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Absorption of nitro-polycyclic aromatic hydrocarbons by biomembrane models: Effect of the medium lipophilicity

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ABSTRACT

To demonstrate the relationship between the structure of nitro-polycyclic aromatic hydrocarbons and their effect on biomembranes, we have investigated the influence of three structurally different nitro-polycyclic aromatic hydrocarbons, 2-nitrofluorene, 2,7-dinitrofluorene and 3-nitrofluoranthene, on the thermotropic behavior of dimyristoylphosphatidylcholine multilamellar vesicles, used as biomembrane models, by means of differential scanning calorimetry. The obtained results indicate that the studied nitro-polycyclic aromatic hydrocarbons affected the thermotropic behavior of multilamellar vesicles to various extents, modifying the pretransition and the main phase transition peaks and shifting them to lower temperatures. The effect of the aqueous and lipophilic medium on the absorption process of these compounds by the biomembrane models has been also investigated revealing that the process is hindered by the aqueous medium but strongly allowed by the lipophilic medium.

1. Introduction

Nitro-polycyclic aromatic hydrocarbons (nitroPAHs) originate primarily as direct or indirect products of incomplete combustion of polycyclic aromatic hydrocarbons (PAHs), which contain two or more aromatic rings (Pitts et al., 1978; Li et al., 2000). Only a few nitroPAHs are produced industrially.

NitroPAHs occur in the environment as a mixture together with parent PAHs and hundreds of other organic compounds either in the vapor phase or adsorbed to particulate matter (Ohnishi et al., 1985). Although a wide variety of bacteria, fungi and algae have been shown to degrade the parent PAHs containing two to five rings, nitro-substituted PAHs are only slowly degraded by indigenous microorganisms and may persist in soils and sediments. The recalcitrance of high molecular weight nitroPAHs is due in part to the strong adsorption to soil organic matter, low solubility, large molecular size and the polar character of the nitro group (Cerniglia and Somerville, 1995).

Experimental evidence indicates that nitro-polycyclic aromatic hydrocarbons are "direct-acting" mutagens in procaryotic and eucaryotic cells (IPCS, Environmental Health Criteria 229, 2003; Pedersen et al., 2005), are carcinogenic (Beije and Moller, 1988; IARC, 1989; Fu, 1990; Cui et al., 1995; Malejka-Giganti et al., 1999; Purohit and Basu, 2000; Lewtas, 2007), are metabolized to derivatives which bind to DNA and proteins (King et al., 1983; Landvik et al., 2007), and induce unscheduled DNA synthesis (Campbell et al., 1981), sister chromatid exchanges (Marshall et al., 1982), and DNA transformation (Lewtas, 2007).

Because of their widespread presence in the environment and genotoxic activities, including mutagenicity and carcinogenicity, many of these compounds may pose a health risk to humans.

To exert their mutagenic activity nitroPAHs have to cross the biological membranes. The cytoplasmic membrane of cells consists of a phospholipid bilayer forming a matrix in which enzymes and transport proteins are embedded. The cytoplasmic membrane permits the solute transport, plays an important role in the maintenance of the energy status of the cell, regulation of the intracellular environment, turgor pressure, signal transduction, and other processes. Although the lipid molecules constitute only a part of the total membrane mass, they form the matrix in which the other components are embedded. The physical properties of the cytoplasmic membrane influence the structure and functioning of the other components.

Few publications deal with the interaction of PAHs with biomembrane models (Castelli et al., 2002; Librando et al., 2003; Farkas et al., 2004) and just very few, to our knowledge, reports on the effect of nitroPAHs on biomembrane model (Castelli et al., 2001). Following the experimental procedure previously reported (Castelli et al., 2002; Librando et al., 2005, 2006), we have studied the interaction of three nitroPAHs (2-nitrofluorene, 2,7-dinitrofluorene and 3-nitrofluoranthene, Scheme 1) with biomembrane models constituted by dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles (MLV) with the aim to better define how structural differences in the compounds can affect their interaction with the biomembranes. Kinetic experiments have been also carried out to

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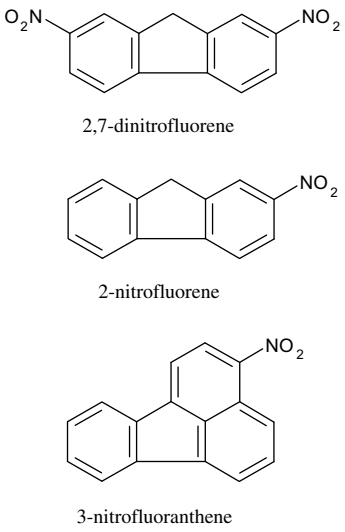

Scheme 1. 2,7-Dinitrofluorene, 2-nitrofluorene and 3-nitrofluoranthene structure.

investigate the effect of the aqueous and lipophilic medium on the absorption of these compounds by the biomembrane models. DMPC MLV undergo, upon heating, to a transition from an ordered or gel state to a disordered or liquid-crystalline state characterized by a well defined temperature called transition temperature (T_m) and an enthalpy change (ΔH). Substances interacting with DMPC MLV can provoke modifications on the T_m and enthalpy change. Differential scanning calorimetry (DSC), which detects these modifications, has been employed to study the interaction and the absorption of the nitroPAHs by biomembrane models. Finally, with the aim to get knowledge on the effect of the NO_2 group on such interaction, the obtained results have been compared with the data of a previous paper of us (Librando et al., 2003), where the interaction of fluorene and fluoranthene with biomembrane models was investigated.

2. Materials and methods

2.1. Chemicals

Dimyristoylphosphatidylcholine was purchased from Genzyme Pharmaceuticals (Liestal, Switzerland). Lipids were chromatographically pure as assessed by two-dimensional thin-layer chromatography. Lipid concentration was determined by the phosphorous analysis (Rouser et al., 1970). 2-Nitrofluorene (purity: 98%) was obtained from Sigma (Germany), 3-nitrofluoranthene (purity: >99%) was obtained from Fluka (Germany), 2,7-dinitrofluorene (purity: 98%) was supplied by Alfa Aesar (Germany). A 50 mM Tris-hydroxymethylaminomethane (Tris) buffer solution, adjusted to pH 7.4, was employed.

2.2. Multilamellar vesicles preparation

Stock solutions of lipid and nitroPAHs were prepared in chloroform:methanol 1:1 (dimyristoylphosphatidylcholine, 2-nitrofluorene, 3-nitrofluoranthene) or in chloroform (2,7-dinitrofluorene). Then appropriate aliquots of lipid and of each nitroPAH were mixed in a glass tube to have 0.010325 mmol of DMPC and the following molar fractions of nitroPAH: 0.00, 0.015, 0.03, 0.045, 0.06, 0.09, 0.12, 0.15 and 0.18. The solvents were evaporated under a nitrogen flow. Further evaporation was carried out by keeping the samples under vacuum for 1 h. Dry lipid films were suspended with 168 μL of Tris pH 7.4 and the multilamellar liposomes were prepared by vortexing the samples for 1 min, keeping at 37 °C (above the gel-to-liquid-crystalline phase transition temperature) 1 min for three times. The obtained MLV were kept, 1 h, at 37 °C to permit their homogenization and the complete partition of each compound between the aqueous and lipid phases. Each preparation was carried out in triplicate.

2.3. Differential scanning calorimetry measurements

The DSC measurements were performed using a Mettler Toledo STAR^e system equipped with a DSC-822^e calorimetric cell and a Mettler TA-STAR^e software. One hundred and sixty microlitres of aluminum pans were used. The samples were submitted, at least four times to check the results reproducibility (indicating the MLV homogeneity), to the following procedure:

(a) A heating scan between 5 and 37 °C at 2 °C/min.
(b) A cooling scan between 37 and 5 °C at 4 °C/min.

The sensitivity was automatically chosen as the maximum possible by the calorimetric system and the reference pan was filled with Tris buffer solution. DSC was calibrated, in temperature and enthalpy changes, by using indium, stearic acid and cyclohexane by following the procedure of the DSC 822 Mettler TA STAR^e instrument.

After the calorimetric analysis, aliquots of all samples were extracted from the calorimetric aluminum pans and used to determine, by the phosphorous assay (Rouser et al., 1970), the exact amount of phospholipids present.

2.4. Permeation kinetic experiments

An exact amount of the examined powdered compounds (to obtain a 0.09 molar fraction with respect to the lipid) was weighted in the bottom of the DSC aluminum pan and 120 μL (0.007375 mmol) of the DMPC MLV aqueous dispersion were added. The aluminum pan was hermetically sealed and the sample submitted to the following calorimetric analysis:

(a) A heating scan between 5 and 37 °C, at the rate of 2 °C/min, to detect any interaction between compound and MLV.
(b) An isothermal scan (1 h) at 37 °C, to permit the compound to eventually dissolve in the medium, reach the MLV surface, penetrate the phospholipid bilayers and interact with them.
(c) A cooling scan between 37 and 5 °C, at the rate of 4 °C, to bring the phospholipid system back to the ordered state.

The procedure was run at least eight times, to follow any variation in the calorimetric curves during the incubation time. The experiments were run in triplicate.

2.5. Transmembrane transfer experiments

Sixty microlitres (0.003687 mmol) of DMPC MLV dispersion prepared in the presence of 0.09 molar fraction of each compound (loaded MLV) were delivered in the DSC aluminum pan and 60 μL of an equimolar DMPC MLV dispersion (empty MLV) were added. The pan was hermetically sealed and the sample was submitted to the calorimetric analysis following the same procedure reported in "Permeation kinetic experiments" section. The experiments were carried out in triplicate.

3. Results and discussion

The nitroPAHs used in this study have been selected on the basis of their toxic properties and structural differences to evaluate the significance of different structural elements of their molecules

on thermotropic properties of DMPC multibilayers such as cooperativity, temperature shift of the pretransition and the main phase transition and changes of enthalpy. We have prepared and analyzed, by DSC, DMPC MLV in the presence of increasing molar fraction of nitroPAHs. The calorimetric curves of all the nitroPAHs–DMPC MLV are compared to that of pure DMPC MLV (Fig. 1). As it is known, DMPC shows two endothermic peaks: a main peak at 24.8 °C and a smaller peak at 15–16 °C. The main peak is related to the transition from a gel phase (L_β) to a liquid-crystalline (L_α) phase. In the gel phase, occurring at lower temperatures, the lipid chains are ordered with all-*trans* conformation. The liquid-crystalline phase, occurring at higher temperatures is characterized by rapid *trans–gauche* isomerism of the lipid chain segments, rotation around the long axis of the lipid molecules; the lateral packing density is reduced considerably, relative to that of the gel phase. Between the L_β phase and the L_α phase, an

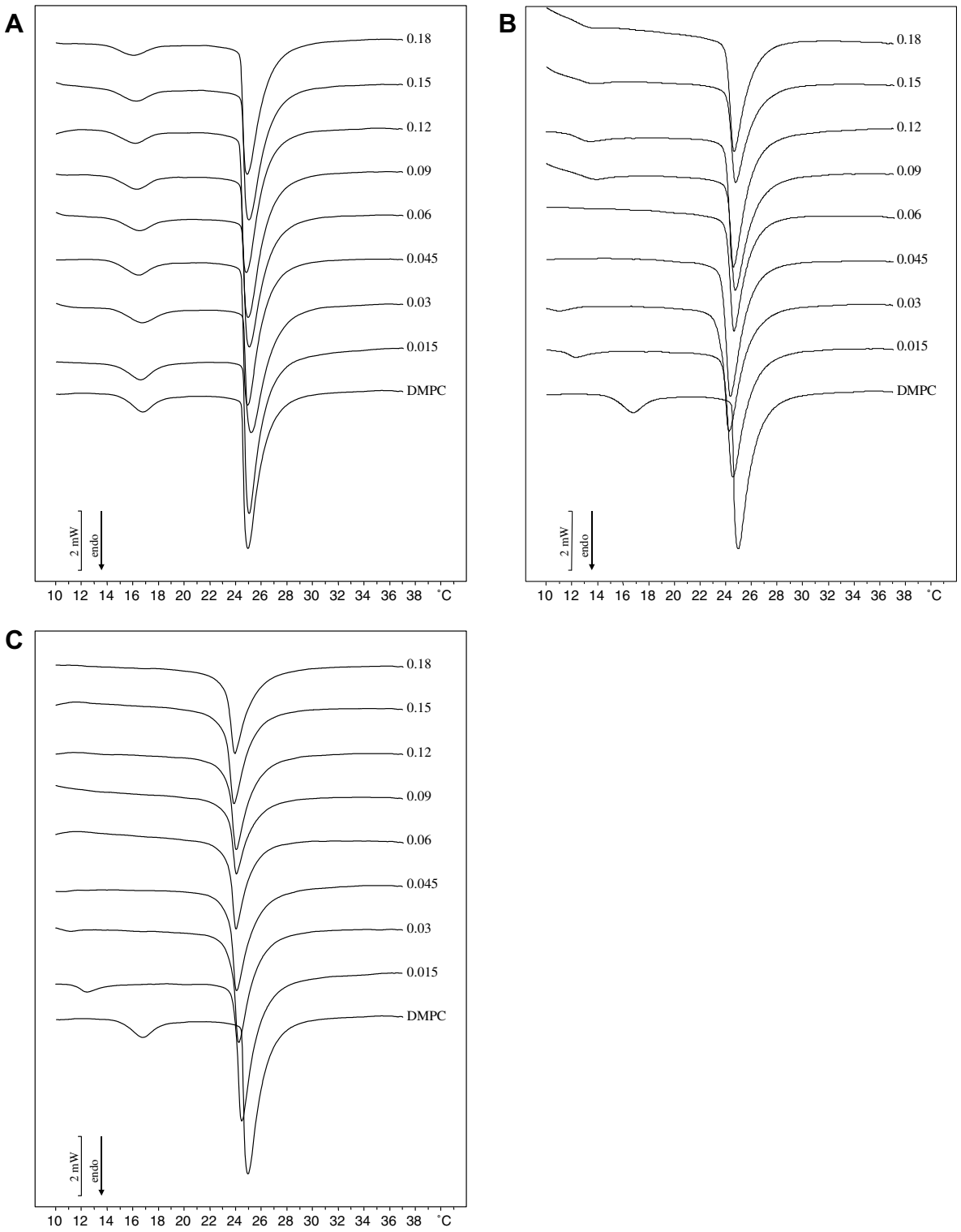

Fig. 1. Calorimetric curves, in heating mode, of DMPC MLV prepared in the presence of increasing molar fractions of (A) 2,7-dinitrofluorene, (B) 2-nitrofluorene and (C) 3-nitrofluoranthene.

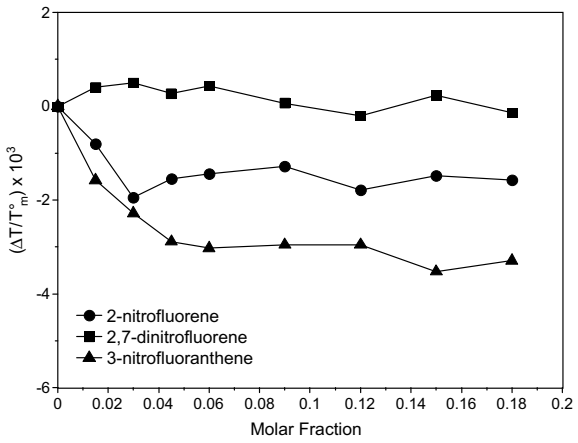

Fig. 2. Transition temperature variations, as $\Delta T/T^0_m$ ($\Delta T = T_m - T^0_m$, where T^0_m is the transition peak temperature of pure DMPC MLV and T_m is the transition peak temperature of DMPC MLV prepared in the presence of compound), as a function of compound molar fractions in the MLV dispersion.

intermediate gel phase, called the ripple phase, is observed in which the bilayer surface is undulated and the lipid chains are tilted with respect to the layer normal. The transition from the gel phase to the ripple phase is called pretransition and is related to the smaller peak called the pretransition peak (Marsh, 1995; Walde, 2004). Any variation in the pure DMPC MLV calorimetric curve indicates that the compounds are able to interact with DMPC bilayers. As far as concern the pretransition peak, there are big differences between 2,7-dinitrofluorene, with two NO_2 groups, and the other two compounds, with one nitro group. In the presence of 2,7-dinitrofluorene (Fig. 1A), the pretransition peak is clearly evident for all the molar fractions even though slightly shifted towards lower temperature. In the case of 2-nitrofluorene (Fig. 1B), the pretransition is barely evident whereas for 3-nitrofluoranthene (Fig. 1C) it is evident but largely shifted to lower temperature up to 0.03 molar fraction then it completely disappears. Speaking about the main peak, 2,7-dinitrofluorene does not cause evident variations neither in its shape nor in its dimension, for all the examined molar fractions, and the transition temperature remains almost unchanged; 2-nitrofluorene, as its molar fraction increases causes

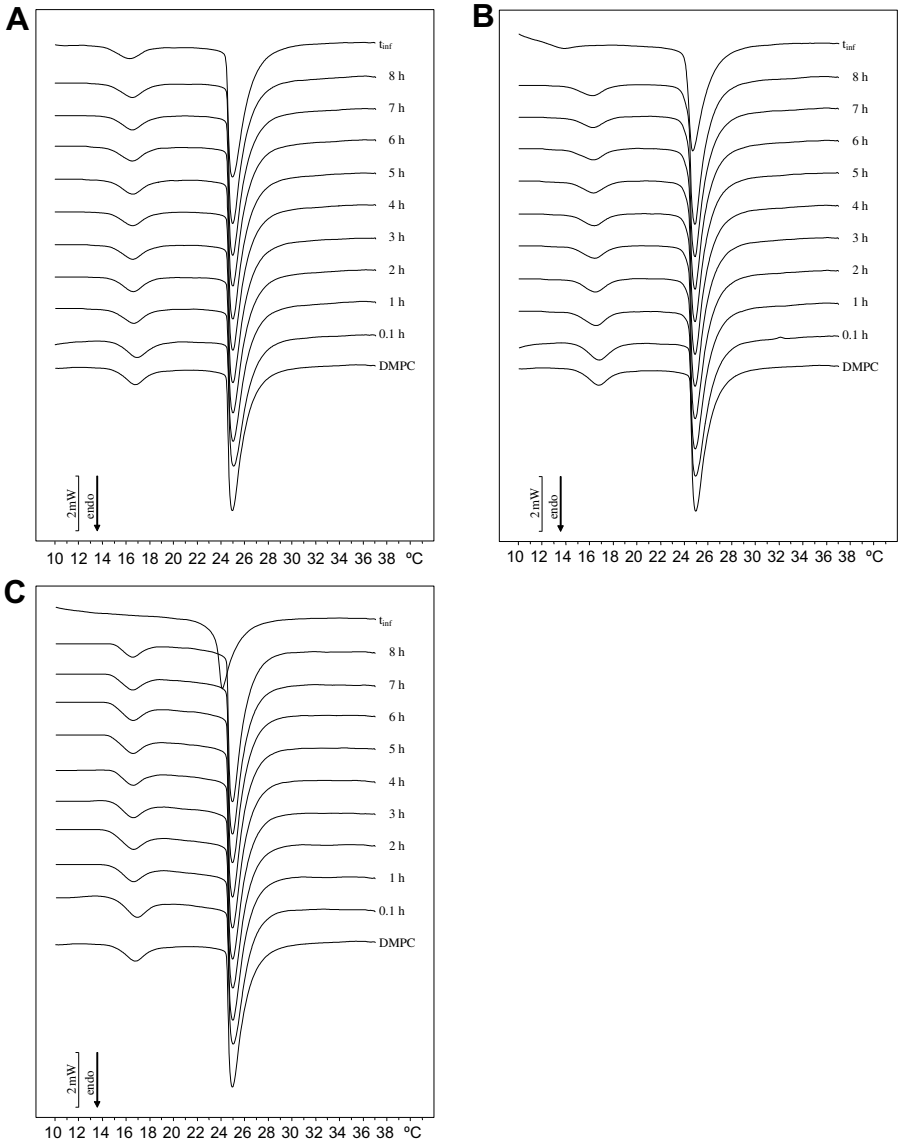

Fig. 3. Calorimetric curves, in heating mode, of DMPC MLV left in contact with a 0.09 molar fraction of (A) 2,7-dinitrofluorene, (B) 2-nitrofluorene and (C) 3-nitrofluoranthene at increasing incubation time. Curves t_{inf} belong to DMPC MLV prepared in the presence of a 0.09 molar fraction of compound.

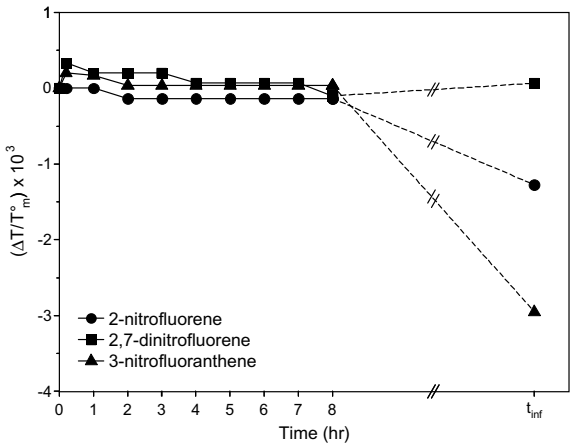

Fig. 4. Transition temperature variations, as $\Delta T/T^0_m$, of DMPC MLV left in contact with a 0.09 molar fraction of compound, as a function of the incubation time. The t_{inf} values represent the transition temperature variation of DMPC MLV prepared in the presence of a 0.09 molar fraction of compound and can be considered as the maximum interaction between compound and MLV.

the main peak to become smaller and to move toward lower temperature (at low molar fraction); 3-nitrofluoranthene causes the broadening, the reduction and the shift to lower temperatures of the main peak. The effects of the studied compounds on the transition temperature are compared in Fig. 2 where the temperature variation is reported as $\Delta T/T^0_m$ ($\Delta T = T_m - T^0_m$, where T_m is the transition temperature of the DMPC MLV prepared in the presence of nitroPAHs and T^0_m is the transition temperature of pure DMPC MLV) as a function of their molar fraction present in the MLV aqueous dispersion. The data are obtained from experiments carried out in triplicate and the standard deviation was less than 1.5%. 2,7-Dinitrofluorene exerts the weakest effect on the transition temperature. The strongest decrease of the temperature of the gel-to-liquid-crystalline phase transition is caused by 3-nitrofluoranthene, followed by 2-nitrofluorene. A part of 2,7-dinitroflourene which shows an almost flat profile, for the other compounds the transition temperature decreases as the molar fraction rises, up to a certain value (that is 0.03 for 2-nitrofluorene and 0.06 for 3-nitrofluoranthene) then no further big variations are seen.

A clear relationship is evident between the compound structure and their interaction with the biomembrane models. In fact, the

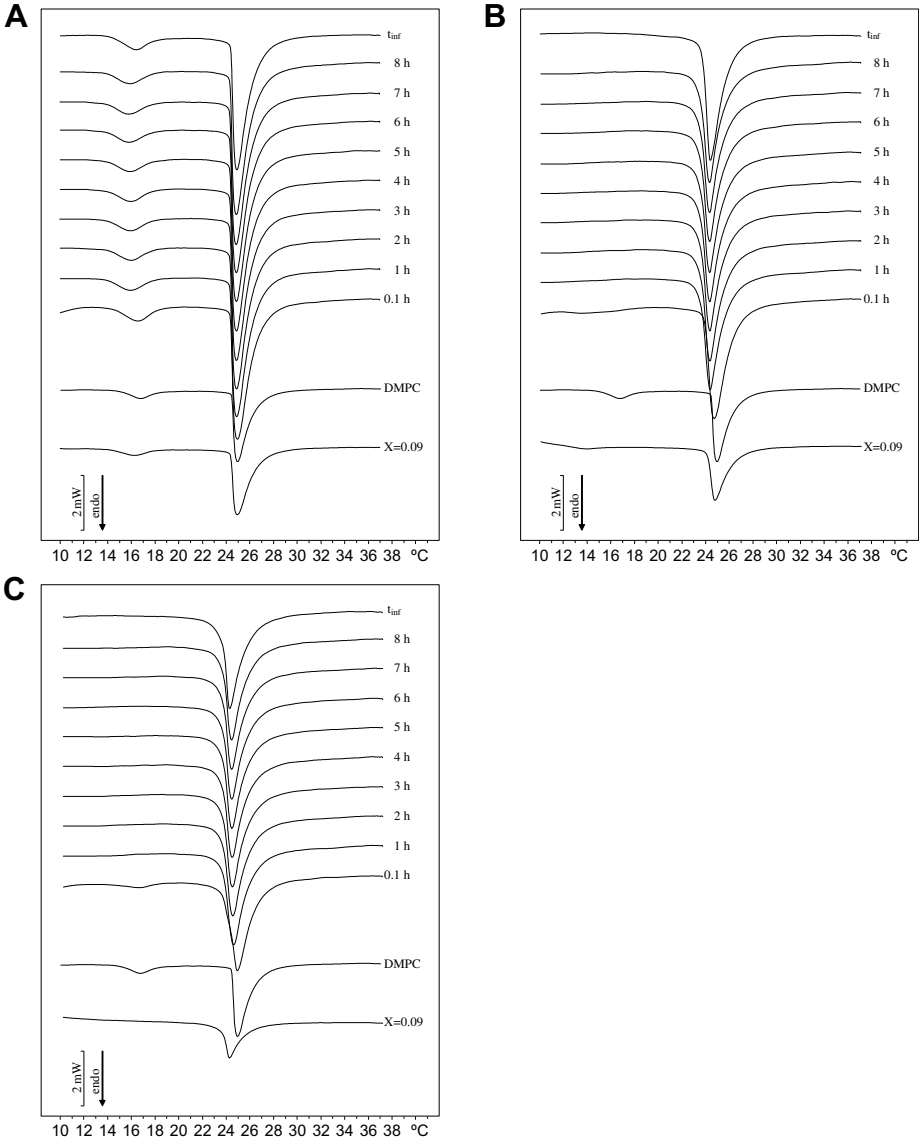

Fig. 5. Calorimetric curves, in heating mode, of DMPC MLV left in contact with an equimolar amount of DMPC MLV prepared in the presence of a 0.09 molar fraction of (A) 2,7-dinitrofluorene, (B) 2-nitrofluorene and (C) 3-nitrofluoranthene at increasing incubation time. Curves t_{inf} belong to DMPC MLV prepared in the presence of a 0.045 molar fraction of compound. Curves DMPC and X = 0.09 were obtained from the analysis of the samples before to be put in contact.

fluoranthene nitro-derivative exerts a bigger effect than the fluorene derivatives. In addition, among the two fluorene nitro-derivatives, 2-nitrofluorene shows a stronger interaction with DMPC with respect to 2,7-dinitrofluorene. As a decrease of the T_m indicates an increase of the phospholipid bilayer fluidization, 3-nitrofluoranthene, among the studied compounds, exerts the maximum fluidization effect. Inside the phospholipid bilayers, compounds can act as interstitial impurities intercalating between the phospholipid molecules and causing a T_m decrease with no modifications in the ΔH values or as substitutional impurities taking the place of the phospholipid molecules and causing a T_m and ΔH decrease (Jorgensen et al., 1991; Tenchov, 1991; Castelli et al., 1992). 2,7-Nitrofluorene does not affect in an evident way the transition temperature nor the enthalpy change (data not shown) whereas 2-nitrofluorene and, even more, 3-nitrofluoranthene cause a decrease of the transition temperature as well as of the enthalpy change (data not shown) and then, it is reasonable to say that they behavior as substitutional impurities. The preparation and stabilization method used for these experiments allows the best contact between nitroPAHs and phospholipids, the distribution of nitroPAHs inside the biomembrane model and, consequently, their complete partition between aqueous and phospholipidic phases; then, the results obtained from these measurements can be considered the maximum interaction between the studied compounds and the biomembrane models and will be used as reference for the following experiments.

To get information on the absorption of nitroPAHs by biomembranes mediated by the aqueous medium we have carried out experiments in which a fixed amount (corresponding to a 0.09 molar fraction with respect to the phospholipid) of powdered nitroPAHs has been weighted in the bottom of the calorimetric pan and DMPC MLV aqueous dispersion has been added and the interaction between the nitroPAHs and the phospholipid bilayers has been monitored at increasing incubation periods. A 0.09 molar fraction was used in these experiments as, in the experiments where MLV were prepared in the presence of increasing molar fraction of nitroPAHs, it gave a good calorimetric peak shifted towards lower temperature due to a good interaction nitroPAHs/MLV. The calorimetric curves are reported in Fig. 3. The curve t_{inf} represents the maximum possible interaction between nitroPAHs and DMPC bilayers as it belongs to DMPC MLV prepared in the presence of 0.09 molar fraction of nitroPAHs as described in MLV preparation (see Fig. 1A–C). If the compounds were able to dissolve and, successively, migrate through the aqueous medium, reaching the MLV surface and interacting with the phospholipid bilayers, the calorimetric curves should gradually become similar to t_{inf} curve. The calorimetric curves of all the studied compounds do not show variations neither in the main nor in the pretransition peak; and the curve t_{inf} is not reached. The transition temperature, as $\Delta T/T_m^0$, has been plotted in Fig. 4 as a function of the incubation time. The data are obtained from experiments carried out in triplicate and the standard deviation was less than 1.5%. No important variations are seen along the incubation time. From the obtained results it is reasonable that the studied nitroPAHs, probably due to their highly lipophilic nature and to their low water solubility (IPCS, Environmental Health Criteria 229, 2003) are not able to dissolve in the aqueous medium and, consequently, do not reach and interact with the phospholipid membranes. Hence, the nitroPAHs absorption by the phospholipid membranes is hindered by the aqueous medium.

A series of experiments has been carried out to verify if the lipophilic medium can favor the nitroPAHs absorption by the biomembranes model. With this aim, nitroPAHs (0.09 molar fraction) loaded MLV, which mimic a lipophilic carrier, have been prepared and put in contact with empty MLV and the transfer of nitroPAHs from loaded to empty MLV has been monitored by DSC analysis at increasing incubation periods. The calorimetric curves have been compared with those of the samples which were put in contact and that of the MLV prepared in the presence of nitroPAHs at 0.045 molar fraction which is considered a reference curve (curve t_{inf}) (Fig. 5). If the nitroPAHs completely transfer from loaded to empty MLV, as the incubation time increases, at the end of the process a MLV population containing a 0.045 molar fraction of nitroPAHs will be present and a curve similar to curve t_{inf} should be obtained. As far as concern 2,7-dinitrofluorene (Fig. 5A) no important variations are visible, in fact, the pretransition as well as the main peak remain almost unchanged. The calorimetric curves related to 2-nitrofluorene (Fig. 5B) show the disappearance of the pretransition peak at the second scan (obtained after 1 h the loaded and empty MLV had been put in contact) and the shift of the main peak toward lower temperature reaching the curve t_{inf}. In the calorimetric curves related to experiments carried out with 3-nitrofluoranthene (Fig. 5C), the pretransition peak is no more present from the second scan (after 1 h incubation) and the main peak shifts toward lower temperature. The curve t_{inf} is almost reached. The transition temperature of these curves is reported in Fig. 6, as $\Delta T/T_m^0$, as a function of the incubation time. t_{inf} values represent the results obtained from the curves t_{inf} (prepared in the presence of 0.045 molar fraction of compounds). The data are obtained from experiments carried out in triplicate and the standard deviation was less than 1.5%. An almost flat line is obtained for 2,7-dinitrofluorene. With regard to 2-nitrofluorene, within the first hour of incubation, the transition temperature largely decreases and then remains constant. Anyway the value t_{inf} is reached. As to 3-nitrofluoranthene, the transition temperature gradually decreases up to 5 h of incubation, then remains almost constant without reaching the value t_{inf}. These results suggest that the nitroPAHs transfer from loaded to unloaded MLV and hence that that their absorption by the biomembrane models is favored by a lipophilic medium more than an aqueous medium does.

These results are in agreement with the water solubility and the octanol–water partition coefficients ($\log K_{ow}$) of the nitroPAHs. In fact, the low water solubility joined to the high $\log K_{ow}$ values (2-fluorene = 3.37; 2,7-dinitrofluorene = 3.35; 3-nitrofluoranthene = 4.69) (IPCS, Environmental Health Criteria 229, 2003), on one hand do not allow the nitroPAHs to solubilize in the aqueous medium

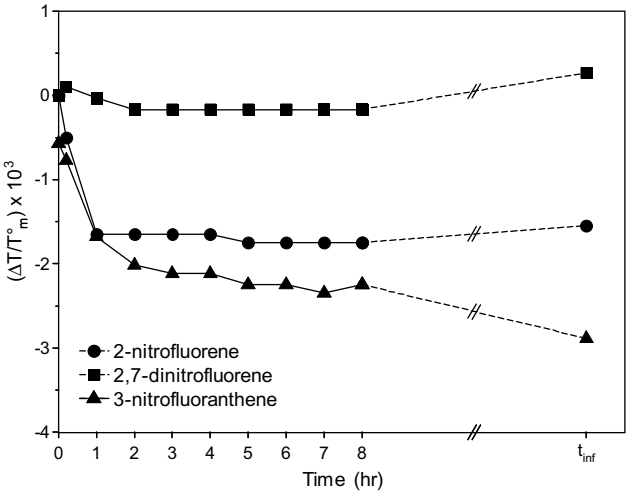

Fig. 6. Transitional temperature variations, as $\Delta T/T_m^0$, of a fixed amount of pure DMPC MLV left in the presence of equimolar DMPC MLV prepared in the presence of a 0.09 molar fraction of compounds, as a function of the incubation time. The t_{inf} values represent the transition temperature variation of DMPC MLV prepared in the presence of a 0.045 molar fraction of compound and can be considered as the maximum interaction between compound and MLV.

and reach and be absorbed by the biomembrane models on the other hand let the nitroPAHs to easily diffuse through the lipophilic medium, get in contact with the MLV surface, be absorbed by the phospholipidic bilayers and interact with them.

To verify if the interaction of these compounds with the biomembrane models can be affected by the NO_2 group, the results obtained in this study have been compared with those of a previous paper of us (Librando et al., 2003) studying the interaction of fluorene and fluoranthene with biomembrane models The comparison highlights two significant differences: (a) the stronger interaction of fluorene and fluoranthene with the biomembrane models with respect to the nitro-derivatives used in the present study; (b) fluorene and fluoranthene interact in a concentration dependent way whereas the nitro-derivatives cause the decrease of the DMPC T_m up to a certain molar fraction and then the T_m remains constant. These results are in agreement with those obtained in a precedent paper of some of us (Castelli et al., 2001) where the interaction of pyrene and nitro-pyrene with biomembrane models was studied.

4. Conclusions

In the present paper the interaction of 2,7-dinitrofluorene, 2-nitrofluorene and 3-nitrofluoranthene with biomembrane models has been studied with the aim to reveal the relationship between the compounds structure and the effect on the biomembrane models. It has been found: (a) the following order of interaction: 3-nitrofluoranthene > 2-nitrofluorene > 2,7-dinitrofluorene; (b) a stronger interaction of PAHs with respect to the corresponding nitro-derivatives.

Moreover, to have insight of the role of the medium on the absorption of the compounds by the biomembrane models kinetic experiments have been carried out which show that in an aqueous medium the nitroPAHs are not absorbed by the biomembrane models whereas when carried by a lipophilic medium the nitroPAHs are strongly taken up by the biomembrane models.

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