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Effects of Tributyltin(IV) Chloride Exposure on Larvae of *Ciona intestinalis* (Urochordata): An Ultrastructural Study

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The effects of tributyltin(IV) chloride (TBT chloride) have been tested on embryos of the ascidian *Ciona intestinalis*, at two different stages of development: (1) before hatching (coiled larval stage) and (2) 2 h after hatching (swimming larval stage). *In vivo* observations carried out with a light microscope showed that embryos at the coiled larval stage did not hatch following exposure to TBT chloride. Severe anomalies in the swimming larva, mainly concerning the morphology of the tail, which appeared twisted and squatter than in the controls, were observed. Such anomalies were also found at a functional level, i.e. contractile movements were poor so that the larvae appeared motionless. Ultrastructural investigations carried out using a transmission electron microscope (TEM) evidenced that the muscle cells of the tail were damaged. Modifications mainly occurred in mitochondria and myofibrils, i.e. the energetic and enzymic centres. This fact is probably the main cause of the loss of mobility of the larvae.

Keywords: tributyltin; embryo toxicity; ascidians

INTRODUCTION

The effects of heavy-metal derivatives on marine and terrestrial organisms have been the subject of many reports in recent years.

In this context, organometallic, in particular organotin(IV) derivatives, have received particular attention, due to their high cytotoxicity evaluated on different seawater and freshwater animal groups (i.e. fish, crustaceans, tunicates). Numerous research reports exist on the effects of organometallic derivatives on normal metabolic processes and generally, the survivability of seawater organisms at the adult stage.[1,2] However, few data are available on the effects of these compounds on early developing embryos of the same organisms. In particular, studies of this kind carried out on gametes and on developing embryos of ascidians[3–5] have shown that tributyltin(IV) species were more toxic than the dibutyltin(IV) (DBT) derivatives.

The exposure of embryos to tributyltin(IV) compounds (TBT) produced irreversible arrest of their development owing to the strong ultrastructural modifications which they underwent. Furthermore, exposure to TBT chloride determines the loss of mobility of the larva, which does not then proceed to metamorphosis. Analogous effects have been observed, following exposure to toxic agents, by marine invertebrates.[6–8] Ascidians are marine animals, of the sessile phylum. At the adult stage, they are sessil and live by the shoreline, fixed to the walls of harbours etc. and to the keels and sides of boats and ships. The adult stage is preceded by a larval stage, during which the animals are free and can swim in the marine environment (swimming larvae).

The aim of this research is to test the effects of tributyltin(IV) chloride (TBT chloride) towards *Ciona intestinalis* embryos at the larval stage. In particular,

(1) we have analysed the coiled larva (before hatching) and the swimming larva; and
(2) we have described alterations involving the organs, the cells and the cell structures of the larva, following exposure to TBT chloride.

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EXPERIMENTAL

Adult specimens of *Ciona intestinalis* were collected, between April and June, from the Gulf of Palermo, Termini and Terrasini harbours (Palermo). Specimens were transferred in a water carrier and were maintained there at 18–20 °C. Sperm and eggs of several animals were removed from the gonoducts and transferred into Syracuse dishes containing filtered and sterilized seawater. After fertilization the embryos were followed up to the swimming larval stage. In particular, observations were carried out by both light and electron microscopy on the following samples.

Lot A

(a) Controls — coiled larvae (before hatching) maintained in seawater, filtered and sterilized, up to the swimming larvae stage.
(b) Coiled larvae (before hatching) incubated for 1 h in 10^{-7} mol dm^{-3} TBT chloride solution and then transferred to filtered and sterilized seawater.

Lot B

(a) Controls — swimming larvae (2 h after hatching) maintained in filtered and sterilized seawater up to the beginning of metamorphosis.
(b) Swimming larvae (2 h after hatching) incubated for 1 h in a 10^{-7} mol dm^{-3} TBT chloride solution and then transferred to filtered and sterilized seawater.

Tributyltin(IV) chloride (TBT chloride) was a gift from Witco GmbH (Bergkamen, Germany). Concentrated stock solutions were freshly prepared by dissolving stoichiometric amounts of the compound in Millipore-filtered seawater (MFSW). Working solutions (pH 7.25–8.50) were obtained by further dilution of the concentrated stock in MFSW. Solution stability and tin content were checked as previously reported.[9]

Light and transmission electron microscopy

Ciona intestinalis larvae from Lots A and B, vitally stained for 5 min with Nile Blue sulphate (1 : 10^5 in MFSW) and maintained in culture at 18 °C, were observed with a Leitz Orthoplan microscope, and photographed using an Ilford FP4 Plus film.

Larvae of Lots A and B were fixed with 2.5% glutaraldehyde in 0.2 mol dm^{-3} phosphate buffer (pH 7.5) solution and postfixed with 1% osmium tetroxide in the same buffer solution. The fixed material was dehydrated in alcohol of various fixed concentrations and embedded in Epon 812.[10]

Semithin sections (1–2 μm thick) obtained with an Ultracut Reichert ultramicrotome, were stained with 1% Toluidine Blue at pH 2.5.[11] Sections were observed and photographed with a Leitz Orthoplan microscope, using an Ilford FP4 Plus film.

For ultrastructural observation, thin sections obtained with an Ultracut Reichert ultramicrotome were contrasted with uranyl acetate and lead citrate,[12] and then photographed with a Philips EM 410 electron microscope, using Kodak electron microscope film (Estar thick base 4489).

RESULTS

Light and electron microscopy observations

Swimming larva controls

Observations made with a light microscope on *Ciona intestinalis* swimming larvae (2 h after hatching), vitally stained with Nile Blue sulphate, showed that they consisted of a cephalic region (cephalenteron) and of a long tail (Fig. 1).

The tail movement consisted of strong side thrusts which rotated the larva around its principal axis. The larva started to swim with long periods of activity, interspersed with short periods of rest. In its anterior region, the cephalenteron contained three palps, which allowed the larva to adhere to the substrates before metamorphosis began. In the medial region, the cephalenteron displayed sensorial organs ('ocellus' and 'otolith') (Fig. 1). Inside the tail, and along its length, an axile structure, the notochord, could be observed. The larva was completely covered by a coat (the tunic or test). Several test cells were noted adhering to the test (Fig. 1).

Analysis carried out on a semithin section showed the presence of elongated muscle cells in the caudal region, between the ectodermic external layer and the notochord (Fig. 2).

Each muscle cell had elongated and become elliptical in shape, showing a relatively large and irregular nucleus with the cytoplasm almost completely occupied by mitochondria with an

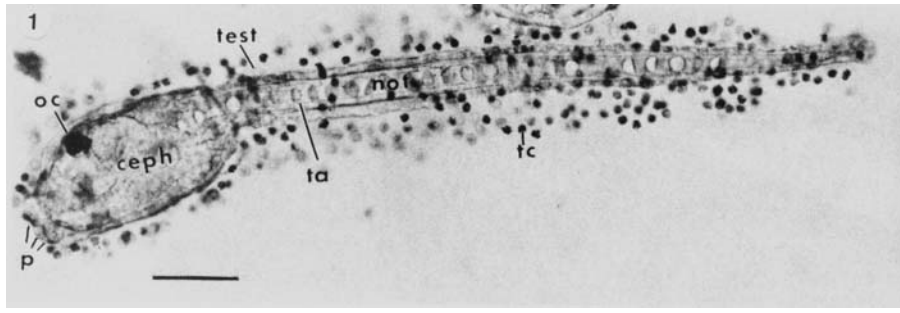

Figure 1 Control: *Ciona intestinalis* swimming larva vitally stained with Nile Blue sulphate. The larva consists of a cephalic region or cephalenteron (**ceph**) and a tail (**ta**) for swimming in seawater. Inside the tail, and along its length, an axil structure, the notochord, can be observed. The cephalenteron presents, in its anterior region, three palps (**p**) and in its median region, the ocellus (**oc**), a sense organ. The larva is completely covered by a coat called the test (**test**). Several test cells (**tc**) adhere to the test. (Bar=100 μm.)

orthodox configuration. Below the cytoplasmic membrane (sarcolemma) a single layer of myofibrils, parallel to the central axis of the cell, was observed (Figs 3 and 4).

Each myofibril showed the ultrastructure typical of those described in ascidian muscle cells.[13, 14] Myofibrils were made up by a sequence of 9 to 15 sarcomeres, and A, H and I bands were present (Fig. 5). Each sarcomere, 1.8 μm long, was delimited by two Z lines.

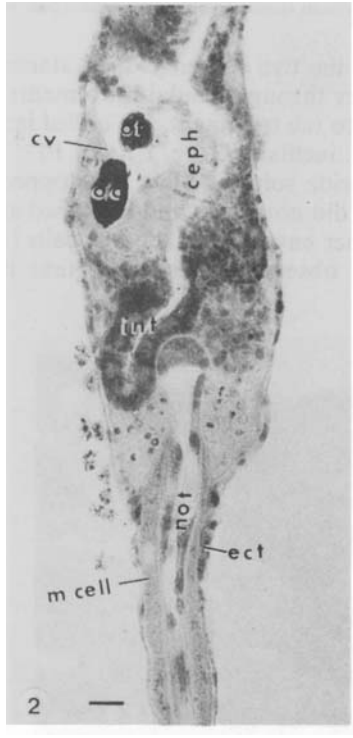

Figure 2 Control: semithin section of *Ciona intestinalis* swimming larva stained with 1% Toluidine Blue. In the cephalenteron (**ceph**), the intestine (**int**) and the cerebral vesicles (**cv**) containing the two sense organs, ocellus (**oc**) and otolith (**ot**), can be seen. In the tail between the layer of ectodermal cells (**ect**) and the notochord (**not**), the elongated cells, muscle cells (**m cell**) are present. (Bar=10 μm.)

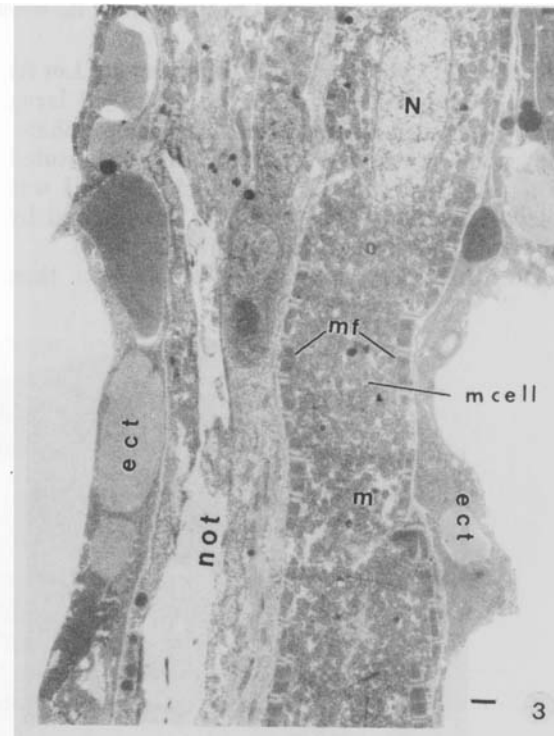

Figure 3 Control: caudal region of *Ciona intestinalis* swimming larva (2 h after hatching) observed by TEM. Between the layer of ectodermal cells (**ect**) and the notochord (**not**), a muscle cell (**m cell**), elongated in shape, is seen. An irregular nucleus (**N**) is present in the cytoplasm, almost completely filled with mitochondria (**m**). Below the cytoplasmic membrane, two single layers of myofibrils (**mf**) are observed. (Bar=1 μm.)

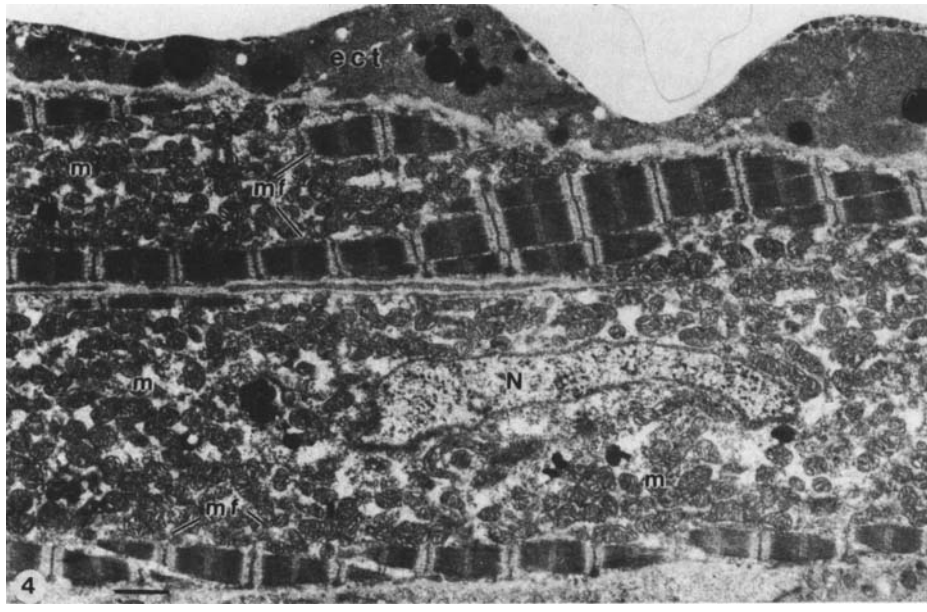

Figure 4 Control: muscle cells of the caudal region of *Ciona intestinalis* swimming larva. The cytoplasm is occupied by numerous mitochondria (**m**). Below the cytoplasmic membrane, the myofibrils (**mf**) with typical bands can be observed. (Bar = 1 μm)

Effects of TBT chloride on the coiled larvae (Lot A) Figure 6 shows a *Ciona intestinalis* coiled larva (control) stained vitally with Nile Blue sulphate. The larva was ready to hatch and was constituted by a cephalenteron around which the tail was coiled 1.75 times. The larva was surrounded by two ovular envelopes (test and follicle cells).

After hatching, the larva, which was then devoid of the two cell envelopes, started to swim in seawater through caudal movements.

Owing to the treatment, the coiled larvae which had been incubated for 1 h in 10^{-7} mol dm^{-3} TBT chloride solution (Lot A) stopped developing. They did not hatch and remained surrounded by the inner envelope of the test cells (Fig. 7).

T.E.M. observations showed that the muscle

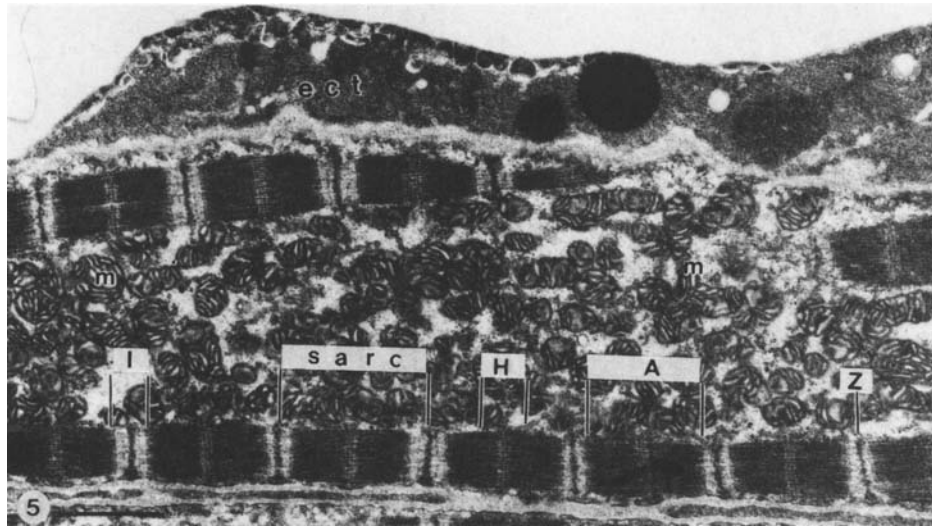

Figure 5 Control: muscle cells of the caudal region of *Ciona intestinalis* swimming larva. Each myofibril (**mf**) is characterized by a sequence of sarcomeres (**sarc**) and **A**, **H** and **I** bands. Each sarcomere is delimited by two **Z** lines. (Bar=1 μm.)

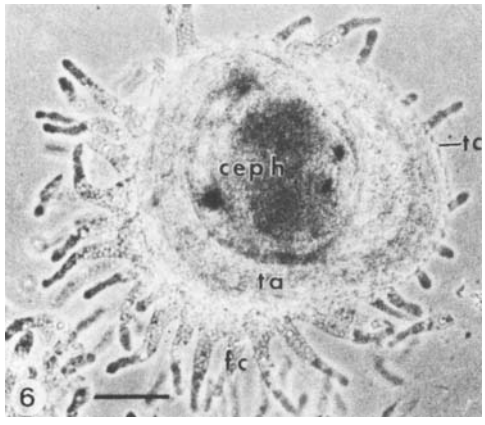

Figure 6 Control: *Ciona intestinalis* coiled larva vitally stained with Nile Blue sulphate. The larva is surrounded by two ovular envelopes. The outer envelope is constituted by large and elongated follicle cells (**fc**); the inner envelope is formed by smaller and round-shaped test cells (**tc**). The larva is made up of a cephalenteron (**ceph**) around which the tail (**ta**) was coiled in 1.75 turns. (Bar=100 μm.)

cells were no longer than in the control and presented an irregularly shaped nucleus in a central position (Fig. 8).

Contrary to the controls, in which cells were present as an organized layer with a linear arrangement above the notochord, cells in treated larvae were grouped without any organization. In

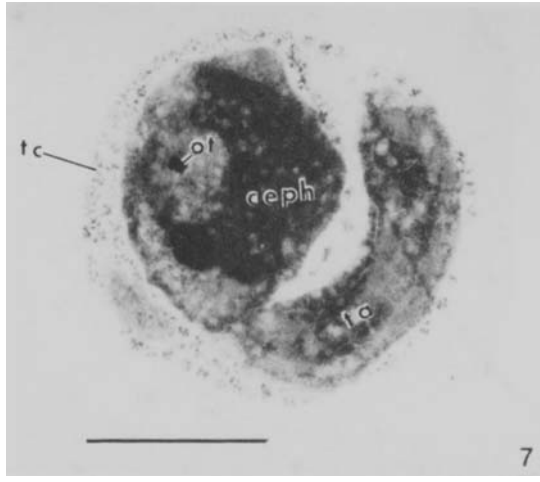

Figure 7 Lot A: *Ciona intestinalis* coiled larva incubated for 1 h in 10^{-7} mol dm^{-3} TBT chloride solution and then transferred to seawater. Semithin section stained with 1% Toluidine Blue. The larva stops developing, does not hatch and remains surrounded by the inner envelope of test cells (**tc**). The tail (**ta**) shows severe anomalies and appears squat and short. (Bar=100 μm.)

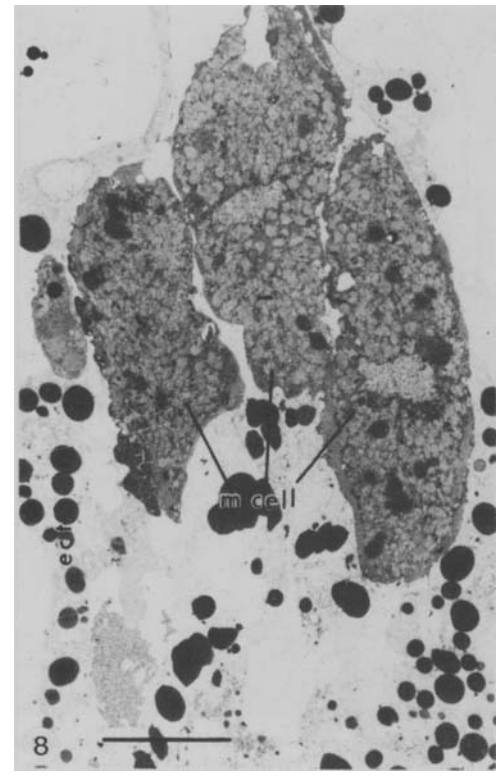

Figure 8 Lot A: *Ciona intestinalis* coiled larva incubated for 1 h in 10^{-7} mol dm^{-3} TBT chloride solution. Transverse section of dorsal region in the tail. The muscle cells (**m cell**) are not organized as a linear layer below the ectoderm (**ect**). (Bar=10 μm.)

the cytoplasm of these cells, the mitochondria were irregular in shape and strongly swollen. The cytoplasmic spaces between the mitochondria were so reduced that they seemed to have merged and their outer membranes were not distinguishable (Fig. 9). The cristae, ultrastructurally, were strongly irregular and appeared as tubular vesicles dispersed in the mitochondrial matrix. Myofibrils, occurring below the cell membrane, underwent even heavier modifications. They completely lost their ultrastructural organization: the sarcomeres and the characteristic band sequence were no longer present (Fig. 9). The myofibrils were thus replaced by structures devoid of ultrastructural organization and made up of amorphous material of half the electron density.

Effects of TBT chloride on swimming larvae (Lot B)

Observations of *Ciona intestinalis* swimming larvae incubated for 1 h in 10^{-7} mol dm^{-3} TBT

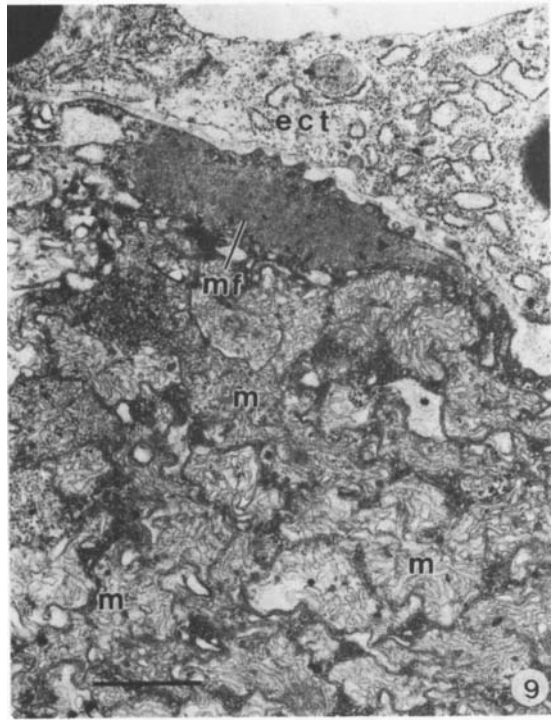

Figure 9 Lot A: *Ciona intestinalis* coiled larva incubated for 1 h in 10^{-7} mol dm^{-3} TBT chloride solution. Transverse section of the tail. Muscle cell beneath the ectoderm layer (**ect**). In the cytoplasm, the mitochondria (**m**) present severe ultrastructural modifications and their cristae appear as tubular vesicles in the matrix. Also, the myofibrils completely lose their ultrastructural organization and are constituted of amorphous material of medium electrondensity. (Bar=1 μm.)

chloride solution (Lot B) suggested that the larval cephalenteron did not present particular anomalies. In fact, both the sense organs and the three adhesive papillae could be observed (Fig. 10).

Severe morphological anomalies occurred in the tail, resulting in a strongly twisted appearance in this region. Moreover, the tails in treated embryos were shorter than in the controls (Fig. 10). Consequently the larvae were not able to move regularly in the marine environment.

Ultrastructural modifications in the caudal muscle cells were as observed in the preceding experiments, with slight modifications, mainly involving mitochondria and myofibrils. Some mitochondria appeared, although in a reduced number, with an orthodox configuration (Fig. 11). Several sectors of myofibrils did not present their typical linear arrangement but were bent (Figs 12, 14).

Owing to the folding, several myofibril sectors

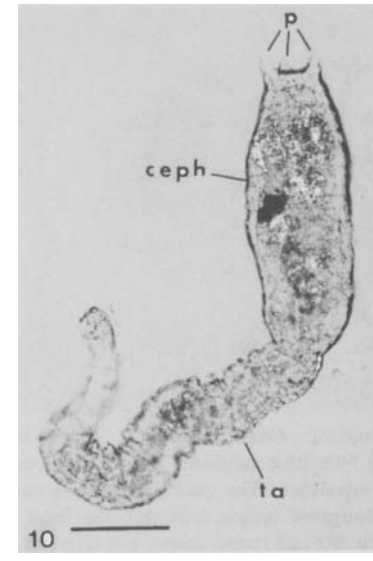

Figure 10 Lot B: *Ciona intestinalis* swimming larva incubated for 1 h in 10^{-7} mol dm^{-3} TBT chloride solution and then transferred to seawater. The larva, vitally stained with Nile Blue sulphate, presents severe anomalies in the tail (**ta**), which is strongly twisted; it makes weak and brief movements and the larva is not able to swim. (Bar=100 μm.)

appeared no more as linear bundles of myofilaments, but as point structures (Fig. 13).

Several sectors of myofibrils completely lost

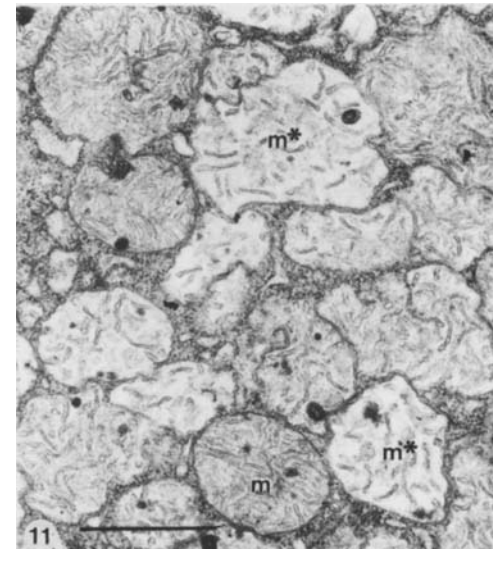

Figure 11 Lot B: *Ciona intestinalis* swimming larva incubated for 1 h in 10^{-7} mol dm^{-3} TBT chloride solution. Longitudinal section of the muscle cell. In the cytoplasm, mitochondria with an orthodox configuration (**m**) and swollen and irregularly shaped mitochondria (**m***) are present. (Bar=1 μm.)

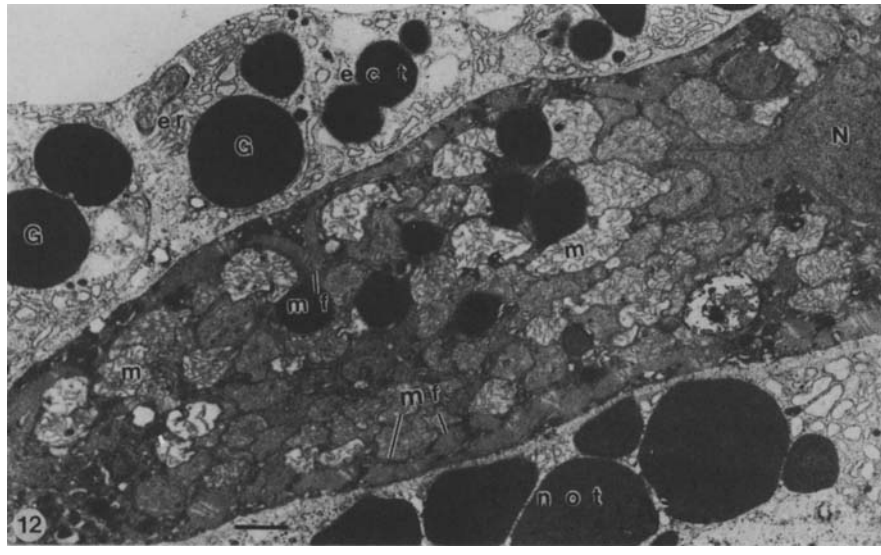

Figure 12 Lot B: *Ciona intestinalis* swimming larva incubated for 1 h in 10^{-7} mol dm^{-3} TBT chloride solution. Ultrastructural modifications are evident in the myofibrils. Several sectors of myofibrils do not present their typical linear arrangement, but bend till they form an angle. In the ectoderm (**ect**), yolk granules (**G**) and r.e.r. vesicles (**er**) can be seen. (Bar=1 μm.)

their ultrastructural organization and were averted in all directions (Fig. 15).

The cells of ectodermic layer, arranged on the muscle cells, did not show severe damage from TBTchloride exposure. Their cytoplasm was very rich in reticulum endoplasmic rough (r.e.r.) vesicles and presented a large nucleus.

DISCUSSION

Water containing heavy metals might provoke blockage of normal development or the death of the planktonic larvae,[15] delay in development[16] or a strong decrease in the movement rate.[6] Data on ascidian embryos[3,5,17] treated with TBT chloride,

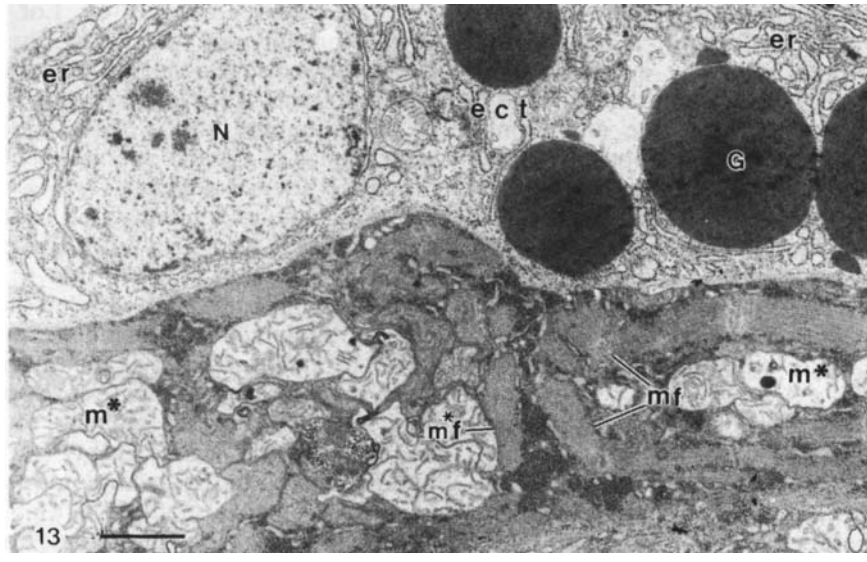

Figure 13 Lot B: *Ciona intestinalis* swimming larva incubated for 1 h in 10^{-7} mol dm^{-3} TBT chloride solution. Longitudinal section of the muscle cell. Owing to the folding, several sectors of myofibrils (**m*f**) appear as point structures. Some mitochondria (**m***) show a strongly modified ultrastructure. The cells of the ectodermal layer (**ect**) present a large nucleus (**N**), yolk granules (**G**) and are very rich in r.e.r. vesicles (**er**). (Bar=1 μm.)

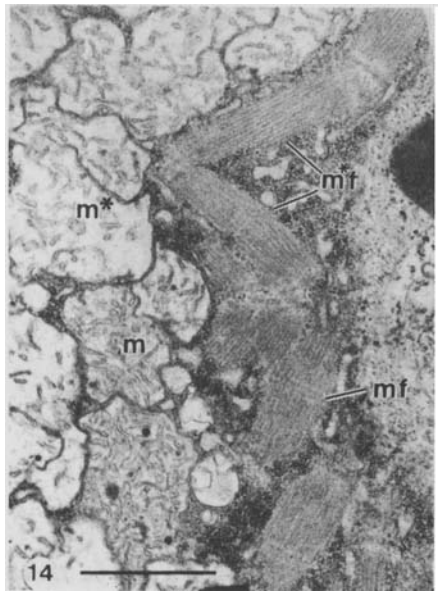

Figure 14 Lot B: *Ciona intestinalis* swimming larva incubated for 1 h in 10^{-7}mol dm^{-3} TBT chloride solution. The myofibrils (**m*f**) have a modified ultrastructural organization, and some sectors are bent. (Bar=1 μm.)

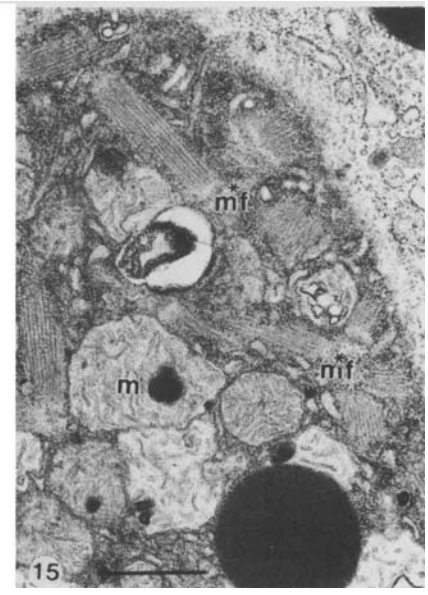

Figure 15 Lot B: *Ciona intestinalis* swimming larva incubated for 1 h in 10^{-7} mol dm^{-3} TBT chloride solution. The sarcomeres no longer present a linear arrangement and are oriented in all directions. **m**, mitochondria with an orthodox configuration; **m***, ultrastructurally modified mitochondria. Bar=1 μm.)

at the neurula and gastrula stages, indicated that these chemicals stopped development, caused fusion of the blastomeres in polynucleated cell masses and prevented the formation of a normal mitotic spindle.

Ultrastructural investigations, carried out with an electron microscope, suggested that the blockage of normal development is related to a degenerative process involving cytoplasmatic, nuclear and mitochondrial membranes. Vitturi *et al.* have evaluated chromosome alterations of spermatocytes in the mesogastropod *Truncatella subcylindrica* (Mollusca) following exposure to dibutyltin(IV) dichloride (DBT dichloride) and tributyltin(IV) chloride (TBT chloride).[9]

It has also been reported that TBT chloride has effects on the development of ascidian embryos which may be closely correlated with the incubation time and with the TBT chloride concentration.[3–5]

According to these reports,[5–8] the parameters determining the toxic activity of TBT chloride resulting in heavy morphological anomalies of the embryos were established at a concentration of TBT chloride between 10^{-5} and 10^{-7} mol dm^{-3} and at incubation times ranging from 1 to 2 h.

In the present paper, investigations have been carried out, by light and electron microscopy, on larvae before hatching (Lot A) and on swimming larvae (Lot B) both incubated in 10^{-7} mol dm^{-3} TBT chloride for 1 h.

The light-microscopy observations suggested that the treated larvae of Lot A stopped developing and did not hatch. The tails of the larvae showed severe morphological anomalies; in particular, they appeared squat and shorter than in the swimming larvae. Also, larvae of Lot B were damaged by the action of TBT chloride, though to a lesser degree.

Ultrastructural modifications of mitochondria and myofibrils were also noted and produced the following results.

(1) The tails did not develop normally and did not show the movements necessary for the larva to hatch freely in the marine environment, by breaking the two envelopes of accessory cells. The loss of mobility of the tail can be related to the lack of energy (ATP) produced by the mitochondria, whose ultrastructure appeared strongly modified. This conclusion agrees with that of Engel and Fowler,[18] who demonstrated, following exposure to cadmium and copper derivatives, ultrastructural modifications in the mitochondria of molluscan gill tissue. In addition, this conclusion is also in

agreement with that of Reverberi,[19] who maintained that sodium azide, sodium malonate and sodium selenite inhibited mitochondrial enzymes (cytochrome oxidase and succinic dehydrogenase), thereby producing severe anomalies in larval development.

(2) Ultrastructural investigations showed that damage involving muscle cells in Lot B were not as severe as those observed in larvae of Lot A.

We conclude that our results confirm previous research,[3–5] according to which damage induced by toxicants is more important in early developing embryos. To explain this feature, we suggest that the coiled larvae stage, in which muscle cells are not completely differentiated, is more sensitive to toxicant action than the swimming larva stage, where cell differentiation is more advanced.

In conclusion, we propose that the toxic behaviour of the organotin(IV) derivatives could inhibit the functionality of several proteins, both functional and enzymic.

Similar results were reported by Mansueto *et al.*,[3–5] who suggested that the toxic effects of TBT chloride on embryo development would inhibit the functional activity of proteins of the cytomembrane, of the cytoskeleton and of tubulin. Also, Longwell and Hughes[20] showed, in *Scomber scombrus* mitosis blocked by the action of DBT dichloride, which prevented the formation of the mitotic spindle due to the inhibition of tubulin polymerization.

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