APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2005; **19**: 1073–1078
Bioorganometallic Chemistry AOC
Published online 9 September 2005 in Wiley InterScience (www.interscience.wiley.com). DOI:10.1002/aoc.970

Di- and tri-phenyltin chlorides transfer across a model lipid bilayer

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Received 10 May 2005; Revised 22 June 2005; Accepted 25 June 2005

A compound's ability to penetrate the plasma membrane of a cell is the critical parameter that determines its potential to become a biologically potent factor. A well-known group of organotin compounds that exhibit toxic properties in relation to biological systems are phenyltins. There are as yet no studies that in a direct manner have established whether organotin compounds such as diphenyltin dichloride (DPhT) and triphenyltin chloride (TPhT) diffuse, or not, through the lipid bilayer, although we know that at least some organotins absorb in both liposome and biological membranes. In this paper we present a series of experiments that show transfer of these compounds across the lipid membrane using the stopped-flow technique. The results obtained demonstrate that DPhT and TPhT first adsorb onto the lipid bilayer surface, in a diffusion-controlled manner and within a very short time (0.05 s), whereas the membrane crossing was observed to be on the order of a minute. The adsorption process was easily fitted with a single exponential for both the compounds studied, indicating a single process phenomenon. The longer time kinetics (characteristic of membrane crossing) showed a complex dependence on compound concentration and the presence of cholesterol in the membrane. On passing from the outer to the inner surface of the bilayer, organotins undergo desorption and enter the liposome interior, which has been shown in lipid monolayer desorption studies. In conclusion, it can be stated that amphiphilic DPhT and TPhT permeate the liposome membrane. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: diphenyltin dichloride; triphenyltin chloride; adsorption; diffusion; bilayer; stopped-flow; fluorimetry

INTRODUCTION

Organotin derivatives are toxic compounds that occur in the human environment due to their various industrial applications.[1–9] The exposure of living organisms to even small quantities of these compounds may lead to diverse pathological changes.[10–14] Their toxicity depends on various factors; however, the most important seems to be connected with their capability to cross biological membranes.[13,15,16] There is no straightforward evidence showing the transfer of such compounds through a membrane, despite the fact that indirect evidence is numerous.[11,12,17,18] The aim of our studies was to demonstrate a direct passage of the compounds across lipid membranes (and thus across the lipid phase of biological membranes). There are a variety of experimental approaches that have been employed to investigate the effect of phenyltins on the stability and organization of model and biological membranes.[19–24] Our previous studies, as well as others, show that diphenyltin dichloride ($((C_6H_5)_2SnCl_2)$ or DPhT) and triphenyltin chloride ($((C_6H_5)_3SnCl$ or TPhT) are adsorbed onto the lipid bilayer. There are substantial differences between the two compounds. DPhT intercalates into the hydrophobic region of the lipid bilayer, whereas TPhT, due to steric constraints, resides mainly in the membrane interfacial region.[25–27] Those results, along with data obtained using haemolytic tests,[28] show that the two compounds' toxicity levels may result from differences in their location within the cell plasma membrane.[25] The destabilization of the plasma

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Contract/grant sponsor: KBN; Contract/grant number: 2 P04G 089 27.

membrane is, however, not the only way phenyltins may affect cell function. It has been shown previously that such compounds interfere with enzyme functions when examined *in vitro*.[29,30] In order to do so in the cell, they have to pass the plasma membrane barrier. The most likely possibility of doing that is by passive transport through the lipid bilayer. In this paper we show that DPhT and TPhT not only adsorb on the lipid bilayer surface, but are also capable of crossing it.

MATERIALS AND METHODS

Chemicals

Egg phosphatidylcholine (egg-PC), and cholesterol were purchased from Avanti Polar Lipids Inc. (Birmingham, AL, USA) and synthetic DPPC phosphatidylcholine from Sigma (Deisenhofen, Germany). All measurements with liposome membranes were performed in phosphate-buffered saline (pH 6.5) with 147 mM NaCl, whereas the monolayer studies were performed in a 4:1 (v/v) chloroform:methanol solution. The organotin compounds DPhT and TPhT were purchased from Alfa Products (Karlsruhe, Germany). Fluorescein-PE was obtained from Molecular Probes (Eugene, OR, USA). Chloroform, methanol and benzene of analytical grade were purchased from POCH (Gliwice, Poland).

Liposomes preparation

Small unilamellar vesicles were prepared by the extrusion method, as described elsewhere.[31] In short, the lipids and fluorescent dye (both dissolved in chloroform) were mixed in appropriate quantities and the solvent was evaporated under an argon stream. The lipid film was kept under vacuum for 2 h and hydrated with the buffer. Next, the sample was vortexed for 4 min to obtain a milky multilamellar vesicle suspension. The extrusion was performed through a polycarbonate filter with pore size of 100 nm. Organotin compound (DPhT or TPhT) in ethanol solution (10 mM) was dissolved in the buffer to obtain the desired concentrations (10–50 μM).

Stopped-flow measurements

Stopped-flow measurements were performed on an SF-61 stopped-flow spectrofluorimeter from Hi-Tech Scientific (Salisbury, UK). Changes in fluorescence intensity were detected at right angles to the incident light beam when mixing the vesicle suspension with an equal volume of a phenyltin solution at a temperature of $22 \pm 1\,^{\circ}C$. The excitation wavelength was 485 nm; emission light was measured after passing through the cut-off filter (type OG 530). To determine the reproducibility of the experimental kinetic traces, each measurement was repeated at least three times.

Monolayers formation and desorption study

Monomolecular surface layers of DPPC:DPhT or DPPC:TPhT were formed on a bidistilled water (subphase) surface in standard equipment for the study of monomolecular lipid layers.[32] It consisted of a Teflon rectangular Langmuir trough, a strain gauge to measure surface pressure and a Wilhelmi plate. To ensure the required purity, the measurement vessel was rinsed each time with an organic solvent (chloroform:methanol:benzene, 1:1:1 v/v/v) of analytical-grade purity. The Wilhelmi plate was exposed to an ethanol flame for ~1–2 min. Monolayers were formed using a Hamilton syringe by putting about 10 μl of the compounds studied on the water surface, dissolved in methanol:chloroform (1:1 v/v) mixed in the proper ratio with DPPC to a given mole fraction. The surface pressure was controlled and kept constant at 25 mN m^{-1}. After monolayer formation (methanol/chloroform evaporated immediately), 5 ml samples of the water subphase were taken at 1, 10 and 60 min time intervals and subjected to spectrophotometric analysis for tin content. The analysis was done with a plasma photometer with inductively coupled plasma (ICP) mass spectrometry (MS) mass detection, which was computer controlled in collaboration with an UltraMass 700 (Varian) analytic system. The analysis is based on creating ions of the substance being studied in the flame of an induced argon plasma at 10 000 K. The ions obtained are then separated according to mass and detected in proportion to their number. The subphase samples were spectrally mineralized with 10% nitrous acid of analytical-grade purity with addition of hydrogen peroxide at 10 MPa pressure in a Milestone (Italy) microwave apparatus. Quantitative analyses were done based on calibration curves made with ICP standards (Pronochem). The limit of tin detection was 0.4×10^{-9} kg l^{-1}. The assay was made three times for each sample.

RESULTS AND DISCUSSION

As shown previously, DPhT and TPhT are amphiphilic compounds that adsorb on the lipid bilayer surface in a different manner.[23,25,26,33] In addition, the two compounds' effects on the erythrocyte plasma membrane also differ in a way that can be correlated with their intra-membrane location. TPhT disturbs the membrane more efficiently than DPhT.[34] The haemolytic experiments, as well as those carried out on other cells, gave information regarding the overall cell response without differentiating between simple destabilization of plasma membrane integrity and interference with metabolic processes. In order to affect the metabolic process, a toxic compound must interact with important macromolecules, i.e. proteins and/or nucleic acids of the cell or other barriers, e.g. the 'blood–brain' barrier; the latter is known as a real barrier for ions, but not for amphiphilic compounds. This is why organic tin (and lead) compounds are supposed to be more toxic than inorganic ones. This is only possible when the compound is able to cross the plasma membrane barrier. The first obvious possibility is to see whether the compound crosses the lipid bilayer via simple diffusion. To investigate this possibility, a test on the

model lipid bilayer is sufficient. One possible indication of the compound crossing the lipid bilayer is the character of the compound–membrane interaction kinetics under non-equilibrium conditions. In order to determine such kinetics, stopped-flow experiments using fluorescence-labelled lipid bilayers were performed. The lipid bilayer was symmetrically labelled with fluorescein-PE. That dye is sensitive to the local pH, which is a function of the electrostatic surface potential.[35] A change of this potential caused by positively charged amphiphilic phenyltins appearance on each lipid bilayer surface can be followed as a function of time. Such a model system allows for monitoring of both the kinetics of the compound adsorption and the lipid bilayer penetration.

The kinetics of fluorescence changes was determined after mixing either DPhT or TPhT with a vesicles suspension in a stopped-flow mixing chamber. The measurements were performed on two different time scales. Within the time range 0–0.05 s the compound adsorbs onto the lipid bilayer surface, and at longer periods (0.05–500 s) the permeability processes have been observed. Examples of such traces are presented in Fig. 1. The adsorption kinetics traces were fitted with a single exponential decay, $F \approx \exp(-t/\tau)$, from which the time constant τ was calculated. The dependence of this time constant on phenyltin concentration is shown in Fig. 2. There is no difference in the calculated time constants for the two phenyltins. The observed change in fluorescence intensities shows the accumulation of surface electrostatic charges upon compounds adsorption.[34] This process is controlled predominantly by diffusion of the phenyltins towards the membrane surface from the exterior aqueous phase; hence, there are no differences in time constants observed (Fig. 2). In addition to the fast process, there are other processes that are detected in the time range of minutes (Fig. 3). These can be associated with redistribution of phenyltins within the lipid bilayer itself. In the case of TPhT, the kinetic traces obtained for all concentrations used can be satisfactorily fitted with a single exponential, indicating that a single process is observed (Fig. 3). The calculated time constants τ_1 are plotted as a function of TPhT concentration, and their values change from 50 s for low TPhT concentration (10 μM), up to 120 s at the higher concentration (40 μM); see Fig. 4. These data show that the increasing amount of TPhT changes the lipid bilayer organization, resulting in reduced permeability of the compound. It has been shown previously that TPhT has a high

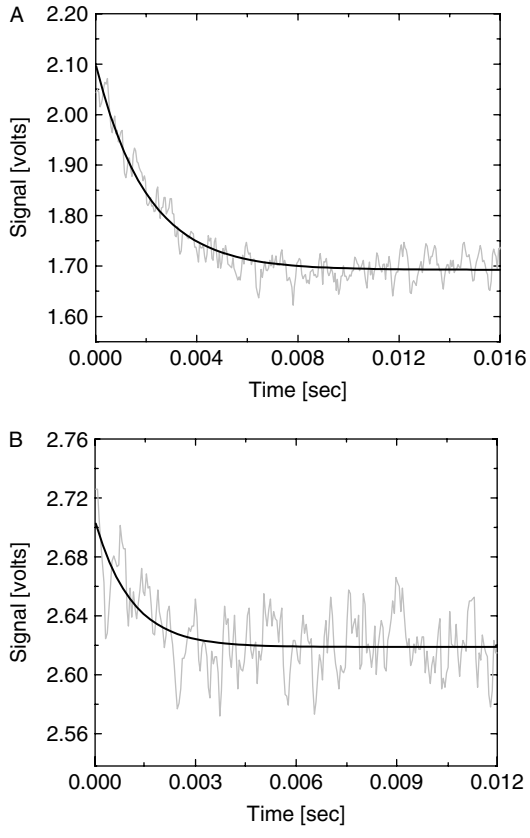

Figure 1. Fluorescence intensity changes with time for egg-PC–fluorescein-PE (2 mol%) mixed with 30 μM DPhT (A) and 30 μM TPhT (B). Lipid concentration in both cases was 131.5 μM. A single exponential function was used for approximation of the kinetic trace, and time constants were obtained. For the results presented, $\tau = 0.0020$ s (A) and $\tau = 0.0011$ s (B).

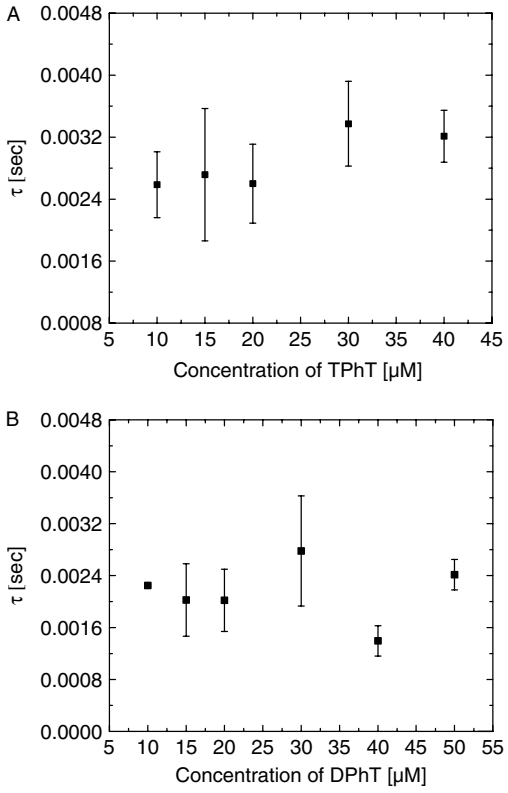

Figure 2. The dependence of the calculated time constant τ associated with the adsorption process on TPhT (A) and DPhT (B) concentration. Egg-PC concentration was equal to 131.5 μM.

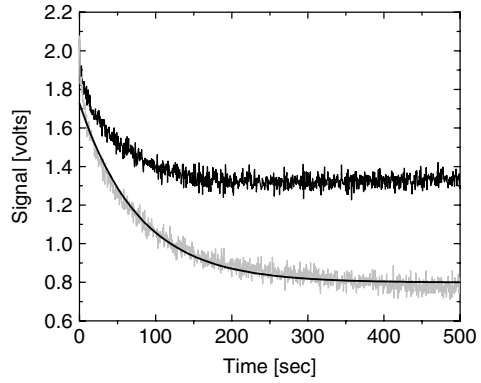

Figure 3. Example of kinetic traces for 15 μM (black) and 30 μM TPhT (light grey) mixed with fluorescein-labelled egg-PC liposomes. The smooth curve is the single-exponential nonlinear least-squares best fit.

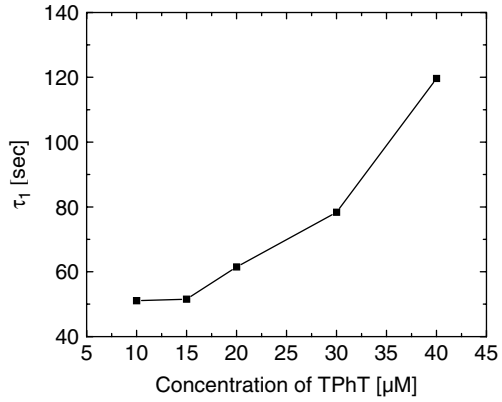

Figure 4. The dependence of the time constant τ_1 associated with diffusion through the egg-PC lipid bilayer on TPhT concentration.

potency to disturb the lipid bilayer organization, especially at the glycerol level.[23] The detailed correlation between the lipid bilayer organization and permeability of TPhT requires further studies with molecular-level resolution.

In contrast to TPhT, kinetic traces obtained over longer times for DPhT require two exponentials to obtain a satisfactory fit. Figure 5 shows an example of the dependence of fluorescence time changes when the labelled lipid bilayer interacts with diphenyltin along with a single- (Fig. 5A) and a two-exponential (Fig. 5B) approximation. The need for the two-exponential fitting of the experimental data indicates that more than one process is involved. Consequently, two time constants were obtained from each kinetic trace. The shorter one (τ_1) equals 12 s and does not depend on DPhT concentration; the longer one (τ_2) is dependent on diphenyltin concentration. The lack of DPhT concentration dependence suggests that dissociation processes are involved. It can be reasonably postulated that DPhT is in the form of aggregates, but that only the monomer is capable of

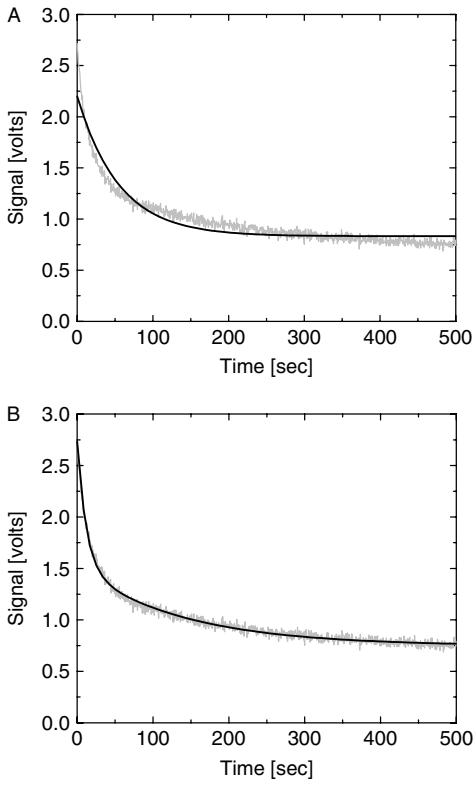

Figure 5. Example of fluorescence intensity change of fluorescein-PE in egg-PC bilayer treated with DPhT. Sample contained 131.5 μM lipid and 30 μM DPhT. The fluorescence trace was fitted with (A) one exponential function ($\tau = 55$ s) and (B) two exponential functions ($\tau_1 = 12$ s, $\tau_2 = 139$ s).

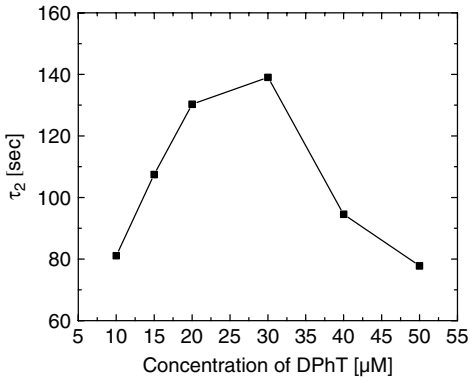

Figure 6. Dependence of the time constant associated with diphenyltin diffusion through the lipid bilayer on DPhT concentration. Samples contained 131.5 μM of egg-PC lipids.

crossing the lipid bilayer. This hypothesis needs to be validated with additional experimental data. The dependence of the longer time constant (τ_2) on DPhT concentration is presented in Fig. 6. For concentrations below 30 μM, the plot of the time constant resembles that for TPhT: the lipid

bilayer permeability decreases when the concentration of phenyltin rises. However, the calculated time constants, e.g. for phenyltins at 10 μM concentration, are larger for DPhT, i.e. 80 s versus 50 s for TPhT. However, above 30 μM the permeability of DPhT increases again, indicating that the lipid bilayer loses its barrier properties. This difference in the effect of the two phenyltins on lipid bilayer permeability correlates with their location within the membrane. TPhT located in the interface has little chance to alter the lipid bilayer hydrophobic core, whereas increased pressure in the interface region may increase the hydrocarbon chain organization.[25] Diphenyltin, which penetrates the hydrocarbon region, may act in a detergent-like manner, destabilizing the integrity of the membrane and, therefore, increasing its permeability.[25]

To show that the time constant assigned to the compound diffusion through the lipid bilayer was chosen correctly, the lipid bilayer was modified with 30 mol% cholesterol. Cholesterol is known to make the lipid bilayer less permeable by altering the organization of hydrocarbon chains and extending the thickness of the hydrophobic membrane core.[36–39] Consequently, the time constant associated with a compound's cross-membrane diffusion should rise. At the same time, the kinetics of phenyltin adsorption should remain unaltered. Examples of fluorescence time traces observed for triphenyltin treated membranes with and without cholesterol are shown in Fig. 7.

As expected, the presence of cholesterol extended the slow process, indicating a reduced lipid bilayer permeability. The calculated time constant for 30 μM TPhT extended from 78 s, when membrane was formed from egg-PC alone, to 144 s when it contained 30 mol% cholesterol. A similar increase was obtained when the permeability of DPhT was measured (Fig. 7B). In that case, since two exponential functions are needed, both time constants are increasing, which implies that the two processes (diffusion through the lipid bilayer and the other unidentified process) depend on the membrane properties; therefore, the other process should occur after DPhT adsorption.

Summarizing, the data presented show that both the phenyltins are able to cross the lipid bilayer by means of passive diffusion within a few minutes. This implies that those compounds may influence not only the plasma membrane integrity, but also may interact with intracellular structures affecting various metabolic processes. Therefore, it is very likely that, whereas the phenyltin concentration required to perturb the plasma membrane is relatively high (micromolar range), the other processes may become affected at much lower concentrations, as has been shown in various studies *in vitro* with a number of different proteins.[40] Moreover, since the two compounds are positively charged with high affinities to the membrane interfaces, they may affect the signalling pathways mediated by anionic lipids and/or they may interfere with interactions between membrane-associated proteins and the inner plasma membrane leaflet.[41,42] Experiments on desorption of both the tin compounds from the lipid monolayer to the water subphase indicate that not only are the compounds transported within both phases, but they also undergo desorption to the subphase (Fig. 8). This means that phenyltin molecules can be passively transported across the lipid barrier of both liposomes and biological cell membranes, e.g. the blood–brain lipid barrier.

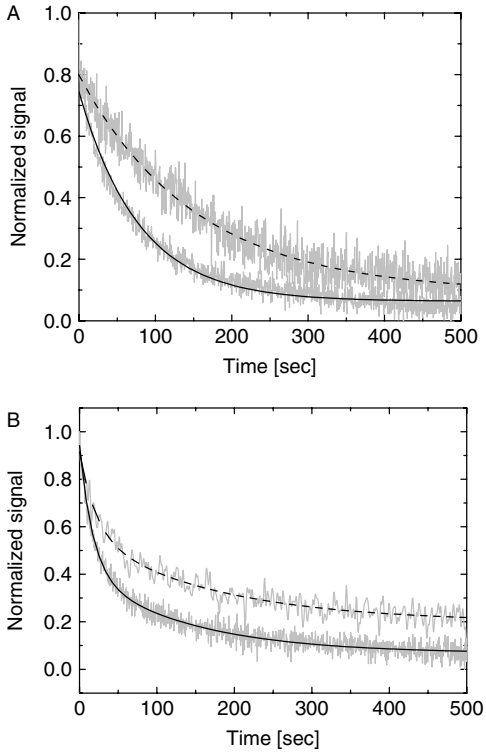

Figure 7. Normalized fluorescence intensities in the absence (straight line) and presence (dashed line) of cholesterol in the egg-PC lipid membrane after addition of 30 μM (A) TPhT and (B) DPhT. The calculated time constants were equal to 78 s and 144 s respectively for TPhT and 138 s and 168 s respectively for DPhT.

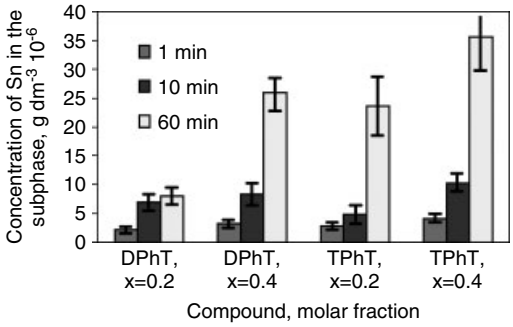

Figure 8. Concentration of tin in the subphase as a function of time (for 1, 10 and 60 min, as indicated) in the presence of DPhT or TPhT (in 0.2 and 0.4 molar fractions) in the mixed DPPC–phenyltin monolayer.

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