Mutagenesis vol.12 no.4 pp.265–269, 1997

Genotoxicity of tetrodotoxin from puffer fish tested in root meristem cells of *Allium cepa* L.

Samanta S.Khora[1], Kamal K.Panda[2,3] and Brahma B.Panda[1]

[1]Department of Marine Sciences and [2]Genetic Toxicology Laboratory, Department of Botany, Berhampur University, Berhampur-760 007, Orissa, India

[3]To whom correspondence should be addressed

Tetrodotoxin (TTX) extracted and purified from puffer fish *Arothron nigropunctatus* was tested for genotoxicity employing the root meristem cells of *Allium cepa* as the assay system. The genotoxicity endpoints investigated were mitotic index (MI), meta-anaphases with spindle aberrations, interphases with micronuclei (MNC) and sister chromatid exchanges (SCE) in metaphase chromosomes. The results demonstrated that TTX inhibited mitosis at concentrations of ≥30 μM as evident by the fall of MI, but failed to induce MNC at significant levels at any of the concentrations tested (10–100 μM). TTX was thus proved to be neither clastogenic nor aneugenic in the present study. It was, however, noteworthy that TTX at far lower concentrations, 0.1–5.0 μM, significantly enhanced the frequencies of SCE which indicated possible interference of the toxin in DNA replication and repair.

Introduction

Tetrodotoxin (TTX) is one of the best known non-protein marine toxins because of its frequent involvement in fatal food poisoning (Khara, 1994; Ghosh *et al.*, 1994). The toxin derives its name from the puffer fish family Tetraodontidae, but studies have revealed its wide distribution in both terrestrial and aquatic animals (Fuhrman, 1986). The food-chain transmission of TTX in certain marine ecosystems involving fish, crabs, annelids, algae and bacteria have been reported (Yasumoto *et al.*, 1986), underscoring the possible ecotoxicological implications. With its unusual chemical structure (Figure 1), TTX is an established neurotoxin known to selectively block sodium channels of excitable membranes (Kao, 1986). The genotoxicity of TTX is however not known. Information with respect to the genotoxicity of TTX from puffer fish is vital because many of them have been valued as food for a long time and pose a potential threat to human and environmental health.

Plants are very useful as first-tier genotoxicity assays because of their simplicity, low relative cost, versatility and minimal laboratory facilities required for their performance, so much so that they have been the assay of choice for genetic toxicological testing in developing countries (Plewa, 1985; Grant, 1994). Furthermore, due to the high degree of concordance between genotoxicity results obtained from mammalian assays and plant assays (Ennever *et al.*, 1988; Sandhu *et al.*, 1994), the latter were used in the present study. TTX extracted for the first time from the species of puffer fish *Arothron nigropunctatus* (Abe, 1984) was tested for genotoxicity employing micronuclei (MNC) and sister chromatid exchange (SCE) assays in root meristem cells of *Allium cepa*. The genotoxicity endpoints of MNC and SCE were selected to find out whether TTX was aneugenic and/or clastogenic (Reddy *et al.*, 1995) and whether it interfered with the fidelity of DNA replication and repair (Tucker *et al.*, 1993).

Materials and methods

Extraction and purification of TTX

Weighed specimens of puffer fish, *A.nigropunctatus* (Bloch and Schneider) were homogenized and extracted in boiling 0.1 N acetic acid. After defatting with diethyl ether, the extract was adjusted to pH 5.5 with dilute sodium hydroxide and passed through an activated charcoal column. The column was washed with deionized water and the toxin was eluted with an aqueous solution containing 20% ethanol and 1% acetic acid. After evaporation of the solvent, the residue was dissolved in distilled water and chromatographed successively on a series of columns: Bio-Gel P-2 (2.5×90 cm), Bio-Rex 70 (1×84 cm) and Hitachi Gel 3011C (0.5×40 cm) (Nakamura and Yasumoto, 1985). The columns were washed with distilled water and the toxin eluted with 0.05 N acetic acid. The eluate was then lyophilized and redissolved in distilled water prior to subsequent chromatography. The eluate was analysed for TTX by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) as follows.

A fluorimetric HPLC analyser was employed for separation of TTX on a Develosil ODS-5 column (0.46×25 cm) by paired ion chromatography with 0.05 M acetic acid–ammonium hydroxide buffer, pH 5, containing 3% acetonitrile and 0.06 M heptafluorobutyric acid, at a flow rate of 0.5 ml/min, and the fluorescence intensities of toxin products were measured (Yasumoto and Michishita, 1985; Yotsu *et al.*, 1989). The final purification of the TTX was accomplished under identical conditions.

TLC of the toxin was performed on precoated silica gel 60 plates (Merck, Germany) using the solvent pyridine–ethyl acetate–acetic acid–water (15:7:3:6). TTX was detected as fluorescent spots with 10% potassium hydroxide in methanol.

The toxin isolated and purified from puffer extract as above was confirmed by comparing the chromatographic properties on TLC and fluorometric HPLC with those of reference TTX (Yasumoto *et al.*, 1988). The purified TTX from puffer fish was lyophilized, weighed and sealed in ampoules for further toxicity testing. A stock solution of TTX was prepared by dissolving a weighed amount of the toxin in slightly acidified distilled water and experimental solutions of desired concentrations were prepared by dilution with tap water, pH 7.

Assay system

The growing root meristems of local onion, *A.cepa* L. (2n = 16), were used as the assay system. Healthy bulbs of *A.cepa* were allowed to germinate by placing five bulbs per cylindrical plastic receptacle containing 250 ml of tap water at 24 ± 1°C in the dark. Air was bubbled through continuously at a rate of 10–20 cm^3/min and the tap water was renewed daily.

MNC assay

The growing root meristems of *A.cepa*, 2–3 cm long, were treated with the experimental solutions containing 10, 30, 60 or 100 μM of TTX for 1 h followed by recovery in tap water. During recovery the root tips were excised

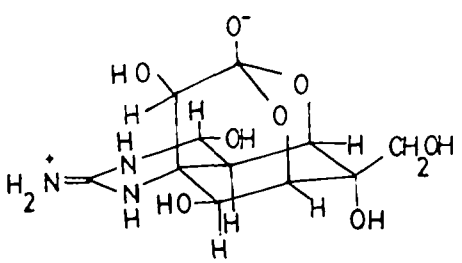

Fig. 1. Tetrodotoxin (CAS No. 4368-28-9).

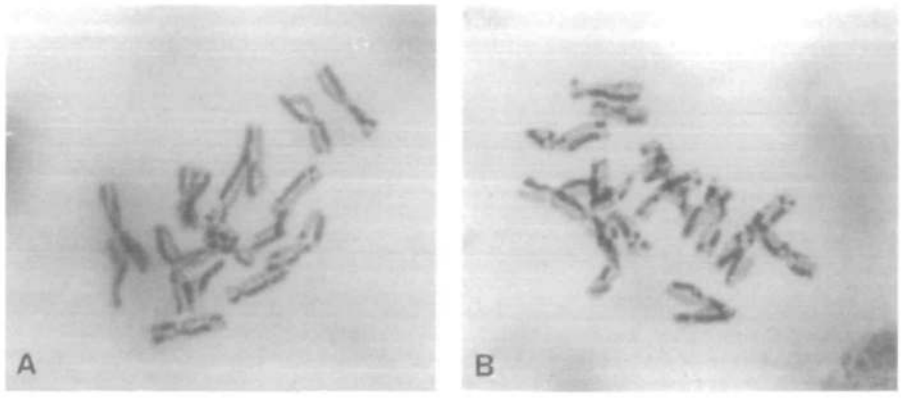

Fig. 2. SCE in chromosomes of *A.cepa* stained with haematoxylin procedure. (**A**) Control. (**B**) Induced by TTX, 0.5 μM.

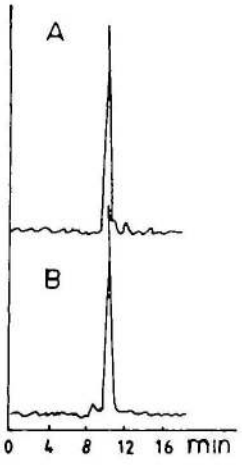

Fig. 3. Fluorometric HPLC chromatograms of (**A**) reference TTX and (**B**) puffer fish TTX

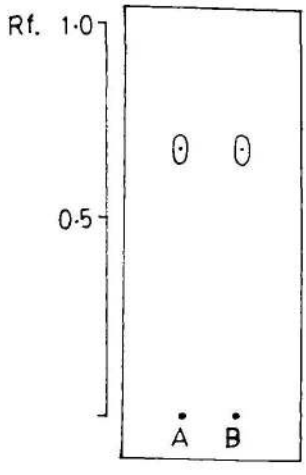

Fig. 4. TLC chromatograms of (**A**) reference TTX and (**B**) puffer fish TTX.

and fixed in acetic acid–ethanol, 1:3, at 6 h intervals from 6 to 48 h. The root meristems were then processed for cytology following the haematoxylin schedule (Sharma and Sharma, 1980) to determine mitotic index (MI, percentage of mitoses), frequencies of meta-anaphases with spindle aberrations, represented by C-metaphases, star anaphases, multipolar anaphases and anaphases with lagging chromosomes (Dash *et al.*, 1988) and interphases with MNC (Reddy *et al.*, 1995).

SCE assay

The growing root meristems of *A cepa*, 2–3 cm long, were treated with 100 μM 5-bromodeoxy uridine (BrdUrd), 0.1 μM 5-fluorodeoxyuridine (FdUrd) and 5 μM uridine (Urd) (Sigma, New Delhi, India) for 20 h (approximately one cell cycle) followed by treatment with 0.1–5 μM TTX for 1 h in the presence of 100 μM deoxythymidine (dT) (Loba-Chemie, Mumbai, India) and 5 μM Urd. After a brief wash, the roots were allowed to grow for another round of treatment with dT and Urd for 19 h. The treatments were terminated by washing the roots under running tap water and treating them with 0.05% colchicine (Loba-Chemie) for 2.5 h. All the above treatments were performed in plastic receptacles containing 250 ml of experimental solution under continuous bubbling. Roots were washed, excised and fixed in acetic acid: methanol, 1:3, for 7 h and preserved at 4°C. The root meristems were processed cytologically for SCE analysis (Panda *et al.*, 1996) with the following modifications. The meristems were hydrolysed in 5 N HCl at 25°C for 92 min and stained with haematoxylin (Sharma and Sharma, 1980) for at least 5 h followed by moderation with 0.5% ferric chloride for 15 min. The stained root meristems were washed in distilled water, squashed in a drop of 45% acetic acid and tapped for metaphase chromosome separation under coverslips. The coverslips were sealed with nail polish. The SCEs were scored at ×400 magnification and representative chromosome spreads were photographed at ×1000 magnification (Figure 2) using a Zeiss Standard photomicroscope.

Tap water controls were included in both assays and analysis was carried out using coded slides. Increases in the frequency of cells with spindle aberrations, MNC or SCE over controls were tested statistically using the one-tailed *t*-tested. Dose–response curves obtained for SCE were subjected to regression analysis

Results and discussion

The toxin extracted, isolated and purified from puffer fish was identified and confirmed to be TTX (Figure 1) following HPLC (Figure 3) and TLC (Figure 4), which were compared with that of reference TTX. From 1.2 kg of homogenized and extracted puffer, the TTX obtained in pure form was ~2 mg. The R_f value and the retention time (t_R) so determined for puffer TTX were 0.70 and 10 min 20 s respectively by TLC and HPLC, which were comparable with that of reference TTX. The toxin was subsequently tested for genetic toxicity in the present study.

Results with respect to the effect of TTX on mitosis are presented in Table I. At 10 μM TTX the MI recorded at different recovery times varied between 7 and 9, which was comparable with those recorded for the controls. A gradual fall in MI was evident however with the increase of concentration to 30 and 60 μM, the MI being in the range of 5–7 and 3–5 respectively. The induction of cells with spindle aberrations that included C-metaphases and C-anaphases were significant ($P \leq 0.05$–0.01) at only one of the concentrations of TTX (30 μM), and only at 6 and 12 h post-treatment. The frequencies of cells with spindle aberrations recorded for the rest of the concentrations were not significant ($P \leq 0.05$). The frequencies of cells with MNC induced by TTX were insignificant at all concentrations and recovery hours. TTX was tested for

Table I. Frequency of cells with spindle aberration and MNC induced by TTX in root meristems of *A.cepa*

Chemical exposure (concentration) (μM)	Recovery (h)	MI ± SEM	Metaphases analysed	Anaphases analysed	Meta-anaphases analysed	C-metaphases	Anaphases[a]	Cells with spindle aberration	Cells with spindle aberration n ± SEM	Interphases analysed	Cells with MNC	MNC/1000 cells ± SEM
0 (control)	6	6.32 ± 0.28	231	105	336	0	0	0	0	4958	1	0.2 ± 0.2
	12	5.59 ± 0.44	210	118	328	0	0	0	0	5321	0	0
	18	6.49 ± 0.22	195	102	297	0	1	1	0.28 ± 0.28	5468	1	0.18 ± 0.18
	24	7.45 ± 0.18	218	87	305	1	0	1	0.3 ± 0.3	4550	0	0
	30	8.78 ± 0.66	136	96	232	0	0	0	0	5184	1	0.18 ± 0.18
	36	9.42 ± 0.25	218	87	305	0	0	0	0	4320	1	0.22 ± 0.22
	42	8.48 ± 0.48	144	108	252	0	0	0	0	5321	1	0.17 ± 0.17
	48	8.38 ± 0.41	235	112	347	1	1	2	0.57 ± 0.2	4320	1	0.2 ± 0.2
10	6	8.52 ± 0.54	197	111	308	2	0	2	0.55 ± 0.05	5727	1	0.17 ± 0.11
	12	7.39 ± 0.59	126	59	185	0	0	0	0	5170	1	0.19 ± 0.19
	18	7.58 ± 0.23	261	107	368	0	1	1	0	5362	0	0
	24	8.42 ± 0.21	212	122	334	0	0	0	0	6221	1	0.17 ± 0.17
	30	9.83 ± 0.28	208	141	349	1	0	1	0.28 ± 0.28	6142	2	0.32 ± 0.28
	36	7.45 ± 0.13	198	153	351	1	0	1	0.3 ± 0.3	4978	1	0.18 ± 0.10
	42	8.29 ± 0.4	194	96	290	0	0	0	0	5885	1	0.16 ± 0.16
	48	9.18 ± 0.51	190	158	348	1	1	2	0.6 ± 0.27	6561	2	0.16 ± 0.16
30	6	5.14 ± 0.39	166	107	273	6	8	14	4.65 ± 0.71[b]	7758	16	2.34 ± 1.41
	12	5.87 ± 0.42	180	108	288	5	4	9	3.13 ± 0.75[c]	7507	7	1.15 ± 0.18
	18	5.52 ± 0.43	165	89	254	3	1	4	1.03 ± 0.46	7527	10	1.39 ± 0.43
	24	4.93 ± 0.22	140	98	238	4	1	5	2.13 ± 0.75	7221	9	1.31 ± 0.19
	30	5.69 ± 0.3	147	146	293	2	0	2	0.84 ± 0.53	8808	6	0.68 ± 0.02
	36	7.00 ± 0.56	191	175	366	2	0	2	0.6 ± 0.38	9245	7	0.7 ± 0.02
	42	7.33 ± 0.33	202	180	382	4	0	4	0.5 ± 0.32	8527	4	0.5 ± 0.32
	48	7.23 ± 0.25	117	169	286	0	0	0	0	8243	6	0.77 ± 0.02
60	6	4.66 ± 0.55	129	113	242	3	0	3	1.2 ± 0.93	8054	5	0.6 ± 0.12
	12	4.89 ± 0.12	141	78	219	2	1	3	1.6 ± 0.7	6331	2	0.31 ± 0.10
	18	4.14 ± 0.78	130	88	218	3	0	3	1.3 ± 0.8	5381	1	0.18 ± 0.18
	24	4.6 ± 0.62	129	100	229	0	2	2	1.8 ± 0.6	5571	4	0.71 ± 0.4
	30	3.9 ± 0.52	109	73	182	3	1	4	2.19 ± 0.3	9553	2	0.3 ± 0.2
	36	4.21 ± 0.38	139	70	209	3	2	5	2.03 ± 0.9	9308	3	0.28 ± 0.26
	42	4.08 ± 0.18	140	82	222	1	1	2	1.3 ± 0.6	8756	4	0.43 ± 0.18
	48	3.02 ± 0.52	144	62	206	2	1	3	1.9 ± 0.4	7655	2	0.22 ± 0.2
100	6	0	No cell division									
	12	0										
	18	0										
	24	0										
	30	0										
	36	0										
	48	0										

[a]Anaphase includes star anaphase, multipolar anaphase and anaphase with lagging chromosome(s).
[b]$P \leq 0.01$; [c]$P \leq 0.05$ compared with control.

Table II. Dose–response induction of SCE in root meristem cells of *A cepa* by TTX

Concentration (μM)	No. of metaphases analysed (no. of roots)	No. of chromosomes analysed	Total no. of SCEs	SCE/cell ± SEM	SCE/chromosome ± SEM
0 (control)	36(6)	553	1205	38 15 ± 0.11	2.4 ± 0.27
0.1	45(7)	802	1215	36.49 ± 0.1	2 3 ± 0.27
0.5	38(6)	660	1640	47.09 ± 0.16[a]	3 47 ± 0.17[a]
1.0	63(9)	908	2821	46.09 ± 0.16[a]	3 6 ± 0.27[a]
3.0	40(7)	585	3092	96.1 ± 0 35[a]	6 06 ± 0 02[a]
5.0	SCEs were too many to be scored				

[a]$P \leq 0.001$ compared with control

-induction of SCE at much lower concentrations ranging from 0.1 to 5 μM. The frequencies of SCE calculated either per cell or per chromosome (Table II) induced by TTX from 0.5 μM onwards were significantly higher than those recorded for the controls. Furthermore, the SCE frequency was double the control value at 3 μM. At the next higher dose (5 μm) the SCE induced by TTX were not only too many but also too minute and therefore could not be scored. The increase of SCE induced by TTX followed a dose–response ($r = 0.91$, $P \leq 0.05$).

Puffer fish are mainly tropical in their distribution, although some species are found in temperate regions (Ghosh *et al.*, 1994). Puffer poisoning appears to be a major health hazard in Japan, lower California and the Mediterranean. In India, human poisoning due to puffer fish has been reported from the Bengal and Kerala coasts (Khora, 1994). Notwithstanding the danger of TTX-poisoning, seafoods prepared from puffer are consumed as a delicacy, so much so that the puffer fish is an important commercial commodity in Japan (Ghosh *et al.*, 1994). The neurotoxicity of TTX at extremely low concentrations is attributed to its selective and high affinity blockade of the voltage-gated sodium channels of excitable membranes and, therefore, has been an important tool in electrophysiological and neurobiological research, and more particularly in understanding the molecular biology of the sodium channel (Kao and Levinson, 1986). This toxin has been incomparably useful as a legend that enables the purification of sodium channel peptides and as a pharmacological agent that permits the precise reduction or elimination of Na^+ currents in excitable cells (Ritchie and Rogart, 1977; Agnew *et al.*, 1978).

In the present study TTX is demonstrated to be an inhibitor of mitosis as indicated by the fall of MI but only at higher concentrations of ≥30 μM. At specific concentrations, the toxin interfered with spindle function, as noted at 30 μM and 6 and 12 h of recovery in the present study. The frequencies of cells with MNC induced by TTX were, however, insignificant at any of the concentrations tested. The *Allium* MNC assay thus provided evidence that TTX was neither an aneugen nor a clastogen (Reddy *et al.*, 1995).

The SCE assay has been proved to be one of the most sensitive short-term genotoxicity assays because of its ability to detect genotoxins at very low concentrations (Tucker *et al.*, 1993). Although the exact mechanism(s) of induction of SCE is still a subject of discussion (Panda *et al.*, 1996), SCEs are widely believed to represent the interchanges of DNA replication products at apparently homologous loci, and involve DNA breakage and reunion (Latt *et al.*, 1981). TTX at far lower concentrations (0.1–5 μM) enhanced SCE significantly over the control that not only followed a dose–response but doubled the frequency at 3 μM, thereby establishing TTX as clearly positive in the SCE assay (Tucker *et al.*, 1995). Although the implications of this positive response with the SCE assay may not be clear, the present findings nevertheless provide evidence that TTX possibly interfered with DNA replication and its repair process, thereby warranting further research.

Acknowledgements

The authors are grateful to the authorities of Berhampur University for providing institutional facilities to carry out the present research Extraction and purification of TTX was carried out at the Laboratory of Food Hygiene, Faculty of Agriculture, Tohoku University, Japan, by S.S.K. while working with Professor T Yasumoto, supported by a grant from the Ministry of Education, Science and Culture, Japan. The present work was supported by CSIR, New Delhi, through the award of research associateships to S S.K and K.K.P.

References

Abe,T. (1984) *Pufferfishes Available in Japan — An Illustrated Guide to their Identification*. Environmental Health Bureau, Ministry of Health and Welfare, Japan, Tokyo, pp. 85.

Agnew,W S , Levinson,S.R , Brabson,J.S. and Rafferty,M.A. (1978) Purification of the tetrodotoxin-binding component associated with voltage-sensitive sodium channel from *Electrophorus electricus* electroplax membranes *Proc Natl Acad Sci USA*, **75**, 2606–2610.

Dash,S , Panda,K K and Panda,B.B. (1988) Biomonitoring of low levels of mercurial derivatives in water and soil by *Allium* micronucleus assay. *Mutat Res.*, **203**, 11–21.

Ennever,F.K., Andeano,G and Rosenkranz,H.S. (1988) The ability of plant genotoxicity assays to predict carcinogenicity. *Mutat. Res.*, **205**, 99–105.

Fuhrman,F.A. (1986) Tetrodotoxin, tarichatoxin, and chiriquitoxin· historical perspectives. *Ann NY Acad Sci.*, **479**, 1–14

Ghosh,S., Hazra,A.K., Mitra,S.K and Mukherjee,B. (1994) Poisonous fishes: potential begetters of bioactive substances. In Devadasan,S , Mukundan,M.K., Antony.P D , Nair.P G , Perigreen,P A. and Joseph,J. (eds), *Nutrients and Bioactive Substances in Aquatic Organisms*. Society of Fisheries Technologists (India), Cochin, pp. 44–58.

Grant,W.F. (1994) The present status of higher plant bioassays for the detection of environmental mutagens *Mutat. Res* , **310**, 175–185.

Kao,C.Y. (1986) Structure–activity relations of tetrodotoxin, saxitoxin, and analogues. *Ann. NY Acad. Sci.*, **479**, 52–67.

Kao.C Y and Levinson.S R. (eds) (1986) Tetrodotoxin, saxitoxin and the molecular biology of the sodium channel. *Ann. NY Acad. Sci.*, **479**, 448.

Khora,S.S (1994) Puffer fish toxins. In Devadasan,S., Mukundan,M.K., Antony,P.D., Nair,P.G.V., Perigreen,P.A. and Joseph,J. (eds), *Nutrients and Bioactive Substances in Aquatic Organisms*. Society of Fisheries Technologists (India), Cochin, pp. 171–176.

Latt,S.A., Allen,J., Bloom,S.E., Carrono,A., Falke,E., Kram,D., Schneider,E., Schreck,R., Tice,R., Whitefield,B and Wolff,S. (1981) Sister chromatid exchange· a report of the Gen-Tox program. *Mutat. Res* , **87**, 17–62.

Nakamura,M and Yasumoto,T. (1985) Tetrodotoxin derivatives in puffer fish. *Toxicon*, **23**, 271–276.

Panda,K K., Patra,J. and Panda,B.B. (1996) Induction of sister chromatid exchanges by heavy metal salts in root meristem cells of *Allium cepa Biol Plantarum*, **38**, 555–561.

Plewa,M J (1985) Plant genetic assays and their use in studies on environmental mutagenesis in developing countries. In Muhammed,A. and von Borstel,R.C (eds), *Basic and Applied Mutagenesis with Special*

Reference to Agricultural Chemicals in Developing Countries. Plenum Press, New York, pp. 249–268.

Reddy,N.M., Panda,K.K., Subhadra,A.V. and Panda,B.B. (1995) The *Allium* micronucleus (MNC) assay may be used to distinguish clastogens from aneugens. *Biol. Zent. Bl.*, **114**, 358–368.

Ritchie,J.M. and Rogart,R.B. (1977) The binding of saxitoxin and tetrodotoxin to excitable tissue. *Rev. Physiol. Biochem. Pharmacol.*, **79**, 1–50.

Sandhu,S.S., de Serres,F.J., Gopalan,H.B.B., Grant,W.F., Svendsgaard,D., Veleminsky,J. and Becking,G.C. (1994) Results and recommendations. *Mutat. Res.*, **310**, 257–263.

Sharma,A.K. and Sharma,A. (1980) *Chromosome Techniques. Theory and Practice*. Butterworths, London, pp. 711.

Tucker,J.D., Auletta,A., Cimido,M.C., Dearfield,K.L., Jacobson-Kram,D., Tice,R.R. and Carrano,A.V. (1995) Sister-chromatid exchange: second report of the Gen-Tox program. *Mutat. Res.*, **291**, 101–180.

Yasumoto,T. and Michishita,T. (1985) Fluorometric determination of tetrodotoxin by high performance liquid chromatography. *Agric. Biol. Chem.*, **49**, 3077–3080.

Yasumoto,T., Nagai,H., Yasumura,D., Michishita,T., Endo,A., Yotsu,M. and Kotaki,Y. (1986) Interspecies distribution and possible origin of tetrodotoxin. *Ann. NY Acad. Sci.*, **479**, 44–51.

Yasumoto,T., Yotsu,M., Murata,M. and Naoki,H. (1988) New tetrodotoxin analogues from the newt *Cynops ensicauda*. *J. Am. Chem. Soc.*, **110**, 2344–2345.

Yotsu,M., Endo,A. and Yasumoto,T. (1989) An improved tetrodotoxin analyzer. *Agric. Biol. Chem.*, **53**, 893–895.

Received on December 16, 1996; accepted on March 25, 1997