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Fundamental and Molecular Mechanisms of Mutagenesis

Analysis of mitotic recombination induced by several mono- and bifunctional alkylating agents in the Drosophila wing-spot test

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Abstract

Mitotic recombination induced by six alkylating agents has been studied in the wing-spot test of *Drosophila melanogaster*. The model mutagens chosen have different modes of action at the DNA level. These are: the direct-acting small alkylating agent methylmethanesulfonate (MMS), the small promutagens *N*-dimethylnitrosamine (DMN), and *N*-diethylnitrosamine (DEN), the bifunctional cross-linking alkylating agents mitomycin C (MMC), chlorambucil (CLA) and monocrotaline (MCT). Flies of the standard cross ($flr^3/TM3$, Bd^S females and *mwh* males) were used to produce the larvae to be treated. Three-day-old Drosophila larvae were exposed by chronic feeding for 48 h to three different concentrations of all six alkylating agents. Acute feeding for only 2 h was used in addition with DEN and MMC. Wings of the marker-heterozygous ($mwh + / + flr^3$) as well as of the balancer-heterozygous ($mwh + /TM3$, Bd^S) progeny were analysed. The ranking of the compounds with respect to their genotoxic potency, based on *mwh* clone formation frequency in marker-heterozygous wings, was: MMS > MMC > DMN > CLA ~ MCT > DEN. The ranking with respect to the induction of twin spots, which are produced by mitotic recombination exclusively, was: MMS > DMN > MMC > MCT > CLA > DEN. The quantitative determination of recombinagenic activity, based on *mwh* clone formation frequencies obtained in both types of wings, gave the following values: MMS, 93%; MCT, 87%; CLA, 80%; MMC, 73%; DMN, 67%; DEN, 22%. A clear relationship exists between the extent of *N*-alkylation of DNA and the efficiency of the monofunctional agents MMS and DMN as well of the bifunctional agents MCT, CLA and MMC to induce mitotic recombination. This contrasts with the ethylation of base oxygen atoms and the resulting lower efficiency of DEN to produce mitotic recombination.

Keywords: Somatic cell; Genetoxicity test; Mitotic recombination; *Drosophila melanogaster*; Chlorambucil; *N*-Diethylnitrosamine; *N*-Dimethylnitrosamine; Methyl methanesulfonate; Mitomycin C; Monocrotaline

1. Introduction

Mitotic recombination was first discovered in *Drosophila melanogaster* by Stern (1936). X-ray-induced mitotic crossing-over has been extensively

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used to study the development of various imaginal discs in the fly (Garcia-Bellido and Merriam, 1971a, b; Garcia-Bellido, 1972; Garcia-Bellido and Dapena, 1974).

In Drosophila, the study of chemically induced recombination has gained new interest in connection with the development of the wing-spot test, which detects both somatic mutation and mitotic recombination in the left arm of the third chromosome in somatic cells, and which has proven useful as a test for the detection of a wide variety of both direct acting mutagens and those requiring metabolic activation (Graf et al., 1984; Würgler, 1991; Van Schaik and Graf, 1993).

Alkylating agents are known for their ability to generate methylating or ethylating species that interact with different types of macromolecules such as DNA, either directly (methyl methanesulfonate, MMS) or after metabolic activation (dimethylnitrosamine, DMN; diethylnitrosamine, DEN). However, in the case of biotransformation, it has been shown that DMN produces a reactive monomethylnitrosamine intermediate (Haggerty and Holsapple, 1990), and results in at least two different mutagenic metabolites (Vogel, 1989a). The molecular mechanisms of action of alkylating agents are well known. Previous studies on small alkylating agents such as MMS revealed that it is possible to correlate specific types of DNA adducts with various genetic end points in Drosophila (Vogel and Natarajan, 1979a, b, 1982; Vogel et al., 1986) and in mammalian cells (Heflish et al., 1982; Natarajan et al., 1984; Zdzienicka and Simons, 1986). In both systems a strong correlation was observed between the extent of ethylation at the DNA base oxygen atoms and the efficiency of DEN for inducing point mutations as opposed to structural changes (Vogel, 1986, 1989b). On the other hand, the clastogenicity of monofunctional alkylating agents may result predominantly from their reaction with nitrogen atoms in DNA (Vogel et al., 1990). The group of bifunctional agents can form promutagenic monoadducts (Hemminki and Ludlum, 1986) as well as intra- and interstrand cross-links (Singer and Grunberger, 1983). Their high carcinogenic potency may be attributable to their ability to cross-link DNA (Vogel et al., 1990).

In order to gain information about the efficiency of inducing mitotic recombination by several model mutagens with different modes of action at the DNA level, these experiments were performed using the wing-spot test of *Drosophila melanogaster*. The monofunctional alkylating agents tested were the small direct-acting mutagen methyl methanesulfonate (MMS) and the small procarcinogens dimethylnitrosamine (DMN) and diethylnitrosamine (DEN). The bifunctional cross-linking agents were the procarcinogens mitomycin C (MMC), chlorambucil (CLA) and the pyrrolizidine alkaloid monocrotaline (MCT).

2. Materials and methods

2.1. Chemical compounds

Methylmetanesulfonate (MMS, CAS Registry Number 66-27-3), *N*-methyl-*N*-nitrosamine (DMN, 62-75-9), *N*-ethyl-*N*-nitrosamine, (DEN, 55-18-5), mitomycin C (MMC, 50-07-7) and CB 1348, chlorambucil (CLA, 305-03-3) were purchased from Sigma (St. Louis, MO, USA). The pyrrolizidine alkaloid monocrotaline (MCT, 315-22-0) was a gift by Prof. E.W. Vogel (Leiden, The Netherlands). For the feeding of larvae the compounds were dissolved either in 5% sucrose (MMS, DMN, DEN, MMC) or in a mixture of 1% Tween 80 (Sigma) plus 3% ethanol (Merck) in 5% sucrose (CLA, MCT).

2.2. Larval feeding

Eggs were collected for 8 h in culture bottles. Three days later, the larvae were washed out of these bottles with tap water through a fine-meshed stainless steel strainer and then used for treatments of various durations.

For chronic feeding, small vials with 1.5 g Drosophila Instant Medium (Carolina Biological Supply Co., Burlington, NC, USA) and 5 ml of the respective mutagen solution were used. Negative water and solvent controls were always included. The larvae were fed on this medium for the rest of their development which corresponds to approx. 48 h (Van Schaik and Graf, 1993).

For acute feeding, the larvae were collected and distributed into plexiglass tubes which have the lower end covered with fine nylon gauze. These tubes are

then placed into 100 ml beakers containing 0.3 g of powered cellulose (Merck) and 1.5 ml of the mutagen solution. The larvae were fed on the mutagen-cellulose suspension through the gauze for 2 h. After that period the mutagen solution was washed out, and the larvae were put into vials containing 1.5 g dry Instant Medium wetted with 5 ml distilled water (Graf et al., 1989).

2.3. Somatic mutation and recombination test

The following cross of flies carrying markers on the left arm of chromosome 3 was used: *flr*^{3}/*In (3LR)TM3, ri p*^{P} *sep bx*^{34e} *e*^{S} *Bd*^{S} females mated to *mwh* males. For detailed information on the genetic markers see Lindsley and Zimm (1992). The flies from the vials were collected and stored in 70% ethanol. The wings of both types of progeny, namely the marker-heterozugous (*mwh* + / + *flr*^{3}) and the balancer-heterozygous (*mwh* + /*TM3*, *Bd*^{S}) were mounted in Faure's solution. Both surfaces of the wings were scored under a compound microscope at 400 × magnification for the occurrence of spots. In the marker-heterozygous wings three categories of spots are recorded separately: (1) *mwh* single spots, (2) *flr* single spots, and (3) twin spots with adjacent *mwh* and *flr* areas. Because there is a correlation between the time of induction of a genetic change in the somatic cells and the size of the resulting spot (Graf, 1995), spot sizes are also recorded in addition to spot frequencies. Single spots are produced by somatic point mutations, deletions and mitotic recombinations occurring between the two markers. Twin spots are produced exclusively by mitotic recombinations occurring between the proximal marker *flr* and the centromere of chromosome 3. In the *TM3* balancer-heterozygous wings, all recombination events are eliminated due to the multiple inversions present so that all *mwh* single spots recovered are produced by non-crossover type mutational events exclusively. For more details on the techniques see Graf (1995) and Graf et al. (1984, 1989).

2.4. Data evaluation and statistical analysis

The wing-spot data were evaluated with the computer programme SMART (Würgler, unpublished). For the statistical analysis the spots were grouped in the following three types: (1) small single spots of 1 or 2 cells in size (*mwh* or *flr*), (2) large single spots of 3 or more cells, and (3) twin spots with a *mwh* and a *flr* clone. These three types of spot are evaluated separately. For the frequencies of spots per wing a multiple-decision procedure was used to decide whether a result is positive, weakly positive, inconclusive or negative (Frei and Würgler, 1988). For the calculations, the conditional binomial test according to Kastenbaum and Bowman (1970) was used with significance levels $\alpha = \beta = 0.05$. Based on the number of *mwh* clones (i.e., *mwh* single spots and the *mwh* parts of twin spots), the number of wings analysed, and the number of cells scored in each wing (ca. 24 400), the clone formation frequency per cell cycle and 10^5 cells was calculated. The clone formation frequencies were corrected for differences in mean clone size according to the formula proposed by Frei et al. (1992). The proportion of mitotic recombination versus somatic mutation was calculated based on these corrected clone formation frequencies by linear interpolation of the dose-response relationships obtained for the two types of wings (see also Graf et al., 1992).

3. Results

Two different treatment methods were used in these studies, namely chronic feeding (48 h) and acute feeding (2 h). Chronic feeding was employed for all six compounds, and in addition acute exposure was used for DEN and MMC. Three concentrations in the chronic studies and two in the acute treatments were tested for each compound. With each compound and treatment, two independent experiments were performed with concurrent negative solvent controls. The data presented are the sum of these two experiments, which were pooled after verifying the homogeneity of the two data sets.

Table 1 summarises the results obtained in the Drosophila wing-spot test after chronic exposure with the six alkylating agents. The spot data are given both for the marker-heterozygous and the balancer-heterozygous wings. The results obtained after acute exposure of larvae with DEN and MMC in both types of wings are shown in Table 2. For the statistical evaluation of the effects, the results obtained

Table 1
Summary of results obtained in the Drosophila wing somatic mutation and recombination test. Chronic feeding of 3-day-old larvae with 6 alkylating agents for 48 h

Geno-type [a]	Conc. (mM)	No.of wings (*N*)	Spots per wing (No. of spots) Stat. diagn. [c]				Spots with *mwh* clone [d] (*n*)	Mean *mwh* clone size class [d,e] (*i*)	Clone formation frequencies (per 10^5 cells per cell div.)	
			Small single spots (1–2 cells) [b] [*m* = 2.0]	Large single spots (> 2 cells) [b] [*m* = 5.0]	Twin spots [*m* = 5.0]	Total spots [*m* = 2.0]			Without size correction [e,f] (*n*/*NC*)	With clone-size corr.e,f ($2^i/2^2$) ×(*n*/*NC*)
Methylmethanesulfonate										
MH	0.00	154	0.31 (47)	0.06 (9)	0.03 (5)	0.40 (61)	61	1.85 [0.00]	1.6 [0.0]	1.5 [0.0]
	0.50	144	2.94 (423)+	2.31 (332)+	1.56 (225)+	6.81 (980)+	911	2.64 [2.69]	26.0 [24.3]	40.5 [39.3]
	1.00	66	5.64 (372)+	6.42 (424)+	3.98 (263)+	16.05 (1059)+	971	2.95 [2.98]	60.3 [58.7]	116.3 [115.6]
	2.00	60	4.15 (249)+	7.78 (467)+	4.93 (296)+	16.87 (1012)+	881	3.52 [3.57]	60.2 [58.6]	172.8 [173.7]
BH	0.00	160	0.18 (29)	0.01 (1)		0.19 (30)	30	1.53 [0.00]	0.8 [0.0]	0.6 [0.0]
	0.50	160	0.85 (136)+	0.26 (41)+		1.11 (177)+	177	1.93 [2.04]	4.5 [3.8]	4.3 [3.9]
	1.00	160	1.67 (265)+	0.67 (107)+		2.33 (372)+	372	2.07 [2.13]	9.5 [8.8]	10.0 [9.6]
	2.00	160	1.77 (284)+	0.92 (146)+		2.69 (430)+	430	2.24 [2.31]	11.0 [10.3]	13.0 [12.7]
N-Nitrosodimethylamine										
MH	0.00	80	0.20 (16)	0.01 (1)	0.03 (2)	0.24 (19)	19	1.84 [0.00]	1.0 [0.0]	0.9 [0.0]
	0.25	76	2.46 (187)+	1.49 (113)+	0.43 (33)+	4.38 (333)+	280	2.26 [2.29]	15.1 [14.1]	18.1 [17.3]
	0.50	104	3.57 (371)+	3.69 (384)+	0.82 (85)+	8.08 (840)+	616	2.44 [2.47]	24.3 [23.3]	33.0 [32.3]
	1.00	24	5.96 (143)+	4.67 (112)+	0.92 (22)	11.54 (277)+	190	2.22 [2.23]	32.5 [31.5]	37.8 [37.0]
BH	0.00	80	0.12 (10)	0.14 (11)		0.26 (21)	21	2.76 [0.00]	1.1 [0.0]	1.8 [0.0]
	0.25	80	1.23 (98)+	0.33 (26)+		1.55 (124)+	124	1.79 [1.59]	6.4 [5.3]	5.5 [4.0]
	0.50	132	1.33 (175)+	0.54 (71)+		1.86 (246)+	246	1.98 [1.85]	7.6 [6.6]	7.5 [5.9]
	1.00	42	3.12 (131)+	0.86 (36)+		3.98 (167)+	167	1.77 [1.70]	16.3 [15.2]	13.9 [12.4]
N-Nitrosodiethylamine										
MH	0.00	120	0.17 (20)	0.01 (1)	0.02 (2)	0.19 (23)	23	1.70 [0.00]	0.8 [0.0]	0.6 [0.0]
	1.00	154	0.36 (55)+	0.16 (25)+	0.08 (13)+	0.60 (93)+	88	2.38 [2.72]	2.3 [1.6]	3.0 [2.6]
	2.50	80	0.25 (20)i	0.17 (14)+	0.00 (0)i	0.43 (34)+	27	2.19 [2.83]	1.4 [0.6]	1.6 [1.1]
	5.00	56	1.30 (73)+	0.25 (14)+	0.11 (6)+	1.66 (93)+	89	1.70 [1.70]	6.5 [5.7]	5.3 [4.6]
BH	0.00	38	0.18 (7)	0.00 (0)		0.18 (7)	7	1.00 [0.00]	0.8 [0.0]	0.4 [0.0]
	1.00	160	0.16 (26)i	0.03 (4)i		0.19 (30)i	30	1.50 [29.5]	0.8 [0.0]	0.5 [0.0]
	2.50	80	0.17 (13)i	0.08 (6)+		0.24 (19)+	19	1.95 [5.22]	1.0 [0.2]	0.9 [0.5]
	5.00	40	1.42 (57)+	0.05 (2)i		1.48 (59)+	59	1.20 [1.23]	6.1 [5.3]	3.5 [3.1]

Mitomycin C															
MH	0.00	78	0.28	(22)	0.04	(3)	0.01	(1)	0.33	(26)	25	1.60 [0.00]	1.3	[0.0]	1.0 [0.0]
	0.31	80	0.51	(41)+	0.32	(26)+	0.08	(6)i	0.91	(73)+	51	1.92 [2.25]	2.6	[1.3]	2.5 [1.5]
	0.62	78	1.26	(98)+	1.22	(95)+	0.33	(26)+	2.81	(213)+	195	2.62 [2.76]	10.3	[8.9]	15.7 [15.2]
	1.20	62	1.52	(94)+	2.81	(174)+	0.79	(49)+	5.11	(317)+	272	3.58 [3.74]	18.0	[16.7]	53.8 [55.6]
BH	0.00	78	0.21	(16)	0.00	(0)			0.21	(16)	16	1.19 [0.00]	0.8	[0.0]	0.5 [0.0]
	0.31	78	0.51	(40)+	0.14	(11)+			0.65	(51)+	51	1.82 [2.11]	2.7	[1.8]	2.4 [2.0]
	0.62	80	0.75	(60)+	0.39	(31)+			1.14	(91)+	91	2.20 [2.42]	4.7	[3.8]	5.4 [5.1]
	1.20	80	1.39	(111)+	1.17	(94)+			2.56	(205)+	205	2.54 [2.66]	10.5	[9.7]	15.3 [15.3]
Chlorambucil															
MH	0.00	80	0.22	(18)	0.04	(3)	0.00	(0)	0.26	(21)	21	1.9 [0.00]	1.1	[0.0]	1.0 [0.0]
	2.00	80	0.50	(40)+	0.10	(8)i	0.01	(1)i	0.61	(49)+	49	1.71 [1.57]	2.5	[1.4]	2.1 [1.1]
	4.00	80	0.94	(75)+	0.30	(24)+	0.10	(8)+	1.34	(107)+	106	2.11 [2.16]	5.4	[4.4]	5.9 [4.9]
	8.00	40	3.35	(134)+	1.52	(61)+	0.37	(15)+	5.25	(210)+	205	2.32 [2.34]	21.0	[19.9]	26.3 [25.3]
BH	0.00	80	0.15	(12)	0.01	(1)			0.16	(13)	13	1.62 [0.00]	0.7	[0.0]	0.5 [0.0]
	2.00	80	0.41	(33)+	0.00	(0)i			0.41	(33)+	33	1.03 [0.65]	1.7	[1.0]	0.9 [0.4]
	4.00	80	0.32	(26)+	0.08	(6)i			0.40	(32)+	32	1.72 [1.79]	1.6	[1.0]	1.4 [0.8]
	8.00	80	1.49	(19)+	0.28	(22)+			1.76	(141)+	141	1.66 [1.66]	7.2	[6.6]	5.7 [5.2]
Monocrotaline															
MH	0.00	80	0.24	(19)	0.05	(4)	0.01	(1)	0.30	(24)	23	1.74 [0.00]	1.2	[0.0]	1.0 [0.0]
	1.25	80	0.34	(27)i	0.44	(35)+	0.20	(16)+	0.98	(78)+	72	3.08 [3.71]	3.7	[2.5]	7.8 [8.2]
	2.50	54	1.69	(91)+	1.37	(74)+	0.43	(23)+	3.48	(188)+	179	2.79 [2.89]	13.6	[12.4]	23.5 [23.1]
	5.00	74	1.72	(127)+	1.16	(86)+	0.62	(46)+	3.50	(259)+	254	2.68 [2.77]	14.1	[12.9]	22.6 [21.9]
BH	0.00	80	0.28	(22)	0.00	(0)			0.28	(22)	22	1.05 [0.00]	1.1	[0.0]	0.6 [0.0]
	1.25	80	0.20	(18)−	0.02	(2)i			0.23	(20)−	20	1.45 [0.00]	1.0	[−0.1]	0.7 [−0.1]
	2.50	80	0.43	(34)i	0.17	(14)+			0.60	(48)+	48	2.19 [3.15]	2.5	[1.3]	2.8 [3.0]
	5.00	80	0.56	(45)+	0.20	(16)+			0.76	(61)+	61	2.00 [2.54]	3.1	[2.0]	3.1 [2.9]

[a] MH, marker heterozygous (*mwh* + / + *flr*^{3}); BH, balancer-heterozygous (*mwh* + / *TM3*, *Bd*^{S}).

[b] Including *flr*^{3} single spots.

[c] Statistical diagnoses according to Frei and Würgler (1988): +, positive; −, negative; i, inconclusive. m, multiplication factor for the assessment of significantly negative results. Kastenbaum-Bowman test, significance levels $\alpha = \beta = 0.05$.

[d] Considering *mwh* clones from *mwh* single spots and from twin spots.

[e] Numbers in square brackets are control-corrected.

[f] $C = 24400$, i.e., approximate number of cells examined per wing.

[g] Only *mwh* single spots can be recovered in *mwh* / *TM3* balancer-heterozygotes as *TM3* does not carry *flr*^{3}.

Table 2
Summary of results obtained in the Drosophila wing somatic mutation and recombination test. Acute feeding of 3-day-old larvae with 2 alkylating agents for 2 h

Geno-type [a]	Conc. (mM)	No of wings (N)	Spots per wing (No. of spots) Stat. diagn. [c]				Spots with *mwh* clone [d] (n)	Mean *mwh* clone size class [d,e] ($\hat{i}$)	Clone formation frequencies(per 10^5 cells per cell div.) [e,f] (n/NC)
			Small single spots (1–2 cells) [b] [$m = 2.0$]	Large single spots(> 2 cells) [b] [$m = 5.0$]	Twin spots [$m = 5.0$]	Total spots [$m = 2.0$]			
N-Nitrosodiethylamine									
MH	0.0	40	0.32 (13)	0.00 (0)	0.00 (0)	0.32 (13)	13	1.08 [–]	1.3 [0.0]
	10.0	40	0.55 (22)i	0.32 (13) +	0.03 (1)i	0.90 (36) +	36	2.39 [ ]	3.7 [2.4]
	20.0	40	0.53 (20)i	0.32 (13) +	0.05 (2)i	0.92 (35) +	35	2.69 [ ]	3.8 [2.5]
BH	0.0	40	0.10 (4)	0.00 (0)		0.10 (4)	4	1.00 [–]	0.4 [0.0]
	10.0	40	0.35 (14) +	0.00 (0)		0.35 (14) +	14	1.07 [ ]	1.4 [1.0]
	20.0	40	0.32 (13) +	0.03 (1)i		0.35 (14) +	14	1.29 [ ]	1.4 [1.0]
Mitomycin C									
MH	0.0	40	0.28 (11)	0.12 (5)	0.03 (1)	0.43 (17)	17	2.41 [–]	1.7 [0.0]
	5.0	40	0.62 (25) +	0.37 (15) +	0.10 (4)i	1.10 (44) +	44	2.93 [ ]	4.5 [2.8]
	10.0	40	0.60 (24) +	1.77 (71) +	0.32 (13) +	2.70 (108) +	104	4.46 [ ]	10.7 [8.9]
BH	0.0	40	0.20 (8)	0.00 (0)		0.20 (8)	8	1.25 [–]	0.8 [0.0]
	5.0	40	0.40 (16)i	0.08 (3)i		0.47 (19) +	19	1.68 [ ]	2.0 [1.1]
	10.0	40	0.45 (18) +	0.15 (6) +		0.60 (24) +	24	2.12 [ ]	2.5 [1.6]

[a] MH, marker-heterozygous (*mwh* + / + *flr*^{3}); BH, balancer-heterozygous (*mwh* + /*TM3*, *Bd*^{S}).
[b] Including *flr*^{3} single spots.
[c] Statistical diagnoses according to Frei and Würgler (1988): +, positive, –, negative; i, inconclusive, m, multiplication factor for the assessment of significantly negative results. Kastenbaum-Bowman test, significance levels $\alpha = \beta = 0.05$.
[d] Considering *mwh* clones from *mwh* single spots and from twin spots.
[e] Numbers in square brackets are control-corrected.
[f] $C = 24400$, i.e., approximate number of cells examined per wing.
[g] Only *mwh* single spots can be recovered in *mwh* / *TM3* balancer-heterozygotes as *TM3* does not carry *flr*^{3}.

were always compared to the corresponding concurrent controls. The frequencies of spontaneous spots obtained with the 5% sucrose control series (MMS, DMN, DEN, MMC) and with the 1% Tween 80 plus 3% ethanol control series (CLA, MCT) are very similar.

The size distributions of the *mwh* clones (i.e., *mwh* single spots and *mwh* part of twin spots) are plotted in Fig. 1 always for the highest concentration analysed with each compound using chronic feeding and for both types of wings. As can be seen from Fig. 1 as well as from the mean *mwh* clone size class given in Table 1, the average clone size recorded in the two genotypes is different for all six compounds. Therefore, the *mwh* clone formation frequencies were corrected for clone size according to Frei et al. (1992) and are also given in Tables 1 and 2. The resulting dose-response curves for size-corrected clone formation frequencies are plotted in Fig. 2. The two dose-response curves obtained with each compound for the two genotypes were compared by linear regression analysis as shown in Table 3. The slopes of the two curves were used to determine the proportion of recombination versus mutation for each compound and treatment (also given in Table 3).

MMS. This small direct-acting monofunctional alkylating agent proves to be a very potent inducer of all types of spots after chronic feeding (Table 1). At the highest concentration, *mwh* clones of a size between 5 and 8 cells were preferentially induced in the marker-heterozygous wings while in the balancer-heterozygous wings *mwh* clones of 1 cell were the most frequent ones (Fig. 1). Of all the six compounds evaluated, MMS has the highest genotoxic activity, as can be seen from the dose-response curves for clone formation frequencies given in Fig.

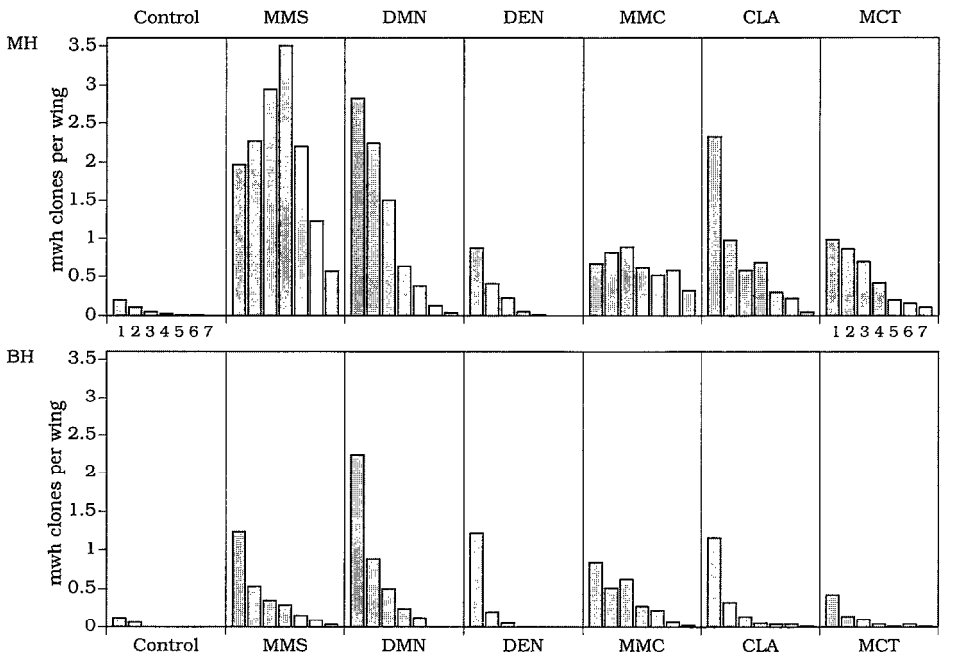

Fig. 1. Size distributions for *mwh* clones recorded on marker-heterozygous (MH) and on balancer-heterozygous (BH) wings after chronic feeding with the highest concentration of six alkylating agents. In each panel the clone sizes are: 1, 1 cell; 2, 2 cells; 3, 3–4 cells; 4, 5–8 cells; 5, 9–16 cells; 6, 17–32 cells; 7, more than 32 cells.

2. The proportion of recombination determined was 92.9% (Table 3).

DMN. This indirect-acting monofunctional methylating agent induced all three types of spot with a clear dose-response relationship over the three concentrations tested (Table 1 and Fig. 2). However, its

Table 3
Linear regression analysis of dose response curves for clone formation frequencies per 10^5 cells (size corrected) and proportion of recombination

Compound	Type*	Slope	Intercept	Mutation (%)	Recombination (%)
MMS	MH	88.56	5.66		
	BH	6.28	1.48	7.1	92.9
MCT	MH	4.52	3.92		
	BH	0.57	0.56	12.6	87.4
CLA	MH	3.28	−2.66		
	BH	0.65	−0.20	19.8	80.2
MMC	MH	46.73	−6.41		
	BH	12.49	−0.93	26.7	73.3
DMN	MH	35.83	6.80		
	BH	11.75	2.06	32.8	67.2
DEN	MH	0.84	0.95		
	BH	0.65	0.08	77.8	22.2
Acute feeding					
MMC	MH	0.22	0.29		
	BH	0.04	0.22	18.4	81.6
DEN	MH	0.30	0.41		
	BH	0.13	0.14	43.3	56.7

* MH, marker-heterozygous; BH, balancer-heterozygous.

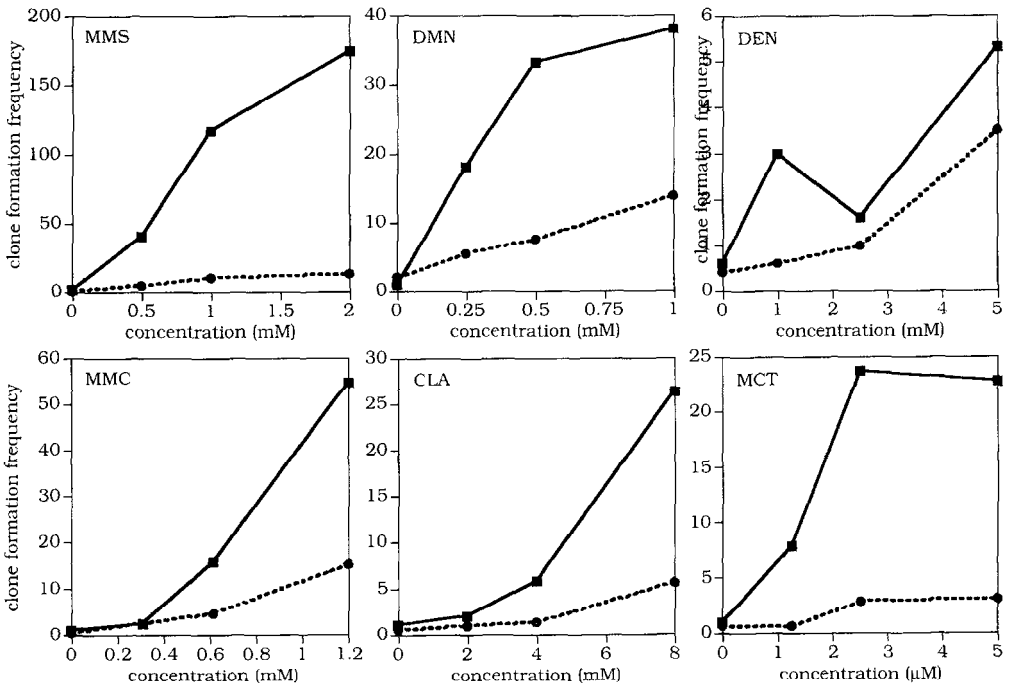

Fig. 2. Dose response relationships for clone formation frequencies per 10^5 cells recorded on marker-heterozygous (squares) and on balancer-heterozygous (circles) wings after chronic feeding with different concentrations of si × alkylating agents.

genotoxic activity is far lower than that of MMS. The percentage of recombination obtained was 67.2% (Table 3).

DEN. This promutagenic ethylating agent was tested in chronic as well as in acute exposures. No clear dose-response relationships were observed for both treatments. As can be seen in Fig. 2, the curve for chronic feeding shows an unusual shape. This is probably due to the fact that this compound exhibits the lowest genotoxic activity of all six compounds, effect that could be related to its biotransformation. Very low frequencies of twin spots were obtained in both chronic and acute feeding exposures (see Tables 1 and 2). The proportion of recombination obtained after acute treatment was 56.7%, whereas it was only 22.2% after chronic treatment. This extremely low value is mainly due to the unusual shape of the dose-response curve which does not allow for a good estimation of recombinagenic activity. However, the recombinagenicity of DEN is the lowest of all six compounds investigated.

MMC. This bifunctional cross-linking alkylating agent was also tested by two different techniques. In the chronic exposures as well in the acute treatments all types of spots were produced in a dose-related manner. Its overall genotoxic activity is the second highest observed (see Fig. 2). The percentage of recombination was 73.3% for the chronic feeding experiments and 81.6% for the acute exposure (Table 3).

CLA. This second cross-linking alkylating agent exhibited a genotoxic activity which corresponds to approximately one half of that observed for MMC (see Fig. 2). The proportion of recombination was 80.2% (Table 3).

MCT. This pyrrolizidine alkaloid acts as a cross-linking agent, after metabolic activation by mixed-function oxidases (Culvenor and Jago, 1979). In the marker-heterozygous wings it induced all three types of spots with a levelling-off at the two highest concentrations. In the balancer-heterozygous wings it gave a positive result only at the two highest concentrations tested (Table 1). Its overall genotoxic activity is slightly lower than that of CLA (see Fig. 2). The percentage of recombination was 87.4% (Table 3).

4. Discussion

Five of the compounds tested in the present series of experiments were procarcinogens, only one (MMS) was a direct-acting and rather powerful reference mutagen. The mutagens requiring metabolic activation were efficiently detected in chronic as well as in acute feeding treatments. However, according to the frequencies of clone formation given in Tables 1 and 2 for DEN and MMC, the chronic treatments are considerably more efficient that the acute feedings because higher values are obtained with lower concentrations of the compounds. Furthermore, for twin spots the acute exposures always yield lower spot frequencies than the chronic exposures.

Different spot size distributions are expected with chronic feedings compared to acute ones. After chronic treatment, in both types of wings the small spots predominate and larger spots continuously decrease in frequency (Graf et al., 1984). Our results are in agreement with this expectation with the exception of MMS and MMC where the size distributions are different (see Fig. 1). These are the two compounds which show the highest genotoxic activity of all six compounds tested, and they produce spot size distributions which resemble those of acute treatments as observed with MMC and DEN (data not shown) (see also Graf et al., 1984). The spot size distributions for acute treatments are characterized by a larger average clone size than those for chronic treatments (see Tables 1 and 2). Furthermore, with all six compounds the average clone size is always lower in the balancer-heterozygous wings than in the marker-heterozygous ones (see Table 1, Table 2 and Fig. 1). In addition, the analysis of the balancer-heterozygous wings demonstrates a reduction in the frequency of induced spots after both feeding exposures. With MMC, the two values for the proportion of induced recombination are in good agreement (81.6% for acute and 73.3% for chronic feeding). This is not the case for DEN (56.7% vs. 22.2%) where the peculiar shape of the dose-response curve after feeding precludes a precise determination of the recombinagenic activity.

Based on the numbers and sizes of *mwh* clones observed it is possible to calculate the clone formation frequency (Szabad et al., 1983; Frei et al., 1992) which gives a useful estimate for the ranking of the compounds with respect to their genotoxic potency. For the marker-heterozygous wings the following ranking order was obtained: MMS > MMC > DMN > CLA ~ MCT > DEN (see Fig. 2). In this context, it has to be noted that DMN and DEN are promutagens and that, therefore, their genotoxic potency depends on the bioactivation capacity of the tester larvae. The use of different strains or crosses (e.g., high bioactivation cross, see Graf and van Schaik, 1992) changes their ranking drastically.

The scoring of single and twin spots in marker-heterozygous and of *mwh* single spots in balancer-heterozygous wings permits, at least partly, the study of the genetic end points produced. In marker-heterozygous wings the origin of spots can be due to gene mutations, deletions, certain types of chromosome aberrations as well as mitotic recombination and possibly gene conversion; in balancer-heterozygous wings all recombinational events are eliminated due to the multiple inversions present in the *TM3* chromosome (Graf et al., 1989). In the marker-heterozygous wings, the *mwh* spots may arise both from mutation and recombination. Therefore, it is impossible to determine the relative contributions of these two mechanisms to total spot induction, although the number of twin spots may give an indication as these are exclusively the result of recombination produced between *flr* and the centromere. In the balancer-heterozygous wings, mitotic crossing-over leads to anomalous karyotypic configurations that are inviable. Balancer chromosome heterozygosity is able to suppress the induction of spots originating from mitotic crossing-over (Frei et al., 1992). We always found a reduction in the frequency of spots, especially of large single spots (Tables 1 and 2). However, from the significantly increased frequencies of single spots over the control frequencies in balancer-heterozygous wings obtained with all six compounds, we conclude that in addition to mitotic recombination and inequal intrachromosomal recombination, also various types of point mutations as well as certain types of chromosome aberrations contribute to the overall genotoxic activity.

The peculiar shape of the dose-response curves for some AAs, i.e., the levelling-off at higher concentrations, could be explained by the fact that at these concentrations proportionately more chromo-

some aberrations and fewer point mutations are induced than at lower ones (see Fig. 2).

Another result of this study is that in the balancer-heterozygous flies the clones are smaller on average than in the marker-heterozygous flies (see Fig. 1). Previous studies suggested that induced segmental aneuploidy leads to clones which divide poorly, if at all (Baker et al., 1978; Graf et al., 1984). As can be seen from the results shown in Table 3, in marker-heterozygous flies induced mitotic recombination represents an important fraction of the total genotoxic activity (Graf et al., 1992). The larger mean clone size would indicate that on average mitotic recombination leads to clones in which proliferation is not or is less impaired than in chromosomally deficient ones originating from mutational events. A shift in average size to smaller clones in balancer-heterozygous flies seems to be a general phenomenon which was also observed with other genotoxic compounds (Hara et al., 1987; Frei et al., 1992; Graf et al., 1992).

It is well established that semilethal chromosomal aberrations, such as loss of the normal third chromosome or deletion of the terminal segment including the normal allele of *mwh*, are responsible for the occurrence of small *mwh* clones (Merriam and Garcia-Bellido, 1972; Baker et al., 1978; Kennison and Ripoll, 1981). Recently, Ayaki et al. (1990) have suggested that if mitotic recombinational repair, a G2-specific one, works to efficiently convert double strand breaks to tolerable DNA aberrations (crossing-over, gene conversion), it must be accompanied by repair errors, producing semitolerable DNA aberrations at the cellular level. Whatever the mechanism is that produces small *mwh* single spots, the rank order in increase above control levels induced by the model mutagens at the highest concentration tested is DMN > CLA > MMS > MMC > MCT = DEN for the marker heterozygous wings (see Table 1). It has been hypothesised that large *mwh* single spots composed of three or more cells result practically exclusively from crossing-over (Ayaki et al., 1990). The rank order in increase above control levels of large *mwh* spots observed for the six alkylating agents tested is DMN > MMS > MMC > CLA > MCT > DEN for the marker-heterozygous wings (Table 1). This is essentially the same order as observed for the small *mwh* spots from which we may conclude that the conclusion drawn by Ayaki et al. (1990) is probably not generally valid. The flr^3 single spots in the *mwh* +/+ flr^3 wings are very infrequent because they probably arise from relatively rare events like point mutations at the locus, interstitial deletions and perhaps double crossing-overs (Frei et al., 1992). It has also been suggested that they may be produced by single crossing-over between the flr^3 locus and the centromere where recombinants occasionally fail to express phenotypically the homozygous marker *mwh* (Garcia-Bellido and Dapena, 1974).

It is well known that the formation of a twin spot is predominantly the consequence of chromosome breakage followed by mitotic recombination. A high yield of twin spots can be regarded as the result of particularly high ability for breaking chromosomes and inducing a rearrangement that generates a mitotic recombinational event. The fold of increase above control level for the twin spots obtained at the highest concentration with the six compounds gives the following rank order: MMS > MMC > MCT > CLA > DMN > DEN (Table 1). However, it has been shown that the recovery of twin spots is strongly biased because of two non-ideal properties of the flr^3 marker. Firstly, it is lethal in large clones, and secondly it is only weakly expressed in small clones (Szabad et al., 1983; Graf, 1995). For this reason, the quantitative determination of recombinagenic activity has to be based on a comparison of the *mwh* clone formation frequencies observed in both types of wings. According to this procedure, the data given in Table 3 give the following rank order: MMS > MCT > CLA > MMC > DMN > DEN. These data also show that with the exception of DEN all the other alkylating agents give proportions of recombinagenicity that are higher than approximately two thirds of the total genotoxic activity observed.

Previous studies with monofunctional alkylating agents have revealed that a positive relationship exists between carcinogenic potency in rodents (Bartsch et al., 1983), hypermutability in exr^- Drosophila strains and nucleophilic selectivity (Vogel, 1989a). In contrast, a series of cross-linking agents, mostly anti-tumour drugs, did not follow this relationship (Barbin and Bartsch, 1989; Vogel et al., 1990). The mutational spectra obtained by Vogel (1989b) for several carcinogens demonstrate different modes of

action: (1) Direct miscoding mutagenesis leading to mutation is the predominant mechanism for diethylnitrosamine (DEN). (2) Depurinisation leading to apurinic sites as mutagenic intermediates is a frequent mutagenic pathway for AAs with high nucleophilic selectivity, i.e., MMS. (3) Agents capable of cross-linking DNA produce mono-adducts and intra- as well interstrand cross-links.

MMS-induced *N*-alkylations in DNA followed by the formation of apurinic sites have been shown to accumulate in a time-related manner (Singer and Grunberger, 1983; Loeb, 1985). The clastogenicity of monofunctional alkylating agents may result predominantly from their reaction with nitrogen atoms in DNA (Vogel et al., 1993). This mechanism may explain the results obtained for MMS and DMN in the present study. Monofunctional alkylating agents are also able to alkylate oxygen atoms. It has been shown that this type of alkylation induces preferentially point mutations (Vogel and Natarajan, 1979a, b, 1982; Nivard et al., 1992) which is in agreement with the pattern of predominantly mutagenic activity found for DEN in our study. The cross-linking, bifunctional agents, which are extremely potent inducers of mitotic recombination, probably act through the formation of premutagenic DNA monoadducts or intrastrand cross-links (Hemminki and Ludlum, 1986) after *N*-alkylation of DNA (Barbin and Bartsch, 1989).

Finally, as shown in Fig. 3, there is an excellent correlation between the nucleophilic selectivity of the alkylating agents (*s* value) and the recombinagenic activity produced by them. The main conclusion from our study is that there exists a strong relationship between the extent of *N*-alkylation of DNA and the efficiency of the monofunctional agents MMS and DMN as well as the bifunctional agents MCT, CLA and MMC to induce mitotic recombination. This contrasts with the ethylation of base oxygens atoms and the lower efficiency of DEN to produce mitotic recombination. The low recombinagenicity of DEN is consistent with the suggestion that it produces mainly point mutations. The results obtained in our study demonstrate that the wing somatic mutation and recombination test of *Drosophila melanogaster* is an ideal tool for the study of the relationships between structure and reactivity of chemical compounds and the resulting spectra of different genotoxic events. It is especially well suited for a quantitative determination of the recombinagenic activity of genotoxins.

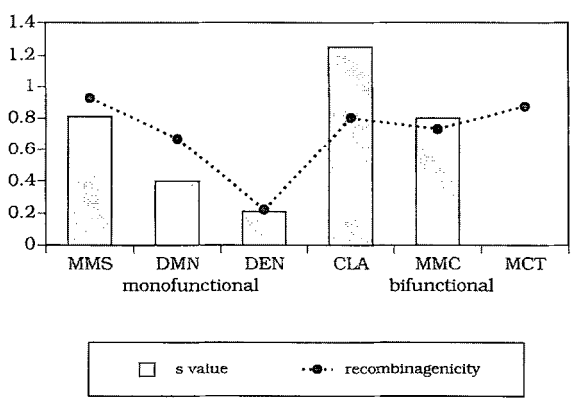

Fig. 3. Relationship between the nucleophilic selectivity (s value) and the recombinagenic activity of six alkylating agents in the wing spot test.

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