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Effect of triorganotin compounds on membrane permeability

Antonio Ortiz, José A. Teruel, Francisco J. Aranda *

Departamento de Bioquímica y Biología Molecular 'A', Facultad de Veterinaria, Universidad de Murcia, Campus de Espinardo, E-30100, Murcia, Spain

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Abstract

Organotin compounds are widely distributed toxicants. They are membrane-active molecules with broad biological toxicity. In this contribution, we study the effect of triorganotin compounds on membrane permeability using phospholipid model membranes and human erythrocytes. Tributyltin and triphenyltin are able to induce the release of entrapped carboxyfluorescein from large unilamellar vesicles. The rate of release is similar for phosphatidylcholine and phosphatidylserine systems and the presence of equimolar cholesterol decreases the rate of the process. Release of carboxyfluorescein is almost abolished when a non-diffusible anion like gluconate is present in the external medium, and it is restored by addition of chloride. Tributyltin is able to cause hemolysis of human erythrocytes in a dose-dependent manner. Relative kinetics determination shows that potassium leakage occurs simultaneously with hemoglobin release. Hemolysis is reduced when erythrocytes are suspended in a gluconate medium. These results indicate that triorganotin compounds are able to transport organic anions like carboxyfluorescein across phospholipids bilayers by exchange diffusion with chloride and suggest that anion exchange through erythrocyte membrane could be related to the process of hemolysis.

Keywords: Organotin compound; Carboxyfluorescein release; Model membrane; Hemolysis

1. Introduction

Triorganotin compounds are used extensively as stabilizers in the production of plastic, agricultural pesticides, preservatives of paper and textiles and antifoulant paints [1,2]. The high biological activity of these compounds towards aquatic organisms has a deleterious impact on aquatic ecosystems [3]. The toxicity of triorganotin compounds is very broad, they cause neurotoxicity in animals and humans [4] and are known to have detrimental effects on the immune response [5].

The extensively used triorganotin compounds tributyltin (TBT) and triphenyltin (TPT) are two of the most toxic species to mammalian cells. These compounds are membrane active molecules, and their mechanism of action appears to be strongly dependent on organotin lipophilicity [6,7]. They function as ionophores [8] and produce hemolysis [7], release of calcium from sarcoplasmic reticulum [9], alteration of phosphatidylserine-induced histamine release [10], alteration of mitochondrial membrane permeability [11], perturbation of membrane enzymes [12,13], and induction of apoptosis in lymphocytes [14]. Organotin compounds have been shown to affect cell signalling, they activate protein kinase C [15] and increase free arachidonic acid through the activation of phospholipase A_2 [16].

Hydrophobicity of organotin compounds suggests that interaction with membranes may play an important role in their toxic mechanism. In this respect, the understanding of the interaction between organotin compounds and the lipid component of membranes is of considerable interest. TBT has been shown to affect the thermotropic properties of dipalmitoylphosphatidylcholine, suggesting a location of the toxicant in the hydrophobic region of the membrane [17,18]. Triorganotin compounds affect the degree of hydration of the carbonyl moiety of phosphatidylcholine systems [19], they promote the formation of nonlamellar phases in unsaturated phosphatidylethanolamines [20] and disorder the packing of phosphatidylserine bilayers [21].

In order to get insight into the mechanism of the toxic action of triorganotin compounds, we present a study of the effect of these toxicants on the permeability of different phospholipid membranes and extent the study to the hemolitic activity of these compounds.

Abbreviations: CF, carboxyfluorescein; CHOL, cholesterol; FITC, fluorescein isothiocyanate; PC, phosphatidylcholine; PS, phosphatidylserine; TBT, tri-*n*-butyltin chloride; TPT, tri-*n*-phenyltin chloride

* Corresponding author. Fax: +34 968 364147.
E-mail address: fjam@um.es (F.J. Aranda).

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2. Materials and methods

2.1. Materials

Egg yolk L-α-Lecithin (phosphatidylcholine, PC) and bovine brain phosphatidylserine (PS) were obtained from Avanti Polar Lipids (Birminham, AL). Cholesterol, 5(6)-Carboxyfluorescein (CF), tri-*n*-butyltin chloride (TBT), tri-*n*-phenyltin chloride (TPT), and FITC-dextrans were obtained from Sigma-Aldrich (Spain). All other reagents were of the highest purity available. Twice-distilled and deionized water was used. Stock solution of lipids were prepared in chloroform/methanol (1:1) and stored at −20 °C.

2.2. Release of vesicle contents

Vesicles were prepared by combining the appropriate amount of lipids in chloroform/methanol (1:1). Organic solutions were dried under a stream of N_2 and last traces of solvent were removed by 3 h desiccation under high vacuum. To the dry samples, 1 ml of a medium containing 50 mM CF, 5 mM HEPES pH 7.4 was added and multilamellar vesicles were formed by vortexing the mixture. Large unilamellar vesicles were prepared by repeated extrusion of the multilamellar vesicles through polycarbonate filters (Nuclepore, 0.1 μm pore size) using a LiposoFast (Avestin, Inc, Canada). Vesicles were separated from nonencapsulated CF by gel filtration on Shephadex G-50, using 100 mM NaCl (or potassium gluconate when indicated), 0.1 mM EDTA, 5 mM HEPES pH 7.4 as elution buffer. Lipid vesicle samples were analyzed for phospholipid phosphorous according to the method of Böttcher [22].

Release of CF was measured at 25 °C in a Biotek SFM-25 spectrofluorometer. Excitation and emission wavelengths were 430 and 520 nm, respectively. For calibration of the fluorescence scale, maximum release was induced by lysing the vesicles with 1% Triton X-100. TBT or TPT were added to the vesicle suspensions in buffer from a stock solution in DMSO. The volume of DMSO added was always less than 5% of the total buffer volume and control experiments showed that DMSO by itself did not produce any release.

2.3. Erythrocytes

Human erythrocytes were freshly prepared right before the experiments from just outdated red blood cell concentrates obtained from a local blood bank. Cells were washed twice with buffer (150 mM NaCl or potassium gluconate when indicated, 5 mM HEPES, pH 7.4), and finally suspended in this same buffer at a hematocrit of 24%. All the operations were carried out at 4 °C.

2.4. K^+ leakage and hemolysis

The leakage of K^+ from erythrocytes was measured using a K^+ selective electrode (Jenway, UK) as follows. Erythrocytes were suspended in 10 ml of 150 mM NaCl, 5 mM HEPES, pH 7.4 at a hematocrit of 0.36%. TBT was added from a stock solution in DMSO (the volume of DMSO added was always less than 0.5% of the total buffer volume and control experiments showed that DMSO by itself did not produce any hemolysis), and the potassium efflux was continuously monitored using a recorder. The total amount of K^+ was determined by disrupting the cells with sodium cholate. The solutions were continuously stirred using a magnetic device. Measurements were done in a jacketed vessel and temperature was kept constant at 37 °C using a circulating water bath. Upon addition of TBT under different conditions as indicated, hemolysis was determined by measuring the absorbance of released hemoglobin at 540 nm after pelleting the membranes by centrifugation for 2 min in a bench microfuge. The total amount of hemoglobin was established by lysing the erythrocytes with water.

3. Results and discussion

3.1. Release of carboxyfluorescein from phospholipid vesicles

The ability of TBT to induce release of CF from phosphatidylcholine vesicles is shown in Fig. 1. Low μM concentrations of TBT, corresponding to molar fractions of TBT ranging from 0.01 to 0.1, produced a rapid release of CF which was complete within seconds for samples containing a TBT concentration higher than 1 μM.

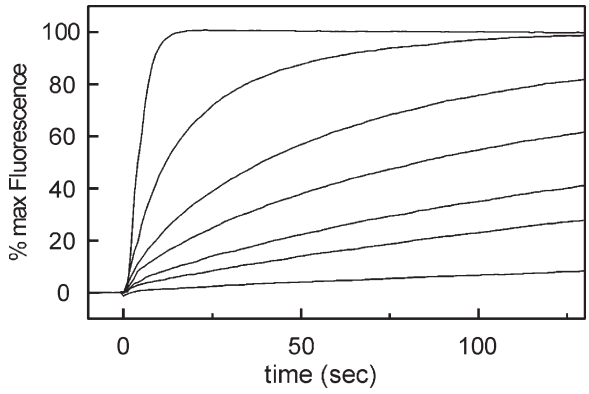

Fig. 1. Time dependence of TBT induced release of CF from phosphatidylcholine vesicles. TBT was added to a phosphatidylcholine vesicle suspension (25 μM) at different concentration, from top to bottom: 1.31, 0.38, 0.25, 0.17, 0.12, 0.07, 0.02 μM.

Fig. 2 shows the initial rate of CF release from vesicles of different phospholipid composition after addition of TBT. Initial rates of CF release were very similar for vesicles composed by phosphatidylcholine and phosphatidylserine, while the presence of cholesterol (1:1) produced a drastic decrease. The inset of Fig. 2 shows the time course of CF release after addition of 0.51 μM TBT. Considering that phosphatidylcholine is a neutral phospholipid and phosphatidylserine bears a negative charge at pH 7.4, it seems that the composition of the polar head group of the phospholipid is not important for the mechanism of release of entrapped CF. It has been suggested that triorganotin compounds are located in the upper part of the hydrophobic region of the membrane [17,19,23] where cholesterol is also located. The effects of cholesterol on membrane permeability are well established [24]. Two parameters have been identified which are related to structural discontinuities and defects in the lipid bilayer. These factors are related to the “free volume” in which small permeating molecules can reside and move with this free volume [25]. At the molecular level, there are kinks formed due to trans-gauche isomerization which runs along the hydrocarbon chain, and at the lateral organization level, there are membrane defects formed at the phase boundary under conditions that lateral phase separation occurs [26]. The presence of cholesterol in the membrane minimizes free volume, thereby reducing membrane permeability [27]. These effects could explain the observed reduction of CF release in the presence of cholesterol.

TPT was also able to release CF from phospholipid vesicles and similar qualitatively results were obtained concerning the presence of cholesterol (not shown). It is interesting to observe that for any given triorganotin concentration the release of CF in the presence of TPT was an order of magnitude less efficient than in the presence of TBT. The latter is in accordance with the observation that TPT is less toxic [28] and induces less drastic lesions [29] than TBT.

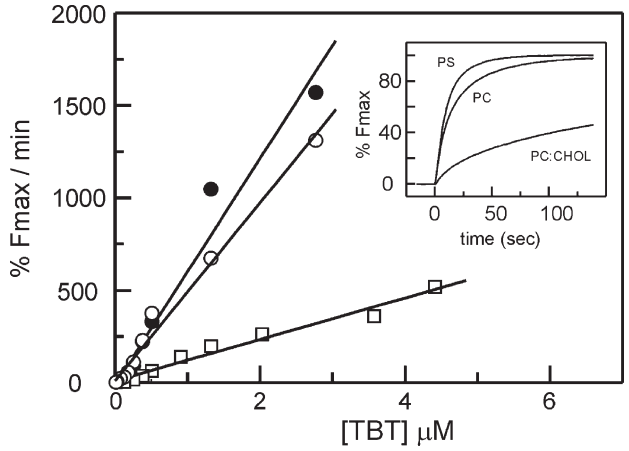

Fig. 2. Effect of increasing concentration of TBT on the initial rate of CF release from phospholipid vesicles (25 μM) of different composition, phosphatidylserine (●), phosphatidylcholine (○), and phosphatidylcholine/ cholesterol (1:1) (□). Data were fitted by linear regression using Microcal Origin 5.0 software. The inset shows the time dependence of CF release from phospholipid vesicles of different composition after addition of TBT (0.51 mM), phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylcholine/cholesterol (1:1) (PC/CHOL).

3.2. Effect of external non-diffusible anion on the release of CF from phospholipid vesicles

It is known that triorganotin compounds can mediate exchange diffusion of halides and other inorganic anions across biological and model membranes [8,30,31]. To test the possibility that the organic anion, CF, which is encapsulated inside phospholipid vesicles, could be released through exchange diffusion with external chloride, we measured release of CF under two different conditions: samples with chloride present in the external medium and samples in which chloride was replaced by gluconate. Fig. 3 clearly shows that in the absence of chloride and in the presence of gluconate, there is almost no release of CF from phosphatidylcholine vesicles. Similar qualitatively

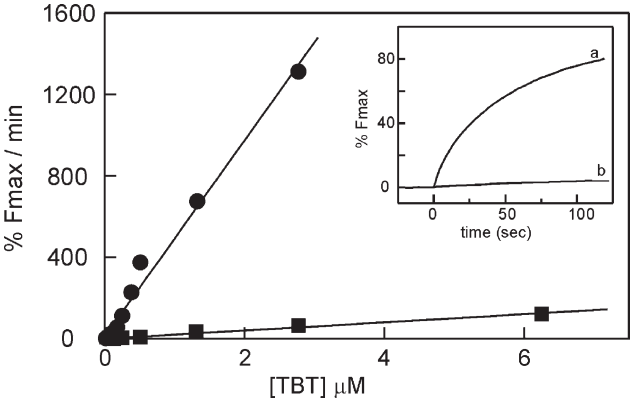

Fig. 3. Effect of increasing concentration of TBT on the initial rate of CF release from phosphatidylcholine vesicles (25 μM) placed in a medium containing chloride (●) or gluconate (■). Data were fitted by linear regression using Microcal Origin 5.0 software. The inset shows the time dependence of CF release from phosphatidylcholine vesicles placed in a medium containing chloride (curve a) or gluconate (curve b) after addition of TBT (0.25 μM).

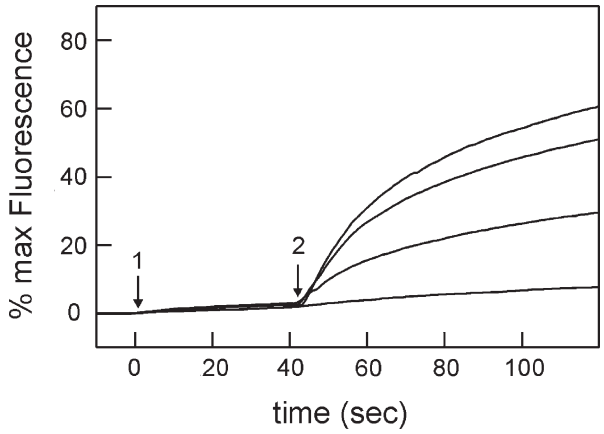

Fig. 4. Time dependence of CF release from phosphatidylcholine vesicles (25 μM) placed in a medium containing gluconate after addition of TBT and subsequent addition of chloride. (1) Initial addition of TBT (0.25 μM); (2) subsequent addition of sodium chloride at different concentration, from top to bottom: 100, 50, 16.6 and 3.3 mM.

results were obtained for TPT samples (not shown). The addition of chloride to the external medium containing gluconate restored the ability to release encapsulated CF, the rate of release being dependent of the concentration of added chloride (Fig. 4). TBT is able to exchange chloride and CF, but as shown in Fig. 5, this exchange is not produced when a larger molecule, like FITC-dextran, is encapsulated inside the vesicles. These results indicate that triorganotin compounds are able to transport organic anions across the lipid bilayer by a mechanism of exchange diffusion with chloride.

In order to test the possibility that transport through exchange diffusion with chloride could be related to the known hemolytic ability of TBT, we will next characterize the release of hemoglobin after TBT addition and then study the effect of a non-diffusible anion on the process.

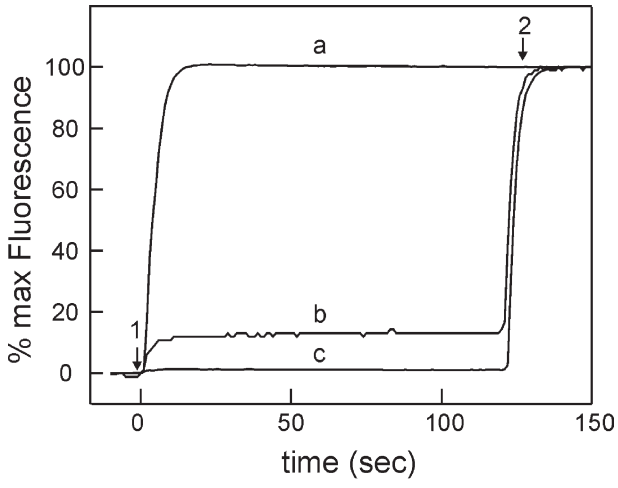

Fig. 5. Effect of TBT on time dependence of release of contents from phosphatidylcholine vesicles (50 μM) containing CF (curve a), FITC-dextran 4 (curve b), and FITC-dextran 10 (curve c). (1) Addition of TBT (1.31 μM) and (2) addition of triton X-100.

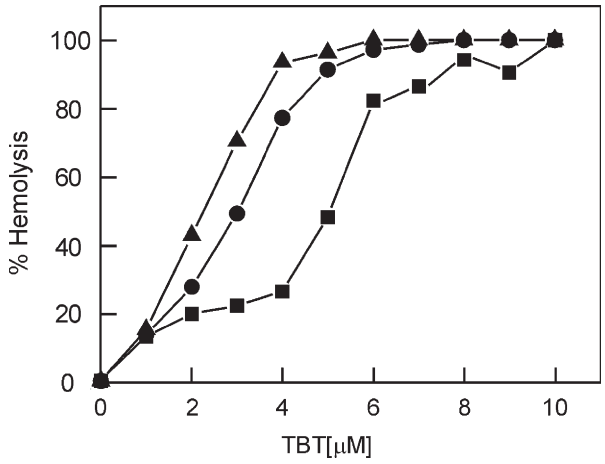

Fig. 6. Dependence of the relative hemolysis of human erythrocytes on the concentration of TBT, at different incubation times: 3 min (■), 7 min (●) and 11 min (▲).

3.3. Hemolytic activity

The ability of triorganotin compounds to cause hemolysis of human and other species erythrocytes has been reported before [28,32,33]. The kinetics of TBT induced hemolysis has been described to be very different depending on the erythrocyte species, whether whole blood or red cells concentrate are used, and also on the effective concentration of erythrocytes in the incubation mixture. The dependence of hemolysis on TBT concentration at different incubation times is presented in Fig. 6. It is seen that low μM concentration of TBT produced a relatively fast liberation of hemoglobin which was complete within 10 min.

Upon interaction with erythrocytes, TBT also induced the leakage of K^+ ions in a concentration dependent manner. Initial rates of K^+ leakage were obtained and they are shown in Fig. 7. It can be seen that increasing concentration of TBT produced an

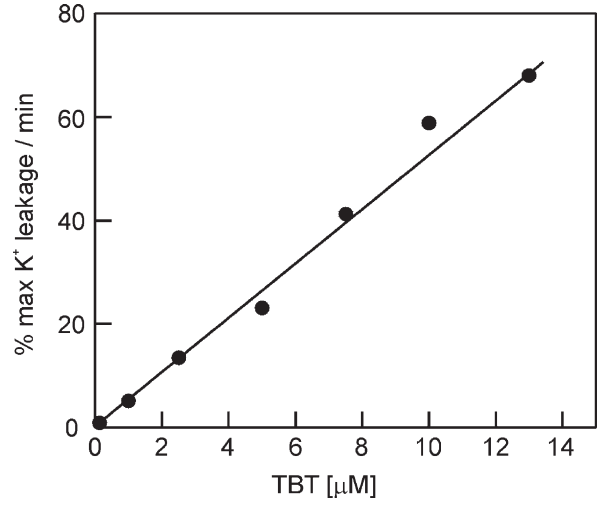

Fig. 7. Effect of increasing concentration of TBT on the initial rate of K^+ leakage from erythrocytes. Data were fitted by linear regression using Microcal Origin 5.0 software.

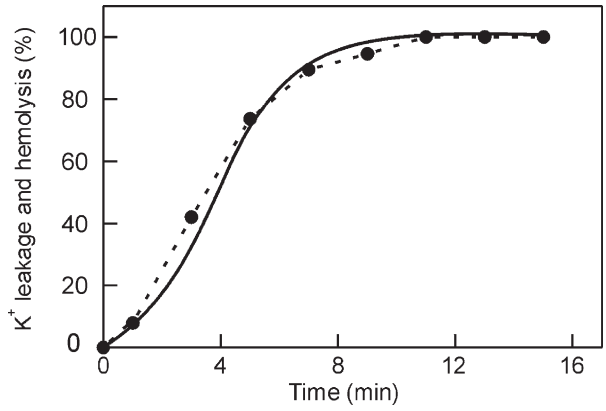

Fig. 8. Relative kinetics of K^+ leakage (continuous solid line) and hemolysis (●, dashed) induced by TBT (5 μM).

increase in the initial rate of K^+ leakage, showing a linear dependence with no saturation in the range of TBT under study.

The relative kinetics of K^+ leakage and hemolysis of human erythrocytes were measured and compared (Fig. 8). As shown in this figure, the K^+ leakage curve resulting from incubation of erythrocyte suspension with 5 μM TBT is very close to the hemolysis curve. The sigmoidal kinetic pattern has been taken as an indication of a complex process representing accumulation of sufficient membrane damage to permit leakage of hemoglobin from cells [34]. Several hemolytic agents, including melittin [35], ethanol [36] and iturin A [37], have been described to cause hemolysis by the colloid-osmotic mechanism. These agents form pores or small lesions in the membrane producing the rapid leakage of K^+, water enters due to the osmotic gradient created by hemoglobin trapped inside, the erythrocyte swells, further damage occurs and finally hemoglobin is lost. The similar rate of K^+ leakage and hemoglobin release observed if Fig. 8 strongly excludes the formation of pores and consequent colloid-osmotic lysis. Rather, the results support the concept or TBT-induced hemolysis via direct membrane disruption. It has been reported that organotin compounds induce erythrocyte shape transformation and form tin-

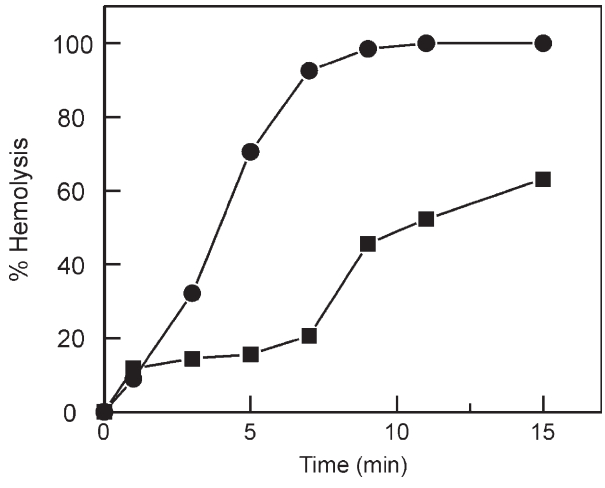

Fig. 9. Dependence of the relative hemolysis of human erythrocytes after addition of TBT (5 μM) in chloride (●) or gluconate (■) buffer.

containing aggregates within the plasma membrane which have been associated with hemolysis [38].

Erythrocyte membrane disruption is in contrast with the lack of membrane alteration described above for model membranes. This may reflect the more complex nature of the red cell as compared to phospholipid vesicles. The different erythrocyte membrane composition and the presence of several ions and osmotic active molecules like hemoglobin into the erythrocyte aqueous inner space may be the cause of the distinct response to TBT.

The detailed mechanism for TBT induced hemolysis has not been determined. It has been proposed that a rapid ATP depletion, possibly due to inhibition of membrane calcium or sodium–potassium pumps, could produce a rapid erythrocyte shape transformation followed by lysis [39]. TBT has been claimed to disrupt the cytoskeletal–lipid bilayer interactions leading to shape transformation and lysis [40]. The possibility that TBT could act producing free radicals resulting in membrane alteration and lysis has also been presented [41].

Hemolysis was measured with erythrocytes suspended in medium with a non-diffusible anion, gluconate, and compared with those suspended in a medium with chloride. Fig. 9 shows that relative hemolysis is decreased when erythrocytes were in a medium in the absence of chloride, suggesting a protective role for gluconate against TBT induced hemolysis. We have described above that the protective effect of gluconate against CF release from phospholipids vesicles can be reversed by the presence of chloride. Although the possibility of an electrostatic interaction between TBT and gluconate which could prevent the interaction of TBT with the erythrocyte membrane cannot be ruled out, the results suggest that gluconate may protect erythrocyte from hemolysis simply because it cannot be easily transported across the membrane. The data suggest that transport of anions across the membrane could be related to the mechanism of hemolysis. Organic and inorganic anion transport across the erythrocyte membrane may alter ionic gradients and led to the reported shape transformation and lysis, producing the simultaneous release of hemoglobin and K^+. Whether anionic transport is the primary event in hemolysis or it acts complementing other proposed mechanisms will require further study.

4. Concluding remarks

In this work, we have shown that triorganotin compounds are able to transport organic anions across the phospholipid bilayer through an exchange diffusion mechanism with chloride. This transport is not affected by the nature of the polar head group of the phospholipids, but it is reduced by the presence of cholesterol which could make the diffusion of triorganotin compounds more difficult given its known permeability-decreasing effect on membranes. The study of the hemolytic activity of TBT showed that the toxicant induces the release of hemoglobin from erythrocytes at a similar rate that the release of K^+ which indicates that TBT is not forming small lesions in the membrane. The results suggest that anion transport might be related to the hemolysis mechanism.

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