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Tannic acid is not mutagenic in germ cells but weakly genotoxic in somatic cells of *Drosophila melanogaster*

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Tannic acid (TA) was tested for genotoxic activity in three different assays (1–3) in *Drosophila melanogaster* by feeding of larvae or adult flies. TA did not induce sex-linked recessive lethals (1) nor sex-chromosome loss, mosaicism or non-disjunction (2) in male germ cells. In the wing somatic mutation and recombination test (SMART) (3) TA was found to be toxic for larvae of the high bioactivation cross and produced a weak positive response. These results suggest that this compound, when administered orally to larvae or adults of *D.melanogaster*, is not mutagenic and clastogenic in male germ cells, but weakly genotoxic in somatic cells of the wing imaginal disk.

Introduction

Tannic acid (TA) is a phenolic compound that occurs naturally in wood, bark, leaves and roots of a variety of plants, including mono- and dicotyledons (Enomoto, 1987). Several hundred tons of TA per year are prepared commercially for pharmaceutical and industrial uses [International Agency for Research on Cancer (IARC) 1976]. In the human diet it is found in beverages such as green and black tea, cocoa, coffee, red and rosé wine and beer. In addition, TA is widely used as a flavouring agent in non-alcoholic beverages, ice cream, sweets, baked goods and liquors (Bichel and Bach, 1968; Hartman and Shankel, 1990). It is estimated that the human consumption of tannins is >1 g/day per person (Ramel *et al.*, 1986).

Earlier studies suggest that TA displays acute hepatotoxic effects and can induce liver tumours and sarcomas in laboratory rodents (Korpássy and Mosonyi, 1950; Kirby, 1960; Korpássy, 1961; Bichel and Bach, 1968). In spite of these observations, no sufficient data exist which clearly indicate that TA has carcinogenic activity in humans (Enomoto, 1987).

Experimental evidence on the mutagenic or clastogenic effects of TA is scarce and controversial. There are some indications that this compound has clastogenic activity in mammalian cells both *in vivo* and *in vitro* (Sharma *et al.*, 1982; Stich and Rosin, 1984; Stich and Dunn, 1986). A further indication for the interaction of TA with genetic material comes from the work of Szakmary and Knasmüller (1991) who found that it has mutagenic activity in somatic cells of *Drosophila melanogaster*.

In contrast to these findings, a large number of studies have also characterized this chemical as an antimutagenic agent acting via various mechanisms (Huang *et al.*, 1983; Das *et al.*, 1987; Sasaki *et al.*, 1988, 1989, 1990; Ito *et al.*, 1989). Furthermore, studies done in our own laboratory have demonstrated that TA shows a co-mutagenic effect on ring-X chromosome loss induced by mitomycin C in sperm cells of *D.melanogaster* (Cunha *et al.*, 1994). The existence of conflicting results on the genotoxic effects of TA observed in different test systems prompted us to study its genotoxicity in *D.melanogaster* in more detail. We have tested TA in male germ cells by means of the sex-linked recessive lethal (SLRL) and the sex-chromosome loss, mosaicism and non-disjunction (RXL) assays, as well as in somatic cells by means of the wing somatic mutation and recombination test (SMART). The SMARTs using either eye or wing imaginal disk cells offer several advantages over the germ line assays. Due to the fact that they are one-generation tests, they are much faster and far less laborious than the germ line assays. The major advantage, however, is the fact that they detect not only mutagenic activity but also recombinogenic activity in somatic cells.

Materials and methods

Chemicals

Tannic acid (TA, CAS No. 1401-55-4) was purchased from Vetec Quimica Fina Ltda, São Paulo, Brazil. It was dissolved in 0 03 M phosphate buffer, pH 6.8, containing 5% sucrose (Merck, Darmstadt, Germany).

Treatment of larvae

Eggs were collected over 8 h in culture bottles containing a solid agar base (5% w/v agar–agar in water) covered with a 5 mm thick layer of live baker's yeast supplemented with sucrose. Three days later, the larvae were washed out of the bottles using tap water and then transferred into vials containing Drosophila Instant Medium (Formula 4-24, Carolina Biological Supply Co., Burlington, NC, USA) which was rehydrated with 5 ml of the different TA solutions. Negative buffer controls were included. The larvae were fed this medium for the rest of their development until pupation (Graf *et al.*, 1984).

Treatment of adults

Three-day-old adult males were starved at room temperature for 4 h and then fed for 2 days with the different solutions using the standard feeding procedure (Würgler *et al.*, 1984). The solutions were renewed after 24 h.

Genetic designations

A description of the genetic symbols used can be found in Lindsley and Zimm (1992).

Sex-linked recessive lethal test

One-day-old males (treated as larvae) or 3-day-old males (exposed as adults) from the wild-type stock *Oregon R* were mated to virgin *Basc* females, and the X chromosomes were tested in the standard way for the presence of sex-linked recessive lethals (Würgler *et al.*, 1984). Each treated adult male was crossed to three virgin females per brood for two successive broods of 3 and 2 days duration, respectively. For the males exposed as larvae, the brooding pattern was different: each male was mated twice with a harem of three females for only 1 day (Valencia *et al.*, 1989). The statistical evaluation of the SLRL data was performed with the conditional binomial test according to Kastenbaum and Bowman (1970) (two-sided tests, $\alpha = \beta = 0.05$).

Sex-chromosome loss test

Males of the genotype *C(1)2, y B / y⁺ Y B^S*, treated as larvae or as adults, were crossed to *y w sn³* virgin females. The brood patterns used in this assay were identical to those described above. The progeny of each vial were scored separately, and the scoring continued until the 14th day after starting the cultures. The following classes of progeny were recorded, where X_f and ring-X_m represent X chromosomes from the female and the male parent, respectively: regular females (X_f/ring-X_m) and regular males (X_f/Y); exceptional males resulting from complete ring-X loss (X_f/0) and from partial Y

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chromosome loss (X_f/Y, *Df(B^S)Y*) and X_f/Y, *Df(y^+)Y*); mosaic flies resulting from complete ring-X chromosome loss (X_f/ring-X_m + $X_f/0$) and partial Y chromosome loss (X_f/Y+ X_f/Y, *Df(y^+)Y*); as well as exceptional females resulting from paternal nondisjunction (X_f/ring-X_m/Y). The statistical analysis of the data was done with the conditional binomial test according to Kastenbaum and Bowman (1970) (two-sided tests, $\alpha = \beta = 0.05$).

Somatic mutation and recombination test

In this test, a high bioactivation cross was used: *ORR/ORR; flr^3/In(3LR) TM3, r^i p^p sep l(3)89Aa bx^{34e} e Bd^S* females mated to *mwh* males. This cross is described in Graf and van Schaik (1992) and Graf and Singer (1992). The wing spot test was performed according to the description given by Graf *et al.* (1984, 1989). Only the wings of trans-heterozygous individuals (*mwh flr^+/mwh^+ flr^3*) were mounted and scored for the occurrence of spots (i.e., *mwh* or *flr^3* single spots or *mwh//flr^3* twin spots). The size of the mutant spots was determined by counting the number of mutant cells. Equal numbers of wings from female and male flies were analysed in each series, and control and treated series were always of identical size, as outlined by Frei and Würgler (1995). The evaluation of the wing spot data was performed with the computer programme SMART (F.E.Würgler, unpublished). The frequencies of total spots per wing were analysed statistically using a multiple-decision methodology (Frei and Würgler, 1988). The conditional binomial test according to Kastenbaum and Bowman (1970) was used (two-sided, $\alpha = \beta = 0.05$). Based on the number of *mwh* clones, the number of wings analysed, and the number of cells scored in each wing (~24 400), the clone formation frequency per cell cycle and 10^5 cells was calculated (for details see Frei *et al.*, 1992a,b).

Results and discussion

In a first approach we investigated the possible genotoxic activity of TA in male germ line cells of larvae and of adults using two different assays in order to detect the mutagenic and/or clastogenic activity of TA. Table I gives the results of the SLRL test after the exposure of *Oregon R* male larvae to 0, 25, 50 or 100 mM TA, respectively. All lethals occurred as single mutations in both TA-treated and negative control groups. The data recorded in Table II provide similar results on the induction of SLRLs in adult males treated with the same three concentrations of TA. The results summarized in Tables I and II demonstrate that feeding of TA to larvae as well as to adults had no genotoxic effect in germ cells. This is true for metabolically inert mature sperm (Table II, brood 1) as well as for metabolically active immature male germ cells (Table I, broods 1 and 2; Table II, brood 2).

In the RXL test, genotoxicity was assessed by four criteria: (i) complete sex-chromosome loss; (ii) partial Y chromosome loss; (iii) mosaicism for chromosome loss; and (iv) non-disjunction. Feeding of *C(1)2, y B / y^+ Y B^S* larvae was only possible with concentrations between 10 and 25 mM, whereas for the feeding of adult males concentrations up to 200 mM could be applied. In preliminary experiments, it was determined that these concentrations are all at or below the LD_{50}; for this reason, large numbers of progeny were obtained in all series. The data shown in Tables III and IV demonstrate that TA treatment does not increase the occurrence of the four categories of genotoxic events after feeding of larvae or adult males. In particular, the frequencies of complete sex-chromosome loss are not significantly increased.

Therefore, none of the two male germ cell assays which measure different genetic end-points were able to detect any genotoxic activity of TA after oral treatment of adults or larvae. Early reports demonstrated hepatotoxic, cirrhogenic and/or carcinogenic activity of TA in mice, rats, rabbits and goats only when applied via the subcutaneous, intraperitoneal or intravascular routes (Baker and Handler, 1943; Korpássy, 1959, 1961). These effects were not observed when TA was given orally (Handler and Baker, 1944; Korpássy and Mosonyi, 1950). Thus, a possible genotoxic activity of TA in male

Table I. Sex-linked recessive lethal test in *D.melanogaster* with tannic acid fed to male larvae

TA (mM)	Lethals/chromosomes [frequency (%)][a]		
	Brood 1 (1 day)	Brood 2 (1 day)	Total (2 days)
0	3/1266 (0.24)	4/1253 (0.32)	7/2519 (0.28)
25	2/1617 (0.12)	2/1668 (0.12)	4/3285 (0.12)
50	2/1637 (0.12)	3/1812 (0.17)	5/3449 (0.14)
100	1/1545 (0.06)	3/1776 (0.17)	4/3321 (0.12)

[a]Differences between TA treatments and negative control are not significant (Kastenbaum and Bowman, 1970).

Table II. Sex-linked recessive lethal test in *D.melanogaster* with tannic acid fed to adult males

TA (mM)	Lethals/chromosomes [frequency (%)][a]		
	Brood 1 (3 days)	Brood 2 (2 days)	Total (5 days)
0	3/1873 (0.16)	3/1841 (0.16)	6/3714 (0.16)
25	3/1475 (0.20)	1/1496 (0.07)	4/2971 (0.13)
50	5/1387 (0.36)	4/1564 (0.26)	9/2951 (0.30)
100	3/1456 (0.21)	1/1518 (0.07)	4/2974 (0.13)

[a]Differences between TA treatments and negative control are not significant (Kastenbaum and Bowman, 1970).

germ line cells might be observed only when using injection treatments.

In a second series of experiments TA was assayed in somatic cells of the wing imaginal disk by feeding of larvae derived from the high bioactivation cross. This recently developed high bioactivation cross produces larvae which possess constitutively increased cytochrome P-450-dependent enzyme activities and therefore make the wing spot test more sensitive for the detection of promutagens and procarcinogens (Graf and Singer, 1992; Graf and van Schaik, 1992). The data on the induction of somatic mutation and mitotic recombination are recorded in Table V, together with a laboratory historic control series for comparison purposes. Two separate experiments of identical size were performed. TA was quite toxic for these larvae: only a few flies survived at a concentration of 70 mM, and 50 mM was the highest testable concentration. In the first experiment, positive results were obtained for the 25 and 50 mM series, with the 10 mM series giving an inconclusive diagnosis. In the second experiment, only the 50 mM series gave a positive result, the remaining ones again being inconclusive. Pooling of the two identical experiments results in sample sizes of 120 wings per series, which is sufficient for optimal statistical conditions (Frei and Würgler, 1995). After pooling, all three concentrations gave a positive result. However, the dose–response observed is rather weak. The dose–response relationships for each experiment and for the pooled data are shown in Figure 1. A levelling-off of the genotoxic effects at the highest concentration is observed, which may be due to limited bioactivation capacity at higher concentrations. Furthermore, the data presented in Table V show that the genotoxic effects are mainly due to increases in the frequencies of the small single spots, with the large single spots and the twin spots remaining mostly unaffected. Small single spots may either be the consequence of genotoxic events occurring very late in larval development or they may be due

Table III. Sex-chromosome loss test in *D.melanogaster* with tannic acid fed to male larvae

TA (mM)	Brood 1 (1 day)[a]							Brood 2 (1 day)[a]						
	Regul. prog.		Exceptional. prog.				CL[b] (%)	Regul. prog.		Exceptional. prog.				CL[b] (%)
	Fem.	Male	CL	PL	M	ND		Fem.	Male	CL	PL	M	ND	
0	2753	2225	45	5	9	4	1.61	2128	1672	41	3	3	3	1.89
10	3175	2420	69	1	4	2	2.13	2135	1784	33	1	5	5	1.52
15	3372	2821	78	3	16	6	2.26	2298	2003	57	2	4	4	2.42
20	4149	3189	63	2	5	4	1.50	2051	1381	34	4	3	3	1.63
25	2955	2150	54	1	6	3	1.79	1705	1096	44	1	3	3	2.52

Abbreviations: Regul. prog., regular progeny (females and males); Exceptional prog., exceptional progeny; CL, complete loss; PL, partial loss; M, mosaicism; ND, non-disjunction in male.
[a]Differences between treatments and control are not significant (Kastenbaum and Bowman, 1970).
[b]Complete loss calculated as percentage of regular females plus CL.

Table IV. Sex-chromosome loss test in *D.melanogaster* with tannic acid fed to adult males

TA (mM)	Brood 1 (1 day)[a]							Brood 2 (1 day)[a]						
	Regul. prog.		Exceptional. prog.				CL[b] (%)	Regul. prog.		Exceptional. prog.				CL[b] (%)
	Fem.	Male	CL	PL	M	ND		Fem.	Male	CL	PL	M	ND	
0	4037	4663	75	3	8	4	1.82	3207	3266	52	4	7	6	1.60
50	4590	5025	94	2	6	10	2.01	3286	3350	52	2	6	7	1.56
100	5297	5641	106	0	5	12	1.96	3599	3366	64	0	10	8	1.75
150	4110	4413	88	2	5	11	2.10	2208	2058	41	2	7	7	1.82
200	3834	4083	79	2	7	10	2.02	3779	3957	67	4	6	10	1.74

Abbreviations: Regul. prog., regular progeny (females and males); Exceptional prog., exceptional progeny; CL, complete loss; PL, partial loss; M, mosaicism; ND, non-disjunction in male.
[a]Differences between treatments and control are not significant (Kastenbaum and Bowman, 1970).
[b]Complete loss calculated as percentage of regular females plus CL.

Table V. Wing spot data obtained after exposure of *ORR/+; mwh flr$^+$/mwh$^+$ flr^3* trans-heterozygous larvae of *D.melanogaster* with tannic acid

TA (mM)	Number of wings	Small single spots (1–2 cells)	Large single spots (>2 cells)	Twin spots	Total spots			Spots with *mwh* clone	Mean clone size class	Frequency of clone formation per 10^5 cells[b]	
					no.	freq.	diag.[a]			Obs.	Ctrl
Laboratory historic control (water)											
0	232	62	8	1	71	0.31		70	1.5	1.2	
Experiment 1											
0	60	15	1	0	16	0.27		16	1.3	1.1	
10	60	19	2	0	21	0.35	i	20	2.0	1.4	0.3
25	60	38	2	1	41	0.68	+	41	1.6	2.8	1.7
50	60	34	2	0	36	0.60	+	35	1.3	2.4	1.3
Experiment 2											
0	60	17	0	0	17	0.28		17	1.1	1.2	
10	60	25	4	0	29	0.48	i	29	1.5	2.0	0.8
25	60	26	1	2	29	0.48	i	29	1.4	2.0	0.8
50	60	24	4	2	30	0.50	+	30	1.9	2.1	0.9
Pooled experiments											
0	120	32	1	0	33	0.28		33	1.2	1.1	
10	120	44	6	0	50	0.42	+	49	1.7	1.7	0.6
25	120	64	3	3	70	0.58	+	70	1.5	2.4	1.3
50	120	58	6	2	66	0.55	+	65	1.6	2.2	1.1

Abbreviations: Obs., observed; Ctrl, control corrected.
[a]Statistical diagnoses according to Frei and Würgler (1988): +, positive; i, inconclusive; multiplication factor $m = 2$. Kastenbaum–Bowman tests, two-sided. Probability levels: $\alpha = \beta = 0.05$.
[b]Frequency of clone formation: *mwh* clones/wings/24 400 cells (without size correction).

to cells with partial aneuploidies which do not divide normally (Frei *et al.*, 1992a). All in all, the results demonstrate that TA has a weak genotoxic activity in high bioactivation larvae of the wing spot test. These positive results are in line with those reported by Szakmary and Knasmüller (1991) who also found genotoxic activity of TA in somatic cells using the *white/white$^+$* eye spot test. In their experiments, however, treatments with TA (2.5–15 mmol/l) resulted in a moderate and dose-

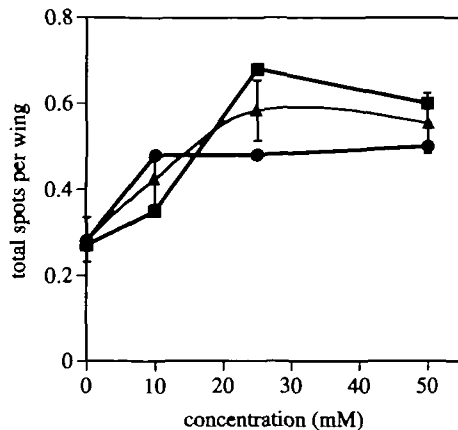

Fig. 1. Somatic mutation and recombination test. Dose–response relationships for total spots per wing after chronic treatments (48 h) with different concentrations of tannic acid. Squares, experiment 1; circles, experiment 2; triangles, pooled data (bars, standard error of the mean).

dependent induction of mosaic spots. One can assume that the genetic background of the larvae used for treatment has an influence on the genotoxic activity of TA. The strains of the eye and the wing spot tests have different genetic backgrounds, and this may explain the difference in the responses.

Szakmary and Knasmüller (1991) observed a pronounced synergistic effect of TA on mutagenicity when administered simultaneously with methyl methanesulphonate (MMS), 4-nitro-quinoline-*N*-oxide (4NQO) and *cis*-platinum (*cis*-DDP) to male larvae. In a recent study, Knasmüller *et al.* (1992) used the Tradescantia micronucleus assay to demonstrate that TA alone has a clastogenic effect and a synergistic effect when combined with X-ray treatment . The synergistic effects observed are rather unexpected since, in the literature, des-mutagenic (Stich *et al.*, 1982; Conney, 1982; Huang *et al.*, 1985; Das *et al.*, 1987) or bio-antimutagenic effects (Shimoi *et al.*, 1985; Sasaki *et al.*, 1988, 1989, 1990; Ito *et al.*, 1989; Imanishi *et al.*, 1991) of TA have been reported. However, in a recent study we have also demonstrated that TA (from the same source as in the present experiments) displays a significant co-mutagenic action on the frequency of sex-chromosome losses when females exposed to different TA concentrations were crossed with mitomycin-C (MMC)-treated males carrying a ring-X chromosome (Cunha *et al.*, 1994). This finding shows that, although TA has no clastogenic activity in *D.melanogaster* germ cells, it has a potentiating effect on MMC-induced damage in sperm DNA.

We conclude that TA may show mutagenic, co-mutagenic, des-mutagenic or bio-antimutagenic activity, depending on the particular experimental situation. It is well known that TA is composed of different moieties (Versole and Delahaye, 1983; Hagerman and Butlon, 1989; Hagerman *et al.*, 1993). Actually, tannic acids from different commercial sources behaved differently toward various tannin assays (redox, metal complexing and protein precipitation assays). Different structures of the tannin moieties in the various preparations of TA may lead to different physico-chemical properties that affect the response in biological test systems (Makkar and Becker, 1993). Chemical diversity may explain the controversial results obtained with respect to the mutagenic and/or antimutagenic action of TA. It seems that a comparative evaluation of the biological effects of TA from different sources would be indicated. TA from our source may also be non-genotoxic or co-mutagenic depending on the experimental protocol and assay system used. The antimutagenic and anticarcinogenic properties of TA require further evaluation to determine the possible risks and/or protective potentials that could be associated with the exposure of living organisms to this compound.

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