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Relationship between Hemolytic Toxicity and Signal Intensity of Various Organotin Compounds by a Spin-labeling Technique

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Various organotin compounds caused strong hemolysis of erythrocytes. To investigate the mechanism of hemolysis, spin-labeling techniques with electron spin resonance (ESR) spectroscopy were used and the relationship between hemolytic toxicity and signal intensity was investigated. Two kinds of spin-labeled stearic acid in which the paramagnetic center was located at different sites on the alkyl chain (5- and 12-doxyl-stearic acids; 5- and 12-NS) were used. It became clear that the decrease of ESR signal intensity was related to the hemolytic toxicity of the organotin compounds. © 1997 by John Wiley & Sons, Ltd.

Keywords: electron spin resonance; spin-labeling; doxyl-stearic acid; erythrocyte; organotin compounds; hemolysis

INTRODUCTION

Various organotin compounds act as environmental pollutants.[1–3] We have investigated the hemolytic effects of organotin compounds and demonstrated clearly that tri-n-butyltins and triphenyltins have higher hemolytic activities than sodium-n-dodecyl sulfate (SDS).[4] The hemolysis may occur by damage to the biomembranes of erythrocytes.

Spin labeling techniques with ESR spectroscopy is a useful technique for investigation of the nature of biomembrances.[5,6]

In this paper, we investigated the effects of various organotin compounds on biomembrances of erythrocytes using two kinds of spin labeled stearic acid in which the paramagnetic center was located at C5 and C12 on the fatty-acid alkyl chain, and we compared the hemolytic toxicity with the ESR signal intensity.

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MATERIALS AND METHODS

Chemicals

Methyl, butyl and phenyl compounds of mono-, di-, tri- and tetra-organotins were used. Bis(tri-n-butyltin) oxide (TBTO) was also used. The compounds tested are shown in Table 1. These organotin compounds were dissolved in ethanol.

5- and 12-doxylstearic acids (5- and 12-NS) were obtained from Aldrich.

Table 1. Organotin compounds tested

Compound	Abbreviation	Source[a]
Methyltin compounds		
Methyltin trichloride	$MTCl_3$	A
Dimethyltin dichloride	$DMTCl_2$	M
Trimethyltin chloride	TMTCl	K
Tetramethyltin	tetraMT	K
Butyltin compounds		
n-Butyltin trichloride	$BTCl_3$	A
Di-n-butyltin dichloride	$DBTCl_2$	M
Tri-n-butyltin chloride	TBTCl	K
Tetra-n-butyltin	tetraBT	M
Bis(tri-n-butyltin) oxide	TBTO	W
Phenyltin compounds		
Phenyltin trichloride	$PTCl_3$	A
Diphenyltin dichloride	$DPTCl_2$	A
Triphenyltin chloride	TPTCl	K
Tetraphenyltin	tetraPT	N

[a]K, Kantoh Chemical Co.; M, Merck; N, Nakarai Tesque Co.; W, Wako Pure Chemicals Co.; A, Aldrich.

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Preparation of erythrocyte suspension

Blood (10 ml) was exsanguinated from Japanese white rabbits (body weight *ca* 4 kg) into a test-tube containing 50 U of heparin solution. The blood was diluted with 5–10 ml of Tris buffer saline (0.075 M NaCl/5 mM Tris–NCl buffer, pH 7.4), centrifuged (2000 rpm, 4 °C) for 5 min and the plasma was removed. Tris buffer saline was added to the precipitated cells and the cells were resuspended. The resuspension was centrifuged and the supernatant was removed. Tris buffer saline was added to the washed erythrocytes to become a 1:1 (v/v) suspension.

Preparation of labeled erythrocyte suspension

Spin labeling reagent (0.77 mg) was dissolved in 2 ml of chloroform in a test-tube and a fine membrane of spin labeling reagent was formed on the inner surface of the test-tube by removing the chloroform with a rotary evaporator. Erythrocyte suspension (4 ml) was added and incubated at 37 °C for 16 h in darkness with slow shaking. Tris buffer saline (5–10 ml) was added and centrifuged (2000 rpm, 4°C, 5 min) and the supernatant was decanted. Tris buffer saline was added to the precipitate and the new suspension was centrifuged and the supernatant was removed. This procedure was repeated twice; then Tris buffer saline was added to the precipitated erythrocyte to achieve a four fold dilution.

ESR measurement

Twenty microliters of various concentrations of organotin compound solutions and 180 μl of labeled erythrocyte suspension or labeling reagent (4 μM) were mixed and incubated at 20 °C for 60 min.

The ESR signal was measured under the following conditions: apparatus, JEOL JES-REIX; center field, 322 ± 10 mT; field modulation width, 0.1; receiver gain, 1000; time constant, 1 s; sweep time, 90 mm min^{-1}; microwave power, 10 mW.

The ratio of signal intensity was calculated by means of Eqn [1].

$$\text{Ratio of signal intensity} = (S_s/M_s)/(S_o/M_o) \quad [1]$$

where S_s = central signal height of nitroxide radical with organotin and labeled erythrocyte
M_s = signal height of manganese (marker)
S_o = central signal height of nitroxide radical with no organotin (the control)
M_o = signal height of manganese in the control

The experiments were repeated twice or three times and the mean was calculated.

RESULTS

At first the organotin compound and the spin labeling reagent (ethanol solution) were mixed and the ESR signal was investigated. However, the signal heights with organotins were the same as with no organotin (the control). The organotin compounds and the spin labeling reagents must not react directly.

In Fig. 1 the ESR spectra of 5-NS and 12-NS incorporated into erythrocytes are shown. The shapes of the two spectra were different, but the positions and shapes of the central signals were similar.

The effects of various organotin compounds on the labeled erythrocyte were investigated. The

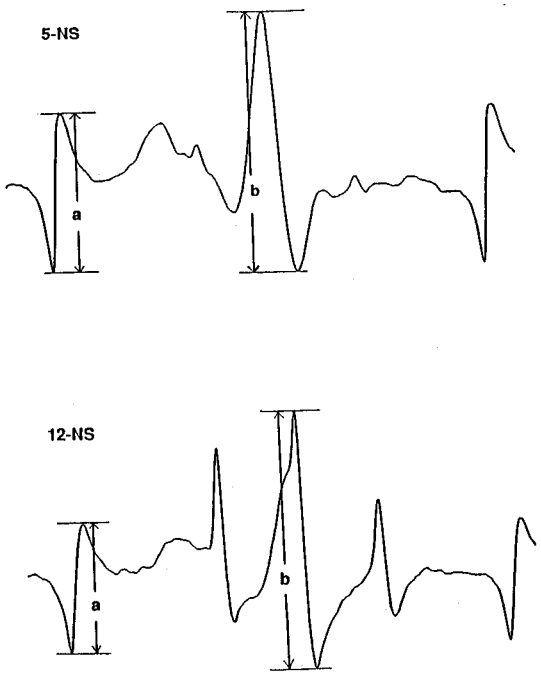

Figure 1 ESR spectra of 5-NS and 12-NS incorporated into rabbit red blood cells: a, height of marker signal (manganese); b, height of central signal of nitroxide radical.

results with methyltin compounds are shown in Fig. 2. No methyltins affected the signal intensity with 5-NS. However, the intensity of the $MTCl_3$ and $DMTCl_2$ signals decreased with 12-NS. The nitroxide probe of 12-NS is located more centrally in the bilayer than that of 5-NS. These organotins may attack from the middle of the erythrocyte membrane.

The results with butyltin compounds are shown in Fig. 3. A decrease in signal intensity was observed with mono, di- and tri-butyl compounds with both 5-NS and 12-NS. Tetrabutyl compounds did not reduce signal intensity. As in the case of methyltins, the decrease in signal intensity for 12-NS was larger than that for 5-NS. Butyltin compounds may also exert there influence from the middle of the membrane.

The results for phenyltin compounds are shown in Fig. 4. A decrease in signal intensity was observed for mono-, di- and triphenyl compounds with both spin labeling reagents. Tetraphenyl compounds did not decrease the signal intensity.

The EC_{20} and EC_{50} of the signal intensity with 5-NS and 12-NS were measured as the organotin

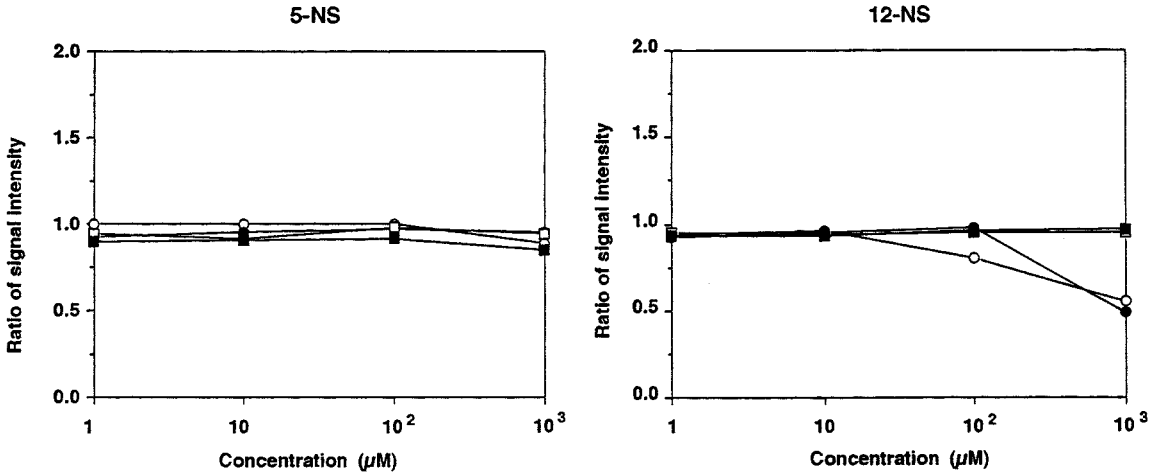

Figure 2 Effect of methyltin compounds on signal intensity of NS incorporated into rabbit red blood cells: ○, $MTCl_3$; ●, $DMTCl_2$; □, TMTCl; ■, tetraMT.

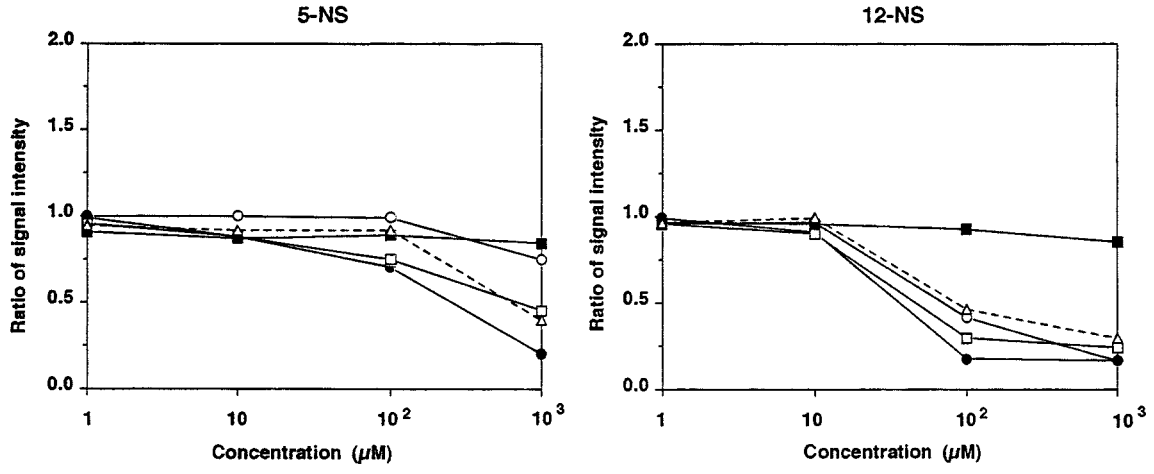

Figure 3 Effect of butyltin compounds on signal intensity of NS incorporated into rabbit red blood cells: ○, $BTCl_3$; ●, $DBTCl_2$; □, TBTCl; ■, tetraBT; △, TBTO.

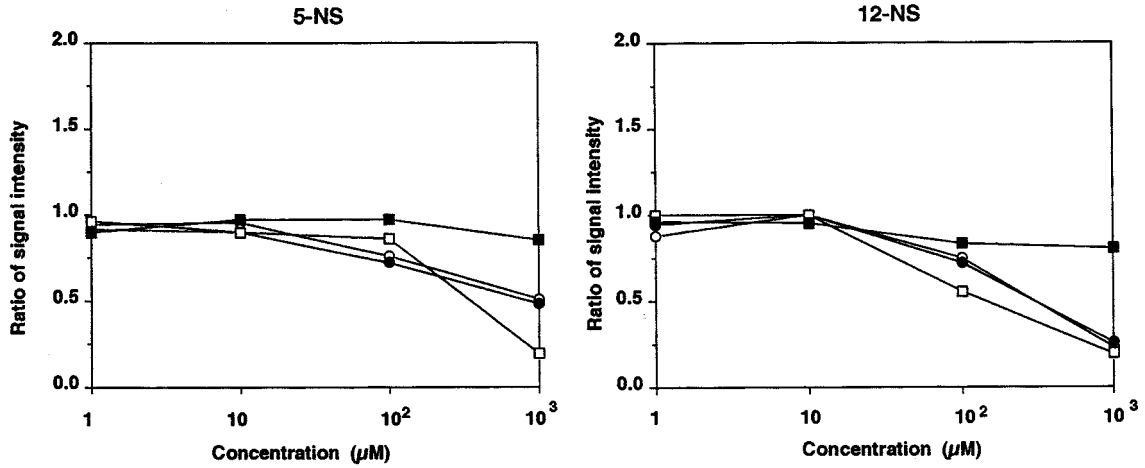

Figure 4 Effect of phenyltin compounds on signal intensity of NS incorporated into rabbit red blood cells: ○, $PTCl_3$; ●, $DPTCl_2$; □, TPTCl; ■, tetraPT.

concentrations at which the signal intensity decreases to 80% and 50% respectively. The results are shown in Table 2.

There is a possibility that the decrease in signal intensity is related to hemolytic toxicity. The correlation coefficients between $\log(1/EC_{20})$ or $\log(1/EC_{50})$ of the signal intensity and $\log(1/EC_{20})$ or $\log(1/EC_{50})$ of hemolysis were calculated. The hemolysis data were reported in a previous paper.[4] The results are shown in Table 3. When EC_{20} or EC_{50} were over 1000 μM, the correlation coefficients were calculated as for 1000 μM.

The decrease in signal intensity was related to hemolytic toxicity, because correlation coefficients between various $\log(1/EC_{20})$ or $\log(1/EC_{50})$ of the signal intensity and those of hemolytic toxicity were significant with this statistical method ($P < 0.05$).

It can also be observed that the correlation coefficient between $\log(1/EC_{20})$ and $\log(1/EC_{50})$ of 5-NS or 12-NS is significant statistically. There is also a relationship between $\log(1/EC_{20})$ or $\log(1/EC_{50})$ of 5-NS and 12-NS.

Table 2. EC_{20} and EC_{50} (μM) of organotin compounds

	5-NS		12-NS	
	EC_{20}	EC_{50}	EC_{20}	EC_{50}
$MTCl_3$	>1000	>1000	>1000	>1000
$DMTCl_2$	>1000	>1000	230	950
TMTCl	>1000	>1000	>1000	>1000
tetraMT	>1000	>1000	>1000	>1000
$BTCl_3$	640	>1000	20	71
$DBTC_2$	27	250	14	37
TBTCl	44	670	14.5	47
tetraBT	>1000	>1000	>1000	>1000
TBTO	170	640	26	85
$PTCl_3$	63	>1000	64	300
$DPTCl_2$	37	840	52	300
TPTCl	125	340	17	145
tetraPT	>1000	>1000	>1000	>1000

DISCUSSION

In a previous paper,[4] we investigated the hemolytic effect of various organotin compounds and observed that tri-n-butyltin chloride showed the highest hemolytic activity, and the methyltin compounds were less active than any other organotin compounds. We also observed that various EC_{50} values differed with their chemical structures.

There are many reports which have investigated the structure of the erythrocyte membrane using a spin label method with ESR,[5,6] but theoretical interpretation of signal changes is difficult.

In our experiments, the shape of the ESR signal did not change with the organotin compounds; only the peak height decreased. If the shapes of the ESR signals are the same, the peak height reflects the signal intensity even when the ESR signal is depicted as differential form. Also,

Table 3. Correlation coefficients between various $\log(1/EC_{20})$ or/and $\log(1/EC_{50})$

		5-NS		12-NS		Hemolysis	
		$\log(1/EC_{20})$	$\log(1/EC_{50})$	$\log(1/EC_{20})$	$\log(1/EC_{50})$	$\log(1/EC_{20})$	$\log(1/EC_{50})$
5-NS	$\log(1/EC_{20})$		0.644*	0.796**	—	0.670*	—
	$\log(1/EC_{50})$			—	0.679*	—	0.577*
12-NS	$\log(1/EC_{20})$				0.942**	0.727*	—
	$\log(1/EC_{50})$					—	0.588*
hemolysis	$\log(1/EC_{20})$						—
	$\log(1/EC_{50})$						

* $p < 0.05$, ** $p < 0.01$

when 5- and 12-NS are incorporated in erythrocyte membranes, the decrease of signal intensity of the nitroxide radical may reflect the reduction or loss of radical and the damage to the biomembrane. We observed that there is a significant correlation between the decrease in signal intensity and the hemolytic toxicity.

Sandberg and Piette[7] reported that the erythrocyte membrane is probably altered upon hemolysis and all of the membrane components may not be retained, but the spin label spectrum is always the same. Holmes and Piette[8] reported that the relative signal intensity is decreased by chlorpromazine (a representative phenothiazine derivative), urea and isotonic NaCl with the nitroxide spin label probe technique. Leterrier *et al*.[9] reported that some correlation could be found between the intensity of the spin label spectroscopic response and the pharmacological activity of phenothiazine derivatives. Benga *et al*.[10] reported that 3 mM chlorpromazine reduced the amplitude of the spectrum by 40% and these spectral changes may be caused by the reduction by chlorpromazine of the nitroxide free radical.

When 12-NS is used, the nitroxide probe is located in the membrane closer to the middle of the bilayer than in the case of 5-NS. We observed that the decrease in signal intensity for 12-NS was generally larger than those for 5-NS with several organotin compounds. Organotin compounds may attack the biomembrane from the inner regions.

More research will be required in order to reveal the molecular mechanism of the membrane toxicity of various organotin compounds.

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