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GENOTOXICITY TESTING OF PROMUTAGENS IN THE WING SOMATIC MUTATION AND RECOMBINATION TEST IN *Drosophila melanogaster*

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ABSTRACT

The wing Somatic Mutation and Recombination Test (SMART) in *Drosophila melanogaster* has been used to analyse five different promutagens for genotoxicity using the standard cross and the high bioactivation cross. Benz(a)anthracene and N-nitrosodiphenylamine were weakly positive in the standard cross both after 48 h and 72 h chronic feeding. However, the dose-response relationship showed a plateau. Of the two naphthylamines, the carcinogen 2-naphthylamine was also weakly positive in the standard cross at the highest concentration tested. The carcinogen N-nitrosopyrrolidine was tested in the standard cross using both 48 h and 72 h chronic feeding as well as 6 h acute feeding. It proved to be highly mutagenic and recombinagenic with a clear-cut dose-response. The genotoxicity of this promutagen can be enhanced by a factor of two to four when the high bioactivation cross is used or when the larvae are pretreated for 24 h with phenobarbital. These results demonstrate the sensitivity and versatility of the Drosophila wing spot test for the evaluation of genotoxic activity of promutagens and procarcinogens.

RESUMEN

La Prueba de Mutación y Recombinación Somáticas (SMART) en el ala de *Drosophila melanogaster* ha sido empleada para verificar la actividad genotóxica de cinco diferentes promutágenos usando las cruzas estándar y de alta bioactivación. Benzo(a)antraceno y N-nitrosodifenilamina fueron débilmente positivos en la cruza estándar con ambos tratamientos de 48 h y 72 h; sin embargo la relación de dosis-respuesta se expresó en forma de meseta. De las dos naftilaminas, el carcinógeno 2-naftilamina fue también débilmente positivo en la cruza estándar con la concentración más alta empleada. El carcinógeno N-nitrosopirrolidina fue analizado en la cruza estándar con 48 h y 72 h de tratamiento crónico y con 6 h de tratamiento agudo; este compuesto se manifestó altamente mutagénico y recombinagénico en una curva de dosis-respuesta muy clara. La genotoxicidad de este promutágeno se incrementa de dos a cuatro veces cuando se usa la cruza de alta bioactivación o cuando las larvas se pretratan por 24 h con fenobarbital. Los resultados obtenidos mostraron la alta sensibilidad y la versatilidad de la prueba de manchas en el ala de Drosophila para la evaluación de la actividad genotóxica de promutágenos y procarcinógenos.

INTRODUCTION

The fruit fly *Drosophila melanogaster* is no doubt an ideal organism for the screening of chemical compounds for mutagenic activity. This eukaryote offers the advantages of having a short generation time (approx. 10 days at 25°C), needing very inexpensive culture media and allowing the breeding of large numbers of animals using simple facilities (for practical details, see Graf *et al.* 1992b). A series of well-defined mutagenicity tests have been developed which are able to measure the whole spectrum of genetic end points in germ cells as well as in somatic cells (Würgler *et al.* 1984). Furthermore, it is well documented that Drosophila possesses an effi-

cient system for the metabolism of xenobiotics (Baars 1980, Hällström *et al.* 1984, Vogel 1980). A large number of chemicals, including both carcinogens and noncarcinogens, have been screened for mutagenicity in *D. melanogaster* using mainly the well-established test for sex-linked recessive lethals (Lee *et al.* 1983). However, this germ cell test system has the disadvantage that for weak mutagens or nonmutagens large numbers of chromosomes need to be tested. In addition, this test takes at least two fly generations (i.e. approx. 1 month) to complete an experiment. For those reasons, the use of somatic cells rather than germ cells for mutagenicity testing in *D. melanogaster* has been proposed (for more details, see Vogel 1987).

The somatic cells of *D. melanogaster* offer the possibility to develop very fast and flexible short-term assays which are able to detect a wide spectrum of genotoxic agents. The use of trans-heterozygous recessive cell markers permits extension of the spectrum of genetic end points scored: in addition to the types of mutation recorded in the sex-linked recessive lethal assay (point mutations, deletions, and certain types of chromosome aberrations), the somatic tests can detect mitotic recombination and gene conversion as well (Graf *et al.* 1984, Vogel *et al.* 1980, Vogel and Zijlstra 1987). The characteristics and validation status of the three most widely used somatic assays in *D. melanogaster* have been reviewed by Würgler and Vogel (1986). The wing Somatic Mutation and Recombination Test (SMART) using larvae trans-heterozygous for the third chromosome markers *mwh* (multiple wing hairs) and *flr*^{3} (flare-3) has proved to be an efficient assay for genotoxicity screening (Alonso Moraga and Graf 1989, Frei *et al.* 1992, Graf *et al.* 1983, 1984, 1989, 1992a,c, van Schaik and Graf 1991). The standard cross for producing the trans-heterozygous larvae makes use of the *mwh* and the *flr*^{3}/*TM3, Ser* strains. In an attempt to improve the wing spot test for the detection of promutagens activated via cytochrome P450-dependent metabolism, Frölich and Würgler (1989) constructed two new tester strains. These strains carry chromosomes 1 and 2 from a DDT-resistant Oregon R(R) line (Dapkus and Merell 1977) which is characterized by a high constitutive level of cytochrome P450 (Hällström and Blanck 1985). A number of promutagens show increased genotoxicity when the high bioactivation cross (*ORR*; *mwh* females and *ORR*; *flr*^{3} males) is used, compared to the standard cross (*mwh* females and *flr*^{3} males) (Frölich and Würgler 1989, 1990a,b, Graf and van Schaik 1992).

The wing spot test offers the possibility to use a number of different routes of application of the test compound, namely feeding, inhalation or injection (for more details, see Graf *et al.* 1984, 1989). The standard treatment is chronic feeding of 3-day-old larvae for the rest of the larval development (approx. 48 h). In special cases it is also possible to start the feeding period already at a larval age of 2 days (corresponding to a feeding period of 72 h). Acute feeding of 3-day-old larvae for up to 6 h can be used as well with powerful genotoxic agents. With all these feeding protocols dose-response relationships can be studied by using a range of different concentrations of the test compound.

To contribute further to the validation of the wing spot test, we present here the results obtained with five promutagens requiring metabolic activation. The polycyclic aromatic hydrocarbon benz(a)anthracene and the nitroso compund N-nitrosodiphenylamine were tested in larvae derived from the standard cross with chronic feeding for 48 h and 72 h. The two structurally related aromatic amines 1-naphthylamine and 2-naphthylamine were analyzed in larvae of the standard cross with 48 h chronic feeding alone. The very potent mutagen and carcinogen N-nitrosopyrrolidine was tested with all three feeding protocols in the standard cross. In addition, it was also assayed in the high bioactivation cross. Furthermore, an attempt was made to increase the sensitivity of the larvae of the standard cross for this promutagen by pretreating them for 24 h with the enzyme-inducing agent phenobarbital (Zijlstra *et al.* 1984).

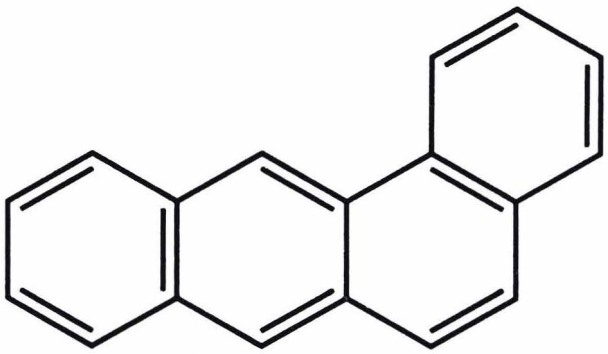
MATERIALS AND METHODS

Chemical compounds

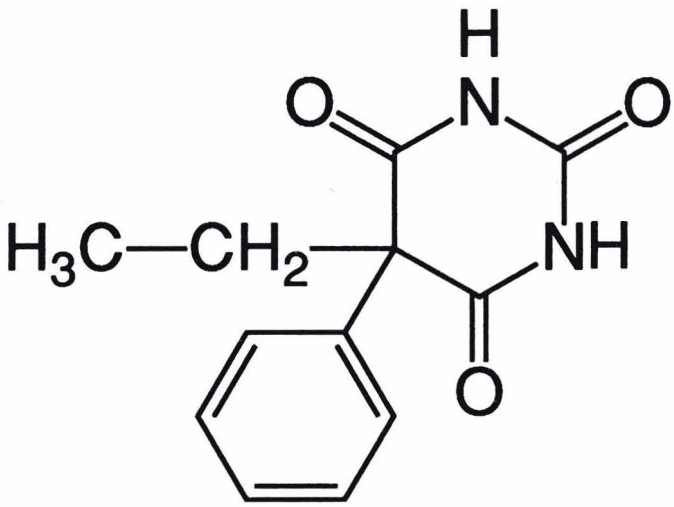
Benz(a)anthracene (BA, CAS Registry Number 56-55-3), 1-naphthylamine (1-NA, 134-32-7), N-nitrosodiphenylamine (NDA, 86-30-6), N-nitrosopyrrolidine (NNP, 930-55-2), and phenobarbital (PB, 50-06-6) were purchased from Fluka, Buchs, Switzerland; 2-naphthylamine (2-NA, 92-59-8) was from Sigma, Deisenhof, FRG. All six compounds were dissolved in a mixture of 1% Tween-80 (Serva, Heidelberg, FRG) plus 3% ethanol (Merck, Darmstadt, FRG) in distilled water. The chemical structures of the test compounds are shown in figure 1.

Larval feeding

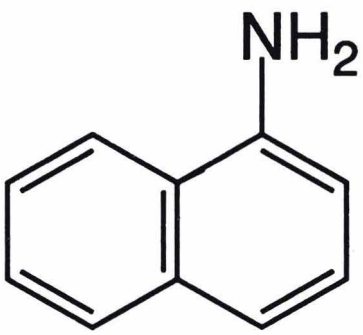
For the three different crosses (see below) the females were collected as virgins and aged in bottles contai-



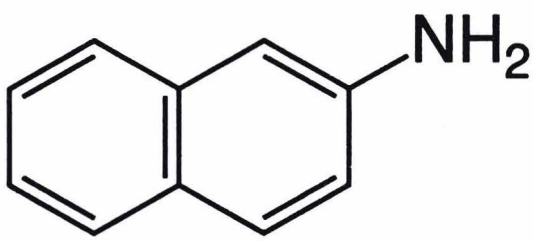
Benz(a)anthracene



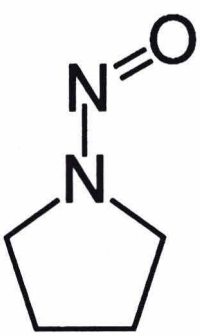
Phenobarbital



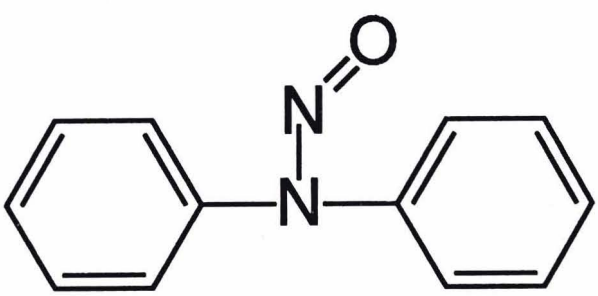
1-Naphthylamine



2-Naphthylamine



N-Nitrosopyrrolidine



N-Nitrosodiphenylamine

Fig. 1. Structural formulas of the test compounds

ning standard medium and fresh yeast for 2 to 3 days; males were collected and kept in isolation for 1 day in the same type of bottles. On day 3 the flies were crossed in standard culture bottles (approx. 80 females and 80 males per bottle). One day later, eggs were collected from these flies by shaking them without anesthesia into special egg collection bottles. These bottles contain a solid agar base (4% [w/v] agar-agar in water) which is covered completely with an approx. 5 mm layer of fermenting fresh baker's yeast supplemented with sucrose (Graf *et al.* 1991, Magnusson and Ramel 1990). These egg collection bottles were then kept undisturbed in the dark for 8 h. After removing the flies from these bottles, they were stored again in standard culture bottles and used several times more for the collection of eggs. Three (or two) days after egg collection, the larvae were washed out of the bottles with tap water (at room temperature!) through a fine-meshed stainless steel strainer. They were thoroughly washed free of yeast with tap water while still in the strainer. This procedure eliminates the use of a separation funnel and of sodium chloride solution. The larvae were then used for chronic or acute feedings.

For the chronic treatments, the larvae were transferred in equal batches from the strainer into plastic vials with the help of a small spatula. The vials contained Drosophila Instant Medium (Formula 4-24, Carolina Biological Supply Co., Burlington, NC, USA) prepared with the solutions of the test compounds. 5 ml of the solutions of the various compounds were always added to 1.5 g of dry Instant Medium. Negative solvent controls were included. A small piece of folded filter paper was added to each vial to absorb excess humidity and to increase space for pupation of the larvae. The larvae were fed on this Instant Medium for the rest of their development (approx. 48 h in the case of 3-day-old larvae, approx. 72 h in the case of 2-day-old larvae).

For the acute feedings, the 3-day-old larvae were introduced in batches into plexiglass tubes which have the lower end covered with nylon gauze. The larvae on the gauze were then immersed in a mixture of 1.5 ml test solution and 300 mg powdered cellulose (Merck, Darmstadt, FRG) contained in a 100 ml beaker. The larvae feed on the mutagen-cellulose suspension through the gauze. After 6 h they were lifted out of the solution, washed clean with tap water and transferred directly with 5 ml distilled water into plastic vials containing 1.5 g dry Instant Medium where they continued their development.

The culturing of the flies, the collection of the eggs as well as the treatments of the larvae were carried out at a temperature of 25°C (± 0.5°C) and at a relative humidity of approx. 65%. The maintaining of a constant culture temperature is important in order to obtain reproducible treatment conditions and because the expression of the *mwh* marker is affected by temperature fluctuations (Graf 1986). A high humidity allows production of large numbers of eggs from the females in only 8 h; it also avoids desiccation of the treatment vials.

Somatic mutation and recombination test

The following three crosses of flies carrying the markers *mwh* (3-0.3) and *flr*^{3} (3-38.8) on the left arm of chromosome 3 were set up:

1) Standard cross: *flr*^{3}/*In(3LR)TM3, ri pp sep bx*^{34e} *e*^{s} *Ser* females mated to *mwh* males. This is the reciprocal cross of the standard cross used previously (Alonso Moraga and Graf 1989, Graf *et al.* 1989, van Schaik and Graf 1991).

2) High bioactivation (HB) cross: *ORR*; *flr*^{3}/*TM3* females crossed with *ORR*; *mwh* males. This is the reciprocal cross of the one described by Frölich and Würgler (1989).

3) Improved HB cross: *ORR*; *flr*^{3}/*TM3* females and *mwh* males (Graf and van Schaik 1992).

Detailed information on the genetic markers as well as the balancer chromosome is found in Lindsley and Grell (1968) or in Lindsley and Zimm (1985, 1990). Experience has shown that *mwh* females are much less fertile than *flr*^{3}/*TM3* females. The use of the reciprocal crosses of those previously used facilitates collection of ample numbers of larvae.

The surviving flies were collected from the treatment vials on days 10 to 12 after egg laying and stored in 70% ethanol. In all three crosses the trans-heterozygous (*mwh flr*$^+$/*mwh*$^+$ *flr*^{3}) progeny can be distinguished from the inversion-heterozygous progeny based on the *Ser* marker. The wings were mounted in Faure's solution (gum arabic 30 g, glycerol 20 ml, chloral hydrate 50 g and water 50 ml). Both surfaces of the wings were scored under a compound microscope at 400x magnification for the ocurrence of spots. In each case, the size of a spot was determined by counting the number of wing cells exhibiting the *mwh* or *flr*^{3} phenotype. Three ca-

tegories of spots were recorded separately: 1) *mwh* single spots, 2) *flr*^{3} single spots, and 3) twin spots showing adjacent *mwh* and *flr*^{3} areas. Single spots are produced either by somatic gene mutation, deletion or other change at one or the other marker locus or by mitotic recombination occurring between the two markers; twin spots are produced exclusively by mitotic recombination occurring between the proximal marker *flr*^{3} and the centromere. A more detailed description of the wing spot test is given by Graf *et al.* (1984).

Data evaluation and statistical analysis

The wing spot data were evaluated with the computer program SMART (Würgler, unpublished). For the statistical analysis, the spots were grouped according to the following three types: 1) small single spots of 1 or 2 cells in size (*mwh* or *flr*^{3}), 2) large single spots of 3 or more cells (*mwh* or *flr*^{3}), and 3) twin spots with an *mwh* and a *flr*^{3} area. These three types of spots were 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 small single spots (with the highest spontaneous frequency) a multiplication factor of 2 was used; for the large single spots and the twin spots (with a low spontaneous frequency) a multiplication factor of 5 was chosen. For the calculations the Kastenbaum-Bowman test was used with $P = 0.05$.

For the chronic treatments, an induction frequency per 10^5 cells can be calculated based on the number of clones showing the *mwh* phenotype (i.e. *mwh* single spots and the *mwh* part of twin spots). This frequency is obtained as the number of *mwh* clones divided by the number of wings analyzed divided by 24,400 (which is the number of cells contained in the area of the wing that is inspected for the presence of spots). More details on these calculations can be found in Frei *et al.* (1992).

RESULTS AND DISCUSSION

The data collected in the wing spot test in *Drosophila melanogaster* with the four compounds BA, 1-NA, 2-NA, and NDA using the standard cross are presented in table I. A total of 2,462 wings were analyzed. The frequencies of spontaneous spots recorded in the two solvent controls (1% Tween-80 plus 3% ethanol) with 48 h and 72 h feedings are in the normal range as observed previously for the standard cross (Alonso Moraga and Graf 1989, Graf *et al.* 1989, 1992a,c). The two compounds BA and NDA were tested in three independent experiments with 48 h and 72 h chronic feedings. BA proved to be equally toxic to the larvae with both types of treatment; the highest concentration tested is 100 mM. In the 48 h series BA is positive for the frequency of total spots per wing only at the highest concentration tested. With the longer treatment of 72 h it is also positive at 75 mM. From this result one can conclude that under conditions of low toxicity a feeding period longer than the standard treatment for 48 h may be advantageous. This allows more time for the metabolism of the xenobiotic to be induced and to be active. The frequencies of total spots per wing induced by the treatments with BA are rather low and show no clear dose-response relationship. As shown by Frölich and Würgler (1990a), the genotoxicity of BA can be enhanced by a factor of 2 to 3 when the high bioactivation cross is used.

In contrast to BA, NDA was more toxic with the longer treatment period: With 48 h feeding 200 mM could be assayed, with 72 h feeding the highest concentration tested was 100 mM. However, in all the six separate treatment series this compound induced significantly increased frequencies of spots, predominantly of small single spots. The twin spots were positive with both treatments at the highest concentration tested. This indicates that this nitroso compound has not only mutagenic but also recombinagenic activity in somatic cells of Drosophila. The frequencies of total spots show a plateau at a level of approximately 0.5 spots/wing. This may be a reflection of the limited bioactivation capacity of the larvae of the standard cross for this type of compound. The same phenomenon has been observed previously with polycyclic aromatic hydrocarbons (Frölich and Würgler 1990a).

The two naphthylamines were tested in two separate experiments. These compounds are considerably more toxic than the previous two. The concentrations assayed were 3.5 and 7.0 mM. The noncarcinogen 1-NA is clearly negative at both concentrations for all categories of spots. The carcinogen 2-NA produced a significantly higher frequency of total spots at the higher concentration. Again, this frequency is rather low, most probably due to limited bioactivation capacity for this type of compound in the larvae of the standard cross. In fact, Vogel *et al.* (1991) have demonstrated that the genotoxicity of this aromatic amine in eye somatic cells is strongly dependent on the genotype of the larvae used for treatment: In the w/w^+ assay, 2.0 mM 2-NA produced significantly increased frequencies of mosaic

TABLE I. SUMMARY OF RESULTS OBTAINED IN THE DROSOPHILA WING SPOT TEST WITH FOUR DIFFERENT COMPOUNDS USING THE STANDARD CROSS

Compound Conc. (mM)	Number of wings	Spots per Wing (Number of Spots) Stat. Diagn.* Small single spots (1—2 cells) m = 2.0	Large single spots (>2 cells) m = 5.0	Twin spots m = 5.0	Total spots m = 2.0	Spots with mwh clone	Mean clone size class	Frequency of clone formation per 10(5) cells** observed	control corrected
48 h Chronic Feeding									
Control	(1% Tween-80 + 3% ethanol)								
0	478	0.23 (110)	0.05 (26)	0.01 (7)	0.30 (143)	141	1.89	1.2	
Benz(a) anthracene									
50	120	0.24 (29)—	0.05 (6)—	0.04 (5)i	0.33 (40)—	40	2.17	1.4	0.2
75	120	0.26 (31)—	0.08 (9)—	0.04 (5)i	0.38 (45)—	44	2.05	1.5	0.3
100	120	0.36 (43)+	0.06 (7)—	0.08 (9)+	0.49 (59)+	59	1.98	2.0	0.8
1-Naphthylamine									
3.5	68	0.12 (8)—	0.01 (1)—	0.00 (0)—	0.13 (9)—	9	1.78	0.5	—0.7
7.0	160	0.14 (23)—	0.05 (8)—	0.02 (3)—	0.21 (34)—	31	2.10	0.8	—0.4
2-Naphthylamine									
3.5	72	0.26 (19)—	0.06 (4)—	0.01 (1)i	0.33 (24)—	24	1.79	1.4	0.2
7.0	86	0.30 (26)—	0.08 (7)—	0.06 (5)+	0.44 (38)+	34	2.00	1.6	0.4
N-Nitrosodiphenylamine									
25	160	0.47 (75)+	0.04 (6)—	0.03 (4)i	0.53 (85)+	85	1.54	2.2	1.0
50	160	0.46 (73)+	0.05 (8)—	0.04 (6)i	0.54 (87)+	87	1.91	2.2	1.0
100	160	0.41 (65)+	0.06 (10)—	0.04 (6)i	0.51 (81)+	81	1.90	2.1	0.9
200	160	0.39 (62)+	0.05 (8)—	0.05 (8)+	0.49 (78)+	78	1.82	2.0	0.8
72 h Chronic Feeding									
Control	(1% Tween-80 + 3% ethanol)								
0	120	0.21 (25)	0.02 (2)	0.00 (0)	0.22 (27)	27	1.85	0.9	
Benz(a) anthracene									
75	72	0.28 (20)i	0.08 (6)+	0.01 (1)i	0.38 (27)+	25	1.96	1.4	0.5
100	112	0.27 (30)i	0.06 (7)i	0.03 (3)i	0.36 (40)+	40	1.95	1.5	0.5
N-Nitrosodiphenylamine									
50	164	0.37 (60)+	0.05 (9)i	0.02 (3)i	0.44 (72)+	71	1.70	1.8	0.9
100	130	0.41 (53)+	0.06 (8)i	0.04 (5)+	0.51 (66)+	66	1.70	2.1	1.2

* Statistical diagnoses according to Frei and Würgler (1988) for comparison with corresponding control: + = positive, — = negative, i = inconclusive, m = multiplication factor. Kastenbaum-Bowman test, one-sided. Probability levels: alpha = beta = 0.05

** Frequency of clone formation: mwh clones/wings/24,400 cells (without size correction)

light spots in the strains Hikone-R, Oregon-K and 91-C, whereas this same treatment was negative in the strains Berlin-K, Haag-79 and 91-R.

In contrast to the results discussed so far, the effects of the carcinogen NNP in the wing spot test are of a different order of magnitude (Graf and Singer 1989). The data collected with all three treatment protocols are given in table II. Both with 48 h and 72 h chronic feeding significantly increased frequencies of all three categories of spots are induced in a dose-dependent manner. This compound possesses both mutagenic as well as recombinagenic activity. The highest frequency of spots recorded in the 48 h feeding series is 6.35 (50 mM) whereas it is 4.40 (20 mM) in the 72 h feeding series. With this compound acute feeding for 6 h was performed as well. Due to the good solubility of NNP in 1% Tween-80 plus 3% ethanol, concentrations as high as 750 mM could be tested. These did not induce marked toxicity in the treated larvae with this short treatment period. This type of treatment proved to be effective at all concentrations analyzed. Clear-cut dose-response relationships are found for all three categories of spots. The highest frequency recorded was 7.40 spots/wing. It is evident that this nitroso compound is metabolized very efficiently in the larvae of the stan-

TABLE II. SUMMARY OF RESULTS OBTAINED IN THE DROSOPHILA WING SPOT TEST WITH N-NITROSOPYRROLIDINE USING THE STANDARD CROSS

		*Spots per Wing (Number of Spots) Stat. Diagn.**						*Frequency of clone formation per 10(5) cells***	
Compound Conc. (mM)	*Number of wings*	*Small single spots (1—2 cells) m = 2.0*	*Large single spots (>2 cells) m = 5.0*	*Twin spots m = 5.0*	*Total spots m = 2.0*	*Spots with mwh clone*	*Mean clone size class*	*observed*	*control corrected*
Control		*(1% Tween-80 + 3% ethanol)*							
0	228	0.31 (70)	0.03 (7)	0.02 (5)	0.36 (82)	82	1.84	1.5	
48 h Chronic Feeding									
5	40	0.43 (17)i	0.10 (4)i	0.05 (2)i	0.57 (23)+	22	2.09	2.3	0.8
10	40	0.90 (36)+	0.15 (6)+	0.05 (2)i	1.10 (44)+	43	1.81	4.4	2.9
20	40	2.58 (103)+	0.15 (6)+	0.05 (2)i	2.78 (111)+	110	1.45	11.3	9.8
50	60	5.60 (336)+	0.58 (35)+	0.17 (10)+	6.35 (381)+	375	1.60	25.6	24.2
72 h Chronic Feeding									
5	40	0.70 (28)+	0.08 (3)i	0.08 (3)i	0.85 (34)+	33	2.21	3.4	1.9
10	38	1.34 (51)+	0.13 (5)+	0.08 (3)i	1.55 (59)+	57	1.77	6.2	4.7
20	40	3.10 (124)+	1.00 (40)+	0.30 (12)+	4.40 (176)+	167	2.16	17.1	15.7
6 h Acute Feeding									
20	40	0.55 (22)+	0.10 (4)i	0.13 (5)+	0.77 (31)+	31	2.06		
50	40	0.52 (21)+	0.17 (7)+	0.28 (11)+	0.98 (39)+	39	2.51		
100	40	0.52 (21)+	0.43 (17)+	0.28 (11)+	1.23 (49)+	47	2.87		
200	40	0.90 (36)+	0.65 (26)+	0.28 (11)+	1.83 (73)+	69	2.75		
300	20	1.30 (26)+	1.85 (37)+	0.55 (11)+	3.70 (74)+	64	3.44		
500	20	1.90 (38)+	2.00 (40)+	1.60 (32)+	5.50 (110)+	104	3.39		
750	20	2.30 (46)+	4.00 (80)+	1.10 (22)+	7.40 (148)+	143	3.79		

* Statistical diagnoses according to Frei and Würgler (1988): + = positive, i = inconclusive, m = multiplication factor. Kastenbaum-Bowman test, one-sided. Probability levels: alpha = beta = 0.05

** Frequency of clone formation for chronic treatments: mwh clones/wings/24,400 cells (without size correction)

dard cross leading to reactive metabolites immediately. This is then reflected in the considerably larger size of the *mwh* clones induced: with the chronic treatments the mean size class is aproximately 2 whereas with the acute treatment sizes close to class 4 can be obtained (see Table II). This basic difference in the average size of the spots depending on the type of treatment is best seen in the spot size distributions plotted in figure 2.

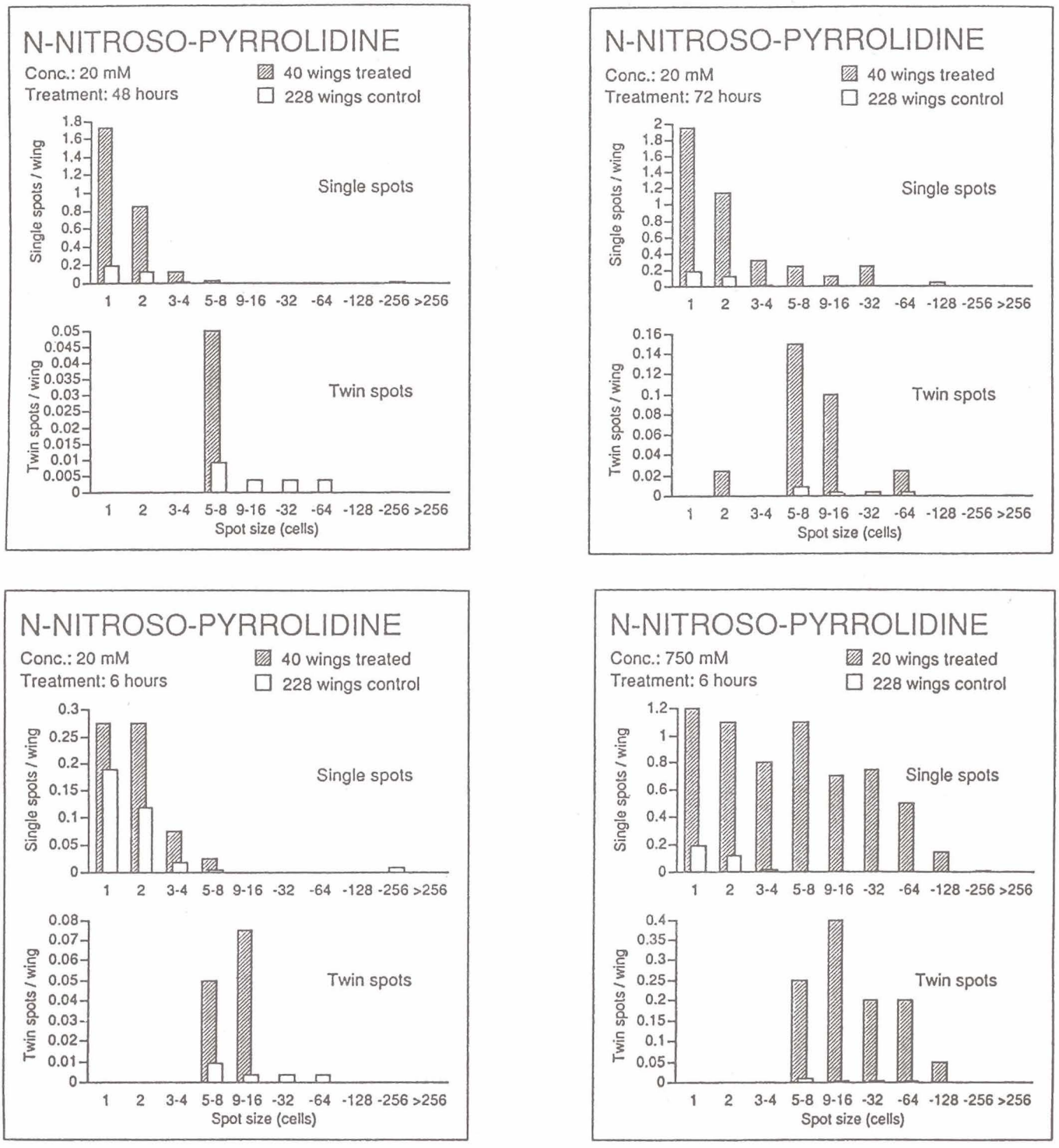


Fig. 2. Spot size distributions for single and twin spots obtained with different feeding protocols with N-nitrosopyrrolidine. Standard cross

TABLE III. SUMMARY OF RESULTS OBTAINED IN THE DROSOPHILA WING SPOT TEST WITH N-NITROSOPYRROLIDINE USING THE TWO HIGH BIOACTIVATION CROSSES

		*Spots per Wing (Number of Spots) Stat. Diagn.**						*Frequency of clone formation per 10(5) cells***	
Compound Conc. (mM)	*Number of wings*	*Small single spots (1—2 cells) m = 2.0*	*Large single spots (>2 cells) m = 5.0*	*Twin spots m = 5.0*	*Total spots m = 2.0*	*Spots with mwh clone*	*Mean clone size class*	*observed*	*control corrected*
A. High Bioactivation Cross									
Control		(1% Tween-80 + 3% ethanol)		(72 h)					
0	160	0.29 (46)	0.03 (4)	0.04 (6)	0.35 (56)	56	1.75	1.4	
48 h	Chronic Feeding								
0.5	42	0.38 (16)i	0.02 (1)i	0.02 (1)—	0.43 (18)—	18	1.67	1.8	0.3
1.0	36	0.28 (10)—	0.17 (6)+	0.11 (4)i	0.56 (20)i	19	2.53	2.2	0.7
5.0	30	1.23 (37)+	0.80 (24)+	0.43 (13)+	2.47 (74)+	68	2.78	9.3	7.9
10.0	20	0.90 (18)+	1.45 (29)+	0.55 (11)+	2.90 (58)+	49	3.12	10.1	8.6
72 h	Chronic Feeding								
0.5	26	0.27 (7)—	0.15 (4)+	0.00 (0)—	0.42 (11)i	10	2.00	1.6	0.1
1.0	58	0.38 (22)i	0.41 (24)+	0.09 (5)i	0.88 (51)+	46	3.37	3.3	1.8
5.0	36	1.06 (38)+	1.44 (52)+	0.22 (8)+	2.72 (98)+	92	3.70	10.5	9.0
B. Improved High Bioactivation Cross									
Control		(1% Tween-80 + 3% ethanol)		(48 h)					
0	80	0.28 (22)	0.03 (2)	0.03 (2)	0.32 (26)	26	2.46	1.3	
48 h	Chronic Feeding								
5.0	40	1.25 (50)+	1.25 (50)+	0.40 (16)+	2.90 (116)+	98	3.01	10.1	8.7
10.0	44	2.07 (91)+	1.86 (82)+	0.95 (42)+	4.89 (215)+	197	3.03	18.4	17.0

* Statistical diagnoses according to Frei and Würgler (1988): + = positive, — = negative, i = inconclusive, m = multiplication factor. Kastenbaum-Bowman test, one-sided. Probability levels: alpha = beta = 0.05

** Frequency of clone formation: mwh clones/wings/24,400 cells (without size correction)

NNP was also tested in the high bioactivation cross with 48 h and 72 h treatments (see Table III). The larvae of this cross are considerably more sensitive to the toxic action of this promutagen. The highest concentrations assayed were 10 mM (48 h feeding) and 5.0 mM (72 h feeding), respectively. The frequencies of total spots per wing of 2.90 and 2.72, respectively, induced at these concentrations are approximately threefold higher with the larvae of the high bioactivation cross than with the larvae of the standard cross. Furthermore, in the former type of larvae the average size of the *mwh* clones induced is considerably larger as well than in the latter type. These effects demonstrate that the increased cytochrome P450-dependent bioactivation capacity present in these larvae leads to significantly increased genotoxic effects. However, this high bioactivation cross is characterized by some disadvantages in its practical application: The wings of the resulting flies exhibit regularly a serious disturbance in the wing hair pattern (Frölich and Würgler 1989, 1991). This makes the scoring of these wings more difficult and tedious. For this reason, we have developed an improved high bioactivation cross which has a completely normal wing hair pattern like the standard cross (Graf *et al.* 1991). As can be seen from the data given in table III, 48 h feeding of larvae of the improved high bioactivation cross with 10 mM NNP leads to a frequency of total spots per wing of 4.89. This is an in-

crease of over four times over the frequency obtained with the standard cross. And again, the average size of the *mwh* clones is larger in the improved high bioactivation cross than in the standard cross. With three more promutagens, namely diethylnitrosamine, dimethylbenz(a)anthracene and urethane, identical increases in genotoxic activity were observed with this improved high bioactivation cross as with the original high bioactivation cross (Graf and van Schaik 1992).

Another approach to increase the sensitivity of the larvae for promutagens consists of giving them a pretreatment with an inducing agent (Zijlstra *et al.* 1984). We have performed one experiment with the standard cross where 2-day-old larvae were collected and fed for 24 h on a standard medium containing 10 or 20 mM phenobarbital, respectively. After this pretreatment, the larvae were collected again from the vials and transferred to fresh vials containing Instant Medium with 10 mM NNP where they were fed for the remaining 48 h of their development. The results of this experiment are presented in table IV. It can be seen that the 24 h treatment with phenobarbital alone has no genotoxic effects at all. However, this pretreatment leads to frequencies of spots induced with NNP which are approximately 2 to 2.5 times higher than with NNP alone. This demonstrates that an increased xenobiotic metabolism can be induced in the larvae of the standard cross.

The different ways of modulating the genotoxic effects of NNP by varying the treatment conditions or the genotype of the larvae used, are illustrated best by comparing the dose-response curves for the frequencies of total spots per wing which are plotted in figure 3. It is quite impressive to see that for this compound the concentration range which can be analyzed depending on these variables spans nearly three orders of magnitude.

In conclusion, the somatic cells of the imaginal discs of *Drosophila melanogaster* are no doubt a very efficient and sensitive tissue to demonstrate the genotoxic activity of NNP and of other types of promutagens. The wing spot test with its great flexibility in treatment conditions and with its high bioactivation cross

TABLE IV. SUMMARY OF RESULTS OBTAINED IN THE DROSOPHILA WING SPOT TEST AFTER TREATMENTS WITH PHENOBARBITAL AND N-NITROSOPYRROLIDINE USING THE STANDARD CROSS

Compound Conc. (mM) A B#	*Number of wings*	*Spots per Wing (Number of Spots) Stat. Diagn.**				*Spots with mwh clone*	*Mean clone size class*	*Frequency of clone formation per 10(5) cells***	
		Small single spots (1—2 cells) m = 2.0	*Large single spots (>2 cells) m = 5.0*	*Twin spots m = 5.0*	*Total spots m = 2.0*			*observed*	*control corrected*
Control (1% Tween-80 + 3% ethanol) (72 h)									
0 — 0	126	0.16 (20)	0.01 (1)	0.02 (2)	0.18 (23)	23	1.78	0.7	
Phenobarbital (24 h)									
10 — 0	40	0.17 (7)i	0.00 (0)i	0.00 (0)i	0.17 (7)i	7	1.43	0.7	0.0
20 — 0	40	0.22 (9)i	0.05 (2)i	0.00 (0)i	0.28 (11)i	11	2.09	1.1	0.4
N-Nitrosopyrrolidine (48 h)									
0 — 10	34	0.56 (19)+	0.21 (7)+	0.06 (2)i	0.82 (28)+	27	2.11	3.3	2.5
Phenobarbital (24 h) — N-Nitrosopyrrolidine (48 h)									
10 — 10	40	1.15 (46)+	0.22 (9)+	0.13 (5)+	1.50 (60)+	59	2.03	6.1	5.3
20 — 10	40	1.65 (66)+	0.22 (9)+	0.10 (4)+	1.98 (79)+	76	1.80	7.8	7.0

* Statistical diagnoses according to Frei and Würgler (1988): + = positive, i = inconclusive, m = multiplication factor. Kastenbaum-Bowman tests, one-sided. Probability levels: alpha = beta = 0.05

** Frequency of clone formation: mwh clones/wings/24,400 cells (without size correction)

A = pretreatment (24 h); B = normal treatment (48 h)

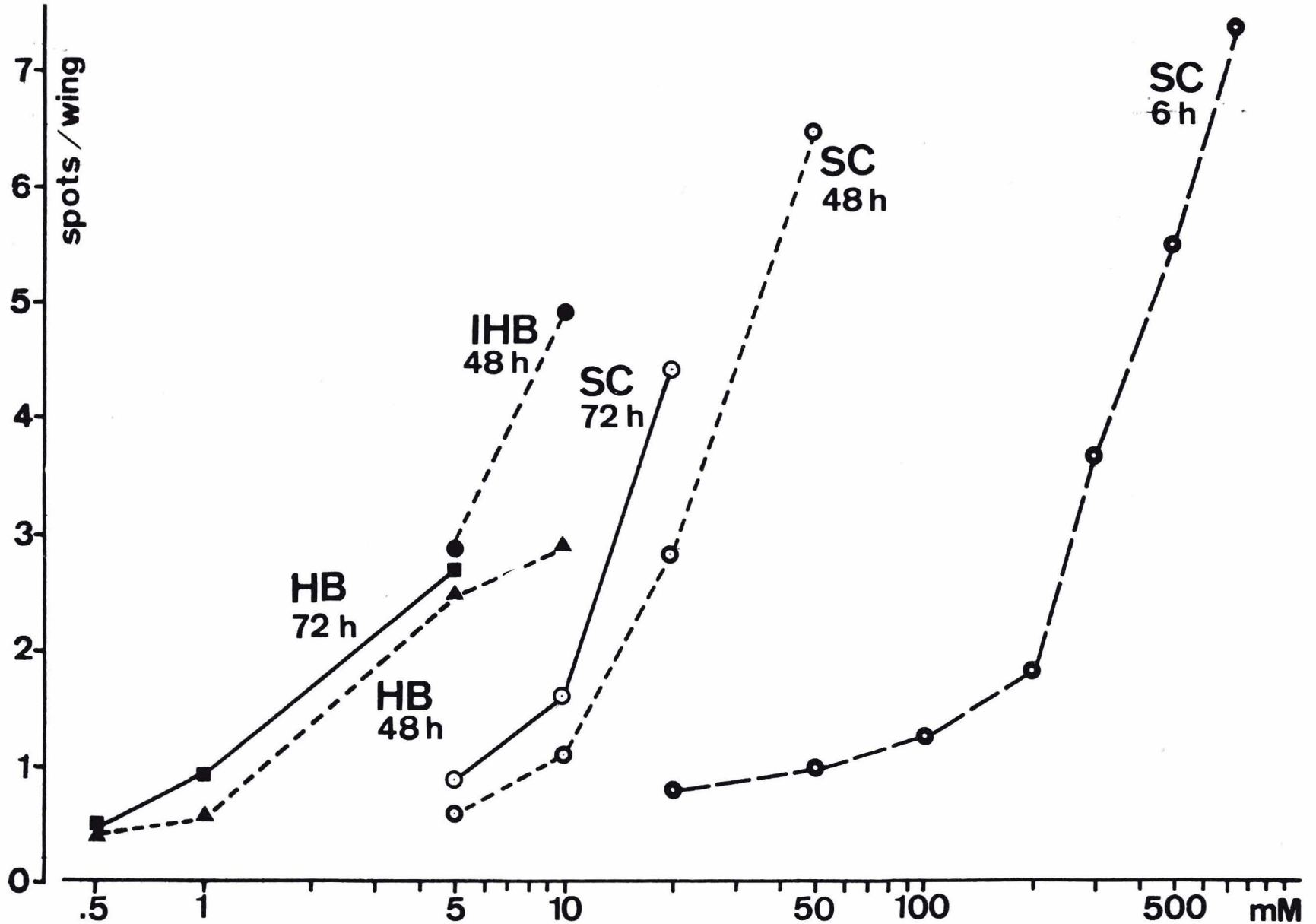


Fig. 3. Dose-response relationships for total spots per wing recorded with N-nitrosopyrrolidine using different treatment periods and different crosses. SC = standard cross, HB = high bioactivation cross, IHB = improved high bioactivation cross

is certainly ideally suited for the analysis of promutagens and procarcinogens.

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