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Organometallic Complexes with Biological Molecules. XIV. Biological Activity of Dialkyl and Trialkyltin(IV) [Meso-tetra(4-carboxyphenyl)porphinate] Derivatives

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The effects of several organotin(IV) *meso*-tetra(4-carboxyphenyl)porphinate] derivatives with the general formula $(R_2Sn)_2$TPPC and $(R_3Sn)_4$TPPC (R = Me, Bu, Ph) were tested *in vivo* on ascidian embryonic development. Embryos at the two-cell stage were incubated in 1×10^{-5} or 1×10^{-7} M solutions of various compounds. The ligand, [*meso*-tetra(4-carboxyphenyl)porphine] (H_4TPPC) was toxic at 1×10^{-5} M, because development was blocked at an early gastrula stage, whereas 1×10^{-7} M H_4TPPC allowed the eggs to develop up to the larva stage. The most toxic among the tested compounds was tributyltin(IV) [*meso*-tetra (4-carboxyphenyl)porphinate], $(Bu_3Sn)_4$TPPC, since the fertilized eggs were unable to divide into two cells, even at a concentration of 1×10^{-7} M. To correlate this embryonic arrest with the metabolic pathway, and especially to understand why cellular organelles first underwent chemical damage, 10^{-5} and 10^{-7} M $(Bu_3Sn)_4$TPPC-cultured fertilized eggs were tested for DNA, RNA, protein, glucose, lipid and ATP contents, comparing the values obtained with those of control culture fertilized egg contents. The higher concentration (1×10^{-5} M) reduced the content of all the tested compounds, but the lower one (1×10^{-7} M), even if still unable to allow cleavage, reduced only the lipids and the ATP contents. A hypothesis concerning initial damage to mitochondrial membrane is proposed. Copyright © 2000 John Wiley & Sons, Ltd.

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INTRODUCTION

The acute effects of organotin(IV) compounds, used as biocides on a number of organisms, have been extensively investigated. All these studies indicate that sublethal effects can have more serious long-term consequences in various processes which can ultimately affect the survival and propagation of the species. Therefore, there is considerable current interest in understanding the mechanism through which these compounds exert their toxic action on the organisms. It has been demonstrated that some of these derivatives are immunotoxic, neurotoxic, etc.[1] Moreover, they inhibit phagocytosis and exocytosis in the rat.[2] In *Ciona intestinalis*, phagocytosis inhibition by tributyltin(IV) chloride (TBT) is irreversible, as demonstrated by Cooper *et al.*[3] TBT also affects the chromosome structure of Mollusca and Isopoda.[4,5] Furthermore, it is well known that organotin(IV) compounds may inhibit mitochondriae enzyme and hexokinase activity.[6,7]

Developmental systems could be a suitable field of study because they share certain fundamental features, including:

(1) storage and transfer of developmental information,

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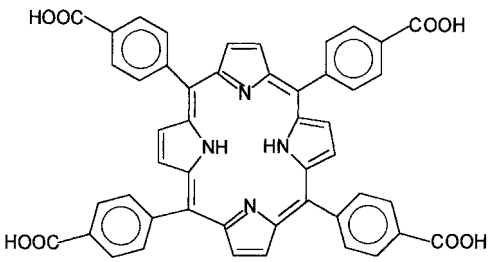

Figure 1 [*meso*-tetra(4-carboxyphenyl)porphine] (H_4TPPC).

(2) molecular, cellular or organism growth,
(3) morphogenesis, and
(4) differentiation, the emergence of a functionally specialized state.

Recent studies in ascidian embryonic development after exposure to organotin(IV) derivatives demonstrated reduced fertility, arrest of the mitosis process, mortality of embryos and inhibition of larval movement, which implies reduction of the intrinsic rate of population growth. At an ultrastructural level it has been proved that cell membranes, mitochondriae structures[8] and muscle cells are altered by TBT.[9]

At this point, an understanding of the effects of the organotin(IV) derivatives on the development of ascidian eggs was needed. The aim of the present work is to assess the morphological effects of short-term exposure to diorganotin(IV) and triorganotin-(IV) derivatives of (Fig. 1) [*meso*-tetra(4-carboxyphenyl)porphine] with the general formula $(R_2Sn)_2$TPPC and $(R_3Sn)_4$TPPC (R = Me, Bu, and Ph) at all the developmental stages of ascidian eggs, and to determine any subsequent change in biochemical composition associated with the exposure to these organotin(IV) derivatives. Development of ascidians proceeds as follows: the ascidians release gametes into the sea water solution, where development occurs; after fertilization, the eggs segment into two, four, eight cells, etc., up to gastrulae, then neurulae, coiled larvae, swimming larvae and finally metamorphosed larvae.

MATERIALS AND METHODS

Experiments *in vivo*

Ciona intestinalis adults were collected from Palermo harbour. The eggs removed from the oviducts were fertilized. After 90 min, the eggs divided into two blastomeres. Five batches of eggs at the two-cell stage were incubated in seawater solutions of 1×10^{-5} and 1×10^{-7} M organotin(IV)[*meso*-tetra(4-carboxyphenyl)porphinate], $(R_2Sn)_2$TPPC and $(R_3Sn)_4$TPPC, previously synthesized and characterized according to literature reports,[10] and were left to develop at 22 °C. Several cells at the two-cell stage, from each batch, were incubated in normal seawater (controls). The pH of the solution was controlled and maintained within the range 7.76–8.00 (seawater pH). Observations *in vivo* were made with a Leitz Ortoplan microscope using an Ilford FP4 Plus film.

Biochemical experiments

Culture

The fertilized eggs of *Ciona intestinalis* were divided into three batches, of which one was cultured in pasteurized seawater and used as a control, and two were cultured in pasteurized seawater containing 1×10^{-5} and 1×10^{-7} M tributyltin(IV) [*meso*-tetra(4-carboxyphenyl) porphinate], $(Bu_3Sn)_4$TPPC.

As at least 97% of the control-fertilized eggs reached the two-cell stage, the developmental stage of the eggs treated with $(Bu_3Sn)_4$TPPC solutions was monitored. Soon afterwards, eggs of all the batches were washed several times with pasteurised seawater, collected by low-speed centrifugation, and immediately frozen at −80 °C, pending biochemical studies on the total extracted DNA, RNA, proteins, glucose, lipids and ATP.

Extraction

Procedures for extraction of DNA, RNA, protein, glucose, lipid and ATP fractions extraction were followed according to modified Schneider methods.[11]

Briefly, 0.2 ml of cultured fertilized eggs were homogenized in a small volume of distilled water by sonication for 1 min at 2 Hz. An aliquot was withdrawn for subsequent protein extraction. The remaining volume was centrifuged at 5000 g, at room temperature. From the supernatant, an aliquot was withdrawn for the glucose determination. The remaining supernatant was mixed with the solid residue, and cold trichloroacetic acid (TCA) was added up to 6% (w/v) final concentration. The solution was first maintained for 10 min in an ice bath, then centrifuged at 4000 g for 15 min at 4 °C. The supernatant was withdrawn and stored at

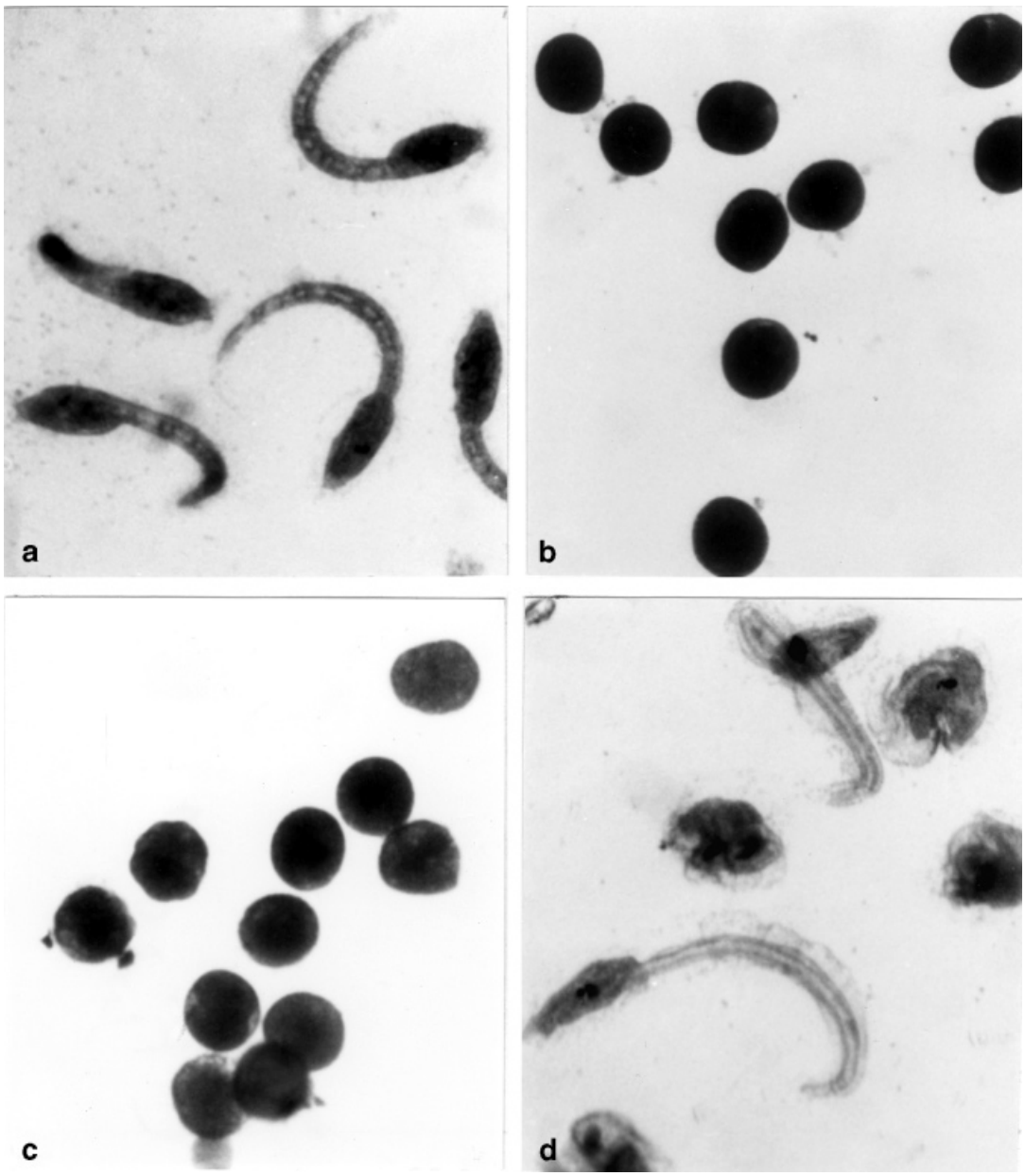

Figure 2 (a) *Ciona intestinalis* swimming larvae. (b) Larvae originating from two-cell stage eggs incubated in 10^{-7} M $(Bu_3Sn)_4$ [*meso*-tetra(4-carboxyphenyl)porphinate] solution. Some larvae have twisted tails in membranes. (c) Blocked two-cell stage eggs incubated in 10^{-5} M $(Bu_3Sn)_4$[meso−tetra(4-carboxyphenyl)porphinate] solution. The blastomeres failed to develop and the eggs appear to be unsegmented. (d) Anomalous blocked 2–4-cell stage eggs incubated in 10^{-7} M $(Bu_3Sn)_4$[*meso*-tetra-(4-carboxyphenyl)porphinate] solution. The blastomeres have an abnormal spatial disposition. Magnification ×56.

−80 °C for the subsequent ATP determination. Pellets were used for separation of lipids.

Extraction was performed in five steps: two steps in 95% ethanol and three steps in ethanol–ether (3:1, v/v) solutions. For each step, the 5000 g supernatant fraction was withdrawn. Final pellets were processed for RNA and DNA fraction preparations. The pellets were incubated into

Table 1 Development of fertilized eggs incubated in seawater solutions of diorgano- and triorganotin(IV) [*meso*-tetra(4-carboxyphenyl)porphinate][a].

Compound	Concn (M)	Development stage[b],%					
		2–4 cells	4–8 cells	Gastrulae	Anomalous embryos	Anomalous larvae	Swimming larvae
H_4TPPC	10^{-5}			100			
	10^{-7}					100	
$(Me_2Sn)_2$TPPC	10^{-5}			100			
	10^{-7}					100	
$(Bu_2Sn)_2$TPPC	10^{-5}		100				
	10^{-7}					80	20
$(Ph_2Sn)_2$TPPC	10^{-5}			100			
	10^{-7}				50	50	
$(Me_3Sn)_4$TPPC	10^{-5}			100			
	10^{-7}					50	50
$(Bu_3Sn)_4$TPPC	10^{-5}	100					
	10^{-7}		100				
$(Ph_3Sn)_4$TPPC	10^{-5}			100			
	10^{-7}				50	50	

[a] H_4TPPC, [*meso*-tetra(4-carboxyphenyl)porphine]; $TPPC^{4-}$, [*meso*-tetra(4-carboxyphenyl) porphinate]; Me, methyl; Bu, butyl; Ph, phenyl.
[b] The control gave rise to 90% swimming larvae. Results are presented as the percentage of developed eggs, arrested at an intermediate stage, or developed to swimming larvae, averaged from five experiments.

0.3 M KOH for 20 h at 37 °C, then neutralized with 6 M $HClO_4$ (PCA) and the $KClO_4$ salt formed was removed by low-speed centrifugation.

DNA was precipitated by a 5% PCA (v/v, final concentration) solution, and collected as a pellet after 10 min of centrifugation at 10000 g at 4 °C.

The supernatant fraction was used for RNA determination. The pellet was hydrolysed with 5% PCA solution in Sovirel tubes at 90 °C for 15 min; the DNA fraction was taken as the supernatant of a 10 min centrifugation at 10000 g at 4 °C.

Methods of determination

DNA, RNA and lipid contents were determined according to standard methods already described by Snell,[12] Brown,[13] and Marsh and Weinstein,[14] respectively.

Protein contents were determined according to the modified Lowry method,[15] with a P5656 kit (Sigma Chemical Co., St. Louis, MS, USA) after precipitation with 5% TCA and collection of the insoluble material as a pellet from a 10000 g centrifugation.

Finally, glucose and ATP contents were determined according to the GOD–POD–PAP method with a 1131.4 Sentinel CH (Milano, Italy) kit, and to the PGK–GAPD–NADH method with a 366 Sigma Chemical Co. kit, respectively.

All cultures and extractions were performed three times, while each determination was carried out twice.

RESULTS

Observations *in vivo*

Controls

The fertilized *Ciona intestinalis* eggs develop into two-, four-, eight-, 16-cell, etc., blastomeres, then into gastrulae, neurulae and swimming larvae (Fig. 2a). These possess a neural tube, myotomes, pharyngeal gills, notochord, an ocellus or simple eye and an otolith, a gravity receptor. The development time is about 24 h: 90 min from fertilization to the two-cell stage; 5–6 h up to the gastrulae stage, 7.5 h up to the neurulae; and 24 h up to the larva.

Incubation of two-cell stage eggs in diorganotin(IV) and triorganotin(IV) [*meso*-tetra(4-carboxyphenyl)porphinate]

The results of incubation of two-cell stage eggs in diorganotin(IV) and triorganotin(IV) [*meso*-tetra(4-carboxyphenyl)porphinate], $(R_2Sn)_2$TPPC and

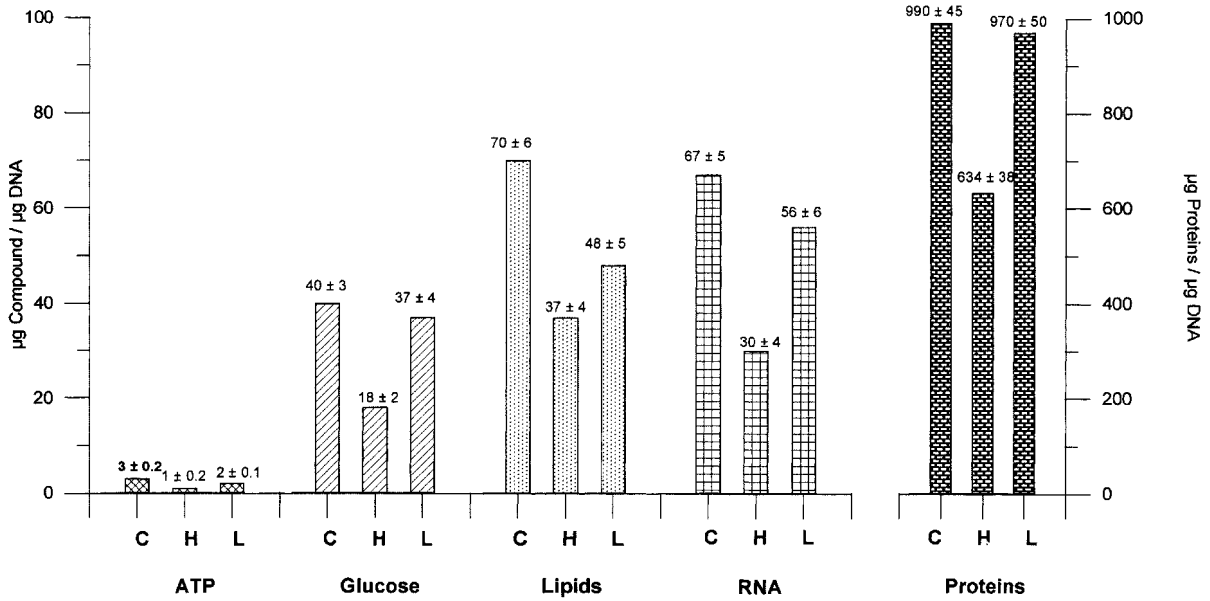

Figure 3 ATP/DNA, glucose/DNA, lipids/DNA, RNA/DNA and proteins/DNA ratios determined at two different concentrations of $(Bu_3Sn)_4$[*meso*-tetra(4-carboxyphenyl)porphinate]: H, 1×10^{-5} M; L, 1×10^{-7} M; C, control.

$(R_3Sn)_4TPPC$ (R = Me, Bu, Ph) solutions are reported in Table 1, which shows that while the free ligand, [*meso*-tetra(4-carboxyphenyl)porphine], was toxic at 1×10^{-5} M concentration because of the egg development arrest at the gastrulae stage, in 1×10^{-7} M ligand concentration the fertilized eggs developed up to anomalous larvae with short tails. An analogous result was obtained in 1×10^{-5} M solutions of $(R_2Sn)_2TPPC$ (R = Me, Ph) and $(Ph_3Sn)_4TPPC$, while undifferentiated embryos and anomalous larvae were obtained in 1×10^{-7} M solutions of the same derivatives, (Table 1).

In 1×10^{-5} M $(Me_3Sn)_4TPPC$ solution, the eggs blocked at the early gastrulae stage, while those incubated in 1×10^{-7} M solution gave rise to 50% normal larvae and 50% anomalous larvae, (Fig. 2b).

The incubation of eggs at the two-cell stage in 1×10^{-5} M $(Bu_2Sn)_2TPPC$ solution allowed them to undergo only two to three mitosis, while in 1×10^{-7} M solution the eggs developed into 20% normal larvae and 80% anomalous larvae.

Finally, the embryos at the two-cell stage stopped developing in 1×10^{-5} M tributyltin(IV)-[meso-tetra(4-carboxyphenyl)porphinate] [$(Bu_3Sn)_4TPPC$], solution (Fig. 2c), whereas in 1×10^{-7} M solution the eggs achieved the 4–16-cell stage (Fig. 2d). In this latter case the blastomeres presented an anomalous spatial disposition pattern.

Biochemical results

These are represented by histograms in Figs 3 and 4. In Fig. 3, the biochemical entities analysed are expressed as ratios relative to the DNA present per cell, i.e. as the ratios ATP/DNA, glucose/DNA, lipids/DNA, RNA/DNA and proteins/DNA. As expected for the high cytotoxicity of $(Bu_3Sn)_4TPPC$, all the ratios obtained from the determinations in the $(Bu_3Sn)_4TPPC$-cultured fertilized eggs were lower than those determined in the fertilized control eggs. Furthermore, the ratios of the H cultures [1×10^{-5} M $(Bu_3Sn)_4TPPC$ solution] were lower than the ratio of the L cultures [1×10^{-7} M $(Bu_3Sn)_4TPPC$ solution]. Figure 4 shows the same data tabulated as percentages, with respect to the control (100%).

The results show that the ATP, glucose, lipid, protein and RNA contents obtained for the H cultures were around 50% or less than those from the control. On the contrary, the contents of proteins, glucose and RNA obtained from the L cultures were very close to those of the controls, whereas the ATP and lipid contents were less than 70% of those in the control.

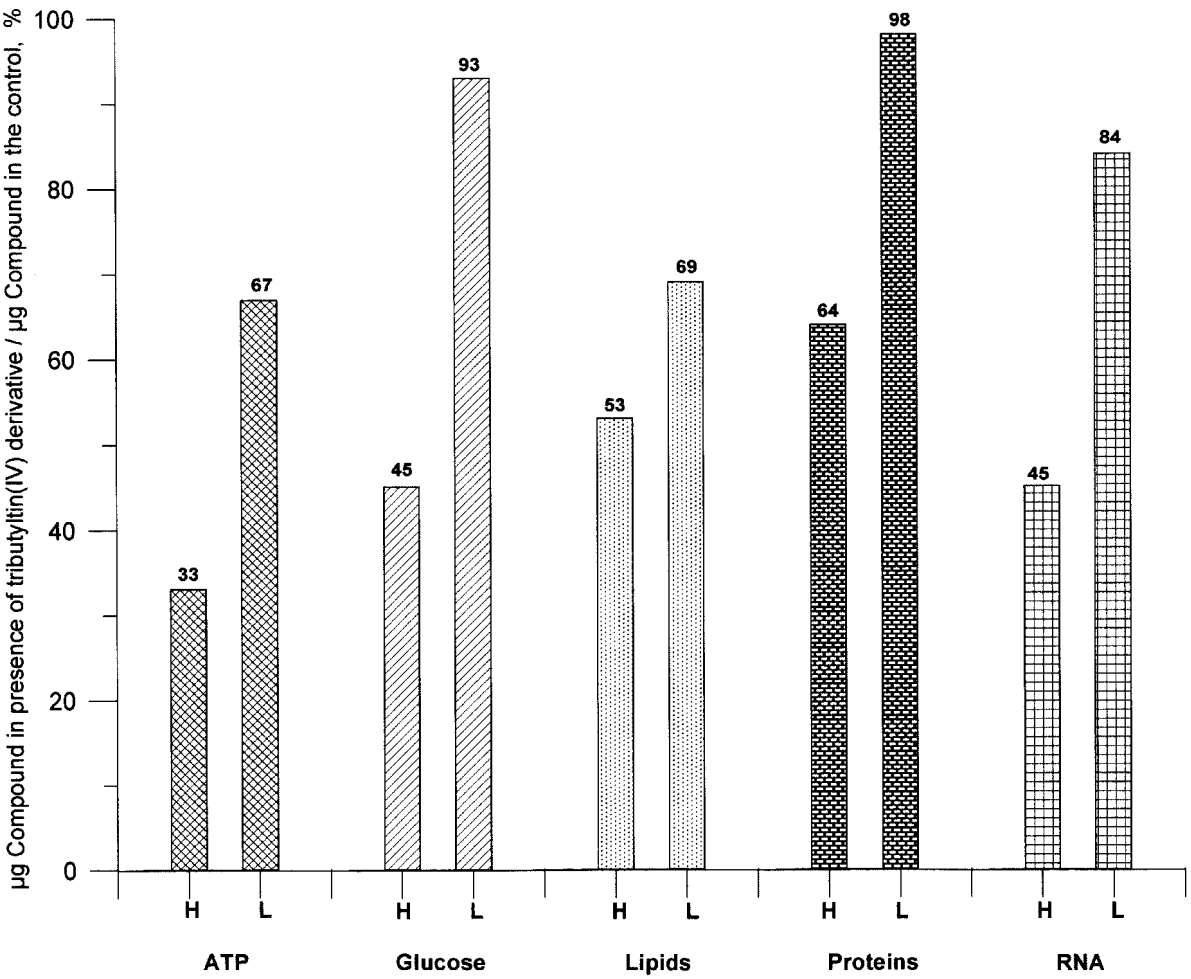

Figure 4 Percentage of ATP, glucose, lipids, RNA and proteins in the presence of $(Bu_3Sn)_4$[*meso*-tetra(4-carboxyphenyl)porphinate] calculated relative to the same compounds present in the control.

DISCUSSION

The data reported in this paper all support the conclusion that some of the organotin(IV) [*meso*-tetra(4-carboxyphenyl)porphinate] under investigation act, like other previously tested derivatives,[15–17] as heavy toxicants. The cytotoxicity of the complexes decreased according to the following trend:

$$(Bu_3Sn)_4TPPC \gg (Bu_2Sn)_2TPPC > (Ph_3Sn)_4TPPC = (Ph_2Sn)_2TPPC > (Me_3Sn)_4TPPC > (Me_2Sn)_2TPPC$$

The most toxic derivative, $(Bu_3Sn)_4TPPC$, acted during mitosis. The less toxic derivatives were effective during the gastrulae stage, or at lower concentrations they acted later in development: the larvae were anomalous, with short tails in membranes. The cytotoxicity of the $(Bu_3Sn)_4TPPC$ derivative included molecular mechanisms: once the compound migrates inside the cell, it may immediately disrupt the cell metabolism of RNA, proteins, lipids, glucose and ATP.

The choice of presenting the data as ratios (μg of investigated compound/ μg of DNA per cell) arose from the consideration that only the DNA content is a cellular constant. Another point to consider was that the amount of DNA in the control appears to be double that in uncleaved $(Bu_3Sn)_4TPPC$-treated fertilized eggs. This can be explained on the basis that whereas in the control the fertilized eggs were

at the two-cell stage, the uncleaved fertilized eggs from organotin-treated egg cultures not only maintained the one-cell morphology but also, when observed under the electron microscope, did not show any nuclear area,[8] thereby suggesting an inability to synthesize DNA. The problem now is to explain the molecular reason for the blockage of cleavage by $(Bu_3Sn)_4TPPC$. A concentration of 1×10^{-5} M blocked cleavage and drastically reduced the amounts of all the compounds determined; but when the concentration employed was 1×10^{-7} M, the situation appeared different: RNA, protein and glucose values reached almost those of controls, thus showing that such compounds were not directly involved in the $(Bu_3Sn)_4TPPC$ mechanism. As far as the lipids and ATP are concerned, even at this low $(Bu_3Sn)_4TPPC$ concentration, the values obtained were still quite low compared with those found in controls. This could mean that the blockage of cleavage was not connected either with the decrease in RNA or with the protein synthesis or glucose breakdown, but it was linked to the energy inefficiency (ATP loss) and/or to the loss of lipids. As already known, ATP contents mainly arise from the Krebs cycle, even from glucose breakdown, but only subsequently from other sources such as lipids. In our experiments, while the lipid and ATP contents decreased, the glucose content did not vary appreciably and it was maintained almost at the control level. This could imply that a lipid source of this ATP is lost. Some explanations could be given at this point: i.e., cleavage did not occur because of the lack of energy, or an ATP shortage provoked a cAMP deficiency. The latter idea could be interesting, since cAMP comes from ATP, by the action of adenylate cyclase located on the cell membrane, and cAMP is necessary to phosphorylate protein kinase which in turn is necessary to phosphorylate tubulin for mitotic spindle formation and egg cleavage. But lipids and ATP are also present in mitochondria, which were found to be seriously damaged by $(Bu_3Sn)_4TPPC$ compounds; meanwhile, peroxisomes increased,[8] probably in order to use the excess of cellular O_2 caused by a reduced respiratory activity. Another possibility was that, inside the mitochondria, the porphyrin group of $(Bu_3Sn)_4TPPC$ might compete with the porphyrin group of cytochrome and therefore it might damage the H-pump for ATP production.

In conclusion, whatever the molecular mechanism of $(Bu_3Sn)_4TPPC$ that blocks fertilized-egg cleavage, the primary effect seems to be damage to the molecular membrane structure, i.e. to the mitochondrial membrane, or cellular membrane, or both.

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