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Organometallic complexes with biological molecules: XVI. Endocrine disruption effects of tributyltin(IV)chloride on metamorphosis of the ascidian larva

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The effects of tributyltin(IV)chloride (TBT) on the metamorphosis of ascidian larvae of *Ciona intestinalis*, 2 h after hatching, were investigated. Ascidians are protochordates that lack thyroid follicles and possess thyroid hormones (THs) and their precursors, 3-monoiodo-tyrosine (MIT) and 3,5-diiodo-tyrosine (DIT), in their endostyle. According to recent findings, these hormones are also present at larval stages, localize in mesenchymal cells and their function seems to be mainly related to larval transformations. Here, we investigate the effects of TBT on thyroxine (T_4) content and localization by exposing larvae of *C. intestinalis* for different times to TBT concentrations known to block metamorphosis. The result is a blocking of the retraction of the tail, and larval immobility. As detected by immmunohistochemistry, in normal larvae, T_4 is found in all mesenchymal cells spread out in the body cavity, under the adhesive papillae and around the intestine. By contrast, in larvae exposed to 10^{-5} M and 10^{-7} M TBT, T_4 is detected only in 5% and 25% of total mesenchymal cells respectively. Radioimmunoassay shows a 70% decrease of T_4 content in TBT-exposed larvae, with respect to unexposed larvae. In addition, neosynthesis of THs is inhibited. Even if parallelism does not exist between the endocrine systems of invertebrates and vertebrates, however, a close similarity exists for functions such as reproduction and metabolism. Our results indicate that TBT could behave as an endocrine disrupter (ED) in ascidians and could impair T_4 metabolism. These findings suggest that the ED activity of TBT could be conserved from invertebrates to vertebrates. Copyright © 2001 John Wiley & Sons, Ltd.

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INTRODUCTION

Among the chemical endocrine disrupters (EDs), polychlorinated biphenyls and dioxins are implicated in impairing thyroid function.[1–5] In some vertebrates, like fish, birds and mammals, and in some invertebrates, like shellfish and gastropods, exposure to endocrine-disrupting chemicals present in the environment has been associated with abnormal thyroid function, decreased fertility, masculinization, male feminization, and alteration of immune function.[6]

Endocrine-disruptive effects of tributyltin(IV)-chloride (TBT) have been reported for females of some marine gastropod snails, causing the so called 'imposex condition', i.e. the development of male primary sexual characteristics.[7] TBT is one of the most toxic sea pollutants[8–11] and can also be considered an ED, as suggested by toxicological

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data and observations on animals and humans, where specific compounds (such as pesticides, etc.) are potentially capable of disrupting endocrine systems, provoking severe reproductive impairment, such as genital tract malformations, sexual behaviour alterations and reduced fertility.[6,12,13] At high concentrations of TBT Bryan *et al.*[8] reported reduced reproduction in field populations of *Nucella lapillus* (Muricidae). In this group of marine gonochorist snails, TBT is assumed to have interfered with normal androgen metabolism. In the Mediterranean sea, *Hexaplex trunculus* (Muricidae) has been studied by Terlizzi *et al.*[9] These authors report that TBT causes early anomalies of the genital system and that females are affected by imposex.

Endocrine-disruptive effects of TBT are not reported for other wildlife organisms. Experiments on laboratory animals, e.g. rats of different strains, should indicate any alteration of the endocrine system induced by TBT. Indeed, rats given food containing high dosages of bis(tri-*n*-butyltin)oxide show alteration of certain hormone levels, i.e. insulin, thyroxine (T_4) and thyroid stimulating hormone.[10,11]

The effects of TBT exposure on early embryonic stages, from egg fertilization to larva, have been analysed in ascidians, which are marine protochordates and are thus considered ancestors of vertebrates.[14–17] These studies have shown that all development stages are affected; moreover, the TBT-exposed larvae remain motionless and do not metamorphose.

Ascidian metamorphosis is a complex process in which various mechanisms seem to be involved. We have shown the presence and localization of thyroid harmones (THs) involved in larval metamorphosis, through biochemical and immunohistochemical means.[18–20]

THs and their precursors were detected many years ago, mainly in the endostyle, of adult ascidians, lacking thyroid follicles.[21] Considering the known TBT-inhibitory effect on metamorphosis,[14,17] the focus of this study is to investigate the possible action of TBT on larval TH metabolism.

MATERIALS AND METHODS

Biological material

Adult specimens of *Ciona intestinalis* were collected from the coasts of Palermo and Sciacca (Sicily). Female and male gametes were removed from the gonoducts of dissected animals and transferred into Syracuse dishes with Millipore filtered sea water (MFSW) at pH to a final suspension of 7–8. Dry sperm was diluted before insemination approximately 0.1% v/v. The experiments were performed at 22 °C.

Swimming larvae were collected 24 and 48 h after fertilization by gentle centrifugation and used for subsequent TBT-exposure experiments.

Exposure to TBT solutions

TBT was a kind gift from Witco GmbH (Bergkamen, Germany).

Concentrated stock solutions were obtained by dissolving stoichiometric amounts of the compound in 0.07% dimethylsulfoxide (DMSO) containing MFSW. The total tin content was checked as previously reported.[22] Working solutions (pH 7.25–8.5) were obtained by further dilution of the stocks in MFSW.

Freshly prepared 10^{-5} and 10^{-7} M TBT solutions were used.

Larvae of *C. intestinalis*, after hatching (24 h after fertilization), were transferred and reared in the two solutions of TBT for different time periods; some lots were cultured for 3 h, others for 24 h, and then cultured in MFSW until the control larvae were metamorphosed. To verify potential reversibility of TBT-induced effects, after exposure the larvae were washed multiple times with TBT-free MFSW, transferred to TBT-free MFSW and analysed for possible recovery.

Histological and immunocytochemical processing

Larvae of *C. intestinalis* were fixed with cold methanol or with 10% buffered formaldehyde. After dehydratation, the specimens were embedded with paraffin using standard procedures. Serial 4 μm thick sections were prepared for haematoxylin/eosin staining.[23] For immunohistochemical staining, the sections were washed with phosphate-buffered saline (PBS) and permeabilized for 30 min with 0.2% Triton X-100 and 0.1% Tween 20 in PBS. The sections were incubated in 0.1% gelatin in PBS for 1 h to block aspecific binding sites. After washing in PBS and Tween 20, the intrinsic peroxidase was inactivated with 0.01% H_2O_2–methanol solution at room temperature (RT) for 1 h. After an additional wash in PBS and Tween 20, the sections were incubated with a rabbit polyclonal

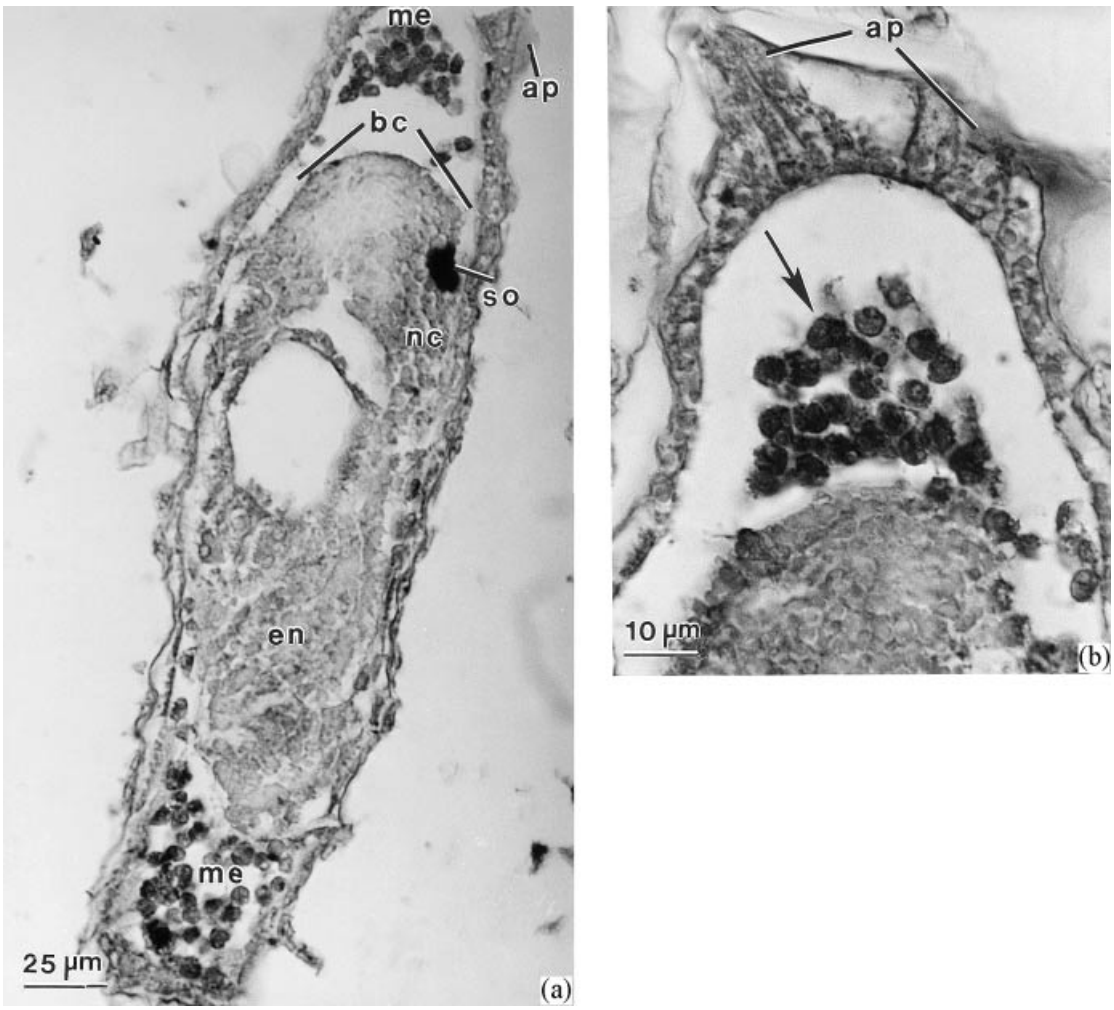

Figure 1 Longitudinal sections of *C. intestinalis* larvae showing immunocytological localization of T_4. (a) Immunohistochemical staining of mesenchymal cells spread out in body cavity and under the adhesive papillae. (b) Arrow indicates mesenchymal cells positive to immunoperoxidase reaction: ap = adhesive papillae; bc = body cavity; en = endodermal cells; me = mesenchyme cells; nc = nervous cells; so = sensory organs.

anti L-T_4 antibody (Sigma) at 1:20 dilution at 4 °C overnight. Detection of bound antibodies was carried out with a horseradish-peroxidase-conjugated goat anti-rabbit IgG secondary antibody (BioRad) used at 1:50 dilution for 30 min at RT, followed by visualization with 0.05% 3.3′-diaminobenzidine tetrahydrochloride solution (Sigma) in Tris–HCl buffer (0.05 M, pH 6.8) containing 0.05% H_2O_2, at 20 °C for 20 min and blocked in distilled water.

In controls, the primary antibody was omitted.

Extraction of THs

Extraction of THs was carried out essentially as described by Gordon *et al.*[24] with some modifications. Swimming larvae, 24 and 48 h after fertilization, as well as TBT-exposed larvae, were collected by centrifugation at 400*g* for 15 min. The pellet was homogenized with three to five ml of a 2:1 chloroform/methanol mixture. The supernatant was obtained by centrifugation at 1940*g* for 10 min at 4 °C. A one-fifth volume of 0.05% $CaCl_2$ was

added to the supernatant, which was mixed and allowed to stand on ice until separation of the two phases. After methanol removal, the extracts were dissolved in 50 µl of 0.01 M NaOH and neutralized with 100 µl of 0.1 M Tris–HCl buffer (pH 8.2) and 50 µl of 0.01 M HCl for radioimmunoassay (RIA).

RIA

RIA of T_4 was performed from extracts of control larvae (24 and 48 h after fertilization) and from those exposed to 10^{-5} and 10^{-7} M TBT solutions for 3 or 24 h. The samples for each experiment (six) were assayed three times and in duplicate. T_4 content was determined using a Cambridge Life Sciences plc, UK, (CLS) FT_4RIA kit according to the directions provided for its use. T_4 (Sigma) was dissolved in small amounts of 0.05 M NaOH, then diluted to various concentrations and used as standard solutions.

Determination of protein content

Total protein contents of control and TBT-exposed larvae were determined according to Bradford,[25] using bovine serum albumin (BSA) as a standard.

RESULTS

The first steps of ascidian metamorphosis

Almost all solitary ascidians have an indirect development with a planktonic free-swimming larva. The larva has a single body plan; it is formed by a trunk and a tail and consists of a few thousand cells and only six different tissues: epidermis, endoderm, nervous system, notochord, muscle, and mesenchyme. The ascidian larva is considered a prototype of the ancestral chordate.[26,27] Despite the reduced complexity, the larva exhibits the hallmarks of a chordate: a dorsal central nervous system, a notochord and a ventral gut.

The transition between pelagic and benthonic existence involves two processes: settlement and metamorphosis. Settlement is the process of locating and affixing to the juvenile habitat; it generally precedes metamorphosis and includes the attachment to a substrate by a sticky cementing substance secreted from the adhesive papillae at the anterior end of the head.

Metamorphosis is the sequence of morphological events that transform the larva into a sessile, feeding juvenile. This process includes: resorption of the tail, a 90 ° rotation of the trunk, migration of blood cells from the haemocoele to the tunic, retraction of the sensory vesicle and destruction of larval structures.[28–30]

We have previously shown that metamorphosis is controlled by THs.[19,20] In these studies, T_4 was found to be present in all mesenchymal cells of the larva (Fig. 1) and its synthesis inhibited by 0.5 to 2 mM thiourea (TU) solutions. Furthermore, ascidian larvae reared in Tu medium do not metamorphose and the content of T_4 is decreased. The results show purified T_4 concentrations of 0.287 ± 0.114 ng mg^{-1} in normal larvae and 0.176 ± 0.031 ng mg^{-1} protein in TU-treated larvae.

Exposure of larvae to TBT

Swimming larvae of *C. intestinalis*, 2 h after hatching, were collected, transferred to 10^{-5} and 10^{-7} M TBT solutions and reared in these media for different time periods, from 2 to 24 h. After 30 min of exposure to 10^{-5} M TBT, the larvae were motionless at the bottom of the culture dish and remained in this condition without metamorphosing. After a further 3 h of TBT-exposure the larvae appear corroded and in the course of cytolysis. Only a few larvae retract a quarter of the tail (Fig. 2a–c).

The histological sections of the larvae, stained with haematoxylin/eosin, show destruction of the nervous system and the endoderm and dilatation of the haemocoele cavity. A thin epithelial layer covers the larval body, and is almost completely devoid of structures. In some sections, remnants of sensory organs are visible. However, it is evident that almost all the mesenchymal cells are intact, and they seem to be more numerous than in the controls (Fig. 3a and b).

Chordal cells and muscle cells are present in the tail but are damaged (Fig. 3a). The damage is permanent, even in larvae exposed to TBT for only a few hours, as no recovery is observed after multiple washes and transfer to TBT-free MFSW.

Larvae exposed to 10^{-7} M TBT react better (Fig. 3d). They continue to swim slowly or contract for several hours before falling to the bottom of the culture dish. About 50% of them begin to retract half of the tail, 10% retract all the tail and the remainder show immobility.

The sections of these larvae show that all tissues are intact, even if a certain defective structure is evident; a few cells are destroyed and the haemocoele cavity is a little dilated (Fig. 3c and d).

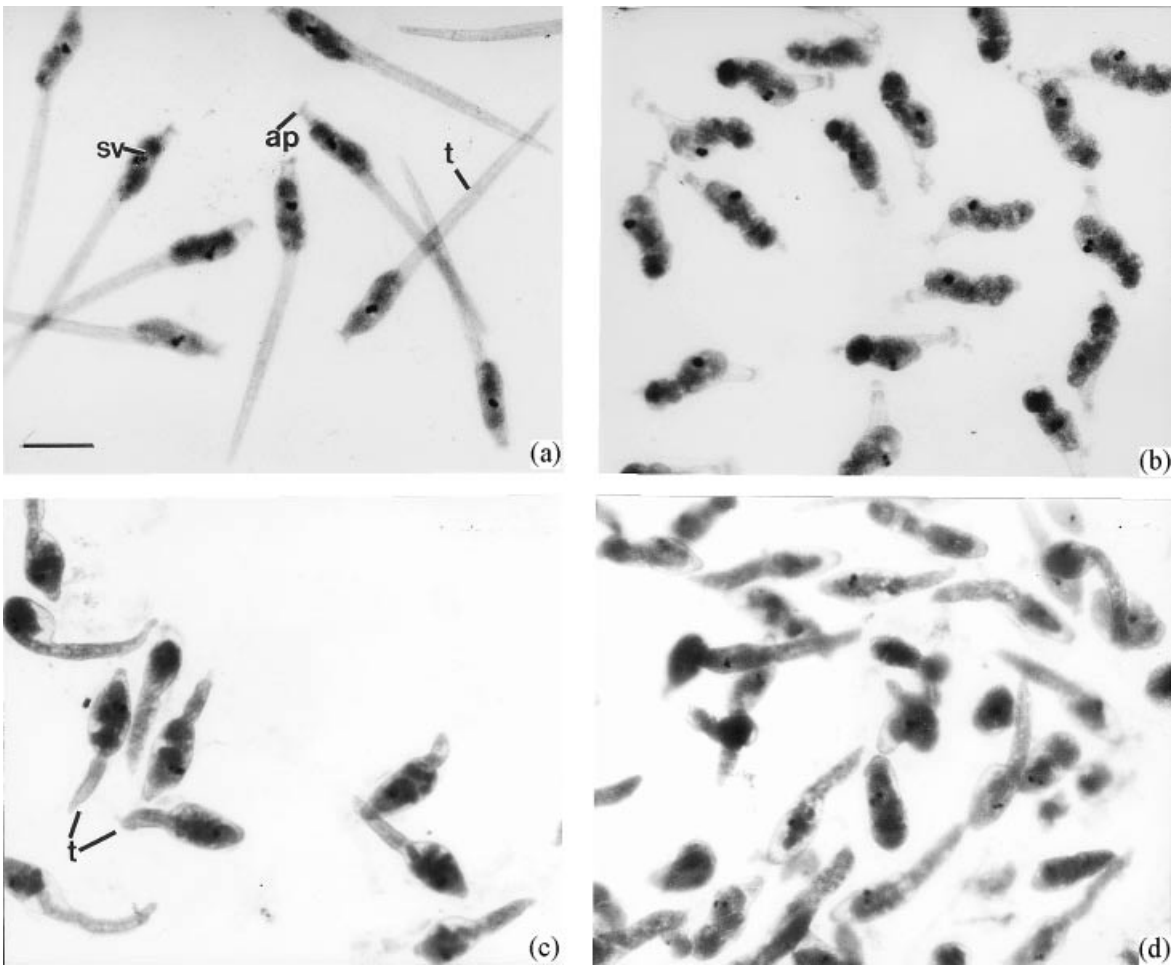

Figure 2 Larvae of *C. intestinalis*. Control larvae (a) 24 h and (b) 48 h after fertilization. Larvae exposed for 24 h to (c) 10^{-5} M and (d) 10^{-7} M TBT solutions: ap = adhesive papillae; sv = sensory vesicle; t = tail. Scale bar represents 200 μm and refers to all figure parts.

By contrast with the larvae exposed to 10^{-5} M TBT, larvae exposed to 10^{-7} M TBT for a few hours are able to recover and retract the tail after multiple washes and transfer to TBT-free MFSW.

Immunohistochemical localization of T_4

Sections of TBT-exposed larvae were stained with anti-T_4 polyclonal antibody and detection of bound antibodies was carried out with a horseradish-peroxidase-conjugated secondary antibody followed by visualization with an appropriate reaction substrate. Exposed larvae show staining of only a few cells of the trunk. Larvae exposed to 10^{-5} M TBT show dots or pockets of lightly stained material in about 5% of mesenchymal cells spread out in the haemocoele cavity (Fig. 3a and b); larvae exposed to 10^{-7} M TBT show a much more dense spotted staining in about 25% of mesenchymal cells (Fig. 3c and d).

RIA for T_4

Determination of T_4 content in extracts of TBT-exposed and unexposed (control) larvae of *C. intestinalis* was carried out with RIA. The T_4 content in control larvae 24 h and 48 h after fertilization is 0.28 ± 0.10 ng mg^{-1} and 0.37 ± 0.15 ng mg^{-1} of total protein respectively. Larvae exposed to 10^{-5} M TBT for 3 h and 24 h show a T_4 content of 0.10 ± 0.02 ng mg^{-1} and $0.09 \pm$

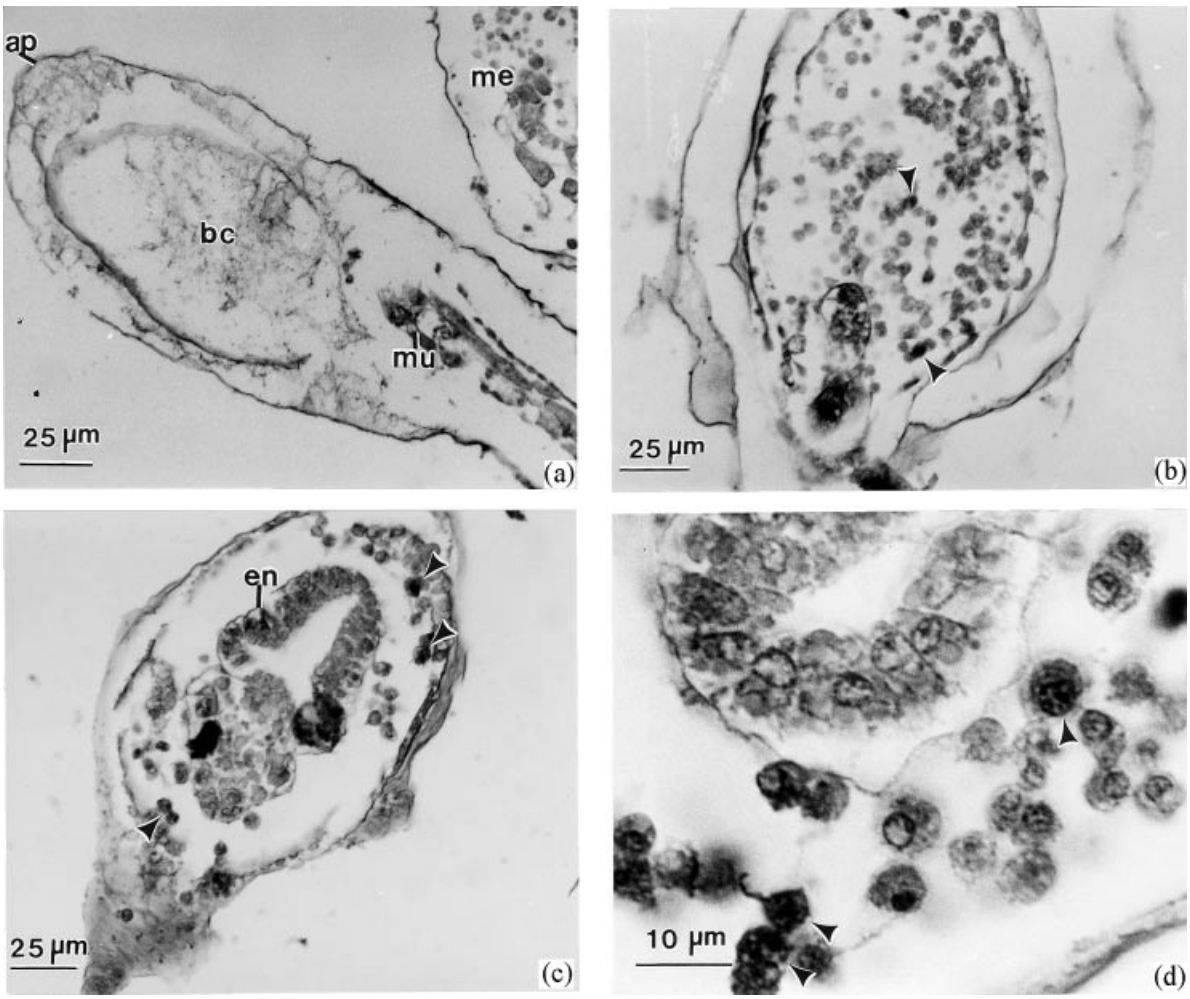

Figure 3 Longitudinal sections of larvae of *C. intestinalis* exposed to TBT solutions. (a,b) Larvae treated for 24 h with 10^{-5} M TBT solution in which the haemocoelic cavity is enlarged and is full of mesenchyme cells. Arrowheads indicate only a few cells stained with anti-T_4 antibody (about 5%). (c,d) Larvae treated with 10^{-7} M TBT solution; arrowheads indicate slightly more numerous stained mesenchymal cells (about 25%). ap = adhesive papillae; bc = body cavity; en = endodermal cells; me = mesenchyme cells; mu = muscle cells.

$0.02\ ng\ mg^{-1}$ total protein respectively. Larvae exposed to 10^{-7} M TBT for 3 and 24 h, show a T_4 content of $0.12 \pm 0.03\ ng\ mg^{-1}$ and $0.12 \pm 0.05\ ng\ mg^{-1}$ total protein respectively. The data are summarized in Fig. 4, in which the content of T_4 is compared with the T_4 standard curve.

DISCUSSION

We have found that ascidian larvae possess T_4, a TH that has been associated with metamorphic processes.[18–20] As in amphibians, THs also control larval transformations in ascidians, as shown by the results obtained by exposing larvae of *Ascidia malaca* to exogenous T_4[18] and by inhibition of tail retraction and resorption by exposure of larvae to Tu.[19,20]

Using immunohistochemistry, the presence of T_4 in normal larvae of *C. intestinalis* has been localized to mesenchymal cells, many of which will be the future blood cells. In this study, we demonstrate that T_4 molecules of the larvae of these protochordates are strongly affected by TBT, which not only blocks metamorphosis, but also reduces by

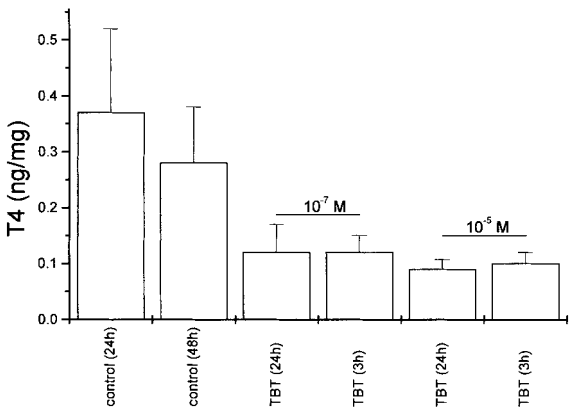

Figure 4 T_4 content in methanol–chloroform extracts of control and TBT-exposed larvae of *C. intestinalis*. The values represent the average of three samples determined in duplicate.

70% the amount of the hormone. In vertebrates, endocrine-disrupting chemicals act on thyroid biosynthesis, impairing the production of THs, or blocking hormone-receptor binding. TBT is a compound that can also react directly or indirectly with a hormone in invertebrates, altering its structure or interfering with its biosynthesis; indeed, our data indicate that TBT is an ED in ascidians, invertebrates lacking thyroid follicles, which possess, however, THs in larval tissue.

This xenobiotic probably alters and destroys almost all T_4 molecules present in mesenchymal cells and blocks its neosynthesis. Even in larvae exposed to the lowest TBT concentration used in this study, despite the integrity of all tissues, T_4 is found only in a few cells and the content of the hormone is substantially decreased, as independently confirmed by RIA even after 3 h of exposure.

These data clearly indicate that, in addition to the many drastic effects induced by the xenobiotic on embryo development, a major portion of TBT toxicity is attributable to its ED function.

Indeed, the toxic effects of TBT on early stages of ascidian embryonic development have been examined by electron microscopy and biochemical analyses.[14–17,31,32] TBT-exposed embryos presented strong anomalies and blocking of development. The hypothesis suggested by previous authors was based on TBT-induced cytoskeletal and chromosomal damage, alteration of cytoplasmic organelles and cell metabolism, leading to inhibition of larval movement. The subsequent events of metamorphosis are clearly linked to the endocrine-disrupting effect of the chemical.

TBT also induces high embryonic mortality and malformations, reduction of veliger and post-larval length and absence of metamorphosis in bivalve mussels.[33–36]

SIGNIFICANCE

THs are present in ascidian larvae (Urochordatae), and their function is related to the control of metamorphosis. Invertebrates do not have thyroid tissues; nevertheless, some of them possess thyroid hormones and their precursors (T_3, T_4, MIT, and DIT).[37] Among the invertebrates able to synthesize THs, adult ascidians have phylogenetic importance, as the body plan of their larvae is a basic model of vertebrate morphogenesis.

Ascidians and amphioxus, which are protochordates, together with the ammocoete of the lamprey, a primitive chordate, concentrate iodide and synthesize THs in a subpharyngeal afollicular endostyle. This structure is considered a thyroid homologue. In the larva of the lamprey, the endostyle reorganizes into a follicular thyroid at metamorphosis to the adult, but in protochordates it never transforms into a follicle. A close histological resemblance of the ammocoete and the protochordates shows the homology of these organs. The endostyle is able to carry out thyroid biosynthesis, and the conclusion is that the characteristic molecules of the thyroid gland are already present in protochordates, the ancestors of vertebrates. In the present study, we have demonstrated that THs of ascidian larvae are strongly affected by TBT, which is an ED compound, destroying the thyroid molecule and blocking its neosynthesis. As the clinical use of some potent synthetic oestrogen diethylstilbestrol provides human data that can be compared with those obtained in experimental systems, we hypothesize that TBT could also block and destroy thyroid molecules in man.

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