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**REVIEW**

# Organotins: toxicology and biological effects

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**Organotins are currently one of the most studied groups of organometallic compounds; their novel and often unique chemical properties have intrigued chemists for over 100 years and, today, many of these compounds find extensive use in agriculture and industry. Over the years, however, a number of the organotins have been demonstrated to be toxic and there is now increasing concern that their widespread use may cause adverse effects within environmental and biological systems. This article reviews and updates the current literature concerning organotin toxicology. It identifies the various target organs and systems, discusses mechanisms and species susceptibility, and directs the reader to additional sources of more specialized information as appropriate.**

**Keywords: Review, organotin compounds, toxicity, biological susceptibility, target organs/systems, mechanisms, human exposure**

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## HISTORICAL PERSPECTIVE

Organotins are a series of compounds containing at least one covalent tin–carbon bond, with the tin atom acting as either a bi- or tetra-valent atom. The former species exist as dialkly or diaryl derivatives and diethyltin, $(C_2H_5)_2Sn$, synthesized by Lowig in 1852 has the distinction of being the first organotin (II) compound to be described in the literature (interested readers are directed to the work by Krause and Von-Gross,[1] which covers the relevant literature up to 1936). The mixed compound $(C_2H_5)_2SnI_2$ was prepared by Frankland in 1849 and in 1853 Frankland[1] also prepared tetraethyltin $(C_2H_5)_4Sn$; these were the starting points for the tetravalent organotin series where four basic configurations are possible, viz. $R_4Sn$, $R_3SnX$, $R_2SnX_2$ and $RSnX_3$: R may be a simple or substituted aliphatic or aromatic hydrocarbon radical, and X may be a halide, hydroxide, alkoxide, sulphydride, acyl sulphur–alkyl, or other group. Indeed, it was during these early pioneering studies that the toxic nature of some of the organotins became apparent, and many of the workers, including Frankland, witnessed their noxious effects at first hand. In 1858, Buxton[1] synthesized triethyltin chloride and also remarked on its irritant properties.

The search for novel organotin continued throughout the early part of the twentieth century, although it was

not until 1925 that the first commercial application of an organotin was recorded (as a mothproofing agent). Seven years later, tetra-alkyltins found industrial application as hydrogen chloride scavengers in chlorinated hydrocarbons used as insulators in heavy-duty transformers and capacitors. In the early 1950s, attention focused on more generalized commercial applications of organotins, most notably following the systematic investigations of Van der Kerk at Utrecht, The Netherlands, under the sponsorship of the International Tin Research Institute. As a consequence, the annual world consumption of organotins has grown rapidly from 500 tons in 1950 to around 50 000 tons in 1986.

Today, by far the largest usage of organotin compounds is in the stabilization of poly(vinyl chloride) (PVC) polymers. PVC is used extensively by numerous construction and consumer industries for flooring, piping and fabrication, and in the food-processing and packaging industry for bottles, packages and coverage films. PVC is both heat- and UV light-sensitive, with prolonged exposure causing brittleness and discolouration and, consequently, stabilizers are required in all transparent food-contact or packaging PVC products. Dialkyltin(IV) derivatives, in particular di-n-butyltin(IV) and di-n-octyltin(IV) species, find extensive use in this area in that they protect against heat and light both during extrusion as well as during subsequent use. More recently, dimethyltin(IV) and the diestertin(IV) derivatives have found increasing applications in the food package and processing industry. Further industrial applications of these compounds include their use as additives to polysiloxanes; as additives to improve water repellency of fibrous materials; as corrosion inhibitors in organosilicon polymers; as additives to confer sunlight resistance to cellulose acetate fibres; as additives to control pore size in polyurethane foam production; and also as additives to improve the curing of silicone and epoxy resins. In rubber products and paints, organotins find application as antioxidants and anticracking agents to retard rubber deterioration; in siloxane rubber vulcanization; and as stabilizers of chlorinated rubbers and chlorinated paints.

A wealth of literature[1-32] now exists which catalogues the discovery of new compounds and details the chemistry of individual molecules.[1-23] There are also numerous reviews detailing specific industrial applications[1,14-22] and agricultural usage, including some describing the more recent environmental effects[24-32] (see also Conclusions). For further information, interested readers are referred to relevant publications and literature surveys, e.g. those obtainable through the International Tin Research Institute, London.[29] The present article concentrates on the biological effects of organotins. It is clearly not feasible within the confines of a short text to review fully all of the possible interactions that may occur between individual organotin compounds and the numerous components that constitute the biosphere. Discussion is therefore focused on those interactions which have more attracted the attention of biologists, biochemists and toxicologists in recent years.

## BIOLOGICAL INTERACTIONS

Tin in the inorganic state is generally accepted as being relatively non-toxic; the oxides are unreactive and the metal is essentially non-ionized at physiological pH.[25] The addition of one or more organic groups to the tin atom, however, has a profound effect in terms of overall biological activity, the nature and magnitude of which is determined by the number and configuration of the organic substituents.

In general, the biological activity within each class of organotins is related to the number of carbon atoms per side chain. Variation of the inorganic group, X, within any given $R_3SnX$ series is usually found to have no significant effect on the overall bioactivity of the molecule. The bioactivity profile resulting from substitutions within $R_nSnX_{4-n}$ may therefore be most easily appreciated by considering the effects of sequential changes in R and $n$. It is now well established that the progressive introduction of organic groups into the above model produces maximal toxicity when $n=3$. However, the impact of R varies with the biological test system. The highest mammalian toxicity is seen when R is ethyl. The tributyl- and triphenyl-tins, on the other hand, show the highest activity against fungi and have been applied commercially without apparent adverse effects to those applying them (see Human exposure, below). Conversely, the same compounds are highly toxic to fish and phytoplankton, and inadvertent agricultural run-off and excessive marine applications are now linked with adverse environmental effects.[28-32] Whereas trimethyltins are most effective

against insects, it is the tricyclohexyltin derivatives that are most effective against spiders and mites. Similarly, tripropyltins are toxic for Gram-negative bacteria and tributyltin is most effective against Gram-positive bacteria. Further increases in n-alkyl chain length produce a rapid reduction in bioactivity and the tri-n-octyltins are considered essentially non-toxic. Below, we discuss the effects of organotins in a number of biological systems, including mammalian toxicity and associated mechanisms.

## Phytotoxicity versus plant protection

The majority of data in this area stem from two principal sources: firstly, trials designed to assess fungicidal efficacy against plant pathogens and, secondly, studies designed to assess toxicity, antifeedant and chemosterilizing effects in insects. Readers particularly interested in the current status of organotins in agriculture are directed to the recent review by Crowe 1987.[31] Accordingly, we restrict our attention here to a brief outline of the emergence of organotins in agriculture and insect control.

The first commercial organotin products for agricultural use were introduced during the early 1960s. These include 'Brestan' (fentin acetate, triphenyltin actetate $Ph_3SnOAc$) and a sister compound 'Brestanol' (fentin chloride, triphenyltin chloride, $Ph_3SnCl$) by Hoechst, and 'Duter' (fentin hydroxide, triphenyltin hydroxide, $Ph_3SnOH$) by Philips Duphar. All have found use as general-purpose fungicides and have been used for algae control in paddy fields and preventing leaf spot fungi in a variety of plant types. 'Plictran' (tricyclohexyltin hydroxide, $(C_6H_{11}Sn)_3OH$) produced by The Dow Chemical Company entered the market a few years later, being advocated for the control of pathogenic mites. Additional mite control agents in the form of 'Vendex' [bis(trineophyltin) oxide, $[(C_6H_5(CH_3)_2CCH_2)_3Sn]_2O$] and 'Peropal' [tricyclohexyltin 1,2,4-triazole ($c-C_6H_{11})_3Sn\ C_2H_2N_3$] were subsequently introduced by Shell and Bayer respectively. Today, these six agents find extensive use under various trade names in over 15 countries (see Ref. 31 for full details).

Organotins as plant protection agents offer a number of advantages over conventional fungicides and pesticides, especially those based on mercury, copper or arsenic. These latter elements are permanantly toxic, their residues remaining behind on the crops, and repeated spraying is now raising concern over their accumulation within the environment and food chains. With organotin compounds, toxic residues are considered to be less of a problem since the toxicity of the compounds resides in the $R_3Sn$ radical. This is gradually broken down by UV light and weathering to $R_2Sn$, and, in due course, to $SnO_2$, which, like other inorganic tin compounds, is essentially without biocidal activity.[25] Consequently, it has been suggested that spraying of crops to a defined schedule could ensure that residues will be converted to an inert form, prior to the crop being harvested.

These benefits, however, have to be weighed against the phytotoxic effects of the compounds. Whereas trialkyltins are some 10 to 20 times more fungicidal than triphenyltins in the laboratory,[33] they are essentially excluded from agricultural applications because of their phytotoxicity.[34,35] The triphenyltins are considerably less phytotoxic, in both laboratory and field trials, but a number of crops are selectively sensitive. In plants, phytotoxicity is influenced by X in $R_3SnX$ compounds and particularly so for the triphenyltin compounds, the toxicity being most acute in the chloride and sulphate, and decreasing down the series acetate, hydroxide, oxalate and *p*-toluenesulphonamide.[36] This effect is in accordance with the earlier results of Bauman,[37] who, working in greenhouse conditions with tomatoes, demonstrated that, whereas tributyl- and tripropyl-tin acetates were highly phytotoxic, as judged by burn damage, triphenyltin acetate and hydroxide were significantly less toxic. Bauman also examined the effects of triphenyltin acetate in a series of unrelated plants and established that phytotoxicity varied considerably between the taxa. Whereas grape and hop plants were rapidly damaged by this compound, beet, potato and celery were almost totally resistant. Byrdy *et al.*[38] subjected mustard, *Sinapis alba*, to various triphenyltin compounds. The degree of damage and weight loss were correlated with the compounds' effect on *Venturia inaequalis*, *Altenaria tenuis* and *Phytophera infestans*. In this system, triphenyltin hydroxide displayed greatest phytotoxicity and lowest fungicidal activity.

Efforts to overcome phytotoxicity often fail because substitution of X in triphenyltins, in such a way as to reduce phytotoxicity, often significantly reduces the fungicidal properties of the compound. Thus bis(triphenyltin) sulphide $[(Ph_3Sn)_2S]$ and the disulphide are much less phytotoxic than triphenyltin

acetate.[34,39] However, whereas triphenyltin acetate is an effective wide-spectrum fungicide,[35] the sulphide and disulphide have restricted fungicidal activity.[34] Phytotoxicity is also significantly influenced by the nature of the formulation of the produce and, in particular, the particle size of the active component, the pH of the carrier, its formulation and concentration, and its mode of application.

A balance is therefore usually struck between effective fungicidal control and acceptable plant damage, this being influenced by the individual susceptibilities of the plant and pathogen respectively. Since there is no apparent correlation between phytotoxicity and fungitoxicity, treatment regimens can only be determined after extensive greenhouse and field studies. This is clearly worthwhile for cash-crops. Using *Pernospora* on vines as the model system, Haertel, in 1962,[40] using a triphenyltin perchlorate ($Ph_3SnClO_4$) treatment at phytotoxic levels, demonstrated that grape yield was unaffected and even apparently enhanced. The latter point is of particular interest, since a number of authors have suggested that some organotin applications may actually possess growth-promoting effects.[31,41–44] Others, however, ascribe the increased crop yields to the beneficial fungicidal effects of the compound. Conflict also exists as to whether the tin compounds enter the tissues and act systemically in plants. Studies using[113] Sn-labelled compounds have shown that radioactivity is not absorbed and translocated into sugar beet.[45] However, Solel, in 1971[46] using triphenyltin acetate also in sugar beet, demonstrated that the application of this compound to one side of the plant conferred protection against infection to other untreated leaves. He concluded that systemic transport of the organotin must have occurred. These questions must remain undecided until further and more detailed studies are performed.

## Insect control studies

During the early 1930s, Hartmann and his coworkers[47] patented a series of compounds, including a series of alkyl- and aryl-substituted tins linked to inorganic acid radicals (e.g. triethyltin fluoride), as well as tetrabenzyltin and tetraphenyltin, as mothproofing agents. In 1950, Shell introduced an ethyl-, methyl- and propyl-tin chloride package for application as an insecticide/ovicide to be used in conjunction with DDT and Pyrethrum for protection against pests in general.[48] Soon interest focused on the possibility of combining these anti-insect and anti-fungal properties for the protection of textiles, a particular target being *Anthrenus vorax*, the carpet beetle. In their early studies, Hueck and Luitjen[49] examined a series of organotin compounds for efficacy. Of the many tested, only the alkyl-tin derivatives were sufficiently active, with triethyltin-*p*-toluenesulphonamide [$(C_2H_5)_3SnNHSO_2C_6H_4CH_3$], triethyltin ethoxide [$(C_2H_5)_3SnOC_2H_5$] and tributyltin oxide [$(C_4H_9)_3Sn)_2O$], all affording good protection. Indeed, tributyltin oxide paralleled DDT in terms of toxicity to both the clothes-moth and carpet beetle. The tetraalkyl and -aryltins were relatively non-toxic, as were the mono- and dialkyl-tin derivatives.

Unfortunately, it soon became apparent that fabric fastness (the ability to survive repeated washing) limited the suitability of many organotins in textile applications. Others searched for more suitable uses, Becker[50] demonstrating tributyltin acetate [$(C_4H_9)_3SnOAc$] to be effective against the furniture beetle and Richardson[51] using tributyltin oxide [$(C_4H_9)_3SnO$] at 0.1% in powdered biscuit to control the larvae of *Stegobium paniceum*, the drug-store beetle. Blum and Bower[52] demonstrated that triethyltin hydroxide [$(C_2H_5)_3SnOH$], and related esters induced rapid paralysis in DDT-resistant house flies.

Stannous carboxylates (octanoate or oleate) and monoalkyl or monoaryl derivatives of tin(IV) (e.g. phenyltin trichloride, ($PhSnCl_3$) and even tin tetrachloride itself were essentially non-toxic, with dialkyl- and diaryl-tins exhibiting only slightly raised toxicity.[52] Again, these findings are in accordance with studies in fungi and mammals in which the monoalkyltins always prove to be the least toxic among the alkyl-substituted tins.

As in fungi and mammals, toxicity is associated with the trisubstitution at the central tin atom, with toxicity not being significantly influenced by the nature of the X anion. Overall chain length does, however, have interesting effects: in fungi, maximal toxicity is attained with 9–12 carbon atoms divided between the three organic chains, the decreasing order of activity being tri-n-butyl > tri-isopropyl, tri-n-propyl > triethyl > trimethyl > trioctyl.[33] This pattern holds true even for non-symmetrical compounds, such as dimethyl-n-octyltin acetate.[53]

Nevertheless, whereas trimethyltin has relatively low

toxicity against fungi, trimethyltin bromide, for example, is highly toxic in house flies. Similarly, in mammals, such as the rat, oral toxicity of $R_2SnX$ in terms of $LD_{50}$ occurs in the order: triethyl > trimethyl > tri-isopropyl > tributyl > trihexyl > trioctyl[54] (see below).

As a consequence, many of the organotins that have been tested are far too toxic in mammals to be considered for domestic use, either as pesticides or as fly-killers. It was therefore somewhat fortuitous that field trial studies with 'Duter', fentin hydroxide ($Ph_3SnOH$), a triphenyltin of relatively low mammalian toxicity, showed it to have dramatic effects for the control of a number of plant pests including the citrus rust mite, *Phyllocoptruta oleivora*[55] and the pink rust mite, *Aculus pelekassi,* the cabbage army worm, *Mamestra brassicae,* and the tobacco horn worm, *Protoparce sexta.*[56]

Additionally, triphenyltins have also found application in the control of insects by anti-feeding. Anti-feeding refers to the ability of a compound not to kill the insect but to inhibit feeding. Thus, application of 'Brestan' ($Ph_3SnOAc$) as an agricultural fungicide has also been shown to protect plants from a number of important insect pests, including the Egyptian cotton leafworm, *Prodenia litura,* the larvae of the potato tuber moth *Gnorimosschema operculella* and the larvae of *Agrotis ypsilon,* a related noctuid.[57,58] Laboratory studies using sugar beet leaves and *Prodenia litura* confirmed that the protective effect was anti-feeding rather than a toxic effect in that leaves dipped in 'Brestan' were protected, even from starved larvae.[58] Tributyltin oxide has similarly been shown to protect exposed wood from termite attack for up to one year.[59] More recent studies concerning the anti-feeding and chemosterilant effects of organotin compounds are discussed in Refs 31 and 60.

## MAMMALIAN TOXICITY

Unlike the organo-leads and -mercurials, in which compounds show a general toxicity, the organotins seem to follow no general rules. Toxic effects are often highly species-specific.[61,62] Dibutyltin oxide [$(C_4H_9)_2SnO$], for example, has an $LD_{50}$ of 100–200 mg $kg^{-1}$ in the rat[63,64] but the same compound is used at high doses as a helminthicide in chickens.[65] Dioctyltin dichloride [$(C_8H_{17})_2SnCl_2$] induces rapid thymic atrophy in rats whereas mice, rabbits and guinea-pigs are relatively resistant (see Immunotoxic effects, below). Triphenyltin acetate is approximately 10 times more toxic in guinea-pigs and rabbits than in rats; conversely, guinea-pigs are more resistant to tetraethyltin than rats and rabbits. These effects are ascribed to differences in diet, absorption, passage time through the intestine, passage across the blood–brain barrier and differing susceptibilities of target organs and enzyme systems.[61,62,66]

Some distinction can be made, however, between the general toxic effects of the di-, tri- and tetra-substituted compounds. It is established that certain of the trisubstituted compounds act within the central nervous system, producing cerebral oedema. The disubstituted compounds, on the other hand, have no neurological effects but are powerful irritants and cause hepatic and biliary inflammation following ingestion. The tetraorganotins resemble their triorganotin counterparts, although there is some delay before their effects become apparent. It has been suggested that this lag period stems from the fact that the tetraorganotin form is converted to a triorganotin derivative following metabolism in the liver[67] and/or gastrointestinal tract.[68]

As we have seen in previous sections, (insect and plant) toxicity within each class of the organotins correlates with the number of carbon atoms within each side chain, with the X anion (in $R_3SnX$) having only a minor influence on overall bioactivity, although in $R_2SnX_2$ compounds the X group *does* show an effect on bioactivity. Within the triorganotin series, highest mammalian toxicity is seen with the tri-methyl and -ethyl compounds, toxicity decreasing as the chain length increases, with the trioctyltin compounds being essentially non-toxic and triphenyltin compounds equating with tributyltins in terms of overall toxicity.

A considerable amount of literature has now accumulated relating to the toxicity of organotins; organotin toxicity up to 1959 has been reviewed by Barnes and Stoner[54] and, more recently, by Duncan[69] and by the World Health Organization (WHO).[25]

Because of the limitations in undertaking human studies (volunteer studies are reported under Human exposure below), tests have been performed either in animals or in *in-vitro* cell systems. The laboratory rat is the most commonly used experimental animal, although some studies have also used rabbits, mice, guinea-pigs and, occasionally, dogs.

In studies of acute toxicity, the injury induced by

a single toxic dose is determined following one or more routes of administration (oral, dermal, inhalation), usually chosen to reflect the most likely routes of exposure in man. Usually, the test chemical is administered to at least three groups of animals (five males and five females per group) as a single toxic dose with levels chosen from range-finding studies so as to produce a spread of toxic signs or changes over a two-week study period. Post mortems are performed in conjunction with histopathology in order to determine the nature of the toxic injury, target organ or tissue specificity and the cause of death. Such data are invaluable in setting safety levels and when dealing with cases of accidental human exposure and poisoning.

In order to compare toxicity between compounds, an $LD_{50}$ is determined, this being the dose of a particular compound which causes death in 50% of treated animals. A list of acute $LD_{50}$ values for a range of the better-researched organotin compounds is presented in Table 1.

The apparent inverse relationship between chain length and oral toxicity appears to be the consequence of the poor absorption of the higher trialkyltin compounds from the gastrointestinal tract. Thus, in rats, Stoner *et al.*[70] demonstrated that triethyltin sulphate ($[(C_2H_5)_3Sn]_2SO_4$) was equally toxic after intraperitoneal (i.p.), intravenous (i.v.) and oral administration, with 40 mg $kg^{-1}$ causing death within 2 h and 10 mg $kg^{-1}$ causing death within 4–5 days. Tricyclohexyltin hydroxide, on the other hand, has an oral $LD_{50}$ of between 100 and 1000 mg $kg^{-1}$ for most species, whereas 20 mg $kg^{-1}$ is lethal following i.p. or i.v. administration. The $LD_{50}$ value is a useful index of toxicity. However, alone it gives no indication of the mechanism of the effect and there is now a trend to abandon the $LD_{50}$ approach in favour of more reasoned analytical approaches.

In some respects the minimal effective and no-observed-effect doses of compounds are more significant than the $LD_{50}$ value. When dibutyltin dichloride was fed to rats for 90 days at 10, 20, 40, and 80 mg $kg^{-1}$, the no-effect level was set at 40 mg $kg^{-1}$. After 6 months' exposure, the no-effect dose was judged to be 20 mg $kg^{-1}$,[54] thus indicating an effect of cumulative toxicity. Dibutyltin sulphide administered orally to rats at doses of 1.0, 0.1, 0.01, and 0.001 mg $kg^{-1}$ $day^{-1}$ for 7 months was tolerated without any adverse effect, at all doses up to 0.01 mg $kg^{-1}$ $day^{-1}$.[71] By comparison, the dioctyltin compounds did not produce toxicity in mice, rats, or guinea-pigs at doses up to 400 mg $kg^{-1}$ body weight per day when given for 3–4 successive days, nor were there any apparent ill effects when dioctyltin compounds were included in the diet of rats for 4 months at a rate of 200 mg $kg^{-1}$.[54] (See Immunotoxic effects, below, for specific effects on the thymus.)

Data on the effects of trialkytin compounds are, in the main, limited to the effects of triphenyltin derivatives and tricyclohexyltin hydroxide. In rats, triphenyltin acetate administered at 5, 10, 25, and 50 mg $kg^{-1}$ for up to 17 days produced no apparent adverse effects up to the 25 mg $kg^{-1}$ dose level. Deaths from infection, however, occurred in the 50 mg $kg^{-1}$ dose group.[72]. In a two-year study in guinea-pigs using triphenyltin acetate at doses up to 200 mg $kg^{-1}$, the no-effect level was reported to be 5 mg $kg^{-1}$. At increased levels, there was growth retardation and an increased incidence of mortality associated with fatty degeneration of the heart and liver.[73]. In rats, a two-year study investigating the effects of triphenyltin hydroxide exposure up to 10 mg $kg^{-1}$ gave the no-observable-effect dose to be 2 mg $kg^{-1}$, equivalent to about 0.1 mg $kg^{-1}$ $day^{-1}$. Using tricyclohexyltin hydroxide, no toxicity was observed in rats at doses up to 12.5 mg $kg^{-1}$ day for 19 days. However, in a two-year study at the same dose levels, the no-observable-effect dose level was reduced to 3 mg $kg^{-1}$ day. Dogs appear to be more susceptible to tricyclohexyltin hydroxide, in that exposure to this compound at doses up to 12 mg $kg^{-1}$ $day^{-1}$ for 6–12 months resulted in acute weight loss and death from starvation in the top dose group (whether this effect is toxicity as such, or just that the dog rejected the compound is difficult to decide). Nevertheless, the no-observable-effect level for dogs was established at 0.75 mg $kg^{-1}$ $day^{-1}$.[73] Data from pesticide-related studies investigating the suitability of aqueous applications of tricyclohexyltin hydroxide in yearling cattle, sheep and goats demonstrated that yearling cattle tolerated suspensions up to 0.5%, whereas 1% was associated with anorexia; young sheep and goats tolerated 0.1%. However, transient anorexia and eye irritation were noted in goats exposed to a 0.2% suspension of this compound.

Although it is always possible to establish a 'no-observable-effect' limit, more subtle changes can occur even in the absence of growth retardation or overt pathological changes. This is particularly relevant with

respect to the immune system, where modern approaches can demonstrate treatment-induced changes in the levels of immunologically important molecules within hours of exposure. Below, we discuss the effects of organotins on defined target organs and the immune system.

**Table 1** Acute toxicity of selected organotin compounds

| Compound | $LD_{50}$(mg $kg^{-1}$) | Species | Reference |
|---|---|---|---|
| Butylstannoic acid | 6000 (oral) | Mouse | 87 |
| Butyltin trichloride | 1400 (oral) | Mouse | 87 |
| Butyltin *S,S′S″*-tris(isoethyl-mercaptoacetate) | 1520 (oral) | Mouse | 87 |
| Octyltin *S,S′S″*-tris(2-ethylhexyl-mercaptoacetate) | 1500 (oral) | Rat (male) | 87 |
| Dibutyltin dichloride | 182 (oral) | Rat (male) | 71 |
| | 112 (oral) | Rat (female) | |
| | 35 (oral) | Mouse | |
| | 190 (oral) | Guinea-pig | |
| Dibutyltin sulphide | 24 (oral) | Mouse | 71 |
| | 145 (oral) | Rat | |
| | 180 (oral) | Rat (female) | |
| Dioctyltin dibutylmaleate | 3750 (oral) | Mouse | 87 |
| Dioctyltin *S,S′*-bis(butyl-mercaptoacetate) | 1140 (oral) | Mouse | 87 |
| Dioctyltin bis(dodecyl-mercaptide) | 4000 (oral) | Mouse | 87 |
| Dioctyltin *S,S′*-bis(2-ethylhexyl-mercaptoacetate) | 2010 (oral) | Mouse | 87 |
| Triethyltin acetate | 4 (oral) | Rat (female) | 97 |
| Triethyltin sulphate | 5 (i.p.) | Rat (male) | 97 |
| | 5 (i.p.) | Guinea-pig | |
| Tributyltin acetate | 46 (oral) | Mouse (male) | 89 |
| | 99 (oral) | Mouse (male) | |
| Tributyltin benzoate | 108 (oral) | Mouse | 89 |
| Tributyltin chloride | 117 (oral) | Mouse | 89 |
| Trihexyltin acetate | 1000 (oral) | Rat | 54 |
| Triphenyltin acetate | 21 (oral) | Guinea-pig | 66 |
| | 24 (oral) | Guinea-pig | 84 |
| | 136 (oral) | Rat | 66 |
| | 81 (oral) | Mouse (male) | 73 |
| | 7 (i.p.) | Mouse (male) | 97 |
| | 136 (oral) | Rat (male) | 72 |
| | 491 (oral) | Rat (female) | 97 |
| | 450 (oral) | Rat (male) | 66 |
| | 8 (i.p.) | Rat (female) | 97 |
| | 11 (i.p.) | Rat (female) | 97 |
| | 13 (oral) | Rat (male) | 72 |
| Triphenyltin chloride | 80 (oral) | Rat (male) | 73 |
| | 135 (oral) | Rat (female) | 73 |
| Triphenyltin hydroxide | 245 (oral) | Mouse (male) | 73 |
| | 209 (oral) | Mouse (female) | 73 |
| | 27 (oral) | Guinea-pig (male) | 73 |
| | 37 (oral) | Guinea-pig (female) | 73 |
| | 171 (oral) | Rat (male) | 87 |
| | 268 (oral) | Rat (female) | 87 |
| Tetraethyltin | 40 (oral) | Mouse | 71 |
| | 9 (oral) | Rat | |
| | 40 (oral) | Guinea-pig | |
| | 7 (oral) | Rabbit | |

See Ref. 25 for further listings.

## Local effects: dermal and ocular toxicity

It was established early in the history of industrial organotin production that a number of di- and tri-organotin compounds could irritate the eyes, skin and mucous membranes. Indeed, a number of such compounds were even considered for use as chemical warfare agents.[74–76]

Early studies by Lyle in 1958[77] using human volunteers demonstrated that, of the dialkytin compounds examined, only dibutyltin dichloride was an irritant, all tributyletin compounds examined (chloride, acetate, alurate and oxide) produced chemical burns, whereas tetrabutyltin was inactive. Dimethyl-, diethyl-, dipropyl-, and dibutyl-tin laurate [$R_2Sn(OCoC_{11}H_{23})_2$] caused irritation in rats.[13] Dipropyl-, di-isopropyl-, and dibutyl-tins produce both deep-seated skin lesions and systemic bile duct damage. Dipentyl- and dihexyl-tins had no effect on the skin, but did produce systemic effects. Dioctylin dilaurate and dichloride were without effect and were considered non-toxic for these systems. There is, therefore, a direct relationship between the overall chain length and lipophilic nature of each compound and the ensuing toxic effect. Of the water-soluble compounds, dimethyltin derivatives caused rapid necrosis of the superficial layers of rat skin, whereas the more lipophilic compounds, such as dibutyl- and dipropyl-tin compounds, crossed the epidermal barrier and induced deep-seated inflammatory lesions.

Again, species-specific effects are apparent. Dermal application of dibutyltin dichloride at 10 mg $kg^{-1}$ body weight daily for 12 days produced severe tissue damage in rats and mice; guinea-pigs, on the other hand, were more resistant, showing little reaction to 120 mg $kg^{-1}$ daily for 5 days.[54] Also, in rats, a single dose of 167 nmol dibutyltin $cm^{-2}$ produced no apparent adverse effects, whereas an equimolar dose of tributyltin produced rapid epidermal necrosis and dermal oedema.[78] A single aqueous application of bis(tributyltin) oxide to the skin of rats at 0.36–0.95 mg $kg^{-1}$ produced local irritation lasting up to 3 weeks,[79] with higher concentrations producing severe necrosis.[80] This compound was also shown to produce severe eye irritation.[80] Triphenyltin hydroxide was reported not to affect the skin of rabbits[81,82] or guinea-pigs,[70,81–83] but proved to be extremely irritating to the eyes. Tricyclohexyltin irritates both the skin and eyes of rabbits[84] in the absence of systemic effects.[73] With regard to the mechanism of these effects, the consensus of opinion is that dermal or ocular toxicity occurs as a consequence of altered cellular energy production and direct cytotoxicity (see Mechanisms for further details). Thus, when rat skin was exposed to tributyltin *in vitro*, energy metabolism and cellular proliferation in the dermal/epidermal interface was severly disrupted. ATP levels, oxygen consumption and DNA synthesis were all significantly decreased, all effects occurring prior to the observed necrotic changes.[78,85]

## Pulmonary and gastrointestinal effects

Oral administration of dibutyltin dichloride to rats at a dose of 50 mg $kg^{-1}$ $day^{-1}$ for seven days resulted in gastrointestinal distension, fluid accumulation, and diarrhoea. Intravenous administration of a single dose of 10 mg $kg^{-1}$ dibutyltin dichloride or 20 mg $kg^{-1}$ diethyl-, dipropyl-, di-isopropyl- or dipentyl-tin dichloride resulted in severe pulmonary oedema and lung congestion.[54] In sheep, the intraruminal administration of 150 mg $kg^{-1}$ tricyclohexyltin hydroxide also resulted in fluid retention, diarrhoea and pulmonary congestion, with higher doses (500 mg $kg^{-1}$) producing petechial and ecchymotic haemorrhage in the trachea and larynx.[86] Gastroenteritis was also a prominent finding in rats exposed to tricyclohexyltin hydroxide orally at 25 mg $kg^{-1}$ $day^{-1}$ for 19 days[73] and an acute study described varying degrees of gastrointestinal haemorrhagic damage in mice 24 h after the oral administration of 4 g $kg^{-1}$ of butyltin trichloride.[87] However, the biological significance of such an extreme regimen is clearly questionable.

## Renal toxicity

A 90-day study in rats revealed glomerular congestion and toxic nephrosis following dietary exposure to 400 mg $kg^{-1}$ tricyclohexyltin hydroxide.[88] A similar form of toxic effect was observed in rats receiving tricyclohexyltin hydroxide at a dose of 25 mg $kg^{-1}$ for 19 days.[73] Haemorrhagic lesions were also reported in the kidneys of mice following a single 500 mg $kg^{-1}$ oral dose of tributyltin chloride, benzoate, laurate and oleate. Tributyltin acetate, on the other hand, produced no adverse effects. Hyperaemia was noted in all mice, whereas tubular lipid accumulation and fatty degeneration was only evident in mice

receiving the laurate and oleate.[89] Fatty degeneration was also reported in the renal cortical epithelium of mice treated with a single high-dose challenge (4 g $kg^{-1}$ orally) of dioctyltin *S,S'*-bis(2-ethylhexyl-mercaptoacetate) $[(C_8H_{17})_2Sn(SCH_2COO\text{-}i\text{-}C_8H_{17})_2]$, dioctyltin *S,S'*-bis(butyl-mercaptoacetate) $[(C_8H_{17})_2Sn(SCH_2COOC_4H_9)_2]$ butyltin *S,S',S''*-tris(2-ethylhexyl-mercaptoacetate) $[C_4H_9Sn(SCH_2COO\text{-}i\text{-}C_8H_{17})_3]$, butylstannonic acid ($C_4H_9SnOOH$) or butylthiostannonic acid ($C_4H_9SnSOH$).[90] Interestingly, butyltin trichloride produced no fatty degeneration, even though all of the compounds tested produced varying degrees of renal hyperaemia.[87] Kidney weight was also significantly increased in female rats treated with 200 mg dioctyltin *S,S'*-bis(2-ethylhexyl-mercaptoacetate) for 12 months.[91] Similar distrophic changes were also observed in rats treated with dibuyltin dichloride.[71] Whether these effects are indicative of selective organ toxicity or merely a consequence of non-specific toxicity, following concentration and excretion of the test compounds and their metabolites, remains to be determined.

## Biliary tract and hepatotoxicity

A number of monobutyltin compounds, most notably butyltin trichloride, butylstannonic acid, butyltin *S,S'S''*-tris(2-ethylhexyl-mercaptoacetate), and butylthiostannonic acid, all produce steatosis and hepatomegally in mice following oral administration at 4 g $kg^{-1}$.[87] Similarly, Niconorow *et al.*[91] reported a significant mean increase in liver weight in rats exposed orally to 20 mg $kg^{-1}$ dioctyltin *S,S'*-bis(2-ethylhexyl-mercaptoacetate) daily for 90 days.

Nevertheless, on a weight-for-weight basis, dibutyltins are considerably more active compounds which specifically target the liver and more especially the bile duct for toxic effects. In rats, a single oral dose of dibutyltin dichloride at 50 mg $mk^{-1}$ was shown to produce rapid inflammation and congestion of the distal end of the bile duct; repeated dosing over three successive days produced a more severe lesion encompassing the proximal bile duct, the intrahepatic tracts and the portal vessels. Areas of necrosis were apparent within the liver and, in severe cases, peritonitis and pancreatitis occurred as a consequence of bile duct perforation.[92,93] A similar reaction was seen to occur after a single i.v. administration of 5 mg $kg^{-1}$ or a dermal application of 10 mg $kg^{-1}$ of dibutyltin dichloride. In mice, the effects of 20–50 mg $kg^{-1}$ of this compound were essentially the same as those seen in rats, although liver damage was more widespread in the mice. In rabbits, repeated doses of the same compound were fatal but with the absence of biliary or hepatic injury; guinea-pigs tolerated this dose without any adverse effects.[92]

In the dialkyltin series, bile duct damage was produced more effectively by dibutyltins, with diethyl-, dipropyl-. dipentyl- and dihexyl-tins producing lesser damage. In rats treated with dibutyltin, high concentrations of tin were found in the bile.[92,93] However, biliary tin levels were significantly lower in rats treated with dipentyl- and dihexyl-tin and only trace amounts were detected in animals treated with dioctyltin.[94]

Bile duct lesions have also been reported in animals exposed to tributyltin compounds. However, it is relevant to point out that chronic exposure was necessary to produce the effects.[92,95,96] A single oral dose of tributyltin acetate, chloride, benzoate, laurate or oleate at 500 mg $kg^{-1}$ produced liver damage and fatty degeneration in mice[89] and triphenyltin acetate given at 10 mg $kg^{-1}$ over a two-year period resulted in similar lesions in guinea-pigs.[73] Cholangitis was reported in rats treated with 400 mg $kg^{-1}$ dietary tricyclohexyltin hydroxide.[88] Similarly intra- and extra-hepatic cholangitis was reported in rats exposed to 25 mg $kg^{-1}$ tricyclohexyltin hydroxide for 19 days.[83,74]

As a consequence of these species differences, it has been suggested that hepatic lesion occurs only in species in which the bile duct and pancreatic duct share a common course. Whether this is indeed the case or merely a consequence of other species-specific effects will only be resolved by further study. However, since it has been shown that the severity of the lesion correlated with the biliary tin concentration, it seems more likely that the species differences are a consequence of differing absorption and bioavailability rather than differential susceptibilities.

## Neurological effects

Neurolgical intoxication is limited to the lower trialkyltin homologues and, in particular, trimethyl- and triethyl-tin derivatives. Intoxication in animals by triethyltin species is characterized by a general lethargy and weakness, progressing to a state of paralysis which, depending on the particular compound, may also be

accompanied by a generalized tremor. In rabbits, typical responses to 5 mg $kg^{-1}$ triethyltin sulphate administered i.v. (a lethal dose) include prostration and flacid paralysis in conjunction with encephalitis and cerebral oedema.[70,97] In rats exposed to triethyltin hydroxide (20 mg $kg^{-1}$ in the diet), weakness became apparent in the hindquarters after one week of treatment. This progressed to reach a maximum effect after 3–4 weeks, when almost 50% mortality had occurred. The remaining rats recovered within one week following transfer to normal diet. Following exposure at 40 mg $kg^{-1}$, recovery again was observed after transfer to a conventional diet, but, at 80 mg $kg^{-1}$, the rats developed generalized muscular tremors.[70]

Although the neurotoxic capacity of triethyltin compounds was well established, Magee[98] was the first to induce and note a definable lesion within the central nervous system (CNS). Using rats, Magee demonstrated that triethyltin hydroxide administered at a dose of 20 mg $kg^{-1}$ induced interstitial oedema of the white matter of the brain and spinal chord, without causing obvious neuronal damage. Subsequent vascular permeability studies using sodium fluorescein, FITC-dextran (fluorescein isothiocyanate covalently linked to dextran) and horseradish peroxidase as tracer molecules, revealed that fluid accumulated within the myelin, but permeability to molecules greater than 3000 Da was not significantly increased.[99] The oedema became microscopically visible after three days' exposure and progressed to reach a maximum after two weeks. The effect was reversible and was fully resolved after four months following normal diet. High-power electron microscopy studies in affected rabbits revealed that the myelin sheaths had split to form vacuoles in which the fluid (responsible for the oedema) accumulated.[100] Neonatal exposure to triethyltin caused a reduction in brain weight and delayed myeliogenesis with the nursing mothers showing cerebral oedema and paralysis, whilst the young rats were unaffected.[101,102]

More recent studies have confirmed the earlier pathological observations and, in addition, have demonstrated various electrophysiological[103] and behavioural disturbances.[104,105] Muscular weakness, paralysis and neuropathic changes have also been consistently reported in chronic toxicity studies.[106] These responses are also typical of those reported in man following accidental intoxication with triethyl- or trimethyl-tins compounds (see Human exposure, below).

Tetra-alkyltin compounds also possess neurotoxic properties. These compounds undergo metabolic conversion to the active trialkyltin derivatives following exposure to microbial or hepatic microsomal enzymes. Consequently, exposure to these tetra-alkyl agents produces similar, albeit somewhat delayed, effects following ingestion. The toxicity of certain tetra-alkyltin compounds in mice and dogs was studied by Caujolle *et al.*[107] As one might by now expect, the lower homologues were the most potent. Tetraethyltin was the most active with tetramethyltin slightly less so; with other members of the series, toxicity was seen to decrease as molecular weight increased. Interestingly, tetramethyltin toxicity was different from the other members of the series in that this compound preferentially induced tremors and hyperexcitability. The dominant effects of the other members of the tetra-alkyltin series were muscular weakness and paralysis, with death occurring from respiratory failure.

Although the neurological effects of the trimethyltin compounds were well characterized in the early studies of Stoner *et al.* In 1955[70] and Barnes and Stoner in 1958,[54] the nature of the lesions responsible for producing the tremor, hyperexcitability and aggression remained elusive. Indeed, it was some 20 years before Brown *et al.*[108] and Baldwin *et al.*[109] observed localized neuronal necrosis in the hippocampus and pyriform cortex of trimethyltin-treated rats. Additional studies revealed localized neuronal damage in the amygdaloid nucleus, brainstem, neurocortex, spinal chord, sensory neurones and retina.[110–115] Although the exact distribution and degree of damage was species-specific,[116] the sensitivity of the hippocampus was a consistent finding. Similar neurological disturbances were noted in rodents and primates[117–120] and, in man, low-level triethyltin exposure has been reported to induce a spectrum of neurological and motor neurone effects, including irritability, depression, aggressiveness, headache, tremors, visual disturbances and loss of libido.[121,122]

The causative mechanism of the organotin-induced lesion remains both elusive and controversial. Whereas the triethyltins have been demonstrated to have a raised affinity for myelin,[123] these compounds have not been demonstrated to localize in any specific way within the central nervous system.[124–126] Triethyltins do, however, interfere with cellular energy production, and rat brain slices from treated animals were shown to exhibit reduced mitochondrial respiration. However, the effect was common to both white and grey matter

and, consequently, the biochemical and pathological effects may not necessarily be causally related.[124,127]

More recent research examining the differential sensitivity of the white and grey matter was focused on relative neurotransmitter levels[128] and membrane-associated enzymes, including NaATPase, KATPase, adenylate cyclase and cAMP-phosphodiesterase.[129]

Turning to the neurological effects of the trimethyltins, trimethyltin was also shown not to accumulate to any significant degree in the brain, and the amount of tin recovered from the CNS of trimethyltin-treated rats again followed no specific uptake or distribution pattern.[126] Since trimethyltin only marginally inhibits mitochondrial respiration[127] and is only mildly cytotoxic *in vitro*,[130] it seems unlikely that the latter effects are responsible for the discrete neurological effects associated with these compounds. Additional studies have, however, linked trimethyltin exposure with reduced adrenal epinephrine and norepinephrine[130] and also with a reduced hippocampal zinc concentration.[131] Both effects were apparent before the onset of neurotoxicity and could be important clues in the slowly evolving puzzle of organotin-induced neurotoxicity.

## Immunotoxic effects

Despite the large amount of data relating to the general organ toxicity of organotin compounds, it is only relatively recently that attention has focused on the possible adverse effects that organotin compounds may have on the immune system.

Before discussing these effects, however, it is necessary to discuss aspects of the nature and function of the normal mammalian immune system. Within its confines, we find a finely tuned and precisely regulated mechanism involving multiple interactions between ranks of regulatory and effector T-cells (thymus-matured lymphocytes), antibody secreting B-cells (bone-marrow and peripherally-matured lymphocytes) and a variety of antigen processing/presenting accessory cells. These components act either independently or in concert with a spectrum of other cell types including polymorphonuclear leukocytes, natural killer cells, basophils, eosinophils and mast cells. Together, these cells not only provide an effective and versatile defence against invading parasitic or pathogenic micro-organisms, but also a mechanism whereby the host's own body tissues may be continuously monitored for neoplastic events, a concept usually referred to as immune surveillance (see Refs 132–135 for some general reviews).

Normal immune competence is, therefore, the result of adaptive/reactive cellular and biochemical changes designed to ensure the survival of the species in an ever-changing and potentially hostile environment. Unfortunately, the inherent complexity of the system is also its weakness, since even minor perturbations within the immune network can produce dramatic and potentially damaging changes to immune reactivity.

The thymus is a vital lymphoid organ in that it is a prerequisite for normal T-cell differentiation. T-cells are involved in all aspects of immunity, either as direct effector cells in cytotoxicity, as indirect effector cells triggering delayed-type hypersensitivity reactions or as regulatory 'helper' (Th) or 'suppressor' (Ts) cells controlling humoral and cell-mediated responses. T-cell maturation is now reasonably well characterized. Precursor T-cells originating in the haemopoietic tissues of the embryonic liver, and, in adults, the bone marrow, migrate to the thymus where they are triggered to undergo both proliferation and differentiation. The process of differentiation is marked by morphological changes and the expression of characteristic T-cell markers.[136,137] This extensive proliferation is thought to be one mechanism favouring increased genetic variation within the T-cell repertoire producing cells with different immune reactivity patterns. The vast majority of the cells are, however, surplus to requirements and these die within the thymus. Selected cells, approximately 10% of the resident population (a figure now also under debate), emigrate from the thymus. In the neonate, these cells 'seed' the spleen and peripheral lymph nodes. In adult life, emigration continues, though at a reduced rate. The T-cells circulate between the blood and lymph and back again to the blood via the thoracic duct thereby maintaining surveillance within the body tissues as a whole and, even though the thymus involutes post puberty, the organ continues to maintain basal mitosis and 'tops up' the T-cell system as required.[138,139]

Whereas traditional pathological approaches have already implicated various lymphoid tissues as possible organs for toxic effect, it is only relatively recently that the thymus has been demonstrated to be a sensitive and, perhaps, early indicator of toxicity. This increasing awareness stems in part from modern advances in immunology and molecular biology which have provided new and precise techniques for the detection

and analysis of immune dysfunction. These novel approaches have not only increased our understanding of the regulatory mechanisms which govern the normal functions of the immune system but have also raised a note of caution concerning the immunotoxic potential of certain industrial or environmental organotin compounds.

### Effects on lymphoid tissues

Some of the earliest observations of a direct interaction between organotins and the immune system were provided in 1970 by the studies of Pelikan and Cerny,[90] who observed small necrotic areas in the germinal centres of splenic follicles in mice following gavage with butyl- or dioctyl-tin compounds (cited above; see under Renal toxicity). Subsequently, Seinen and Willems in 1976[140] reported that dioctyltin dichloride was particularly active in rats, with the main target organ being the thymus. It was also apparent from these early experiments that the effect was species-specific, in that mice and guinea-pigs were relatively resistant to the effects of this compound. Further experiments by the same authors revealed that the dietary administration of dioctyltin dichloride or dibutyltin dichloride to Wistar rats (40–45 g weanlings) at 50 or 150 mg $kg^{-1}$ for six weeks produced marked dose-dependent thymic atrophy and reduced splenic and lymph node weights. It is noteworthy that these effects occurred with the absence of overt pathological change in other body tissues.[141,142]

Histologically, the decreased lymphoid organ weights were associated with a depletion of lymphocytes in the thymus and corresponding T-dependent areas in the speen (periarteriolar lymphocyte sheaths) and lymph nodes (paracortical areas). The latter effects would be expected since, in the neonates of weanlings, these lymphoid sites are dependent upon the thymus for T-cell seeding. Once seeded, however, the susceptibility of the spleen and lymph nodes decreases, indicating that the primary lesion occurs within the thymus. Comparative studies with various dialkyltin compounds revealed that similar, but less pronounced, effects were produced by diethyl-tin dichloride and dipropyl-tin dichloride, whereas dimethyltin dichloride, didodecyltin dibromide, and dioctyltin dibromide, were shown to have no effect on lymphoid tissues.[141,142]

Subsequent work at the British Industrial Biological Research Associations (BIBRA) laboratories[143–145] indicated that 6–8-week-old PVG-strain rats were equally sensitive to dioctyltin dichloride. Animals exposed to 75 ppm of this compound via their diet developed overt thymic atrophy after two weeks' exposure, almost complete atrophy was apparent after four weeks and, by eight weeks, the thymic remanent consisted almost entirely of brown fat. Thymic atrophy was also accompanied by a reduction in circulating leukocytes. This was first apparent after four weeks of treatment, but showed little further decrease after this time. The remaining cell population, however, demonstrated a reduced resting thymidine incorporation and peripheral blood lymphocytes from treated rats were less responsive to mitogenic stimulation *in vitro*. Body weight gain and food intake were not influenced by the feeding regimen and the animals showed no abnormal behaviour or appearance. Histological examination of thin plastic embedded thymic sections from dioctyltin dichloride-treated rats revealed marked depletion of cortical thymocytes in the absence of apparent cellular destruction, a concurrent loss of the cortico-medullary junction and an increase in the number of vacuolated reticuloepithelial cells. Tissue sections of the spleen, liver, prostrate, and seminal vesicles of the treated rats revealed no abnormal histological features or pathological lesions attributable to treatment, thus indicating a selective thymic sensitivity.

Similar immunotoxic effects have also been observed with certain members of the triorganotin series. Thymic weight reduction and the characteristic depletion of cortical thymocytes produced by dioctyltin treatment also occurred in rats fed tripropyl-, tributyl- and triphenyl-tin compounds.[96,146,147] Tributyl- and triphenyl-tin compounds have also been shown to reduce spleen weight and decrease the number of circulating lymphocytes in rats,[148] mice[149] and guinea-pigs.[150,151]

In a 14-day feeding study, Snoeij *et al.* in 1985[148] examined a series of trialkyltins for immuno- and neuro-toxic capacity. As expected, the lower trialkyltins (trimethyl- and triethyl-tin chloride) were essentially neurotoxic (as above). However, animals exposed to a dietary level of 150 ppm triphenyltin chloride, tripropyltin chloride, or tributyltin chloride, presented 81%, 51%, and 39% reductions in thymus weights, respectively. The atrophy was associated with lymphocyte depletion in the thymic cortex, with only 16% of the normal T-cell contingency being recoverable in the tributyltin chloride-exposed thymus.

Thymic atrophy was completely reversible after two weeks on normal diet. Slight thymic atrophy was apparent in rats treated with 150 ppm trihexyltin chloride, whereas the same dose of trioctyltin chloride was without effect. A dose-related decrease in spleen weight was noted in rats exposed to tributyl- and tripropyl-tin chloride and increased liver weights were apparent in rats receiving tributyl-, trihexyl- and triphenyl-tin chloride. Conversely, animals receiving 100 ppm tripropyl- and tributyl-tin chloride for 28 days presented severe thymic atrophy but normal spleen and liver weights. In summary, the lower trialkyltins (trimethyl- and triethyl-tin chloride) are essentially neurotoxic, the intermediate trialkyltins (tripropyl- and tributyl-tin chloride) and the aromatic triphenyltin chloride are primarily thymotoxic, whereas the higher alkyltins (trihexyl- and trioctyl-tin chloride) are essentially non-toxic at the levels tested.

Funahashi *et al.*[152] also reported that tributyltin oxide produces marked thymic atrophy and generalized, though less severe, atrophy of the spleen and lymph nodes in rats. In mice, tributyltin oxide has also been reported to cause a reduction in spleen weight and a state of leukopenia.[149]

Following these early studies, Vos and his coworkers at The Dutch National Institute of Public Health and Environmental Hygiene examined these effects in more detail.[153] In these studies, young Wistar-strain rats were maintained on a diet containing 5, 10, 20, 80 or 320 mg $kg^{-1}$ tributyltin oxide for four weeks. The results revealed a number of immunological alterations, including a dose-related reduction in thymic weight, circulating leukocyte count and changes in serum immunoglobulin levels. Thymic atropy was the most consistent finding, first being detectable in the 20 mg $kg^{-1}$ group. As with the other lower alkyl homologues, pathology revealed a severe lymphocyte depletion and absence of the cortico–medullary junction. Cell depletion was also apparent in the spleen and particularly the mesenteric lymph node. Immunocytochemistry confirmed the T-cell-specific nature of the lesion.

### Effects on immune reactivity

Chemically induced thymic atrophy has been shown to compromise a number of T-cell-dependent immune responses, especially in neonates and young animals where the smooth running of the immune system later in life depends on an adequate supply of T-cells from the thymus to populate T-dependent areas in the peripheral lymphoid tissues.

Using rats, Seinen *et al*[141,154] demonstrated that dioctyltin dichloride reduced delayed-type hypersensitivity to tuberculin, impaired allograft rejection, reduced humoral responsiveness to T-cell-dependent antigens and reduced graft versus host reactivity. As one would predict, the humoral response against *E.coli* lipopolysacchoride (LPS; a T-independent antigen) was not influenced by the treatment. Additional studies revealed a reduced cellular responsiveness to mitogenic and allogeneic stimuli in the circulating lymphocyte pool[145,155] and that serum from treated rats was able to suppress mitogen-induced proliferation of normal cells *in vitro*.[156]

Studies in mice, a species relatively resistant to dioctyltin dichloride induced thymic atrophy, demonstrated that this compound selectively diminished the humoral immune response to both self-(autoantigenic) determinants and heterologous erythrocyte determinants, thus demonstrating that dioctyltin dichloride can influence immune competence in the absence of overt thymic atrophy.[157]

Studies investigating adverse effects of tributyltin oxide indicated that this compound also appears to influence preferentially T-cell-mediated immunity. Thus, in the studies outlined in the previous section,[154] tributyltin oxide at 20–80 mg $kg^{-1}$ produced profound suppression of the T-cell-dependent delayed-type hypersensitivity response to ovalbumin and tuberculin. This treatment was also shown to reduce host resistance to *Tricinella spirilis*, a parasitic intestinal worm, in a dose-dependent manner. Dietary exposure to tributyltin oxide at 20 and 80 mg $kg^{-1}$ also suppressed phytohaemaglutinin (PHA) and pokeweed mitogen-induced T-cell-specific blastogenesis. However, B-cell responses to mitogenic stimulation by *E.coli* LPS and antigen were unaffected. After nine weeks' exposure, the cell populations were phenotyped and quantified by fluorescence-activated cell sorting. The rats treated with 80 mg $kg^{-1}$ tributyltin oxide presented a normal level of splenic B-cells but a significantly suppressed T-cell population (52–60% of the control); the Ts/Th ratio was, however, not significantly altered. Regarding the mononuclear phagocyte system, Vos demonstrated that splenic clearance of *Listeria monocytogenes* was impaired in the 80 mg $kg^{-1}$-treated rats. This effect is in contrast

with that mediated by triphenyltin hydroxide, which does not affect the splenic clearance of monocytogenes and dibutyl- and dioctyl-tin dichloride which do not influence *in vivo* carbon clearance.[142] *In vivo* studies, designed to determine the effects of tributyltin oxide at the cellular level, revealed that the bactericidal activity of peritoneal macrophages was unaltered, whereas the activity of splenic macrophages was impaired. Furthermore, additional studies demonstrated that the adherent cell population was reduced in the spleen but not the peritoneal cavity. Tributyltin oxide was also shown to reduce activity of natural killer cells in the spleen against a murine lymphoma cell line. Natural killer cell activity is currently believed to play a pivotal role in immune surveillance mechanisms regulating neoplastic expansion processes.

It is clear from the above that certain of the dialkyl- and trialkyl-tin compounds can exert profound regulatory effects on discrete aspects of the immune system. Their capacity to modify T-cell-dependent pathways is well established and a number of studies suggest that other immune components may also be affected.[153,157] However, whether these effects occur as a direct effect of the compound on the effector cells *per se*, or as a consequence of altered regulatory T-cell control, is unclear at present.

The unique sensitivity of the thymus clearly warrants further comment. Results suggest that it is the proliferating thymocytes and the so-called short-lived T-cells that are the sensitive population, the latter probably coming under attack when they are triggered by antigen to undergo clonal expansion within the periphery. Studies designed to elucidate the mechanism(s) whereby these compounds exert their effect have, by and large, yielded few positive indicators. It is clear that the immunotoxic effects of these compounds is not mediated by a stress-related increase in steroid hormones since both dioctyltin dichloride and tributyltin oxide produce immune perturbations in adrenalectomized animals.[143,153] These effects also occur in the absence of other pathological changes and at doses which do not interfere with the growth of the animals.[155]

Perhaps the most promising observations made on the action of the active organotins were those of Miller[143] and Penninks,[158] which demonstrated the ability of dioctyltin dichloride to down-regulate the spontaneous proliferation of thymocytes *in vitro*. Whereas these effects are clearly endorsed by the biochemical and cellular studies outlined below, recent studies at BIBRA have revealed additional information. Using density gradient centrifugation techniques to separate individual thymocyte populations, dioctyltin dichloride preferentially affected cells of intermediate density. Immunohistochemical analysis of these cells, using monoclonal antibodies, demonstrated a reduction in the number of cells expressing the MRC OX18 marker (a common medullary or peripheral T-cell marker) as early as 48 h following treatment.[159] Since dioctyltin dichloride has been shown to deplete a medullary thymocyte population, as well as depressing thymocyte proliferation, we explored the possibility that dioctyltin dichloride may exert its effect by compromising interleukin-2 (IL-2) production. This molecule is produced by medullary thymocytes and serves to initiate and sustain thymocyte proliferation within the thymic cortex. The same molecule is also necessary for antigen-specific T-cell proliferation within the tissues.

Using a gene-probing technique, it has now been demonstrated that dioctyltin dichloride treatment produces a rapid decrease in IL-2 gene expression.[160] These genetic effects were detected within 24 h and thus appeared to precipitate the reduction in the MRC OX18-positive cell population. The effect appeared to be specific for the IL-2 gene, since the so-called housekeeper genes (e.g. the actin gene) were unaffected by the treatment. If the same holds true for IL-2 expression in peripheral T-cells then it may be possible to explain some of the immunomodulatory events associated with organotin exposure in terms of a treatment-associated reduction in IL-2 production. The overall effects may, therefore, involve both antimetabolic processes and discrete lesions in the systems controlling interleukin production. The relationship between these two effects with respect to the compound, its dose and relative time of administration, clearly needs to be established before a full picture of the lesion and its possible significance with respect to human exposure can be fully appreciated.

## MECHANISMS

Of the various biological systems discussed above, mechanistic studies have, by and large, centred on unravelling the cellular and biochemical basis of mam-

malian toxicity. The systems are complex and the reader should be aware that reactions observed *in vitro* do not necessarily correlate with whole-animal effects. Below, we discuss current thoughts on the biological and cellular effects of various organotin compounds and, where possible, related these processes to the toxic effects seen in the organ systems outlined above.

## Tissue distribution, metabolism and biotransformation

The higher alkyltin compounds have been shown to undergo stepwise tin–carbon bond cleavage following ingestion, with both intestinal bacteria and liver enzymes playing active roles in this process. *In vitro*, tetraethyltin undergoes stepwise destannylation to the tri-,[67] di-,[161] and mono-ethyltin derivatives[162] when incubated in the presence of a rat liver microsomal enzyme system. Whereas other trialkyltin compounds have been shown to behave in a similar fashion,[161,163] the aromatic triphenyltin acetate is not metabolished *in vitro* but yields di- and mono-phenyltin derivatives *in vivo*.[164] Following i.v. administration of diethyl- and dibutyl-tin dichloride to mice and rats, the highest concentrations of the test species were found in the liver and kidney, with intact material being excreted in the bile.[92] Further studies revealed that cytochrome *p*-450 was responsible for the primary metabolism and that carbon hydroxylation occurred on the butyl groups of $^{14}$C-labelled tributyltin acetate at the $\alpha$ and $\gamma$ positions.[164,165] Similarly, the primary hydroxylated metabolic products of tetrabutyltin were rapidly destannylated to the tributyltin derivatives.[165] In rats fed a total of 11 mg of triethyltin hydroxide over a period of 89 days, only 0.7 mg of the test species was recovered in the tissues, 40% of this in the blood, 28% in the liver and 29% in the skeletal muscle, with smaller amounts of triethyltin being present within the kidney, brain, heart and spleen.[124]

A number of intriguing species differences have been reported with regard to the distribution of triethyltin. When added to rat blood, almost 90% of the original dose could be recovered from the erythrocytes, with little or none free in the plasma, whereas, in rabbit blood, triethyltin was distributed approximately 33:66% between erythrocytes and plasma respectively,[124] an effect which could explain why triethyltin persists in the blood of treated rats, whereas it rapidly disappears from the blood of treated rabbits.[59,166] Studies in rats, revealed that the end-point of the tricyclohexyltin hydroxide metabolic pathway was inorganic tin.[163]

The role of gut bacteria has been discussed previously.[67,68] Thus, although the oral administration of triphenyl-, tributyl- or trihexyl-tin chloride in rats causes rapid thymic atrophy, intravenous administration of the same compounds has no effect on the thymus. Using $^{14}$C-labelled tributyltin acetate, Snoeij and his colleagues[167] demonstrated that approximately 25% of an administered dose was absorbed through the gut, radioactivity being high in the liver and kidney, but low in the blood and thymus. More than 90% of the administered dose was excreted within four days, approximately 75% in the faeces and 13% via the urine, 8% of the activity remained within the tissues, and the remaining 3% was attributed to expired carbon dioxide ($CO_2$). In mice given a single oral dose of 1.2 mg kg$^{-1}$, Kimmel *et al.*[164] reported faecal and urinary excretion at 52 and 16% repectively at 138 h, with 22% of the dose being recovered as carbon dioxide at 90 h post-dosing. In the rat study,[167] serum analysis at 3 and 27 h after an oral dose of 13 mg kg$^{-1}$-labelled tributyltin acetate, revealed the presence of tri-, di- and mono-butyltin derivatives in the (concentration) range of $10^{-7}$–$10^{-6}$ mol dm$^{-3}$. These data clearly demonstrate the importance of intestinal metabolism and emphasize the fact that tributyltin-induced thymic atrophy is mediated by a dibutyltin metabolite derivative rather than the parent compound.

It was also apparent from the tissue distribution studies that there was no significant thymic accumulation of either the labelled parent compound or its metabolies, an observation in accordance with previous studies using radiolabelled dioctyltin dichloride.[145,168] Although it would appear that thymic atrophy is not associated with the excessive accumulation of labelled compounds within the thymus, it is not inconceivable that metabolism removes the radioactive atom from the labelled molecule.

In a number of the early studies outlined above it was suggested that destannylation (carbon–tin bond cleavage) was the major result of biotransformation. Notably, for example, triethyltin was reported in the tissues of both rats and rabbits given tetraethyltin by the intravenous route.[124] The liver was reported to be most actively involved in this conversion process. Further supportative evidence is provided by Bridges

*et al.*,[162] who reported that dealkylation of diethyltin dichloride occurred both within the gastrointestinal tract and tissues of the rat, and by Herok and Götte,[169] who demonstrated trace amounts of inorganic tin in the milk of sheep treated with triphenyltin acetate. More recent studies using tributyltin acetate have demonstrated the biological oxidation of carbon–hydrogen bonds at $\alpha$, $\beta$, $\gamma$ and $\delta$ carbon positions with regard to the tin atom.[170] This reaction had already been assumed to occur by Casida *et al.*,[161] although no carbon-hydroxylated metabolites were detected when triethyltin derivatives were biologically oxidized *in vitro* to monoethyltin derivatives.

## Biochemical and cellular effects

Organotin compounds are powerful metabolic inhibitors. In this respect, dialkyltins were all shown to interfere with oxygen and substrate consumption in isolated rat liver mitochondria.[127,171] These compounds apparently inhibited the two $\alpha$-ketoacid-oxidizing complexes in mitochrondia, namely pyruvate and $\alpha$-ketoglutarate dehydrogenase, since, in the presence of the organotins, the test substrates failed to be fully oxidized, accumulating instead as their equivalent $\alpha$-ketoacids. It was also demonstrated that the dialkyltin compounds possessed a high chemical binding affinity for dithiol groups, suggesting that the previous observations may be the result of an interaction between the organotin and lipoic or lipoyl dehydrogenase, both of which molecules are essential factors in the $\alpha$-ketoacid oxidation process.[172]

Studies examining the biochemical effects of the trialkyltin compounds revealed three principal interactions with mitochondrial respiration:[127,171,173–176] (1) trialkyltins can exchange halide atoms for hydroxyl ions across the mitochondrial membrane, thereby disrupting membrane potential; (2) trialkyltins have been shown to bind a component of the mitochondrial $Mg^{2+}$ATPase, leading to the direct inhibition of ATP production; and (3) the trialkyltins and, in particular, the more lipophilic members of the series, produce mitochondrial swelling, thereby disrupting the internal architecture and active sites in the organelle. Aldridge[127] performed a comparative study of trialkyltin derivatives and ranked the capacity of each compound to cause 50% inhibition of ATP production in isolated liver mitochonodria as follows: triethyl > tripropyl > tributyl > trihexyl > trimethyl.

More recently, the biological and cellular properties of the di- and tri-alkyltin chlorides have been examined in more detail by Penninks and his coworkers in The Netherlands. Their studies revealed that the treatment of normal thymocytes with dimethyl-, diethyl-, dibutyl- or dioctyl-tin dichloride resulted in a marked increase in glucose and a concurrent accumulation of pyruvate and lactate within the cell cytosol.[172] The observation that glucose-supplemented media protected the cells from dibutyltin dichloride-induced ATP depletion led to the suggestion that the dialkytin, acting upon pyuvate dehydrogenase, blocks the entry of glycolytic end-products into the tricarboxylic acid (TCA) cycle, and, as a consequence, ATP production falls, and pyruvate and lactate accumulate.[177] In response to the reduced TCA cycle activity, glycolytic phosphorylation is increased in order to maintain intracellular ATP. It is the latter process which requires the supply of extracellular glucose. In addition, dialkyltin exposure *in vitro* was reflected in reduced DNA, RNA and protein synthesis.[178] Micromolar amounts of dibutyl- and dioctyl-tin compounds were also shown to affect the proliferation of rabbit chrondrocytes *in vitro*.[179]

All of the trisubstituted organotins have been shown to exert powerful effects on mitochondrial energy systems, the only exceptions being the most hydrophilic and lipophilic homologues: trimethyl- and trioctyl-tin respectively.[180]

The trialkyltins affect the mitochondria in a similar, though not strictly identical, manner to the dialkyltins outlined in (1)–(3) above. Thus, rat thymocytes cultured in the presence of trialkyltin compounds increase glucose consumption and accumulate lactate. Unlike the dialkyltin study, however, pyruvate levels were less significantly influenced. As before, the intracellular ATP levels were reduced, especially under glucose-free conditions.[180,181] These results are in accordance with those obtained using dialkyltin derivatives and may be explained on the basis that the trialkyltin compounds inhibit ATP formation by binding to the ATP-synthase complex and by altering the charge across the mitochondrial membrane. The latter effects block pyruvate transport and, as a consequence, pyruvate is seen to accumulate in the cytoplasm. The lack of effective oxidative phosphorylation is in turn reflected by increased cellular NADH. The accumulation of these products produces a shift in the balance between pyruvate and lactate levels, the

former being rapidly converted to lactate at the expense of cytosolic NADH. Although glycolysis continues, cellular ATP production is inefficient and energy demands outstrip aerobic glycolysis. Therefore, in contrast to dialkyltin-treated cells, trialkyltin-treated cells rapidly succumb, even in the presence of free glucose. Similar antiproliferative effects have been previously reported in studies using a baby hamster kidney cell line,[182] rabbit chondrocytes and rat skin.[179] It also seems likely that the recently reported inhibitory effects of tributyltin oxide on murine embryonic limb bud development *in vitro*[183] occurs as a consequence of mechanisms outlined above.

Whereas the effects of organotins on cellular energy processes have been appreciated for many years, it is only relatively recently that the phenomenon of direct cytolysis has been examined. It is now apparent that certain organotins possess potent membrane-damaging properties and, for example, the *in vitro* exposure of thymocytes to tributyltin chloride at concentrations in excess of 2 $\mu$mol dm$^{-3}$ results in rapid (two hours) cell death following loss of membrane integrity. Using rat thymocytes *in vitro*, Snoeij *et al.*[180,181] have recently investigated the effect of a series of organotins, including tributyltin chloride, tripropyltin chloride, tricyclohexyltin chloride and triphenyltin chloride. Cells were cultured in media containing graded concentrations of each of the test compounds (0.01–10 $\mu$mol dm$^{-3}$) for up to 30 h and cell integrity assessed in terms of dye exclusion and tritiated thymidine incorporation. Of the compounds studied, tributyl-, tripropyl- and triphenyl-tin chloride proved to be the most cytotoxic, significantly reducing thymidine incorporation at concentrations as low as 0.05–1 $\mu$mol dm$^{-3}$. Membrane damage as judged by impaired dye exclusion became apparent at slightly higher levels (1–10 $\mu$mol dm$^{-3}$), with tributyltin chloride and triphenyltin chloride producing effects at 8 $\mu$mol dm$^{-3}$ within 2.5 h. Tritiated thymidine incorporation was the most sensitive index of cell damage with 40–60% inhibition being noted within 5 h at the 0.1–1 $\mu$mol dm$^{-3}$ treatment level. Similar figures were reported by Reinhardt *et al.*,[182] who noted that the cloning efficiency of baby hamster kidney cells was inhibited by 0.1 $\mu$mol dm$^{-3}$ tributyltin chloride, whereas cell detachment (indicative of membrane damage) did not occur with concentrations below 10 $\mu$mol dm$^{-3}$.

Of the various trialkyltin compounds studied to date, different structure–activity relationships are apparent. Studies on human erythrocyte lysis, swelling of rat mitochondria and dye release studies demonstrate a correlation between increasing chain length and membrane damage.[180–184] Lipophilicity is again directive in membrane toxicity, with tributyl- and trimethyl-tin chloride representing the most and least toxic of the trialkyltin homologues.

## HUMAN EXPOSURE

Some of the first harmful effects associated with organotin exposure came to light following early experimental production. Diethyltin dichloride was reported as early as 1858 to possess a 'powerfully pungent odour' and, when heated, produced fumes that 'painfully attack the skin of the face' and respiratory membranes.[185] Similar effects were subsequently reported following exposure to tri- and di-ethyltin chlorides[186] and also triethyltin acetate which, in addition, produced a number of unpleasant systemic symptoms, including headache, nausea and diarrhoea.[74] The subsequent reports of these unpleasant and untoward effects attracted the attention of the military who investigated the possibility of utilizing triethyltin compounds and, in particular, triethyltin iodide in antipersonnel or other chemical warfare applications. Interest in the compounds declined, however, as they failed potency testing and military applications were abandoned.[74–76]

These early studies demonstrated the toxicity of certain of the organotins and established a clear relationship between the structure and activity of organotins in general. Mild concern over gradually increasing production and usage of organotins became much more serious following an incident of mass poisoning in France in 1954, referred to as the 'Stalinon Incident'. A number of deaths were associated with 'Stalinon', a proprietary tin preparation sold throughout France and Algeria for the treatment of furuncles, boils and other unpleasant staphylococcal skin infections. 'Stalinon' supposedly contained diethyltin diiodide admixed with linoleic acid. However, during the period in question, a preparation of 'Stalinon' capsules was sold contaminated with an estimated 10% of toxic impurities, most notably monoethyltin and triethyltin iodides. Out of a group of approximately 1000 people who are believed to have taken the contaminated

'Stalinon', 217 cases of poisoning were reported and of these 100 individuals died. A full report of the legal proceedings which followed this unfortunate incident has been published, and clinical and autopsy data are cited in Ref 24 and 54. Symptoms of intoxication appeared after about four days. Affected individuals presented rapid weight loss and severe and persistent headache, vomiting, nausea, abdominal pain, and visual disturbances. Localized or organ-associated lesions were absent and death occurred apparently from respiratory and cardiac failure.

Adverse effects produced by occupational exposure to di- and tri-butyltin compounds were first reported amongst laboratory and process workers accidentally exposed to the agents in the workplace.[77] The majority of the lesions were skin burns in the form of an itching erythematous reaction which became apparent between one and eight hours after exposure. Cessation of contact with the material resulted in rapid and complete healing. In studies in volunteers in which test compounds were applied to the skin on the back of the hand, skin lesions were produced by a single application of dibutyltin dichloride and by tributyltin chloride, acetate, and oxide; dibutyltin diacetate, dilaurate, oxide and maleate, and tetrabutyltin, were without effect. Irritation was apparent after 8 h and sterile pustules appeared on the second day, these remaining as discrete lesions which gradually healed during the course of one week.

The majority of the recent incidents involving organotin intoxication has occurred following occupational exposure to triphenyltin acetate when used as an agricultural fungicide.[187–191] Reported effects include general malaise, nausea, vomiting, headache, dizziness, confusion and visual disturbances, gastric problems and anorexia. In the past, crop sprayers who handle large quantities of organotin-containing fungicides have been particularly prone to self-intoxication. Systemic toxicity was reported in a crop sprayer who spilt fungicide (containing 60% triphenyltin acetate) on his hands and chest while filling spray-tanks. He first experienced dermal irritation after three hours, followed by induration and vesicular lesions the following day. He subsequently reported general weakness, headache, nausea, and epigastric pain. Clinical chemistry revealed liver damage and elevated hepatic enzymes.[191]

Similarly, Horecek and Demcik[189] reported a case of group intoxication involving a spraying team comprising of two pilots and three mechanics who were again using a 60% triphenyltin acetate formulation. Protective measures were ignored and food was consumed on site with unwashed hands. After 14 days' exposure, various symptoms compatible with triphenyltin acetate intoxication became apparent (as above); the individuals remained unwell for up to six weeks, but all made a complete recovery.

A group of female spray-paint workers using a latex paint containing 20% bis(tributyltin) oxide as a fungicide reported irritation of the eyes and nasal mucosa. The reaction occurred soon after starting the work and was experienced by all of the sprayers. The work continued for a further two weeks, during which time the symptoms became more severe. Medical examination revealed conjunctivitis, rhinitis, hyperaemia and septal damage. The removal of the fungicidal component from the paint effected rapid and complete recovery.[192]

The only fatality in which occupational exposure to organotin was implicated occurred following an incident in which a young woman was accidentally drenched in a heated mixture containing (amongst other things) di- and tri-phenyltin chloride. Death occurred 12 days later from renal failure. However, the woman also suffered significant burns, and, consequently, the final cause of death cannot be ascribed with any degree of certainty.[193]

## CONCLUSIONS

In the light of the above incidents and an increased awareness of the toxic capacity of certain of the organotins currently in use in industry and agriculture, occupational exposure has become more vigorously controlled. At the same time, however, both public and scientific attention has focused on the possible long-term environmental impact of the organotins, especially, for example, on the accumulation of tributyltin oxide in aquatic and marine environments. In this work, we have identified and graded compounds in terms of their mammalian toxicity. We have considered the mechanisms of their effects and have identified both target organs and systems. We have precluded further discussions regarding the environmental effects of these compounds. The interested reader is directed to the Proceedings of the *Oceans 86* and *Oceans 87* conference symposia,[194,195] and, more recently, the review by Maguire regarding the nature of the

current problem, analytical difficulties and recent leglislation.[32]

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