Spermatocyte chromosome alterations in *Truncatella subcylindrica* (L., 1767) (Mollusca, Mesogastropoda) following exposure to dibutyltin(IV) and tributyltin(IV) chlorides

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In order to analyze chromosomes for possible numerical and structural alterations in response to exposure to organotin(IV) compounds, gastropod *Truncatella subcylindrica* specimens were treated with dibutyltin(IV) and tributyltin(IV) chloride solutions with different exposure times.

Experimental evidence suggests that tributyltin(IV) chloride is more toxic to this organism than dibutyltin(IV) dichloride at low concentrations. Furthermore, the toxicity responses to these organotin(IV) derivatives seem to be proportional to both concentration and exposure time.

The following structural lesions have been identified by comparative analysis of spermatocyte chromosomes from untreated specimens and specimens treated with organotin(IV) compounds: (1) breakages; (2) bridging; (3) irregular outline; and (4) light areas after staining with acetic orcein. In this respect, dibutyltin(IV) and tributyltin(IV) chlorides seem to have an effect similar to that of colchicine.

Keywords: Organotin(IV) chlorides, spermatocyte chromosomes, alterations, Mollusca

INTRODUCTION

Experimental evidence strongly suggests that many pollutants, such as heavy metals, aromatic and chlorinated hydrocarbons,[1,2] various pesticides[3,4] and physical agents,[5] all increase the incidence of spontaneous chromosomal aberrations during mitosis in species such as *Umbra limi*,[6] *Salmo gairdneri* (Pisces Perciformes)[7] and *Mytilus edulis* (Mollusca, Bivalvia).[8]

Organotin(IV) compounds are widely used as industrial and agricultural biocides, antifouling agents, and miticides.[9] These chemicals may contaminate seawater surfaces, and exert toxicity towards a range of marine organisms, such as mussels,[10] oysters,[11–13] fish[14,15] and ascidian embryos.[16,17] Since experimental studies concerning the genotoxicity of these chemicals on marine organisms are virtually nonexistent, an investigation of this phenomenon is necessary for quantitative estimates of their effects.

The present research describes results obtained from a wide range of experiments with dibutyltin(IV) and tributyltin(IV) chloride solutions with regard to spermatocyte chromosomes of the mesogastropod *Truncatella subcylindrica*.

Truncatella subcylindrica was selected as the organism for study because:

(1) *Truncatella subcylindrica* is easily cultured, thus providing a continuous supply of experimental material;
(2) previous karyological reports[18,19] suggest that most Mesogastropoda species are characterized as possessing a relatively low number of chromosomes (ranging from $n = 16$ to $n = 18$) that are often large in size;
(3) testes from sexually mature male specimens can supply good chromosome preparations.
(4) spermatocytes are acknowledged to be chemically vulnerable organs.[20]

Owing to the fact that *Truncatella subcylindrica* is cytologically unknown, a preliminary description of spermatocyte chromosomes of this species conventionally stained with acetic orcein has been given.

MATERIALS AND METHODS

About 1000 specimens of *Trucantella subcylindrica*, 3–4 mm in length (Fig. 1), classified according to Parenzan,[21] were collected along the Sicilian Poseidonia beach of Trabia (Palermo) in

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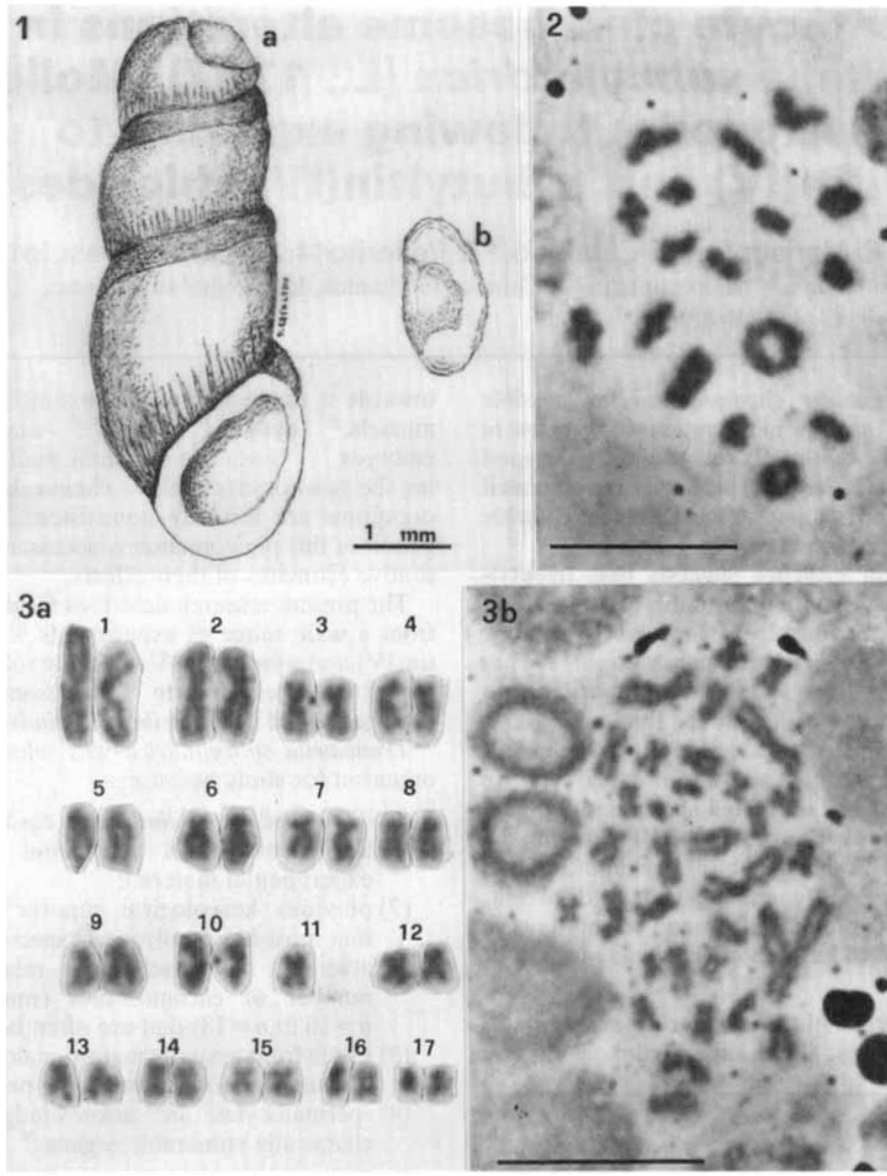

Figure 1 *Truncatela subcylindrica*: a, shell; b, operculum.

Figure 2 Acetic orcein diakinetic chromosomes of *Truncatella subcylindrica*. Acetic orcein is the C.I. Natural Red No. 28. The reported formula is $C_{28}H_{24}N_2O$; chemically it is a mixture of indefinite composition containing α-amino-orcein, iso-α-amino-orcein, α-hydroxyorcein and its tautomers, β-amino-orcein, β-amino-orceinimine, γ-amino-orcein and γ-amino-orceinimine. This histological staining reagent[29] is used in Molier's and Kornhauser's quadruple stains for showing elementary structure of animal tissue,[30, 31] elastic fibers, nuclei and connective tissue.[32, 33]

Figure 3a Representative karyotype of *Truncatella subcylindrica*.

Figure 3b Colchicinized spermatogonial metaphase plate of *Truncatella subcylindrica*.

April, May and June 1988–1989. The voucher shells of 20 specimens were deposited in the Museum of the Institute of Zoology, University of Palermo. Specimens, handled in groups of 50 individuals, were incubated in the presence of light in dibutyltin(IV) and tributyltin(IV) chloride solutions (concentrations and exposure times are documented in Table 1) and sexed by examination of the gonads. Their diet consisted of fragments of *Poseidonia oceanica* (common algae).

In Table 1, the number of sexually mature males analyzed for each experiment is also reported.

The organotin(IV) chlorides were a gift from Schering AG (Bergkamen, Germany). Concentrated stock solutions were obtained by dissolving stoichiometric amounts of each compound in Millipore-filtered seawater (MFSW). Working solutions (pH 7.8–8.0) were obtained by further dilution of the stocks in MFSW.

Freshly prepared Organotin(IV) concentrations in the diluted solutions were used and were stable. They were assayed using a Model 372 Perkin–Elmer atomic absorption spectrophotometer equipped with a graphite furnace. Stability of this species was demonstrated by the observation of single tin(IV) environments by Mössbauer spectroscopy.

The lowest organotin(IV) concentration used was 10^{-11} mol dm^{-3}, in accordance with the Italian legal limit for 'safe' tin concentration in water of 20 ng Sn dm^{-3}.

Since genetic activity of organotin(IV) compounds is virtually unknown, it is useful to test

Table 1 Dibutyltin(IV) and tributyltin(IV) chloride concentrations, incubation times and percentages of different categories of anomalous spreads observed during analysis of 100 spreads per specimen

All specimens were male

	Time interval:	3 h		24 h		48 h		144 h
	No. of specimens, *n*:	3		2		4		2
	Organometal:	DBTD	TBTC	DBTD	TBTC	DBTD	TBTC	DBTD
Anomaly	Concentration (mol dm^{-3}):	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}
Irregular outlines		Normal[b]	45	45	86	82	91	86
Breakages		Normal	19	20	37	40	35	28
Chromosome bridging		Normal	24	26	40	32	36	35
Lightly stained areas		Normal	3	43	78	67	86	84
	No. of specimens, *n*:	3		6		6		2
	Concentration (mol dm^{-3}):	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}	10^{-7}
Irregular outlines		Normal	Normal	7	55	42	85	78
Breakages		Normal	Normal	3	21	15	38	45
Chromosome bridging		Normal	Normal	3	19	15	39	32
Lightly stained areas		Normal	Normal	5	50	33	80	65
	No. of specimens, *n*:	3		6		6		2
	Concentration (mol dm^{-3}):	10^{-9}	10^{-9}	10^{-9}	10^{-9}	10^{-9}	10^{-9}	10^{-9}
Irregular outlines		Normal	Normal	Normal	46	16	60	35
Breakages		Normal	Normal	Normal	14	7	21	15
Chromosome bridging		Normal	Normal	Normal	16	10	26	18
Lightly stained areas		Normal	Normal	Normal	44	15	48	35
	No. of specimens, *n*:	3		6		6		2
	Concentration (mol dm^{-3}):	10^{-11}	10^{-11}	10^{-11}	10^{-11}	10^{-11}	10^{-11}	10^{-11}
Irregular outlines		Normal	Normal	Normal	12	Normal	38	25
Breakages		Normal	Normal	Normal	8	Normal	20	15
Chromosomes bridging		Normal	Normal	Normal	10	Normal	19	16
Lightly stained areas		Normal	Normal	Normal	11	Normal	35	22

[a] DBTD, dibutyltin(IV) dichloride; TBTC, tributyltin(IV) chloride.

[b] The term 'Normal' indicates no difference from the control.

[c] As 100 spreads of each specimen were examined, the percentage values in this Table are calculated from *n* observations in each case, where $n = 100 \times$ no. of specimens.

them also using higher concentrations (10^{-4} mol dm^{-3}) of organotin(IV) derivatives to detect evidence of cytotoxicity.

Chromosome slides were prepared from testes according to previously described methods used for other molluscan species.[19]

Slides were also prepared from control animals which had been incubated in the presence and absence of colchicine (10^{-4} mol dm^{-3} hypotonic solution).

Mitotic chromosomes were classified according to the terminology of Levan *et al.*[22]

Chromosome counts and observations and photomicrographs were made using a Jenamed 2 phase-contrast microscope and Agfa Gevaert AG25 film.

RESULTS

Uncolchicinized chromosomes (controls)

Chromosome observations were made on three sexually mature males per experiment.

At diakinesis (Fig. 2), all the bivalents appeared homogeneously stained, well separated and with regular outlines.

From counts of 100 spreads per specimen, the haploid number was determined to be $n = 17$. Ring- and cross-shaped chromosomes occurred, probably bearing two terminal and two subterminal chiasmata, respectively. Only *ca* 5% of the spreads analyzed showed bivalents which were broken or sticking to one another. Few spreads (from two to five per specimen) were aneuploid due to a lower chromosome number. Mitotic metaphases were absent.

Colchicinized chromosomes (controls)

Diakinetic chromosomes resembled those in uncolchicinized slides and their counts gave the haploid number of 17 with the exception of a few spreads (3–4%) which possessed a lower chromosome number.

From analysis of ten spreads per specimen (ten sexually mature males were analyzed), spermatogonial chromosomes appear homogeneously stained and with regular outlines (Fig. 3b). They showed sister chromatids as distinct elements, so that centromere locations were clearly visible.

All chromosomes were gathered in pairs, 14 of which were bi-armed (M + SM) and 3 mono-armed (ST + T) (Fig. 3a). In these preparations, from three to five mitotic spreads per specimen showed polyploid chromosome numbers (Fig. 4).

Dibutyltin(IV) and tributyltin(IV) chloride-treated chromosomes

The number of sexually mature male specimens examined, the organotin concentrations, the incubation times and the percentage of different categories of anomalous diakinetic plates with respect to the controls, are reported in Table 1.

The most frequently observed anomalies included:

(1) bivalents with irregular outlines (Fig. 5);
(2) breakages (Fig. 6), and
(3) chromosome bridging (Figs. 6, 7).

A high frequency of spreads showed the occurrence of bivalents which were non-uniformly stained due to small unstained regions (Figs. 8, 9) as well as bivalents with irregular outlines (Fig. 5), so that more than one anomaly per spread was routinely observed.

About 4–5% of the spreads analyzed were aneuploid, and, as in control specimens, chromosome numbers were lower than the mode.

In comparison with the controls, meiotic spreads of treated specimens showed diakinetic bivalents which were broken or bivalents which had clumped together, thus initiating chromosome bridging. Sometimes breakage involved more than one bivalent per spread. In some spreads more than one association could be ascertained (Fig. 7).

Among the organotin(IV)-treated specimens, a variable number of spermatogonial metaphase spreads, ranging from 7 to 12 per specimen, were encountered.

Chromosomes in mitotic spreads displayed a regular morphology except for the occurrence of small unstained regions (gaps) (Fig. 10), observed in various elements of each spread. A high incidence, ranging from 15 to 20 spreads per specimen, had polyploid numbers. Contrary to the chromosomes of colchicinized polyploid spreads which were homogeneously stained, the chromosomes of specimens treated with organotin(IV) compounds showed small unstained regions involving several elements per spread (Fig. 11).

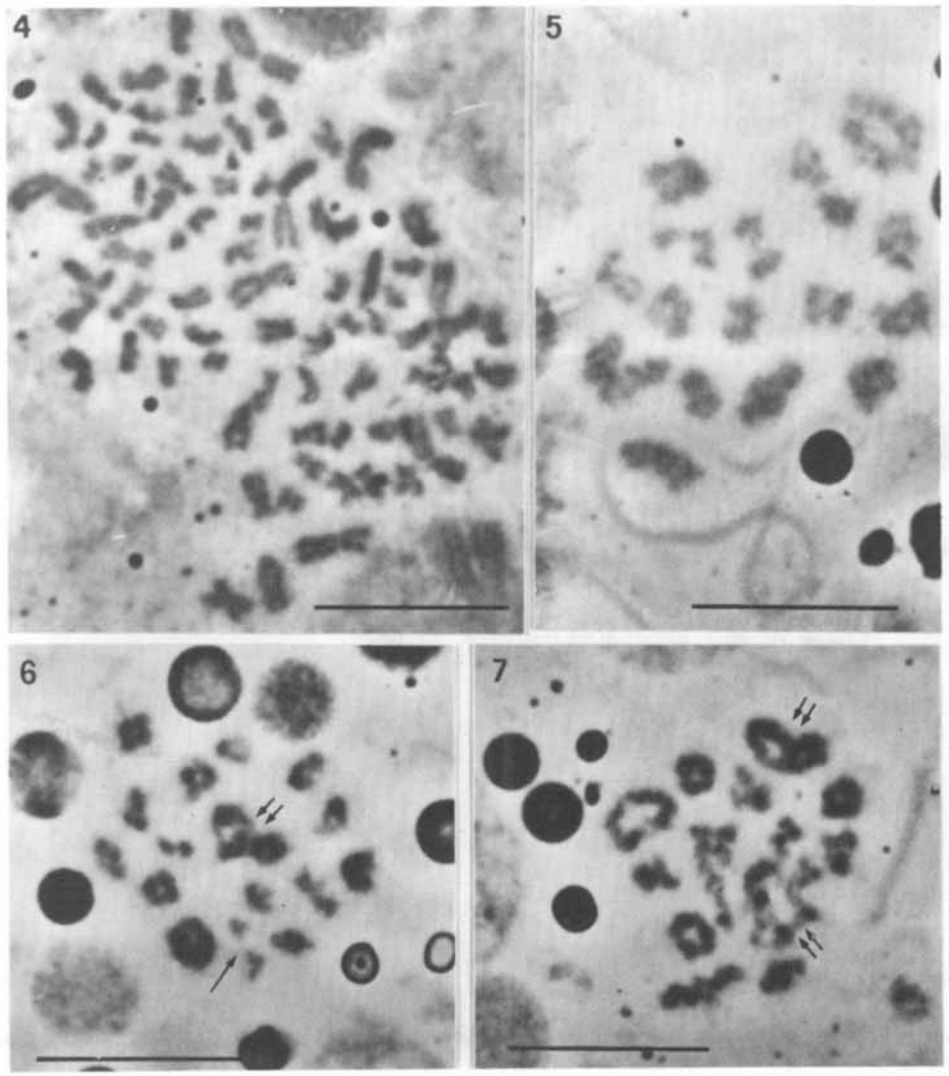

Figure 4 Colchicinized mitotic tetraploid plate of control *Truncatella subcylindrica*.
Figure 5 Diakinetic bivalents of *Truncatella subcylindrica* treated with 10^{-9} mol dm^{-3} $Bu_3Sn(IV)Cl$, for 24 h.
Figure 6 Diakinetic bivalents of *Truncatella subcylindrica* treated with 10^{-11} mol dm^{-3} $Bu_3Sn(IV)Cl$, for 72 h (single arrow indicates a breakage and double arrow indicates the bridging).
Figure 7 Diakinetic bivalents of *Truncatella subcylindrica* treated with 10^{-9} mol dm^{-3} $Bu_3Sn(IV)Cl$ for 72 h (double arrows indicate the bridging). (Bars represent 10 μm.)

DISCUSSION

By counting diakinetic bivalents, we determined the haploid chromosome number to be $n = 17$ for *Truncatella subcylindrica*. The diploid value $2n = 34$ was established from counts of spermatogonial chromosomes at metaphase.

Considering the results from conventionally stained slides obtained from uncolchicinized and colchicinized specimens, we are not surprised to find in *Truncatella subcylindrica* the same cytological characteristics reported for other molluscan species previously investigated,[18,19] i.e., mitotic and meiotic chromosomes which are homogeneously stained and with regular outlines.

Cytological data, listed in Table 1, and data obtained from controls suggest some significant points.

(1) A low background level (≈5%) of spontaneous abnormalities in diakinetic figures

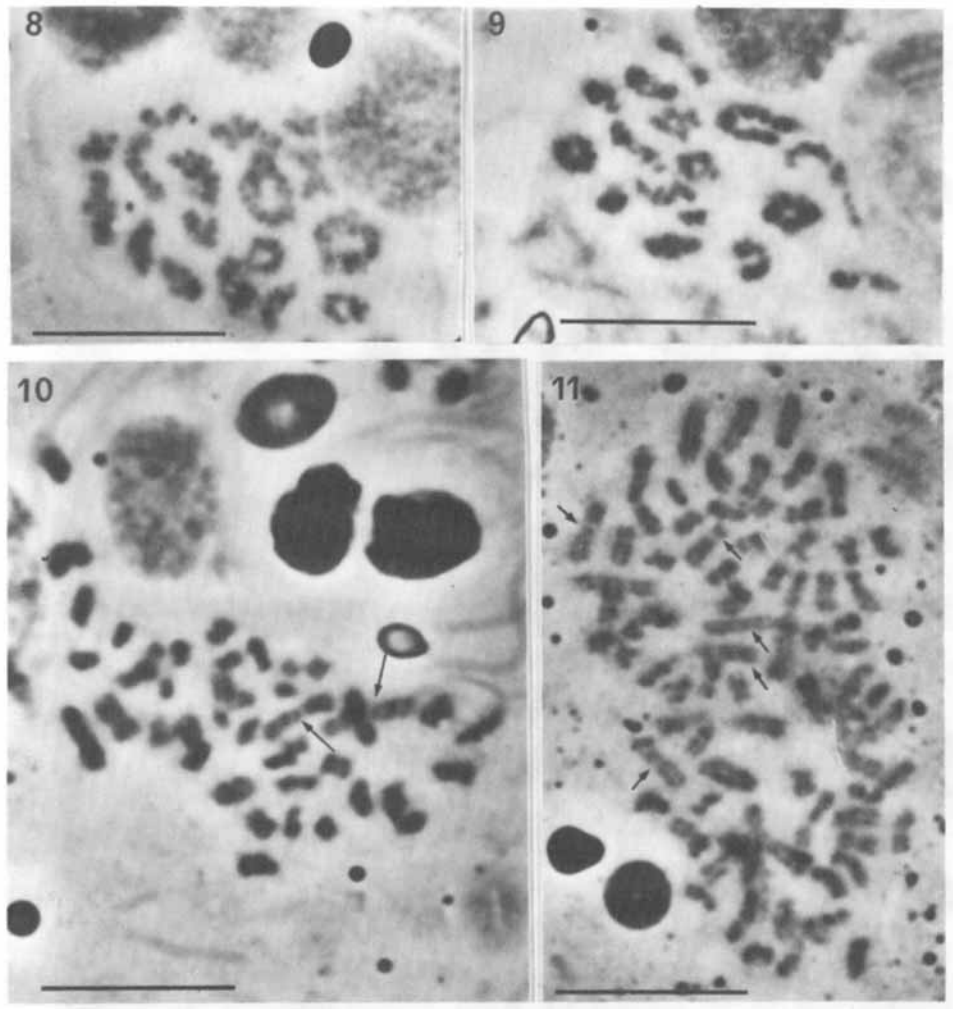

Figure 8 Diakinetic bivalents of *Truncatella subcylindrica* treated with 10^{-9} mol dm^{-3} $Bu_3Sn(IV)Cl$ for 72 h (double arrows indicate the bridging).

Figure 9 Diakinetic bivalents of *Truncatella subcylindrica* treated with 10^{-7} mol dm^{-3} $Bu_2Sn(IV)Cl_2$ for 24 h

Figure 10 Spermatogonal metaphase plate of *Truncatella subcylindrica* treated with 10^{-7} mol dm^{-3} $Bu_2Sn(IV)Cl_2$ for 48 h (arrows indicate lightly stained areas).

Figure 11 Mitotic tetraploid plate of *Truncatella subcylindrica* treated with 10^{-7} mol dm^{-3} $Bu_3Sn(IV)Cl$ for 24 h (arrows indicate chromosomes with lightly stained areas). (Bars represent 10 μm.)

(chromosome breakages and junctions between two or more bivalents) were present in *Truncatella subcylindrica*.

(2) Tributyltin(IV) chloride was more toxic than dibutyltin(IV) dichloride at the lowest concentrations.

(3) Genotoxicity of organotin(IV) compounds, clearly demonstrated using a 10^{-4} mol dm^{-3} solution, seems to parallel both the concentration and length of exposure.

In consequence, this might imply that concentrations of these chemicals, that are lower than those used in our experiments, could result in chromosomal damage after longer exposure times.

Since an approximately equal percentage (4–5%) of aneuploid spreads in both treated and untreated specimens were observed, and because of the relative fragility of hypotonically treated cells, 'aneuploidy' is probably an artifact of the

squashing technique. Further, the data listed in Table 1 indicate that more than one chromosome alteration was routinely present in most of the spreads analyzed and that both organotin(IV) compounds exerted adverse effects at a significant rate for the 48 h treatment when solution concentrations ranged from 10^{-7} to 10^{-9} mol dm^{-3}.

In particular, at these concentrations, when the exposure time rises from 24 h to 48 h, an approximately twofold increase for 'breakages' has been noted.

However, if it is true, as suggested by several authors,[2,5] that 'stickiness' (chromosomal aggregation) in chromosomal groups would result from previous breakages and translocations, followed by rejoining of the broken ends in abnormal patterns, then there might have been a higher number of breakages involving meiotic chromosomes than that reported by us. In fact, in our experiments, numerous spreads displayed chromosomal bridging (see Table 1).

Lightly stained areas occurred either in the bivalents at diakinesis or in metaphase mitotic chromosomes.

Discontinuities within chromatid arms, in which the chromatid region distal to the discontinuity is aligned with the rest of the chromatids, are known as 'gaps' or 'achromatic lesions' and, in some instances, they are sensitive indicators of genotoxicity.

Since chromosomes with unstained regions (gaps) occurred only in specimens treated with organotin(IV) derivatives, we can conclude that the lack of staining is caused by these chemicals.

In order to explain this anomaly, at least three interpretations can be given: the first is that, in accordance with Savage,[5] 'gaps' would be induced by the association of lesions which are close in space and time; and the second is that errors in packing during chromosome condensation could have occurred. However, since these achromatic regions, which appear to involve both sister chromatids, retain alignment, it is suggested that these areas are not empty but possess a very much reduced DNA content. Analogous conclusions have been reported by Scheid and Trout,[24] who, by employing higher-resolution methods (UV) gave clear evidence of chromatin continuity through achromatic regions.

The third interpretation is that an induced gene activation could have occurred. Thereby, changes in gene activity have been observed in response to physical and chemical stresses.[25-27]

By considering the fact that mitotic chromosomes have lightly stained areas but show neither irregular outlines nor breakage, we conclude that they are less prone to modification than meiotic bivalents. However, as observed after treatment with methyl and phenylmercury compounds[2,28] and dibutyltin(IV) and tributyltin(IV) compounds (this work), mitotic chromosomes show modifications similar to those observed after treatment with colchicine. Effects analogous to these have also been observed after treatment with these chemicals in early ascidian embryos.[16,17]

It is known that colchicine action mainly involves the spindle apparatus. As in mitotic chromosomes we found unstained regions, it is possible to conclude that additional structural modifications occurred, probably in response to metabolic disorder (Figs 10, 11).

Finally, since it is impossible to distinguish polyploid spreads from apparent polyploid spreads resulting from closely adjacent diploid cells, we cannot state conclusively that polyploidy is an anomaly induced by exposure to organotin(IV) compounds.

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