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Effect of Phenyltin Compounds on Lipid Bilayer Organization

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Phenyltin compounds are known to be biologically active. Their chemical structure suggests that they are likely to interact with the lipid fraction of cell membranes. Using fluorescence and NMR techniques, the effect of phenyltin compounds on selected regions of model lipid bilayers formed from phosphatidylcholine was studied. The polarization of *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) dipalmitoyl-L-phosphatidylethanolamine and desorption of praseodymium ions was used to probe the polar region, whereas the polarization of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate measured the hydrophobic core of the membrane. In addition, changes in the *N*-(5-fluoresceinthiocarbanoly)dipalmitoyl-L-α-phosphatidylethanolamine fluorescence intensity indicated the amount of charge introduced by organotin compounds to the membrane surface. There were no relevant changes of measured parameters when tetraphenyltin was introduced to the vesicle suspension. Diphenyltin chloride causes changes of the hydrophobic region, whereas the triphenyltin chloride seems to adsorb in the headgroup region of the lipid bilayer. When the hemolytic activity of phenyltin compounds was measured, triphenyltin chloride was the most effective whereas diphenyltin chloride was much less effective. Tetraphenyltin causes little damage. Based on the presented data, a correlation between activity of those compounds to hemolysis (and toxicity) and the location of the compound within the lipid bilayer could be proposed. In order to inflict damage on the plasma membrane, the compound has to penetrate the lipid bilayer. Tetraphenyltin does not partition into the lipid fraction; therefore its destructive effect is negligible. The partition of the compound into the lipid phase is not sufficient enough, by itself, to change the structure of the lipid bilayer to a biologically relevant degree. The hemolytic potency seems to be dependent on the location of the compound within the lipid bilayer. Triphenyltin chloride which adsorbs on the surface of the membrane, causes a high level of hemolysis, whereas diphenyltin chloride, which penetrates much deeper, seems to have only limited potency. © 1997 John Wiley & Sons, Ltd.

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INTRODUCTION

Organotin derivatives are used in various industrial applications. They are applied in wood preservation, food packaging, marine antifouling paints and as fungicides in agriculture.[1,2] The biological effects of phenyl compounds have attracted much attention in recent years.[3–7] It has been shown that many organometallic compounds and their degradation products are frequently hydrophobic; hence they are likely to interact with cell membranes.[8,9] When in the plasma membrane, they can possibly interact with various membrane proteins and/or penetrate further into the cell interior. The presence of organotin compounds in the lipid bilayer does not confirm their biological activity. Various factors should be considered, e.g. the extent of disruption in the hydrophobic membrane core, the type of modification of the membrane surface and how the membrane proteins are affected.

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We selected a series of phenyltin compounds which have different hydrophobicities, net charge and size, so that their interaction with the lipid bilayer would vary. Some work on the biological activity of phenyltin compounds has been published,[10–13] but no systematic studies on the interaction of phenyltin compounds with model membranes have been presented so far. Here we present data showing the effect of triphenyltin chloride (TPhT), diphenyltin dichloride (DPhT) and tetraphenyltin (TTPhT) on the packing of the lipid bilayer and their effectiveness in causing hemolysis of red blood cells. We applied a combination of steady-state ^{1}H NMR and steady-state fluorescence spectroscopy to measure the effects of phenyltin compounds on different regions of the model lipid membrane.

MATERIALS AND METHODS

Materials

Phosphatidylcholine was extracted from egg yolk, as described elsewhere.[14] All the organotin compounds, i.e. $(C_6H_5)_4Sn$—tetraphenyltin (TTPhT), $(C_6H_5)_3SnCl$—triphenyltin chloride (TPhT) and $(C_6H_5)_2SnCl_2$—diphenyltin dichloride (DPhT), were purchased from Alfa Products, Aldrich Chemical Co. and Merck. Praseodymium chloride ($PrCl_3 \cdot 6H_2O$) and heavy water (D_2O) were obtained from Aldrich Chemical Co. and Świerk (Poland), respectively. Fluorescence probes, *N*-(7 - nitrobenze - 2 - oxa - 1,3-diazol - 4 - yl)dipalmitoyl - L-α-phosphatidylethanolamine (NBD-PE), 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5,hexatriene *p*-toluenesulfonate (TMA-DPH) and *N*- (5 - fluoresceinthiocarbamoyl)dipalmitoyl - L - α - phosphatidylethanol - amine (fluorescein-PE), were obtained from Molecular Probes (Eugene, OR, USA).

Fluorescence measurements

Small unilamellar vesicles (SUVs) were prepared as described elsewhere.[15] In short, the lipid together with an appropriate fluorescence probe was dissolved and mixed in chloroform. The chloroform was removed under vacuum, a buffer was added and the sample was vortexed to obtain a milky suspension of multilamellar vesicles. The concentration of lipid (phosphatidylcholine) in the vesicle stock suspension was 1.3 mM. Such a suspension was then sonicated for 30 min with a 20 kHz sonicator (equipped with a titanium probe) to obtain a transparent suspension of SUVs. Each sample of the vesicle suspension was prepared shortly before measurements were made, and was kept on ice during experiments. Before each fluorescence measurement the diluted phosphatidylcholine vesicle suspension (0.26 mM) was thermally equilibrated in a cuvette holder to obtain stable fluorescence (about 3 min). The organotin compound was then added in concentrated ethanol (2×10^{-3} M) to the stirred sample of vesicle suspension. The final concentration of ethanol never exceeded 2% (v/v). Fluorescence polarization was calculated according to the standard formula:[16] $P=(I_{\parallel} - I_{\perp})/(I_{\parallel}+I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are the components of the emitted light that are parallel and perpendicular to the polarization direction of the excitation light, respectively. Fluorescence intensity and polarization were measured before (F_o and P_o) and after (F_i and P_i) each addition of organotin compound. The excitation (λ_{EX}) and emission wavelength (λ_{EM})) were as follows: for fluorescein-PE, $\lambda_{EX}=495$ nm, $\lambda_{EM}=520$ nm; for NBD-PE, $\lambda_{EX}=465$ nm, $\lambda_{EM}=535$ nm; and for TMA-DPH, $\lambda_{EX}=355$ nm, $\lambda_{EM}=430$ nm. Fluorescence intensity and polarization are presented as a fraction of relative change versus initial fluorescence intensity, according to the formulae $(F_i - F_o)/F_o$ and $(P - P_o)/P_o$. Steady-state fluorescence measurements were carried out on a fluorimeter with a built-in polarization attachment (Kontron Instruments, Switzerland). Fluorescence intensities were corrected for the inner filter and dilution effects.[17]

^{1}H NMR experiments

Lecithin was dried under nitrogen and dispersed in D_2O. The final concentration of lecithin was 33 mM. SUVs were prepared as described above. The dispersion of vesicles was divided into samples, to which praseodymium ions and phenyltin compounds dissolved in the deuterated methanol were added. All data were corrected for the effect of deuterated methanol. Praseodymium ions were added to the vesicle suspension from the stock solution in D_2O. The final concentration of praseodymium ions calculated as a molar fraction of lipid in the sample was 1:3.38. In some experiments phenyltin compounds were added during vesicle formation. The ^{1}HNMR spectra were recorded on a Bruker Avance DRX

300 MHz spectrometer at 30 °C. Its parameters were as follows: spectral windows, 6173 Hz; digital resolution, 145.2145 Hz cm^{-1} or 0.48348 ppm cm^{-1}, pulse width, 10.7 μs; acquisition and delay times, 2.65 and 1 s, respectively. All other details were described by Gabrielska and Gruszecki.[18]

Red blood cell hemolysis

In the hemolytic experiments, fresh, heparinized pig blood was used. Erythrocytes collected from plasma were washed in an isotonic phosphate buffer of pH 7.4 (131 mM NaCl, 1.79 mM KCl, 0.86 mM $MgCl_2$, 11.79 mM $Na_2HPO_4 \cdot 2H_2O$, 1.8 mM $NaH_2PO_4 \cdot H_2O$) and incubated with phenyltin compounds at 37 °C. All samples contained 2% (v/v) of packed erythrocytes. The degree of hemolysis was estimated from measurement of the absorption (at 540 nm) of the supernatant taken from a centrifuged sample (Specol 11, Carl Zeiss, Jena, Germany).[19, 20] The amount of hemoglobin in the supernatant is shown as a fraction of the released hemoglobin versus that in the totally hemolyzed sample. All phenyltin compounds were taken from the ethanol stock solution. The ethanol concentration in each sample never exceeded 1% (v/v).

RESULTS

Fluorescence measurements

The fluorescence of fluorescein-PE is sensitive to the amount of charge on the surface of the lipid bilayer.[21] The fluorescent moiety of the probe is located in the aqueous phase adjacent to the membrane; hence it is sensitive to changes in the vicinity of lipid headgroups. Addition of phenyltin compound to the vesicle suspension results in the appearance of an extra charge on the membrane surface due to the adsorption of this compound onto the membrane. Figure 1 shows the dependence of the relative change in fluorescence intensity of fluorescein-PE as a function of the bulk concentration of the phenyltin compound. The results show that TPhT causes the biggest change in fluorescence intensity and that TTPhT does not introduce any additional surface charge. The other probe, NBD-PE, has the fluorescence moiety located in the polar region

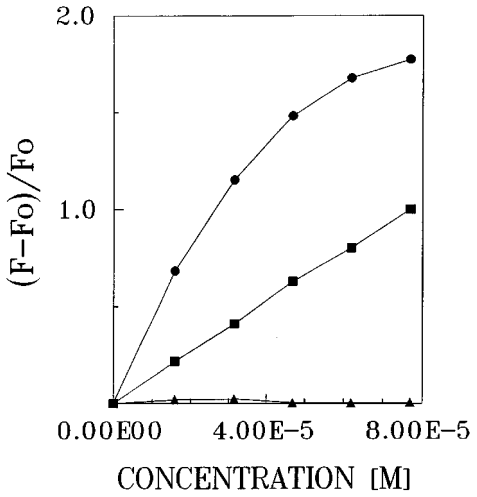

Figure 1 Relative change in fluorescein-PE fluorescence in a phosphatidylcholine bilayer induced by addition of phenyltin compounds. The concentrations given are the totals for a phenyltin compound in the sample. Lipid concentration in the sample was 0.26 mM. Small unilamellar vesicles were suspended in 140 mM NaCl phosphate buffer, pH 7.4. ▲, TTPhT; ●, TPhT; ■, DPhT.

of the membrane. As shown elsewhere, the polarization reflects the mobility of the fluorophore and its fluorescence intensity depends on the polarity of the environment. Figure 2 presents the

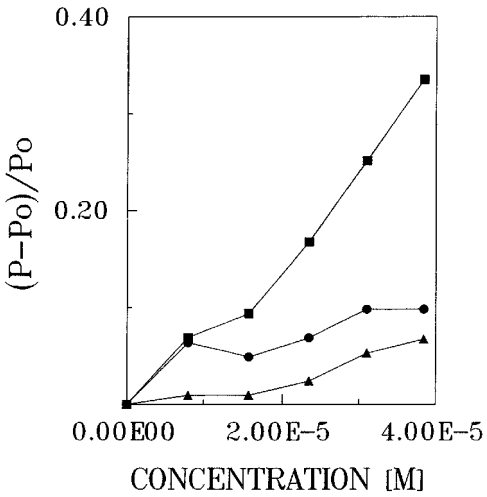

Figure 2 Effect of organotin compounds on the polarization of NBD-PE in a phosphatidylcholine bilayer. The concentration given represents the total amount of the organotin compound in the phosphatidylcholine vesicle suspension. Experimental conditions are the same as those in Fig. 1. ▲, TTPhT; ●, TPhT; ■, DPhT.

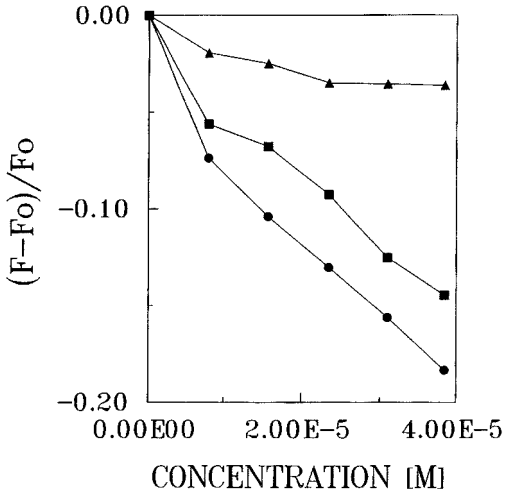

Figure 3 Dependence of relative change in NBD-PE fluorescence on the concentration of phenyltin compounds added to the vesicle suspension. Experimental conditions are the same as those in Fig. 1. ▲, TTPhT; ●, TPhT; ■, DPhT.

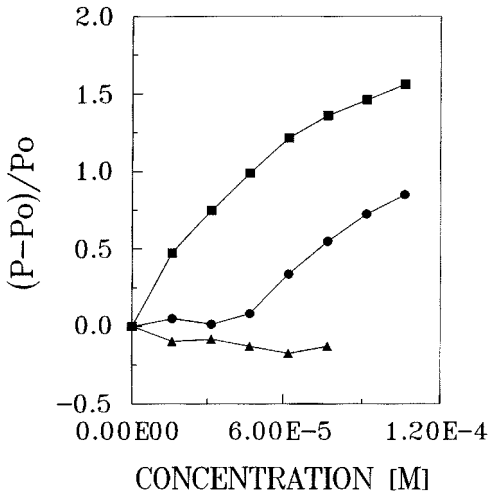

Figure 4 Dependence of the relative change in TMA-DPH polarization on concentration of the phenyltin compound added to the vesicle suspension. Experimental conditions are the same as those in Fig. 1. ▲, TTPhT; ●, TPhT; ■, DPhT.

relative change in polarization as a function of the concentration of phenyltin compound in the sample. As shown, DPhT causes the greatest effect, whereas TPhT and TTPhT induce only minor changes of NBD-PE polarization. Figure 3 shows the relative change in NBD-PE fluorescence intensity. The decreased fluorescence intensity indicates that polarity in the vicinity of the fluorophore increases. Here, TPhT reduces the fluorescence intensity of the dye the most, DPhT is less effective, whereas TTPhT affects it slightly. The polarization of TMA-DPH is sensitive to the 'organization' of the lipid bilayer in the upper part of the hydrocarbon chains.[22] The relative change in polarization as a function of phenyltin compound concentration in the sample is presented in Fig. 4. Here, the greatest effect comes from DPhT. The disturbance caused by TPhT is smaller and there is very little effect caused by TTPhT.

NMR measurements

The ^{1}H NMR spectrum of a vesicle suspension in the presence of 4.03 mM Pr^{3+} without modifications by the phenyltin compound is presented in Fig. 5, curve A. Figure 5, curve B shows an example of the ^{1}H NMR spectrum in the presence of 1.89 mM DPhT. The signal splitting between $-N^+(CH_3)_3$ groups from the inner and the outer lipid layers is proportional to the concentration of adsorbed praseodymium ions on the outer monolayer of the membrane. Adsorption of phenyltin compound molecules to the lipid bilayer causes desorption of praseodymium ions, which shows as a change of signal splitting.[23–25] Therefore, the decrease in signal splitting might be used as a measure of the amount of adsorbed phenyltin compound. Figure 6 shows the percentage of praseodymium ions released from the membrane as a function of the concentration of organotin compound in the sample (the desorption was estimated from the extent of signal splitting). The greatest desorption of praseodymium was caused by DPhT, whereas the effect of TPhT is lower. TTPhT does not cause any measurable changes in signal splitting. In the next experiment (Fig. 7), sorption of praseodymium ions to the lipid bilayer formed in the presence of phenyltin compound was measured. The sorption was estimated on the basis of the extent of signal splitting, as in the previous experiments. The presence of DPhT in the membrane prevents praseodymium ions from associating with membrane. This result agrees well with desorption experiments.

As shown previously, the linewidth of the choline $-N^+(CH_3)_3$ resonance depends on the mobility of the lipid headgroups.[26] The adsorption of the bulky phenyltin molecules should reduce the lipid headgroups' ability to move. The

increase in linewidth of the choline $-N^+(CH_3)_3$ headgroup signal versus concentration of the phenyl compound is presented in Fig. 8. Clearly, TPhT causes the greatest change. Eigenberg and Chan[27] have shown that resonances originating from the choline $-N^+(CH_3)_3$ groups situated in the inner and outer surfaces resolve into a downfield peak (outer monolayer) and an upfield peak (inner monolayer), and that the changes in peak position depend on the headgroup packing. The upfield chemical shifts in the choline $-N^+(CH_3)_3$ headgroup signal from the inner part of SUVs indicates that the packing of the inner monolayer is further increased. The increase in chemical shift of the inner monolayer as a function of concentration of phenyltin compound is shown in Fig. 9. In this case DPhT and TPhT cause similar upfield chemical shifts. There were no such effects when the vesicles were treated with TTPhT (results not shown).

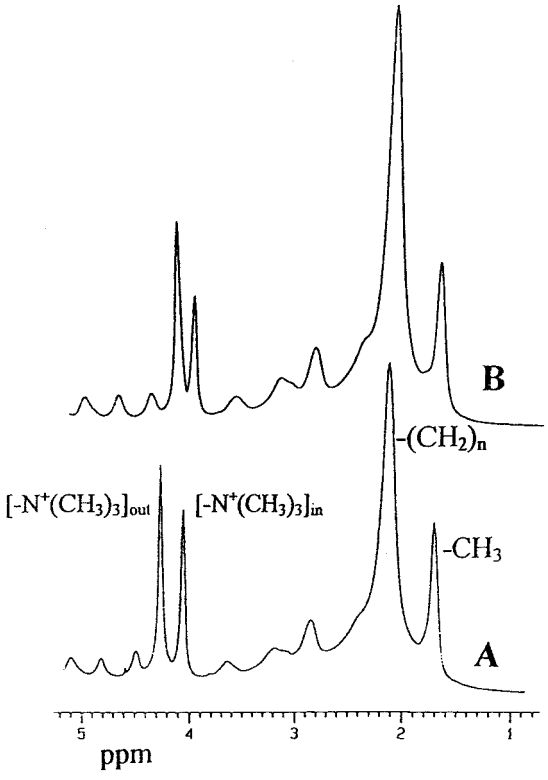

Figure 5 Curve A: An example of a typical 300 MHz ^{1}H NMR spectrum of a sample of egg yolk lecithin liposome suspension (33 mM) pretreated with Pr^{3+} in D_2O at 30 °C. The extravesicular concentration of Pr^{3+} was 4.06 mM. The water peak at 4.700 ppm was used as the reference signal (not seen in this Figure). The main signals come from extravesicular $[-N^+(CH_3)_3)]_{out}$ and intravesicular $[-N^+(CH_3)_3]_{in}$ choline headgroups, acyl chain methylenes $-(CH_2)_n$ and the terminal methyl groups $-CH_3$. Curve B: For praseodymium-pretreated liposomes modified with diphenyltin dichloride (DPhT) at 1.89 mM concentration.

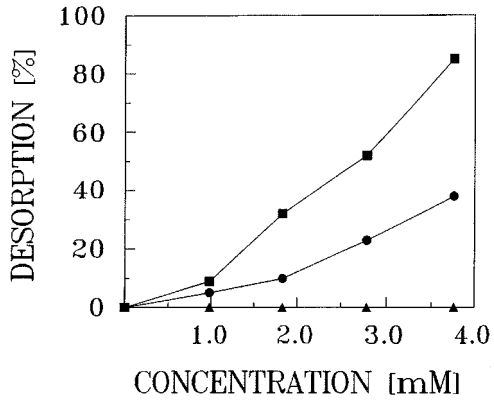

Figure 6 Effect of phenyltin compounds added to a vesicle suspension on the desorption of Pr^{3+} ions. Lipid vesicles were exposed to Pr^{3+} before the addition of the phenyltin compound. The extent of the desorption is shown as a percentage of the Pr^{3+} ions adsorbed on the membrane before the addition of the phenyltin compound. The amount of adsorbed Pr^{3+} ions was estimated from the signal splitting of the ^{1}H NMR spectra. The fraction of Pr^{3+} ions added to lecithin was 1/6.76. The lipid concentration in the sample was 33 mM. ▲, TTPhT; ●, TPhT; ■, DPhT.

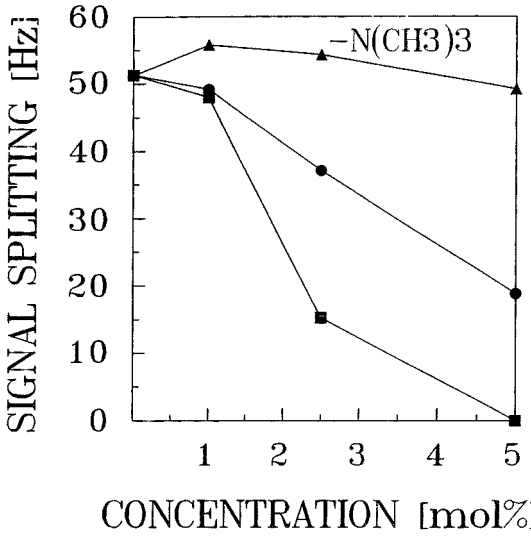

Figure 7 ^{1}H NMR signal splitting of choline headgroups $-N^+(CH_3)_3$ in a vesicle suspension after addition of $PrCl_3$ (pretreated with a phenyltin compound). The concentration of the phenyltin compound is given as a molar fraction of the lipid. The lipid concentration in the sample was 33 mM. ▲, TTPhT; ●, TPhT; ■, DPhT.

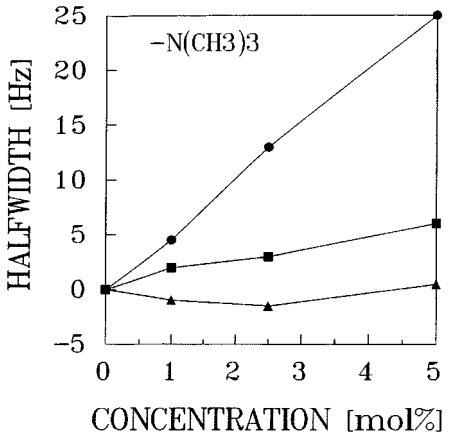

Figure 8 Effect of phenyltin compounds on halfwidth of the choline headgroup $-N^+(CH_3)_3$ resonance peak. The concentration of the phenyltin compound is given as a molar fraction of the lipid. The lipid concentration in the sample was 33 mM. ▲, TTPhT; ●, TPhT; ■, DPhT.

Hemolysis of red blood cells

When phenyltin compounds were added to the suspension of red blood cells, an increased lysis of erythrocytes was observed. The extent of lysis was used as a measure of the toxicity of the phenyltin compound. The extent of hemolysis was estimated from the amount of the extracellular hemoglobin, measured according to standard techniques.[20, 21] Cells were incubated in the presence of 10^{-4} M phenyltin compound added from concentrated stock solution in ethanol. The extent of hemolysis as a function of time is shown in Fig. 10. TTPhT, as in previous experiments on model lipid membranes, caused very little effect. TPhT caused the greatest damage to the plasma membrane of red blood cells, whereas the effect of the presence of DPhT was noticeably lower.

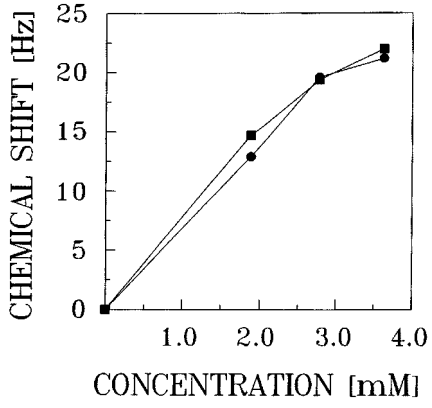

Figure 9 Dependence of the chemical shift of $-N^+(CH_3)_3$ choline headgroups located in the inner monolayer of the lipid bilayer on phenyltin compound concentration. The lipid concentration in the sample was 33 mM. ■, DPhT; ●, TPhT.

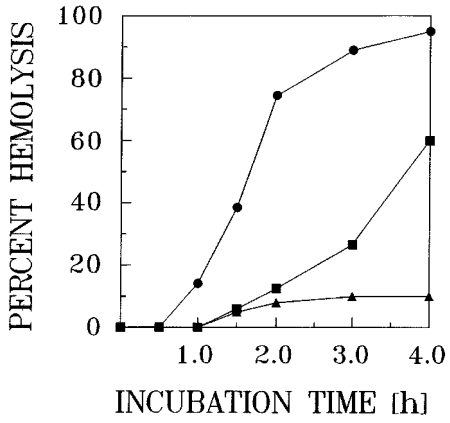

Figure 10 Hemolysis of red blood cells induced by phenyltin compounds. A phenyltin compound was added to the cell suspension (2% v/v packed erythrocytes) and the extent of hemolysis as a function of time was measured. The concentration of the phenyltin compound in the sample was 10^{-4} M. ▲, TTPhT; ●, TPhT; ■, DPhT.

DISCUSSION

The octanol/water partition coefficient is considered to be a measure of compound hydrophobicity. Hydrophobicity is used widely as an indicator of the ability of molecules to penetrate the lipid fraction of the biological membrane. The presence of a hydrophobic molecule in the lipid bilayer is the prerequisite for any biological activity. It has been observed, however, that the effect of compound hydrophobicity on model membranes and the toxicity of the phenyltin compound are not simply correlated. When the octanol/water partition coefficient is measured, a predictable series can be formed: TTPhT > TPhT > DPhT (their respective values of the logarithm of the partition coefficients are 4.39, 2.65 and 1.4)[28]. When the toxicities of the compounds are evaluated, a different sequence is formed. For example, when

toxic effects on the red killifish (*Otyzias latipes*) is measured, the compound potencies are in the order TPhT > TTPhT > DPhT.[28] The lack of the expected correlation is likely to be caused by the fact that the octanol phase is not a good model of the highly organized lipid bilayer. In order to correlate the partition of phenyltin compounds into the lipid bilayer with their toxic potency, the nature of their interaction with the lipid bilayer should be determined.

In this paper we attempt to establish the correlation between hemolytic activity and the effect of phenyltin compounds on lipd bilayer organization. The problem of the effect of the substances studied was not addressed in the case of membrane proteins.

We applied the combination of fluorescence and H^1 NMR techniques to probe various regions of the lipid bilayer. The linewidth of the ^1H NMR peak of the $-N^+(CH_3)_3$ groups in the polar region of the membrane is a measure of the mobility of the choline headgroups. When phenyltin compound is added to the vesicle suspension, a substantial increase in linewidth is observed. This increase shows that the choline groups are less mobile.[26] The greatest loss of mobility is caused by TPhT. The effect of DPhT is lower, whereas the influence of TTPhT is not measureable (Fig. 8). This result shows that TPhT is adsorbed on the surface of the lipid bilayer, affecting it the most. The lower effect caused by DPhT might result from the fact that the compound interacts more weakly with the membrane and/or that it interacts with different regions of the lipid bilayer.

When the upfield shift in $-N^+(CH_3)_3$ groups from the inner layer is measured, for both DPhT and TPhT a similar shift is observed (Fig. 9). This chemical shift depends on the headgroup packing inside the vesicles.[6] It is caused by adsorption of the phenyltin compound on the outer layer of the lipid bilayer, which changes the equilibrium between layers. This result shows that both compounds are being adsorbed on the membrane surface to the same extent. Therefore, DPhT and TPhT bind to different regions of the lipid bilayer. To test the stated theory, we applied two fluorescent probes whose polarization might be used as a measure of lipid packing in the lipid/water interface and in the hydrophobic core of the membrane.

The fluorescent probe NBD-PE has a fluorophore attached to the headgroup of the phosphatidylethanolamine, and was found to be located in the vicinity of glycerol.[29] The polarization of the NBD moiety might be used as a measure of the membrane packing in that region.[30, 31] When labeled vesicles were exposed to phenyltin compounds, the polarization was affected by DPhT more than by TPhT. There was no measurable effect from TTPhT. This result shows that DPhT binds deeper into the lipid bilayer than TPhT (Fig. 2). In addition, changes in fluorescence intensity reflect variations in the polarity of the dye environment[32, 33] (Fig. 3).

The other fluorescent probe is TMA-DPH. TMA-DPH is a linear, amphiphilic dye with a charged amine group on one end. This structure ensures that the probe is located in the hydrophobic core of the membrane, parallel to lipid hydrocarbon chains.[34, 35] The polarization of TMA-DPH reflects its mobility; hence the packing of the hydrophobic core in the bilayer can be estimated. Data obtained with this probe show again that DPhT changes the probe polarization the most. The effect of TPhT is smaller and there is no effect caused by TTPhT (Fig. 4).

The experiments discussed above show that TPhT is adsorbed on the membrane lipid/water interface, little affecting the membrane interior, whereas DPhT penetrates much deeper, causing extensive changes in the hydrophobic core. Simultaneously, DPhT does not perturb the headgroup region to the same extent as TPhT. Since phenyltin chloride dissociates in the aqueous phase, it is likely that while being adsorbed on the membrane surface it introduces additional net charge there. Due to its molecular structure, DPhT should carry a greater charge then TPhT. To measure the amount of net charge on the membrane surface, fluorescein-PE was used. The fluorophore is covalently bound to the headgroup of the phosphatidylethanolamine molecule, which fixes its location with respect to the membrane surface. The fluorophore is known to be located in the water phase adjacent to the membrane.[34] Fluorescein is sensitive to proton concentration, which in turn depends on the amount of the net charge introduced to the surface by the adsorbing phenyltin compound.[20, 36]

The results obtained with fluorescein-PE (Fig. 1) show that TPhT introduces the biggest net surface charge of the three compounds studied (TTPhT causes no effect). The smaller net charge sensed by the dye when vesicles are exposed to DPhT might result from the different location of the molecule within the lipid bilayer. Taking into

account the fact that DPhT penetrates the membrane to the polar region, it is likely that its positive charge is balanced by the negatively charged phosphate group. This is supported by the ^{1}H NMR experiments in which the desorption of Pr^{3+} ions was measured. Pr^{3+} ions, when added to the vesicle suspension, interact with the negatively charged phosphate groups of lipid molecules.[37, 38] Their release from the membrane caused by a phenyltin compound is due to the competition between them for the same binding seats. Data presented in this paper show that DPhT, as expected, releases Pr^{3+} most efficiently (Fig. 6). When Pr^{3+} ions are added to vesicles pretreated with a phenyltin compound, their associations with the lipid bilayer when with DPhT is the smallest (Fig. 7). Neither of the methods used sensed the presence of TTPhT.

Experiments on model membranes show that, although the molecules studied had similar hydrophobicities, their interaction with the lipid bilayer varies. TTPhT does not interact with the membrane at all; we were not able to detect its presence in any region of the lipid bilayer. TPhT interacts only with the choline groups on the surface of the membrane and affects the electrostatic balance of the membrane by changing the polarity of the headgroup region. DPhT penetrates the membrane deeper than TPhT and causes changes in the packing of the hydrophobic part of the membrane, but its effect on the surface properties of the lipid blayer is not great.

Finally, we studied the damage inflicted to the plasma membrane of red blood cells caused by phenyltin compounds (Fig. 10). Here, hemolysis caused by TTPhT is negligible, whereas TPhT causes hemolysis of red blood cells much faster than DPhT.

In summary, TTPhT is not able to penetrate the lipid bilayer at all in spite of its high octanol/water partition coefficient, so its effect on the plasma membrane of cells is negligible. The difference in the location of DPhT and TPhT within the lipid bilayer seems to determine their lytic potency. The data presented in this paper show that TPhT located at the surface of the lipid bilayer is more potent than DPhT which is located deeper in the hydrophobic core of the membrane.

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