory role of cholesterol was tested on DPPC multilamellar liposomes and the results are evidenced in Fig. 3. While cholesterol, at the relatively low concentration we

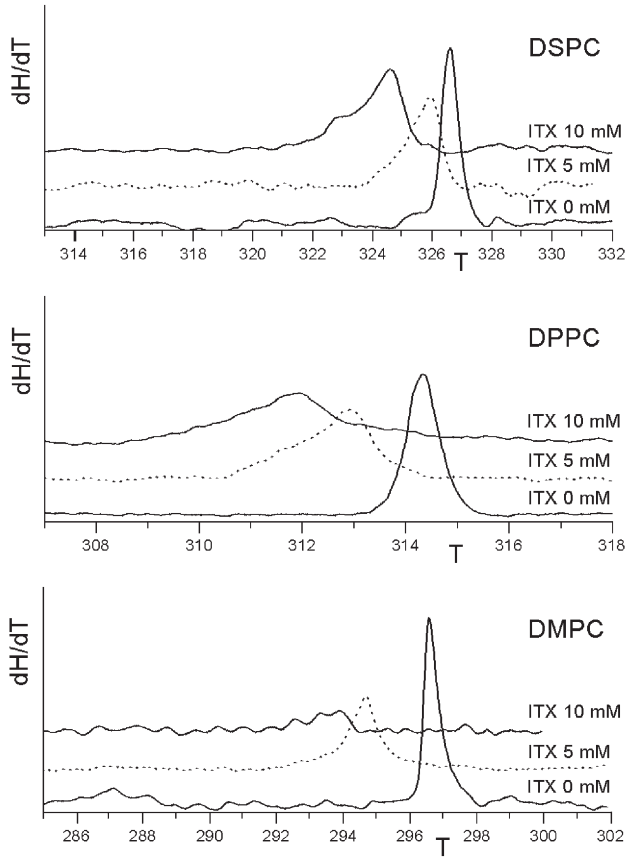

Fig. 2. DSC profiles of the gel-to-fluid state transition of PC multilamellar liposomes at increasing ITX concentrations. *y* axis, dH/dT in arbitrary units; *x* axis, temperature T in Kelvin.

adopted, does not modify significantly temperature and cooperativity of the DPPC transition, its concomitant presence with ITX determines less pronounced and broader transitions, without lowering T_c, than ITX only.

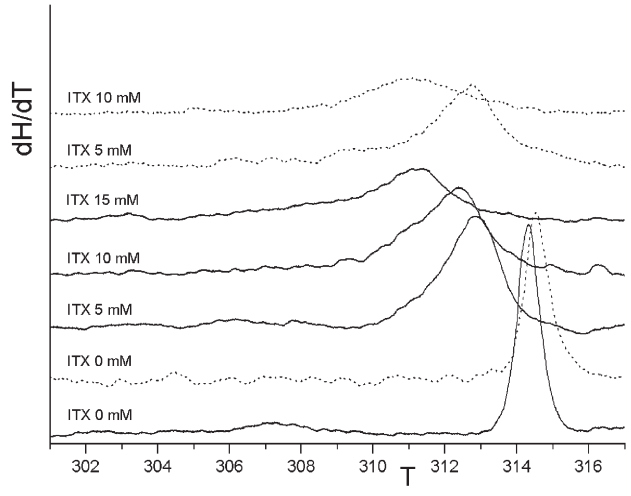

Fig. 3. DSC profiles of the gel-to-fluid state transition of DPPC multilamellar liposomes with (----) and without cholesterol (10 mM) (—), at increasing ITX concentrations. *y* axis, dH/dT in arbitrary units; *x* axis, temperature T in Kelvin.

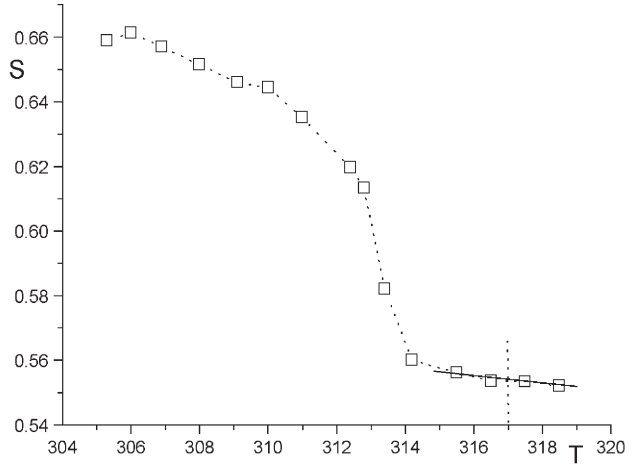

Fig. 4. S values of 5-SASL in multilamellar liposomes of DPPC vs. temperature T in Kelvin.

3.2. Spin labelling

ESR spectra were collected for stearic acids, spin labelled at the 5th, 10th and 16th position, incorporated in DPPC liposomes, with and without the dopant and cholesterol, as described under liposomes preparation. The order parameter S of 5-SASL in liposomes of pure DPPC are reported in Fig. 4. The values of S at 317 K was calculated by linear interpolation of the nearest points. Errors were quoted as the standard

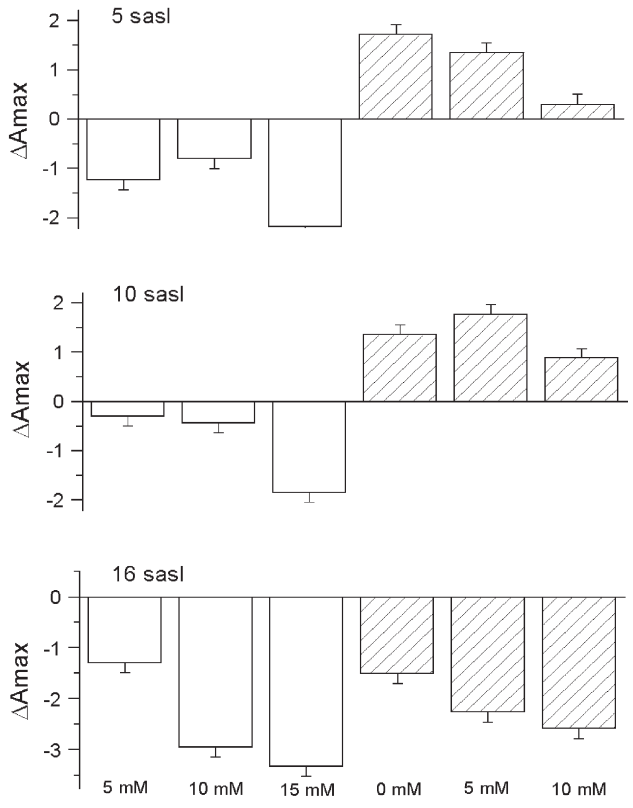

Fig. 5. ΔA_{max} values (in Tesla·10^{-4}) of 5, 10, 16-SASL in multilamellar liposomes of DPPC doped with: ITX; //// ITX + cholesterol 10 mM. *x* axis, ITX concentration.

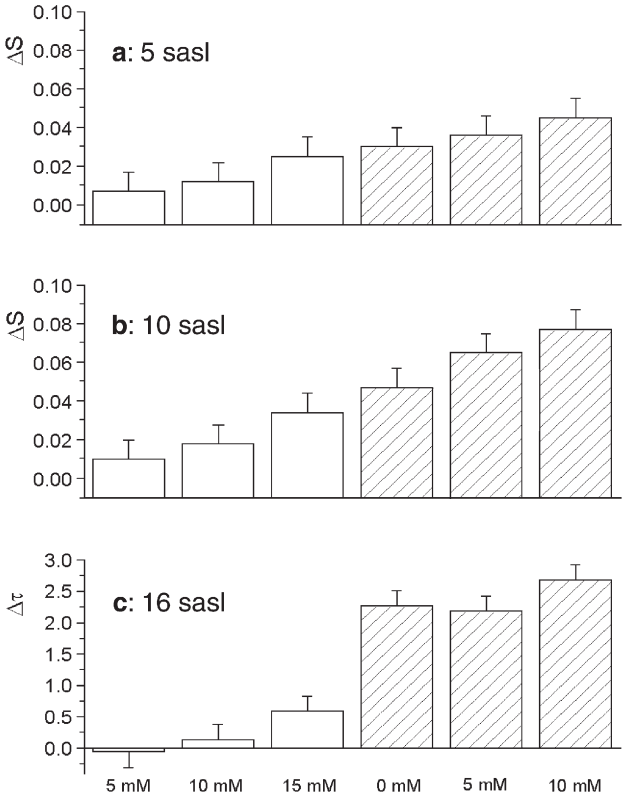

Fig. 6. ΔS values of 5, 10-SASL and $\Delta\tau$ (16-SASL, in $s \cdot 10^{-10}$) in multilamellar liposomes of DPPC doped with: ITX; //// ITX+cholesterol 10 mM. x axis, ITX concentration.

deviation of four independent samples of pure DPPC for each n-SASL. The same procedure was adopted for the values of A_{max} and τ, and repeated for each sample.

The results are summarized in Figs. 5 and 6, in terms of the differences of S, A_{max} and τ from their control values S_0, A_{max0} and τ_0, relative to pure DPPC and same spin label n.

The data make evident that the effects of ITX on the lipid organization are opposite when observed in the gel or fluid state.

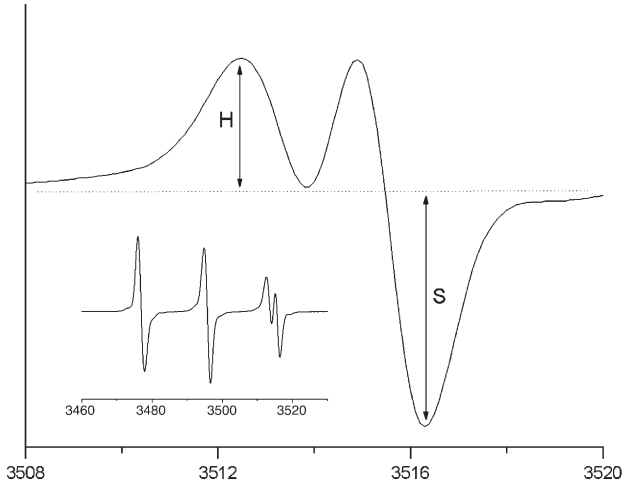

Fig. 7. ESR spectrum of TEMPO in an aqueous dispersion of DSPC liposomes above the gel-to-fluid state transition. x axis, magnetic field in Tesla $\cdot 10^{-4}$. Inset: full ESR spectrum.

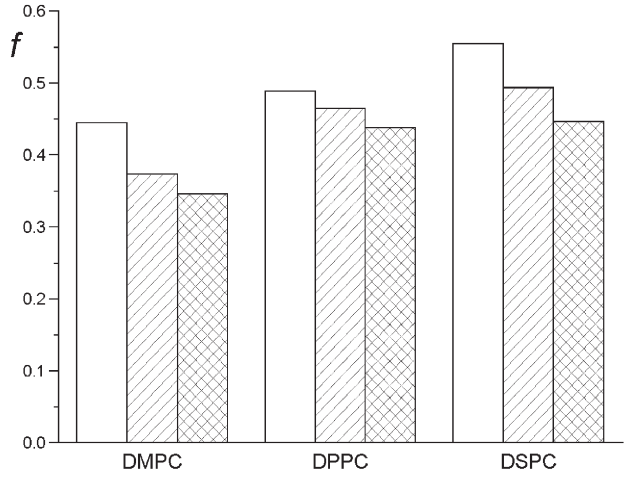

Fig. 8. fraction f of TEMPO dissolved in the fluid phase of multilamellar liposomes of: pure DMPC, DPPC and DSPC; and in the presence of: //// ITX 5 mM; ××× ITX 10 mM.

In the gel phase the perturbations introduced by ITX are described by the variation of A_{max} and represented in Fig. 5. The data demonstrate that ITX is destabilizing for the membrane, increasing the fluidity along the whole acyl chain; the effect is considerable at the level of the 5th carbon position, and even more evident at 16th level, while the 10th position is relatively unaffected by the presence of the drug, at least at concentrations lower than 15 mM.

When cholesterol, at the relatively low 10 mM concentration, is added to DPPC liposomes, the rigidity of the membrane in the gel state increases, with observable effects up to the 10th carbon position [11]; deeper inside the membrane, at the 16th carbon position, the situation is reversed, due to the voids created by cholesterol. In all cases ITX reduces the motional constraints introduced by cholesterol, but again its perturbative

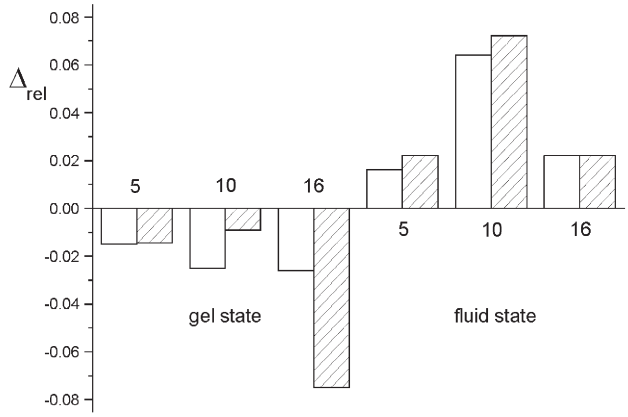

Fig. 9. Gel state: $\Delta A_{max}/A_{max0}$ values vs. spin label position (n-SASL) in multilamellar liposomes of DPPC doped with: anthracene 10 mM; //// ITX 10 mM. T=305 K. Fluid state: $\Delta S/S_0$ values (5, 10-SASL) and $\Delta\tau/\tau_0$ (16-SASL) vs. spin label position in DPPC liposomes doped with: anthracene 10 mM; //// ITX 10 mM. T=317 K. A_{max0}, S_0 and τ_0 are the reference values of pure DPPC. The relative values allow the representation of different parameters on the same scale and do not alter the comparison between each couple of data (anthracene-ITX) in the figure.

effects are lessened in correspondence of the 10th carbon position.

Being clear that an univocal interpretation is difficult, the data seem to indicate an inhomogeneous distribution of the dopant which accumulates preferably in the core of the bilayer and near to the interface with the polar region. Moreover the results are substantially different from those obtained substituting ITX with cholesterol, thus subtracting arguments to the hypothesis of an alignment of the ITX molecules with the long axis of the phospholipids.

In the fluid state the situation is totally reversed, and the ordering effect of ITX is clearly demonstrated by the increasing values of the order parameter S, even in the presence of cholesterol (Fig. 6). On the contrary, the spectra of 16-SASL, interpreted in terms of the rotational correlation time τ, do not show any meaningful perturbation after adding ITX.

A possible conclusion is that the complex interaction of ITX with saturated phospholipids membranes can be explained by a mechanism of relocation, and maybe reorientation, of the drug inside the membrane.

In the gel state the interaction is destabilising, and impairs both the order of the alkyl chains and the cooperativity and packaging of the lipid matrix. This is consistent with the hypothesis, supported by the spin labelling data, that a fraction of the dopant accumulates in the deepest region of the bilayer contributing to the lessening of the packaging, while another fraction locates in the interfacial region, may be oriented parallel to the membrane surface. This would account for the weakening of the cooperativity, as expected when relatively large molecules squat in the polar region, altering the polar head organization up to the glycerol group.

In the fluid state ITX has a cholesterol like behavior which strongly suggests a positioning in the hydrophobic moiety, with the molecules oriented parallel to the phospholipid long axis. As the mobility of 16-SASL is apparently unaffected, the drug is preferably accumulated in the high side of the hydrophobic region, leaving depleted the central core of the bilayer.

4. Partition to TEMPO

The EPR spectra of the spin label TEMPO in an aqueous dispersion of phosphatidylcholine liposomes is made up by the superposition of two distinct components that are partially resolved only in correspondence of the high field hyperfine lines (Fig. 7). Of the two, the outer is assigned to the spin label in the aqueous phase (S), the other to the spin label in the hydrophobic region of the lipid (H); then the parameter $f=H/(H+S)$ represents approximately the fraction of TEMPO dissolved in the membrane and can be used as an empirical measure of the membrane permeability to small organic solutes. In the gel state the permeability is limited, with values of $f\approx 0.1$ for all phospholipids and virtually unmodified by the dopant. In the fluid state (Fig. 8), the adsorbed fraction is about 0.5 with clear trends as a function of the phospholipids' length and the presence of ITX. In particular, the permeability is reduced by ITX in a concentration dependent way, reflecting the restricted mobility conditions of the acyl chains created by the dopant.

5. Conclusions

DSC and spin labelling were used in this first study of the chemical and physical interactions of ITX with phospholipids membranes. The work has no pretence to be an exhaustive investigation but it brought confirmation that the interactions are dominated by a mechanism of relocation when passing from the gel to the fluid state.

The behavior is common to ITX and chlorpromazine, both charged or neutral [5]: the molecules destabilize the ordered organization of lipids due to their inability to interdigitate among the acyl chains in the gel state, which can be ascribed to the rigid structure of rings of the two compounds. In fact it is reasonable to hypothesize that ITX and chlorpromazine, similar but shorter than cholesterol, if aligned to the acyl chains in the gel state, may loosen a number of van der Walls bonds between adjacent chains, such to create a situation energetically unfavorable with respect to a more disordered accumulation.

To test the assumption, we repeated part of the spin labelling experiments with anthracene, a completely non-polar molecule with the same ring structure, and the results are summarized in Fig. 9.

From the comparison between anthracene and ITX data, several differences are evident; anyway the interpretative scheme we suggested for ITX seems to be confirmed, if we observe that also anthracene behaves like a disordering agent in the gel state (negative shifts of the A_{max} values), while it tends to rigidify the membrane (positive shifts of S and τ) over the transition temperature.

References

[1] The EFSA Journal 293 (2005) 1–15.
[2] Flexographic Ink Options Vol. 2 Appendix 3-B US EPA 744-R-02-001B.
[3] Pesticide and Toxic Chemical News 27 (51) (1999).
[4] W. Nerdal, S.A. Gundersen, V. Thorsen, H. Høiland, H. Holmsen, Biochimica et Biophysica Acta 1464 (2000) 165–175.
[5] A. Wisniewska, A. Wolnicka-Glubisz, Biophysical Chemistry 111 (2004) 43–52.
[6] A.B. Hendrich, O. Wesolowska, M. Komorowska, N. Motohashi, K. Michalak, Biophysical Chemistry 98 (2002) 275–285.
[7] A. Kusumi, W.K. Subczynski, J.S. Hyde, Oxygen transport parameter in membranes as deduced by saturation recovery measurements of spin-lattice relaxation times of spin labels, Proceedings of the National Academy of Sciences of the United States of America 79 (1982) 1854–1858.
[8] D. Marsh, Electron Spin Resonance: Spin Labels, in: E. Grell (Ed.), Membrane Spectroscopy, Springer-Verlag, New York, 1981, pp. 51–142.
[9] N. Kocherginsky, H.M. Swartz, Chemical Reactivity of Nitroxides in Nitroxide Spin Label, CRC Press, Boca Raton, 1995.
[10] L.J. Berliner, Spin labeling in enzymology: spin-labeled enzymes and proteins, Methods Enzymol. 49 (1978) 418–480.
[11] W.K. Subczynski, A. Kusumi, Biochimica et Biophysica Acta 854 (1986) 318–320.