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Activity of Aromatic Amines in the Eye: w/w^+ Somatic Assay of *Drosophila melanogaster*

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Aromatic amines represent a category of classical environmental hazards which require biotransformation in order to exert their mutagenic and carcinogenic effects. The mutagenic activity of several aromatic amines was tested by means of the w/w^+ somatic assay of *Drosophila melanogaster* employing a wild-type strain Leiden Standard (LS) and an insecticide-resistant stock Hikone-R (HK-R). Four monocyclic– ortho-anisidine (O-AN), ortho-toluidine (O-TO), 2,4-diaminoanisole (2,4-DAA) and 4-nitro-o-phenylenediamine (4-N-o-PDA), and one bicyclic– 4,4'-oxidianiline (4,4'-ODA) aromatic amines were tested by chronic exposure. Two separate experiments were run, and concurrent controls were treated with the solvent mixture. Results show that the ortho-anilines (O-AN and O-TO) were active in the assay, while the diamines containing a methoxy (2,4-DAA) or a nitro group (4-N-o-PDA) were inactive. The bicyclic aromatic amine, 4,4'-ODA, was the most potent. The genotoxic responsiveness of both strains was very similar.

Key words: aromatic amines, *Drosophila* w/w^+ somatic assay, wild-type (LS), insecticide-resistant (HK-R) strains, mutation

INTRODUCTION

Aromatic amines (AA) are genotoxic in a variety of organisms from bacteria to mammals, although the evaluation of these chemicals has produced conflicting results due in part to different metabolic activation systems and end-points used for evaluation [Aeschbacher and Turesky, 1991]. AA must be metabolically activated in order to exert genotoxicity. N-oxidation by cytochrome P-450 is the first step in this biotransformation of aromatic amines [Miller and Miller, 1969; Lotlikar and Zaleski, 1975; Kato et al., 1983; Yamazoe et al., 1989]. AA may also be activated by peroxidases [Perry et al., 1989; Flammang et al., 1989].

Aniline and its derivatives are widely used as intermediates for dyestuffs and a variety of polyurethane products [IARC, 1982]. o-Anisidine (O-AN), used in the manufacture of azo dyes [Merck Index, 1989], is a potent human bladder carcinogen, genotoxic in vitro and positive in CHO clastogenicity and SCE assays [Galloway et al., 1987]. O-AN is converted to a genotoxin through activation by the enzyme prostaglandin H synthase which has peroxidase activity [Bolcsfoldi and Hellmer, 1992]. Other effects include methhemoglobinaemia [Ashby, 1992] following absorption and oxidation. O-AN was not genotoxic in rodent genotoxicity assays [Ashby et al., 1991].

o-Toluidine (O-TO) is used as an intermediate in the dyeing industry, rubber processing, and production of pharmaceuticals and pesticides [Merck Index, 1989; Danford, 1991]. N-oxidation of O-TO produced a range of compounds, both mutagenic and nonmutagenic, in strain TA-100 of *Salmonella typhimurium* in the presence of S-9 [Gupta et al., 1987]. In *Saccharomyces cereviseae*, O-TO induced "petite" mutants in strain D5 [Ferguson, 1985] but was negative for mitotic crossing over and gene conversion in strain D7 [Metha and von Borstel, 1985; Brooks et al., 1985]. Both positive and negative responses were obtained in the Drosophila somatic cell w/w^{co} test using repair proficient strains [Vogel, 1985; Würgler and Vogel, 1986]. Similar inconclusive results were obtained for the reversion at w/w^z in the excision-repair deficient mei-9 strain [Fujikawa et al., 1985; Batiste-Alentorn et al., 1991]. Both positive and negative results were also obtained in mammalian cytogenetic assays [Lane et al., 1985; Obe et al., 1985; Perry and Thomson, 1981]. The remaining three compounds have not been extensively tested. Ashby and Tennant [1991] reported that 2,4-diaminoanisole (2,4-DAA) was carcinogenic in both rats and mice; 4-nitro-o-phenylenediamine (4-N-o-PDA) was not carcinogenic, and the bicyclic 4,4'-oxydianiline (4,4'-ODA) was a hepatocarcinogen in the mouse.

The induction of hereditary genetic damage by progenotoxins showed genotype dependance in *Drosophila melanogaster* [Hällström et al., 1982; Hällström, 1984; Zijlstra et al., 1984]. A strong intraspecies variability has been recently found for the metabolic conversion of several procarcinogens such as polycyclic aromatic hydrocarbons, ni-

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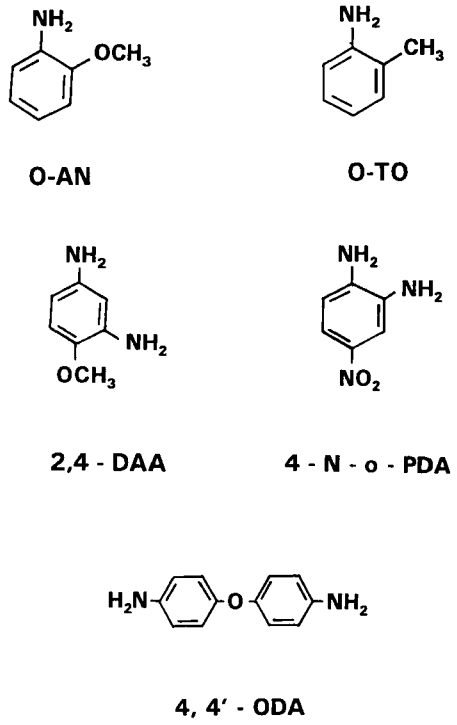

Fig. 1. Structural formula of the compounds tested. O-AN = Ortho-anisidine; O-TO = ortho-toluidine; 2,4-DAA = 2,4-diaminoanisole; 4-N-o-PDA = 4-nitro-O-phenylenediamine; 4,4'-ODA = 4,4'-oxydianiline

trosamines, and the pyrrolizidine alkaloid monocrotaline [Rodriguez-Arnaiz et al., 1993].

Experiments were performed to study the activity of the aromatic amines O-AN, O-TO, 2,4-DAA, 4-N-o-PDA and 4,4'-ODA (see Fig. 1 for the structural formulae) in the eye w/w^+ somatic assay of *Drosophila melanogaster* in a wild-type strain and in an insecticide-resistant stock.

MATERIALS AND METHODS

Chemicals

O-AN (CAS #134-29-2), O-TO (CAS #636-21-5) and 2,4-DAA (CAS #39156-41-7) were purchased from Aldrich, Co., Milwaukee, WI; 4,4'-ODA (CAS #101-80-4) and 4-N-o-PDA (CAS #99-56-9) were obtained from Sigma Chemicals, St. Louis, MO.

Treatment of the Flies

The compounds were administrated by chronic treatment. Twenty-five pairs of flies were permitted to lay eggs for 3 days on standard cornmeal-agar-yeast medium supplemented with the test substance, which was first dissolved in a mixture of 3 parts ethanol: 1 part Tween 80, yielding a final concentration in the medium of 3% ethanol and 1% Tween 80. Growing cultures were exposed to each progenotoxin during all three instar stages of larval development.

Drosophila Strains

Two Drosophila stocks were used: one wild-type strain, Leiden Standard (LS), and one insecticide-resistant strain Hikone-R (HK-R). White females were mated with either yellow (LS) or wild males (HK-R) [Rodriguez-Arnaiz et al., 1993]. The cytochrome P-450 contents in both stocks are very similar [Zijilstra et al., 1984].

Somatic Assay

The w/w^+ assay [Vogel et al., 1991] was used. Newly hatched females were transferred to fresh medium, and scored 1 to 5 days later. The scoring of females was carried out in a liquid consisting of ethanol/Tween 80/water (90:1:9). The eyes of the females were inspected under a dissecting microscope at a magnification of 120×, with optical fiber illumination.

Adult females are heterozygous for white and were inspected for the occurrence of white in their compound eyes. Classification of mosaic light spots was done on the basis of small spots (2–4 ommatidia affected), large spots (> 4 ommatidia affected) and total spots. Spots separated from each other by at least four nonmutated ommatidia were considered as independent events [Vogel and Nivard, 1993]. The genotoxic effectiveness in the eye disc cells was calculated according to the formula $f = 2\ nm/NC$ where m is the mean clone size, n is the total number of mosaic spots, 2 is a correction factor, C is a constant (800 ommatidia) and N the number of eyes analyzed [Szabad et al., 1983; Vogel and Zijlstra, 1987].

In preliminary dose range-finding experiments, toxicity denoted by developmental delay, abnormal ommatidia and/or exposure-related mortality, was the limiting factor for the highest exposure. Different dose levels were tested. For each compound, two separate and independent experiments were conducted at the same doses with each chemical. For each experiment a concurrent control was run, where larvae were treated with the solvent alone. Since no statistically significant differences between the results of repeated experiments were found, the data were pooled. The number of eyes scored varied between 180 and 900 for treated series, and between 500 and 900 for concurrent controls.

Statistical Analysis

Statistical evaluation of the data was done by the chi-square for proportions [Frei and Würgler, 1988].

RESULTS AND DISCUSSION

The w/w^+ assay monitors a diverse set of DNA lesions in somatic cells of treated Drosophila larvae. Loss of heterozygosity of the w^+ gene can, in theory, be due to point mutations and deletions inactivating the w^+ allele, chromosomal aberrations, and various types of recombination events. Homologous interchromosomal mitotic recombination has been shown to represent the major event measured in the assay [Vogel and Szakmáry, 1990; Vogel and Nivard, 1993].

Each of the compounds tested showed a structural alert according to Ashby and Tennant [1991]. O-AN and O-TO have the reactive amino group in the ortho position; 2,4-DAA has the second amino group in the meta position and a methoxy group in para; and 4-N-o-PDA has the nitro group in the para position. The bicyclic aromatic amine has two amino groups.

The data obtained for somatic eye mutations and mitotic recombination using a wild-type strain (LS) and an insecticide-resistant (HK-R) strain, are summarized in Table I. These results showed that amine groups in the ortho position are mutagenic in both strains. On the other hand, the monocyclic diamines containing a nitro or methoxy group in the para position are not mutagenic, presumably because they

TABLE I. Activity of Aromatic Amines in Wild (LS) and in Insecticide-Resistant (HK-R) *w/w*$^+$ Females of *Drosophila melanogaster*

Compound (mM) and stock	Number of eyes	Spots per 100 eyes[a]			Average clone size	Clones per 10^4 cells	Activity[b]
		Small	Large	Total			
O-AN							
LS							
Control	828	8.57	1.44	10.01	3.32	8.32	
0.5	600	20.50	2.83	23.33	3.31	19.29	+
1.0	722	18.42	2.90	21.32	3.74	19.94	+
2.5	572	27.97	6.11	34.08	4.88	40.95	+
5.0	180	44.44	8.33	52.77	4.71	62.15	+
HK-R							
Control	904	9.40	2.54	11.94	4.08	12.10	
0.5	550	17.45	2.54	19.99	6.70	33.50	+
1.0	908	26.65	4.73	31.38	4.01	31.40	+
2.5	502	33.86	7.56	41.42	1.29	45.70	+
5.0	500	40.00	8.20	48.20	4.17	50.20	+
O-TO							
LS							
Control	800	6.12	1.50	7.62	3.75	7.14	
1.0	750	15.30	0.93	16.23	4.32	17.56	+
2.5	502	25.30	2.39	27.69	4.01	27.75	+
5.0	192	34.89	1.56	36.45	2.41	21.75	+
HK-R							
Control	724	8.42	2.62	11.04	6.97	19.25	
1.0	678	15.04	4.27	19.31	5.88	28.40	+
2.5	572	15.03	2.79	17.82	3.17	14.13	+
5.0	480	24.58	5.83	30.41	4.31	32.77	+
2,4-DAA							
LS							
Control	500	9.20	1.40	10.60	2.30	6.09	
0.1	448	10.50	1.78	12.28	3.65	11.20	−
0.25	500	15.00	0.60	15.60	2.41	9.39	−
0.5	500	13.00	1.60	14.60	3.73	13.61	−
HK-4							
Control	640	10.15	1.25	11.40	3.44	9.80	
0.1	500	11.80	1.20	13.00	2.80	9.10	−
0.25	476	13.44	0.80	14.24	2.61	9.18	−
0.5	500	11.40	2.20	13.60	3.48	11.83	−
4-N-o-PDA							
LS							
Control	450	8.40	1.33	9.77	2.79	6.82	
0.1	476	9.45	1.26	10.71	2.51	6.72	−
0.25	444	9.68	0.90	10.58	1.85	4.89	−
0.5	450	9.77	1.11	10.88	2.82	7.68	−
1.0	458	9.82	0.87	10.70	2.73	7.30	−
HK-R							
Control	530	6.98	2.07	8.67	3.35	7.27	
0.1	562	8.54	2.66	11.38	4.17	11.87	−
0.25	592	8.61	2.02	10.64	4.62	12.29	−
0.5	520	10.77	1.92	12.50	4.43	13.84	−
1.0	490	7.55	2.45	10.00	3.00	7.50	−
4,4-ODA							
LS							
Control	500	9.20	1.40	10.60	3.36	8.90	
0.1	506	10.70	1.60	12.30	2.56	7.84	−
0.25	500	18.40	1.80	20.20	3.99	20.15	+
0.5	508	38.00	3.93	41.93	3.00	31.44	+
1.0	476	36.80	2.52	39.32	2.12	30.64	+
5.0	500	84.80	6.80	91.60	2.77	63.43	+

(continued)

TABLE I. Activity of Aromatic Amines in Wild (LS) and in Insecticide-Resistant (HK-R) w/w^+ Females of *Drosophila melanogaster (continued)*

Compound (mM) and stock	Number of eyes	Spots per 100 eyes[a]			Average clone size	Clones per 10^4 cells	Activity[b]
		Small	Large	Total			
HK-R							
Control	540	11.50	3.14	14.64	4.06	14.84	
0.1	446	13.90	2.01	15.91	3.90	15.52	−
0.25	500	23.20	4.60	27.80	3.99	27.93	+
0.5	500	45.40	7.00	52.40	3.60	65.88	+
1.0	436	69.95	6.90	76.85	5.19	97.31	+
5.0	384	131.51	13.02	144.53	3.12	113.34	+

[a]Size classes: Small, clone size 1–4 ommatidia; Large, clone size > 4 ommatidia.
[b]Activity: +, positive; −, inactive.

are not activated by the Drosophila to mutagenic forms. The bicyclic aromatic amine is mutagenic in both LS and HK-R strains.

The majority of clones were small spots representing events which became manifest during the third instar larval stage. As mutation frequencies cannot be exactly determined for somatic cell populations, the frequency of clones per 10^4 cells could be considered an indirect estimation of genotoxic effectiveness. In the present report the order of effectiveness obtained for both strains was 4,4′-ODA > O-AN > O-TO > 2,4-DAA = 4-N-o-PDA.

A correlation has been shown between the structure of aniline derivatives and their mutagenicity or carcinogenicity [Hatch et al., 1992]. The para-substituted anilines have been shown to be the least biologically active compounds, whereas the ortho-substituted anilines are the most active [Sontag, 1981; Suzuki et al., 1983; Shahin, 1989]. In addition, Gentile et al. [1987] have shown that ortho- and meta-phenylenediamines are more mutagenic than para-phenylenediamines. The results reported in this paper support the hypothesis that only the ortho anilines are activated to a mutagenic species by Drosophila.

The number of rings in the compound seems to be another structural factor which modulates genotoxic potency [Hatch et al., 1992]. The results described in this study support this, as it was found that the bicyclic aromatic amine was more potent than any of the monocyclic ones.

Cytochrome P450 is the most efficient enzyme system for activating AA, but other enzyme systems are also capable of activation. These include flavin-containing monoxygenase and peroxidases, where the amines serve as reducing cofactors for prostaglandin H synthase (PHS), an arachidonic acid-dependent peroxidase, and thereby undergo peroxidase metabolism to form reactive species capable of binding covalently to DNA [Aeschbacher and Turesky, 1991].

The strains employed in the present experiments show similar levels of cytochrome P450 [Zijlstra, 1987]. No differences were found in the biotransformation exerted by the LS and HK-R strains for either the monocyclic or bicyclic aromatic amines. In conclusion, the ability of the w/w^+ assay to detect genotoxic aromatic amines which depend on cytochrome P450 conversion to DNA-reactive species, has been demonstrated.

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