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Analysis of the relationship between age of larvae at mutagen treatment and frequency and size of spots in the wing somatic mutation and recombination test in *Drosophila melanogaster*

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Abstract. The relationship between the induction of mutant clones and the time of mutagen treatment was studied in the somatic mutation and recombination test (SMART) in wing cells of *Drosophila melanogaster*. Larvae trans-heterozygous for the recessive marker mutations multiple wing hairs (*mwh*) and flare (*flr*) were produced. Batches of these larvae were then treated with mutagen at different ages spanning all three larval instars. Methyl methanesulfonate was fed acutely for 2 h by immersing the larvae in a solution of the mutagen mixed with powdered cellulose. Wings of the surviving adult flies were mounted and scored for the presence of spots. The frequency and size of single and twin spots were recorded separately. Twin spots are produced exclusively by mitotic recombination, whereas single spots can result from various types of mutational and exchange events. There exists a clear correlation between time of induction and frequency as well as size of the single spots. In young larvae only few but very large spots are induced, whereas in older larvae the frequencies are considerably increased but the sizes are smaller. The twin spots show a different relationship. Practically no twin spots are found in very young and in very old larvae. The results demonstrate that in the wing spot test the optimal age of the larvae for mutagen treatment is 72 h.
Key words. Genotoxicity; somatic cells; spot size; *Drosophila melanogaster*; methyl methanesulfonate.

The somatic mutation and recombination test (SMART) using wing spots in *Drosophila melanogaster* is a rapid and inexpensive in vivo assay which detects genotoxic agents using somatic cells of a higher eukaryote[1-4]. It screens both for somatic mutation and for mitotic recombination, and it is sensitive to a wide variety of both direct-acting mutagens and those requiring bioactivation[5-9]. It is based on the principle that the loss of heterozygosity of suitable marker genes in cells of the imaginal disks in larvae can lead to the formation of clones of mutant cells, which are then expressed as spots on the wings of the adult flies. The two recessive markers multiple wing hairs (*mwh*) and flare (*flr*) on the left arm of chromosome 3 are employed. Different crosses of flies have been developed which produce larvae that are trans-heterozyous for these two markers[3,8,10]. The analysis of two different genotypes (one with structurally normal chromosomes, one with a multiply inverted balancer chromosome) allows for the quantitative determination of the recombinagenic activity of genotoxic agents[11,12]. Standard test protocols for chronic or acute feeding as well as for inhalation treatment of the larvae with the test compounds are available[2]. The wing spot test is also useful for the study of structure-activity relationships of various groups of chemical compounds[13-16]. Several parameters have been studied which can influence the frequency and/or the size of the spots on the wings. For example, it has been shown that the temperature affects the frequency of spontaneous spots considerably[17]. At temperatures below 20 °C and above 27 °C the frequencies of spontaneous small single spots are increased significantly[18].

Another basic aspect which is important for the wing spot test is the relationship between the time of induction of a clone of mutant cells in the imaginal disk and the size of the resulting spot on the wing. The imaginal disks are tissues which grow continuously by mitotic division throughout the whole period of larval development. The wing disks consist of approximately 50 to 100 cells at the time of hatching of the first instar larva and reach a size of about 30,000 cells in the early pupa when wing differentiation starts[19]. Of these cells, 24,400 are analyzed in the scoring of the wings for induced somatic spots[20]. The continuous cell proliferation during the development of the larva leads to an increase in the number of target cells present in an imaginal disk. Therefore, increasing clone induction frequencies are expected with increasing age of the mutagen-treated larva. In contrast to the clone induction frequency, the size of the induced clones is expected to decrease with increasing age of the larva. This inverse relationship between clone size and clone frequency as a function of the age of the larvae has been demonstrated for ionizing radiation by Garcia-Bellido and Merriam[20], and Haynie and Bryant[21].

Here we present the results of a similar investigation that demonstrates the relationship which exists between

the time of treatment with a chemical mutagen and the frequency and size of the resulting single and twin spots. For this purpose, larvae derived from the standard cross were collected at different ages spanning all three larval instars and then treated acutely with methyl methanesulfonate. This direct-acting alkylating agent was chosen because it induces high frequencies of both somatic mutations and mitotic recombinations in acute feedings as short as 2 h.

Materials and methods

Chemical compound. Methyl methanesulfonate (MMS, CAS No. 66-27-3) was obtained from ICN-K&K Co., Plainview NY. It was dissolved in distilled water and was fed to larvae as an 0.1% (v/v) or 0.02% solution which is equivalent to 11.8 mM and 2.35 mM, respectively.

Larval feeding. Eggs were collected in culture bottles for 8 h according to standard procedures[15]. Larvae of different ages (24, 36, 48, 60, 72, 84, 96 and 108 h after egg collection) were washed out of these bottles and placed in small batches in plexiglass tubes which had one end covered with nylon gauze. These tubes were then placed in 100 ml beakers containing 2 ml MMS solution and 300 mg cellulose (microcrystalline; Merck, Darmstadt, Germany). In this way, the larvae are immersed and feed on the cellulose particles and mutagen solution. After 2 h the tubes were removed, and the larvae washed thoroughly with tap water and then transferred to vials containing normal Drosophila Instant Medium (Formula 4-24; Carolina Biological Supply Co., Burlington NC) where they continued their development.

Somatic mutation and recombination test. Virgin *mwh/mwh* females were mated with *flr*^{3}*/In(3LR)TM3, ri p*^{p} *sep l(3)89Aa bx*^{34e} *e Bd*^{S} males (for a description of the markers see ref. 22). This corresponds to the standard cross as it was originally used[13]. The further processing and microscopic analysis of the wings of the trans-heterozygous (*mwh flr*$^+$*/mwh*$^+$ *flr*^{3}) genotype were done as described previously[2,3,23]. The experiments were carried out at 25 °C and 60% relative humidity.

Data evaluation and statistical analysis. The wing spot data were evaluated and the statistical analysis performed in the way previously described[11,24].

Results

Two separate large-scale experiments were performed with 8 lots of larvae of different ages (24 to 108 h after egg laying). Because the eggs were collected over a period of only 8 h, an age variation of approximately ± 4 h is expected in the series with the youngest larvae. This age variation is then expected to increase slowly with progressively older larvae because not all the larvae develop at the same speed. The wing imaginal disk contains between 50 and 100 cells when the first instar larvae hatch from the egg shells[25]. It continues to grow continuously until it reaches its final size before cell differentiation starts. The last cell divisions take place in the young pupae. It is estimated that one cell division cycle in the wing imaginal disks lasts on average about 8 to 10 h[26,27]. Therefore, the feeding of the larvae with

Table. Summary of results obtained in the Drosophila wing somatic mutation and recombination test. Acute feeding of larvae of different ages with 0.1% and 0.02% MMS for 2 h.

Age at treatment (h)	No. of wings (N)	Spots per wing; (no. of spots); stat. diagnoses*				Spots with *mwh* clone (n)	Mean *mwh* clone size (number of cells) (m)	Clone formation in 10,000 cells ($n \times m/N \times 24{,}400$)
		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]			
A. Control								
	500	0.20 (99)	0.04 (19)	0.02 (10)	0.26 (128)	128	2.1	0.2
B. 0.1% MMS								
24	240	0.15 (36) −	0.08 (19)w	0.01 (2) −	0.24 (57) −	56	132.5	12.7
36	240	0.18 (43) −	0.08 (20)w	0.00 (1) −	0.27 (64) −	64	158.4	17.3
48	94	0.39 (37) +	0.82 (77) +	0.15 (14) +	1.36 (128) +	119	39.3	20.4
60	60	0.50 (30) +	2.08 (125) +	1.38 (83) +	3.97 (238) +	226	16.2	25.0
72	38	2.05 (78) +	2.63 (100) +	2.50 (95) +	7.18 (273) +	259	8.9	24.9
84	8	3.63 (29) +	2.63 (21) +	2.13 (17) +	8.38 (67) +	64	3.6	11.8
96	50	8.42 (421) +	1.12 (56) +	0.28 (14) +	9.82 (491) +	490	2.0	8.0
108	38	6.03 (229) +	0.32 (12) +	0.03 (1)i	6.37 (242) +	241	1.6	4.2
C. 0.02% MMS								
48	20	0.15 (3)i	0.10 (2)i	0.00 (0)i	0.25 (5)i	5	25.2	2.6
60	20	0.30 (6)i	0.60 (12) +	0.35 (7) +	1.25 (25) +	24	9.7	4.8
72	20	0.55 (11) +	0.60 (12) +	0.50 (10) +	1.65 (33) +	29	7.3	4.3
84	20	0.85 (17) +	0.05 (1)i	0.00 (0)i	0.90 (18) +	18	2.3	0.9
96	20	0.50 (10) +	0.00 (0) −	0.05 (1)i	0.55 (11) +	12	1.6	0.4
108	20	0.60 (12) +	0.00 (0) −	0.05 (1)i	0.65 (13) +	13	1.5	0.4

*Statistical diagnoses according to Frei and Würgler[24] for comparisons with control: + = positive; w = weak positive; − = negative; i = inconclusive. m = multiplication factor. Kastenbaum-Bowman tests, one-sided. Probability levels: $\alpha = \beta = 0.05$ (ref. 24).

MMS for just 2 h covers only part of a cell cycle. Due to the instability of the compound, it is not expected that significant amounts of MMS will persist for extended periods inside the larval body after termination of the feeding. The procedure is thus an extremely acute treatment that can be compared to an acute irradiation of larvae with ionizing radiation.

In the first experiment all 8 series were fed simultaneously under identical conditions with 0.1% MMS for 2 h. This was repeated identically in the second experiment, where an additional 6 series (48 to 108 h) were also fed with 0.02% MMS. A summary of the pooled results for wings of the trans-heterozygous genotype is given in the table.

The results of the various treated series are compared to a large pooled control comprising 500 wings which were collected over an extended period of time from water controls of 11 separate experiments using the same experimental conditions as in the two experiments presented here. The frequencies of spontaneous spots are comparable to those observed previously with this standard cross[3,13]. When one takes the frequency of total spots as a measure of the efficiency of the treatment with 0.1% MMS, it is evident that in larvae of the first instar (ages 24 and 36 h) it is not possible to find a significant increase over the control even in wing samples as large as 240 wings. Only the frequencies of the large single spots (i.e. spots of a size of 3 or more cells) are increased significantly; however, they do not reach a statistically significant doubling of the spontaneous rate. This results in the statistical diagnosis 'weak positive'. In the series with older larvae, few wings and variable numbers were analyzed. In particular, from the treatments of 84 h old larvae only 8 wings could be obtained. This is due to an enhanced toxicity of the treatment at this particular stage: in the first experiment no flies at all survived, and in the second only 4 flies were collected. In all the series with larvae older than 36 h, the frequencies of small and large single spots are increased significantly. In these larvae of the second and third instar, the frequency of small single spots increases continuously with age up to 96 h where it reaches 8.42. At 108 h a drop in the frequency of small single spots is observed. The frequency of large single spots reaches a

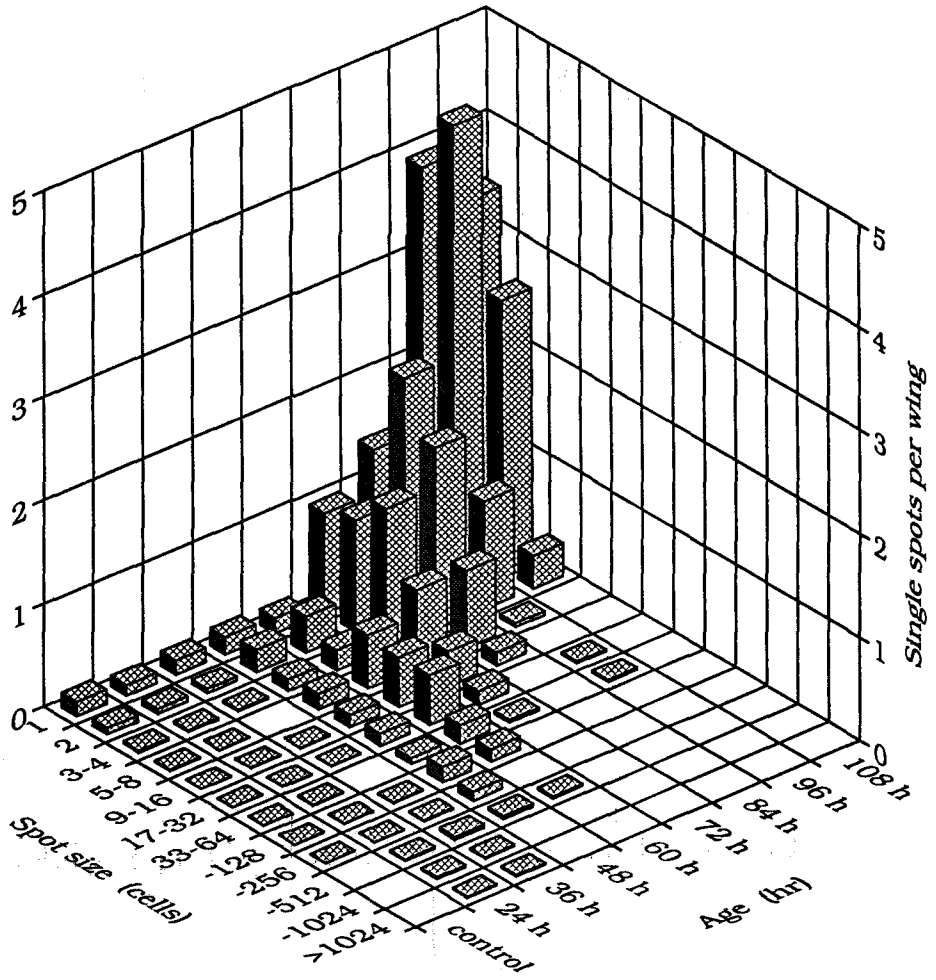

Figure 1. Size distribution of single spots after feeding of larvae of different ages with 0.1% MMS for 2 h.

maximum of 2.63 at 72 and 84 h, respectively, and then drops again. The lack of induced single spots in the 108 h old larvae is probably due to the fact that at this age some of them may already have stopped feeding prior to pupation, which is expected to take place at around 120 h.

In contrast to the single spots, the twin spots show a completely different pattern. Practically no twin spots can be induced in larvae of the first instar (24 and 36 h). Only with 48 h old larvae are the frequencies of the twin spots significantly increased over the control. This frequency continues to increase until the age of 72 h where it reaches a peak of 2.50 twins per wing. This coincides also with the peak frequency of the large single spots. In the older larvae (84 and 96 h), the twin spot frequency drops again and finally falls to the spontaneous rate at 108 h.

All these observations are confirmed when one looks at the results which were obtained after acute treatment with a lower concentration of MMS. In this case, larvae between 48 and 108 h of age were fed with 0.02% MMS for 2 h. Identical samples of 20 wings were analyzed in each age series (table). Again, the highest frequency of spots per wing is found in 72 h old larvae where it reaches 1.65. The highest frequency of twin spots (0.50 twins/wing) is also found in this series. With this low concentration of MMS no significant induction of spots is observed in the 48 h or in the 108 h old larvae.

The different pattern of induction of the two types of spots is best seen when one looks at the spot size distributions recorded in each age series. These spot size distributions for single spots as well as for twin spots after feeding of 0.1% MMS are shown in figures 1 and 2, respectively. Here the spots are grouped into 12 size classes (1 cell, 2 cells, 3–4 cells, etc.) reflecting the number of cell divisions which took place between the occurrence of the genotoxic event and the end of the growth of the wing imaginal disk.

Discussion

In the wing spot test using *Drosophila melanogaster*, two different types of spots can be distinguished, i.e. single spots and twin spots. As discussed elsewhere[2,28], the

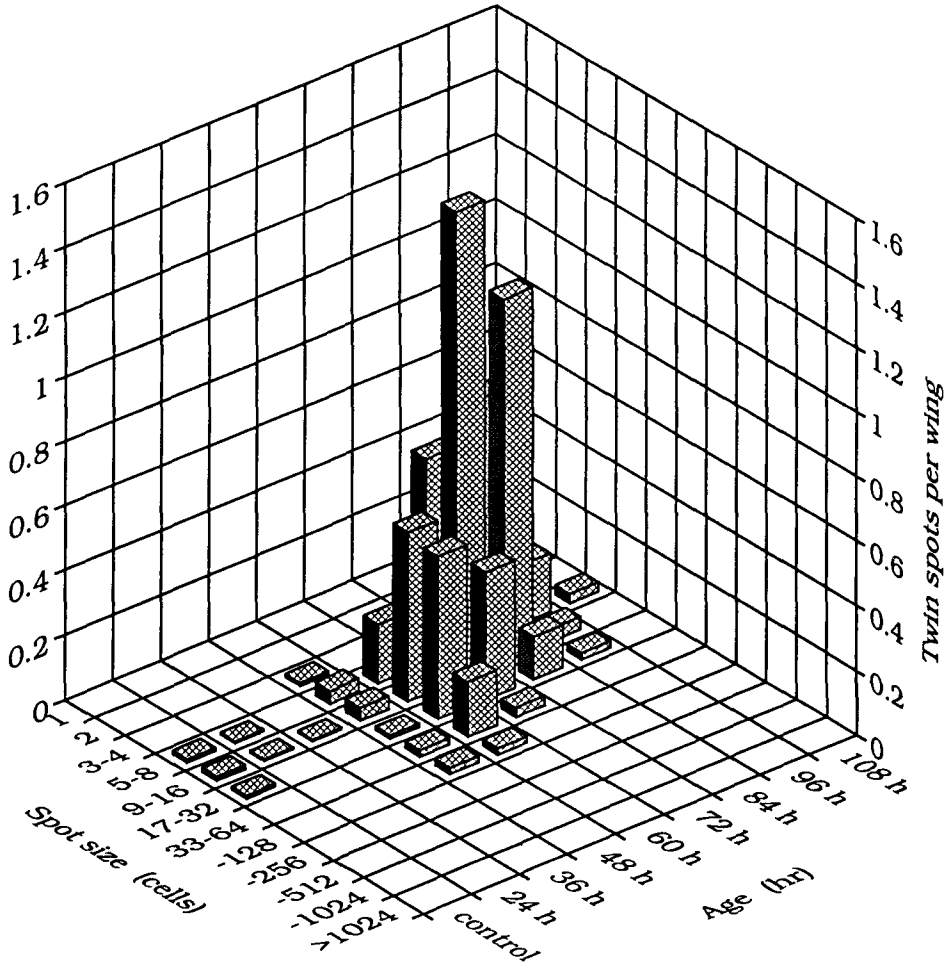

Figure 2. Size distribution of twin spots after feeding of larvae of different ages with 0.1% MMS for 2 h.

single spots (mainly of the *mwh* phenotype, rarely also of the *flr* phenotype) are produced not only by various types of genotoxic events (point mutation, deletion, certain types of translocation, perhaps also gene conversion and monosomy), but also by mitotic recombination occurring between the two markers. In contrast, the twin spots which are composed of a *flr* and an *mwh* clone are exclusively the result of mitotic recombination occurring between the proximal marker *flr* and the centromere of chromosome 3. Consequently, if there is a statistically significant increase in the frequency of twin spots, one can conclude that the corresponding treatment/compound has recombinagenic activity. For this reason, it is useful to classify and quantify the twin spots separately from the single spots. A representative example of a twin spot is shown in figure 3.

The results presented in this paper show that the size distributions for single and twin spots for the various ages exhibit a completely different pattern (figs 1 and 2). Whereas the single spots cover the whole spectrum from the smallest possible spot of 1 cell to extremely large spots of well over 1000 cells, the twin spots are restricted to the size-range between about 3 cells and a maximum of 128 cells. This difference is mainly due to the different properties of phenotypic expression of the two wing cell markers used. The marker *mwh* is expressed properly in clones of only one cell in size, whereas the marker *flr* has a weak and variable expression in small clones. In general, the *flr* phenotype is expressed properly only in clones of 3 or more cells (see ref. 4). This property of the *flr* marker explains why very few twin spots are found at 96 h and no twin spots at all at 108 h. In contrast, the highest frequency of single spots, which are practically all *mwh* single spots, is induced at 96 h. Furthermore, all the *flr* alleles, including flr^3 used in these experiments, are recessive lethals. Large *flr* clones are not able to survive. This contrasts with the marker *mwh* which is viable also in extremely large clones. For these reasons, it is impossible to find increased frequencies of twin spots at the early ages of 24 and 36 h, respectively, where large mutant clones are expected. In contrast, very large *mwh* single spots are obtained with larvae of these ages.

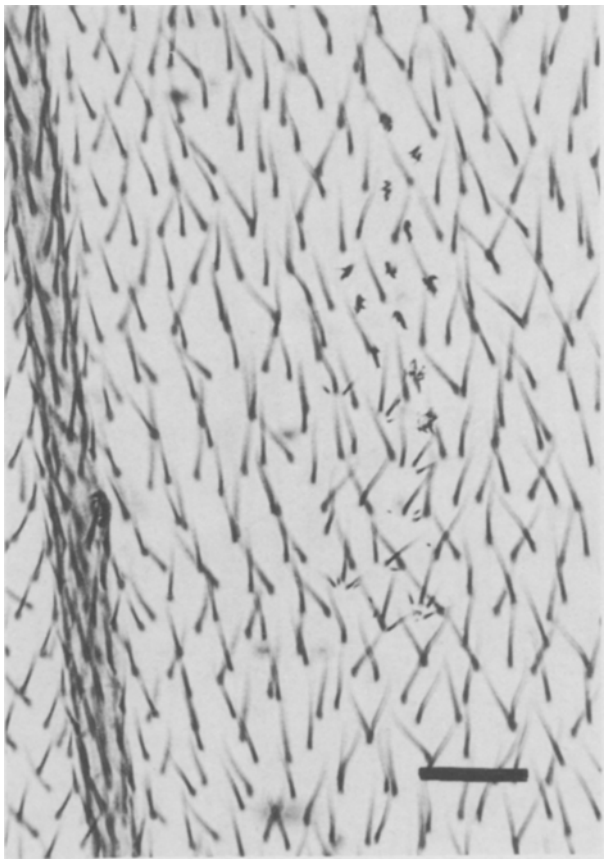

Figure 3. Twin spot. The photograph shows a section of the wing blade with part of a longitudinal vein on the left hand side. In the central part the *mwh* clone (consisting of 9 cells with multiple wing hairs), and above the flr^3 clone (consisting of 12 cells with a flare-shaped hair) of the twin spot can be seen. It is surrounded by wild type cells with one normal hair. Bar = 10 µ. Photograph courtesy of H. Frei.

The highest frequency of twin spots is obtained at 72 h where the frequency of single spots is also already quite high. This is illustrated in figure 4a where the frequencies of these two types of spots are plotted for all the ages tested. This graph demonstrates that the age of 72 h is the optimal one for treating larvae in the wing spot test because it gives the highest sensitivity for recovering twin spots.

For the reasons explained above, a quantitative comparison of the frequencies of induction of genotoxic events in the various age series can only be made with the *mwh* spots as these show the expected size distribution pattern (see refs 11 and 24). Therefore, in the table the numbers of spots with an *mwh* clone are given also together with the mean clone size (number of *mwh* cells). The size distribution of the spontaneously occurring *mwh* clones follows a distribution with a mean size of 2.1 cells. As already discussed above, the largest clones are induced at 24 and 48 h where they reach an average size of 132.5 and 158.4 cells, respectively. Afterwards the mean clone size decreases continuously until the minimum of 1.6 cells is reached at 108 h. The distribution of the mean *mwh* clone sizes is given in figure 4b.

As discussed by Frei et al.[11] and Frei and Würgler[24], in the case of chronic exposures the frequency of clone formation per 10^5 cells can be determined, based on the number of wings analyzed, the number of *mwh* clones recorded, and the number of cells scored in each wing, 24,400. In the case of acute treatments which cover only a small fraction of one cell division cycle, the clone formation frequency can be determined according to the formula given by Szabad et al.[4]. These clone formation frequencies per 10^4 cells are given in the last column of

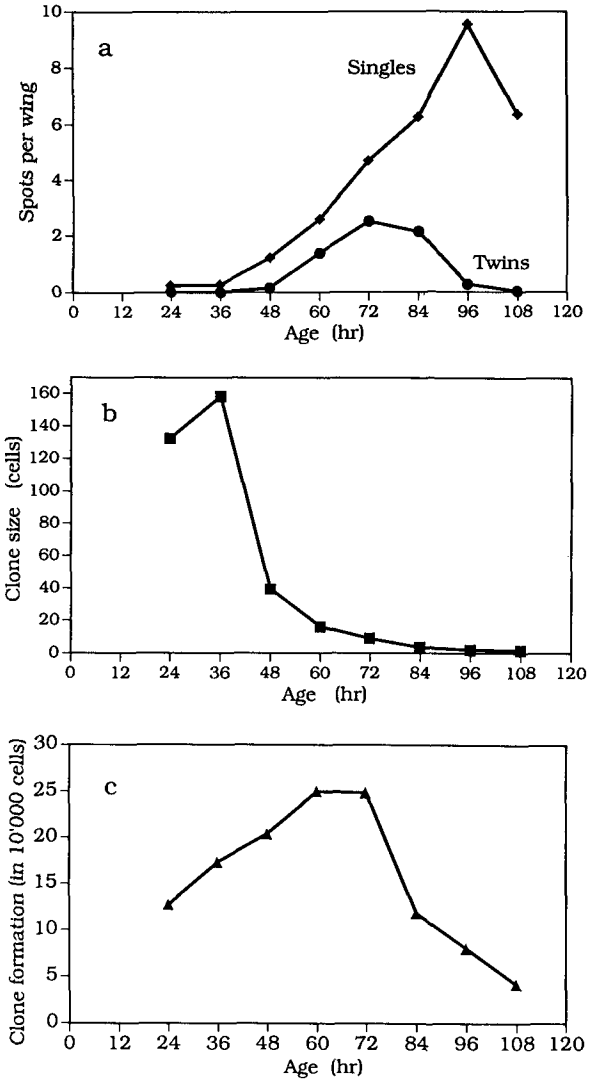

Figure 4. Summary of results obtained after feeding of larvae of ages between 24 and 108 h with 0.1% MMS for 2 h:
a Frequencies of single and twin spots per wing.
b Size distribution of *mwh* clones (numbers of cells).
c Frequency of *mwh* clone formation per 10,000 cells.

the table. With both concentrations of MMS, the highest frequencies of clone formation are observed at the ages of 60 and 72 h, respectively. In older larvae this frequency drops again as can be seen also in figure 4c. The highest clone formation frequencies observed after feeding of 0.1% MMS for 2 h are 25.0 and 24.9 clones per 10,000 cells, respectively. Szabad et al.[4] have recorded a frequency of 45.8 clones per 10,000 cells after irradiation of 22–26-h-old larvae with 1000 R of X-rays. These results then demonstrate that with an acute feeding of larvae with a high concentration of a direct-acting alkylating agent like MMS, effects of the same order of magnitude can be obtained as with ionizing radiation. Of course, the clone formation frequencies normally observed in standard genotoxicity tests with weaker mutagens or promutagens are much lower.

In this connection it is also important to stress that for routine genotoxicity testing of chemical compounds in the Drosophila wing spot test, the preferred treatment is to expose larvae chronically rather than acutely[3]. Compounds with weak genotoxic activity can give false-negative results if they are tested with acute feeding only. The standard treatment, therefore, is a chronic feeding for 48 h, i.e. for the whole third instar.

In conclusion, the wing spot data obtained with the alkylating agent MMS, which is a very powerful genotoxin, demonstrate that the optimal age for treating larvae is 72 h for two reasons. Firstly, the chance of observing the induction of twin spots which are indicative of induced mitotic recombination is highest at this age, and secondly, the frequency of *mwh* clone induction is also high at this age.

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1 Graf, U., Juon, H., Katz, A. J., Frei, H., and Würgler, F. E., Mutat. Res. *120* (1983) 233.
2 Graf, U., Würgler, F. E., Katz, A. J., Frei, H., Juon, H., Hall, C. B., and Kale, P. G., Envir. Mutagen. *6* (1984) 153.
3 Graf, U., Frei, H., Kägi, A., Katz, A. J., and Würgler, F. E., Mutat. Res. *222* (1989) 359.
4 Szabad, J., Soos, I., Polgar, G., and Hejja, G., Mutat. Res. *113* (1983) 117.
5 Frölich, A., and Würgler, F. E., Mutat. Res. *234* (1990) 71.
6 Frölich, A., and Würgler, F. E., Mutat. Res. *244* (1990) 201.
7 Graf, U., and Singler, D., Rev. int. Contam. ambient. *8* (1992) 15.
8 Graf, U., and van Schaik, N., Mutat. Res. *271* (1992) 59.
9 Graf, U., Badoux, M., and Holliger, E., Drosoph. Inf. Serv. *71* (1992) 235.
10 Frölich, A., and Würgler, F. E., Mutat. Res. *216* (1989) 179.
11 Frei, H., Clements, J., Howe, D., and Würgler, F. E., Mutat. Res. *279* (1992) 21.
12 Graf, U., Heo, O., and Olvera Ramirez, O., Mutat. Res. *266* (1992) 197.
13 Alonso Moraga, A., and Graf, U., Mutagenesis *4* (1989) 105.
14 Frei, H., Lüthy, J., Brauchli, J., Zweifel, U., Würgler, F. E., and Schlatter, C., Chem.-biol. Interact. *83* (1992) 1.
15 Graf, U., Wild, D., and Würgler, F. E., Mutagenesis *7* (1992) 145.
16 Van Schaik, N., and Graf, U., Mutat. Res. *286* (1993) 155.
17 Graf, U., Drosoph. Inf. Serv. *63* (1986) 65.
18 Katz, A. J., and Foley, T. A., Envir. molec. Mutagen. *22* (1993) 54.
19 Ransom, R., (ed.) A Handbook of Drosophila Development. Elsevier Biomedical Press, Amsterdam 1982.
20 Garcia-Bellido, A., and Merriam, J. R., Devl Biol. *24* (1971) 61.
21 Haynie, J. L., and Bryant, P. J., Wilhelm Roux Arch. EntwMech. Org. *183* (1977) 85.
22 Lindsley, D. L., and Zimm, G. G., (eds) The genome of *Drosophila melanogaster*. Academic Press, San Diego 1992.
23 Van Schaik, N., and Graf, U., Mutat. Res. *260* (1991) 99.
24 Frei, H., and Würgler, F. E., Mutat. Res. *203* (1988) 297.
25 Postlethwait, J. H., in: The Genetics and Biology of Drosophila, vol. 2c, p. 359. Eds M. Ashburner and T. R. F. Wright. Academic Press, New York 1978.
26 Bryant, P. J., Devl Biol. *22* (1970) 389.
27 Martin, P. F., J. expl Zool. *222* (1982) 97.
28 Würgler, F. E., and Vogel, E. W., in: Chemical Mutagens, Principles and Methods for their Detection, vol. 10, p. 1. Ed. F. J. de Serres. Plenum Press, New York 1986.