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Accumulation and Metabolic Effects of Di-n-Butyltin Dichloride in the Freshwater Clam, *Anodonta anatina*

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Dialkyltin compounds make up a major part of the commercial organotin chemicals (Wilkinson 1984). They are employed as heat stabilizers for polyvinyl chloride (PVC). Dialkyktins leaching from PVC pipe into water constitute a potential environmental hazard (Noland et al. 1983). At the organ level, di-substituted organotins exhibit hepatotoxicity, as evidenced by the occurrence of biliary and intrahepatic lesions in several animal species that had been exposed to dialkyltins (Barnes and Stoner 1958;Barnes and Magee 1958). A second, sensitive parameter of toxicity has been found in the interference with the immune system in rats, as indicated by thymic atrophy and involution (Seinen and Willems 1976; Seinen and Penninks 1979; Penninks 1985). At the molecular level, dialkyltin salts were found to inhibit the activity of 2-oxoacid dehydrogenases, probably by blocking the vicinal thiol group of the lipoic acid coenzyme (Aldridge and Cremer 1955). Dibutyltin dichloride (DBTC) inhibits oxidative phosphorylation, in a manner analogous to the action of oligomycin (Cain et al. 1977), whereas an uncoupling effect has also been reported (Penninks et al. 1983). Within a range of homologous dialkyltin salts, DBTC proved to be the most potent agent in inhibiting the oxygen uptake by isolated rat thymocytes, and evoking anaerobic glycolysis (Penninks and Seinen 1980).

Until now, accumulation and in-vivo effects of DBTC or other dialkyltin compounds in aquatic organisms have not been reported. In the present study, freshwater clams, *Anodonta anatina*, have been subjected to long-term exposure to DBTC, in order to investigate uptake, cellular location, as well as metabolic effects. *Anodonta* is a facultatively anaerobic organism (Holwerda and Veenhof 1984): the animal may be expected to be able to compensate, at least in part, for an impaired oxidative metabolism by turning to an "anaerobic" form of energy supply. We have, therefore, examined some parameters of the energy metabolism of exposed animals. In addition, the accumulation and location of tin in several organs were determined.

MATERIALS AND METHODS

Anodonta anatina L. were collected from a pond near Leiden and acclimatized to laboratory conditions for at least three weeks. Animals were exposed to 0.125 µM DBTC (equivalent to 15 ppb Sn) for 7 months, during the summer. A 40-L volume of tap water (transported internally in polythene conduit), holding 50 animals at the start of the experiment, was spiked with the toxicant dissolved in ethanol (50 ml). Main parameters of water quality were:

Ca	: 34-41 mg/L	Fe	: < 0.1 mg/L	SO_4	: 12-17 mg/L
Mg	: 3-4 mg/L	HCO_3	: 95-115 mg/L	pH	: 7.3-8.0
Na	: 8.5-10 mg/L	Cl	: 13-20 mg/L	dissolved O_2	: 6-9 mg/L

The system was refreshed weekly to the same level. Mortality was 25% over the total period of exposure.

Biochemical parameters - wet weight and dry weight (after lyophilization), carbohydrate and lipid content, and anaerobic end products (lactate, succinate, acetate, and propionate) - were determined for the total soft body mass. In addition, the hemolymph carbohydrate concentration was measured. Hemolymph (2 ml per animal) was taken from the heart with a syringe. Dissection, homogenization, and determination of metabolites were performed as described previously (Holwerda and Veenhof 1984). Carbohydrate was measured using the anthrone method (Holwerda et al. 1977) with D-glucose as the standard. Lipid was assayed using the sulphuric acid/vanillin method (Holwerda et al. 1977) with oleic acid as the standard.

For metal concentration, the wet organs were decomposed in conc. HNO_3 for at least two days, at room temperature. Total tin was measured using graphite furnace atomic absorption spectrometry. Wavelength was set at 235.5 nm, and background correction for non-specific absorption was applied.

Tin was localized light-microscopically with the sulphide-silver technique after Danscher (1981). To visualize glycogen, tissue samples were fixed by freeze-substitution in Gendre (Romeis 1968). Paraffin sections were stained with Periodic Acid-Schiff (PAS).

RESULTS AND DISCUSSION

In the concentration applied, DBTC was not lethal to the clams. By contrast, in preliminary experiments we found that these animals do not survive exposure to bis(tri-n-butyltin) oxide (TBTO, in a concentration equivalent to 5 µg Sn/L) for longer than six weeks. Like DBTC, TBTO has been reported to affect the oxidative metabolism (Cremer 1957). Nevertheless, DBTC significantly influenced parameters that are indicative of delivery and status of energy. First, whereas in control animals the dry weight amounted to 6.8% ± 0.8 (SE, n=6), in exposed animals it had decreased to 3.8% ± 0.6 (n=8). Secondly, carbohydrate stores of exposed animals were reduced to less than 25% (Table 1). The lipid supply seems to diminish considerably less, but it should be kept in mind that the

vanillin method used only measures unsaturated fatty acids. Of course, part of the lipid is structural, and therefore less or not available as an energy substrate.

Table 1. Concentrations of energy substrates, in soft tissue and hemolymph, of DBTC-exposed (7 months, 15 µg Sn/L) and of control animals. Mean value and SE; the number of animals is given in parentheses

	Carbohydrate-hemolymph	Carbohydrate-total tissue	Lipid-total tissue
	µg/ml	mg/g wet weight	
Control	67 ± 4 (4)	4.4 ± 1.0 (4)	2.4 ± 0.3 (4)
Exposed	19 ± 5 (3)	1.0 ± 0.1 (4)	1.2 ± 0.1 (4)

Light-microscopical examination of PAS-stained sections confirmed that, in all tissues containing significant amounts of glycogen in control animals, particularly in mantle, visceral mass and kidney, glycogen was markedly reduced after exposure of the clams to DBTC. Evidently, exposed animals show an increase of metabolic rate (ATP turnover) or they metabolize, with unaffected energy demand, less efficiently. The latter possibility is the more likely one as the levels of lactate (D- and L-), succinate and the volatile fatty acids acetate and propionate, being typical anaerobic end products, were strongly elevated (Table 2).

Table 2. Effect of DBTC-exposure on the concentration of metabolites, involved in anaerobic metabolism; individual values for the control, mean ± SE for exposed animals

	Lactate	Succinate	Acetate	Propionate
	µmol/g dry weight			
Control (n=2)	0.30; 0.36	0.06; 0.06	<0.1;<0.1	n.d*; n.d*
Exposed (n=4)	3.1 ± 0.7	0.24 ± 0.03	3.0 ± 0.8	2.8 ± 0.3

*not detectable (<0.05).

These data indicate that the intoxicated clams relied, at least in part, on an anaerobic type of energy metabolism. However, the metabolic pathway may well differ from the real anaerobic metabolism, as shown by animals when oxygen is deprived (Fig. 1A): there is - except for a short period after the onset of anoxia - no formation of lactate. In the cytoplasm, glycogen is converted into malate. The latter metabolite is transported into the mitochondrion, where it is converted disproportionately - because of the redox balance - into succinate/propionate and acetate. In DBTC-exposed animals too, ATP production will be derived, wholly or partly, from substrate-level phosphorylation, as DBTC inhibits oxidative phosphorylation. However, the conversion of pyruvate

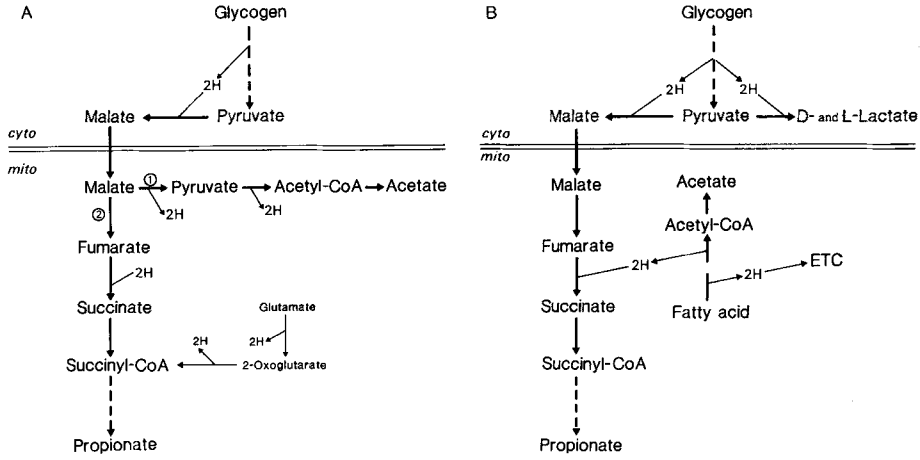

Figure 1A. Metabolic pathways in *Anodonta* during anoxia; ⟶ main pathway, ⟶ minor pathway. Fig 1B. Hypothetical model of energy metabolism in DBTC-exposed animals.

into acetyl-CoA, thought to proceed under anoxia (Fig. 1A), may be inhibited owing to the blockage by DBTC of the pyruvate dehydrogenase complex. Instead, glycogen will be converted into D- and L-lactate and succinate/propionate. The reducing equivalents necessary for fumarate reduction may be derived from fatty acid oxidation, which explains the accumulation of acetate. The latter cannot be further metabolized because of the inhibition of the citric acid cycle enzyme 2-oxoglutarate dehydrogenase. Excess of reducing equivalents, presumably as the reduced acyl-CoA dehydrogenase (FAD dependent) might be oxidized in the mitochondrial electron transfer chain (Fig. 1B). It should be stressed that the tissue concentrations of anaerobic end products (Table 2) cannot be used to construct metabolic balances as these metabolites are differentially released into the medium (Holwerda and Veenhof 1984). Furthermore, the extent of metabolic inhibition will depend on the degree of accumulation of DBTC, or derived compounds, in the individual organs. Finally, it cannot be excluded that the two supposed sites of inhibition, the oxoacid dehydrogenase systems and the oxidative phosphorylation, are affected to a differing degree. Even under the condition of total blockage of 2-oxoglutarate dehydrogenase, i.e. the citric acid cycle being eliminated, ATP could be synthesized via oxidation of $NADH_2$ and $FADH_2$ derived from fatty acid oxidation, provided that oxidative phosphorylation is not totally inhibited or uncoupled.

In Table 3, total-tin concentrations are given for some selected organs, and the remainder of the soft tissues (referred to as "Rest fraction"). By far the highest concentration (on wet weight basis) was found in the kidney. This organ, therefore, serves as the main sink for the organotin compound. Possibly, other organs have little capacity to bind DBTC, and/or the organotin is readily transferred in the direction of the excretory organ.

Table 3. Total-tin concentrations in some selected organs of *A. anatina* after exposure to DBTC (15 µg Sn/L) for 7 months. For 6 individual animals the range of Sn concentration, and the mean value ± SE are given

	Range	Mean
	µg Sn/g wet weight	
Gills	0.66 - 1.39	0.94 ± 0.11
Mantle + Mantle-edge	0.08 - 0.22	0.13 ± 0.02
Foot	0.11 - 0.37	0.22 ± 0.04
Midgut gland	0.40 - 0.79	0.60 ± 0.07
Kidney	17.1 - 31.1	23.5 ± 2.2
Rest fraction	0.14 - 0.98	0.56 ± 0.12

By contrast, simultaneous exposure of *Anodonta* to $CdCl_2$ (16 weeks, 25 ppb Cd) resulted in a quite different distribution of this metal among the various organs (Table 4). This cannot be attributed to the larger concentration factor for Cd (500, for whole animal) compared to that for Sn (50, for whole animal): even in unexposed control animals, the kidney of *Anodonta* did not contain more than 6% of the total Cd body burden (Hemelraad et al. 1985). The relatively low concentration factor for Sn, in combination with the high contribution of the kidney, indicates that the organotin compound is eliminated at a faster rate than inorganic cadmium.

Table 4. Relative distribution of metal (in % of total body burden) in some organs of *A.anatina*, exposed to DBTC (7 months, 15 ppb Sn) and $CdCl_2$ (16 weeks, 25 ppb Cd), respectively

	Sn	Cd
Gills	16	39
Mantle + Mantle-edge	4.6	22
Foot	2.0	3.3
Midgut gland	5.3	4.7
Kidney	40	6.1
Rest fraction	32	25

*derived from Hemelraad et al., 1985.

The histochemical localization of tin is based on the transformation of available metals in the tissues to metal sulphides upon which metallic silver is deposited on development of the sections in a silver nitrate/hydroquinone medium. In unexposed control animals distinct silver grains were seen in specific regions of mantle-edge, foot and gut epithelia, and in particular hemolymph cells (probably granulocytes; Moore and Lowe 1977). Deposits of silver were also observed on extracellular components (supporting rods and calcium concretions) of the gills. In DBTC-exposed animals, a similar silver precipitation pattern was found in the

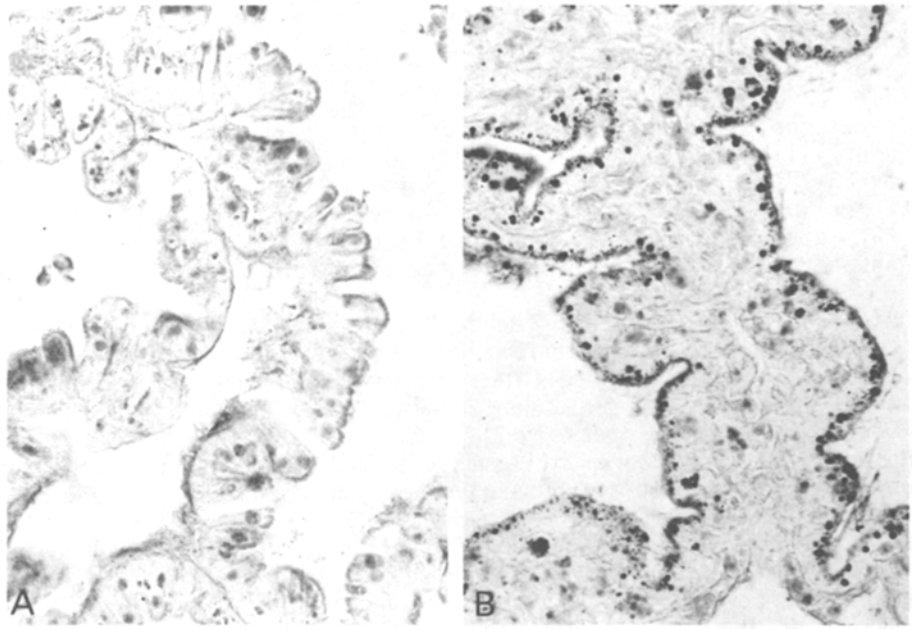

Figure 2. Low-magnification micrographs of kidney tissue of *Anodonta anatina*, treated with the sulphide-silver technique. Magnification: 400 x ; A. Kidney epithelium of unexposed animals. B. Kidney epithelium of DBTC-exposed animals.

organs mentioned. In addition, marked silver deposits were observed in the kidney epithelial cells. The deposits were located mainly in the form of distinct granules in the apical region of the cells (Fig. 2). From the histochemical results it is concluded that the kidney is strongly involved in the accumulation of tin. It must, however, be stressed that the sulphide-silver technique only visualizes metal that can be sulphidated (Danscher 1981). Tin tightly bound to ligands (for instance thionein or other proteins) may escape sulphidation and, therefore, remain histochemically invisible. Apparently, part of the tin accumulated in the kidney is present in a form that can be converted into the metal sulphide.

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