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The Effect of the Dipalmitoylphosphatidylcholine Lipid Bilayer State on the Adsorption of Phenyltins

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The nonspecific adsorption of amphiphilic molecules onto the membrane depends both on the properties of the adsorbate and the state of the lipid bilayer. Electrostatic interactions drive the adsorption of charged molecules and hydrophobicity determines partition of the adsorbate into the membrane, whereas the steric compatibility of the lipid bilayer and the amphiphilic molecule is an additional factor to be accounted for when considering interaction between the adsorbate and the membrane. The adsorption of phenyltins was evaluated from changes in Fluorescein-PE fluorescence intensity. The pH sensitivity of fluorophore, located at the membrane surface, was utilized to detect charges introduced onto the membrane by adsorbing compounds. It has been shown that the state of the membrane affects phenyltin adsorption in accordance with the number of phenyl rings on the molecule. Furthermore, the membrane surface topology determines interfacially located triphenyltin adsorption, with a much weaker effect on deeply embedded diphenyltin. When the dipalmitoylphosphatidylcholine (DPPC) model membrane is in the ripple phase, with complex surface morphology, phenyltin adsorption is greatly enhanced. Results presented in this paper show that steric constraints imposed on rigid and bulky amphiphilic compounds by ordered alkyl chains and membrane surface topology affect nonspecific molecule adsorption onto the membrane. Copyright © 2000 John Wiley & Sons, Ltd.

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INTRODUCTION

When a biologically active compound approaches the cell surface, it can interact with proteins or lipids, depending on its protein specificity and overall affinity with the lipid bilayer; the latter depends mainly on the hydrophobicity of the compound. Most small amphiphilic molecules penetrate the cell plasma membrane via nonspecific partition into the lipid bilayer, affecting its structure in a way similar to that of a detergent filling the free volume of the hydrophobic core. The activity of the compound depends predominantly, in this case, on its membrane partition coefficient and ability to disturb the organization of the hydrocarbon chains. However, there are molecules whose structure restricts their ability to penetrate the ordered lipid bilayer interior. Their location within the membrane depends, in addition to their hydrophobicity, on their steric compatibility with the lipid fraction of the membrane.

For practical purposes, the lipid bilayer can be divided into two compartments: the hydrophobic interior (composed of hydrocarbon chains) and the interface. The interface constitutes almost half of the lipid bilayer's thickness, and is the region between the water phase and the hydrophobic membrane interior, hence depending on the properties of both phases. Interface properties determine the efficiency of amphiphilic molecule adsorption.[1–3] The effect of the state of the interface is especially evident when adsorbing molecules have bulky and rigid residues.[4–6] Rigid rings prevent penetration into the membrane interior, regardless

of hydrophobicity evaluated by the octanol/water partition test. Steric constraints are therefore an additional factor determining the location of a compound within the lipid bilayer. As has been shown previously, phenyltins are examples of molecules whose adsorption onto the lipid bilayer depends on steric constraints.[7] The partition of these molecules is driven not only by their hydrophobicity but also by their steric compatibility with the highly ordered lipid bilayer. Steric constraints cause the less hydrophobic diphenyltin to penetrate the membrane hydrophobic core more effectively than the more hydrophobic triphenyltin. This result is in agreement with the observation that the presence of cholesterol, which modifies the membrane hydrocarbon region, reduces diphenyltin adsorption, whereas the effect of surface-active triphenyltin is complex (paper in preparation). The presence of cholesterol reduces the available free volume, limiting the amount of a compound able to be accommodated in the hydrocarbon region of the membrane.[8,9]

This paper describes studies on the effect of membrane surface topology on the adsorption of diphenyltin and triphenyltin. For that purpose, we have chosen a lipid bilayer formed from dipalmitoylphosphatidylcholine (DPPC) as a model membrane. Its phase behavior is well known and readily available from the literature: at 41 °C the DPPC bilayer undergoes its main phase transition, preceded by pretransition at 35 °C.[10] Below their pretransition temperature, lipids are in a gel phase with well-ordered hydrocarbon chains. Above the main phase transition temperature the membrane is in a liquid-crystalline phase, with increased surface area per lipid molecule and mobile hydrocarbon chains.[11,12] At the main phase transition the two phases coexist and defects, formed at domain boundaries, cause increased membrane permeability and elevated adsorption of some amphiphilic molecules.[13–16] Pretransition is associated with increased mobility of lipid headgroups.[17,18] At temperatures between those of pretransition and main phase transition, the membrane is in the so-called 'ripple phase'. The membrane has ordered hydrocarbon chains in this phase but its topology is altered: the bilayer forms ripples (the β phase) [19–21] Small sawtooth-like asymmetric ripples are characteristic features formed upon heating through pretransition.[19] Our aim in this paper is to establish the effect of the membrane state on the adsorption of amphiphilic molecules which are located differently in the lipid bilayer.

MATERIALS AND METHODS

Materials

DPPC was purchased from Avanti Polar Lipids (Alabaster, GA, USA), and the fluorescence probe *N*-(5-fluoresceinthiocarbamoyl)dipalmitoyl-L-α-phosphatidylethanolamine (Fluorescein-PE) from Molecular Probes (Eugene, OR, USA). All organotin compounds, i.e. $(C_6H_5)_3SnCl$ (triphenyltin chloride), $(C_6H_5)_2SnCl_2$ (diphenyltin dichloride) and $(C_4H_9)_3SnCl$ (tributyltin chloride), were obtained from Aldrich Chemical Co. (Steinheim, Germany) or Alfa Products (Karlsruhe, Germany). The remaining chemicals were of analytical grade.

Fluorescence measurements

Multilamellar vesicles (MLVs) were prepared in a manner described elsewhere.[7] In short, the lipid together with an appropriate amount of Fluorescein-PE was mixed in chloroform. The concentration of the fluorescent probe was below 0.1 mol% of lipid. The chloroform was then removed under vacuum, a phosphate buffer with 140 mM NaCl was added and the sample was vortexed to obtain a milky suspension of MLVs. In experiments where the dependence of fluorescence intensity on temperature was measured, an appropriate amount of an organotin compound was added to the MLVs and the sample was incubated for 15 min. Each sample of the vesicle suspension was prepared shortly before measurements and kept on ice until needed. Before each fluorescence measurement a DPPC vesicle suspension (0.26 mM) was equilibrated thermally (about 5 min) in a thermoregulated cuvette holder, to obtain a stable fluorescence intensity. When the temperature was kept constant throughout the experiment, the organometallic compound was added from a concentrated ethanol stock solution (2×10^{-3} M) to a stirred vesicle suspension in a cuvette holder. The final concentration of ethanol never exceeded 2% (v/v). The excitation (λ_{EX}) and emission (λ_{EM}) wavelengths of Fluorescein-PE were 480 and 530 nm respectively. Fluorescence intensities presented in the paper are expressed as relative changes, calculated as $(F_i - F_o)/F_o$, where F_o is the fluorescence intensity obtained for unmodified vesicles, and F_i fluorescence intensity after the addition of a certain amount of organometallic compound. For presenting the dependence of fluorescence intensity on temperature, the relative fluorescence intensities (Figs 1 and 5) are defined as $(F_i - F_o)/F_o$), where F_o

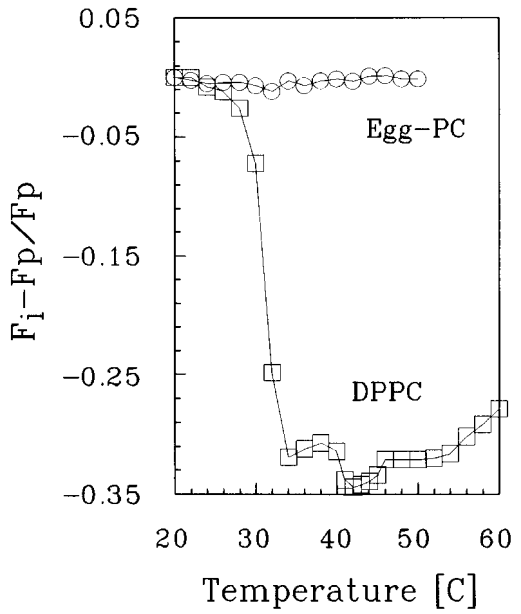

Figure 1 Relative change in Fluorescein-PE fluorescence intensity as a function of temperature. Fluorescein-PE is incorporated into liposomes formed from egg phosphatidylcholine (circles) and DPPC (squares). Fluorescence intensity is normalized to that at 20 °C.

is the fluorescence intensity at 20 °C and F_i is the fluorescence intensity measured at a certain temperature. The fluorescence measurements were carried out on a Kontron fluorimeter (Kontron Instruments, Switzerland). Fluorescence intensities were corrected for inner filter and dilution effects.[22]

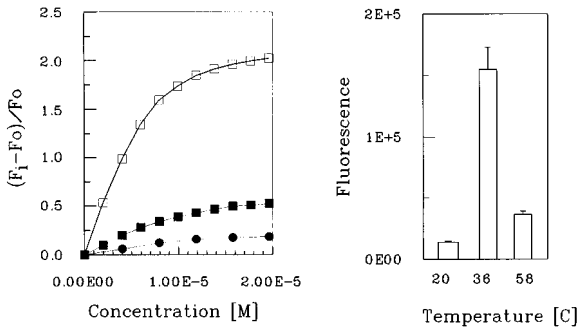

Figure 2 Relative change in fluorescence intensity of Fluorescein-PE induced by triphenyltin adsorption onto the lipid bilayer formed from DPPC, as a function of adsorbing compound concentration (left panel): ■, at 20 °C (gel state); □, at 36 °C (ripple phase); ●, at 50 °C (fluid phase). The right panel shows the slopes of relative fluorescence change calculated for triphenyltin concentration, for the range where the dependence is linear.

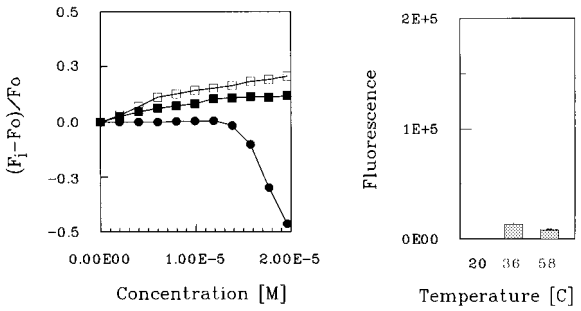

Figure 3 Relative change in fluorescence intensity of Fluorescein-PE induced by diphenyltin adsorption onto the lipid bilayer formed from DPPC. as a function of adsorbing compound concentration (left panel): symbols as in Fig. 2 The right panel shows the slopes of relative fluorescence change calculated for diphenyltin concentration, for the range where the dependence is linear.

RESULTS AND DISCUSSION

We have shown elsewhere that the two phenyl derivatives of tin interact with the lipid bilayer differently. Diphenyltin penetrates the lipid bilayer's hydrophobic core, whereas triphenyltin is adsorbed within the lipid–water interface. The toxicity of the two compounds, evaluated on the basis of the extent of erythrocyte hemolysis, shows that the surface-active triphenyltin is more potent than diphenyltin, which is embedded deeply into the lipid bilayer. The adsorption of phenyltins onto the lipid bilayer surface was detected with the fluorescence probe Fluorescein-PE, The fluorescence intensity of which depends on the local pH.[23,24] The Fluorescein is used to measure changes in the vicinity of the membrane surface as it is covalently attached to the headgroup of a phospholipid molecule (dipalmitoylphosphatidylethanolamine). Its fluorescence intensity depends on, among other factors, the bulk pH, the organization of the lipid's headgroups and the surface charge density.[23–25]

When the lipid bilayer is formed from a zwitterionic lipid (without net surface charge) and there are no conformational changes in the lipid bilayer, then the fluorescence intensity of Fluorescein-PE does not change with temperature, as shown in Fig. 1. The fluorescence probe was

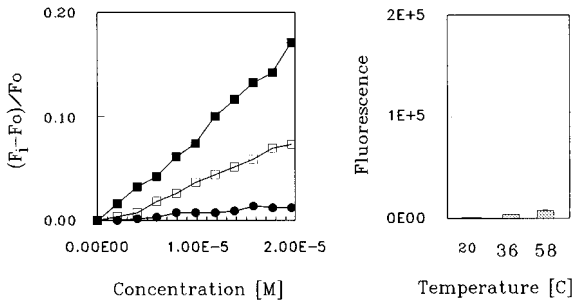

Figure 4 Relative change in fluorescence intensity of Fluorescein-PE induced by tributyltin adsorption onto the lipid bilayer formed from DPPC, as a function of adsorbing compound concentration (left panel): Symbols as in Fig. 2 The right panel shows slopes of relative fluorescence change calculated for tributyltin concentration, for the range where the dependence is linear.

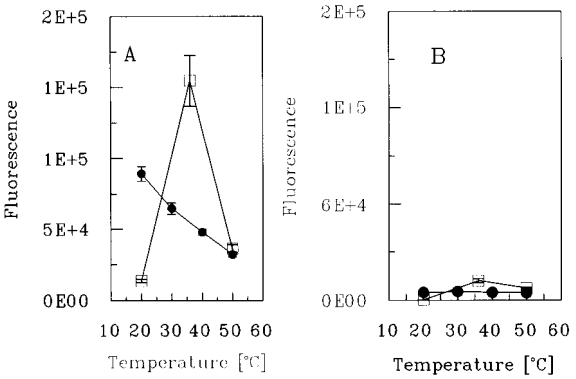

Figure 5 Changes in Fluoresceine-PE fluorescence intensity induced by triphenyltin (A) and diphenyltin (B) adsorption onto the egg-PC membrane (●) and DPPC membranes (□) as a function of temperature. Fluorescence change is represented by slopes obtained from plots of relative change in fluorescence intensity versus compound concentration in the sample.

incorporated into the lipid bilayer formed from Egg-PC (a mixture of phosphatidylcholines with different hydrocarbon chain length and saturation). Such a mixture of lipids forms stable and uniform bilayers without temperature-driven conformational changes, whereas bilayers formed from DPPC, as stated above, have two phase transitions: the pretransition at 35 °C and the main phase transition at 41 °C.[10] The fluorescence intensity of Fluorescein-PE in the DPPC membrane reflects the bilayer's conformational transformations (Fig. 1). When the sample approaches the pretransition temperature, the fluorescence intensity increases, then at temperatures in the vicinity of pretransition (36 °C) it decreases by more than 50%, and it decreases again when the temperature of the main phase transition is reached. The fine details of the dependence of Fluorescein-PE fluorescence on temperature shows a complex behavior, which depends on the thermal history of the sample among other factors. Nevertheless, general trends of the probe fluorescence in the DPPC bilayer were qualitatively similar in all samples. Data presented in Fig. 1 show that Fluorescein-PE fluorescence intensity depends more on the membrane surface morphology (sharp fluorescence drop at pretransition temperature) and less on conformational changes in the lipid bilayer hydrophobic core (main phase transition).[17]

At temperatures between that of pretransition and main phase transition, the so called P_{β}, phase is formed, which has characteristic surface features (ripples). The periodicity of the ripple pattern is membrane lipid-specific.[19,26] Consequently, considering how DPPC bilayer surface topology depends on temperature, there are three states to be examined: a highly ordered gel phase, a ripple phase with complex surface texture, and a liquid-crystal phase, with large entropic effects (surface temporal undulation and high lateral and perpendicular mobility of lipid molecules).[17] Such versatile surface topology should affect the molecule's adsorption, especially when its membrane association is sterically controlled and occurs within the lipid bilayer interface. As shown elsewhere, phenyltins with rigid phenyl rings that sterically restrict their incorporation into the lipid bilayer are examples of such molecules.[7]

We have used differences between DPPC bilayer states to test the effect of surface topology and dynamics on the adsorption of phenyltins when evaluated with surface-charge-sensitive Fluorescein-PE. Diphenyltin and triphenyltin both carry positive charges, which at the membrane interface cause the Fluoresceine-PE fluorescence intensity to rise. The method based on the measurement of fluorescence intensity changes alone does not provide quantitative data but allows one to draw qualitative conclusions regarding changes in the adsorption of the molecules studied.

The character of changes occurring within the lipid bilayer during the pretransition and the main phase transition affects the adsorption of the molecules being studied in a manner that depends on the properties of the molecule and its location within the lipid bilayer. Surface-active molecules should respond more to changes in the membrane surface, whereas the adsorption of molecules

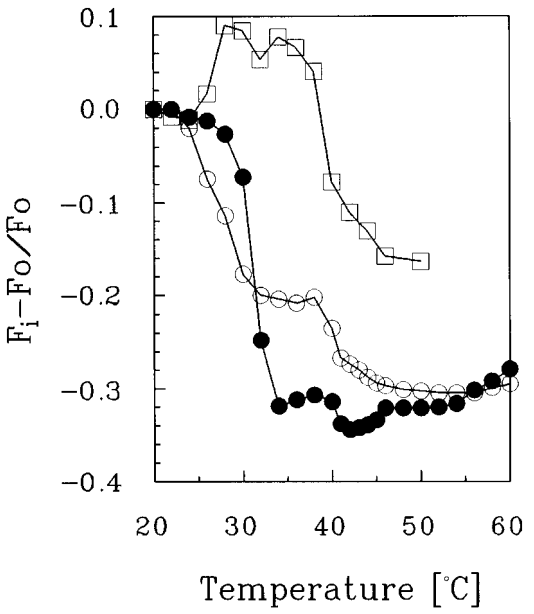

Figure 6 The dependence of the Fluorescein-PE relative fluorescence intensity on temperature when the probe was in an unmodified DPPC membrane (●) and in a membrane modified with diphenyltin (○) and triphenyltin (□). Organometallic and lipid concentrations were 48 μM and 260 μM, respectively. Fluorescence was calculated as change relative to that at 20 °C.

penetrating the interior of the lipid bilayer should be affected by changes in hydrocarbon chain order at the main phase transition.

Figures 2 and 3 show relative changes in the Fluorescein-PE fluorescence intensity as a function of phenyltin concentration. Distinct differences between diphenyltin and triphenyltin are evident. Data obtained for tributyltin, the adsorption of which is driven mainly by the hydrophobicity of flexible hydrocarbon chains, are also presented for comparison. The extent of tributyltin adsorption depends almost exclusively on the state of the membrane hydrophobic core. The lower order of the lipid bilayer, the greater the adsorption of the compound (Fig. 4).

At concentrations below 10 μM the adsorption of diphenyltin causes a rise in the probe's fluorescence intensity depending on the temperature of the DPPC sample. At the gel phase (20 °C), the fluorescence does not change, indicating that the molecule is unable to penetrate the highly ordered hydrocarbon chain region; in the liquid phase (55 °C) diphenyltin penetrates the membrane, causing the fluorescence intensity to increase and unexpectedly, the change in fluorescence intensity is greatest at temperatures where the ripple phase is present (36 °C). When the diphenyltin concentration is higher than 13 μM (Fig. 3) and the membrane is in the gel phase, the fluorescence intensity begins to fall sharply, suggesting changes in the probe's environment. The direction of the induced fluorescence change indicates that it is not caused by the adsorption of positively charged phenyltin alone but by a simultaneous rearrangement of lipids in the membrane. Based on the data presented in Fig. 1, where the fall in fluorescence indicates the appearance of the P_β phase, it can be postulated that diphenyltin forces the lipid bilayer to form the ripple phase at lower temperatures.

The effect of triphenyltin is even more conspicuous. It affects the fluorescence intensity to a greater extent than does diphenyltin (in agreement with our previous observations[7]). Located at the membrane surface, triphenyltin binds even when the membrane is in the gel phase. Its binding to the lipid bilayer in the fluid phase is about three times stronger than that in the gel phase, and similarly, as in the case of diphenyltin, the rise in fluorescence is greatest at the ripple phase. In Figs 2, 3 and 4, the slopes calculated from the left-hand panels for concentrations at which the fluorescence intensity is proportional to the amount of the compound added, i.e. below 10 μM, are presented in the right-hand panels.

When fluorescence intensity changes induced by adsorption of phenyltins onto the membrane formed from Egg-PC are measured, its temperature dependence for the two compounds differs (Fig. 5). Here, the fluorescence change induced by diphenyltin does not depend on temperature, whereas that caused by triphenyltin decreases monotonically with temperature. Such temperature dependence confirms the previously presented observations that triphenyltin adsorbs at the interface. Thermally induced fluctuations of the membrane surface reduce triphenyltin adsorption, whereas deeply imbedded diphenyltin is not affected. When data obtained for the Egg-PC membrane are compared with these for DPPC, there are no qualitative differences between the two phenyltins. At the temperature at which the DPPC membrane is in the gel phase, both di- and tri-phenyltins show depressed changes in fluorescence intensity, compared with that for the Egg-PC membrane. At the liquid-crystalline phase, changes in fluorescence in the DPPC membrane are similar to those in the Egg-PC membrane, indicating that, quantitatively, the two fluid membranes do not differ in their

ability to adsorb the compounds studied. When a DPPC membrane is in the ripple phase, its adsorption of both the compounds greatly exceeds that of the Egg-PC membrane. The effect is very pronounced in the case of triphenyltin.

As shown on Fig. 1, Fluorescein-PE fluorescence intensity itself depends on the state of the lipid bilayer. Fine details of the character of this dependence will be discussed in a future paper. The phenyltins have different effects on the probe fluorescence measured as a function of temperature. Figure 6 shows this dependence for a DPPC membrane and for DPPC membranes with di- or tri-phenyltin. There are quantitative as well as qualitative differences between the temperature characteristics. Triphenyltin modifies probe fluorescence when the membrane is in the ripple phase to such an extent that it exceeds even that of the gel phase. In addition, the major fluorescence drop is now at the main phase transition (around 41 °C). This drastic change in fluorescence indicates modifications in surface properties, caused by the triphenyltin. The effect of diphenyltin at the same concentration (48 μM) causes smaller changes, in agreement with data presented in Figs 2 and 3. The temperature of the main phase transition, when judged by the position of the second fluorescence drop, is not shifted when either compound is present. Similar results were obtained when samples were analyzed with differential scanning calorimetry.[7,27] Both compounds affect pretransition; nonetheless, there are quantitative differences in the change in fluorescence, triphenyltins causing an increase in fluorescence intensity, whereas diphenyltin preserves the general character of the fluorescence dependence on temperature, again in good agreement with data presented in Figs 2 and 3.

There is always the concern that a bulky fluorescent probe may change the lipid bilayer properties and/or interfere in the adsorption of the compounds under study. The effect of the Fluorescein-PE concentration on its fluorescence has been evaluated in separate experiments. Below 0.1 mol%, the dependence of fluorescence intensity on temperature in the DPPC membrane does not depend on probe concentration. In addition, DSC experiments preclude the effect of such Fluorescein-PE concentrations on pretransition or main phase transition. Therefore, these experiments allow us to conclude that such small amounts of probe do not change global lipid bilayer properties.

In order to evaluate the effect of probe concentration on phenyltin adsorption, relative changes in fluorescence intensities induced by their adsorbents were measured for different Fluorescein-PE concentrations. Only when the probe concentration reached 1 mol%, was the measured adsorption enhanced. This effect is likely to be caused by the negative charge associated with the carboxyl group in Fluorescein, which attracts positively charged phenyltin electrostatically. Summarizing, at the probe concentrations used in the experiments that are discussed in this paper, there Fluorescein-PE has no detectable effects on the organization of the lipid bilayer or phenyltin absorption.

Observations presented in this paper were obtained on an artificial DPPC membrane model; nevertheless, they may have some biological relevance. The enhanced binding efficiency of phenyltins to a surface with complex topology shows that rigid residues are then better accommodated. The fact that there is no measurable association of diphenyltin with the lipid bilayer in the gel phase shows that tightly packed, saturated acyl chains hinder the penetration of phenyl rings into the membrane interior, whereas interfacially located triphenytin can still be adsorbed onto a more accessible membrane interface. It is difficult to point out a single driving force for bulky and rigid molecules, that determines their location within the membrane. Most probably, the balance between electrostatic interactions, hydrophobic effects and steric constraints determine a compound's position within the lipid bilayer.

The more charged and less hydrophobic diphenyltin penetrates the membrane much more deeply than the more hydrophobic and less charged triphenyltin, showing that it is mainly steric constraints that determine the location of bulky molecules within the membrane.

For any biologically active compound approaching the cell, the plasma membrane is the first structure it encounters. A molecule may interact with the membrane-embedded proteins or be adsorbed nonspecifically onto the lipid bilayer. When in the lipid bilayer, the compound may remain within the interface or penetrate the hydrophobic core of the membrane. Since the location of triphenyltin is similar to that of tryptophan, i.e. within the lipid bilayer, it may modify the lipid bilayer surface itself or it may interact with peripheral proteins or the interfacial fraction of integral proteins. Furthermore, as shown in this paper, the topology of the altered membrane surface enhances the adsorption of phenyltins onto the lipid bilayer. There is strong evidence that the

cell surface may be a complex lateral and transversal organization of lipids and protein domains[32–36] and that thermally and/or compositionally driven undulations of the membrane are associated with processes leading to endocytosis or vesicle shedding, believed to be preceded by the alteration of the membrane surface morphology.[37,38]

Molecules whose structure predisposes them to interact with the membrane surface in a manner similar to that of phenyltins, can potentially interfere with numerous processes associated with the membrane interface or influence dynamic changes in surface morphology. Many proteins loosely or temporally associated with the membrane via weak hydrophobic and/or electrostatic interactions are prone to such interference. Triphenyltin can interfere, for example, with second messenger cascades, influencing membrane association of surface-active proteins (protein phosphokinase C, phospholipases[39,40] or proteins involved in regulation of lipidic substrate accessibility[41,42]).

CONCLUSION

Phenyltins may interfere with metabolic processes in various fashions depending on their location within the membrane. When the hemolytic potencies of phenyltins were compared, the surface-active triphenyltin was more toxic than membrane-penetrating diphenyltin.[7] This is because the interface can be disturbed in a variety of ways, whereas nonspecific modification of the hydrophobic core requires a relatively high concentration of the perturbing molecule.[28]

Adsorption of bulky amphiphilic molecules depends on their properties and the state of the membrane. A similar dependence has been shown for membrane permeability.[29–31] Data presented in this paper show that the lateral membrane surface morphology affects the adsorption of molecules, whose location within the membrane depends on their steric compatibility with the lipid bilayer surface. The metallo-organics studied contain phenyl rings, which determine the way the compound interacts with the lipid bilayer. As shown elsewhere, the location of phenyltin within the membrane does not follow its hydrophobicity, determined by the octanol/water partition coefficient. Hydrophobic triphenyltin is adsorbed onto the membrane surface, whereas the less hydrophobic diphenyltin penetrates the membrane interior.[7] These discrepancies are the result of steric constraints, which restrict molecule penetration into the ordered lipid bilayer. The data presented show that the adsorption of both compounds is enhanced when the membrane surface topology is altered.

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