APPLIED ORGANOMETALLIC CHEMISTRY
Appl. Organometal. Chem. 2005; **19**: 11–22
Published online in Wiley InterScience (www.interscience.wiley.com). DOI:10.1002/aoc.764

Effects of tri-*n*-butyltin (IV) chloride on neurulation of *Ciona intestinalis* (Tunicata, Ascidiacea): an ultrastructural study

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Received 24 May 2004; Accepted 23 July 2004

This paper reports the cytotoxic effects of tri-*n*-butyltin (IV) chloride, TBTCl, on the neurulation process of the ascidian *Ciona intestinalis*. Exposure of the embryos at early neurula stage in 10^{-5} and 10^{-7} M TBT (IV) chloride solutions for 1–2 h provoked the irreversible arrest of their development. Morphological and ultrastructural observations suggested that most probably there are two principal causes determining the neurulation process block. The first is due to the TBT effects of inhibiting the polymerization and/or degradation of microfilaments and microtubules, proteins that constitute the cytoskeleton. The lack of orientation and extension of both microtubules and microfilaments of actin prevent the shape changes and mobility of neural plate blastomeres indispensable to the neurulation process. The second cause is certainly determined by the ultrastructural modification which mitochondria undergo. The ultrastructural anomalies showed by these organules are so serious as to impede their proper functionality with consequent inhibition of oxidative phosphorylation and ATP synthesis, remarkable metabolic processes that occur during ascidian neurulation. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: tributyltin (IV) chloride; neurulation; ascidian

INTRODUCTION

In recent years there has been a consistent literature accumulation concerning the long-term environmental impact of tri-*n*-butyltin(IV) (TBT) compounds on the survival and reproduction of both marine and terrestrial species.[1,2] TBT is an effective long-action biocide that mainly contaminates aquatic systems owing to its past employment in anti-fouling paints, pesticides and also via discharge of wastewaters and dumping of sewage sludge.[3,4] Recent studies have comprehensively analysed the conditions under which TBT becomes a serious threat to local biodiversity. Much of the focus has been on the harmful role played by TBT compounds on non-target marine invertebrate and vertebrate species.[5–12] The risk posed by concentrated TBT compounds is very high for marine species because of their hydrophobic nature, slight solubility in seawater and highly variable half-life,[13] and they are thus easily accumulated by dietary uptake (biomagnification) and slowly leached from organs or tissues.[14–19]

Numerous investigations have proven chronic and acute effects in filter-feeding benthonic invertebrates, such as bivalves and tunicates, which, after exposure, bioaccumulate into their lipophilic compartments high levels of TBT compounds.[1,3,20] The best documented cases of TBT's harmful effects involve the progressive decline of the production of veligers and recruits in *Crassostrea gigas*[21] (Bay of Arcachon between 1975 and 1982) and simultaneously the presence of abnormally thickened shell in adults. Evidence of the endocrine-disruptive effects of TBTCl have been reported for sexually mature females of some marine gastropods.[22] In particular, TBTCl induces reproductive abnormalities and

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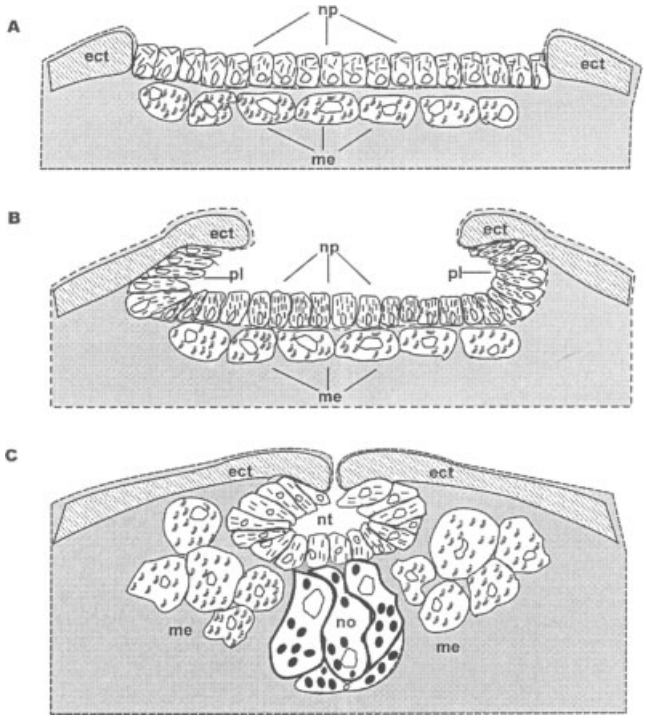
Contract/grant sponsor: Ministero dell'Istruzione, dell'Università e della Ricerca; Contract/grant number: 2001053898_002.
Contract/grant sponsor: Università di Palermo.

sterilization in female specimens, leading to reproductive failure and to population decline in marine gonochoristic gastropods.[23] This phenomenon, which has been called either pseudohermaphroditism[24] or imposex,[25] is characterized by the development of additional male sex organs (penis and/or vas deferent and prostate tissue) in females.[1,26–29] On cultured haemocytes of the colonial ascidian *Botryllus schlosseri*, TBT exposure caused apoptosis processes and internal disorganization of the cytoskeleton.[30] Furthermore, structural chromosomal damages, such as achromatic lesions and chromosome breakages, have been identified on the spermatocyte chromosomes of fish and molluscs.[31–33] TBT compounds lead to inhibition of synthesis of ATP, lipids and nucleic acids while increasing some enzymatic activity in the ovary of *Ciona intestinalis*.[34] Ultrastructural investigations carried out on both female and male gametes of *Styela plicata* showed that exposure to TBT markedly inhibits the mobility of the spermatozoa and produces abnormalities in egg envelopes, all anomalies preventing the fertilization process.[35] Early studies on the biological effect of TBT dealt primarily with consequences on adult specimens,[2] while recently several studies have started to analyse the harmful consequences on early development stages. Harmful biological effects of TBTCl have been detected on larval development of different invertebrates, e.g. bivalves *Mitilus edulis*,[36] and *Crassostrea gigas*[21] and the sea urchin *Paracentrotus lividus*.[37] The impact of TBT compounds on the development and survival of the different embryos[2] is often used as a biological indicator of the degree of TBT sea water contamination.[1,3,20,38] Evidence of an embryonic block in the early developmental stages has been reported, after TBT exposure, for ascidian larvae of *C. intestinalis*.[39–41] Furthermore, there is evidence that TBT derivatives compromise the hatching of coiled larvae of *C. intestinalis* and provoke irreversible mobility loss of their swimming larvae.[42] Recent studies in *C. intestinalis* larvae proved that TBT could behave as an endocrine disrupter. The final result of which was a metamorphosis blocking.[41] The detrimental effects of TBT on all embryonic stages of the ascidian *Styela plicata*, and in particular on the gastrula and neurula stages, have been demonstrated.[43]

However, there is no information at the ultrastructural level concerning the impact and mechanisms of TBT action during the developmental stage of neurulation. From a morphogenetic and organogenetic point of view, neurulation is a crucial moment for the embryonic development as the neural tube and the nervous system are forming. Furthermore, ascidian neurulation involves a series of shape modifications and cellular arrangements that are comparable to those occurring in vertebrates, including mammals.[44]

As a verification, the impact of TBTCl on the neurulation process of the ascidian *Ciona intestinalis* was examined. Morphological and ultrastructural observations were carried out using embryos at early neurula stage as controls. In particular, it was investigated whether TBT compounds may cause possible cellular alterations in embryos at morphological and ultrastructural level.



Scheme 1. Shows the main patterns of ascidian neurula stages. Beginning of neurulation is characterised by the forming of the neural plate (A). In the course of neurulation, cells located at the edges of the neural plate thickened, forming folds (B) that, after raising and converging, folded together to produce the neural tube (C).

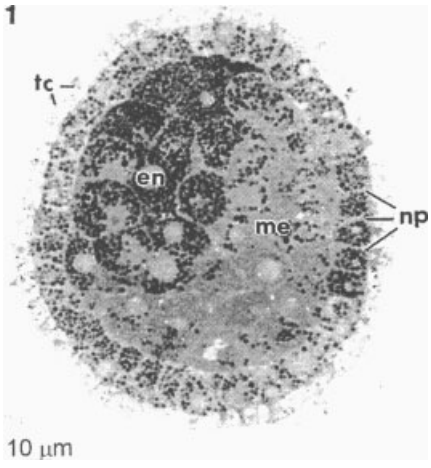
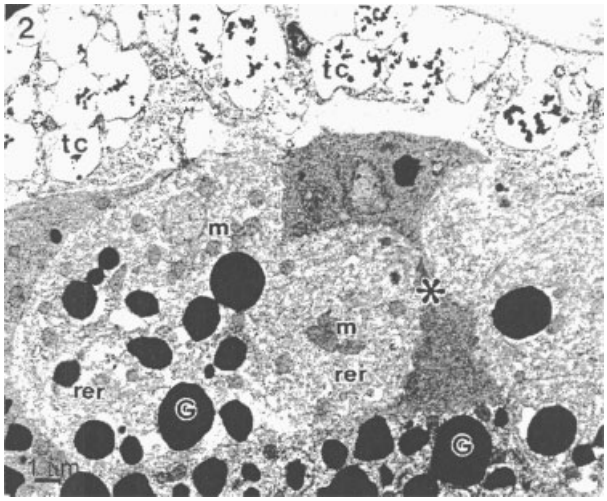
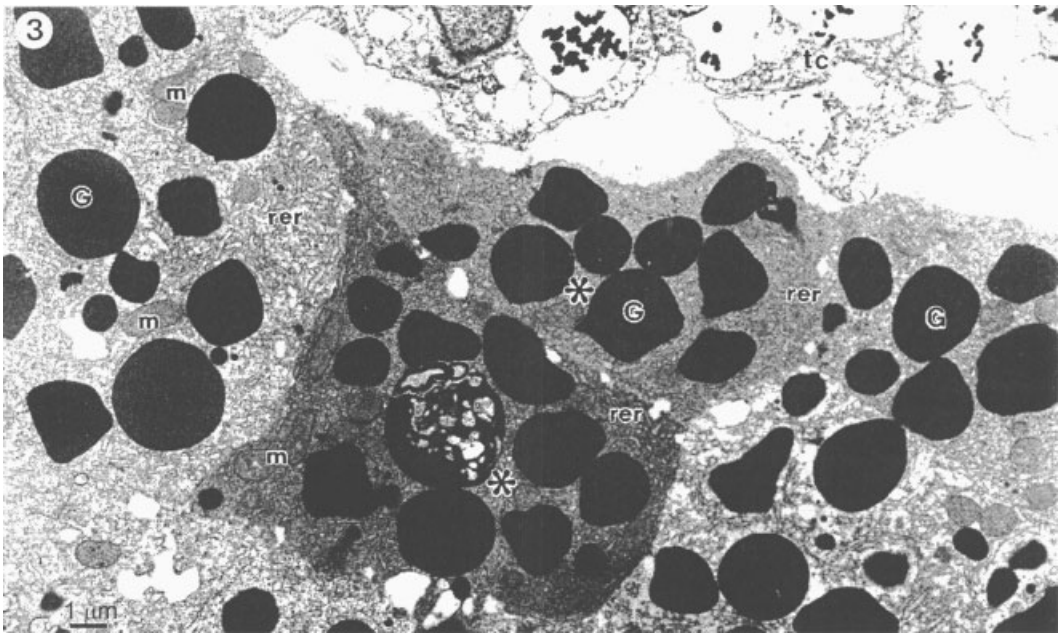


Figure 1. (Lot A.) Semithin parasagittal section of *Ciona intestinalis* embryos at early neurula stage. The embryo is surrounded by a layer of test cells (tc); the neural plate (np) can be noted in the dorsal region made up of a monolayer of cells directly located upon the mesoderm (me). Endodermic cells (en) are present in the ventral region. (Bar = 10 μm).





Figures 2 and 3. (Lot A.) Control *Ciona intestinalis* embryos at early neurula stage. TEM observations show that blastomeres forming the neural plate are characterized by a partly roundish and a partly lengthened shape, with occasional sandglass-shaped cells noted (Fig. 2). Blastomeres present in the marginal areas of the neural plate showed an irregular lengthened shape with occasional bottle-shaped cells (*) (Fig. 3). m, Mitochondria; G, yolk granules; rer, rough endoplasmic reticulum. (Bar = 1 µm.).

MATERIAL AND METHODS

Adult specimens of *Ciona intestinalis* were collected from the Gulf of Palermo and in the harbour of Trabia and Termini Imerese, from July to November 2003. The healthiest specimens were transferred to an aquarium and kept at 16–18 °C. Under these conditions sexually mature individuals could be maintained for up to one month. Following gamete removal, fertilization occurred in Syracuse dishes containing pasteurized and Millipore-filtered sea water (MSFW). After fertilization the embryos were reared to the gastrula and neurula stages and fixed using standard procedures.

In particular, observations were carried out by both light and electron microscopy on the following lots:

- *lot A*—control embryos at neurula stage, developed in filtered and sterilized sea water;
- *lot B*—embryos at early neurula stage maintained for 1 h in 10^{-7} M TBTCl solutions in MSFW containing 0.07% v/v DMSO and then transferred into pure MSFW;
- *lot C*—embryos at early neurula stage maintained for 1 h in 10^{-5} M TBTCl solution in MSFW containing 0.07% v/v DMSO and then transferred into pure MSFW.

The tri-*n*-butyltin(IV)chloride was an Alfa Aesar (Johnson Matthey GmbH, Karlsruhe, Germany), and was used without further purification. Concentrated 10^{-4} M stock solutions were obtained by dissolving stoichiometric amounts of the TBTCl in MFSW containing 0.07% v/v DMSO. Freshly prepared tri-*n*-butyltin (IV) chloride working solutions (pH = 7.25–8.50) were obtained by further dilution of the concentrated stock in MFSW and by adding further DMSO up to 0.07%. TBTCl concentrations and solution stability were checked as previously reported.[31]

Light microscopy

Embryos of *Ciona intestinalis* of lots A, B and C were fixed in solutions containing 2.5% glutaraldehyde, 0.2 M phosphate buffer (pH = 7.5) and postfixed in 1% osmium tetroxide dissolved in the same buffer solution. The fixed material was dehydrated in a graded ethanol series and embedded in Epon 812.[45] Semithin sections (1–2 μm thick) obtained with the Ultracut E (Reichert-Jung) microtome, were stained for 5 min with 5% Toluidine blue at pH = 2.5.[46] Sections were observed and photographed with a Leitz Orthoplan microscope, using Ilford FP4 plus film.

Transmission electron microscopy

For ultrastructural observations

Ciona intestinalis embryos of lots A, B and C were fixed in solutions containing 2.5% glutaraldehyde, 0.2 M phosphate buffer (pH = 7.5) and postfixed in 1% osmium tetroxide dissolved in the same buffer solution. The fixed material was dehydrated in a graded ethanol series and embedded in Epon 812.[45] Ultrathin sections obtained with the Ultracut E (Reichert-Jung) microtome were contrasted with uranyl acetate and lead citrate[47] and photographed with Phillips EM 410 at 80 kV accelerating voltage using Kodak electron microscope film (Estar thick base 4489).

Tannic acid reaction

Ciona intestinalis embryos of lot A were fixed in solutions containing 2.5% glutaraldehyde, 4% tannic acid in 0.2 M phosphate buffer (pH = 7.5) solution and postfixed in 1% osmium tetroxide dissolved in the same buffer solution. The fixed material was dehydrated in a graded ethanol series and embedded in Epon 812.[45] Ultrathin sections obtained with the Ultracut E (Reichert-Jung) microtome were contrasted with uranyl acetate and lead citrate.[47]

According to literature data, tannic acid acts as a supplementary fixing agent and, if used together with glutaraldehyde and osmium tetroxide, highlights the presence of polypeptides, both simple and conjugated as glycoproteins.[48]

RESULTS

Schematic drawing of ascidian neurulation

Scheme 1 shows the main patterns of ascidian neurula stages. The beginning of neurulation was characterized by the neural plate formation, made up of a mono-layer of ectodermic cells immediately dorsally placed upon the mesodermic cells (A). In the course of neurulation, cells located at the edges of the neural plate thickened, forming folds (B) that, after raising and converging, folded together to produce the neural tube (C). At the end of the neurulation process the neural tube was located under a layer of epidermic cells; the mesodermic cells were disposed under the neural tube near the lateral regions of the notochord (C).

Light and electron microscopy observations

Controls: embryos at neurula stage (lot A)

Observations made with a light microscope on semithin sections of control embryos at the early neurula stage of *C. intestinalis*, underlined the position of different presumptive territories of the embryo. The embryo was wholly ringed by test cells and its dorsal region was characterized by the presence of the neural plate, made up of a monolayer of cells directly located upon the mesoderm (Fig. 1). Endoderm cells were present in the ventral region of the embryo directly under the mesodermic cells. Section analysis highlighted some features regarding both the morphology and nature of cytoplasm present in blastomeres of different embryonic regions. Cells of the neural plate showed a partly roundish and a partly columnar, lengthened shape. In their cytoplasm a small quantity of yolk granules was also found (Fig. 1). Mesodermic cells showed a roundish shape and their cytoplasm, in which a large nucleus was present, characterized by

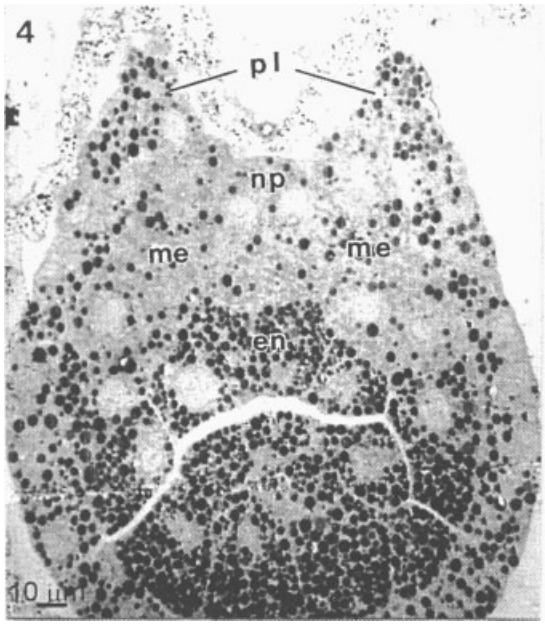
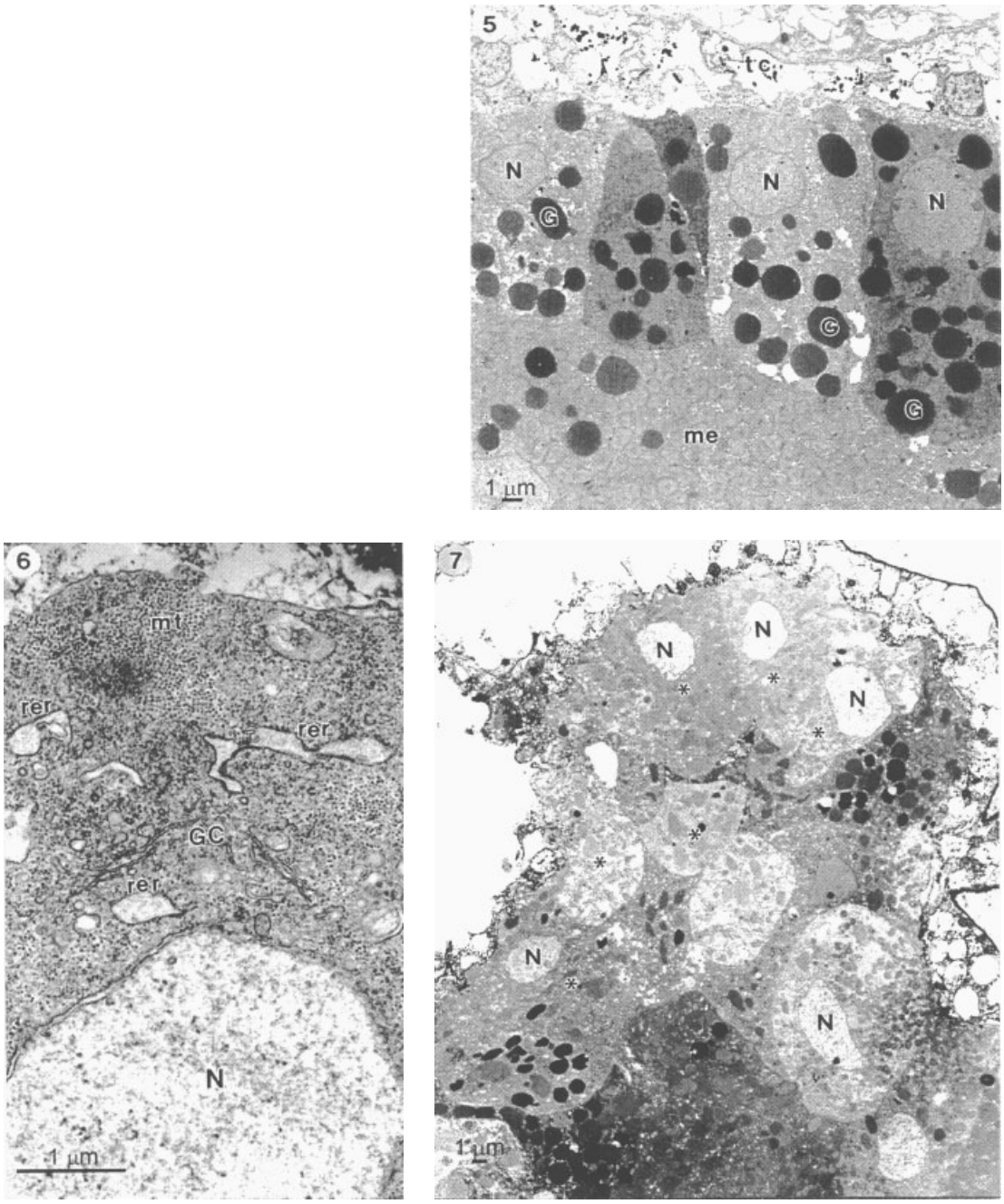


Figure 4. (Lot A.) Semithin transversal section of *Ciona intestinalis* embryos during the neurulation. The progression of neurulation is characterized by the raising of the outer marginal regions of the neural plate and the forming of neural folds (pl). (Bar = 10 μm.).

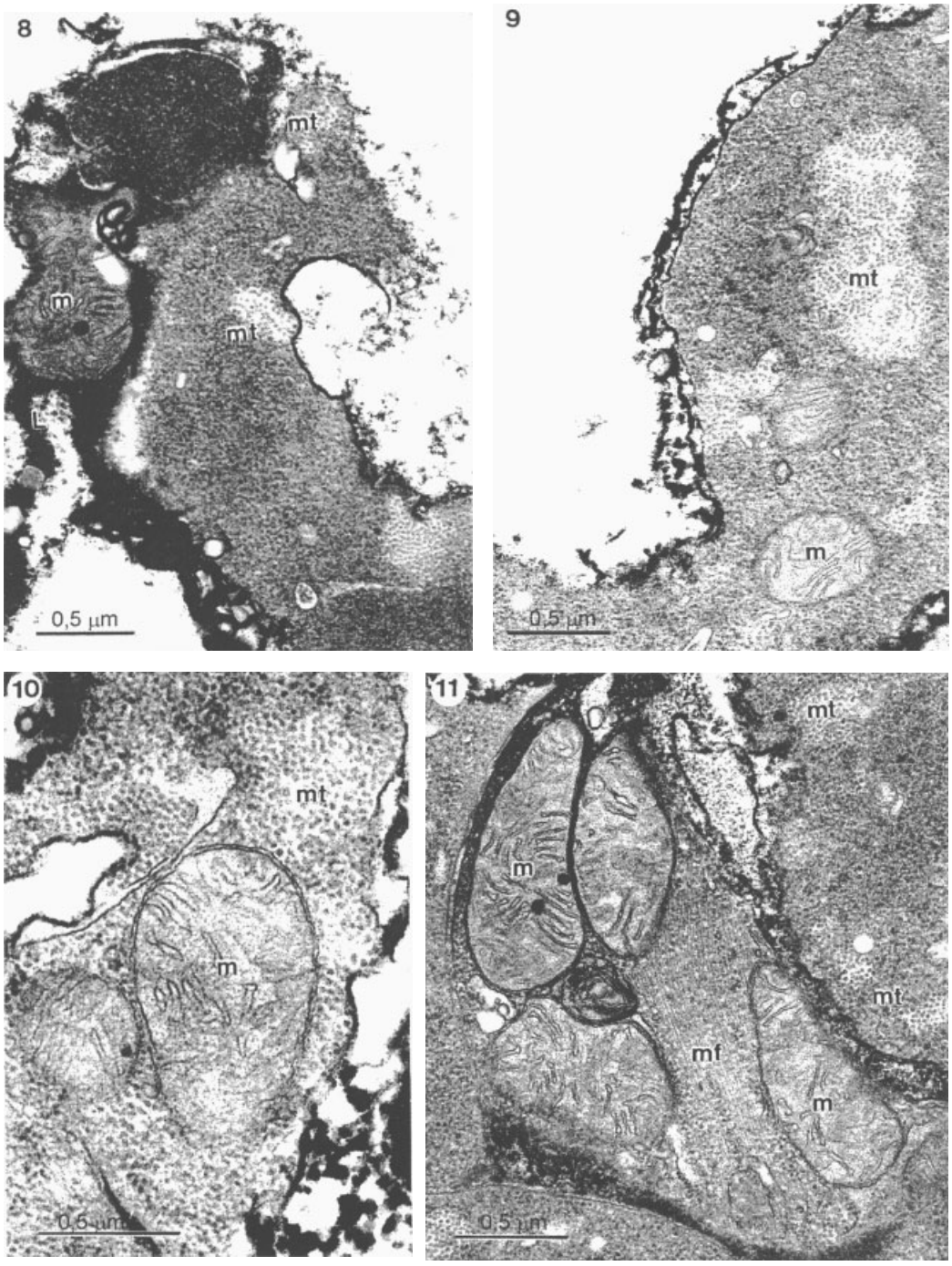


Figures 5–7. (Lot A.) *Ciona intestinalis* embryos during the neurulation. TEM observations have underlined that neural plate blastomeres show a lengthened shape (Fig. 5). Neural plate blastomeres show in their cytoplasm, mithocondria, a Golgi's complex (GC) and vesicles of rough endoplasmatic reticulum (rer). The outer region of the cytoplasm of these cells is characterized by the presence of numerous microtubules (mt) (Fig. 6). Blastomeres (*) are present in the neural fold (Fig. 7). N, nucleus. (Bar = 1 µm.).

a great number of mitochondria. Endodermic cells, present in the ventral region of the embryo, were characterized by the presence of a great number of yolk granules (Fig. 1).

TEM observations confirmed that blastomeres forming the neural plate were characterized by a partly roundish and a partly lengthened shape, with occasional sandglass-shaped cells noted (Fig. 2). Blastomeres present in the marginal areas of the neural plate, involved in the raising of the neural folds, also showed an irregular lengthened shape with occasional bottle-shaped cells noted (Fig. 3).

Light microscope observations showed that process of the neurulation was characterized by the raising of the outer marginal regions of the neural plate and the forming of the neural folds (Fig. 4). TEM observations showed that



Figures 8–11. (Lot A.) *Ciona intestinalis* embryos during the neurulation fixed in tannic acid. Microtubules can be observed inside large extensions of blastomere plasma membrane (Fig. 8). Transversal sections of embryo at neurula stage show that microtubules are similar to ring-shaped structures (20–25 nm in diameter; Figs 9 and 10). Longitudinal sections have also shown the presence of sheaths of actin microfilaments (mf) in the apical cytoplasm of some neural fold blastomeres (Fig. 11). (Bar = 0.5 μm.).

neural plate blastomeres showed a lengthened shape and, in their cytoplasm, yolk granules and a large nucleus were observed (Fig. 5). Blastomeres that were present in the neural fold exhibited an irregular shape that sometimes was lengthened (Fig. 7). Neural plate blastomeres, at high magnification, showed in their cytoplasm mitochondria with orthodox configuration, a Golgi's complex formed by a few flattened lamellae and many swollen vesicles of rough endoplasmatic reticulum (Fig. 6). The outer region of the cytoplasm of these cells was characterized by the presence

of numerous microtubules (Fig. 6). It was also possible to observe microtubules inside large extensions, similar to pseudopodia, of plasma membrane of these cells (Figs. 8–10). The presence of microtubules was pointed out using a post-fixing technique with tannic acid, which emphasized very well cellular structures with both simple and conjugated polypeptides. Transversal sections of neurula specimens fixed with tannic acid showed that the microtubules of blastomeres of the neural plate marginal area are similar to ring-shaped structures (20–25 nm in diameter, Fig. 10). Longitudinal sections also showed, at ultrastructural level, the presence of sheaths of actin microfilaments in the apical cytoplasm of some neural fold blastomeres. These sheaths run parallel to the major cellular axis (Fig. 11).

Effect of 10^{-7} M TBTCl solution on the neurula stage (lot B)

Light microscopy observations carried out on *C. intestinalis* embryos at early neurula stage maintained for 1 h in 10^{-7} M TBTCl (lot B) have reported evidence of an embryonic block in the developmental stage. Observations carried out on embryo sections revealed severe anomalies after TBTCl exposure. In addition to the missed raising of neural folds, in different territories a significant blastomeres disorganization was noted, blastomeres being disaggregated and separated by wide spaces (Fig. 12).

Ultrastructural investigations showed that embryonic cells of the ectodermic neural layer of the dorsal region maintained a roundish shape and did not present particular evaginations,

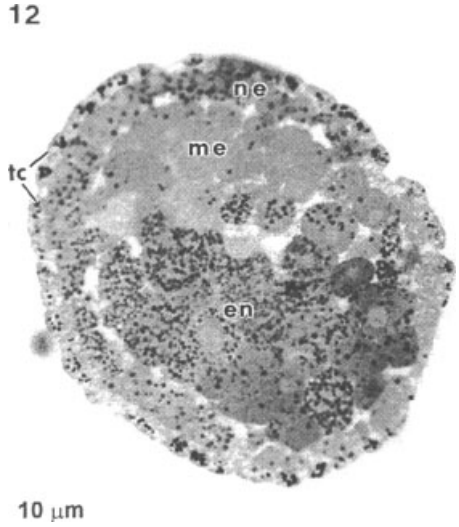


Figure 12. (Lot B.) Semithin parasagittal section of *Ciona intestinalis* embryos at early neurula stage maintained for 1 h in 10^{-7} M TBTCl solution. The embryos reveal severe anomalies and, in addition to the missed raising of neural folds, a significant disorganization and disaggregation of blastomeres can be noted separated by wide spaces. (ne, Neural ectoderm) (Bar = 10 μm.).

such as pseudopodia, in their membrane (Fig. 13). In contrast, the cytoplasm was slack and loosely compacted, and both microtubules and actin microfilaments were missing. The presence of a few vesicles of the rough endoplasmatic reticulum was detected as mitochondria with significant ultrastructural anomalies (Figs 14 and 15). The cristae of these organules appeared swollen and showed an irregular form sometimes elliptical and sometimes spherical (Fig. 15).

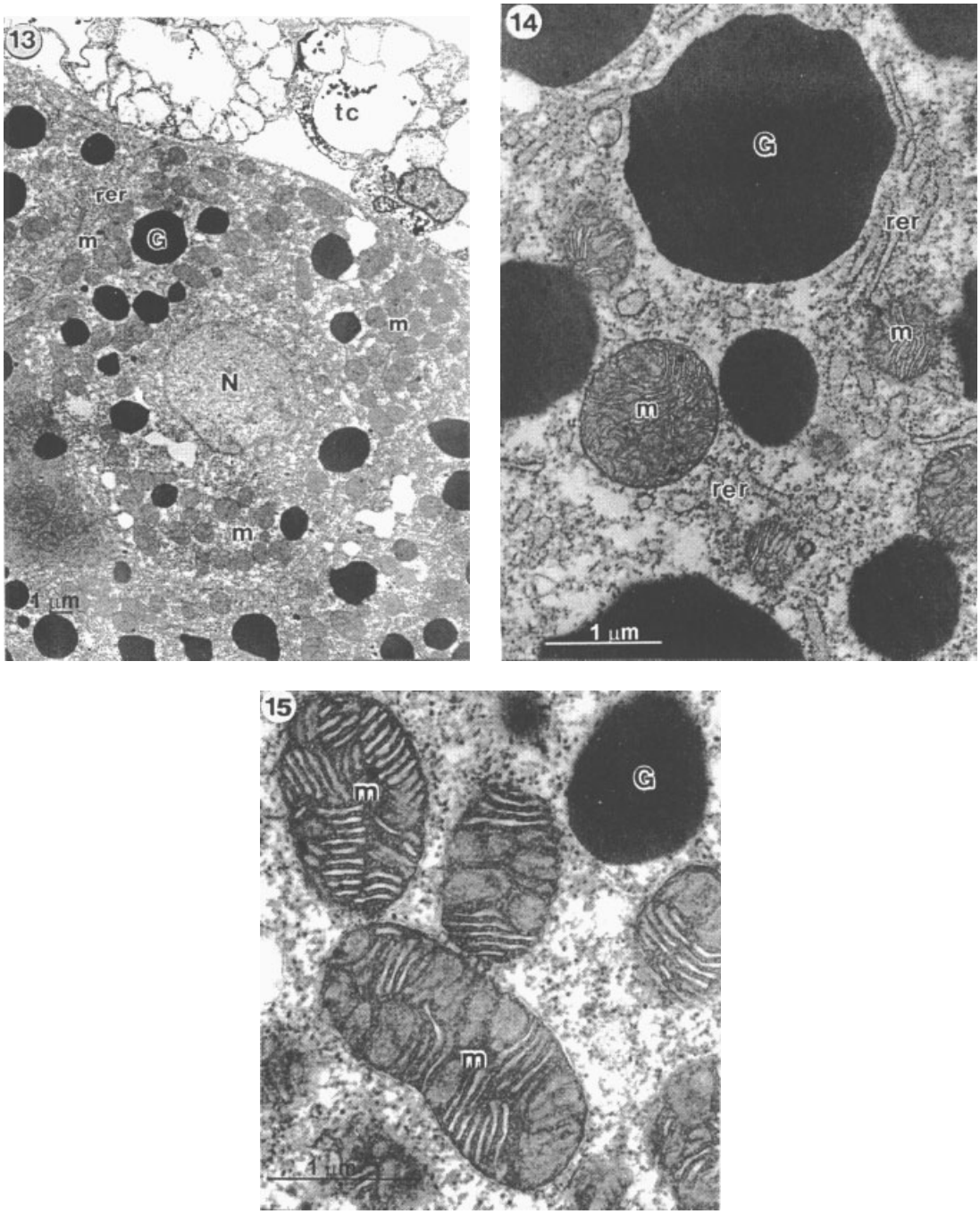
Effect of 10^{-5} M TBTCl solution on the neurula stage (lot C)

Light microscopy observations carried out on *C. intestinalis* embryos at early neurula stage maintained for 1 h in 10^{-5} M TBTCl solution (lot C) demonstrated that TBTCl provoked an irreversible developmental block of the embryos. Light microscope investigations on embryos semithin sections from lot C showed up embryonic anomalies more detrimental than those found in lot B. In different regions, blastomeres increased in size, showed a significant disaggregation and were separated by wide spaces (Fig. 16). Ultrastructural investigations, at cytoplasmatic level, revealed strongly vesiculated cytoplasm, the presence of strongly electro-dense granular precipitates of TBTCl, probably as inorganic tin (Fig. 17), and a large number of mitochondria showing several ultrastructural modifications which inhibited their proper functional activity. Also, in the matrix of these organules electrodense precipitates of TBTCl were noted and the cristae, that in sections looked like circular or tubular vesicles, never exhibited continuity with internal mitochondrial membrane (Fig. 18). Inside the blastomere cytoplasm, the microtubules and actin microfilaments were missing (Fig. 19).

DISCUSSION

Many estuarine and coastal waters, in particular within the Mediterranean Sea, are heavily polluted by organotin compounds. These compounds are a wide class of tin chemicals, which have found commercial applications and are industrially synthesized in large amounts. Their uncontrolled use provoked serious effects and a long-term environmental impact on natural aquatic environments.

Most published data on the effect of TBT on Mediterranean species involve both adult and embryo specimens of different ascidian genera. Ascidians, commonly known as sea squirts, are invertebrates belonging to Urochordata or Tunicata subphylum and are widely considered the ancestors of vertebrates. Their larval stage is a perfect study model because ascidians, belonging to the most primitive line of Protochordata phylum, make a synthesis of the cellular and genomic simpleness of invertebrates and the basic development and morphologic features of vertebrates.[44] The evolutionary relationship of ascidians to chordates can only be seen in their short-lived larvae. Indeed, in the tail of these free-swimming larvae, a neural tube and an axile notochord,



Figures 13–15. (Lot B.) Ultrastructural investigations show that the blastomeres of the ectodermic neural layer maintain a roundish shape (Fig. 13). In their cytoplasm can be observed the presence of mitochondria with significant ultrastructural anomalies (Figs 14 and 15). Microtubules and actina microfilaments are missing. (Bar = 1 µm.).

surrounded by two strings of muscular cells, are present. Furthermore, in the early stages this swimming larvae is, in many aspects, very similar to that of amphibian tadpoles. Only when the larva attaches to the substratum and starts the metamorphosis into a sessile adult does it lose its tail, with annexed notochord, and all analogies with vertebrates cease.

Investigations carried out on development of *C. intestinalis* have proved that all embryonic stages are sensible to pollutant effects and that these incur an irreversibly developmental block.[40] Ultrastructural studies have shown that this developmental block is provoked by the degenerative processes of cellular membranous structures of both the cytoplasm and

nucleus. The most relevant alterations were observed in mitochondria with consequent loss of their functional activity. At molecular level, missed synthesis and/or functioning of both structural and enzymatic proteins was suspected.

TBTCl effects also have been assayed on larvae prior to hatching and free swimming larvae of *C. intestinalis*. Observations carried out *in vivo* with light microscopy have demonstrated that, after TBT exposure, the coiled larva did not hatch. Mobility of swimming larvae became inhibited by significant anomalies of the muscular cells of the tail. According to Gianguzza *et al.*,[42] the main causes of loss of tail motility are connected to ultrastructural modification suffered by muscular cells miofibrills, and a missing energy contribution (ATP) of mithocondria, whose functionality was heavily prejudiced.

In the present paper the effects of TBT (IV) chloride on the neurulation process of the ascidian *C. intestinalis* have been examined using light and electron observations.

Morphological and ultrastructural results in control embryos (lot A) have shown that the ascidian neurulation process, comparable to that of vertebrates, starts with the raising of the neural folds located in the marginal areas of the neural plate. According to literature data,[49–55] at the beginning of neurulation, blastomeres of the neural plate undergo sequential modifications of their shape, changing from a roundish to a irregular lengthened shape. Ultrastructural investigations, made with post-fixing tannic acid technique, have underlined the presence of microtubules in the outer region of blastomeric cytoplasm and inside evaginations, similar to pseudopodia, of the cytoplasmatic membrane. In the cytoplasm of some marginal neural fold blastomeres, in addition to the afore-mentioned microtubules, the presence of microfilament actin sheets was also noted. According to literature data, shape changes occurring in blastomeres of the neural plate seem to be related to the orientation and extension of microtubules, proteinic structures made up of polymerization of α- and β-tubulin. Regarding the raising and converging process of the neural folds to create the neural tube, it is suggested that in this process shape modifications and the mobility of blastomeres are due to contractile strengths produced by microfilaments and other cytoskeleton proteinic structures are made up of actin polymerization.

Data reported in this manuscript showed that embryos of *C. intestinalis* at early neurula stage appeared irreversibly damaged after a minimum incubation time of 1–2 h in 10^{-7}–10^{-5} M TBTCl solutions.

Morphological and ultrastructural analysis carried out on young neurulae of *C. intestinalis* incubated for 1 h in 10^{-7} M TBTCl solution (lot B) showed that typical damage responsible for a developmental block is present. The blastomeres of different embryonal territories are without any specific organization and separated by wide spaces. The most serious ultrastructural anomalies are found in the cristae of mitochondria, whose ultrastructure results are so modified as to compromise their correct functional activity. After exposure to TBTCl, microtubules and actin microfilaments are missing in the outer region of the cytoplasm.

Also, observations carried out on *C. intestinalis* neurulae incubated for 1 h in 10^{-5} M TBTCl solution (lot C) showed that the organotin solution blocked the normal embryonic development. Morphological and ultrastructural investigations showed embryonic damage to be more serious than that found in lot B. Blastomeres of different territories showed a heavy disaggregation and were separated by wide spaces. Their cytoplasm was strongly vesiculated with many electron-dense precipitates of TBTCl. The mitochondrial ultrastructure was so modified that it inhibited proper functional activity. In the mitochondrial matrix it was possible to observe precipitates of TBTCl in the form of electron-dense granules of different sizes. The cristae did not arise from the inner membrane and, in sections, they appeared as tubular vesicles dispersed in the mitochondrial matrix. The fact that the most serious damage was found in lot C embryos confirmed literature data that harmful biological effects of TBTCl are irreversibly dose- and time-dependent.

Morphological and ultrastructural results reported in this paper suggest that TBTCl had a detrimental effect, above all, on mitochondria and on some proteins of cytoskeleton involved in shape modification and cellular movements (microtubules and microfilaments). There is strong literature evidence for cytoskeleton involvement in determining cellular shape modification, regulating cellular motility and, during the mitosis process, chromosomal migration. On the basis of results reported in this paper, as regards the causes determining the block of *C. intestinalis* neurula larvae, it is hypothesized that TBTCl mainly affects the cytoskeleton proteins of the embryonal blastomeres. Polymerization inhibition and/or cytoskeleton protein disaggregation inhibit

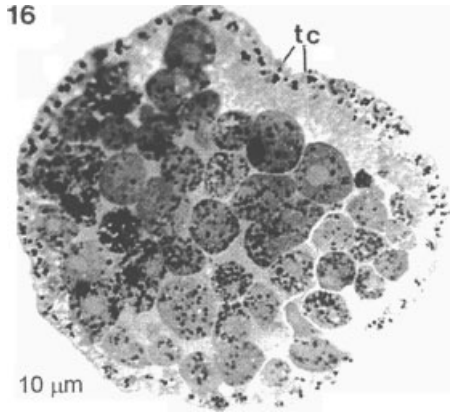
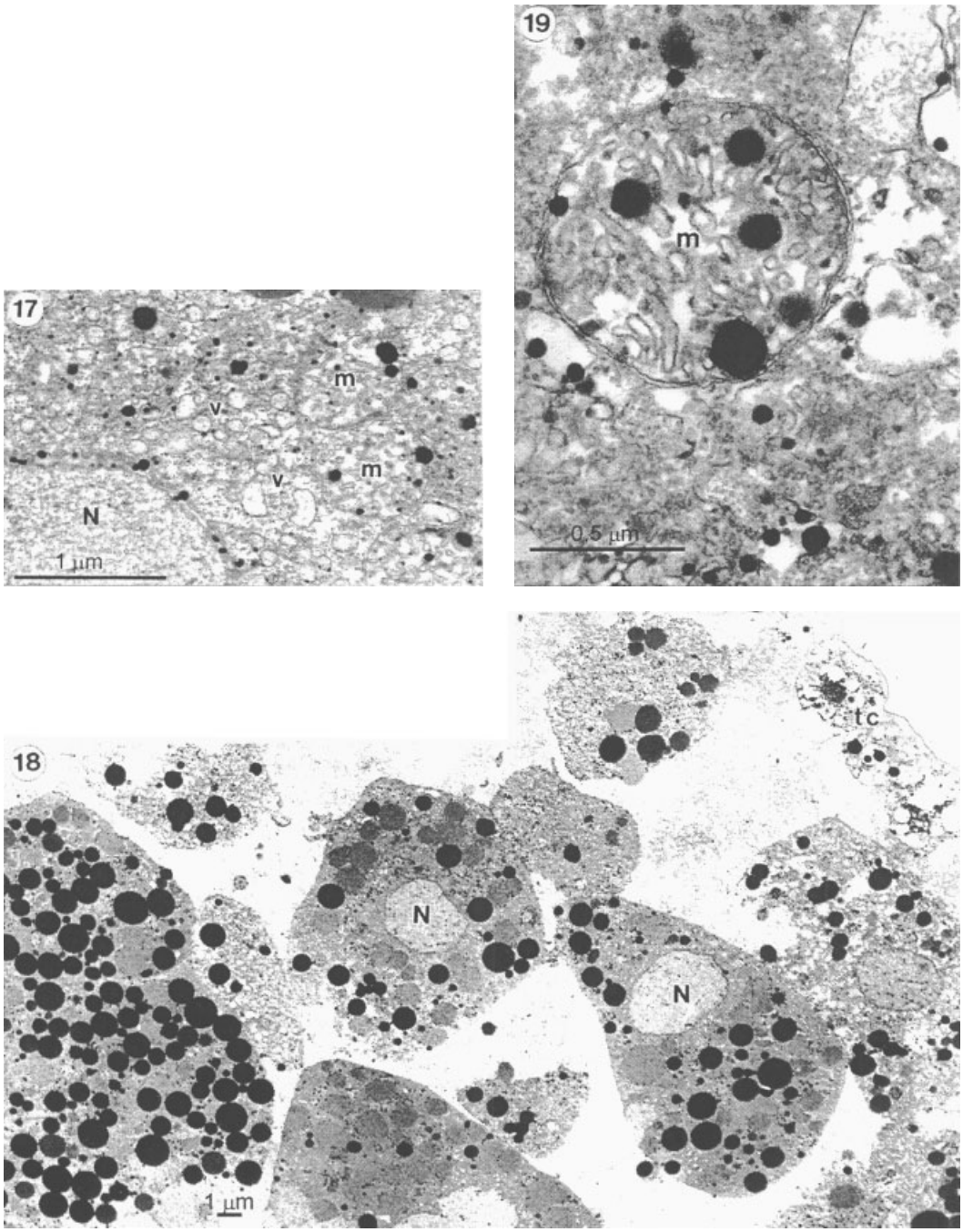


Figure 16. (Lot C.) Semithin parasagittal section of *Ciona intestinalis* embryos at early neurula stage maintained for 1 h in 10^{-5} M TBTCl solution. The embryos are blocked in development; the neural folds are absent and the blastomeres show a significant disaggregation and are separated by wide spaces. (Bar = 10 μm.).



Figures 17–19. (Lot C.) Ultrastructural investigations show the presence of strongly electrondense granular precipitates of TBTCl in the cytoplasm of bastomeres (Fig. 17) and a great number of mitochondria showing several ultrastructural modifications: in the matrix electron-dense precipitates of TBTCl can be noted and the cristae, which in sections looked like circular or tubular vesicles, never exhibited continuity with inner mitochondrial membrane (Fig. 19). Inside the blastomere cytoplasm microtubules and actin microfilaments are not evident (Figs 17–19). (v, Vesicles). (Figs 17 and 18, bar = 1 μm; Fig. 19, bar = 0.5 μm.).

blastomeres shape modification and mobility, basic processes in the neurulation mechanism. Several families of natural and derivative venoms affect the cellular cytoskeleton with specific mechanisms. Recently, however, it has been demonstrated that numerous pollutant substances such as TBTCl directly interact with tubulina and actin, causing cytoskeleton disorganization. Cytoskeleton damage as a result to TBTCl exposure was found in embryos of *Styela plicata*[43] and emocytes of *Botryllus schlosseri*.[30] Organotin compounds cause *in vivo* disaggregations of actin

filaments and inhibition of tubulina polymerization in mammalians.[56]

Another important cause of early embryonic cleavage block is the effect of TBT (IV) chloride on functionality of the mitochondria of neural plate blastomeres. In this study ultrastructural investigations have demonstrated that the mitochondria of blastomeres of the neural plate of lot B embryos underwent ultrastructural modifications and exhibited more detrimental anomalies than those of lot C. Ultrastructural mitochondrial anomalies damage are determinant in compromising proper functional activity and inhibiting oxidative phosphorylation, a remarkable metabolic process that occurs during ascidian gastrulation and neurulation. This finding agrees with investigations carried out on TBTCl-treated gastrula and neurula of *S. plicata*. In agreement with Cima *et al*.,[43] the toxic effects of TBTCl on mitochondrial functionality and oxidative phosphorylation inhibition are thought to be correlated only with the respiratory process of embryonic blastomeres. Meanwhile there is no firm evidence that inhibition of ATP synthesis is correlated to disorganization of blastomeric cytoskeleton microtubules and microfilaments, because ATP and GTP do not seem to be necessary to the polymerization of these cytoskeleton proteins.[43]

In conclusion, data reported in this paper have showed that incubation of *C. intestinalis* neurula larvae in 10^{-7}–10^{-5} M TBTCl solutions for 1–2 h provokes such serious anomalies as to cause an irreversible block of embryonic cleavage.

Morphological and ultrastructural observations reported in this paper suggest that most probably there are two principal causes determining and explaining the block of the *C. intestinalis* neurulation process. The first is due to the TBT effect of inhibiting the polymerization and/or degradation of some proteins that constitute microfilaments and microtubules. The lack of orientation and extension of both microtubules and actin microfilaments prevents those shape changes, and mobility of neural plate cells, indispensable to the neurulation process. The second cause is determined by the ultrastructural modification which mitochondria undergo. The ultrastructural anomalies shown by these organules are so serious as to impede their proper functionality, with consequent inhibition of oxydative phosphorylation and ATP synthesis.

Acknowledgements

The financial support of the Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR, Rome, project no. 2001053898_002), Roma, and of Università di Palermo, is gratefully acknowledged.

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