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Effects of Tributyltin Oxide on the Skeletal Structures of Developing and Regenerating Limbs of the Axolotl Larvae, *Ambystoma mexicanum*

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Organotin compounds including tributyltin (TBT) have recently become more extensively used in industrial and agricultural applications (Gitlitz 1976; Laughlin and Linden 1985; Walsh et al. 1986). Tributyltin is also used as an antifouling agent in marine paints since it is a potent biocide and is very effective at preventing marine organisms such as barnacles from settling on the hulls of boats (Bushong et al. 1988; Weis 1988). However, TBT has created some problems due to its effects on non-target species (particularly bivalves such as clams and oysters) since concentrations in the water of some marinas exceed levels that are lethal to sensitive species (Laughlin and Linden 1987). This has led to limitations or prohibitions on use of TBT in some jurisdictions (Laughlin and Linden 1987: Weis 1988).

Tributyltin is clearly toxic to a variety of marine invertebrates (Laughlin et al. 1983; Lee 1985; Walsh et al. 1986; Weis et al. 1987). Specific morphological and teratological effects have also been noted. For example, tributyltin is known to interfere with the regeneration of the limbs of crabs (Weis and Weis 1988; Weis and Kim 1988). However, the effect of tributyltin on vertebrates has not received as much attention. TBT interferes with the differentiation of mouse limb buds in culture (Krowke et al. 1986) and also acts as a neurotoxin in rats (O'Callaghan and Miller 1988). Boyer (1989) has recently reviewed the toxicity of organotins to humans and experimental animals.

Studies of the teratogenicity of TBT in vertebrates are sparse. Since TBT was known to cause malformations during crab limb regeneration (Weis and Kim 1988) and to interfere with the development of mouse limb buds in culture (Krowke et al. 1986), this study was undertaken to investigate the potential teratogenicity of TBT in vertebrates and in particular to determine if TBT has teratogenic effects on developing or regenerating limbs

of the axolotl, *Ambystoma mexicanum*. Developing hindlimbs and regenerating forelimbs can be studied simultaneously on the same animal since development of the forelimb pattern is complete before hindlimb development begins.

MATERIALS AND METHODS

Axolotl larvae (*Ambystoma mexicanum*) were obtained as eggs or young larvae from the axolotl colony at the University of Ottawa. They were raised in the laboratory in dechlorinated tap water in individual plastic dishes (D-8 cups, Canada Cup Inc., Toronto, Ontario) to prevent damage to the limbs due to the predations of other axolotls. They were fed a diet of brine shrimp, and later chopped or intact *Tubifex* worms as their size increased. The axolotl larvae were used in these experiments when their hind limb buds had reached the cone stage (limb bud longer than it was broad) and the forelimbs were well developed with complete digits (since forelimbs develop earlier than hindlimbs). This allowed observations on both developing hindlimbs and regenerating forelimbs simultaneously.

Axolotl larvae were treated with TBT by adding it to the water in their bowls. Tributyltin [bis(tributyltin) oxide] $\{[CH_3(CH_2)_3]_3\text{-Sn-O-Sn-}[(CH_2)_3CH_3]_3\}$ (Aldrich Chemical Co., Milwaukee, WI) was mixed with acetone (1 mg/mL) (since TBT is not miscible with water) and this solution was then serially diluted with tap water to reach final concentrations of 50, 15, 5, 1.5, or 0.5 µg/L.

Seventy axolotls were anaesthetized in tricaine methane sulphonate neutralized with sodium bicarbonate (Robinson and Scadding 1983), and both forelimbs were amputated through the distal radius and ulna. Axolotls were then distributed into seven groups of ten animals each to provide five experimental groups and two control groups. Each experimental group was immersed in one of the five TBT concentrations listed above. Thus, regenerating forelimbs were exposed to TBT beginning on the day of amputation and the developing hindlimbs were simultaneously exposed beginning at the cone stage of development. One control group was exposed to tap water only, and one to tap water containing acetone (15 µL acetone/L). The TBT and control solutions were changed twice weekly throughout the experiment. Procedures used were consistent with provincial regulations and C.C.A.C. Guidelines (Canadian Council on Animal Care, 1980).

After 49 days, the axolotl larvae were reanaesthetized and all four limbs were removed and fixed in neutral buffered formalin, stained with Victoria Blue B for

cartilage, and cleared in methyl salicylate (for more detailed methods see Scadding and Maden 1986a, b). The skeletal structures were then examined to identify any abnormalities.

RESULTS AND DISCUSSION

TBT was clearly toxic at the higher dose levels employed. All axolotl larvae exposed to 50 µg/L died within 24 hrs and of those exposed to 15 µg/L eight out of ten died within 7 days and only one survived to day 49. Hence, the following results are based on the three lower dose groups, in which all animals survived to day 49.

The normal differentiated hindlimb of an axolotl has a femur, a tibia and a fibula, nine tarsals (including fibulare, tibiale, intermedium, centrale, and five distal tarsals), five metatarsals, and 13 phalanges arranged in a 2.2.3.4.2 pattern (Fig. 1). This was the most common pattern in control axolotls (Table 1). However, variants of this pattern did occur, the most common being the loss of a phalange from digit 4 resulting in a 2.2.3.3.2 pattern or the absence of a tarsal resulting from fusion of distal tarsal 1 with the tibiale (Scadding 1990).

The skeletal patterns of the limbs developing in the presence of 0.5 µg/L TBT were indistinguishable from those of the controls (Table 1). However, the limbs exposed to 1.5 µg/L TBT showed a slightly increased incidence of skeletal deletions. At the 5 µg/L level, only 3 out of 20 limbs were complete and almost all the limbs showed deletions or defects (Table 1). Four limbs showed only 7 tarsals. Two limbs exhibited more extensive defects involving reduction in size of digit 5, and in one of these cases the long bones of the limb were shortened as well (Fig. 2).

The normal differentiated forelimb of an axolotl has a humerus, a radius and an ulna, eight carpals (radiale, ulnare, intermedium, centrale, and four distal carpals), four metacarpals, and nine phalanges in a 2.2.3.2 pattern (Fig. 3). However, distal carpal 1 and the radiale were often joined leading to a pattern of only seven carpals. Occasionally, the intermedium and centrale were joined as well yielding a pattern of 6 carpals. Other variants were seen less frequently. Surprisingly, TBT at concentrations of up to 5 µg/L, did not cause any derangement of the skeletal pattern of the regenerating forelimbs (Table 1) even though the concentration (5 µg/L) approached levels that were lethal (15 µg/L).

The results show that tributyltin is slightly teratogenic to developing axolotl hindlimbs and seems to have no

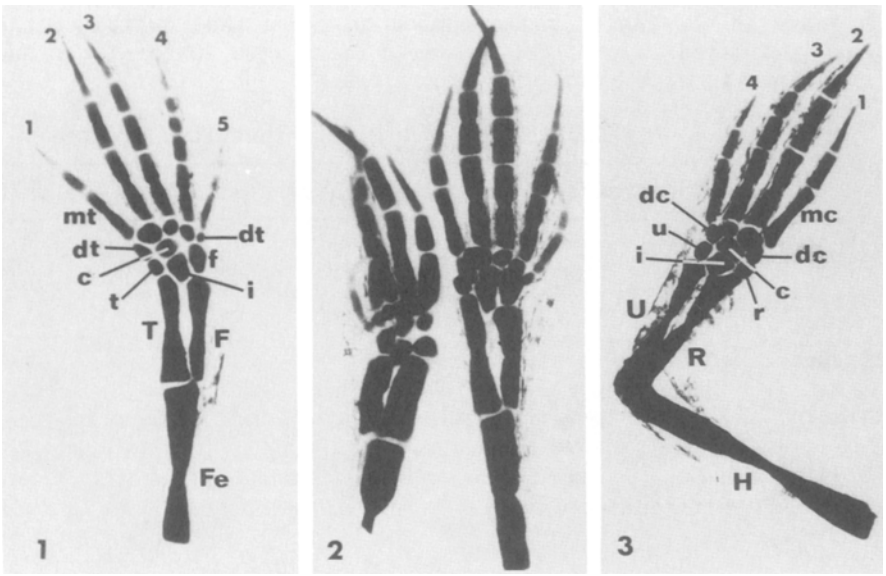

Figure 1. This dorsal view of a right hindlimb of an axolotl from the control group shows the complete normal skeletal pattern consisting of the femur (Fe), the tibia (T) and fibula (F), nine tarsals (including fibulare (f), tibiale (t), intermedium (i), centrale (c), and five distal tarsals (dt)), five metatarsals (mt), and 13 phalanges arranged in a 2.2.3.4.2 pattern on digits 1 to 5 (1-5). X 13.

Figure 2. This dorsal view of the left and right hindlimbs of an axolotl larvae treated with 5 µg/L TBT shows some defects induced by TBT. The right limb is complete except that the phalange formula is 2.2.3.3.2. However, the left limb shows more significant defects: the femur and tibia-fibula are reduced in length, digit 5 is reduced in size, and digit 4 is deflected dorsally and not visible in this photograph. X 14.

Figure 3. This dorsal view of a regenerated left forelimb of an axolotl from the control group shows the complete normal skeletal pattern consisting of the humerus (H), radius (R) and ulna (U), eight carpals (radiale (r), ulnare (u), intermedium (i), centrale (c), and four distal carpals (dc)), four metacarpals (mc), and nine phalanges in a 2.2.3.2 pattern on digits 1 to 4 (1-4). X 12.

Table 1. Effects of tributyltin on skeletal pattern.

Each entry in the table is the number of limbs that exhibited the pattern indicated in the left column. There were 20 forelimbs and 20 hindlimbs in each group of 10 axolotls.

	Controls		Tributyltin treated		
	Untreated	Acetone	0.5 µg/L	1.5 µg/L	5 µg/L
Developing Hindlimbs					
Complete pattern	16	19	18	13	3
Phalanges 2.2.3.3.2	4			3	8
8 tarsals[a]		1	2	4	
8 tarsals[a] & phalanges 2.2.3.3.2					3
7 tarsals[b]					2
7 tarsals[b] & phalanges 2.2.3.3.2					2
Other defects					2
Regenerating Forelimbs					
Complete pattern	7	3	11	6	8
7 carpals[c]	10	11	6	13	7
6 carpals[d]	3	4	3	1	4
5 carpals[e]		1			
9 carpals		1			1

a = distal tarsal 1 and tibiale fused
b = distal tarsal 1 and tibiale; intermedium and centrale fused
c = distal carpal 1 and radiale fused
d = distal carpal 1 and radiale; intermedium and centrale fused
e = distal carpal 1 and radiale; intermedium and centrale; distal carpals 3-4 fused

effect on skeletal pattern in regenerating forelimbs. This result is surprising in the light of the known effects of TBT on limbs in other species (crab - Weis and Kim 1988; mouse - Krowke et al. 1986) and the marked effect on axolotl limbs of known teratogens such as vitamin A (Scadding and Maden 1986a).

The single specimen treated with 15 µg/L TBT and surviving until day 49 showed more extensive derangements of the limb skeleton in both the regenerating forelimbs, where digit 4 was reduced or absent, and in the developing hindlimbs where digits 4 and 5 were reduced in size. Thus it appears that TBT has the potential to be teratogenic in vertebrate limbs, but its other toxic effects are lethal at doses below those necessary to cause major limb malformations. This phenomenon was also observed in mouse embryos where major teratological malformations could not be observed in vivo, but only in limbs in an organ culture system, since embryonic effects of TBT were only evident at maternally toxic dose levels (Krowke et al. 1986).

Toxic levels of TBT observed for axolotl larvae are consistent with those of previous workers in suggesting that vertebrates are generally less sensitive to the toxic effects of TBT than are some invertebrate species (Bushong et al. 1988). The reasons for this are unknown. However, there is great variation in species sensitivity to TBT. The most sensitive fish species tested to date have LC_{50} values as low as 3 µg/L TBT. Concentrations above this level have been reported for water samples from some marinas although levels in marinas were more commonly in the 0.2 to 0.8 µg/L range (Laughlin and Linden 1987). The levels of TBT found in this study to be toxic for axolotl larvae are in the same range as those levels reported to be toxic in fish (LC_{50} of 3-26 µg/L) (Bushong et al. 1988).

The observation that TBT was teratogenic during limb development but not during limb regeneration raises the possibility that the two processes are different in some way allowing differential responses to TBT. Possible differences between regenerating and developing limbs have been considered previously (Scadding and Maden 1986a, b) and could include differential sensitivity of cycling cells, different rates of cell proliferation, or different modes of action of TBT. Developing and regenerating limbs are known to give different responses to other teratogens such as vitamin A (Scadding and Maden 1986a) and acetazolamide (Dinsmore and Maren 1986), suggesting the possibility that patterning mechanisms may differ between developing and regenerating limbs.

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