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Chemically induced chromosome damage in early-developing embryos of *Anilocra physodes* L. (Crustacea, Isopoda) following exposure to bis[dimethyltin(IV)chloro]protoporphyrin IX

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In order to obtain chromosome preparations from early-developing embryos of *Anilocra physodes*, a squash technique has been successfully employed.

Results gathered after exposure of this material to bis[dimethyltin(IV)chloro]protoporphyrin IX {$[(CH_3)_2SnCl]_2$·Protoporphyrin IX} solutions at different exposure times suggest that this chemical complex is capable of producing abnormal metaphase and anaphase figures in proportion to its concentration and not to exposure length.

Essentially, all of the chromosome abnormalities are classifiable as chromosome fragments mainly observed at the metaphase stage; chromosome bridges; and large decondensed chromosome regions.

Keywords: Bis[dimethyltin(IV)chloro]protoporphyrin IX, chromosomes, alterations, Crustacea

INTRODUCTION

Metal porphyrins have recently been studied in the solid phase and in solution.[1,2] Pasternack *et al.*,[3] in particular, found that these compounds bind to nucleic acids and make possible modifications in chromosomal DNA.

In order to verify whether bis[dimethyltin-(IV)chloro]protoporphyrin IX is genotoxic in the solution phase, application of a reliable cytogenetic test for the quantitative evaluation of chromosomal abnormalities was required. Owing to the aquatic orientation of the research in our laboratory, we chose to test this chemical complex using early-developing embryos of *Anilocra physodes* (Crustacea, Isopoda) as the source of mitotic figures. This species was selected for use for three reasons:

(1) *A. physodes* possesses a surprisingly low diploid chromosome number ($2n = 12$),[4] in contrast with most fish and ascidian species which have relatively high numbers of minute chromosomes making it extremely difficult to identify small changes in their chromosome structure;[5–7]
(2) different mitotic stages such as the prophases, metaphase and anaphase–telophase can be easily detected in early embryos of this species; and
(3) the chromosomes of *A. physodes* are also very large in size.

This last consideration is important because it permits a detailed analysis of possible chromosome aberrations.

MATERIAL AND METHODS

Anilocra physodes (Crustacea, isopoda), a parasite on various fish, is a protandrous hermaphrodite species, so that its rudimentary ovary becomes functional only after the testis ceases to function.

Of 70 *A. physodes* specimens, classified according to the guidelines of Riedl,[8] found either attached to the gills or to the fins of *Mugil cephalus* specimens (Pisces, Perciformes) captured in the gulf of Palermo during 1991, 30 were females, each containing 20–30 early-developing embryos.

Specimens were incubated in the presence of light, either in bis[dimethyltin(IV)chloro]protoporphyrin IX solution at different concentrations and exposure times, as documented in Table 1, or in seawater, as controls.

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Table 1 Genotoxic activity: metaphase and anaphase chromosomal damage in *A. physodes* early-developing embryos treated with bis[dimethyltin(IV)chloro]protoporphyrin IX

Compound concentration (M)	Time interval (h)	No. of embryos employed	Total spread[a]	No. of normal[b] metaphases and anaphases		No. of abnormal metaphases[c] and anaphases						
						Fragments		Bridges		Total spread		
				c	d	c	d	c	d	c	d	e
0.956×10^{-5}	24	11	220	10	2	59	3	46	10	104	18	98
						48	2	36	14	80	16	84
2.07×10^{-7}	24	10	200	20	12	50	1	38	14	87	24	89
	48	8	160	23	10	46	4	14	19	62	33	65
1×10^{-9}	24	12	240	63	59	10	5	9	24	78	78	84
	48	8	160	35	42	13	—	5	10	48	52	60
Control	—	12	270	71	78	—	—	2	1	73	79	88

[a] 20 spreads per embryo were analysed. [b] the term 'normal' indicates no difference from the control. [c] No. of metaphases. [d] No. of anaphases. [e] No. of prophases of which chromosome aberrations were not detected.

Bis[dimethyltin(IV)chloro]protoporphyrin IX {$[(CH_3)_2SnCl]_2$·Protoporphyrin IX} has been obtained by a previously described procedure.[9]

Concentrated stock solutions were obtained by dissolving stoichiometric amounts of the compound in Millipore-filtered seawater (MFSW). Working solutions (pH 7.8–8.0) were obtained by further dilution of the stocks in MFSW.

Organotin(IV) concentrations in the diluted solutions were assayed using a model 372 Perkin–Elmer graphite-furnace atomic absorption spectrophotometer.

Chromosome slides were prepared from untreated embryos (controls), always in the absence of colchicine, and embryos were treated with bis[dimethyltin(IV)chloro]protoporphyrin IX according to the following procedure known as squash technique:

(1) Embryos, ≈2 mm in diameter, were washed in freshly prepared 50% acetic acid fixative for 5 min.
(2) Embryos were stained in 50% acetic orcein[10] for about 15 min.
(3) Embryos were washed in 50% acetic acid to remove excess orcein.
(4) Each embryo was gently squeezed between the slide and cover.
(5) Slides were immediately studied under a phase-contrast microscope.

An air-drying technique, described elsewhere,[11] was also attempted but application of this procedure proved to be unsuccessful.

Mitotic chromosomes were classified according to the Levan *et al.*[12] terminology. Chromosome observations and photomicrographs were made using a Jenamed 2 phase-contrast microscope and Agfa Gaevaert AG 25 film.

RESULTS

Untreated chromosomes (controls)

Two embryos per experiment, each supplying a total of 60 different mitotic stages, were examined. Nearly equal numbers of prophases, metaphases and anaphase–telophases were found (Table 1).

At the metaphase stage the chromosomes, homogeneously stained, appeared well separated from one another so that their counts could be easily carried out. The diploid value was $2n = 12$, except for *ca* 2–3% of the metaphases analysed which possessed lower diploid values.

Since kinetocore location was always evident in each element, it was possible to construct a karyotype consisting of four metacentric and two subtelocentric pairs (Fig. 1). Chromosome dimensions ranged from a maximum of *ca* 18 μm in the biggest, to a minimum of 10 μm in the smallest. Very few spreads (*ca* 1–2%) showed chromosomes which were attached to one another.

At the anaphase stage, chromosomes formed two distinct groups directed towards the opposite polar regions of the mitotic spindle (Fig. 2). Association of elements of one group with ele-

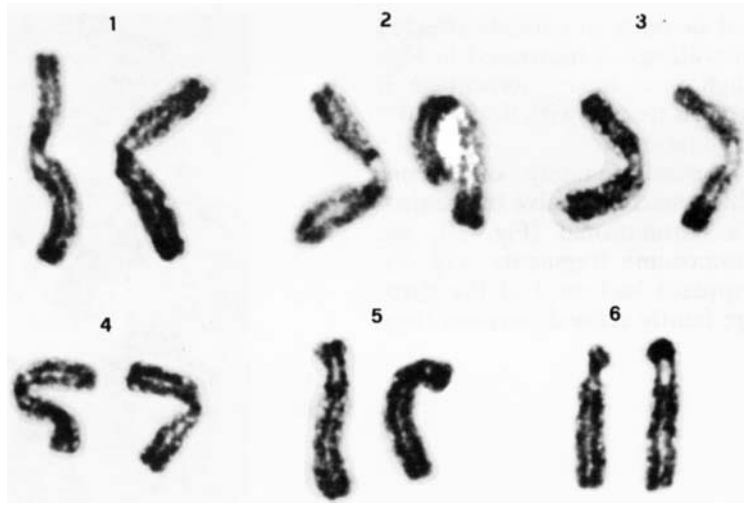

Figure 1 Acetic orcein representative karyotype of *A. physodes*.

ments of the other was occasionally encountered. During the metaphase and anaphase stages, chromosomes with additional structural alterations were not detected.

Chromosomes treated with bis[dimethyltin(IV)chloro]protoporphyrin IX

The data for studies of treated chromosomes including bis[dimethyltin(IV)chloro]protoporphyrin IX concentrations, the incubation times, the number of spreads analysed, and chromosome alterations are reported in Table 1. Nearly equal numbers of prophases and metaphases were found at different concentrations of bis[dimethyltin(IV)chloro]protoporphyrin IX used in our experiments (Table 1). Conversely, as shown in this Table, a very low number of anaphase spreads was encountered at 0.956×10^{-5} M concentration. This number slightly increased after treatment with 2.07×10^{-7} and 1×10^{-9} M solutions.

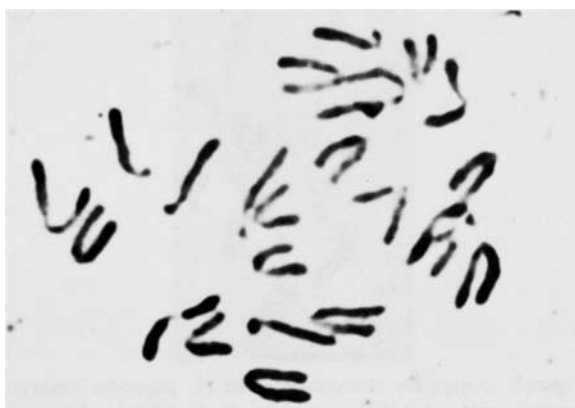

Figure 2 Acetic orcein anaphase spread of *A. physodes* (control).

In comparison with the controls, treated embryos all showed a substantial increase in the number of aberrant metaphases. More precisely, chromosomes of these spreads appeared as irregularly stained bodies with large decondensed portions (Fig. 3), which often showed telomeric connections (Fig. 3, see arrow). Only 3–4% of analysed metaphases were aneuploid and all had a chromosome number lower than the mode.

Probably because of overcondensation of the chromosomes (Fig. 4), other spreads had all elements looking like roundish bodies which were often closely associated in groups. When anaphase–telophase spreads were analysed, they essentially displayed chromosome bridges between daughter chromosomes involving one (Fig. 5, see arrow) or more than one (Fig. 6, see arrow) chromosome pair per spread.

A small percentage of normal anaphase–telophase spreads (Fig. 7) was also observed.

Another type of abnormality involved several metaphase spreads of the embryos treated with the three solutions (Table 1), resulting in chromosome fragments that lagged behind the main body of chromosomes and appeared to be attached by a thin strand of thread-like chromatin (figs 8 and 9; see arrows).

Embryos treated with a 1×10^{-9} M solution

showed a significant increase of spreads affected by chromosome alterations as illustrated in Figs 10 and 11, although in a lower percentage if compared with embryos treated with 0.956×10^{-5} and 2.07×10^{-7} M solutions.

Abnormalities consisted mainly of decondensed areas which seemed to involve the centromeric region of a chromosome (Fig. 10; see arrow) and of chromosome fragments (Fig. 9). Some metaphase spreads had most of the chromosomes with large faintly stained portions (Fig. 11).

DISCUSSION

In order to obtain chromosome preparations for testing genotoxicity of bis[dimethyltin(IV)-chloro]protoporphyrin IX, air-drying and squash techniques have been employed. Since only the latter gave good chromosome spreads, we conclude that the squash method is better for karyological analyses of crustacean early-developing embryos. The failure of the air-drying technique is probably due to the high amount of yolk in *Anilocra physodes* eggs.

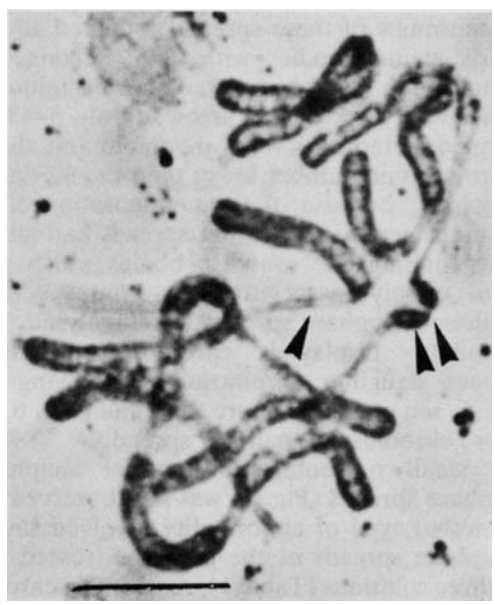

Figure 3 Metaphase chromosomes of *A. physodes* embryo treated with 0.956×10^{-5} M bis[dimethyltin(IV)chloro]protoporphyrin IX for 24 h; the single arrow indicates a chromosome decondensed region and the double arrow indicates a telomeric connection. The bar is 10 μm.

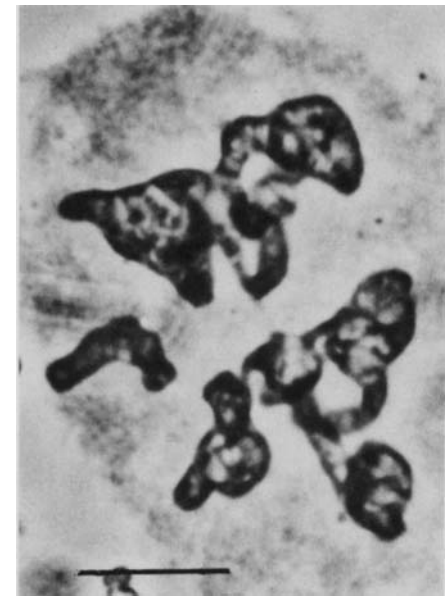

Figure 4 Metaphase chromosomes of *A. physodes* embryo treated with 2.07×10^{-7} M bis[dimethyltin(IV)chloro]protoporphyrin IX for 24 h. The bar is 10 μm.

The fact that the total number of chromosome anomalies observed is higher than the number of cells examined suggests that more than one anomaly per spread occurred routinely. Furthermore, based on numerical chromosomal comparisons among treated embryos and embryos used as controls, a second conclusion

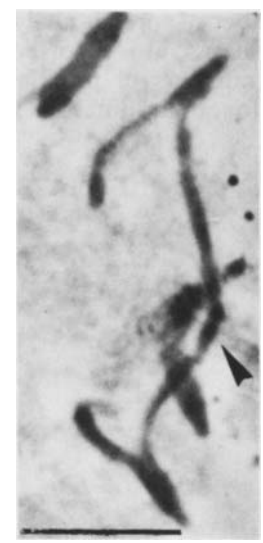

Figure 5 Anaphase chromosomes of *A. physodes* embryo treated with 1×10^{-9} M bis[dimethyltin(IV)chloro]protoporphyrin IX for 24 h; the arrow indicates a chromosome bridge. The bar is 10 μm.

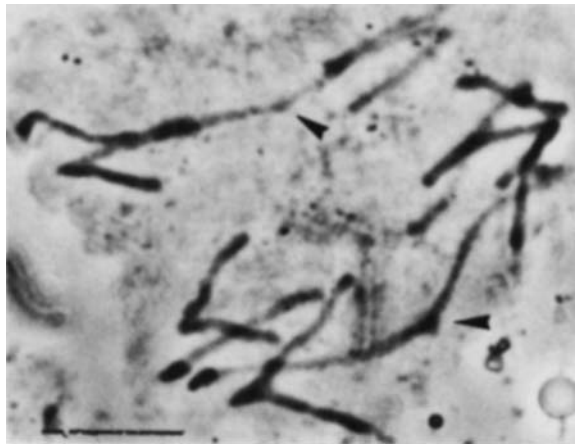

Figure 6 Anaphase chromosomes of *A. physodes* embryo treated with 2.07×10^{-7} M bis[dimethyltin(IV)chloro]protoporphyrin IX for 24 h; the arrows indicate chromosome bridges. The bar is 10 μm.

can be drawn, i.e. that aneuploidy occurring in metaphase spreads must be attributed to the squash technique rather than to the action of the complex tested in this investigation.

This conclusion is supported by the presence of nearly equal percentages of aneuploid metaphases in both treated and untreated embryos, and by the fact that these aneuploid spreads possessed chromosome numbers always lower than the mode. In fact, it is widely accepted that aneuploidy can be established unequivocally[13] only if there is an increase in hyperdiploidy (i.e. extra chromosomes). Conversely, although partial overlapping of numerous elements at the telophase prevented the counting of chromosomes at this stage, we believe that aneuploid telophase nuclei can form under the action of bis[dimethyltin(IV)chloro]protoporphyrin IX. In fact, as reported in Table 1, the presence of this compound significantly increases the rate of chromosome bridging at anaphase. Such events are commonly thought to be mainly responsible for non-disjunction of daughter chromosomes, thus inducing the formation of nuclei with chromosome numbers that deviate from the normal diploid complement.

Further, supposing that these embryos which contain a cell mosaicism may develop into adult forms, this chemical can (at least theoretically) pose a risk to gametogenesis, thereby limiting the fecundity.

Other interesting observations can be drawn from a comparative analysis of the data listed in Table 1. The first is that in treated embryos the number of anaphase spreads is consistently lower than that in controls. This number slightly increases when solution concentrations decrease. Since anaphase processes are closely related to the spindle structure, when they do not occur regularly (as in the case with treated embryos) it must be presumed that bis[dimethyltin(IV)-chloro]protoporphyrin IX interferes with the formation of the mitotic spindle.

Secondly, in the light of the results on chromosome morphology, this compound acts directly on metaphase chromosome structure. This is unambiguously indicated by the finding of either spreads with elements showing unstained areas or spreads with numerous chromosome fragments which regularly occur only in treated embryos.

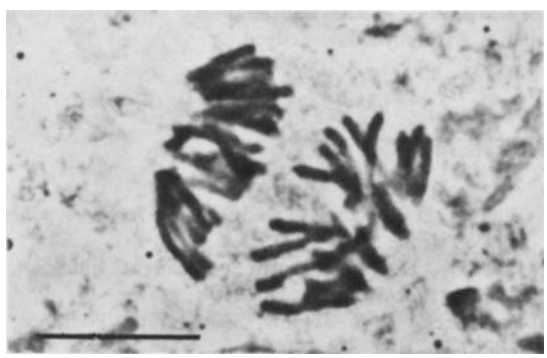

Figure 7 'Normal' anaphase–telophase spread in *A. physodes* embryo treated with 2.07×10^{-7} M bis[dimethyltin(IV)chloro]protoporphyrin IX for 24 h. The bar is 10 μm.

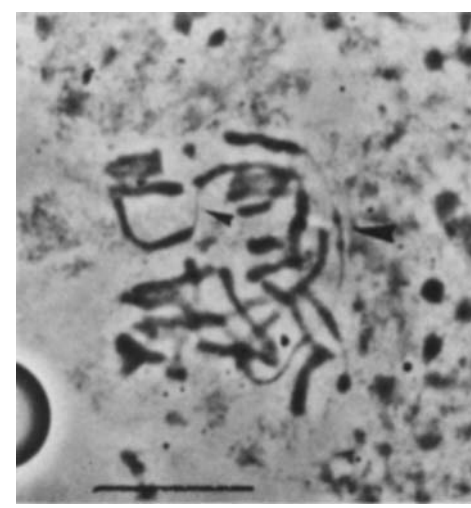

Figure 8 Metaphase spreads of *A. physodes* embryo treated with 1×10^{-9} M bis[dimethyltin(IV)chloro]protoporphyrin IX for 24 h, the arrows indicate chromosome fragments. The bar is 10 μm.

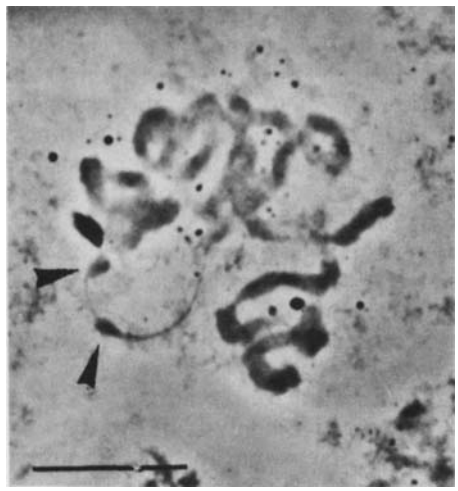

Figure 9 Metaphase spreads of *A. physodes* embryo treated with 2.07×10^{-7} M bis[dimethyltin(IV)chloro]protoporphyrin IX for 48 h; the arrows indicate chromosome fragments. The bar is 10 μm.

Events of the latter kind, previously documented in rainbow trout gonad cells (RTG-2)[14] after treatment with different chemicals such as some aromatic hydrocarbons, 9-aminoacridine (9-AA; Sigma Chemical Co.) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; Sigma Chemical Co.), are deleterious to the cells on account of their capacity for inducing the loss of more or less large DNA portions which can contain gene loci of vital importance to the organism.

The results of this investigation also suggest, interestingly, that bis[dimethyltin(IV)chloro]-protoporphyrin IX is capable of producing abnormal metaphase and anaphase figures in proportion to its concentration and not to the length of exposure (Table 1). The lack of increasing effectiveness at 48 h by this chemical is presumably due to its immediate action on cells.

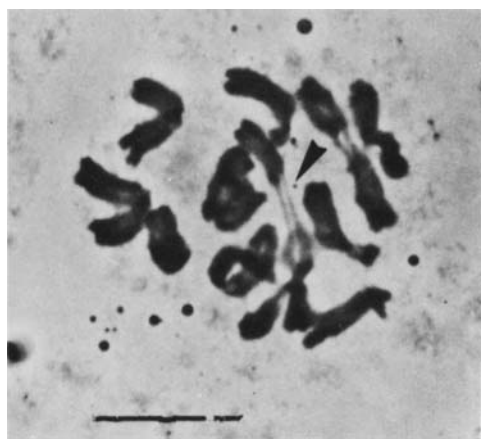

Figure 10 Metaphase spreads of *A. physodes* embryo treated with 1×10^{-9} M bis[dimethyltin(IV)chloro]protoporphyrin IX for 24 h; the arrow indicates a decondensed chromosome region. The bar is 10 μm.

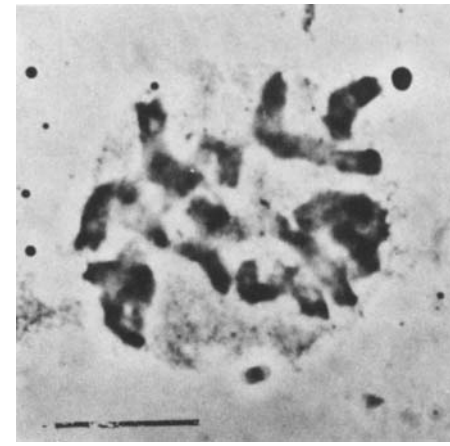

Figure 11 Metaphase spreads of *A. physodes* embryo treated with 1×10^{-9} M bis[dimethyltin(IV)chloro]protoporphyrin IX for 24 h, with large faintly stained regions of chromosomes. The bar is 10 μm.

Finally, although the mechanisms through which the compound under investigation exerts its effects are unknown, it is not unreasonable to consider that its genotoxicity can be attributed to the heavy metal (tin) contained in the complex.[9]

What has been shown is that heavy metals are clastogenic in *in vitro* mammalian cell assays[15] and, owing to their interference with spindle formation, they can induce aneuploidy. Moreover, exposure to heavy metals produced a variety of chromosomal and chromatid aberrations in terrestrial and aquatic animals.[16]

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