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Plasma Membrane Perturbation Induced by Organotins on Erythrocytes from *Salmo irideus* Trout

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Tributyltin chloride and its degradation products monobutyltin and dibutyltin act as water pollutants, owing to the use of tributyltin chloride as a biocide in marine paint formulations. These compounds are lipid-soluble and undergo bioaccumulation and bioconcentration. *Salmo irideus* trout erythrocytes were studied to evaluate the possible effects of these compounds on freshwater fish, which could be exposed to long-term effects due to bioaccumulation of organotins. Data showed that tributyltin increases the haemolysis rate, starting at 10 μM, while dibutyltin has a scant protective effect at each concentration tested. Similar studies were performed in the presence of carbon monoxide (CO), which is protective against membrane oxidative stress due to haemoglobin (Hb) auto-oxidation. In these conditions all the organotins tested induced an increase in the haemolysis rate. These results suggest that the consequence of auto-oxidation of Hb could condition the effects of some organotin compounds. Steady-state fluorescence of probes embedded in the lipidic part of the membrane was used to evaluate the modifications induced by organotins to the physico-chemical state of phospholipids.

Keywords: organotins; trout eythrocytes; lysis; fluorescence; DPH; TMA-DPH

ABBREVIATIONS

TBT	tributyltin
DBT	dibutyltin
MBT	monobutyltin
DPH	1,6-diphenyl-1,3,5-hexatriene
TMA-DPH	1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene
Bu_2 SnBr(of)	DBT-3-hydroxy-flavone bromide
TBTC	TBT chloride
DBTC	DBT chloride
MBTC	MBT chloride
TPTC	triphenyltin chloride
RBC	red blood cells
Hb	haemoglobin

INTRODUCTION

Alkyltin compounds are used in industry mainly as stabilizers for chlorine-containing materials and as biocides, for example in disinfectants or in biocidal preservatives for textiles, paper and paints.[1] In particular, tributyltin compounds (TBT) are used as biocides in marine antifouling paint formulations. Other derivatives, such as monobutyltin (MBT) and dibutyltin (DBT), are used especially as stabilizers for PVC. However, the presence of mono- and di-butyltin species in the environment is mainly the consequence of degradative processes of TBT.[2] TBT and its degradation compounds are found in areas of heavy shipping, due to its use as an antifouling agent in marine paints.[2] In addition, the application of TBTC as a disinfectant agent in some industries may allow contamination of sewage effluents. Organotins are lipid-soluble and undergo bioaccumulation and bioconcentration.[1,3] Sediment concentrations of these compounds may provide a long-term storage reservoir, possibly causing a food-chain accumulation. The toxicity of organotins is determined

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by the number and nature of the organic substituents on tin(IV). In general, the toxicity decreases from tri- to mono-alkyltins.[4,5] Moreover, the toxicity within each class of organotins is determined by the number of carbon atoms in the side chain. In general, elongation of the carbon chain renders the organotins less toxic.[1] The toxicity of alkyltin derivatives is probably linked to their incorporation into the cell. Differences in their intracellular distribution are primarily due to their liposolubility or affinity with the intracellular lipids and lipophilic proteins. Due to the increased application of these compounds, concern about their possible environmental and health effects has increased in the last ten years. The aim of the present investigation is to study the haemolytic activity of some organotin derivatives on *Salmo irideus* trout erythrocytes, in order to evaluate the possible effects of increasing concentrations of these compounds on freshwater fish, which could be exposed to long-term effects due to the bioaccumulation of these organotin derivatives. To evaluate the modifications induced on the physico-chemical state of the phospholipids by organotins, we have measured the steady-state fluorescence of probes embedded in the lipidic part of the membrane. The parallel use of 1,6-diphenyl-1,3,5-hexatriene (DPH) and its charged derivative 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) in the same system allows comparison of membrane physico-chemical characteristics at different depths in the bilayer.

EXPERIMENTAL

Materials

Organotin compounds were obtained from Aldrich. Dibutyltin-3-hydroxyflavone bromide [$Bu_2SnBr(of)$] was synthesized by Dr D. E. Griffiths (Chemistry Department, University of Warwick, UK). DPH and TMA-DPH were obtained from Molecular Probes (Eugene, OR, USA).

Sample preparation

The cells used in this work were collected from *Salmo irideus*, an inbred strain of trout. Blood was extracted by puncturing the lateral tail vein. After removal of plasma and buffy coat by centrifugation, the erythrocytes were washed three times in an isotonic medium (0.1 M phosphate buffer, 0.1 M NaCl, 0.2% citrate, 1 mM EDTA, pH 7.8) and resuspended (2×10^6 RBC$\times$ml) in an appropriate isotonic medium (0.1 M phosphate buffer, 0.1 M NaCl, 0.2% citrate, 1 mM EDTA, at pH 6.5) to follow the lysis.

Haemolytic measurements

The degree of haemolysis was determined as $(100\times A/10\times A_{100\%})$, where A is the optical density (absorbance) of Hb present in the supernatant of a red-cell suspension after centrifugation, and $A_{100\%}$ is the optical density of a red-cell suspension after complete lysis with 10 volumes of distilled water at zero time of incubation. Experiments involving carbon monoxide (CO) were carried out after exposure of the red blood cell suspension (2×10^6 RBC$\times$ml) to a weak vacuum and then to pure CO gas (1/2 atm of CO for 3–5 min). The desired amount of organotin compound (dissolved in ethanol) was added to these suspensions. Controls were prepared with the same concentration of ethanol as was used in samples containing organotins. Samples were maintained at 35 °C. The incubation time was measured starting from the injection of the desired organotin compound in the erythrocyte suspension. Final haemoglobin (Hb) concentration in each sample was about 20 mg ml^{-1}. At least four experiments on new samples for each set of measurements were performed. Results are represented as means ±S.E.M..

Plasma membrane preparation

Haemoglobin and nucleus-free erythrocyte membranes were prepared according to Steer and Levitzki[6] in a discontinuous sucrose gradient (25 and 35%, w/w). The membrane formed a white band between the 25 and 35% layers and the effective absence of the nucleus was monitored by light microscopy, staining the cells by Wright coloration. Membrane protein concentration was obtained by the Lowry method.[7]

Fluorescence measurements

Fluorescence measurements were performed using a Perkin–Elmer MPF-66 spectrofluorimeter equipped with a 7300 data station, on the haemoglobin and nucleus-free erythrocyte mem-

branes. The hydrophobic molecules DPH and its charged derivative TMA-DPH (having Amphipathic properties) were used as fluorescent probes. For steady-state fluorescence anisotropy (r) measurements, the excitation and emission wavelengths were, respectively, 360 nm and 430 nm. The degree of DPH and TMA-DPH anisotropy was obtained by the following equation:

$$r=\frac{I_{\parallel}-I_{\perp}g}{I_{\parallel}+2I_{\perp}g}$$

where g is an instrumental correction factor, and $I_{\parallel}$ and $I_{\perp}$ are, respectively, the emission intensities polarized vertically and horizontally to the direction of polarized light. Steady-state anisotropy has been widely used to monitor membrane fluidity.[8] Results of fluorescence were performed in at least three different samples, with three/five measurements for each sample. Data are reported as means±S.D. Final protein concentration in the sample was 150 μg ml^{-1}, while the probe concentration was 4 μM. Membranes were incubated with probes and organotins at the desired concentration for 2 h at 35 °C, in 0.1 M phosphate buffer at pH 8 in the dark. Measurements were performed at 35 °C.

Statistical analyses

Statistical analysis was performed with Student's t-test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Haemolytic rate

The effect of increasing concentrations of tributyltin chloride (TBTC), dibutyltin dichloride (DBTC) and monobutyltin trichloride (MBTC) on the half-time ($t_{1/2}$) of the haemolytic process is shown in Fig. 1. Trout red blood cells were suspended in an isotonic medium at pH 6.5 and incubated at 35 °C in air. As indicated by Fig. 1, from a typical experiment, DBTC has a scant protective effect at each concentration tested, while TBTC increases haemolysis, starting from 10 μM. MBTC does not present any measurable effect. The haemolytic event, defined as the release of haemoglobin in the supernatant of red

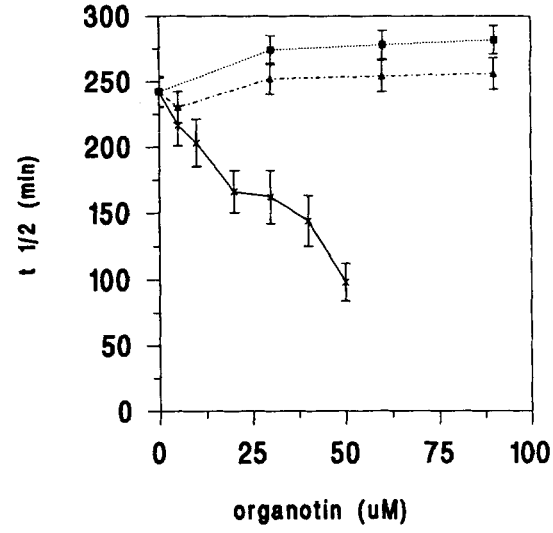

Figure 1 Effect of increasing concentrations of organotins on the half-time, $t_{1/2}$ (min) of haemolysis of trout erythrocytes from *Salmo irideus*. ×, TBTC; ■, DBTC; ▲, MBTC. Incubation was carried out at 35°C at pH 6.5. The degree of haemolysis was determined as ($100\times A/10\times A_{100\%}$). For other experimental details see the Materials and Methods section.

blood cell suspensions, is a complex phenomenon due to the action of different types of biochemical damages. It has been previously reported by us[9] that met-Hb formation in trout red blood cells associates with oxidative haemolysis only when glutathione peroxidase is inhibited. (This enzyme is able to metabolize both hydrogen peroxide and lipid peroxides). Under the experimental conditions of Fig. 1, lysis is also correlated with met-Hb oxidation and inactivation of glutathione peroxidase. Therefore, we undertook parallel experiments in the presence of carbon monoxide (CO). The presence of CO is protective against lysis because carbon monoxy-Hb is a more stable haemoglobin than the oxygenated one, and in these conditions there is no inactivation of glutathione peroxidase or superoxide radical production via met-Hb formation. Table 1 and Figs 2(A) and (B) show the effect of a fixed concentration (30 μM) of different organotins on the haemolytic event ($t_{1/2}$). The experiments were performed in the absence and in the presence of CO, as well as in the presence of 96% ethanol (control) which was used to solubilize the compounds. No significant increase of the haemolysis rate was observed in our experimental conditions (data not shown). When the samples were incubated in the pres-

Table 1 Influence of different organotin derivatives on the haemolysis of *Salmo irideus* erythrocytes[a]

Sample	Haemolysis: $t_{1/2}$ (min)			
	With CO	Δmin (%)	Without CO	Δmin (%)
Control	303±5	—	242±5	—
MBTC	284±5**	−6.3	252±8	+4.1
DBTC	280±4**	−7.6	274±5**	+13.2
TBTC	239±5*	−21.0	162±7*	−33.0
TPTC	275±6**	−9.2	208±9**	−14.0
$Bu_2SnBr(of)$	260±4*	−14.2	225±4**	−7.0

* $P<0.01$; ** $P<0.05$.

[a] Organotin derivatives were 30 μM in each sample. The temperature was 35 °C. The degree of haemolysis was determined as $(100\times A/10\times A_{100\%})$. Δmin is the percentage difference between the $t_{1/2}$ of the sample considered and that of the control for the same set of data. Values are mean±S.E.M. For other experimental details see the Materials and Methods section.

ence of CO, all the organotin compounds tested induced an increase in the rate of *Salmo irideus* erythrocyte haemolysis (Fig. 2B and Table 1). On the contrary, in the absence of CO, the organotins tested displayed varying effects on the haemolysis (Fig. 2A and Table 1). In fact, DBTC presented a small protective action, while in the same conditions, MBTC did not modify the haemolytic rate. Moreover, the flavone derivative appeared to be more effective in the presence of CO; $t_{1/2}$ was decreased by 43 min compared to the control in the presence of CO, while it decreased by 17 min in the absence of CO. These results suggest that the consequence of autoxidation of Hb could differentiate between the effects of organotin compounds on the membrane.

Fluorescence measurements

Steady-state fluorescence was used to investigate the possile modifications induced by organotins on the physico-chemical state of erythrocyte membranes. Anisotropy measurements were performed using two different probes, localized in different regions of the lipid bilayer. DPH is located in the hydrocarbon core of the bilayer,[10] while TMA-DPH is anchored close to the bilayer surface because of its charged amino group.[11, 12] The effect of increasing concentrations of different organotins on the steady-state anisotropy of DPH, embedded in erythrocyte membranes from *Salmo irideus*, is shown in Table 2. In this case the flavone derivative cannot be used for its fluorescence characteristics as it interferes with the DPH fluorescence excitation and emission spectrum. Compared with the control (0.254±0.011), the DPH anisotropy value is decreased by TBTC, reaching 70% of the control value for 50 μM TBTC. Moreover, 50 μM DBTC induces a 12% increase of DPH anisotropy, while 50 μM MBTC produces a 11% decrease of the same parameter. No relevant modifications of DPH anisotropy were shown by lower concentrations of DBTC and MBTC and by triphenyltin chloride (TPTC). No changes induced by the organotins tested were measured in TMA-DPH anisotropy values (Table 3), except for DBTC, showing a significant ($P<0.01$) increase of anisotropy (0.327±0.021), i.e. +11% compared with the control (0.294±0.014).

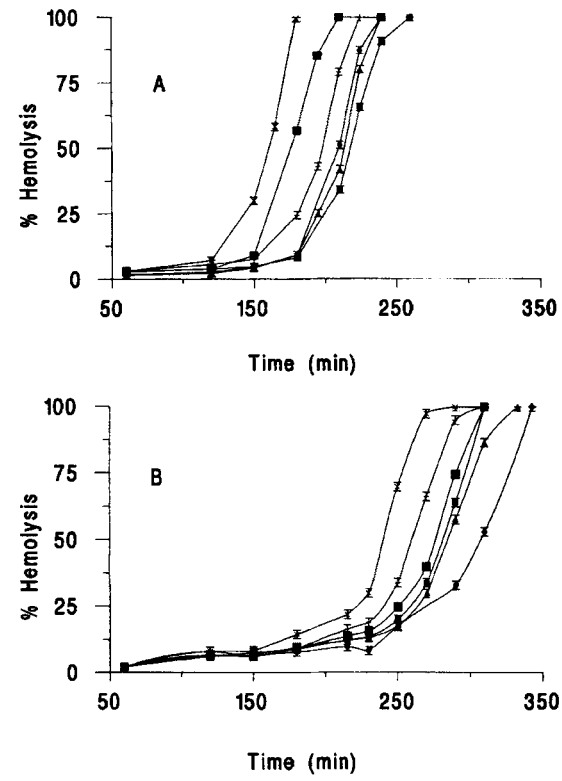

Figure 2 Effect of a fixed concentration (30 μM) of organotin derivatives on trout erythrocytes haemolysis (%). The experiments were performed in air (A) and in a carbon monoxide saturated atmosphere (B), in order to form carboxyhaemoglobin. Incubation was carried out at 35 °C at pH 6.5. For other experimental details see the Materials and Methods section. ◆, Control with 0.6% ethanol; ▲, MBTC; ■, DBTC; ×, TBTC; ■, TPTC; +, $Bu_2SnBr(of)$.

Table 2 Effect of increasing concentrations of different organotin derivatives on steady-state anisotropy (r) of DPH in *Salmo irideus* erythrocyte membranes[a]

Sample concn (μM)	TBTC	DBTC	MBTC	TPTC
0 (control)	0.254±0.011	0.254±0.011	0.254±0.011	0.254±0.011
10	0.208±0.006*	0.227±0.012	0.235±0.007	0.255±0.007
30	0.206±0.016*	0.256±0.020	0.243±0.020	0.236±0.010
50	0.177±0.009*	0.285±0.002*	0.225±0.008*	0.249±0.010

* $P<0.001$.

[a] For steady-state fluorescence anisotropy (r) measurements, the excitation and emission wavelengths were respectively 360 nm and 430 nm. Membranes were incubated for 2 h in 0.1 M phosphate buffer, pH 8, in the dark. Measurements were performed at 35 °C.

DISCUSSION

The haemolytic effect of organotins on mammalian erythrocytes has been extensively studied. Haemolytic activity was demonstrated for TBT and other triorganotin derivatives,[1] although the effect of different organotins can vary with different mammals.[13] The considerable use being made of organotins as antioxidants, surface disinfectants, biocides (i.e. in marine paints) and stabilizers in plastics arouses great interest in the biological effects of these compounds. This interest is increased by the possibility that these compounds may be persistent in the environment due to possible bioaccumulation and bioconcentration in sediments.[1, 3, 14] In particular, TBT is an environmental pollutant, whose food-chain accumulation has been demonstrated in marine organisms.[1]

Previous studies on membrane functions of a marine elasmobranch, *Squalus acanthias*, indicated that organotins are toxic to cell membrane functions which are intimately involved in the movement of electrolytes.[15] Damage at the plasma membrane level may be the cause of the haemolytic action of TBT[1] although the molecular mechanism of action has not yet been clarified. In fact, some hypotheses involve the liposolubility of TBT, suggesting its possible asymmetrical localization inside the bilayer.[16, 17] However, the possibility of an interaction with membrane proteins has also been studied.[18, 19] Inhibition of membrane-associated enzymes was proposed for the cytotoxic effect of TBT and other triorganotins, such as triphenyltin, on a variety of isolated cells.[1]

In contrast, the toxic action of dialkyltins was linked to interaction with biologically important sulphide groups.[1] Moreover, the flavone derivative Bu_2SnBr(of) is an inhibitor of mitochondrial F_1 F_0-ATPase, with a probable apolar binding site on the F_0 subunit.[20]

The solubility in organic solvents of organotins suggests the possibility of their accumulation in lipids, and a possible action at the membrane level.

Within the organotins tested in this work, MBTC was chosen since it is not widely studied, but could be found in the environment as a degradation product of TBTC. In order to test the possible action of increasing concentrations of organotins on cells of an aquatic organism, we studied the RBC haemolysis of *Salmo irideus*, an inbred strain of trout.

The haemolytic effect of TBTC was evident at each concentration tested, starting from 10 μM.

Table 3 Effect of a fixed concentration (30 μM) of organotin derivatives on the steady-state anisotropy (r) of TMA-DPH in *Salmo irideus* erythrocyte membranes[a]

Control	TBTC	DBTC	MBTC	TPTC
0.294±0.014	0.309±0.009	0.327±0.021*	0.283±0.012	0.309±0.020

* $P<0.01$.

For steady-state fluorescence anisotropy (r) measurements, the excitation and emission wavelengths were respectively 360 nm and 430 nm. Membranes were incubated for 2 h in 0.1 M phosphate buffer, pH 8, in the dark. Measurements were performed at 35 °C.

The same process was performed in the presence of carbon monoxide, which is protective, at 37 °C, against haemolysis.[9] Results indicated that the altered steady state of oxygen-reactive species due to Hb auto-oxidation was only slightly involved in the membrane toxicity mechanism due to the presence of TBTC. In fact, TBT action on the red blood cells appears to be slightly more significant in the absence of CO (Table 1).

In order to detect the possible modifications of the physico-chemical characteristics of the bilayer, DPH and TMA-DPH anisotropy studies were performed on isolated erythrocyte membranes. No change was evident on the anisotropy values of TMA-DPH, which is located at the phospholipid headgroup level. In contrast, our results indicate also that relatively low concentrations (10 μM) of TBTC have an effect on the probe located in the hydrophobic core of the bilayer (DPH), suggesting a TBTC localization in this part of the membrane. Data correlated well with the hypothesis of membrane lipid perturbation as a cause of the haemolytic process. This action could be more evident in the presence of oxidative stress due to Hb auto-oxidation. Oxygen radicals produced during its formation (samples without CO) could introduce defects in the hydrocarbon chains, at the double-bond level, modifying membrane permeability.

To compare the relative effectiveness of different organotins, a fixed concentration (30 μM) of each derivative was tested for the haemolysis, both in the absence and in the presence of carbon monoxide (Figs 2A and 2B and Table 1). Comparison of the two experiments (Table 1) showed that all organotins tested increase the haemolysis rate in the presence of CO (samples protected against oxidation). Compounds such as MBTC and $Bu_2SnBr(of)$ were not, or were less, effective in the samples not protected against oxidation (samples without CO). The action of DBTC (Fig. 1) appeared to be slightly protective against the haemolytic process, starting from the lowest concentration used, in the absence of carbon monoxide. In contrast, DBTC increases the haemolysis in the presence of CO (Table 1).

An 'ordering' effect on the lipid bilayer could be the cause of the scant protective effect shown by DBTC in the absence of CO. This hypothesis is in agreement with fluorescence anisotropy studies (Tables 2 and 3) showing that DBTC induces a significant increase of fluorescence anisotropy (related to a decrease of bilayer 'fluidity'), more evident at the headgroup level.

The experimental evidence that all the compounds tested modify the haemolytic rate of *Salmo irideus* erythrocytes under our experimental conditions suggests significant biological effects on this kind of fish. These results could be important, in particular for the evaluation of the environmental risks of TBTC and its degradative products, DBTC and MBTC, for use in marine paint formulations.

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