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Recombinagenic activity of four compounds in the standard and high bioactivation crosses of *Drosophila melanogaster* in the wing spot test

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The wing somatic mutation and recombination test (SMART) using *Drosophila melanogaster* was employed to determine the recombinagenic and mutagenic activity of four chemicals in an *in vivo* eukaryotic system. Two different crosses involving the wing cell markers *mwh* and *flr*^{3} were used: the standard cross and a high bioactivation cross. The high bioactivation cross is characterized by a high constitutive level of cytochromes P450 which leads to an increased sensitivity to a number of promutagens and procarcinogens. Three-day-old larvae derived from both crosses were treated chronically with the oxidizing agent potassium chromate and with the three procarcinogens cyclophosphamide, *p*-dimethylaminoazobenzene and 9,10-dimethylanthracene. From both crosses two types of progeny were obtained: marker-heterozygous and balancer-heterozygous. The wings of both genotypes were analysed for the occurrence of single and twin spots expressing the *mwh* and/or *flr*^{3} mutant phenotypes. In the marker-heterozygous genotype the spots can be due either to mitotic recombination or to mutation. In contrast, in the balancer-heterozygous genotype only mutational events lead to spot formation, all recombination events being eliminated. The oxidizing agent potassium chromate was equally and highly genotoxic in both crosses. Surprisingly, the promutagen cyclophosphamide also showed equal genotoxicity in both crosses, whereas *p*-dimethylaminoazobenzene was negative in the standard cross, but clearly genotoxic in the high bioactivation cross. 9,10-Dimethylanthracene showed a rather weak genotoxicity in the high bioactivation cross. Analyses of the dose–response relationships for *mwh* clones recorded in the two wing genotypes demonstrated that all four compounds are recombinagenic. The fraction of all genotoxic events which are due to mitotic recombination ranged from 83% (9,10-dimethylanthracene) to 99% (*p*-dimethylaminoazobenzene). These results demonstrate that the wing spot test in *Drosophila* is most suited to the detection of recombinagenic activity of genotoxic chemicals.

Introduction

Genetic recombination is a universal phenomenon of importance for all biological systems from viruses to higher eukaryotes. Recombination processes contribute to genetic variability and thus have important evolutionary aspects. As early as 1936 Curt Stern demonstrated that in the fruit fly *Drosophila melanogaster* genetic recombination occurs not only in meiotic cells but also in somatic cells during mitotic cell division (Stern, 1936). More recently it has been shown that recombination phenomena are important for other cellular functions, such as DNA repair and variation in mammalian immunoglobulins (Engler and Strob, 1988).

In genetic toxicology the emphasis has traditionally been placed on screening for classical genetic alterations such as gene mutations, chromosome aberrations and, more recently, aneuploidy. However, tests for the determination of reciprocal mitotic recombination and mitotic gene conversion have also been developed, mainly in yeasts (Zimmermann *et al.*, 1966). It became evident that many agents causing damage to DNA also have recombinagenic activity. Recombinagenic activity has been considered an indirect measure of more general damage to DNA (Hoffmann, 1994). Later it was recognized that recombination may also be responsible for loss of heterozygosity in germline and somatic cells. Loss of heterozygosity can promote the manifestation of recessive heritable diseases or may be involved in the progression of neoplasia. This mechanism has been shown to be operational in the case of various oncogenes and tumour suppressor genes (Bishop, 1991; Marshall, 1991; Sengstag, 1994; Happle, 1999). Awareness of the importance of recombinagenic activity for safety evaluation of chemical agents has led to the development of experimental systems capable of quantitatively measuring mitotic recombination (Würgler, 1992). At present different test systems are available in bacteria, yeasts, *Drosophila*, mammalian cells in culture and a mammal *in vivo* (mouse) (Sengstag, 1994). The tests in *D.melanogaster* present undoubted advantages: they are characterized by rapidity comparable with that of prokaryotic or unicellular *in vitro* systems, but have the predictive value of eukaryotic *in vivo* tests (Vogel, 1992; Vogel *et al*., 1999). One of these assays, the somatic mutation and recombination test (SMART), has been widely used in both a version based on an eye colour marker (Vogel and Zijlstra, 1987a,b; Vogel and Nivard, 1993) and in a version based on two wing cell markers (Graf *et al.*, 1984; Würgler *et al.*, 1985). In both cases loss of heterozygosity leads to uncovering and expression of the recessive marker gene(s) in the larval imaginal disk cells. They give rise to clones of mutant cells showing up as mosaic spots on the eyes or the wings, respectively.

In the wing spot test two different crosses are currently used to produce the experimental larval populations: the standard (ST) cross (Graf *et al.*, 1989) and a high bioactivation (HB) cross (Graf and van Schaik, 1992). The latter is characterized by improved sensitivity to a number of promutagens and procarcinogens owing to high levels of constitutively expressed cytochromes P450. Both crosses produce two types of progeny: (i) marker-heterozygous flies, where wing spots can be due to mitotic recombination or to various types of mutational events; (ii) balancer-heterozygous flies, where only mutational events lead to wing spots because all recombination events are eliminated owing to multiple inversions present on the *TM3*

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Table I. Summary of results obtained in the *Drosophila* wing spot test

Cross[a]	Type[b]	Conc. (mM)	No. of flies	Spots per fly (no. of spots) statistical diagnosis[c]					Mean *mwh* clone size class	Clone formation per 10^5 cells per cell division[d]	
				Small single spots (1–2 cells) $m = 2$	Large single spots (>2 cells) $m = 5$	Twin spots $m = 5$	Total spots $m = 2$	*mwh* clones		Observed	Control corrected
Potassium chromate (48 h)											
ST	MH	0.0	20	0.30 (6)	0.10 (2)	0.05 (1)	0.45 (9)	0.45 (9)	2.33	0.9	
		0.5	20	1.00 (20)++	0.20 (4)[e]	0.90 (18)+++	2.10 (42)+++	2.10 (42)	2.48	4.3	3.4
		1.0	20	3.10 (62)+++	1.45 (29)+++	2.45 (49)+++	7.00 (140)+++	6.80 (136)	2.71	13.9	13.0
		2.0	20	9.40 (188)+++	3.95 (79)+++	6.75 (135)+++	20.10 (402)+++	19.50 (390)	2.59	40.0	39.0
		5.0	3	22.67 (68)+++	17.33 (52)+++	15.33 (46)+++	55.33 (166)+++	53.67 (161)	2.65	110.0	109.1
	BH	0.0	20	0.40 (8)	0.00 (0)	0.00 (0)	0.40 (8)	0.40 (8)	1.13	0.8	
		0.5	20	0.05 (1)– – –	0.00 (0)[e]	0.00 (0)[e]	0.05 (1)– – –	0.05 (1)	1.00	0.1	–0.7
		1.0	20	1.10 (22)++	0.10 (2)[e]	0.00 (0)[e]	1.20 (24)++	1.20 (24)	1.42	2.5	1.6
		2.0	20	1.05 (21)+	0.10 (2)[e]	0.00 (0)[e]	1.15 (23)++	1.15 (23)	1.52	2.4	1.5
		5.0	4	3.50 (14)+++	0.25 (1)[e]	0.00 (0)[e]	3.75 (15)+++	3 .75 (15)	1.47	7.7	6.9
HB	MH	0.0	20	0.45 (9)	0.05 (1)	0.00 (0)	0.50 (10)	0.50 (10)	1.90	1.0	
		0.5	20	0.20 (4)– –	0.30 (6)[e]	0.60 (12)+++	1.10 (22)+	1.05 (21)	3.71	2.2	1.1
		1.0	20	1.40 (28)++	0.70 (14)+++	1.55 (31)+++	3.65 (73)+++	3.45 (69)	2.90	7.1	6.0
		2.0	20	1.90 (38)+++	1.70 (34)+++	3.90 (78)+++	7.50 (150)+++	7.05 (141)	3.28	14.4	13.4
		5.0	2	26.00 (52)+++	12.50 (25)+++	13.00 (26)+++	51.50 (103)+++	51.00 (102)	2.44	104.5	103.5
	BH	0.0	20	0.25 (5)	0.00 (0)	0.00 (0)	0.25 (5)	0.25 (5)	1.00	0.5	
		0.5	20	0.40 (8)[e]	0.00 (0)[e]	0.00 (0)[e]	0.40 (8)[e]	0.40 (8)	1.13	0.8	0.3
		1.0	20	0.40 (8)[e]	0.00 (0)[e]	0.00 (0)[e]	0.40 (8)[e]	0.40 (8)	1.13	0.8	0.3
		2.0	20	0.70 (14)+	0.25 (5)+	0.00 (0)[e]	0.95 (19)++	0.95 (19)	1.95	1.9	1.4
Potassium chromate (6 h)											
ST	MH	0.0	13	0.46 (6)	0.08 (1)	0.00 (0)	0.54 (7)	0.54 (7)	1.71	nc	
		5.0	12	0.83 (10)[e]	0.42 (5)[e]	0.75 (9)++	2.00 (24)+++	2.00 (24)	2.96		
		10.0	19	0.68 (13)[e]	0.37 (7)[e]	1.21 (23)+++	2.26 (43)+++	2.16 (41)	3.37		
		20.0	18	0.50 (9)[e]	0.50 (9)+	0.56 (10)++	1.56 (28)++	1.39 (25)	3.44		
		50.0	17	0.76 (13)[e]	0.76 (13)++	0.71 (12)++	2.24 (38)+++	2.00 (34)	3.82		
		100.0	10	1.00 (10)[e]	0.30 (3)[e]	0.80 (8)++	2.10 (21)+++	2.10 (21)	2.71		
	BH	0.0	20	0.30 (6)	0.00 (0)	0.00 (0)	0.30 (6)	0.30 (6)	1.33	nc	
		5.0	20	0.20 (4)[e]	0.05 (1)[e]	0.00 (0)[e]	0.25 (5)[e]	0.25 (5)	2.60		
		10.0	20	0.20 (4)[e]	0.05 (1)[e]	0.00 (0)[e]	0.25 (5)[e]	0.25 (5)	1.80		
		20.0	20	0.45 (9)[e]	0.00 (0)[e]	0.00 (0)[e]	0.45 (9)[e]	0.45 (9)	1.56		
		50.0	20	0.30 (6)[e]	0.20 (4)[e]	0.00 (0)[e]	0.50 (10)[e]	0.50 (10)	2.20		
		100.0	20	0.40 (8)[e]	0.10 (2)[e]	0.00 (0)[e]	0.50 (10)[e]	0.50 (10)	1.70		
HB	MH	0.0	20	0.25 (5)	0.10 (2)	0.05 (1)	0.40 (8)	0.35 (7)	1.43	nc	
		5.0	20	0.20 (4)[e]	0.40 (8)[e]	0.35 (7)+	0.95 (19)+	0.90 (18)	3.89		
		10.0	20	0.35 (7)[e]	0.35 (7)[e]	0.70 (14)+++	1.40 (28)+++	1.35 (27)	3.52		
		20.0	20	0.80 (16)+	0.40 (8)[e]	0.75 (15)+++	1.95 (39)+++	1.85 (37)	3.32		
		50.0	20	0.70 (14)+	0.40 (8)[e]	0.25 (5)[e]	1.35 (27)+++	1.30 (26)	3.08		
		100.0	10	1.00 (10)++	0.60 (6)+	0.70 (7)++	2.30 (23)+++	2.00 (20)	3.15		
	BH	0.0	20	0.30 (6)	0.00 (0)	0.00 (0)	0.30 (6)	0.30 (6)	1.00	nc	
		5.0	20	0.25 (5)[e]	0.00 (0)[e]	0.00 (0)[e]	0.25 (5)[e]	0.25 (5)	1.00		
		10.0	20	0.40 (8)[e]	0.05 (1)[e]	0.00 (0)[e]	0.45 (9)[e]	0.45 (9)	1.67		
		20.0	20	0.45 (9)[e]	0.05 (1)[e]	0.00 (0)[e]	0.50 (10)[e]	0.50 (10)	1.70		
		50.0	20	0.65 (13)[e]	0.05 (1)[e]	0.00 (0)[e]	0.70 (14)[e]	0.70 (14)	1.43		
		100.0	20	0.45 (9)[e]	0.15 (3)[e]	0.00 (0)[e]	0.60 (12)[e]	0.60 (12)	2.00		

Table I. *Cont.*

Cross[a]	Type[b]	Conc. (mM)	No. of flies	Spots per fly (no. of spots) statistical diagnosis[c]					Mean *mwh* clone size class	Clone formation per 10^5 cells per cell division[d]	
				Small single spots (1–2 cells) $m = 2$	Large single spots (>2 cells) $m = 5$	Twin spots $m = 5$	Total spots $m = 2$	*mwh* clones		Observed	Control corrected
Cyclophosphamide											
ST	MH	0.0	30	0.47 (14)	0.03 (1)	0.03 (1)	0.53 (16)	0.53 (16)	1.63	1.1	
		0.1	20	0.25 (5)– –	0.00 (0)[e]	0.05 (1)[e]	0.30 (6)– –	0.30 (6)	1.67	0.6	–0.5
		0.5	30	1.33 (40)+++	0.23 (7)+	0.07 (2)[e]	1.63 (49)+++	1.60 (48)	1.67	3.3	2.2
		1.0	30	3.67 (110)+++	0.53 (16)+++	0.20 (6)[e]	4.40 (132)+++	4.33 (130)	1.70	8.9	7.8
		2.0	20	5.35 (107)+++	1.20 (24)+++	0.25 (5)+	6.80 (136)+++	6.80 (136)	1.86	13.9	12.8
		3.0	10	9.10 (91)+++	2.30 (23)+++	0.50 (5)++	11.90 (119)+++	11.20 (112)	1.85	23.0	21.9
		5.0	3	56.67 (170)+++	9.00 (27)+++	2.33 (7)+++	68.00 (204)+++	68.00 (204)	1.65	139.3	138.3
	BH	0.0	30	0.47 (14)	0.00 (0)	0.00 (0)	0.47 (14)	0.47 (14)	1.29	1.0	
		0.1	20	0.30 (6)–	0.00 (0)[e]	0.00 (0)[e]	0.30 (6)–	0.30 (6)	1.17	0.6	–0.3
		0.5	29	0.66 (19)[e]	0.03 (1)[e]	0.00 (0)[e]	0.69 (20)[e]	0.69 (20)	1.40	1.4	0.5
		1.0	30	1.27 (38)+++	0.17 (5)+	0.00 (0)[e]	1.43 (43)+++	1.43 (43)	1.63	2.9	2.0
		2.0	30	2.70 (81)+++	0.53 (16)+++	0.00 (0)[e]	3.23 (97)+++	3.23 (97)	1.73	6.6	5.7
		3.0	10	4.40 (44)+++	0.60 (6)+++	0.00 (0)[e]	5.00 (50)+++	5.00 (50)	1.54	0.2	9.3
		5.0	20	6.35 (127)+++	0.70 (14)+++	0.00 (0)[e]	7.05 (141)+++	7.05 (141)	1.52	4.4	3.5
HB	MH	0.0	30	0.47 (14)	0.00 (0)	0.03 (1)	0.50 (15)	0.50 (15)	1.67	1.0	
		0.1	20	0.75 (15)[e]	0.20 (4)+	0.05 (1)[e]	1.00 (20)+	1.00 (20)	1.90	2.0	1.0
		0.5	30	1.50 (45)+++	0.27 (8)++	0.07 (2)[e]	1.83 (55)+++	1.80 (54)	1.80	3.7	2.7
		1.0	30	4.23 (127)+++	1.03 (31)+++	0.27 (8)+	5.53 (166)+++	5.37 (161)	1.89	11.0	10.0
		2.0	21	11.90 (250)+++	3.14 (66)+++	1.33 (28)+++	16.38 (344)+++	6.19 (340)	1.94	33.2	32.2
		3.0	10	21.10 (211)+++	5.80 (58)+++	1.40 (14)+++	28.30 (283)+++	27.30 (273)	1.89	55.9	54.9
	BH	0.0	30	0.37 (11)	0.03 (1)	0.00 (0)	0.40 (12)	0.40 (12)	1.50	0.8	
		0.1	20	0.70 (14)[e]	0.05 (1)[e]	0.00 (0)[e]	0.75 (15)[e]	0.75 (15)	1.53	1.5	0.7
		0.5	30	0.57 (17)[e]	0.03 (1)[e]	0.00 (0)[e]	0.60 (18)[e]	0.60 (18)	1.28	1.2	0.4
		1.0	30	1.40 (42)+++	0.17 (5)[e]	0.00 (0)[e]	1.57 (47)+++	1.57 (47)	1.43	3.2	2.4
		2.0	30	2.47 (74)+++	0.37 (11)++	0.00 (0)[e]	2.83 (85)+++	2.83 (85)	1.54	5.8	5.0
		3.0	10	2.50 (25)+++	0.30 (3)[e]	0.00 (0)[e]	2.80 (28)+++	2.80 (28)	1.57	5.7	4.9
		5.0	20	3.30 (66)+++	0.25 (5)+	0.00 (0)[e]	3.55 (71)+++	3.55 (71)	1.44	7.3	6.5
p–Dimethylaminoazobenzene											
ST	MH	0.0	00	0.46 (46)	0.07 (7)	0.06 (6)	0.59 (59)	0.58 (58)	1.90	1.2	
		0.5	00	0.49 (49)– –	0.13 (13)–	0.05 (5)– –	0.67 (67)– –	0.67 (67)	2.21	1.4	0.2
		1.0	04	0.48 (50)– –	0.09 (9)– –	0.04 (4)– –	0.61 (63)– –	0.61 (63)	1.95	1.2	0.1
		5.0	11	0.36 (4)–	0.00 (0)–	0.00 (0)[e]	0.36 (4)– –	0.36 (4)	1.00	0.7	–0.4
		10.0	13	0.46 (6)[e]	0.08 (1)[e]	0.15 (2)[e]	0.69 (9)[e]	0.69 (9)	2.11	1.4	0.2
HB	MH	0.0	40	0.47 (19)	0.03 (1)	0.03 (1)	0.52 (21)	0.52 (21)	1.43	1.1	
		1.0	50	0.48 (24)–	0.06 (3)[e]	0.04 (2)[e]	0.58 (29)–	0.58 (29)	1.72	1.2	0.1
		2.0	30	0.90 (27)+	0.17 (5)[e]	0.30 (9)++	1.37 (41)+++	1.37 (41)	2.22	2.8	1.7
		5.0	34	1.85 (63)+++	0.47 (16)+++	0.47 (16)+++	2.79 (95)+++	2.74 (93)	2.05	5.6	4.5
		10.0	30	2.43 (73)+++	0.80 (24)+++	0.70 (21)+++	3.93 (118)+++	3.90 (117)	2.15	8.0	6.9
	BH	0.0	40	0.20 (8)	0.03 (1)	0.00 (0)	0.22 (9)	0.22 (9)	1.44	0.5	
		1.0	30	0.37 (11)[e]	0.07 (2)[e]	0.00 (0)[e]	0.43 (13)[e]	0.43 (13)	1.54	0.9	0.4
		2.0	30	0.33 (10)[e]	0.00 (0)[e]	0.00 (0)[e]	0.33 (10)[e]	0.33 (10)	1.10	0.7	0.2
		5.0	50	0.24 (12)[e]	0.00 (0)[e]	0.00 (0)[e]	0.24 (12)[e]	0.24 (12)	1.08	0.5	0.0
		10.0	57	0.35 (20)[e]	0.07 (4)[e]	0.00 (0)[e]	0.42 (24)[e]	0.42 (24)	1.75	0.9	0.4
		20.0	20	0.45 (9)[e]	0.00 (0)[e]	0.00 (0)[e]	0.45 (9)[e]	0.45 (9)	1.11	0.9	0.5

Table I. *Cont.*

Cross[a]	Type[b]	Conc. (mM)	No. of flies	Spots per fly (no. of spots) statistical diagnosis[c]					Mean *mwh* clone size class	Clone formation per 10^5 cells per cell division[d]	
				Small single spots (1–2 cells) $m = 2$	Large single spots (>2 cells) $m = 5$	Twin spots $m = 5$	Total spots $m = 2$	*mwh* clones		Observed	Control corrected
9,10-Dimethylanthracene (Instant Medium)											
HB	MH	0.0	60	0.43 (26)	0.05 (3)	0.00 (0)	0.48 (29)	0.48 (29)	1.76	1.0	
		1.0	60	0.60 (36)[e]	0.15 (9)[e]	0.08 (5)+	0.83 (50)+	0.82 (49)	2.18	1.7	0.7
		5.0	60	0.63 (38)[e]	0.22 (13)+	0.15 (9)++	1.00 (60)+++	0.95 (57)	2.28	1.9	1.0
		10.0	60	0.83 (50)++	0.27 (16)++	0.20 (12)+++	1.30 (78)+++	1.28 (77)	2.34	2.6	1.6
		20.0	60	1.15 (69)+++	0.47 (28)+++	0.27 (16)+++	1.88 (113)+++	1.82 (109)	2.44	3.7	2.7
	BH	0.0	60	0.33 (20)	0.00 (0)	0.00 (0)	0.33 (20)	0.33 (20)	1.20	0.7	
		1.0	60	0.42 (25)[e]	0.02 (1)[e]	0.00 (0)[e]	0.43 (26)[e]	0.43 (26)	1.35	0.9	0.2
		5.0	60	0.60 (36)+	0.05 (3)[e]	0.00 (0)[e]	0.65 (39)++	0.65 (39)	1.44	1.3	0.6
		10.0	60	0.42 (25)[e]	0.10 (6)+	0.00 (0)[e]	0.52 (31)[e]	0.52 (31)	1.90	1.1	0.4
		20.0	60	0.50 (30)[e]	0.15 (9)++	0.00 (0)[e]	0.65 (39)++	0.65 (39)	1.85	1.3	0.6
9,10-Dimethylanthracene (mashed potato flakes)											
HB	MH	0.0	50	0.42 (21)	0.06 (3)	0.06 (3)	0.54 (27)	0.54 (27)	1.78	1.1	
		1.0	60	0.55 (33)[e]	0.13 (8)[e]	0.18 (11)[e]	0.87 (52)+	0.82 (49)	2.12	1.7	0.6
		5.0	60	0.83 (50)++	0.22 (13)+	0.18 (11)[e]	1.23 (74)+++	1.22 (73)	2.21	2.5	1.4
		10.0	60	0.95 (57)+++	0.30 (18)++	0.23 (14)+	1.48 (89)+++	1.47 (88)	2.51	3.0	1.9
		20.0	60	1.12 (67)+++	0.68 (41)+++	0.15 (9)[e]	1.95 (117)+++	1.93 (116)	2.59	4.0	2.9
	BH	0.0	60	0.48 (29)	0.07 (4)	0.00 (0)	0.55 (33)	0.55 (33)	1.52	1.1	
		1.0	60	0.40 (24)– –	0.05 (3)–	0.00 (0)[e]	0.45 (27)– – –	0.45 (27)	1.59	0.9	–0.2
		5.0	60	0.50 (30)– –	0.07 (4)–	0.00 (0)[e]	0.57 (34)– –	0.57 (34)	1.53	1.2	0.0
		10.0	60	0.57 (34)–	0.08 (5)–	0.00 (0)[e]	0.65 (39)–	0.65 (39)	1.56	1.3	0.2
		20.0	60	0.75 (45)+	0.08 (5)–	0.00 (0)[e]	0.83 (50)+	0.83 (50)	1.64	1.7	0.6

nc, not calculated because the number of wing precursor cells exposed during the 6 h acute feeding period was not determined.
[a]ST, standard cross; HB, high bioactivation cross.
[b]MH, marker-heterozygous wings; BH, balancer-heterozygous wings.
[c]Statistical diagnoses according to Frei and Würgler (1988). *m*, minimal risk multiplication factor for the assessment of negative results. One-sided binomial tests, significance levels α and β: positive results, + ($\alpha \leq 0.05$), ++ ($\alpha \leq 0.01$), +++ ($\alpha \leq 0.001$); negative results, – ($\beta \leq 0.05$), – – ($\beta \leq 0.01$), – – – ($\beta \leq 0.001$); inconclusive results, $\alpha > 0.05$, $\beta > 0.05$.
[d]Clone frequencies per fly divided by the number of cells examined per fly (48 800) gives an estimate of formation frequencies per cell and per cell division in chronic exposure experiments (Frei and Würgler, 1988).
[e]Inconclusive.

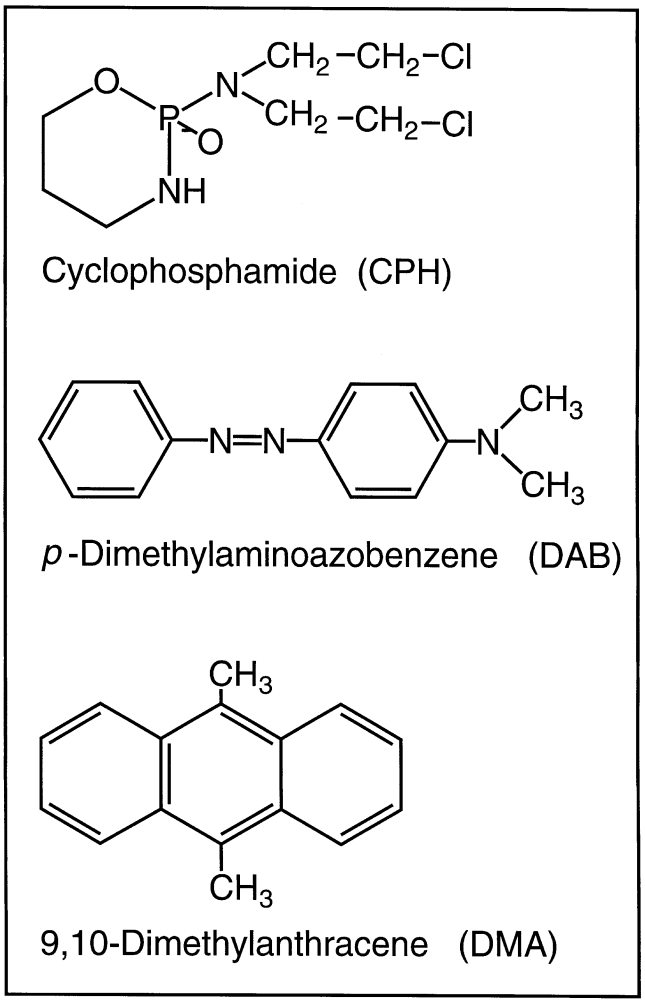

Fig. 1. Structural formulae of the chemical compounds.

balancer chromosome. To determine the fraction of recombinational events responsible for induced wing spots both types of wings of surviving flies were analysed (Frei *et al.*, 1992; Graf *et al.*, 1992b).

This study investigates the recombinagenic activity of chemical compounds belonging to different chemical classes. Larvae from both crosses were treated acutely or chronically with the oxidizing agent potassium chromate (POC), which acts mainly by generating active oxygen species. Three different promutagens that interact with DNA only after metabolic activation were tested by chronic feeding: the bifunctional alkylating cytostatic drug cyclophosphamide (CPH), the procarcinogen *p*-dimethylaminoazobenzene (DAB) and the polycyclic aromatic hydrocarbon 9,10-dimethylanthracene (DMA). DMA was applied in parallel in two different media (*Drosophila* Instant Medium and mashed potato flakes) to investigate the influence of this parameter on the test results.

Materials and methods

Chemicals

DAB (CAS 60-11-7), DMA (CAS 781-43-1) and POC (CAS 7789-00-6) were purchased from Fluka (Buchs, Switzerland). CPH (Endoxan, CAS 50-18-0) was obtained from the Hospital Pharmacy, University of Zurich, Switzerland. CPH and POC were dissolved in distilled water; DAB and DMA were dissolved in a mixture of 1% Tween 80 (Serva, Heidelberg, Germany) and 3% ethanol (Merck, Darmstadt, Germany). The structural formulae of the three promutagens CPH, DAB and DMA are shown in Figure 1.

Genes, flies and crosses

The markers multiple wing hairs (*mwh*, 3-0.3) and flare-3 (*flr*^{3}, 3-38.8) used in the wing spot test are at the tip and roughly in the middle of the left arm of chromosome 3, respectively. Two crosses were carried out to produce the experimental larval progeny: the ST cross, *flr*^{3}/*In(3LR)TM3, ri p*^{p} *sep l(3)89Aa bx*^{34e} *e Bd*^{S} females crossed with *mwh* males; the HB cross, *ORR*; *flr*^{3}/*In(3LR)TM3, ri p*^{p} *sep l(3)89Aa bx*^{34e} *e Bd*^{S} females crossed with *mwh* males. Graf *et al.* (1984, 1989) described the basic methods and the ST cross. The HB cross is described in more detail in Graf and van Schaik (1992). For genetic descriptions and symbols we refer to Lindsley and Zimm (1992).

Larval feeding

From the two crosses eggs were collected for 8 h in culture bottles with an agar–agar base (3% w/v) topped with a thick layer of fermenting live baker's yeast supplemented with sucrose. Three days later the larvae were washed out of the bottles with tap water and collected in a stainless steel strainer. For chronic feeding series of vials were prepared with 1.5 g dry *Drosophila* Instant Medium (Carolina Biological Supply Co., Burlington, NC) and 5 ml of the test solutions. Equal batches of 3-day-old larvae were then distributed into the vials where they fed for the remainder of their larval life (~48 h), pupated and hatched as adult flies. With DMA a separate chronic feeding series was performed in which the *Drosophila* Instant Medium was replaced by mashed potato flakes (Stocki; Knorr, Thayngen, Switzerland). In this case 1 g mashed potato flakes was combined with 5 ml of the test solutions. For acute feeding with POC 300 mg microcrystalline cellulose (Merck, Darmstadt, Germany) were mixed with 2 ml of the test solutions in a small beaker. The larvae were then introduced into these beakers at the bottom of plexiglass tubes which had their lower end covered with nylon gauze. The larvae were fed for 6 h with the mutagen/cellulose suspension entering the tubes through the gauze. Afterwards they were washed free of mutagen solution and transferred to vials containing normal Instant Medium prepared with distilled water. There they continued to develop to adulthood. All experiments comprised negative controls prepared with water or the corresponding solvents. The experiments were carried out at 25°C and 65% relative humidity. More details on the feeding techniques may be found in Graf *et al.* (1984) and Graf (1995).

Somatic mutation and recombination test

The hatched flies were stored in 70% ethanol. Each cross produces two types of progeny, i.e. marker-heterozygous (*mwh flr*$^+$/*mwh*$^+$ *flr*^{3}) and balancer-heterozygous (*mwh flr*$^+$/*mwh*$^+$ *TM3*, *Bd*^{S}) flies. The dominant *Bd*^{S} marker allows the wings of these two genotypes to be distinguished. Wings were mounted on slides and examined for spots at 400× magnification. Frequency and size of single and twin spots were recorded. Single spots (mostly *mwh* but rarely also *flr*^{3}) can result from various types of mutational events (deletions, point mutations, specific chromosome aberrations, etc.) or from recombination if mitotic crossing-over takes place between the two marker genes. Twin spots (composed of a *mwh* and a *flr*^{3} area) are produced by mitotic recombination between the proximal marker *flr*^{3} and the centromere of chromosome 3. Only *mwh* single spots can be recovered on the wings of balancer-heterozygous flies. They are all due to mutational events because recombinational events are suppressed in inversion-heterozygous cells with the multiply inverted *TM3* balancer chromosome.

Data evaluation and statistical analysis

The data were evaluated according to the procedure described by Frei and Würgler (1988, 1995). Accordingly, we distinguished small single spots (1–2 cells in size), large single spots (>2 cells) and twin spots, as this makes sense biologically. The frequencies of *mwh* clones (*mwh* single spots and the *mwh* part of twin spots) were used to calculate the clone formation frequencies per 10^5 cells per cell generation (see also Frei *et al.*, 1992; Frei and Würgler, 1995). Dose dependence of clone formation frequency was determined in linear regressions for the marker-heterozygous and balancer-heterozygous genotypes. The differences between the slopes of the two regressions provided a measure of the relative recombinagenic activity of the genotoxic test compounds.

Results

All four compounds were tested in two independent experiments. The data were pooled after verifying that there were no significant differences between repetitions. A negative control (water or solvents) was included in all experiments. Table I summarizes the results. The spot data for small single spots, large single spots and twin spots together with the total number of spots are given for both marker-heterozygous and

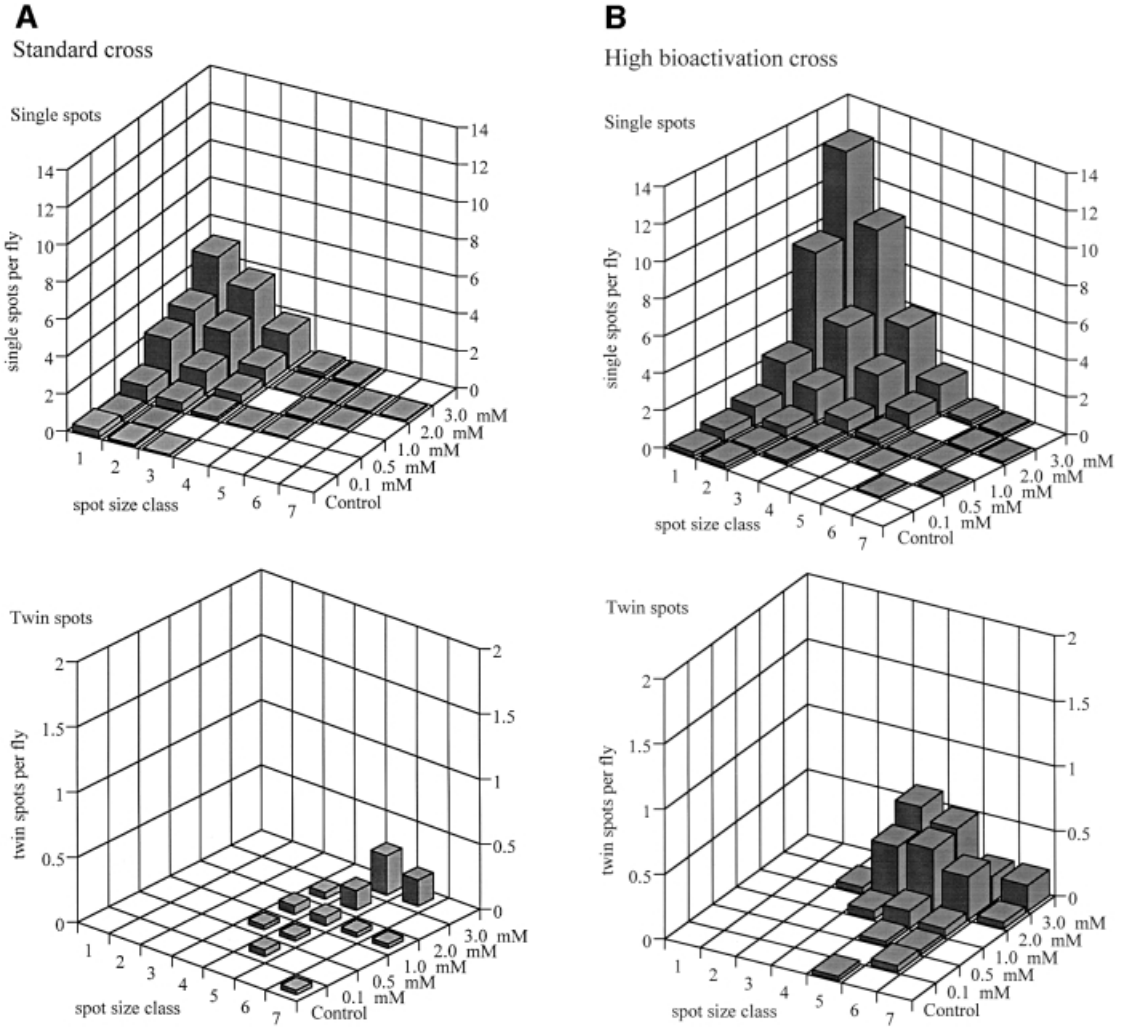

Fig. 2. Size distributions for single and twin spots after chronic treatments with different concentrations of cyclophosphamide. (**A**) ST cross; (**B**) HB cross.

balancer-heterozygous individuals. For the statistical evaluation the results were compared with the corresponding controls.

POC was tested with two different exposure methods: chronic feeding (48 h) and acute feeding (6 h). The ST cross as well as the HB cross were used in parallel with both exposure methods so that the larvae derived from both crosses were treated under identical conditions. Four (0.5–5.0 mM) and five (5.0–100.0 mM) different concentrations were tested with chronic feeding and acute feeding, respectively. The highest concentrations in both treatments were toxic, reducing the numbers of surviving flies (see Table I). Only a few marker-heterozygous and no balancer-heterozygous flies were obtained in the HB cross at the highest exposure with 5.0 mM POC for 48 h. Chronic treatments with POC led to very high frequencies of spots in both types of wings in both crosses. In contrast, acute feeding induced spots only in the marker-heterozygous wings of the two crosses, whereas in the balancer-heterozygous wings the results were inconclusive.

CPH, DAB and DMA were studied in chronic feeding experiments. Six different concentrations of CPH (0.1–5.0 mM) were tested in both crosses. The highest concentration (5.0 mM) was toxic: only a few flies survived in the ST cross, while none survived in the HB cross. This cytostatic drug produced dose-related genotoxic effects in both types of wings and in both crosses.

The size distributions for single spots and twin spots recorded on marker-heterozygous wings after chronic treatment of larvae with 0.1–3.0 mM CPH are shown in Figure 2A and B for the two crosses. The figures show that the size distributions for single spots and twin spots recorded on marker-heterozygous wings after feeding with CPH are completely different. There are very few twin spots, particularly in the small size classes. As has been shown previously (Szabad *et al.*, 1983; Graf *et al.*, 1984; Würgler and Vogel, 1986; Graf, 1995), a lack of twin spots in classes 2 and 3 (2–4 cells in size) is basically due to a property of the *flr* marker, which is not expressed in small clones. Although in principle twin spots are exclusively due to recombination, their frequency of recovery would be inadequate to determine the recombinagenic activity of a compound, because small twin spots cannot be unambiguously identified, being confused with small *mwh* single spots. As outlined elsewhere, an appropriate determination should be based on the frequency of *mwh* clones of single and twin spots induced in marker-heterozygous and balancer-heterozygous wing primordia (Frei *et al.*, 1992; Graf *et al.*, 1992b; Frei and Würgler, 1995).

Four different concentrations of DAB (0.5–10.0 mM) were tested with larvae of the ST cross. The low concentrations gave negative results and the highest one (10.0 mM) an inconclusive result. Because no positive effects were obtained

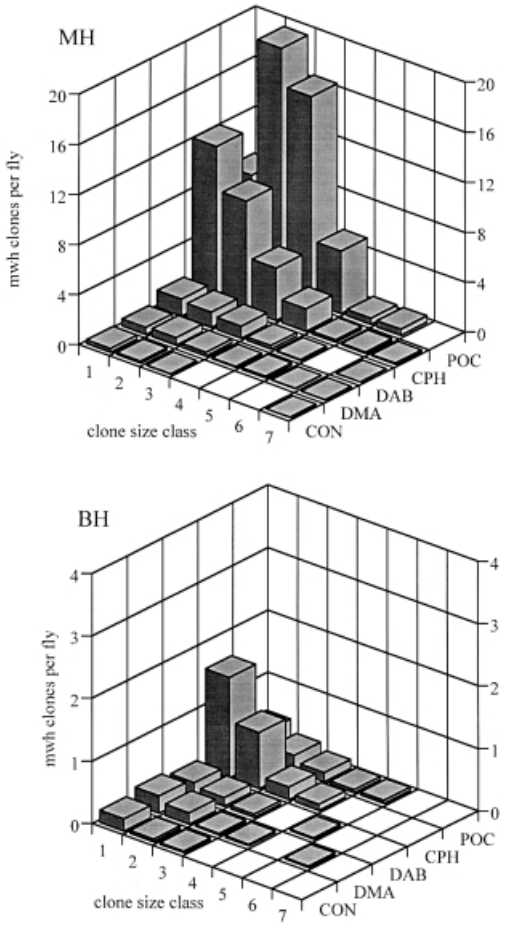

Fig. 3. Size distributions for *mwh* clones on marker-heterozygous (MH) and balancer-heterozygous (BH) wings after chronic treatment of larvae of the HB cross with the highest concentration of each compound.

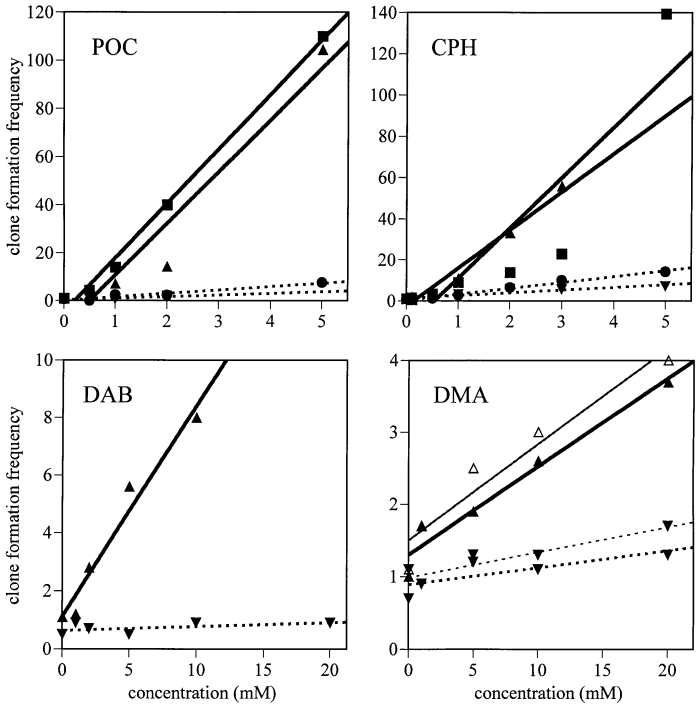

Fig. 4. Linear extrapolation of dose–response relationships obtained with the four compounds. Squares, ST cross, marker-heterozygous wings; circles, ST cross, balancer-heterozygous wings; triangles, HB cross, marker-heterozygous wings; inverted triangles, HB cross, balancer-heterozygous wings; closed symbols, Instant Medium; open symbols, mashed potato medium.

for this compound in the marker-heterozygous wings of the ST cross the corresponding balancer-heterozygous wings were not analysed. Five different concentrations of DAB (1.0–20.0 mM) were tested with larvae of the HB cross. The lowest concentration (1.0 mM) gave a negative result, while the next three concentrations (2.0, 5.0 and 10.0 mM) were clearly genotoxic in the marker-heterozygous wings with positive results for all categories of spots. The highest concentration (20.0 mM) was very toxic for the marker-heterozygous larvae and no flies survived. A very small number of balancer-heterozygous flies survived this highest concentration, but in the balancer-heterozygous wings all treatments led to inconclusive results.

DMA was tested at four concentrations (1.0–20.0 mM) with larvae of the HB cross only but with two different culture media (Instant Medium and mashed potato flakes). The two sets of results are very similar. DMA was weakly but significantly genotoxic in marker-heterozygous flies. The highest concentration also gave a positive result in balancer-heterozygous flies.

Table I also gives the sizes of the *mwh* clones recorded in single and twin spots on both types of wings for all four compounds. The sizes of the *mwh* clones are given as mean size class, whereby class 1 represents the smallest clone size possible, i.e. 1 cell, class 2 corresponds to 2 cells, class 3 to 3–4 cells, class 4 to 5–8 cells, etc. Hence, the size class represents the number of cell division cycles that occurred between the time of induction of the clone in the larval imaginal disk cells and the beginning of differentiation of the wing. It is evident that the *mwh* clones are always smaller in the balancer-heterozygous wings than in the corresponding marker-heterozygous wings. This difference in the average *mwh* clone size is illustrated in Figure 3 by the clone size distributions obtained with the highest concentrations of the four compounds. As suggested in earlier studies, many of the small clones recovered in balancer-heterozygous flies may represent cases of induced segmental aneuploidy with reduced proliferation capacity (Frei *et al*., 1992; Frei and Würgler, 1996).

Based on the frequencies of the *mwh* clones, the clone formation frequencies per cell and cell division without and with correction for control frequencies were calculated, as given in Table I. These clone formation frequencies can then be used to determine the recombinagenic activity of the four compounds. The linear regressions calculated represent first approximations to the corresponding dose–response curves, as shown in Figure 4. Based on the slopes of the regression lines, the percentage of recombinational events among total genotoxic activity was estimated for each compound, as shown in Table II. These estimations are relatively crude: a linear approximation for the dose–response curves was used, but this is not necessarily a true model and may not always be fulfilled. Keeping these limitations in mind, it appears that the percentages of recombination determined for each compound are quite similar in the ST and HB crosses. It is evident that there is a high contribution of recombination to total wing spot induction for all compounds tested (between 83 and 99% recombination).

Table II. Linear extrapolation of the dose–response relationships for *mwh* clone formation frequencies per 10^5 cells per cell division and percentage of recombinational events

Cross[a]	Marker-heterozygous[b]			Balancer-heterozygous[b]			Recombination[c] (%)
	A_1	B_1	r^2	A_2	B_2	r^2	
Potassium chromate							
ST	–4.76	22.69	0.99	0.24	1.45	0.93	93.6
HB	–10.73	21.51	0.93	0.40	0.69	0.90	96.8
Cyclophosphamide							
ST	–13.29	24.40	0.79	0.50	2.89	0.99	88.2
HB	–2.58	18.52	0.97	1.42	1.34	0.88	92.8
p-Dimethylaminoazobenzene							
HB	1.12	0.73	0.96	0.64	0.01	0.28	98.6
9,10-Dimethylanthracene							
HB (Instant Medium)	1.30	0.12	0.96	0.89	0.02	0.54	83.3
HB (mashed potato flakes)	1.50	0.13	0.94	0.99	0.03	0.91	76.9

[a]ST, standard cross; HB, high bioactivation cross.
[b]A_1 and A_2, *y*-axis intercept; B_1 and B_2, slopes; r^2, coefficient of determination.
[c]Percentage of recombinational events $= (B_1 - B_2)/B_1$.

Discussion

Genotoxicity

The chronic treatments (48 h) with the direct acting agent POC and the promutagen CPH revealed that these compounds are highly genotoxic in the ST and HB crosses, with a clear-cut dose–response effect for all spot types distinguished. In contrast, the promutagen DAB was negative in the ST cross, but significantly genotoxic in the HB cross, inducing both single and twin spots. The promutagen DMA was tested in the HB cross only. It showed a rather weak genotoxicity with both types of medium, but induced all types of spots at significant frequencies. The frequency of spots induced in marker-heterozygous individuals in relation to the molar concentration of the genotoxins gives a ranking order for their respective genotoxic effectiveness, POC $\approx$ CPH $>$ DAB $>$ DMA.

With POC larvae of the same age were also given an acute treatment of 6 h at higher concentrations. In this set of experiments the induction of small and large single spots was frequently statistically inconclusive in both crosses, but, nevertheless, several positive results were obtained for these spot categories. On the other hand, clear positive responses were obtained throughout for induction of twin spots in both crosses.

The two types of exposure for POC allow a comparison of the effects of chronic and acute feeding. From Table I it is obvious that the chronic feeding method was more efficient than the acute one: much higher frequencies of spots were induced and at lower concentrations of POC. Furthermore, the spot frequencies achieved with increasing concentrations of POC in the acute feedings seemed to level off at ~1.0 spot per wing. It is a well-known phenomenon that in acute feeding experiments the larvae may stop feeding prematurely if the solutions of the test compounds are very concentrated and therefore not palatable. Consequently, they resume feeding only after transfer to drug-free medium. The present data demonstrate once more that acute feeding protocols are not advisable for routine genotoxicity screening of chemical compounds or mixtures. Acute treatments may be successful in a sufficiently reproducible manner only if very potent genotoxins, such as the oxidizing agent POC, or other powerful direct acting alkylating agents are tested (Graf, 1995; Rodriguez-Arnaiz *et al*., 1996).

Theoretically, different spot size distributions are expected after chronic and acute exposures (Graf, 1995). In line with Graf *et al*. (1984), chronic treatment for 48 h results in a distribution in which small spots predominate and the larger spots are present at decreasing frequency with increasing size, as shown in Figure 2 for CPH. The strong genotoxin POC showed a somewhat different spot size distribution (see Figure 3). The low number of the smallest class of spots (1 cell) observed with POC in chronic feedings may be due to rapid turnover and clearance of the compound in the individuals after pupation. Similar effects have been obtained with other compounds that show very high genotoxic activity, such as 1-((5-nitrofurfurylidene)amino)adamantane (Moraga and Graf, 1989), methyl methanesulfonate and mitomycin C (Rodriguez-Arnaiz *et al*., 1996).

In previous studies CPH was tested in the ST cross by chronic exposures of different durations up to the maximum tolerated concentration (Graf *et al*., 1989). A 48 h feeding interval was obviously optimal for this compound. The present experiments with CPH aimed at a quantitative evaluation of its genotoxicity in the ST and HB crosses. CPH was found to be equally potent for dose-related spot induction in the marker-heterozygous individuals of both crosses. However, the highest concentration was very toxic to larvae of the ST cross, reducing the number of flies available for analysis. The same concentration was lethal for larvae of the HB cross, which are apparently more sensitive to the toxic action of this promutagen. Maybe, as a consequence of this difference in toxicity of CPH in the two crosses, the shapes of the dose–response curves for clone formation are different (Figure 4). The high toxicity observed in larvae of the HB cross is most probably due to the constitutively high levels of cytochromes P450, especially CYP6A2, in these individuals. This may lead to increased production of toxic metabolites (Saner *et al*., 1996). In conclusion, it appears that not only larvae derived from the HB cross but also those from the ST cross have sufficient bioactivation capacity to produce the genotoxic metabolites of CPH that lead to the high genotoxic effects.

CPH has been tested for genotoxicity in transgenic larvae

expressing the mammalian cytochrome P450 CYP2B1 using the wing spot test and an injection protocol (Jowett *et al*., 1991). The transgenic larvae were hypersensitive to CPH compared with control larvae. However, the frequencies of total spots observed in these experiments were much lower than those obtained with either cross in our experiments with chronic feeding of larvae. The injection protocol may have its limits, not only because injection is a relatively elaborate technique, but also because exposure of the target cells is acute rather than protracted. Particularly important for indirectly acting compounds, it may also have reduced sensitivity because it bypasses a most relevant compartment for biotransformation, namely the epithelial cells of the gut.

The promutagen DAB proved to be much more toxic to larvae of the ST cross than to those of the HB cross. Furthermore, it was not genotoxic at all in the former, while it was positive in the latter, with a clear dose–response effect. Similar results were obtained in previous experiments (Graf *et al*., 1992a). This result again demonstrates the utility of the HB cross, with increased levels of cytochromes P450, for detection of genotoxic activity of compounds depending on metabolic activation. With respect to results obtained with other *Drosophila* assay systems, we may recall that DAB induced sex-linked recessive lethal mutations in male germ cells (Angus, 1985; Parry and Sinclair, 1985). It was also weakly positive in the *white/white*$^+$ SMART of the eye (Vogel and Nivard, 1993).

DMA has also been tested previously in the *Drosophila* wing spot test, but only with the ST cross (Graf *et al*., 1989). Chronic exposure (48 h) with 5.0 and 10.0 mM DMA produced positive results for all three types of spots. However, the frequencies of spots at the highest concentration were generally no higher than those observed at the lower one. It was concluded that larval metabolic capacity for this procarcinogen was probably already at its limits at the lower concentration. DMA also produced positive results in the *w/w*$^+$ SMART of the eye in experiments with several different *Drosophila* tester strains (Rodriguez-Arnaiz *et al*., 1993). Based on these prior results, the present experiments were performed only with the HB cross. In contrast to the results recorded with the ST cross, a clear dose–response effect was obtained with the HB cross (see Figure 4), which may be attributed to the increased metabolic transformation capacity of this genotype.

In a study that analysed the combined effects of methyl urea and sodium nitrite increased spot frequencies were obtained with mashed potato flakes as compared with *Drosophila* Instant Medium (Guzmán-Rincón and Graf, 1995; Guzmán-Rincón *et al*., 1998). This prompted us to explore the utility of mashed potato flakes in a series of experiments with DMA. The results obtained with *Drosophila* Instant Medium and mashed potato flakes were rather similar, indicating that the composition of the feeding medium does not seem to influence the genotoxic activity of this compound. Mashed potato flakes may therefore be a cheaper alternative to Instant Medium for the testing of genotoxins.

Recombinagenicity

In marker-heterozygous individuals single spots (*mwh* or *flr*) can be produced either by point mutation, certain types of chromosome breakage event (deletion) or by mitotic recombination. However, we cannot tell how much each mechanism contributes to the total of spots recovered. On the other hand, the presence of twin spots proves that mitotic recombination is induced, as these spots result from mitotic crossing-over between the *flr* locus and the centromere. From the significant induction of twin spots recorded on marker-heterozygous wings with the test compounds studied here it can be concluded that they all have recombinagenic activity. In balancer-heterozygous individuals the *flr* marker is absent. The spots that can be recovered are therefore *mwh* single spots. All recombination events are suppressed or eliminated owing to inversion heterozygosity brought about by the presence of the multiply inverted balancer chromosome. As a rule, therefore, the frequencies of *mwh* clones observed on the wings of balancer-heterozygous flies are always lower than those observed on the wings of marker-heterozygous flies (Graf *et al*., 1984; Frei and Würgler, 1996).

After chronic treatments with POC, CPH and DMA it was obvious that the frequencies of *mwh* clones observed on balancer-heterozygous wings were considerably lower than those observed on marker-heterozygous wings. Nevertheless, in balancer-heterozygous wings spot induction by these compounds was statistically significant compared with those of the respective negative controls. Hence, we may conclude that although the large majority of the spots induced by POC, CPH and DMA in the wings of marker-heterozygous flies are due to mitotic recombination, a small but significant number of them have other causes and may be due to point mutation or chromosome breakage (deletion). With DAB no significant spot induction could be demonstrated in balancer-heterozygous flies, in spite of a slight suggestive trend in the dose–response effect. It is possible that this compound is an exclusive or almost exclusive recombinagen. Such compounds do exist, as shown earlier for ellipticine (Frei and Würgler, 1996). Based on the percentages shown in Table II, the ranking order of the compounds with respect to their relative recombinagenic efficiency was DAB > POC > CPH > DMA. Most genotoxins so far studied for recombinagenicity in the wing spot test show values of ~70%, in the range ~50–90%. Thus the values found for DAB (99%), POC (97%), CPH (93%) and DMA (83%) are not unusual, but clearly in the higher range.

It is now well documented that there is a link between the recombinagenic activity of chemical compounds and their carcinogenicity (Cairns, 1981). On account of this and in consideration of all the other advantages offered by rapid somatic assays in *D.melanogaster* it seems a profitable strategy to screen for genotoxic and recombinagenic activity of compounds in the first instance with this type of assay. On comparing the sensitivities of different somatic assays in *Drosophila*, such as genetic instabilities in the *zeste-white* or the *white-ivory* eye spot test on the one hand and the wing spot test on the other, it became clear that these somatic test systems are not equivalent with respect to the spectra of genotoxic agents they are able to detect (Batiste-Alentorn *et al*., 1995; Graf and Würgler, 1996). The SMART approach appears to be the most potent and, moreover, the wing spot test represents a rapid and inexpensive test method that allows quantitative determination of both the genotoxic and recombinagenic activities of chemical compounds or complex mixtures (Magnusson and Ramel, 1990; Frei *et al*., 1992; Graf *et al*., 1992b; Marec and Gelbic, 1994; Guzmán-Rincón and Graf, 1995; Frei and Würgler, 1996; Graf and Würgler, 1996; Rodriguez-Arnaiz *et al*., 1996; González-César and Ramos-Morales, 1997; Vogel *et al*., 1999).

General conclusions

In summary, all four compounds tested are genotoxins showing clear-cut dose–response effects, but they differ in genotoxic effectiveness. In addition, they are characterized by relatively high proportions of recombinagenic activity (83–99%). It seems likely, therefore, that they produce primary damage in DNA that is to a considerable extent further processed by recombinational DNA repair pathways. The present and previous reports show that *D.melanogaster* offers a very valuable possibility to combine eukaryotic *in vivo* genotoxicity testing with a versatile metabolic capacity for xenobiotics and that the wing SMART method is an efficient and quick procedure for quantitative determination of the recombinagenic potential of genotoxic agents.

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