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# Organotin biocides XV:* Modelling the interactions of triorganotins with cell membranes

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**The interaction of triorganotins with cell membranes has been examined using synthetic phospholipid membranes (vesicles). Electron microscopy and NMR methods indicate that the organotin is in a five-coordinate environment at the membrane surface, and is associated weakly with the phosphate headgroups of the vesicle components. Both trimethyl- and tributyl-tin cause extensive modifications to the vesicles including fusion, aggregation, blebbing and total rupture, these effects being initiated at concentrations as low as 25 $\mu$mol dm$^{-3}$.**

**Keywords: Triorganotins, cell membranes, synthetic membranes, phospholipids, vesicles, electron microscopy, NMR**

## INTRODUCTION

Despite recent concerns over the environmental impact of triorganotin ($R_3Sn$) antifouling paints (specifically those based upon tributyltin compounds) this class of compounds still finds a variety of commercial applications, many of which are associated with biocidal activity.[1] At the molecular level, the biocidal effects of the $R_3Sn$ moiety manifest themselves in two distinct ways, namely the oligomycin-like inhibition of oxidative phosphorylation (observable in the absence of chloride ($Cl^-$) ions)[2] and the mediation of trans-membrane chloride/hydroxide ($Cl^-/OH^-$) exchange.[3] Whilst the former process has attracted considerable interest in terms of the protein binding sites favoured by the metal (cysteine and histidine residues),[4,5] studies of the latter have focused on the ionophoric properties of the organometal rather than the specifics of its interactions with the components of the cell membrane.[3] A recent report, however, has detailed that tributyltin interactions with human erythrocytes (red blood cells, RBC) rapidly lead to morphological transformations and ultimately haemolysis of the cell. These structural changes were apparently associated with the occurrence of electron-dense aggregates (*ca* 70 nm diameter) on the erythrocyte membrane, presumably arising from clusters of the organometallic moieties.[6]

Our interest in the synthesis and properties of novel triorganotin compounds[7] has caused us to consider in more detail the specific nature of the organometallic associated with the cell membrane. The coordination number and the nature of the ligands surrounding tin at the cell surface have clear implications for any proposed mechanism for trans-membrane ion transport, as indeed do the presence of the unique polymetallic aggregates reported above. The difficulty in studying the molecular nature of the organotin–membrane interaction lies in the complexity and lability of natural biological membranes. For this reason, in an attempt to enhance our understanding of the *in vivo* chemistry of triorganotins we have sought to model the environment of tin at the cell wall using artificial phospholipid membranes (unilamellar vesicles, ULV) and study the nature of this interaction by a combination of electron microscopy and NMR techniques. We report our findings herein.

## EXPERIMENTAL

Trimethyltin chloride ($Me_3SnCl$) and tributyltin ethanesulphonate ($Bu_3SnOSO_2Et$) were prepared and purified by literature methods.[8,9] Product purity was determined at >99% by NMR methods. ULVs were prepared by the following method. Ampoules containing 500 mg of egg yolk lecithin (Grade 1) dissolved in chloroform/methanol (4:1) were obtained from Lipid Products. The contents of one ampoule were diluted

*For part XIV of this series see Ref. 7.
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to 20 $cm^3$ using the above solvent system to form a standard solution which was 34 mmol $dm^{-3}$ with respect to phosphatidylcholine (the major constituent of egg yolk). This solution was stored in the dark at 4°C until required. A 2 $cm^3$ aliquot of this solution was transferred to a round-bottomed flask and evaporated to dryness under reduced pressure, and the resulting film shaken with 2 $cm^3$ $D_2O$ (Aldrich) and three glass beads to produce a multilamellar dispersion of the vesicle. This dispersion was cooled in an ice bath during sonication for 15 min with a Soniprep 150 ultrasonic disintegrator (transducer amplitude of 9 $\mu$m peak-to-peak) to yield a suspension of single-compartment, bilayer vesicles [Fig. 1(a)].[10]

$^1H$ *and* $^{13}C$ NMR spectra were recorded as $D_2O$ solutions on a JEOL GX270 spectrometer. $^{31}P$ and $^{119}Sn$ NMR spectra of the same solutions were recorded on a JEOL GX400 spectrometer, the latter nucleus under nuclear Overhauser effect (nOe) suppressed conditions. In a typical experiment 0.05 $cm^3$ of 1 mol $dm^{-3}$ $Me_3SnCl$ in $D_2O$ was added to 2 $cm^3$ of unilamellar vesicle prepared as above. Liposome samples were taken from the NMR solutions immediately following their preparation.

For the electron microscopy study, these vesicle suspensions were loaded onto formvar (plastic support film) coated, carbon-reinforced copper grids made hydrophilic by exposure to ultraviolet radiation. After 1 min excess fluid was drawn off from the side of the grid with filter paper. The grid was then stained by addition of a drop of staining solution (either 2% uranyl acetate, 4% methylamine tungstate or 2% ammonium molybdate) with excess stain removed after 30 s by means of absorbent paper. The grids were then allowed to air-dry for 30 min before examination.

All samples were examined in a JEOL 2000FX transmission electron microscope (TEM) operating at 200 keV with a liquid nitrogen-cooled anti-contamination device attached. Energy-dispersive X-ray spectroscopy (EDXA) was performed with a lithium-drifted silicon detector interfaced to a Link systems AN10000 processor.

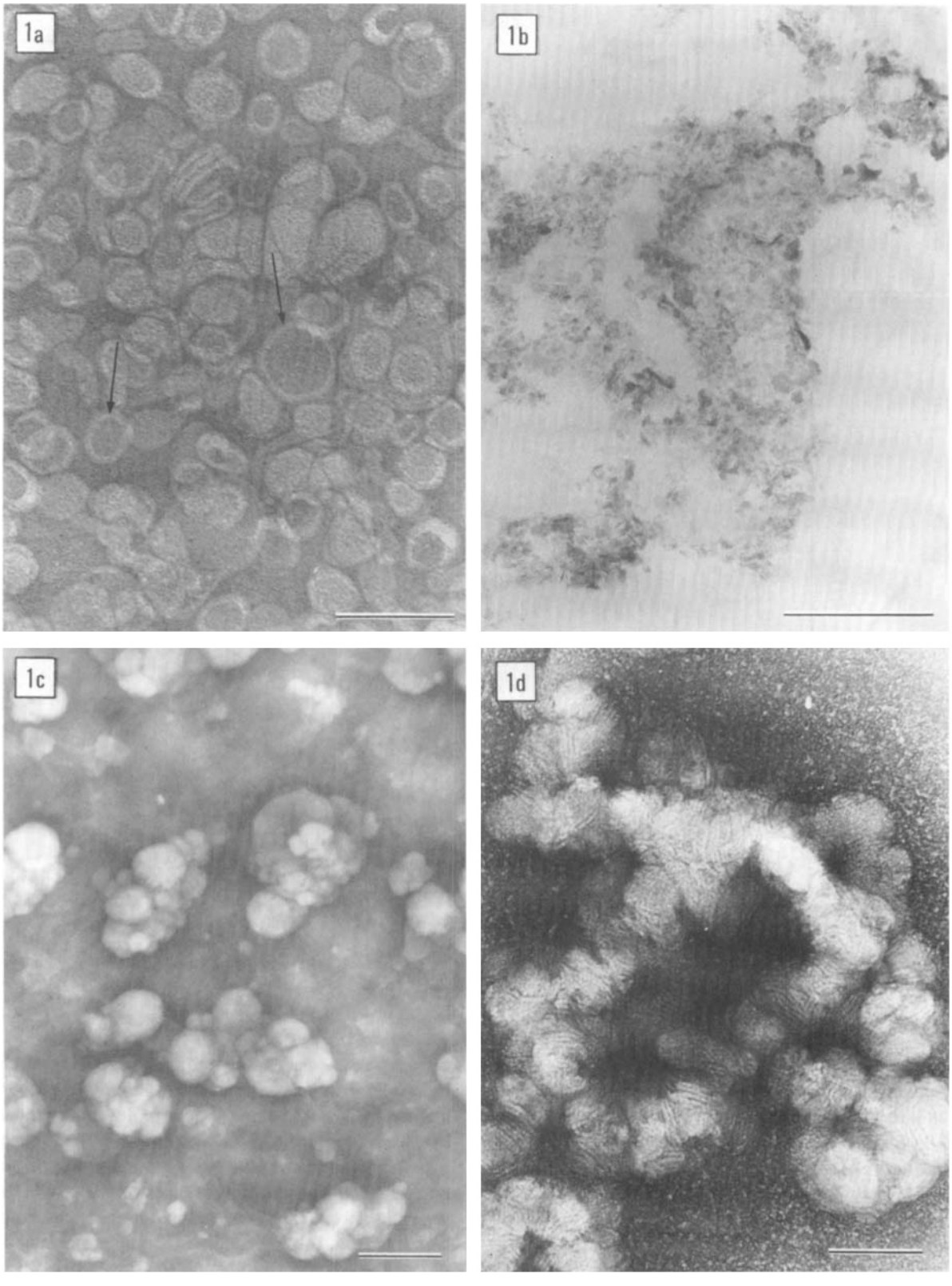
## RESULTS AND DISCUSSION

A homogeneous solution of small (30–60 nm diameter) unilamellar vesicles was produced by sonication of a suspension of multilamellar vesicles comprising phosphatidylcholine molecules. An electron micrograph of a typical sample is shown in Fig. 1(a).

When an aqueous solution of trimethyltin chloride is added to the ULVs, electron micrographs of unstained samples showed that the organotin is associated with the vesicles [Fig. 1(b)]. This is confirmed by the EDXA analysis of representative grid areas which show the presence of tin, phosphorus and chlorine (Fig. 2). Information regarding changes in vesicle morphology, size and membrane thickness brought about by the addition of trimethyltin chloride can be gained from negative staining of the grid sample, by which the stain penetrates the aqueous compartments of the vesicle[11] and when examined in the TEM the unstained lipid bilayers contrast with the stained aqueous phase.[12] It can be seen in Fig. 1(c, d) that the presence of trimethyltin causes extensive clustering and aggregation of the synthetic vesicles and also, to some extent, fusion of ULVs into plurilamellar liposomes, i.e. membranes comprising two or more lamellae. EDXA analysis of the stained samples shows that both tin and chlorine are no longer associated with the vesicles [Fig. 2(c)]. The negative stains used in these experiments (uranyl acetate, methylamine tungstate, ammonium molybdate) are known to have a strong affinity for phosphate groups and will displace other groups bound to the membrane via this part of the phosphatidylcholine moiety.[13–15]

For comparison, we have also studied the interaction of the tributyltin moiety, probably the most commercially used and thus the most environmentally significant of the triorganotins, with the biomimetic liposome system. In general, tributyltin compounds are markedly less soluble in water than their trimethyltin analogues, but we have successfully employed the ethanesulphonate derivative $Bu_3SnOSO_2Et$ which has *ca* 3%

**Figure 1** (a) TEM micrograph of phosphatidylcholine liposomes after sonication. Negative staining reveals small, unilamellar vesicles (↑). Bar = 0.1 $\mu$m. (b) Electron micrograph of phosphatidylcholine vesicles after incubation with trimethyltin chloride. The liposomes were imaged through the inherent electron scattering ability of the organometallic. Specific details of liposome morphology cannot, however, be discerned. Bar = 0.1 $\mu$m. (c) Electron micrograph of negatively stained vesicle sample after incubation with trimethyltin chloride. Extensive clustering of the liposomes can be observed. Bar = 0.5 $\mu$m. (d) TEM micrograph of phospholipid vesicles treated with trimethyltin chloride. At higher magnification, negative staining reveals the extent of the aggregation induced by the organometallic. Bar = 0.1 $\mu$m.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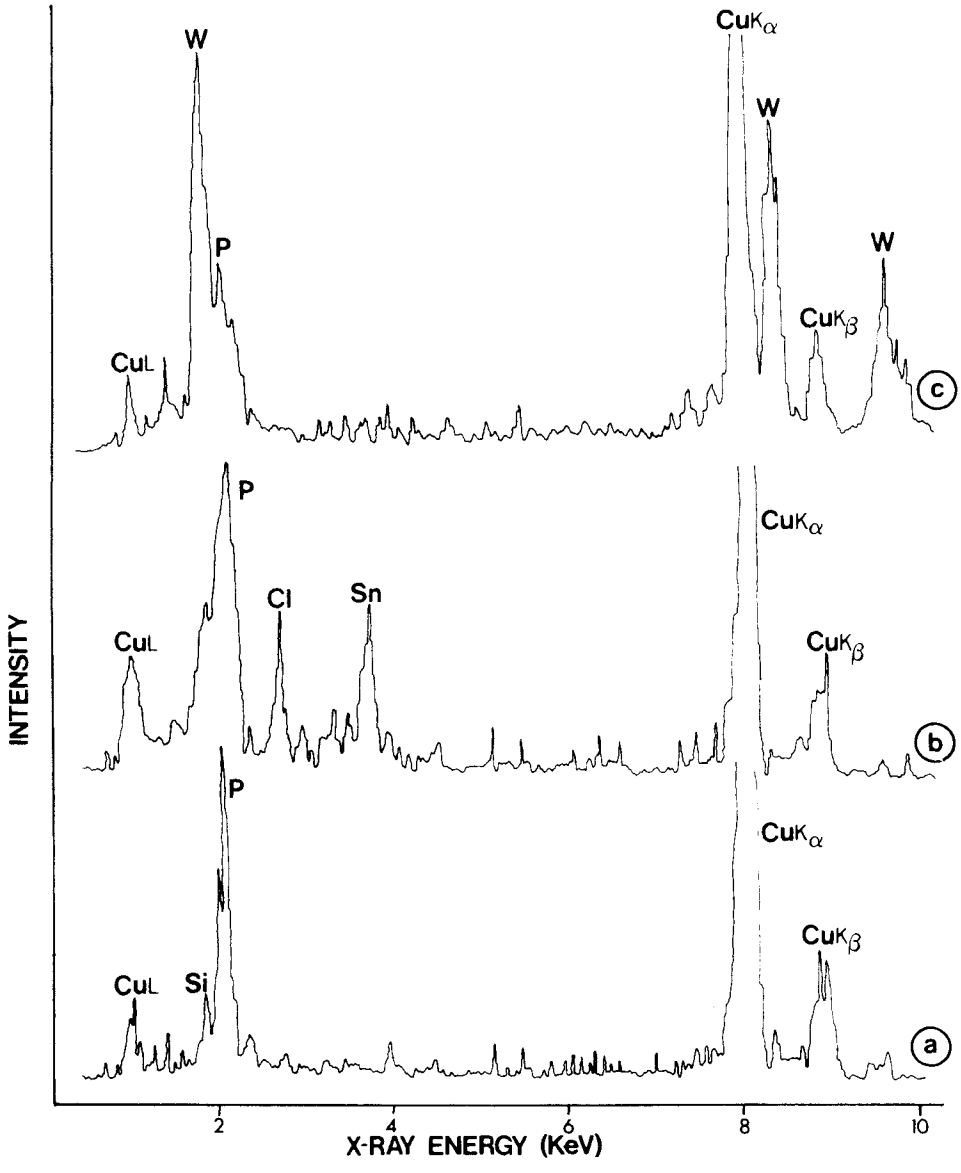


**Figure 2** EDXA spectra: (a) Sonicated phosphatidylcholine (PC) vesicles – unstained; (b) PC vesicles incubated with trimethyltin chloride – unstained; (c) PC vesicles incubated with trimethyltin chloride – negatively stained with 4% methylamine tungstate.

w/w solubility.[9,16] When aqueous tributyltin ethanesulphonate is added to an aqueous solution of ULVs, an immediate flocculation of the solution is observed. The unstained images of the vesicles under these conditions are essentially similar to those in the corresponding trimethyltin experiment [Fig. 1(b)], and EDXA analysis again shows that the organotin is associated with the membrane [Fig. 3(a)]. However, in contrast to the effects of trimethyltin upon the vesicle, tributyltin cations wreak considerably more damage. Negatively stained images of these preparations suggest that severe membrane disruption and loss of molecular architecture have accompanied the interaction of the organometallic with the vesicle. A significant number of multilamellar liposomes were observed [Fig. 4(a)] as well as vesicles exhibiting structural anomalies, e.g. blebbing [Fig. 4(b)]. In addition, complete disruption of several vesicles was also noted [Fig. 4(b)]. These

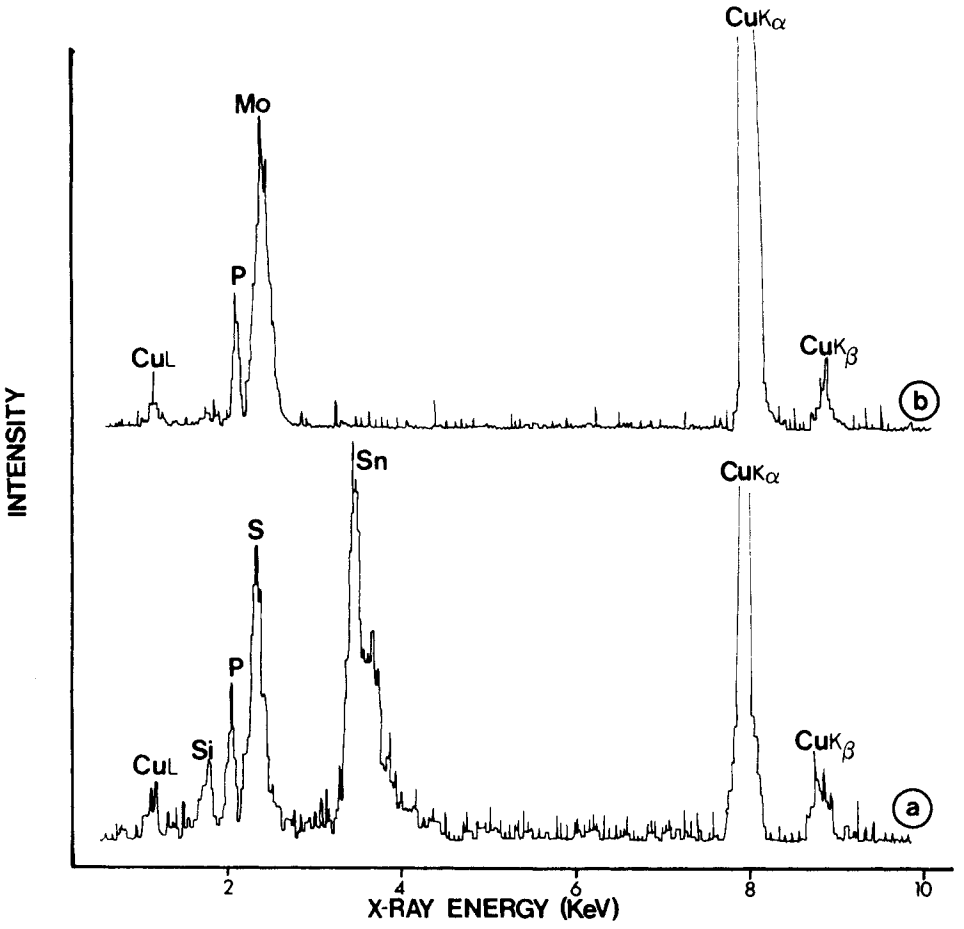


**Figure 3** EDXA spectra of phosphatidylcholine vesicles incubated with tributyltin ethanesulphonate, (a) unstained and (b) negatively stained with 2% ammonium molybdate.

observations are not a result of interactions associated with the negative staining process,[17] as a number of different stains all produced the same result.

Other workers have shown that a variety of divalent cations (calcium(II), barium(II), strontium(II), zinc(II), cadmium(II), mercury(II), etc.) also induce fusion of phospholipid vesicles.[18,19]

The implications of the staining experiments described above are that the organotin is associated with the vesicle via the phosphate headgroups, and we have attempted to explore this possibility further by multinuclear NMR experiments (Table 1). The $^{1}H$ NMR spectrum of $Me_3SnCl$ in $D_2O$ shows a singlet flanked by satellites due to coupling to the $I = 1/2$ $^{119,117}Sn$ nuclei. The $^{2}J(^{119}Sn-^{1}H)$ coupling (68 Hz), along with the $^{1}J(^{119}Sn-^{13}C)$ coupling from the $^{13}C$ spectrum (504 Hz), can be used to determine the Sn–C bond angles in the species present in aqueous solution. The calculated values from the two NMR experiments are 118° and 121.0° respectively[20,21] and this correlates with a five-coordinated *trans*-$X_2SnR_3$ environment about tin which almost certainly arises from the deuterated cation $(CH_3)_3Sn(D_2O)_2^{+}$. The $^{119}Sn$ chemical shift of this species (37.56 ppm) also correlates with its coordination number of five. A similar analysis for aqueous solutions of $Bu_3SnOSO_2Et$ yields $\angle C—Sn—C = 120.0°$ from the $^{119}Sn-^{13}C$ coupling data,[21] and both this and the tin chemical shift (18.23 ppm) are consistent with an analogous aquated tributyltin cation. This latter species has been crystallographically confirmed as its penta(methoxycarbonyl)-cyclopentadienyl salt.[23]

When assessing the NMR data arising from solutions containing both organotin and ULV, two questions

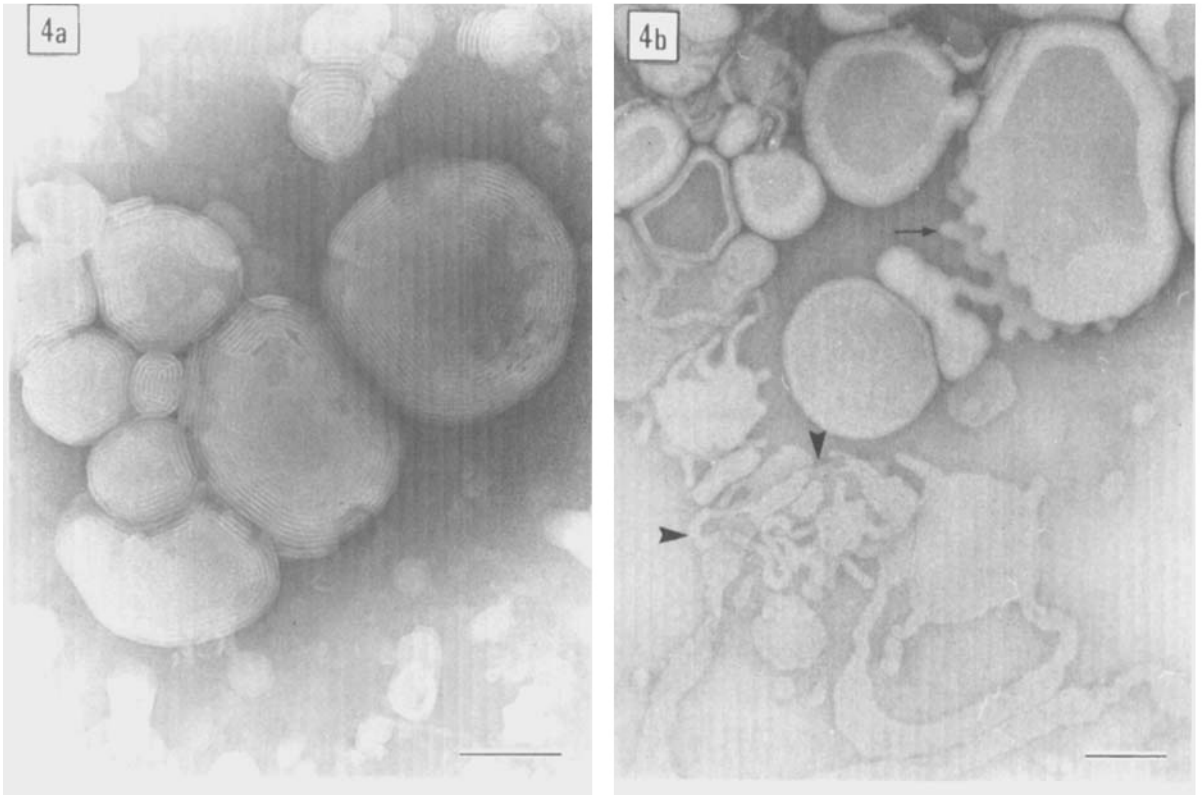


**Figure 4** (a) TEM micrograph of phosphatidylcholine liposomes treated with tributyltin ethanesulphonate. Multilamellar vesicles can be identified in the liposome population. Bar = 0.1 $\mu$m. (b) Electron micrograph showing the structurally anomalous liposomes observed after treatment with the tributyltin complex. Blebbing of the bilayers (↑) and complete disruption of some vesicles (►) is apparent. Bar = 0.1 $\mu$m.

**Table 1** NMR data[a]

| | $^{1}H$ | $^{13}C$ | $^{31}P$ | $^{119}Sn$ |
|---|---|---|---|---|
| $ULV/D_2O$ | | | −1.09(70) | |
| $Me_3SnCl/D_2O$ | 0.59[b] | −1.76[c] | | 37.56(22) |
| $Me_3SnCl/ULV/D_2O$ | 0.56[d] | | −2.38(99) | 34.23(894) |
| $Bu_3SnOSO_2Et/D_2O$ | | 18.23[e,f] | | 20.01(113)[g] |
| $Bu_3SnOSO_2Et/ULV/D_2O$ | | | −6.91(294) | 19.66(565) |

[a] Chemical shifts in ppm with respect to $Me_4Si(^{1}H, ^{13}C)$, $H_3PO_4$ ($^{31}P$), and $Me_4Sn$ ($^{119}Sn$). Full width at half height given in parentheses. [b] $^{2}J(^{119}Sn-^{1}H)$ = 68 Hz. [c] $^{1}J(^{119}Sn-^{13}C)$ = 504 Hz. [d] $^{2}J(^{119}Sn-^{1}H)$ = 73 Hz. [e] $\alpha$-$CH_2$. [f] $^{1}J(^{119}Sn-^{13}C)$ = 452 Hz. [g] Quoted as 21.5 ppm in Ref. 16.

need to be addressed. Firstly, is there evidence to support an interaction between the tin and the phosphate headgroup, and secondly, if such an interaction exists what is the resultant molecular species? The $^{31}P$ NMR chemical shifts of the vesicles in the presence of added triorganotin are different from that of the free ULV. However, while this in itself suggests a perturbation of the phosphorus environment, presumably from an interaction with the tin, the magnitude of this interaction must be weak since the changes in $\delta^{31}P$ are small (1−4 ppm). Similarly, changes in the tin chemical shifts are also small (0.5−3 ppm) so it seems unlikely that the arrangement of ligands about tin is significantly altered. No $^{2}J(^{31}P-O-^{119}Sn)$ coupling

could be seen in the NMR spectra of either nucleus, although the observed linewidths (*vide supra*) are of the same order of magnitude as reported two-bond tin–phosphorus couplings (~ 100 Hz).[24] These data taken collectively imply that the tin is bound to the membrane surface via an anion–cation pair rather than as a result of a reaction in which the phosphate oxygens displace water from the axial sites of its coordination polyhedron, although such an arrangement is a common feature of organotin derivatives of oxyphosphorous acids.[25,26] This relatively weak interaction would also be in keeping with the ease with which the organotin is displaced from the membrane during the negative staining procedure (Figs 2 and 3).

One feature of the NMR spectra of the mixed organotin/ULV solutions which differs significantly from their respective precursors is the resonance linewidths. In the case of $^{31}P$, this is caused by a combination of effects, namely the presence of liposomes in differing stages of disruption and secondly the reduced mobility of the phosphatidylcholine in multilamellar species (decreasing $T_2$ relaxation time). At least with respect to the first of these phenomena, it is interesting to note that the most significant line broadening occurs in the case of added tributyltin. The $^{119}Sn$ resonances also broaden considerably which we attribute to the reduced mobility of the organotin associated with the bulky liposome, thereby decreasing $T_2$ in a manner similar to that of the multilamellar vesicles above.

While our results broadly parallel those of Porvaznik *et al.* on the interactions and effects of tributyltin with RBCs, we can find no evidence for the formation of large polymetallic aggregates. Instead, our electron micrographs show the tin evenly distributed around the membrane surface. This, however, cannot be taken as evidence against the formation of such aggregates, as

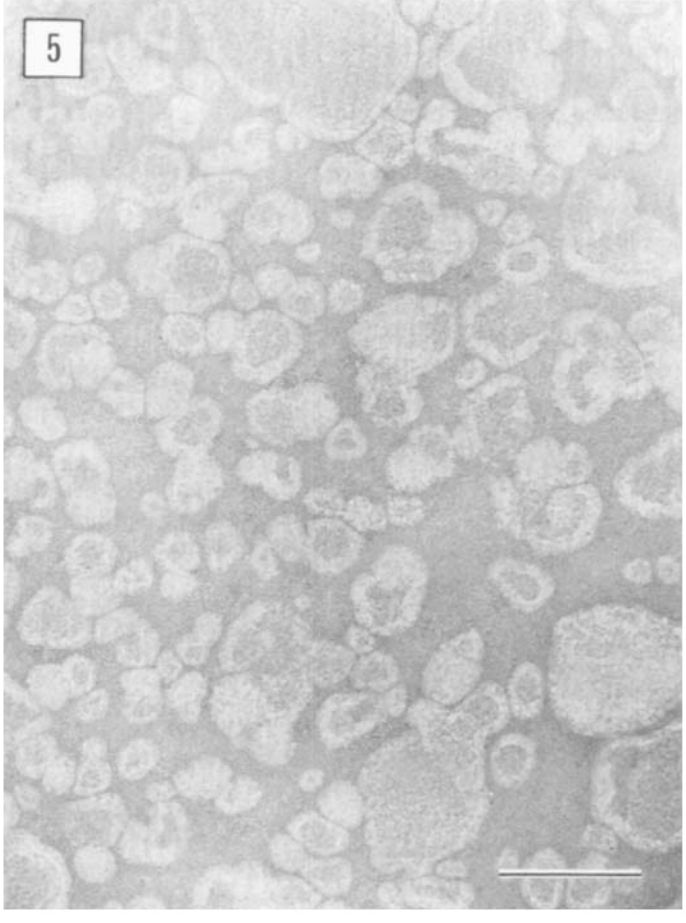


**Figure 5** Electron micrograph of negatively stained phosphatidylcholine vesicles after incubation with 25 $\mu$mol dm$^{-3}$ tributyltin ethanesulphonate. Compare with Fig. 1(a). Bar = 0.1 $\mu$m.

of necessity our experiments were carried out at far greater concentrations of organotin than the above (to allow parallel electron microscopy and NMR analysis of the same samples). However, even at 25 $\mu$mol $dm^{-3}$ concentrations of added tin (similar to that of Ref. 6) no electron dense spheres could be observed with the microscope and methodology available to us. It is worth noting, though, that even at this exceedingly low concentration of tin some structural disruption of the vesicles can still be seen to take place (Fig. 5).

In relation to the formation of tributyltin aggregates at the membrane surface, we have observed that, when $Bu_3SnOSO_2Et$ is added to saline solution, flocculation occurs immediately and $^{119}Sn$ NMR indicates the formation of $Bu_3SnCl$. Thus, while the formation of tributyltin globules will naturally follow when the solubility of the tin species in water is exceeded, the concentrations used by Porvaznik (*ca* $10^{-5}$ mol $dm^{-3}$)[6] should be less than that quoted for $Bu_3SnCl$ in seawater (50 ppm; $1.5 \times 10^{-4}$ mol $dm^{-3}$) which can be used for comparison.[27] Thus, it is tempting to speculate that the formation of localized organotin domains on the membrane surface requires the presence of specific membrane proteins which occur in the natural RBCs but not in our synthetic vesicles. Such speculation awaits future experimental exploration.

At this point we also can only speculate as to the molecular origin of the enhanced vesicle destruction caused by the tributyltin cation over that of the trimethyltin analogue. However, a reasonable explanation would appear to centre on the disposition of the lipophilic hydrocarbon chains, which could be imagined as burying into the lipid membrane, thereby disrupting the equilibrium state of the vesicle bilayer.

## CONCLUSIONS

The interaction of triorganotin cations with synthetic unilamellar vesicles has been examined. The organotin appears to be loosely associated with the membrane surface via a cation–anion pairing with the phosphate groups of the vesicle. Tributyltin cations cause greater structural damage to the membrane than do trimethyltins, which can be postulated as originating from a burying of the hydrophobic hydrocarbon groups into the lipid bilayer.

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## REFERENCES

1. Blunden, S J, Cusack, P A and Hill, R *The Industrial Uses of Tin Compounds*, The Royal Society of Chemistry, London, 1985
2. Aldridge, W N *Organotin Compounds: New Chemistry and Applications*, Adv. Chem. Ser., 1976, 157: 186, and references therein
3. Selwyn, M J *Organotin Compounds: New Chemistry and Applications*, Adv. Chem. Ser., 1976, 157: 204, and references therein
4. Elliot, B M, Aldridge, W N and Bridges, J W *Biochem. J.*, 1979, 177: 461
5. Taketa, F, Siebenlist, K, Kasten-Jolly, J and Polasaari, N *Biochem. Biophys.*, 1980, 203: 466
6. Porvaznik, M, Gray, B H, Mattie, D, Jackson, A G and Omlor, R E *Lab. Invest.*, 1986, 54: 254
7. Mahon, M F, Molloy, K C and Waterfield, P W *J. Organomet. Chem.*, 1989, 361: C5
8. Arbusov, B A and Pudovic, A N *J. Gen. Chem. USSR*, 1947, 17: 2158
9. Blunden, S J, Chapman, A H, Crowe, A J and Smith, P J *J. Pest Control*, 1978, 20: 5
10. Szoka, F and Papahadjopoulos, D *Annu. Rev. Biophys. Bioeng.*, 1980, 9: 467
11. Jousma, H, Talsma, H, Spies, F, Joosten, J G H, Junginger, H E and Crommelin, D J A *Int. J. Pharm.*, 1987, 35: 263
12. Bangham, A D and Horne, R W *Mol. Biol.*, 1964, 8: 660
13. Agostini, B and Haselbach, W *Histochemie*, 1971, 27: 303
14. Raeymakers, L, Agostini, L and Haselbach, W *Histochemistry*, 1981, 70: 139
15. Heywood, B R and Eanes, E *Calcif. Tiss. Int.*, 1987, 41: 192
16. Blunden, S J and Hill, R *Inorg. Chim. Acta*, 1984, 87: 83
17. Baskin, R J *J. Cell Biol.*, 1971, 48: 49
18. Bentz, J and Düzgünes, N *Biochemistry*, 1985, 24: 5436
19. Barfield, K D and Bevan, D R *Biochem. Biophys. Res. Commun.*, 1985, 128: 389
20. Lockhart, T P and Manders, W F *Inorg. Chem.*, 1986, 25: 892
21. Lockhart, T P, Manders, W F and Zuckerman, J J *J. Am. Chem. Soc.*, 1985, 107: 4546
22. Holecek, J and Lycka, A *Inorg. Chim. Acta*, 1986, 118: L15
23. Davies, A G, Goddard, J P, Hursthouse, M B and Walker, N P C *J. Chem. Soc., Chem. Commun.*, 1983: 597
24. Blunden, S J, Hill, R and Gillies, D G *J. Organomet. Chem.*, 1984, 270: 39
25. Molloy, K C and Zuckerman, J J *Acc. Chem. Res.*, 1983, 16: 386, and references therein
26. Cusack, P A, Smith, P J, Donaldson, J D and Grimes, S M *A Bibliography of Crystal Structures of Tin Compounds*, Tin Research Institute, London, 1981, Publication 588
27. Blunden, S J, Hobbs, L A and Smith, P J *Environmental Chemistry*, Bowers, H J M (ed), The Royal Society of Chemistry, London, 1984, p 66