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Protection of Erythrocytes Against Organometals-Induced Hemolysis[1]

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The hemolytic toxicity of tributyllead (TBL) and triphenyllead (TPhL) chlorides and its prevention by dithiotreitol (DTT), diethylenetriaminepentamethylenephosphonic acid pentasodium (PMP) and sodium disulfide (Na_2S) was studied. It was found that both TBL and TPhL efficiently hemolyzed pig erythrocytes when used in micromolar concentrations; tributyllead chloride being about twice more efficient than triphenyllead chloride. The hemolytic efficiency of these compounds was blocked by PMP, DTT and Na_2S in a concentration-dependent manner. However, significant differences in anti-hemolytic efficiency of these compounds were found. Namely, DTT and Na_2S were very efficiently protecting erythrocytes against the action of organoleads, while the PMP protection was weak. Also, differences between DTT and Na_2S protective eficiency were found. They more efficiently prevented erythrocyte hemolysis by TPhL than by TBL. Moreover, erythrocytes were better protected against the action of TBL by Na_2S than by DTT. Such differentiation may be connected with possible differences in localization of the organolead compounds and protective agents in the erythrocyte membrane. To check these possibilities a series of experiments was performed using the fluorescence technique and various fluorimetric probes. These measurements enabled to determine fluidity changes induced in erythrocyte membranes by the organoleads and the protective compounds and to formulate some remarks concerning the differences in the mechanism of interaction of the organoleads with these membranes.

KEY WORDS: Organolead chlorides; erythrocyte hemolysis; fluorescence anisotropy; interaction mechanism.

INTRODUCTION

Organic derivatives of lead belong to toxic compounds that often occur in the environment. Mostly, they originate from lead antiknock additives to gasoline, but some of them are purposefully introduced into the environment as biocides [1–3]. Their invasion into a living organism may lead to multiple and diverse pathological changes [2,4–6]. However, to induce these changes organoleads must come into contact with natural cell or tissue barriers and some toxic effects are the consequence of this contact [7,8]. Toxicity of organolead compounds depends on various factors. The most important seems to be lipophilicity [1,2,9,10], a good measure of toxicity of organoleads being their ability to hemolyse erythrocytes, as it was already been shown [8,11–13].

This work contains the results of studies on the interaction of tributyllead (TBL) and triphenyllead (TPhL) chlorides with erythrocytes (RBC) and erythrocyte ghosts and its main aim was to determine what is the possible mechanism of the interaction of these compounds with the erythrocyte membrane. The kinetics was studied of hemolysis induced by TBL and TPhL and its prevention by dithiotreitol (DTT), sodium disulfide (Na_2S) and diethylenetriaminepentamethylenephosphonic acid pentasodium (PMP). DTT and Na_2S are compounds thad readily react with the thiol groups of proteins, while PMP is used to remove metal ions, and may have potential biological properties as a chelating compound. The thiol

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groups were found to be the reactive sites for organolead compounds [4,7,8,14]. Hemolysis prevention by DTT, Na_2S and PMP indicates that the interaction of the organoleads with the erythrocyte membrane was limited or, under proper conditions, completely eliminated.

It must be noted that the level of lead in blood depends on a large number of factors and the highest reported mean value is about 400 μg/dm^3 [15]. Concentrations of TBL and TPhL in our experiments were about one to two orders of magnitude higher and were intended to induce erythrocyte hemolysis. Similar micromolar concentrations are used in other experiments with human cells [16].

The studies on RBC membrane fluidity changes induced by both the organoleads and preventive compounds were also performed to find out about the mechanism and site of interaction of organoleads with the erythrocyte membrane. These were fluorescence measurements with the use of different fluorescent probes which incorporated in different parts of membrane and thus enabled to determine the anisotropy coefficients for the membrane regions.

EXPERIMENTAL

The triorganolead compounds studied (chlorides of tributyllead—TBL and triephenyllead—TPhL) were purchased from Alfa Products (Germany).

Fresh heparinized pig blood was used in the hemolytic experiments. Blood was centrifuged for 3 min at 1000 $\times$ g, the plasma removed and the cells washed twice with isotonic phosphate buffer solution (131 mM NaCl, 1.79 mM KCl, 0.86 mM $MgCl_2$, 11.80 mM $Na_2HPO_4{\cdot}2H_2O$, 1.80 mM $Na_2HPO_4{\cdot}H_2O$) of pH 7.4. The erythrocytes were then incubated in the same solution containing a chosen concentration of organoleads. The hemolytic experiments enabled to determine TBL and TPhL concentrations that caused 100% or near 100% hemolysis of erythrocytes for 4 h. These were 40 and 80 μM for TBL and TPhL, respectively, and were used later on in experiments aimed at finding the efficiencies of diethylenetriaminepentamethylenephosphonic acid pentasodium (PMP), dithiotreitol (DTT) and sodium disulfide (Na_2S) in protecting erythrocyte membranes against hemolysis by the organoleads. The protective compounds were used in concentrations as shown in Figs. 1 and 2 (PMP concentration, not shown in the Figures changed from 50 to 500 μM). Hemolysis of erythrocytes without (control) and with protective compounds, added to the suspension solution simultaneously will organoleads, was observed for 4 h. The hematocrit was 2% and the temperature 37°C. Percent of hemolysis was measured in 1 mL samples, taken after 0.5, 1, 1.5, 2, 3, and 4 hr. The samples were centrifuged and the hemoglobin content in the supernatant measured with a spectrophotometer (Specol 11, Carl Zeiss, Jena) at 540 nm wavelength. Hemoglobin concentration was expressed as percentage of hemolysed cells, calculated relative to a sample that contained totally water-hemolysed erythrocytes. All triorganolead compounds were dissolved in ethanol, of concentration not exceeding 1% (v/v). Good mixing of the suspension during the entire procedure was insured.

The fluidity experiments were done on erythrocyte ghosts, which were subjected to the action of the compounds studied. Three fluorescent probes were used; 1,6-diphenyl-1,3,5-hexatriene (DPH), *N*-((4-(6-phenyl-(1,3,5-hexatrienyl)phenyl(propyl)trimethylammonium, *p*-toluenesulfonate) (TMAP-DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate (TMA-DPH), all at 1 μM concentration. The erythrocyte ghosts were incubated with the probes for 24 h at 23°C, then organoleads (control) or their mixtures with preventive compounds were added. Protein concentration in the samples was ca. 100 μg/ml. The incubation time was 1 hr. The experiments were performed 5 times and each sample was measured 5 times giving a pool of 25 values for determination of standard deviation. The measurements were performed with an SFM 25 spectrofluorimeter (KONTRON, Switzerland) at 25°C. The excitation and emission wavelengths were 354 nm and 429 nm (TMAP-DPH), 358 nm and 428 nm (TMA-DPH), and 360 nm and 425 nm (DPH). The anisotropy coefficient A was calculated according to the formula [17–19]:

$$A = (I_{\mathrm{II}} - GI_{\perp})/(I_{\mathrm{II}} + 2GI_{\perp}) \qquad (1)$$

where I_{II} is the intensity of fluorescence emitted in a direction parallel to the polarization plane of the exciting light, $I_{\perp}$is the intensity of fluorescence emitted in the perpendicular direction, and G is the diffraction constant.

All reagents used were of analytical grade. The fluorescent probes were purchased from Molecular Probes Inc. (Eugene, USA).

RESULTS AND DISCUSSION

Figure 1 shows percentage of erythrocyte hemolysis induced by triphenyllead chloride and its prevention by increasing concentrations of sodium disulfide and dithiotreitol. Note that hemolysis was completely blocked by 50 μM of Na_2S. Efficiency of DTT was not so good, but very significant differences were found betwen the blocking efficiencies of these two compounds against hemolysis induced by tributyllead. It is apparent (Fig. 2) that

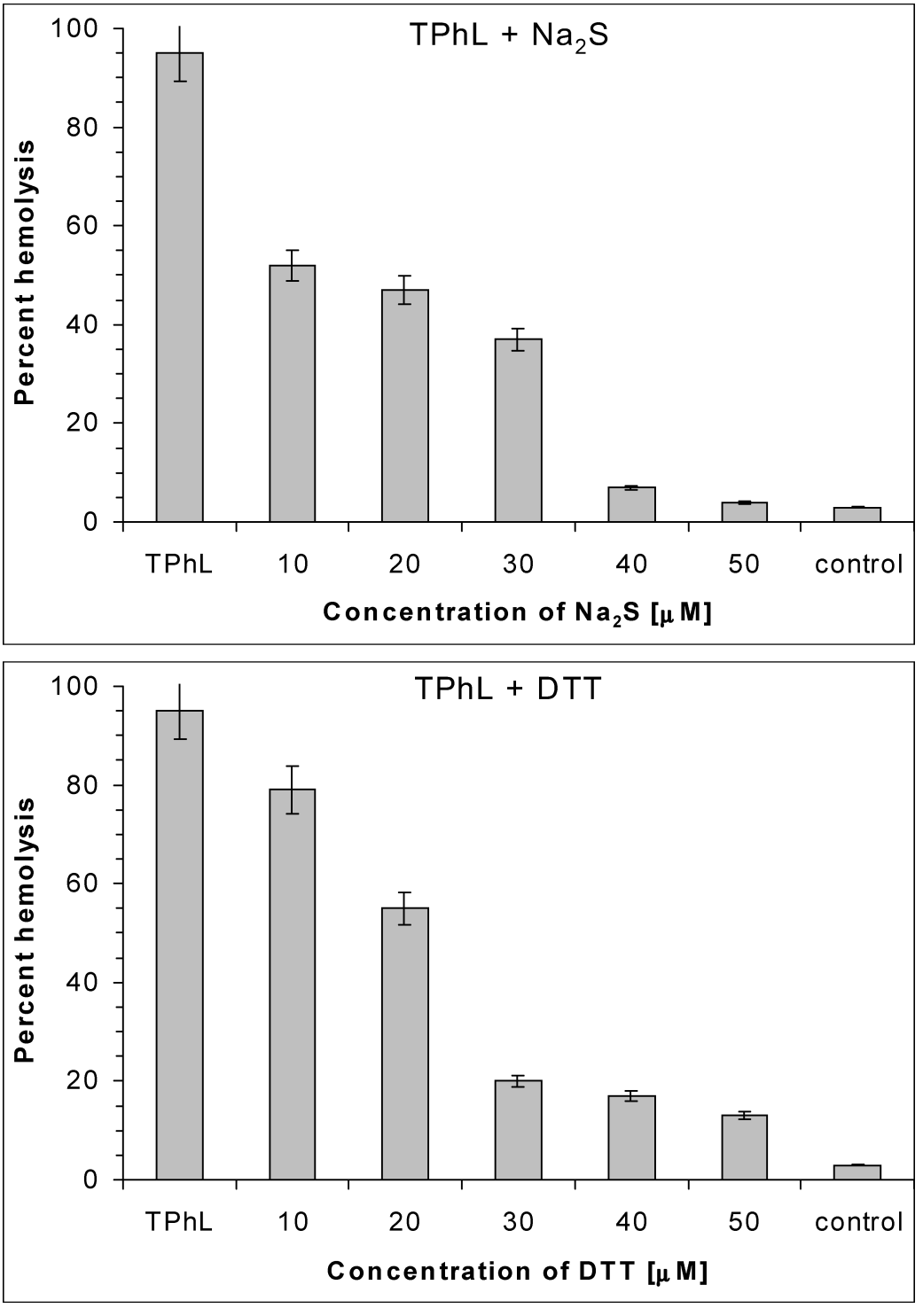

Fig. 1. Hemolysis of erythrocytes by 80 μM triphenyllead chloride (TPhL) and its prevention by different concentrations of sodium disulfide (Na_2S) and dithiotreitol (DTT).

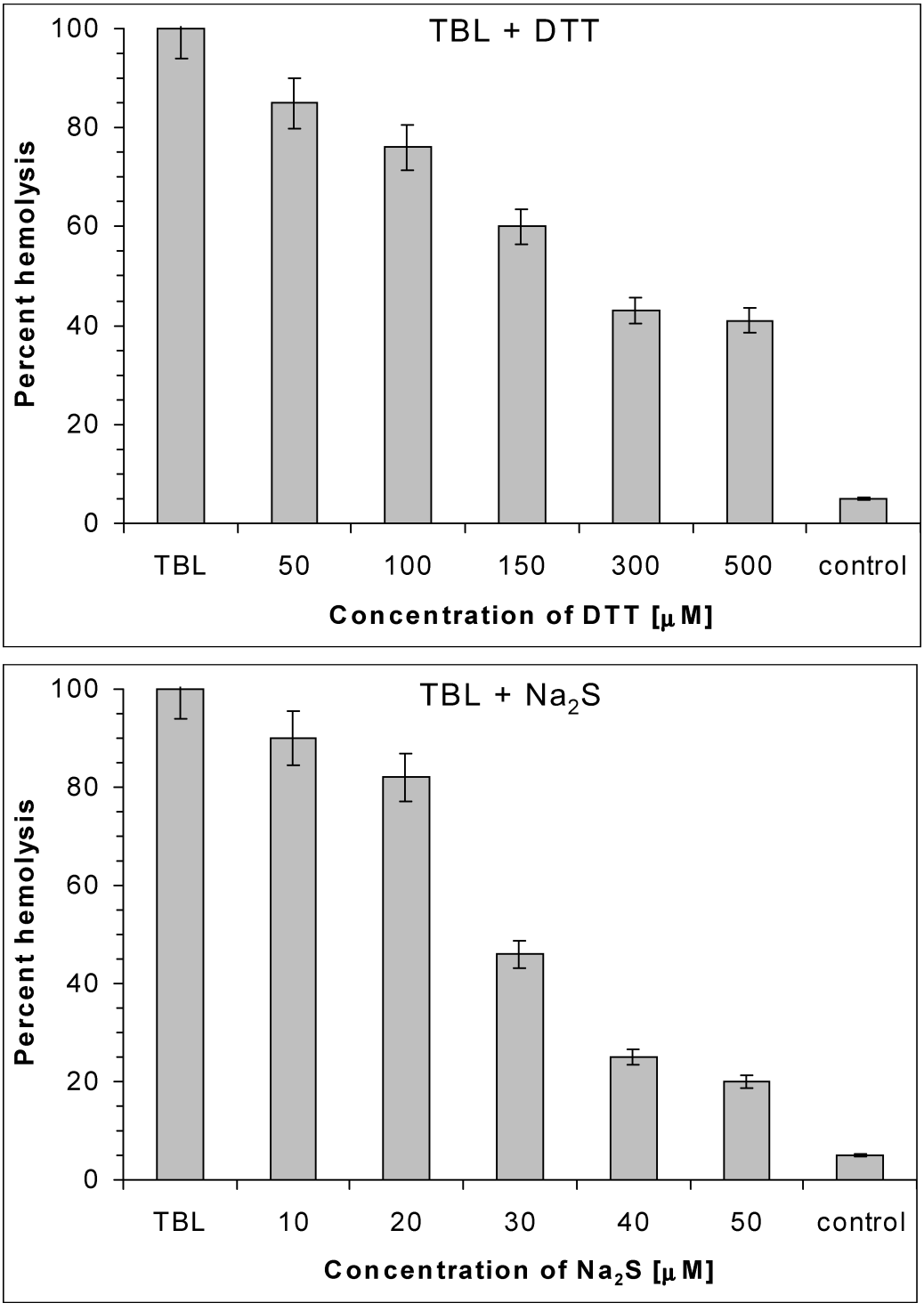

Fig. 2. Hemolysis of erythrocytes by 40 μM tributyllead chloride (TBL) and its prevention by different concentrations of sodium disulfide (Na_2S) and dithiotreitol (DTT).

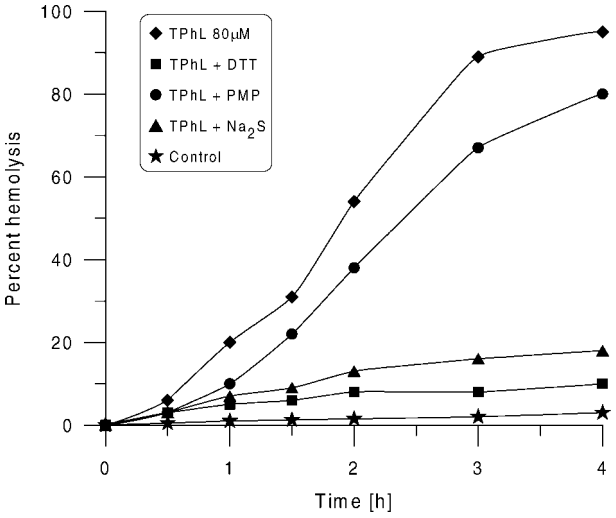

Fig. 3. Kinetics of hemolysis by 80 μM triphenyllead (TPhL) and its prevention by diethylenetriaminepentamethylenephosphonic acid pentasodium (PMP), sodium disulfide (Na_2S), and dithiotreitol (DTT).

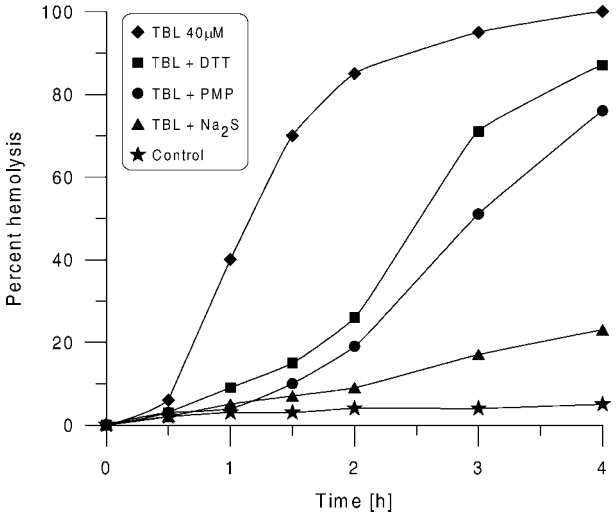

Fig. 4. Kinetics of hemolysis by 40 μM tributyllead chloride (TBL) and its prevention by diethylenetriaminepentamethylenephosphonic acid pentasodium (PMP), sodium disulfide (Na_2S), and dithiotreitol (DTT).

DTT blocked the hemolysis about seven times weaker than Na_2S. The protective properties of PMP are best seen in the next figure which shows the kinetics of erythrocyte hemolysis. The erythrocyte protection by PMP was slightly better in the case of TBL (Fig. 4) than that by DTT and very weak in the case of TPhL (Fig. 3).

The results obtained show that trialkylleads were effectively interacting with the erythrocyte membrane. They caused 100% (TBL) or almost 100% (TPhL) hemolysis in 4 hr. However, in order to do it the concentration of TPhL had to be much higher than that of TBL (80 and 40 μM, respectively). These differences in the hemolytic efficiencies of TBL and TPhL (see Figs. 1–4) could be due to their different lipophilicity. There is also the possibility that both the compounds bind preferentially to different parts of the erythrocyte membrane or there are more then one binding site in the membrane. Such possibility was suggested by Burda *et al.* [20] in the case of studies on hemolysis of erythrocytes by some organoleads and organotins and the proposed alternative sites were thiol groups within the proteins of the membrane skeleton. If the protecting compounds have different possibilities to block these sites, the result can be, as observed in this work, their differentiated antihemolytic efficiency (DTT and Na_2S). As already mentioned, the prevention of hemolysis by PMP was better in the case of TBL. Possibly, such behaviour is not only connected with PMP blocking the TBL and TPhL binding sites but also with its chelating properties.

To explain the mentioned possibilities and try to postulate a mechanism of the interaction of the organoleads with the erythrocyte membrane, a series of fluorescence experiments was performed. The results are collected in Tables I and II. There are the values of anisotropy coefficients determined for all the compounds interacting with ghost erythrocytes individually and in pairs: organolead—protective compound. It can be seen that

Table I. Values of the Anisotropy Coefficients Measured for Probes 1,6-Diphenyl-1,3,5-hexatriene (DPH), 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-Toluenesulfonate (TMA-DPH) and *N*-((4-(6-Phenyl-1,3,5-hexatrienyl)phenyl)propyl)trimethylammonium *p*-tolue-nesulfonate) (TMAP-DPH) for Erythrocyte Ghosts Treated with 5 μM Tributyllead Chloride (TBL), Dithiotreitol (DTT), Diethylenetriaminepentamethylenephosphonic Acid Pentasodium (PMP) and Sodium Sulfide (Na_2S)

	Anisotropy							
Probes	Control	TBL	DTT	PMP	Na_2S	TBL + DTT	TBL + PMP	TBL + Na_2S
DPH	0.254	0.192	0.177	0.179	0.157	0.151	0.153	0.129
TMA-DPH	0.222	0.210	0.214	0.154	0.210	0.191	0.141	0.199
TMAP-DPH	0.279	0.211	0.183	0.212	0.181	0.209	0.201	0.170

Note. Standard deviation ± 0.04.

Table II. Values of the Anisotropy Coefficients Measured for Probes 1,6-Diphenyl-1,3,5-hexatriene (DPH), 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate (TMA-DPH) and *N*-((4-(6-Phenyl-1,3,5-hexatrienyl)phenyl)propyl)trimethylammonium *p*-toluenesulfonate) (TMAP-DPH) for Erythrocyte Ghosts Treated with 5 μM Triphenyllead Chloride, Dithiotreitol (DTT), Diethylenetriaminepentamethylenephosphonic Acid Pentasodium (PMP) and Sodium Sulfide (Na_2S)

	Anisotropy							
Probes	Control	TPhL	DTT	PMP	Na_2S	TPhL + DTT	TPhL + PMP	TPhL + Na_2S
DPH	0.254	0.186	0.172	0.179	0.148	0.141	0.165	0.144
TMA-DPH	0.222	0.150	0.215	0.154	0.224	0.167	0.156	0.182
TMAP-DPH	0.279	0.174	0.175	0.218	0.194	0.141	0.185	0.189

Note. Standard deviation ± 0.04.

all the compounds, no matter if used individually or in pairs, were diminishing membrane fluidity, which was evidenced by decreases in anisotropy coefficient. Although there are not significant differences in anisotropy coefficients (A) obtained for TBL and TPhL when erythrocytes were incubated in solution containing also antihemolytic compounds (values of A differed by less than 10%), there can be seen a tendency for all probes to show slightly higher values of A for TBL present in the incubation solution. Perhaps this is most evident in the case of DPH probe. This could be an evidence for a better efficiency of TBL to disturb the lipid phase of the RBC membrane (to increase membrane fluidity) or, as previously indicated, TBL and TPhL may interact with the membrane at different binding sites. Another possibility is that TBL and TPhL incorporate into lipid membranes to different depths, and that causes the differentiated efficiencies of the antihemolytic compounds to block that action. The results obtained do not permit a very credible formulation and the problem needs further investigation.

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