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Genotoxicity of mycotoxins in an improved drosophila wing spot test and other short-term effects

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of mutagens with different modes of action are detected by the wing assay. Cross linking agents such as mitomycin C and Trenimom; frameshift mutagens such as acridine orange as well as low molecular aldehydes such as formaldehyde are genotoxic in the assay. Non-disjunction is detected by the wing spot test since vinblastine, a spindle poison interfering with tubulin assembly, induces single spots but not twin spots. Methotrexate, an antimetabolite, is a strong genotoxin in this assay inducing wing spots at high frequencies (Graf *et al.*, 1984).

Strong, direct-acting mutagens such as methylating and ethylating mono-functional alkylating agents are also readily detected in the wing spot test. The following promutagens activated via various metabolic pathways proved to be active in SMART: cyclophosphamide, seneciphylline, procarbazine, DMN, and AFB1. Unstable mutagenic compounds are also easily detected in the wing assay (Graf *et al.*, 1984).

By 1986, some 120 compounds had been tested in the wing spot test. The compounds belong to 27 different chemical classes and include at least fifty compounds that needed metabolic activation. Furthermore, SMART has also been used successfully for the screening of complex mixtures such as herbal teas, coffees and extracts from medicinal plants. For a total of 76 compounds tested in SMART, data were also available for the SLRL assay and, with the majority of compounds, the results were similar in the two test systems. The following five compounds have proved positive in the somatic cell but were negative in the germ cell system: cytosine arabinoside (Katz, 1985),

hydralazine (Juon, unpublished), methotrexate (Würgler *et al.*, 1983), safrole (Würgler *et al.*, 1985) and strychnine (Kägi, unpublished) (in Graf and Würgler, 1986).

SMART tests with *Drosophila* are easy to perform, and what is important for their use in less developed countries is that they do not need expensive laboratory equipment nor sophisticated items. Extensive validation and calibration studies have been performed in three different laboratories during the last ten years (Vogel and Würgler, 1990, unpublished). The Vogel-Würgler (1990, unpublished) table summarizes the test results obtained for 330 chemicals, including some complex mixtures. The file includes all the published and unpublished data from the three laboratories.

10.3.3.2 The Advantages of the SMART assay

Since the essential changes that make cells cancerous are now thought to be primarily somatic mutations (the somatic mutation theory of cancer), the advantages of using this somatic cell assay for screening the environment for potential carcinogens, are manifold. The test detects genotoxins with a broad spectrum of DNA interactions and genetic effects. In addition to individual chemicals, complex mixtures can also be tested for genotoxic activity. This is true for both direct acting agents as well as for compounds requiring metabolic activation. The problem of low detection of polycyclic aromatic hydrocarbons in the SLRL assay in germ cells, has been solved for SMART by the construction of sensitive tester strains with high bioctivation capacity (Frölich and Würgler, 1989).

The somatic assays have been able to detect as mutagens all established *Drosophila* germ line mutagens (mostly tested by the SLRL assay). In addition, classes and individual cases of genotoxins found negative in the germ line assays, such as metabolic inhibitors, were picked up by the SMART assays (Vogel and Würgler, 1990).

By and large, SMART constitutes a broad spectrum assay, both in terms of the different types of genotoxins detected, as well as the different genetic end points scored. The conclusion by Vogel and Würgler (1990), based on analysis of the Gene-Tox report and the outcome of international collaborative studies, was that, when comparing the SLRL assay as a predictor of mammalian genotoxicity with the SMART assay, test performance (including sensitivity and accuracy) of the SMART assays are clearly better than that of the SLRL test.

In 1988, Vogel coordinated a workgroup which considered results from seven laboratories. The ability of four polycyclic hydrocarbons to induce both heritable and somatic genetic alterations in *Drosophila* larvae was assayed. One objective of the experimental procedure was to compile results from germinal versus somatic cells, using the same treatment protocol in larvae. Germ line assays on treated male larvae failed to detect as mutagens any of the four test chemicals (Vogel, 1988). With the four compounds, the testing for somatic mutation and recombination is a more sensitive *in vivo* assay than the SLRL test (Vogel, 1988). The aromatic amines 2-aminofluorene (Graf *et al.*, 1990), benzidine (Graf *et al.*, 1989), as well as IQ (Yoo *et al.*, 1985),

induced somatic mutations and mitotic recombinations in the wing spot test in *Drosophila* and the improved efficiency of the SMART test compared with the SLRL test to detect the genotoxic activity of the aromatic amine IQ was reported by Graf and colleagues (1992c).

Two collaborative studies on the "Evaluation of Short-Term Tests for Carcinogens" conducted under the auspices of the International programme on Chemical safety with the participation of three laboratories, clearly demonstrated that the SMART assays can successfully be adopted for routine use by other testing laboratories, and that repeated experiments within a laboratory as well as interlaboratory comparisons, give reproducible results. The relative ease with which the assay can be adopted by other *Drosophila* workers and also by genetic toxicologists not familiar with *Drosophila* genetics, has been experienced at three international training courses (Switzerland in 1988, Mexico in 1989 and Brazil in 1990). There are presently 65 laboratories all over the world with the knowledge and means to conduct the SMART assays (Vogel and Würgler, 1990).

Other factors which have contributed to the still increasing acceptance of SMART are the short time needed for test performance, the low costs of the test (10-15% of the SLRL assay) and the flexibility of SMART with respect to variation of exposure conditions and exposure levels. With the SMART test the mutagenic as well as the recombinogenic activity of chemicals can be studied. Experiments have shown that the wing spot test is also suited for the study of dose-response relationships and it lends itself to the analysis of

structure-activity relationships of chemicals (Graf and Würgler, 1986). Such structure-activity relationship studies offer the prospect of identifying the critical parameters in a molecular structure that influence genotoxic and carcinogenic activities. This approach is also of great applied importance because it can facilitate the development of safe chemical products and aid in the search for alternatives to genotoxic compounds whose uses are indispensable.

The SMART test also has some technical advantages. The incredible amount of work involved in preparing thousands of vials and collecting thousands of virgins for the SLRL assay and the heritable translocation test is avoided. The surviving flies can be stored in 70% ethanol until it is convenient to begin mounting, so the slides can be prepared at any time. Furthermore, as the system uses wing tissue, permanent preparations of the wings can be made, making verification and reconsideration possible (Graf *et al.*, 1984) and this allows for re-inspection of critical samples several months later. In my opinion, this fast, *in vivo* eukaryotic short-term test has great potential for use in the genotoxicity screening of chemicals. It is cheap and easy to perform and results can be obtained in under two weeks. The large numbers of experimental animals which can be used, without being considered inhumane by the Animal Ethics Committee, reduces the possibility of missing effects and of other inaccuracies resulting from small sample size, making statistical evaluation of the results possible. One of the main advantages of the SMART test is the fact that large numbers of cells can be scored on the wing blade allowing for the detection of rare events (Graf and Würgler, 1988).

The SMART test detected both the genotoxic and recombinogenic activity of all aflatoxins, sterigmatocystin and zearalenone and the structure-activity series obtained with the aflatoxins was, in decreasing potency, AFB1 > AFG1 > AFB2 > AFG2 which is in agreement with the order previously reported by Wogan and colleagues (1971). Aflatoxin G1 was also tested in the *Salmonella*/mammalian microsome assay, the heritable translocations test and the sex-linked recessive lethals test. In the *Salmonella*/mammalian microsome assay, AFG1 reverted both strains TA98 and TA100 only in the presence of S9 fraction. Aflatoxin G2 and the animal carcinogen zearalenone, however, are negative in the *Salmonella*/mammalian microsome assay (McCann *et al.*, 1975a; Wehner *et al.*, 1978), but positive in SMART, indicating the increased sensitivity of the SMART assay for promutagens. AFG1 was also mutagenic in the SLRL test and induced translocations in the heritable translocations test, but the mutagenic activity of AFG1 was only detected in early spermatids in both tests. These data also provided good evidence for a correlation of genotoxic and chromosome breakage effects of AFG1 in germinal and somatic cells, and for the practical advantage of the wing spot test, but no additional information was gained by performing the other tests together with SMART. Although the powerful promutagen AFG1 exhibited genotoxic activity in all tests, the cost and labour involved in the assays made it clear that SMART is by far the best short-term mutagenicity test available.

When using SMART, it is not necessary to include many other tests in the test battery as not much information can be gained by supplementing this economical and well validated assay with other assays because SMART is

able to detect the full spectrum of genetic effects. A germ cell assay may be included to determine if the test chemical poses a risk to future generations as well as a mammalian short-term test (such as a mammalian tissue culture test) to determine if differences between mammalian and insect metabolism affect the genotoxicity of the compound. Chemicals in the environment should be screened using an *in vivo* short-term test such as SMART and genotoxic chemicals that show consistent mutagenic activity in this short-term test should then be regarded as potential carcinogens due to the mutagenicity/carcinogenicity relationship. Only then should the chemical be tested in a long term rodent carcinogenicity assay in order to label it a mammalian carcinogen and to determine the target organ of such an agent.

10.3.3.3 Improvement of SMART

Most emphasis has been placed on the study of DNA excision repair defective mutants. A problem, however, is that these mutants are often sterile. Furthermore, as shown by Graf and Würgler (1986), and Graf, Hall and van Schaik (1990) the use of strains carrying different DNA repair defects, to improve the detection capacity of the test, is not recommended. Use of phenobarbital to induce the different enzyme activities relevant to xenobiotic metabolism has not been found to increase the sensitivity of the test (Zjilstra *et al.*, 1984; Frölich and Würgler, 1990 a). The best way, so far, to improve the detection capacity of SMART is by using tester strains with high bioactivation capacity. The results of this sudy showed that the high bioactivation crosses were more efficient than the standard cross in detecting the genotoxic effects of the border line promutagens, AFG2 and AFB2. What

has to be done now is to map some more genes involved in xenobiotic metabolism, to isolate mutants where these genes are amplified or where these enzymes are overproduced and to try to transfer these to the tester strains to make them even more sensitive and to characterize them further metabolically.

Due to the metabolism of xenobiotics in *Drosophila*, results obtained with this insect system are very likely to be relevant for predicting adverse genetic effects in mammals and man. Therefore, positive results in this *Drosophila* test, point to a potential risk for human somatic cells and signal potential genotoxic carcinogens. It is, however, not possible to quantitatively extrapolate screening data from *Drosophila* to man and mammals as possible differences in metabolism must be taken into account.

In 1991, Jowett and colleagues generated transgenic *Drosophila* larvae which express mammalian cytochrome CYP2B1, which is a member of a large gene family encoding several important drug metabolizing enzymes. The gene was fused to a *Drosophila* promoter which confers expression in the larval fat body. The authors used the SMART test to demonstrate that transgenic larvae expressing the mammalian cytochrome P450 are hypersensitive to cyclophosphamide, a procarcinogen activated by the enzyme. Cyclophosphamide caused a highly significant increase in the frequency of total spots in the wings of both the transgenic *a*CYP2B1 strain and control strains, but the increase was almost two fold higher in the CYP2B1 strain. With the development of this gene-targeted P element-induced mutation, the

authors concluded that it will be possible to generate *Drosophila* strains in which endogenous genes for xenobiotic metabolizing enzymes have been replaced by human genes (Jowett *et al.*, 1991).

The problem with this study is that the authors tested a procarcinogen which could be activated by the endogenous P450 cytochrome enzyme system of *Drosophila*. Instead of cyclophosphamide, the authors should have chosen to test a polycyclic aromatic hydrocarbon that is non-genotoxic in untransformed *Drosophila* strains but positive in transgenic *a*CYP2B1 strains expressing mammalian cytochrome P450 before they conclude that this does in fact improve the sensitivity and detecting capacity of the test.

CHAPTER 11

11 CONCLUSION

When screening toxic chemicals for genotoxicity in short-term tests it becomes desirable to test them at sub-toxic exposure levels. The problem is that at these non-toxic levels, genotoxic effects will only be detected if the test system is optimally sensitive. The *Drosophila* somatic mutation and recombination wing spot test (SMART) is a convenient and well validated short-term genotoxicity test and in 1989 Frölich and Würgler increased the sensitivity of the test for promutagens by introducing high bioactivation capacity from the *OR-R* DDT resistant strain into the SMART tester strains. The *OR-R* strain carries the *RI* gene at position 2-65,0 and this gene is responsible for the high constitutive expression of cytochrome P450-dependent activities characteristic of this strain. As the activation by cytochrome P450-dependent enzymes is a very relevant pathway for promutagens, the introduction of this high bioactivation capacity into the tester strains would increase the detectability of promutagens. A drawback of the high bioactivation lines is the presence of an irregular whorling of the trichomes on wing blades which interferes with scoring the wing for mutant spots. The gene responsible for this whorling effect, which I have named paisley (*ply*), was shown to be recessive and located around position 90 on chromosome 2 and is thus linked to the *RI* gene responsible for the high bioactivation. In 1992, Graf and van Schaik established and evaluated an improved HB cross producing *ORR* heterozygous individuals. These have a

normal, undisturbed wing hair pattern while exhibiting P450-dependent bioactivation capacity equal to or even slightly higher than that of the original HB cross. Only promutagens that were detected positive even in the standard cross have been tested in the improved HB cross. Although the IHB cross showed an increased sensitivity for these potent promutagens, animal carcinogens, borderline and weak promutagens that are negative in the SMART standard cross but positive in other short-term tests of mutagenicity should be tested in this cross, to show that the cross is in fact an improvement on the standard cross. In this study, the borderline promutagens AFB2 and AFG2 were tested in the improved HB cross, and although the improved HB cross was more sensitive than the standard cross and helped to confirm the genotoxicity and recombinogenic activity of these compounds, AFB2 and AFG2 were also positive in the standard cross at the higher concentrations. The disadvantage of using the hybrid larvae of the IHB cross is that they are heterozygous for the *RI* and *ply* genes. Several events can eliminate the wild type paisley gene (ply^+) resulting in the expression of whorling in certain individuals. Furthermore, tests of DDT resistance (section 3.3) seem to suggest that the *RI* gene may not be completely dominant and hence *RI* homozygotes would be more sensitive to the effects of promutagens than *RI* heterozygotes. Tests of DDT resistance and results of the biochemical assays, as well as the findings of Graf and van Schaik (1992), seem to indicate that the original *ORR;mwh* stock does not carry the *RI* gene on chromosome two, athough it may carry resistance alleles on the first chromosome. Hence, progeny from the original HB cross may also be heterozygous for the *RI* gene.

It therefore became necessary to construct new HB strains that are homozygous for the *RI* gene but whorl-free and this was done by separating the *ply* gene from the *RI* gene. These new SMART HB strains were shown to have higher bioactivation capacity and improved sensitivity for the detection of borderline promutagens than the standard cross and improved HB cross. AFG2 and sterigmatocystin, which were only detected positive at the high concentrations in the other crosses, were positive at sub-toxic exposure levels in the new HB cross. It was interesting to note that the animal carcinogen, zearalenone, was negative at all concentrations in the SMART standard cross but was detected positive at the two higher concentrations by the new HB cross, demonstrating the improved sensitivity of the new HB cross.

Biochemical assays (section 5.3) provided direct evidence that both the newly constructed stocks *ORR;flr*^{3} and *ORR;mwh* overexpressed P450 cytochrome-dependent activities. The pNA demethylase activity in the new HB tester strains and original *ORR;flr*^{3} strain was similar to that in the *OR-R* strain (from which they were constructed) and 8-10 fold higher than in the standard and original *ORR;mwh* tester strains. The pNA demethylase activity in third instar larvae from the new HB cross was also 5 fold higher than in larval microsomes from the standard cross.

The main advantage of the new HB cross is to combine the high bioactivation capacity of *RI* homozygous individuals, with the ease of scoring the wings using the same criteria as for the standard cross. This cross has, however, been validated with a small number of chemical mutagens and it should

therefore be used in parallel with the standard cross which has been validated with over 330 chemicals so far. Promutagens previously reported negative in *Drosophila* should now be retested using the sensitive new HB cross to further characterize the properties of this new SMART cross.

In addition to the characterization and location of the whorling gene, several characteristics and properties of DDT resistance and the *RI* gene were analysed. The lethal (critical) DDT concentrations of various stocks were determined and it was found that overall males were more DDT resistant than females, larvae were more DDT resistant than adults and that resistance alieles were located on chromosome one, two and three in *OR-R* strains. The maternal effect of the *RI* gene was investigated and since adult and larval progeny from the cross resistant females to sensitive males were always more resistant than larval and adult progeny from the reciprocal cross, it was concluded that maternal imprinting was probably involved. Cytogenetic analysis of salivary gland chromosomes of *RI* heterozygotes from various crosses, revealed regions of asynapsis near the right arm of chromosome 2 (2R) and along the X chromosome. The asynaptic region near 2R spanned position 65 where the *RI* gene is located. This difference in length of RI^+ and *RI* genes seemed to suggest that amplification of the *RI* gene may be involved in insecticide resistance and high bioactivation capacity, but more conclusive molecular evidence is required.

CHAPTER 12

12.1 APPENDIX A

THE *SALMONELLA*/MAMMALIAN MICROSOME ASSAY

Recipes for stock solutions and media

(Adapted from Maron and Ames (1983)

Vogel-Bonner medium E (50X)

Use: Minimal agar

Ingredient	*Per litre*
Warm distilled H_2O (45°C)	670 ml
Magnesium sulphate ($MgSO_4.7H_2O$)	10 g
Citric acid monohydrate	100 g
Potassium phosphate,dibasic (anhydrous) (K_2HPO_4)	500 g
Sodium ammonium phosphate ($NaHNH_4(PO_4.4H_2O$)	175 g

Salts were added in the order indicated to warm water in a 2 litre flask placed on a magnetic stirring hot plate. Each salt was allowed to dissolve completely before adding the next. The volume was adjusted to one litre and then the medium was distributed into two one-litre glass bottles. The medium was autoclaved in the two loosely capped bottles, for 20min at 121°C. When the solutions had cooled, the blue tops were tightened.

0,5 mM histidino/biotin solution

Use: Mutagenicity assay (add 10ml to 100ml of top agar)

Ingredient	*Per 250 ml*
D-Biotin (F.W.247,3)	30,9 mg
L-Histidine.HCl(F.W.191,7)	24,0 mg
Distilled H_2O	250,0 ml

The biotin was dissolved by heating the water to the boiling point. The solution was autoclaved for 20 min at 121°C and the solution was stored in a glass bottle at 4°C.

Top agar

Use: Mutagenicity assay

Ingredient	*Per litre*
Agar	6 g
sodium chloride (NaCl)	5 g
Distilled H_2O	1000 ml

Top agar, containing 0,6% agar and 0,5% NaCl, was autoclaved in 250 ml glass bottles with rubber-lined plastic screw caps (blue tops) in volumes of 100 ml. Before autoclaving, the cap was loosened to prevent pressure from building up, but afterwards the top agar was stored at room temperature with well tightened tops to prevent evaporation. Before use, the cap was loosened

again, the agar was melted in a microwave oven and the bottle was then kept in a water bath.

Ten millilitres of a sterile solution of 0,5 mM L-histidine.HCl/0,5 mM biotin was added to 100 ml of molten agar and mixed thoroughly by swirling. Two millilitre aliquots of top agar were then pipetted aseptically into culture tubes (13 mm x 100 mm) fitted with plastic caps (Bellco).

Salt Solution (1,65 M KCl + 0,4M $MgCl_2$)

Use:S9 mix for mutagenicity assay

Ingredient	*Per 500 ml*
Potassium chloride (KCl)	61,5g
Magnesium chloride ($Mg_2Cl.6H_2O$)	40,7
Distilled water to final volume of	500ml

The ingredients were dissolved in water and then the solution was autoclaved for 20 min at 121°C. The salt solution was stored in glass bottles in the refrigerator.

0,2 mM sodium phosphate buffer, pH 7,4

Use: S9 mix for mutagenicity assay

Ingredient	*Per 500 ml*
0,2M sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$) (13,8g/500ml)	[illegible]
0,2M disodium hydrogen phosphate (Na_2HPO_4) (14.2g/500ml)	440ml

The pH was tested and if it was lower than 7,4, more 0,2M disodium hydrogen phosphate was added. The buffer was sterilized by autoclaving for 20 min at 121°C.

1M NADP solution (nicotine adenine dinucleotide phosphate)

Use: S9 for mutagenicity assay

Ingredient	*Per 5 ml*
NADP (F.W. 787,4)	393,7 mg
sterile distilled water	5 ml

The NADP was added to pre-weighed sterile glass tubes with screw caps. The tubes were wrapped with metal foil to protect against light and the tubes were labelled with the correct weight and calculated volume of water to give a 0,1M solution. The tubes of weighed NADP should be placed in a jar with a tight fitting lid. Silica gel dessicant was placed at the bottom of the jar and the jar was stored in a -70°C freezer. When needed for making up the S9 mix, a tube was removed from the jar and the specified amount of water was

added and mixed by vortexing until the NADP had dissolved and then the tube was placed in an ice bath.

1M glucose-6-phosphate

Use: S9 mix for mutagenicity assay

Ingredient	*Per 10 ml*
Glucose-6-phosphate (G-6-P)	2,82 g
Sterile distilled water	10 ml

Pre-weighed aliquots of glucose-6-phosphate were prepared as described for NADP and stored in dessicated jars in the freezer. Solutions of G-6-P stored in the freezer are stable for 6 months.

S9 mix (rat liver microsomal enzymes and cofactors)

Use: Mutagenicity assay

Ingredient	*Per litre*	
	Standard S9 mix	High S9 mix
Rat liver S9 (Aroclor-1254-induced)	2,0 ml (4%)	5,0 ml (10%)
$MgCl_2$-KCl salts	1,0 ml	1,0 ml
1 M glucose-6-phosphate	0,25 ml	0,25 ml
0,1 M NADP	2,0 ml	2,0 ml

0,2 M phosphate buffer (pH 7,4)	25,0 ml	25,0 ml
Sterile distilled water	19,75 ml	19,75ml

The ingredients were added in the reverse order indicated above so that the liver was added to a buffered solution. The solution was prepared fresh and kept on ice. All ingredients were chilled before use. Any left over S9 or S9 mix was discarded as S9 should never be refrozen.

Ampicillin solution (8mg/ml)

Use: Tests of ampicillin resistance

Master plates for R-factor strains

Ingredient	*Per litre*
Ampicillin trihydrate	0,8 g
Sodium hydroxide (0,02N)	100 ml

The solution was stored in a glass bottle at 4°C.

Crystal violet solution (0,1%)

Use: Tests for crystal violet sensitivity (to confirm *rfa* mutation)

Ingredient	*Per litre*
Crystal violet	0,1 g

Distilled water	100 ml

This solution was stored at 4°C in a glass bottle with a screw cap. The bottle was wrapped in metal foil to protect against light

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Minimal glucose plates

Use: Mutagenicity assay

Ingredient	*Per litre*
Agar	15 g
Distilled water	930 ml
50X VB salts	20 ml
40% glucose	50 ml

This medium consists of 1,5% agar and 2% glucose in Vogel-Bonner medium E (50X).

The agar (15g) was added to the distilled water in a 2-litre flask. This was autoclaved for 20 min using low exhaust. When the solution had cooled slightly, the 20ml of sterile 50X VB salts and 50ml of sterile 40% glucose were added. A large magnetic stir bar was added to the flask before autoclaving for mixing. After all the ingredients were added and the solution thoroughly stirred, 30ml was poured into each petriplate.

Histidine/biotin plates

Use: Master plates for non R-factor strains

Tests for histidine requirement

Ingredient	*Per litre*
Agar	15 g
Distilled water	914 ml
50X VB salts	20 ml
40% glucose	50 ml
Sterile histidine.HCl.H_2O (2g per 400ml H_2O)	10 ml
Sterile 0,5 mM biotin	6 ml

The agar and water were autoclaved and then the sterile 40% glucose, 50X VB salts and histidine were added to the hot agar solution. The solution was allowed to cool slightly and then the biotin was added. After mixing thoroughly, the plates were poured.

Ampicillin plates and ampicillin/tetracycline plates

Use: Master plates for strains carrying the plasmids pKM101 and pKM101 + pAQ1

Ingredients	*Per litre*	*Plate concentration*
Agar	15 g	1,5%
Distilled water	910 ml	-
50X VB salts	20 ml	1X
40% glucose	50 ml	2,0%

Sterile histidine.HCl.H_2O	10 ml	260 μM
(2g per 400ml H_2O)		
Sterile 0,5 mM biotin	6 ml	3 μM
Sterile ampicillin solution		
(8mg/ml 0,02 N NaOH)	3,15ml	25 μg/ml
Sterile tetracycline solution		
(8mg/ml 0,02 N HCl)	0,25ml	2 μg/ml

The water and agar were autoclaved for 20 min and then the sterile glucose, 50X VB salts and histidine were added to the hot solution. The solution was mixed and allowed to cool to approximately 50°C. The sterile biotin and ampicillin solutions were added aseptically. Tetracycline was added only for use with TA102 which is tetracycline resistant.

The 50X VB salts and 40% glucose solutions were autoclaved separately by autoclaving for 20 min. Histidine and biotin solutions were also autoclaved. Plates to be used for tests of tetracycline and/or ampicillin resistance can be stored for approximately 2 months at 4°C.

Nutrient agar plates

Use: 1. Tests for genotypes

(a) crystal violet sensitivity (*rfa*)

(b) UV sensitivity (*uvrB*)

2. Tests for viability of bacteria

Ingredient	*Per litre*
Oxoid nutrient broth	25 g
Agar	15 g
Distilled water	1000 ml

The ingredients were added to a 2-litre flask containing a magnetic stir bar and autoclaved for 30 min at low exhaust. The contents were mixed and the plates were poured.

LB agar plates

Use: Growing the strains for lyophilization

Ingredient	*Per litre*
Bacto tryptone	10 g
Bacto yeast extract	5 g
NaCl	10 g
Agar	15 g
Distilled water	1000 ml

The ingredients were dissolved in water. The pH was adjusted to 7,0 with 1N NaOH (approximately 2ml/l). The ingredients were added to a 2-litre flask containing a magnetic stir bar. The solution was autoclaved for 30 min at 121°C at low exhaust. The solution was mixed and then the plates were poured. The agar is allowed to cool slightly before pouring the plates to avoid condensation.

12.2 APPENDIX B

MAMMALIAN LIVER S9

Prepared by Dr R. Kfir and Miss B. Genthe, Department of Water Technology, C.S.I.R., according to the procedure published by Maron and Ames (1983).

Induction of Rat Liver Enzymes

They used Sprague-Dawley male rats weighing approximately 200g. Aroclor 1254 was diluted in maize oil to a concentration of 200 mg/ml and a single i.p. injection of 500mg/kg was administered to each rat 5 days before they were killed. The rats were given drinking water *ad libitum* and Purina Laboratory Chow until 12 hours before sacrifice when the food (but not the water) was removed. On the fifth day of induction the rats were killed by cervical dislocation.

Removal of liver from rats

The livers were removed aseptically using sterile surgical tools. After the animal was killed, it was placed on its back on an autopsy board and the feet were secured with pins. The fur was swabbed thoroughly with 70% ethanol and the skin was cut using sterile pointed scissors. The skin flaps were folded back and pinned to the autopsy board to avoid getting fur into the abdominal cavity. The muscle layer was swabbed with ethanol and then this layer was cut through and the liver was excised.

Preparation of liver S9 fraction

The preparation was based on the procedure by Garner *et al.* (1972). All steps of the procedure were carried out at 4°C using cold sterile solutions and glassware. The freshly excised livers were placed in pre-weighed beakers containing approximately 1ml of chilled 0.1 M KCl per gram of wet liver. After the livers were weighed, they were washed several times in fresh, chilled KCl. Successive washings in KCl are essential to ensure a sterile preparation and to remove haemoglobin which can inhibit the activity of the cytochrome P450 enzymes. The washed livers were transferred to a beaker containing 3 volumes of 0.15 M KCl (3 ml/g wet liver) and were minced with sterile scissors and homogenized (with a Polytron homogenizer). The homogenate was centrifuged for 10 minutes at 9000g and the supernatant (S9 fraction) was decanted and saved.

12.3 APPENDIX C

PROTEIN DETERMINATION

Reagents used in Protein Determination using Lowry's technique

Reagent A: 10 ml of 2% (w/v) sodium potassium tartar
(or 0,2 g potassium tartrate + 10 ml water)
10 ml of 1% (w/v) $CuSO_4.5H_2O$
(or 0,1 g $CuSO_4.5H_2O$ + 10 ml water)
1 000 ml of 2% (w/v) Na_2CO_3 on 0,1 M NaOH
(or 10 g Na_2CO_3 in 500 ml of 0,1 M NaOH)

Reagent B: Folin diluted with 2 volumes of water

Bottles were covered with aluminium foil because both reagents are light sensitive.

12.4 APPENDIX D

FAURE'S SOLUTION

Recipe for Faure's solution

Ingredients	*Per 50 ml*
gum arabic	30 g
glycerol	20 ml
chloral hydrate	50 g
distilled water	50 ml

The chloral hydrate was dissolved in water and then the glycerol was added and the ingredients mixed. The gum arabic was suspended in a small gauze bag and allowed to dissolve completely. This solution should not be filtered. Faure's solution should be kept in a cool, dark place in the undiluted form. The solution is ditributed to small plastic containers and is diluted approximately 1:3 in distilled water and allowed to settle for a few days before use. The solution should be fluid and clear hence the solution is left to stand so that the impurities can settle at the bottom of the container. The container should not be shaken and it is recommended that the tip of the pasteur pipette should not touch the bottom of the container when pipetting Faure's solution onto well slides.

12.5 APPENDIX E

DROSOPHILA MEDIA

Recipe for *Drosophila* culture medium

Ingredients	*Per 10 bottles*
agar	10 g
water	240 ml
mealie meal	120 ml
water	200 ml
syrup	60 ml
molasses	15 ml
water	400 ml
yeast	5 g

4g Nipasol M (propyl p hydroxybenzoate) in 4ml 70% ethanol (Nipa laboratories Ltd, Great Britain).

The agar, syrup, molasses and water (240ml + 400ml) were brought to the boil in a large pot. The mealie meal, yeast, and water (200ml) were mixed separately and then added to the boiling mixture. The nipasol was then added to inhibit fungal growth on the medium. About 80 ml medium was then poured into stock culture bottles (volume 400 ml).

For smaller cultures, such as pair matings during the location of the paisley gene crosses and for the heritable translocations and sex-linked recessive

lethals assay, vials (40 ml) were used. These glass tubes are about 7,5 cm high and have a diameter of about 2,5 cm. About 10 ml of medium is poured into each culture vial. The surface of the medium in culture bottles and in vials is allowed to set and dry for 24 hours under a gauze cover to prevent stray flies from laying eggs in the medium. The following day, the surface of the medium is covered with a layer of live yeast. The cultures were stoppered with plastic foam plugs. The yeast is allowed to dry for another 24 hours and the bottles are then ready for use. Vials and stoppers are carefully washed prior to use. Culture bottles are autoclaved before and after use.

New egg-laying medium

This medium is used for the collection of larvae according to the method suggested by Magnusson and Ramel (1990).A solid agar base was made of 5% agar-agar in tap water. The sugar is omitted from the agar base to prevent the larvae from burrowing into the medium. Nipasol is added to the agar and then the agar and water is autoclaved in a large flask for 20 min at 121°C. The flask should be placed in another large container and the flask should be stoppered with cotton wool to prevent the agar from boiling over in the autoclave. The agar base was allowed to dry for twenty four hours and was then completely covered with a thick layer (approximately 5 mm) of a paste of fresh baker's yeast with a little sucrose added. This is allowed to dry under a gauze cover for 24 hours before the introduction of the parents for the egg laying.The agar base bottles without yeast keep well for up to 3 weeks covered with laboratory parafilm (American can company, Greenwich) and kept in the refrigerator at 4°C.

12.6 APPENDIX F

THE SCANNING ELECTRON MICROSCOPE (SEM)

The flies were first killed in a vial by placing cottonwool soaked in acetone over the opening for 5 minutes. Specimens were dried in acetone vapour for two hours by placing cottonwool dabbed with acetone under a circle of filter-paper (Whatman number 1) in a petri dish. The flies were placed on the filter-paper and then the petri dish cover was replaced. After two hours, the wings were removed using fine watchmaker forceps and mounted on SEM aluminium stubs with MIGB conducting glue (a silver paste) (10 wings) and graphite dissolved in ethanol (2 wings). Graphite was very difficult to use because it dries very quickly and there is not enough time to mount the wings. The white glue was spread thinly on the stub, small sections at a time, and then the wings were stuck with the aid of a dissecting microscope.

The specimens were then coated with gold-palladium alloy and after 2 days they were loaded into the Jeol JSM-840 scanning microscope. The acceleration voltage was adjusted to 30 KV and the working distance to 15 mm. The probe current was set at 10 amp 10^{-6}. Brightness and contrast were adjusted and the wings were scanned for mutant spots at a low magnification (550x). When a mutant spot was located, the magnification was increased (up to 10 000x) and the fine focus was adjusted. The stigmators were also used to give the best resolution. Before a photograph was taken, brightness and contrast were again adjusted.

12.7 APPENDIX G

MAINTENANCE OF OR-R STOCKS ON DDT

As directed by Dr Phillip Barnes, Connecticut College, U.S.A.; according to the procedure of Dr Merrell (in Dapkus and Merrell, 1977).

The OR-R stocks were exposed to DDT almost every generation. A stock solution of DDT in acetone (150 mg/ml) was prepared following the same guidelines as for the laboratory use of mycotoxins (Appendix A). A mechanical pipetting aid was used to soak the 25 x 75 mm filter-paper strips (Whatman number 1) with the solution. First 0,5 ml were applied per strip and allowed to dry, and then another 0,5 ml were applied so that a total of 1 ml of DDT solution was applied per strip. If the entire 1 ml is applied at once, then some of it will drip off the strip. After ensuring that the strips were dry, they were added to the culture bottles by placing them at an angle, from one edge of the food bottle interface to the side of the bottle, with the DDT-coated side up. The flies were then transferred to the bottles. The adult flies walk over the strips and eventually settle down on the food surface. The adults lay eggs and then the larvae are exposed to the DDT.

12.8 APPENDIX H

PREPARATION OF SOLUTIONS USED IN CYTOGENETIC OBSERVATION OF SALIVARY GLAND CHROMOSOMES

Insect Ringer's Solution

Ingredient	*Per litre*
NaCl	6,5 g
KCl	0,25 g
$CaCl_2$	0,3 g
sodium bicarbonate	0,2 g
(approximate to give a pH of 7,4)	
distilled water	1000 ml

Acid-washed coverslips

The coverslips were placed in 1N HCl for twenty minutes and then rinsed in distilled water. They were then soaked in distilled water for ten minutes and then placed and stored in 70% ethanol.

Propio Carmine

Ingredient	*Per 100 ml*
glacial propionic acid	45 ml
distilled water	55 ml
carmine	0,5 g

This was boiled gently for several hours in a reflux condenser. It was then shaken well and filtered when cool.

12.9 APPENDIX I

ABBREVIATIONS

2-acetylaminofluorene	2-AAF
2-aminofluorene	2-AF
aflatoxin B1	AFB1
aflatoxin B2	AFB2
aflatoxin G1	AFG1
aflatoxin G2	AFG2
aflatoxin M1	AFM1
2-amino-3-methylimidazo [4,5-f]quinoline	IQ
benzo [a] pyrene	BP
carbon dioxide gas	CO_2
cytochalasin B	CYTB
cytochalasin F	CYTF
cytochalasin J	CYTJ
1,1-bis(p-chlorophenyl)-2,2,2-trichloroethane	DDT
deoxynivalenol	DEOX
diacetoxyscirpenol	DIAC
diethylnitrosamine	DEN
dimethylnitrosamine	DMN
dimethyl sulphoxide	DMSO
deoxyribonucleic acid	DNA
ethylnitrosourea	ENU

fumonisin B1	FB1
fumonisin B2	FB2
Johannesburg	JHB
molecular weight	MW
nicotinamide adenine dinucleotide phosphate	NADP
(reduced form)	NADPH
phenobarbitone/phenobarbital (salt)	PB
p-nitroanisole	pNA
polychlorinated biphenyls	PCBs
ribonucleic acid	RNA
roridin A	RORA
South Africa	SA
South African Institute for Medical Research	SAIMR
sterigmatocystin	STM
ultraviolet	UV
United States of America	USA
verrucarin A	VRCA
vinyl chloride	VC
University of the Witwatersrand	WITS
zearalenone	ZEA

12.10 APPENDIX J

TABLE 12.10.1 SOURCES FROM WHICH TESTER STRAINS AND STOCK CULTURES WERE OBTAINED

TESTER STRAIN	SOURCE
TA97 TA98 TA100 TA102	Miss Heidi Grimmer, Department of Biochemistry, University of the Witwatersrand, Johannesburg.
STOCK CULTURE	**SOURCE**
mwh *flr*^{3}/*TM3Ser* *Basc;Cy/Pm* Lego wild type *bw st* p^p	Institute of Toxicology, Swiss Federal Institute of Technology and University of Zürich, Schwerzenbach, Switzerland.
Cy/Pm Cy/Pm; Sb/Ser *Basc* *Basc; Cy/Pm* *pr* *vg* *pi mwh* Oregon R wild type	Department of Genetics, University of the Witwatersrand.
OR-R	Dr P. Barnes, Connecticut College, U.S.A.
ORR;mwh *ORR;flr*^{3}	Dr A. Nöthlich, Institute of Toxicology, Swiss Federal Institute of Technology and University of Zürich, Schwerzenbach, Switzerland.

BALANCER CHROMOSOMES

First Chromosome Balancers

The Basc chromosome carries the easily recognizable genetic markers Bar eyes and white apricot. Bar produces a narrow eye shape in the homozygous or hemizygous condition and a kidney shaped eye when heterozygous in females. White-apricot flies differ from wild type flies in that the eye pigment is changed to light orange. The *Basc* flies homozygous for Bar and white apricot

are thus characterized by narrow-shaped, light orange eyes. In addition to these visible markers, the *Basc* chromosome contains a combination of two scute inversions: the left hand part of sc^{s1} and the right hand part of *sc*, covering the entire X chromosome, and a smaller inner inversion *In-S* (Spencer and Stern, 1948) [*Basc*: *In* (1) sc^{s1L} sc^{8R} + *In (1) s*, sc^{s1} sc^{8} w^{a} *B* (please refer to Lindsley and Grell for a description of the genetic markers)]. All In (1) sc^{s1L} and sc^{8R} derivatives are duplicated for sc. All are viable and fertile in hemizygous males and homozygous females.

Second chromosome balancers

Cy (Curly wings) and *Pm* (Plum eye colour) are dominant markers located on the second chromosome. Both are homozygous lethals and in this line, the chromosome carries large complex inversions which act as crossover suppressors.

The majority of second chromosome balancers are based on *In(2L)Cy (=In(2L)22D1.2; 33F5-34A1) plus In(2R)Cy(=In (2R) 42 A2,3; 58 A4-B1).* Both of these inversions are spontaneous in origin and the dominant Curly mutation is associated with the 22D1.2 breakpoint of In(2L)Cy. The simplest *Cy* balancer is *In (2L)Cy + In (2R)Cy.*

Other chromosomes useful for balancing chromosome two belong to the Plum family. These inversions act as dominant variegators of brown (2-104,5). Of these, *In (2LR)* bw^{V} = *In (2LR)Pm* is perhaps the most useful. It is *In (2LR) 21 C8-D1-60D1.2 + In (2LR) 40F; 59D4 - E1* and normally carries bw^{V}.

Third chromosome balancers

The third multiple (TM) series

The third multiple series of balancers were designed to provide convenient and effective balancing for chromosome three. All except TM1 are marked with alleles of the bithorax complex.

TM3 is one of the most widely used balancers for chromosome three, although it is far from ideal. It is derived from *T(1;3)sc*$^{260\text{-}20}$, a translocation of the tip of the X chromosome (carrying y^+) to the tip of 3L. This translocation was, itself, induced on *In (3LR) sep + In (3R) C; rip sep bx*^{34e} *e*^{5} [*In (3LR) sep = In (3LR) 65E; 85E*]. The product was irradiated to induce three other inversions *In (3LR) 71C; 94 D-F, In (3LR) 76C; 93A, and In (3LR) 79E; 100C.* TM3 usually carries both Stubble *(Sb)* and Serrate *(Ser)* as dominant markers, although chromosomes with only one of these are available. In the heterozygous form *Sb* leads to bristles on the thorax that are shorter and thicker than normal bristles, while *Ser* leads to wings missing a section of the wing

TABLE 12.10.2 SOURCES OF CHEMICALS USED

CHEMICAL	SOURCE
GENERAL	
Acetone	Central Stores, University of the Witwatersrand, Johannesburg, SA.
Ethanol	
Glycerol	
Propionic acid	
S/M M ASSAY*	
Sodium azide	Miss Heidi Grimmer, Department of Biochemistry, University of the Witwatersrand, Johannesburg.
2-AF	
Agar	Oxoid
Ampicillin trihydrate	Sigma
D-biotin	BDH Chemicals, England.
BSA	Boehringer Mannheim, Germany.
DMSO	Saarchem, SA.
D-glucose-6-phosphate	Sigma, USA.
L-histidine.HCl	Sigma, USA.
NADP	Boehringer Mannheim, Germany.
Nutrient broth	Oxoid
S9 Fraction	Dr Kfir, Department of Water Technology, CSIR, SA.
Tetracycline	Sigma, USA.
SMART TEST	
Biofilm	American Can Company, USA.
CO_2 gas	Afrox
Chloral hydrate	Saarchem, SA.
Instant medium	Carolina Biological Supply Company, USA.
Faure's solution	Made up according to the recipe in APPENDIX D by the Institute of Toxicology (Switzerland) and R. Hillier, V. Phillips and R. Pacella (WITS,Johannesburg,SA).
Filter paper	Whatman Number 1
Gum Arabic	Saarchem, SA.
Mycotoxins	Drs Vleggaar and Horak, Department of Food Technology CSIR,SA
Nipasol	Nipa Laboratories, South Wales, Great Britain.
Sucrose	Saarchem, SA.
Tween 80	Saarchem, SA.
Yeast	Anchor, Epping, SA.
CYTOGENETICS	
Carmine	BDH Chemicals, England.
Euparal	BDH Chemicals, England.
Film	Fujicolor Super HR100, Japan. Kodak technical pan film 2415, ESTAR-AH base, TP 135-36.
DDT TESTS	
DDT	Sigma, USA.
BIOCHEMISTRY	
NADPH	Boehringer Mannheim, Germany.
pNA	Aldrich
Phenobarbital	Be-Tabs Pharmaceuticals, SA.
Sodium dithionite	Saarchem

*The *Salmonella*/mammalian microsome assay

TABLE 12.10.3 SOURCES FROM WHICH EQUIPMENT WERE OBTAINED

EQUIPMENT	SOURCE
Centrifuge, Beckman, model J2-21, rotor type JA20	Beckman
Centrifuge, Beckman, model L5-50, rotor type 50Ti	Beckman
Compound microscope	Olympus, Japan.
Homogenizer	Virtis,Gardiner, N.Y.
Spectrophotometer	Cary model 210, Varian.
Stereo microscope	Olympus, Japan.

CHAPTER 13

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