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GENOTOXICITY OF MYCOTOXINS IN AN IMPROVED *DROSOPHILA* WING SPOT TEST AND OTHER SHORT-TERM TESTS

Rosana Elizabe Pacella

A Thesis Submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg in Fulfilment of the Requirements for the Degree of Doctor of Philosophy.

Johannesburg, 1992

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Rosaria Elizabeth Pacella

On the 18th day of Nov , 1992

To my family for their support and understanding.

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ABSTRACT

Attempts have been made recently to improve the *Drosophila* Somatic Mutation and Recombination wing spot test (SMART) by including insecticide resistance genes that confer high bioactivation (HB) to SMART tester strains. A drawback of the HB lines is the presence of whorling of wing hairs which interferes with scoring 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 constitutive expression of cytochrome P450-dependent activities typical of HB strains. The *ply* gene was separated from the *RI* gene and then new *RI* homozygous, paisley-free HB tester strains were constructed by replacing the SMART wing cell markers flr^3 and *mwh*.

Cytogenetic analysis of salivary gland chromosomes in *RI* heterozygotes from various crosses, revealed regions of asynapsis near the right arm of chromosome two (2R) and along the X chromosome. The asynaptic region near 2R spanned position 65 where the *RI* gene has been mapped. This suggested that amplification of the *RI* gene may be involved in DDT resistance and high bioactivation capacity, but more conclusive evidence is required.

A series of mycotoxins was tested in SMART using 48-hour chronic treatments of 72±4 hour old larvae with two or more of these four different crosses: the standard cross (flr^3 ♀ x *mwh* ♂), the high bioactivation (HB) cross (*ORR;*flr^3 ♀ x *ORR?;mwh* ♂) the improved HB cross (IHB) (*ORR;*flr^3 ♀ x *mwh*

♂) and the new HB cross (*ORR;flr*^{3} ♀ x *ORR;mwh* ♂). Biochemical assays and tests of DDT resistance indicated that the original *ORR;mwh* line may not carry the *RI* gene so that only the new HB cross produced *RI* homozygous progeny. The data indicated that the aflatoxins have both genotoxic and recombinogenic activity in the four crosses and about 70-80% of all induced spots were due to mitotic recombination. Roridin A, Cytochalasin H and J, Fumonisins B1 and B2, diacetoxyscirpenol and deoxynivalenol were tested in SMART using the standard and IHB crosses and were negative at all concentrations. Verrucarin A, aflatoxin (AF) M1, AFG1 and cytochalasin B were tested using the standard and HB crosses and only AFM1 and AFG1 showed genotoxic activity. Aflatoxins B1 and G1 were clearly positive in both the standard and IHB crosses but AFG2 and AFB2 were detected as genotoxic only in the IHB cross, demonstrating the increased sensitivity of the IHB cross for the detection of borderline promutagens. Aflatoxin G1, cytochalasin B, ochratoxin A and aflatoxin G2 were also retested in the new HB cross and sterigmatocystin and zearalenone were tested in the standard cross and the new HB cross. Cytochalsin B and ochratoxin A were also negative in the new HB cross. The new HB lines were the most sensitive to the genotoxic effects of aflatoxin G2 and sterigmatocystin, detecting positive results even at the lower concentrations. Overall, the results indicated that the new HB cross may significantly increase the detectability of genotoxic activities of weak and borderline promutagens compared to the standard and original and improved HB crosses. The main advantage of the new HB cross is to combine the high bioactivation capacity of progeny when both parents of the cross overproduce

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CHAPTER ONE

1 INTRODUCTION

Our environment contains a vast variety of agents which are detrimental to human health. Agents which are acutely toxic are readily detected because the immediate effects allow rapid identification of the source of toxicity. On the other hand, some effects of genotoxic or carcinogenic agents, such as cancer, may only be detected many years after exposure. In humans, the latent period for induction may be as long as 20 years.

The public is mainly concerned with damage caused by industrial chemicals and there is a tendency to consider our "natural" environment safe. The first mutagens discovered in the environment were products of the chemical industry and the reaction of many people (as they realized the hazards of modern society) was to "go back to nature" (Zimmerman and Taylor-Mayer, 1985). It has now become apparent that various natural toxins, some of which have been present throughout vertebrate evolutionary history, are potent mutagens that can cause cancer in vertebrates.

Among 52 naturally occurring compounds tested in at least one species in chronic cancer tests, about half (27/52) are carcinogenic (Ames *et al.*, 1990). Even though the overwhelming weight and number of the chemicals humans ingest are natural, only 77 natural chemicals have been tested in both rats and

mice and again about half (37/77) are rodent carcinogens (Ames and Gold, 1990).

Among 16 mycotoxins tested for carcinogenicity, 11 were positive. Carcinogens included aflatoxin, 5 azacytidine, azaserine, citrinin, griseofulvin, luteoskyrin, mitomycin C, ochratoxin A, sterigmatocystin, streptozotocin and zearalenone. Non-carcinogens included erythromycin stearate, fusarenon X, oxytetracycline hydrochloride and penicillin K (Ames *et al.*, 1990). Nineteen mould toxins have been shown to be clastogenic (Ishidate, *et al.*, 1988).

Mycotoxins are fungal secondary metabolites which cause illness and sometimes death in man and his domesticated animals, following consumption of mycotoxin-contaminated food. Exposure to mycotoxins is difficult to avoid because fungal growth in foods is not easy to prevent. Some of the mycotoxins have been shown to have potent acute and chronic biologic effects *in vitro* and *in vivo*.

Consumption of foods heavily contaminated with mycotoxins has resulted in acute intoxication episodes in human populations. The major concern, however, has focused on the possible contribution of mycotoxins to the development of cancer resulting from long term exposure to low levels of mycotoxins in the food supply. Therefore, preventative action is called for. Our natural and industrial environment has to be monitored for mutagenic and carcinogenic hazards. The goal of genetic toxicology is to identify chemicals which cause genetic damage and genetic alterations at sub-toxic exposure

levels. Short-term mutagenicity tests are now available and are used to achieve this goal. One of the aims of this research project is to encourage the development and application of testing and monitoring procedures in our country, in order to avert human exposure to mutagenic agents in our diet, such as the mycotoxins.

1.1 Genotoxins in our Diet

It was Doll (1977) that suggested that damage to DNA by environmental mutagens (both natural and man-made) is a major cause of cancer and genetic birth defects. Damage to the DNA of germ cells can result in genetic effects that may appear in future generations, while somatic mutation in the other cells of the body may give rise to cancerous cells by altering the normal cellular mechanisms coded for in the DNA. It is now thought that 80% of cancers are lifestyle and environment related (Epstein, 1974; Albrecht, 1991; Weinstein and Groopman, 1991). What people eat and drink and their use of tobacco and other related materials, constitutes part of the lifestyle that characterizes each society and some of these practices can carry hazards (Tomatis, 1990).

Extensive studies in experimental animals and in humans, beginning with the pioneering studies of Tannenbaum (1942), provide evidence that dietary factors play an important role in cancer causation. Epidemiological (Doll, 1977; Willett *et al.*, 1990) and migrant studies (Haenszel and Kurihara, 1968) have shown different incidence rates for certain types of cancer in different parts of the world and that the incidence of cancer in various organs of the

human body is related to diet. Food is a major source of carcinogen uptake since by the age of fifty an average individual has already eaten approximately ten tons dry weight of food (Sugimura, 1978).

Nagao and his colleagues (1985) identified various mutagens in our "normal diets". Mutagenic compounds were found in grilled fish and fried hamburgers, vegetables containig flavonoids, pickles, spices and bracken. Promutagens that are activated after nitrite treatment were found in soy sauce, fava bean, pepper, fish (mackerel-pike) and chinese cabbage. Mutagens in beverages included mutagens in fresh and instant coffee and in alcoholic drinks (Nagao *et al.*, 1985).

Natural chemicals in food which may pose a carcinogenic risk to humans include: compounds released during the chewing of betel nuts with tobacco that have been correlated with oral cancer (Hirono, 1987); the phorbol esters present in the *Euphorbiaceae*, used as folk remedies and herbal teas, are potent mutagens and are thought to be the cause of nasopharyngeal cancer in China and oesophageal cancer in Curacao. Pyrrolizidine toxins are mutagens found in comfrey tea, various herbal medicines and some foods; they are hepatocarcinogens in rats and may cause liver cirrhosis and other pathological states in humans (Hirono, 1987).

It is often assumed that, because naturally occurring compounds are part of human evolutionary history, whereas synthetic chemicals are recent, animals have evolved mechanisms to cope with the toxicity of natural chemicals but,

according to Ames and colleagues (1990), these defense mechanisms are of a general type. Plants and fungi have been evolving and refining their chemical weapons for millions of years and incur large fitness costs in producing their toxins (Ames *et al.*, 1990). If these chemicals were not effective in deterring predators or in conveying a competitive advantage, these toxigenic organisms would not have been naturally selected to produce them (Ames *et al.*, 1990). The human diet has changed drastically in the last thousand years and most humans are eating many recently introduced plants that their ancestors did not. Humans have not had time to evolve into a "toxic harmony" with all the contaminants in their diet (Ames *et al.*, 1990). These findings may lead in the long run to changes in our eating and drinking habits as well as in our way of preparing food.

1.2 Metabolic Activation

Even though damage to DNA by environmental mutagens is a major cause of cancer, there are a few forms of cancer in which genetic make up is the predominant element that determines whether a person will suffer from the disease. Genetic factors are also important in terms of influencing an individual's susceptibility to the effects of environmental agents.

The cytochrome P450 mono-oxygenases provide our first line of defence against the toxic and genotoxic effects of environmental chemicals. However, these enzymes also convert normally inert chemicals into their ultimately toxic and carcinogenic forms (Wolf, 1986) as it is during these P450 cytochrome-mediated metabolic processes that chemically inert carcinogens and mutagens

(pro-carcinogens and pro-mutagens) may be transformed into reactive metabolites (electrophiles) that are capable of reacting with DNA and thus possibly produce genetic damage. The delicate balance between P450-mediated activation and deactivation will determine the toxicological outcome (Wolf, 1990).

The P450 enzymes are widely distributed in nature. They occur in mammals, birds, fish, reptiles, insects, plants, yeast and a few bacteria (Zijistra *et al.*, 1987). In mammals, genes encoding cytochrome P450 proteins have diverged into a series of multigene families and subfamilies scattered throughout the genome. This divergence appears to be a consequence of the selection advantage gained by the increased ability to combat the vast range of structurally diverse toxic compounds to which we are exposed (Wolf, 1990).

There is a great deal of variation in how individual members of a population respond to specific toxic agents and this variation is related to genetically determined increases or decreases in cytochrome P450 expression. There are numerous examples where individual differences in cytochrome P450 expression can explain differences in toxicological response (Wolf, 1990). The observation that the fungal metabolite, aflatoxin B1, is much more toxic and carcinogenic in male than in female rats (Newberne and Wogan, 1968) has been attributed to the expression of a male predominant form of cytochrome P450 with high activity in its metabolic activation (Ishii *et al.*, 1986). This trend is also observed in humans (see Table 1.6 and 1.7) and is

also thought to be due to differences in metabolism between males and females. Profound sex differences in the nephrotoxic and carcinogenic effects of a wide variety of compounds in the mouse have been associated with differential rates of metabolic activation resulting from the sexual dimorphism of kidney cytochrome P450 expression.

1.3 Mycotoxins in Food

Mycotoxins are found on a large variety of foods contaminated with toxin producing fungi, but mostly in fungi-damaged, agricultural produce such as maize, peanuts and rice. Although ingestion of foods and feeds containing mycotoxins is the usual route of exposure, dermal or inhalation exposure may also occur. Mycotoxins can enter the food chain directly when an animal eats food contaminated with mycotoxins which then results in illness (primary mycotoxicosis) or, alternatively, an individual may consume products (milk/meat) from an animal which had ingested mycotoxins (secondary mycotoxicosis). Consequently, there is a growing awareness of the potential hazards of these substances as contaminants of food and feed as the diet of humans and farm animals is made up largely of plant and animal products which are targets of contamination with mycotoxins.

The mycotoxins are now recognized as a major cause of food hygiene problems world wide. In 1985, Mannon and Johnson stated that a quarter of all farm commodities contain mycotoxins and a quarter of the world food crops are ruined by mycotoxins. They also added that 10-50% of the grain crops in Africa and the Far East are contaminated. The presence of

mycotoxin-producing fungi on a commodity, however, is not necessarily indicative of mycotoxin contamination. The chemical composition, pH and moisture content of the substrate, as well as environmental factors such as humidity and temperature must be favourable for mycotoxin production to occur. Almost all food and feed are susceptible to mould growth at some stage during their production, processing, transport and storage (Nyathi *et al.*, 1991).

The impact of fungal toxins upon animals extends beyond their obvious and direct effect in producing death in the wide variety of animals that are likely to consume mycotoxin contaminated grain or feeds. The economic impact of lowered productivity, reduced weight gain, reduced egg and meat production, greater disease incidence because of immune system suppression, damage to body organs and interferences with reproduction is many times greater than that of immediate morbidity and lethality. Furthermore, potential threats of cancer induced by mycotoxins in feeds and human foods are coupled to the universal concerns about health risks.

It is well documented that mycotoxin contamination is more regularly associated with low grade cereals which normally do not enter the human food chain in developed countries. Regrettably, this is not true in many developing countries where the better quality cereals are often exported. In countries where foods are in such short supply, mouldy foodstuffs have to be used to avoid starvation. Periodic episodes of mycotoxicoses are more likely to occur in tropical regions of African and Asian underdeveloped countries

where the environmental conditions, necessary for mycotoxin production, are likely to prevail. In addition, scarcity of food, poverty and lack of regulatory systems to monitor and control mycotoxins, result in ingestion of mycotoxin-contaminated food.

Such occurrences should not occur often in South Africa because, although environmental conditions favour toxigenic fungal growth, heavily contaminated and uncontrolled food is not permitted in the market. In developed countries there are stringent, legislated regulatory mechanisms to ensure that food and feed supplies are of high quality and contain lesser amounts of mycotoxins. Nevertheless, there is concern of possible adverse effects in humans resulting from chronic, low-level exposure to potentially carcinogenic mycotoxins in the food supply (Nyathi *et al.*, 1991). In rural South African areas, however, food shortage is often a problem, and mouldy food is often consumed. A few years ago, there was also a case of a well known South African breakfast cereal which contained high levels of aflatoxins (Albrecht, 1991).

The few estimates of the economic costs associated with mycotoxins that have been made indicate that these losses are staggering. In 1984, Linstrom estimated that losses in the export market world wide for only five items (rice, maize, peanuts, barley and cottonseed) would approach 1,5 billion dollars in 1985. Losses are due to harvest rejects, poor health and death of farm animals and, in developing countries, losses are also derived from regulatory programmes to reduce the risk to animals and human health. Taking all these

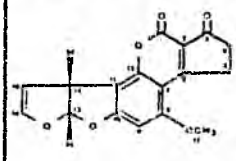
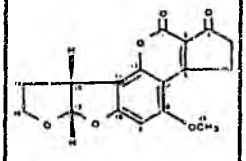
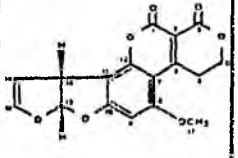
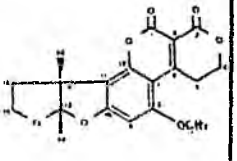
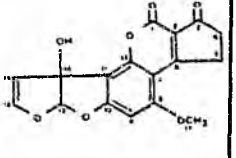
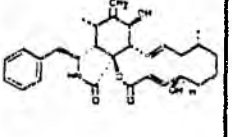
losses into account the economic burden resulting from mycotoxin contamination is truly immense (Pohland and Wood, 1987).

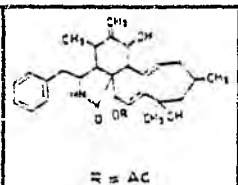
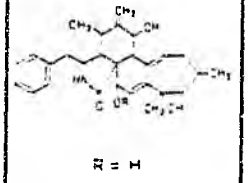
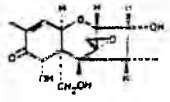
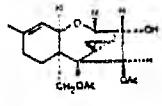
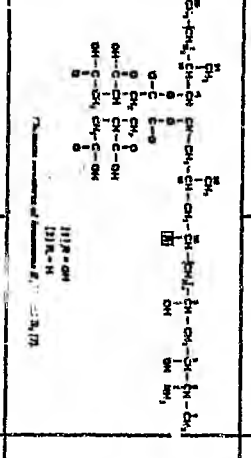
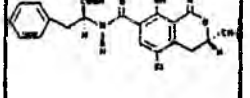
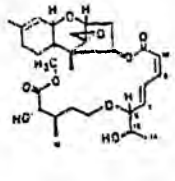
The structure and properties of the mycotoxins examined in this research project are listed in Table 1.1. The effects of these mycotoxins in humans and animals, their possible involvement in human illnesses, together with the commodities in which they have been found are listed in Table 1.2. These mycotoxins were chosen because they are currently considered to pose the greatest potential risk to human and animal health as food and feed contaminants. Although their toxic effects are well documented, very little work has been done to establish their genotoxicity in short-term, *in vivo* tests.

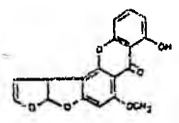
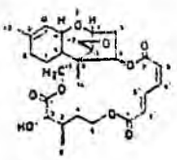
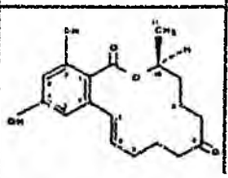
Aflatoxins are produced primarily by some strains of *Aspergillus flavus* and most strains of *A. parasiticus* plus the related species *A. nomius* (Kurtzman *et al.*, 1987). Under favourable conditions of temperature (25,7-27°C) and humidity, these fungi grow on certain foodstuffs and produce aflatoxins (Nyathi *et al.*, 1991). The warm ambient temperatures and prolonged drought conditions essential for the pre-harvest mould growth and production of aflatoxin in peanuts and maize are typical of Southern Africa. Post harvest production of aflatoxins on maize and peanuts is favoured by warm temperatures and high humidity typical of Mozambique, Thailand, Phillipines, Natal in South Africa and many other parts of the world.

Aflatoxins are potent liver toxins and aflatoxicosis is primarily a hepatic disease. These toxins may be lethal when consumed in large doses.

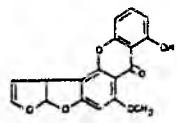
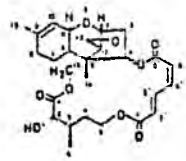
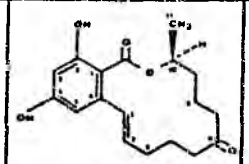
TABLE 1.1 PRODUCING ORGANISMS AND PROPERTIES OF THE MYCOTOXINS TESTED

MYCOTOXIN	MOLECULAR WEIGHT	FORMULA	STRUCTURAL FORMULA	PRODUCING ORGANISMS
AFLATOXIN B1	312,29	$C_{17}H_{12}O_6$		Aspergillus flavus Link ex Fries, A.parasiticus Speare, A.nomius
AFLATOXIN B2	314,31	$C_{17}H_{14}O_6$		Aspergillus flavus Link ex Fries, A.parasiticus Speare, A.nomius
AFLATOXIN G1	328,29	$C_{17}H_{12}O_7$		Aspergillus flavus Link ex Fries, A.parasiticus Speare, A.nomius
AFLATOXIN G2	330,31	$C_{17}H_{14}O_7$		Aspergillus flavus Link ex Fries, A.parasiticus Speare, A.nomius
AFLATOXIN M1	328,29	$C_{17}H_{12}O_7$		Aspergillus flavus Link ex Fries, A.parasiticus Speare, A.nomius
CYTOCHALASIN B	479	$C_{29}H_{37}NO_5$		Phoma exigua, Helminthosporium dematioideum, Hormicium spp

CYTOCHALASIN H	493	$C_{30}H_{39}NO_6$	 R = AC	Phomopsis paspalli
CYTOCHALASIN J	451	$C_{28}H_{37}NO_4$	 R = H	Phomopsis paspalli
DEOXYNIVALENOL	296	$C_{15}H_{20}O_6$		Fusarium roseum
DIACETOXY-SCIRPENOL	366	$C_{19}H_{26}O_7$		Fusarium equiseti, F.scirpi, F.solani, F.diversisporum, F.tricinctum, F.anguioides, Gibberella intricans
FUMONISIN B1	719,92	$C_{34}H_{66}NO_{16}$		Fusarium moniliforme
FUMONISIN B2	703,92	$C_{34}H_{66}NO_{14}$		Fusarium moniliforme
OCHRATOXIN A	403,843	$C_{20}H_{18}NO_6Cl$		Aspergillus ochraceus Penicillium viridicatum
RORIDIN A	532	$C_{29}H_{40}O_9$		Myrothecium verrucaria, M.roridum

STERIGMATO-CYSTIN	524,30	$C_{18}H_{12}O_6$		A.versicolor
VERRUCARIN A	502	$C_{27}H_{34}O_8$		Myrothecium verrucaria, M.roridum
ZEARALENONE	318,39	$C_{18}H_{22}O_5$		Fusarium graminearum F.roseum

Sublethal doses produce a chronic toxicity and low levels of chronic exposure can result in cancer (Wogan and Newberne, 1967), primarily liver cancer in most animal species (Wogan, 1973; Busby and Wogan, 1984). The carcinogenicity of AFB1 in various animal studies is presented in Table 1.3.

STERIGMATO-CYSTIN	324,30	$C_{18}H_{12}O_6$		A.versicolor
VERRUCARIN A	502	$C_{27}H_{34}O_9$		Myrothecium verrucaria, M.roridum
ZEARALENONE	318,39	$C_{18}H_{22}O_5$		Fusarium graminearum F.roseum

Sublethal doses produce a chronic toxicity and low levels of chronic exposure can result in cancer (Wogan and Newberne, 1967), primarily liver cancer in most animal species (Wogan, 1973; Busby and Wogan, 1984). The carcinogenicity of AFB1 in various animal studies is presented in Table 1.3.

TABLE 1.2 COMMODITIES IN WHICH MYCOTOXIN CONTAMINATION HAS BEEN FOUND AND THE RESULTING EFFECTS ON ANIMALS AND HUMANS

MYCOTOXIN	COMMODITIES FOUND CONTAMINATED	AFFECTED SPECIES	PATHOLOGICAL EFFECTS OF MYCOTOXINS
AFLATOXINS (B1, B2, G1, G2, M1)	Peanuts, maize, wheat, rice, cottonseed, nuts copra, various foods, milk, eggs, cheese	BIRDS Turkey, poult, quail, pheasant chick, duckling, mature chicken MAMMALS Young pigs, cat pregnant sows dog, calf, sheep, mature cattle, monkey, human FISH LABORATORY ANIMALS	Hepatoxicity (liver damage) Bile duct hyperplasia Haemorrhage (intestinal tract, kidneys) Carcinogenesis (liver tumours)
OCHRATOXIN A	Cereal grains (wheat,barley, oats, maize), dry beans, mouldy peanuts, cheese, tissues of swine	Swine, dog, rat, duckling, chicken, human	Nephrotoxicity (tubular necrosis of kidney) Porcine nephropathy Mild liver damage Enteritis Teratogenesis Carcinogenesis (kidney tumours)
STERIGMATOCYSTIN	Green coffee, mouldy wheat, Dutch cheeses	Mouse, rat	Carcinogenesis Hepatotoxicity
FUMONISIN B1	Maize, wheat, mixed feed	Horse, rat, humans?	Equine leukoencephalomalacia, cancer promoting activity in rats, oesophageal cancer?
TRICHOTHECENES (diacetoxyscirpenol, deoxynivalenol, verrucarin A, roridin A)	Maize, wheat, commercial cattle feed, mixed feed	Swine, cattle, dog, cat, turkey, mouse, rat, chicken, human	Digestive disorders (emesis, diarrhoea, refusal to eat) Haemorrhage (Stomach, heart, intestines, lungs,bladder, kidney) Edema, Oral lesions Dermatitis, Blood disorders

ZEARALENONE	Maize, mouldy hay, pelleted commercial feed	Swine, dairy cattle, chicken, turkey, rat, mouse, guinea pig, lamb	Oestrogenic effects (edema of vulva, prolapse of vagina enlargement of uterus) Atrophy of testicles Atrophy of ovaries, enlargement of mammary glands Abortion Cervical cancer?

Adapted from Bullerman, 1979, 1981, 1986.

TABLE 1.3 CARCINOGENICITY OF AFB1 IN VARIOUS ANIMALS

(oral administration)

ANIMAL	DOSE (µg/kg diet)	EXPOSURE DURATION	LIVER TUMOUR FREQUENCY	REFERENCE
Mouse	1000	70 weeks	0%	Wogan, 1969
Porton rats	5000	9 weeks	100% (14/14)	Butler and Barnes, 1968
Fischer rats*	15	68-80 weeks	100% (12/12)	Wogan and Newberne, 1967
Trout*	0,1	20 months	10%	Halver, 1969
Salmon	12	?	45%	Wales and Sinnhuber, 1972
Guppy*	6000	11 months	56% (9/16)	Sato et al., 1973
Duck	30	14 months	73% (8/11)	Carnaghan, 1965
Marmosets	2000	9-55 weeks	11% (1/9)	Lin et al., 1974
Tree shrews	2000	74-172 weeks	♀ 60% (6/10) ♂ 38% (3/8)	Reddy and Svoboda, 1975
Monkey	500 mg (total dose)	6 years	100% (1/1)	Adamson et al., 1973

*These studies mention that no tumours occurred in control animals
?exposure duration not specified

Data from IARC monographs on the evaluation of the carcinogenic risk of chemicals to man: Some naturally occurring compounds, vol. 10, pp 51-72 (1975).

In 1975 the evidence of a causal relationship between aflatoxin intake and liver cancer incidence was considered circumstantial (IARC, 1975). Since then, however, the evidence has become conclusive and in 1987, aflatoxin B1 was labelled a group 1 carcinogen (IARC, 1987b). Aflatoxins appear high on the list of 52 agents that have been shown to be causally related to human cancer. Group 1 carcinogens are established human carcinogens and the target organ of aflatoxin B1 is the liver (Vainio and Wilbourn, 1991).

The carcinogenicity of AFB1 is believed to be due to metabolic activation of the compound by microsomal enzymes (P450 cytochromes) in the mammalian liver that generate an epoxide (Garner, 1973a; Swenson *et al.*, 1974). This electrophilic intermediate metabolite binds to macromolecules (DNA, RNA, proteins) within hepatocytes (Garner, 1973b; Swenson *et al.*, 1974). In *in vitro* mutagenicity tests, AFB1 activated by rat liver microsomal preparations has caused point mutations and frame shift mutations in host cell DNA (Berry, 1988). AFB1 also binds covalently to DNA *in vitro* and this binding can cause structural alterations in the DNA. The genotoxic activity of AFB1 in various short-term tests and mammalian *in vitro* and *in vivo* tests is compiled in Tables 1.4 and 1.5, respectively.

Primary hepatocellular carcinoma (liver cancer) is the seventh most common cancer in the world and is one of the leading causes of cancer mortality in Sub-Saharan Africa and Asia (Parkin *et al.*, 1984). The age-standardized incidence rates per 100 000 of liver cancer in african males are recorded in Table 1.6. Incidence rates for males in Canada and the U.S.A. are drastically

In 1975 the evidence of a causal relationship between aflatoxin intake and liver cancer incidence was considered circumstantial (IARC, 1975). Since then, however, the evidence has become conclusive and in 1987, aflatoxin B1 was labelled a group 1 carcinogen (IARC, 1987b). Aflatoxins appear high on the list of 52 agents that have been shown to be causally related to human cancer. Group 1 carcinogens are established human carcinogens and the target organ of aflatoxin B1 is the liver (Vainio and Wilbourn, 1991).

The carcinogenicity of AFB1 is believed to be due to metabolic activation of the compound by microsomal enzymes (P450 cytochromes) in the mammalian liver that generate an epoxide (Garner, 1973a; Swenson *et al.*, 1974). This electrophilic intermediate metabolite binds to macromolecules (DNA, RNA, proteins) within hepatocytes (Garner, 1973b; Swenson *et al.*, 1974). In *in vitro* mutagenicity tests, AFB1 activated by rat liver microsomal preparations has caused point mutations and frame shift mutations in host cell DNA (Berry, 1988). AFB1 also binds covalently to DNA *in vitro* and this binding can cause structural alterations in the DNA. The genotoxic activity of AFB1 in various short-term tests and mammalian *in vitro* and *in vivo* tests is compiled in Tables 1.4 and 1.5, respectively.

Primary hepatocellular carcinoma (liver cancer) is the seventh most common cancer in the world and is one of the leading causes of cancer mortality in Sub-Saharan Africa and Asia (Parkin *et al.*, 1984). The age-standardized incidence rates per 100 000 of liver cancer in african males are recorded in Table 1.6. Incidence rates for males in Canada and the U.S.A. are drastically

lower and are included in the table for comparative purposes. In South Africa the incidence of liver cancer is among the highest in the world, and cancer of the liver is one of the most common cancers amongst black males (Cancer Registry of South Africa, Annual Report compiled by Metz and colleagues 1987). Table 1.6 indicates that the incidence of liver cancer in South Africa, although still high, has dropped considerably over the last few years, probably due to the present careful monitoring of aflatoxin in foods. Nevertheless, the higher incidence in South African blacks suggests that aflatoxin contamination of foods may still be occurring in black rural areas. Liver cancer rates vary worldwide by at least 500-1 000 fold (Okuda and Beasly, 1982). Those regions with the highest liver cancer rate correspond to areas where the climate favours contamination of foodstuffs by aflatoxins. There have been a number of epidemiological studies designed to obtain information on the relationship between the currently estimated dietary intake of aflatoxins and the incidence of liver cancer.

Information is available from Uganda (Alpert *et al.*, 1968, 1971), the Phillipines (Campbell and Salamut, 1970), Swaziland (Peers and Linsell, 1973; Peers *et al.*, 1987), Thailand (Shank *et al.*, 1972a and b), Kenya (Peers and Linsell, 1973), Mali (Bayo *et al.*, 1990), Transkei (van Rensburg *et al.*, 1990), Zimbabwe (Nyathi *et al.*, 1987), and Mozambique (van Rensburg, *et al.*, 1985). The latter country had the highest incidence of liver cancer in the world at the time of the study (1985) as well as the highest estimated daily per capita ingestion of aflatoxins. There was a positive association between high intakes of aflatoxin and high cancer incidence rates (Table 1.7). The

most apparent in adult men (Carlborg, 1979). The incidence of liver cancer in these studies was a linear function of the log dietary intake of aflatoxins (Shank, 1977; Linsell and Peers, 1977).

TABLE 1.4 MUTAGENICITY OF AFLATOXINS IN SHORT-TERM TESTS

AFLATOXIN	BACTERIA (a) MUTATION	BACTERIA (a) DNA DAMAGE	FUNGI (b)	YEAST (c)	SLRL (d)	SMART (d)
B1	+	+	+		+	+
B2	+	-	-	-		
G1	+	+	+	-		
G2	?	-	-			
M1	+					

(a) *Salmonella typhimurium*
(b) *Neurospora crassa* (Ong, 1971)
(c) *Saccharomyces cerevisiae*
(d) *Drosophila melanogaster* (Lamb and Lilly, 1971; Graf *et al.*, 1984)
(+) = positive result
(-) = negative result
(?) = conflicting results

Data taken from IARC monographs on the evaluation of carcinogenic risk to humans, Supplemant 6 pp 40-56 (1987), supplement 7 (1987), pp 83-87 (supplement 7 is an updating to volumes 1-42). World Health Organization/International Agency for Research on Cancer (IARC), Lyon, France.

TABLE 1.5 GENETIC EFFECTS OF AFLATOXINS IN VARIOUS MAMMALIAN *in vivo* AND *in vitro* ASSAYS

AFLATOXIN	CHROM. ABER.	CHROM. BREAK.	MICRO-NUCLEI	SISTER CHROMATID EXCHANGES	UNSCH. DNA SYNTH.	COV. BIND. DNA	CELL TRANS
AFB1							
Human cells in vitro	-		+	+	+	+	
Rodent cells in vitro	+	+		+	+		+
Rodent cells in vivo	+	+	+	+	+	+	
AFB2							
Human cells in vitro					-		
Rodent cells in vitro				+	+	+	
Rodent cells in vivo						+	
AFG1							
Human cells in vitro					+		
Rodent cells in vitro	+		+		+		
Rodent cells in vivo	+					+	
AFG2							
Human cells in vitro					-		
Rodent cells in vitro				+	+		
AFM1							
Rodent cells in vitro					+		

(+) = positive result
(-) = negative result
CHROM. ABER. = Chromosome aberrations
CHROM. BREAK. = Chromosome breakage
UNSCH. DNA SYNTH. = Unscheduled DNA synthesis
COV. BIND. DNA = Covalent binding to DNA
CELL TRANS. = Cell Transformation

Data taken from IARC monographs on the evaluation of carcinogenic risk to humans, Supplement 6 pp 40-56 (1987), supplement 7 (1987), pp 83-87 (supplement 7 is an updating to volumes 1-42). World Health Organization/International Agency for Research on Cancer (IARC), Lyon, France.

TABLE 1.6 LIVER CANCER IN MALES IN AFRICA SOUTH OF THE SAHARA

REGION	AGE-STANDARDIZED INCIDENCE RATES/ 100 000
Sudan (Khartoum 1970-71)	3
Sudan (Port Sudan 1966-69)	21
Ivory Coast (Dabou 1968-73)	15
Ghana (Agogo 1972-73)	17
Nigeria (Ibadan 1960-65)	12
Nigeria (Ilesha 1954-67)	9
Sn Nigeria (Abeokuta, Takum, Egbe, Anua 1972-73)	14
Mid-Nigeria (Vom 1968-73; Tungan 1972-73; Wusasa 1972-73)	13
Cameroon (Ebolova 1972-73)	13
Gabon (Lambarene 1950-65)	12
Zaire (Nyakunde 1972-73)	4
Zaire (Katana 1953-60)	21
Zaire (Kellensburger 1972-73)	18
Rwanda and Burundi (1968-73)	13
Uganda (Kyadondo 1964-68)	15
Kenya (Central Nyanza 1965-66)	14
Kenya (Nairobi 1968-70)	9
Tanzania (Dar es Salaam 1969-71)	13
Malawi (Blantyre 1968-72)	11
Zambia (Lusaka 1968-71)	27
Zambia (Kitwe 1968-71)	18
Zimbabwe (rural 1972-73)	18
Zimbabwe (southern, Bulawayo 1963-67)	35
Botswana (1960-72)	18
Mozambique (Lourenco Marques 1956-60)	104
Swaziland (1964-1968)	32
South Africa (Johannesburg 1953-55)	20
South Africa (Johannesburg blacks 1953-55)	14,2*
South Africa (Johannesburg blacks 1953-55, 70 year age group)	119,0*
South Africa (Acornhoek 1957-66)	36
South Africa (Natal 1964-66)	28
South Africa (rural Natal and Transkei 1972-73)	37
South Africa (Cape Province 1956-59)	25
South Africa (whole male population 1986)	4,8
South Africa (whole male population 1987)	4,4
South Africa (black males 1986)	6,0
South Africa (black males 1987)	6,1
South Africa (black females 1987)	1,7
South Africa (whites 1986)	2,5
South Africa (whites 1987)	1,5
South Africa (coloureds 1987)	1,3
South Africa (asians 1987)	0,2
Canada (Newfoundland 1978-82)	1,2a
U.S.A. (Connecticut: white 1960-67)	2,7*a
U.S.A. (Connecticut: white 1981-85)	2,5a
U.S.A. (California, Los Angeles County: white 1978-82)	2,3a
China (Shanghai 1981-85)	34,4a
Phillipines (1981-85)	17,5a

* = average annual incidence per 100 000 (not standardized)
a = these values are included for comparative purposes
Sn = Southern

Data from Cook-Mozaffari and Burkitt (1990), Metz and colleagues (1986, 1987); Doll *et al.* (1970); Waterhouse *et al.* (1976, 1982) and Muir *et al.* (1987).

TABLE 1.7 INGESTION OF AFLATOXINS AND LIVER CANCER INCIDENCE IN HUMANS

Population	Dietary Aflatoxin intake (mg/kg of body weight/d	Cases of liver cancer in adults >15 years old			
		Men		Women	
		N°/100 000 population/ year	Incidence	N°/100 000 population/ year	Incidence
Kenya					
High alt.	3-5	1	3,1	0	0
Medium	6-8	13	10,8	6	3,3
Low alt.	10-15	16	12,9	9	5,4
Swaziland					
Highveld	5-9	9	7,9	2	1,4
Middle veld	9-14	24	14,8	5	2,2
Lebombo	15-20	4	18,7	0	0
Lowveld	43-53	35	26,7	7	5,6
Thailand					
Songkhala	5-8	-	-	-	-
Ratburi	45-77	-	-	-	-
Mozambique	222	-	35	-	15,7

Adapted from Szmuness (1978)
alt. = altitude
d = day
Data were compiled for one year in Thailand, three years in Mozambique, and four years in Kenya and Swaziland.

A characteristic of aflatoxin exposure in dairy cattle is the conversion of AFB1 to the hydroxylated metabolite AFM1 and the excretion of AFM1 in milk. AFM1 has been found in milk from a variety of species and is hepatocarcinogenic in rats and trout (Busby and Wogan, 1984). In countries with a high rate of liver cancer, a peak incidence is seen at a relatively early age. In Inhambane Province in Mozambique, the age specific incidence rate for males

in the 20-30 year old age group per 100 000 is 43,5 (van Rensburg *et al.*, 1985) with a similar trend in other high incidence countries including Zimbabwe (Munoz and Bosch, 1987). It is possible that this incidence pattern could be influenced by environmental carcinogens early in life. Wild and colleagues (1987, 1990) detected the presence of AFM1 in breast milk from mothers in Zimbabwe and Thailand. It seems that in countries where aflatoxin contamination is likely to be highest, the period of breast feeding is longer than two years. This may present a source of prolonged exposure to this hepatocarcinogen. Taking into account that young animals are more susceptible to the adverse effects of aflatoxins (Vesselnovitch *et al.*, 1972) this may explain the early onset of liver cancer in these regions. AFM1 is stable in raw milk and processed milk products and is unaffected by pasteurization or processing into cheese and yoghurts. In a study of AFM1 incidence in cow's milk by Brown (1982), 23% of samples in South Africa were positive. Relatively high levels in some American milk samples prompted the American Food and Drug Administration (FDA) to set a maximum allowable AFM1 level of 0,5 µg/l of milk. AFM1 in milk is a health hazard and regulations covering milk for consumption by babies is strict in most countries, especially in Switzerland where the level of AFM1 in reconstituted powdered milk is limited to 10 ng/ml.

The mycotoxin sterigmatocystin is produced by several species of *Aspergillus*. Sterigmatocystin is a precursor in the biosynthesis of aflatoxin and chemically resembles the aflatoxins (Hsieh *et al.*, 1973). It has been detected at low

concentrations in green coffee, mouldy wheat and in the rind of hard Dutch cheese (Bullerman, 1981; Scott, 1985; Vesonder and Horn, 1985).

Sterigmatocystin is mutagenic in various *in vitro* tests such as the Ames test, the *Rec* assay and the *Bacillus subtilis* assay (Berry, 1988). Tumorigenicity of this mycotoxin was considerably lower than that of AFB1 in rats (0,1 to 0,001) (Berry, 1988). Sterigmatocystin bonded covalently to DNA at more or less 20-30% of the level observed with AFB1 and caused liver cell carcinoma in laboratory rats (Purchase and van der Watt, 1970).

The trichothecenes are a family of over 148 structurally related compounds produced by several fungal genera (*Fusarium, Cephalosporium, Myrothecium, Stachybotrys* and *Trichoderma*). There are several naturally occurring trichothecene mycotoxins produced in foods and feeds by *Fusarium* species including verrucarin A, roridin A, deoxynivalenol and diacetoxyscirpenol. Deoxynivalenol contamination of maize and wheat has been significant in some crop years. In 1980 and 1981 in Canada, and in 1982 in the United States, deoxynivalenol was found in wheat as a result of severe infestations with the wheat scab fungus *F. graminearum*. The toxicological feature of the trichothecenes (depletion of lymphoid cells) is clearly connected with the development of immunological disorders, and the immunosuppressive effects on humans exposed to these toxins for long periods of time may result in the development of aetiologically unknown diseases. The possibility that these trichothecenes may enhance the carcinogenicity of other mycotoxins (such as AFB1) through their immunosuppressive effects when they occur simul-

taneously in wheat cannot be eliminated. The Canadian government has set tolerance levels below 2 ppm of deoxynivalenol for wheat intended for human consumption and of 1 ppm for all grains for use in infant foods. Similarly in Russia, the maximum tolerated level for trichothecenes has been set at the lowest detection limit (Schuller *et al.*, 1982).

Zearalenone, an oestrogenic mycotoxin causes vulvo-vaginitis and oestrogenic responses in swine. Zearalenone is produced primarily by *Fusarium graminearum* occurring naturally in high moisture maize, mouldy hay and pelleted feeds. Zearalenone has been detected in maize meal and beer in a number of African countries (Lovelace and Nyathi, 1977; Marasas *et al.*, 1977; Martin and Keen, 1978) and in corn flakes used for human consumption in Canada and the United States (Scott *et al.*, 1978) so it is essential to monitor its presence in the human diet and assess the long term effects of exposure (Smith and Moss, 1985), especially since zearalenone has been shown to be carcinogenic (Ames *et al.*, 1990). In Belgium, zearalenone tolerance levels have been set at 0 ng/g.

Ochratoxins are a group of structurally related metabolites that are produced by *Aspergillus ochraceus* and related species as well as certain *Penicillium* species. Ochratoxins are primarily nephrotoxic causing kidney damage in rats, dogs and swine. The major mycotoxin in this group is ochratoxin A.

Although the mutagenicity of ochratoxin A has not been detected by several *in vitro* assays including the Ames test and *rec* assay, tumorigenesis/

carcinogenesis was reported in laboratory animals (Ueno, 1984). Renal and hepatic neoplasms have been induced in mice fed 40 ppm ochratoxin A for 20 months (Ueno, 1984). These results were confirmed in a study performed under the direction of the American National Institute of Environmental Health Sciences as a function of the American National Toxicology Program (Boorman, 1988).

Ochratoxin A has been suggested to be a factor in the aetiology of a human disease known as Balkan endemic nephropathy (Krogh, 1977; Smith and Moss, 1985). Then, in 1980, Stark reported a 40% increase in the incidence of urinary tract cancer in eastern europeans with Balkan nephropathy. This condition is now believed to be the result of chronic ingestion of ochratoxin-contaminated grains grown in these eastern european regions. In view of its possible association with cancer of the urinary tract, the maximum tolerated level of ochratoxin A in all foods has been set at 0 ng/g in Russia and Belgium (Schuller *et al.*, 1982), at 25 ng/g in pork meats and at 10 ng/g in pork liver and kidney in Denmark.

The cytochalasins are a group of fungal secondary metabolites which affect the functions of microtubules (polymers of tubulin) and microfilaments (polymers of actin), the two cytoskeletons that regulate locomotion, cell division, cell membrane phenomena and other cellular functions. Some forty fungal metabolites are known to possess this cytoskeleton-modifying activity and these are collectively called "cytochalasins" (Hsieh, 1987). These cytotoxins inhibit locomotion and cytoplasmic cleavage (cytokinesis) causing

nuclear extrusion and changes in cell shape (Carter, 1967). As spindle fibres are made up of microtubules, these compounds could also interfere with the movement of chromosomes during cell division and perhaps cause non-disjunction.

Fumonisins are newly described mycotoxins isolated from *F. moniliforme* (Gelderblom *et al.*, 1988). This organism is a frequent, almost universal, inhabitant of maize and is involved in producing equine leukoencephalomalacia, a disease of horses (Marasas *et al.*, 1976) and is hepatocarcinogenic in rats (Marasas *et al.*, 1984; Jaskiewicz *et al.*, 1987). Fumonisin B1 was described as being able to reproduce the disease in horses (Marasas *et al.*, 1988). In addition, fumonisin B1, isolated from a culture of *F. moniliforme* was shown to have cancer promoting activity in rats (Gelderblom *et al.*, 1988).

All these dangerous mycotoxins should, if possible, be eliminated from food supplies. Prevention of liver cancer should be a priority in cancer prevention as this disease accounts for high mortality in the African continent; its therapy is costly and the results are poor. The introduction of programmes to reduce aflatoxin intake by better storage of grains and peanuts and by monitoring aflatoxin content of foods is considered one of the best prevention strategies.

In South Africa, government laboratories and the Oil Seeds Board, working in conjunction with the Department of Health, continuously monitor the raw products of the peanut butter industry to ensure that aflatoxin concentrations

remain far below danger levels. According to Mr Lui Verhoef at the Oil Seeds Board, several million rands are spent annually to keep peanut butter products safe for the consumer. There are seventeen selection plants where farmers are required to send their peanuts to be tested. Blemished and unhealthy peanuts are thrown out and, after selection, samples of the raw material are tested to ensure that the level of aflatoxin B1 is not higher than 5 μg and total aflatoxin not higher than 10 μg, per kg of peanuts. These regulations were set down by the Department of Health according to the limits set by the FDA - in the U.S.A. (FDA, 1978; Dichter, 1984).

The problem is that the end products, the peanut butter itself, milk and breakfast cereals, are not regularly tested for genotoxicity in this country to ensure that what the consumer eats is actually safe. In fact, there is no genotoxicity testing for any component of the daily diet in South Africa, nor of food preservatives, additives or colourants. The detection of so many potent naturally occurring genotoxins, such as the aflatoxins, has lead to an international growing concern about environmental mutagenesis and the need to screen the environment for mutagenic activities and hazards (Zimmerman and Taylor-Mayer, 1985).

1.4 Carcinogenicity Testing in Mammals

Mammals have played an indispensable role in studies aimed at the determination of the genetic risk of an exposed human population (Ehling, 1980). Rodent bioassays have been extremely useful. Almost all the known human carcinogens tested positive in animals, and many compounds that cause

cancer in rats or mice, later turned out to be human carcinogens. Rodent assays are now the only way to identify chemicals that cause tumours. In current strategies to prevent human cancer, chronic rodent bioassays are the major source of information used to predict the risk to humans from chemical exposures.

However, screening with mammals should be avoided in accordance with recommendations to minimize the number of animals used in research (Würgler, 1983). Recently, with pressure from organizations such as the Society for the Prevention of Cruelty to Animals to decrease the large number of animals used in experiments, there has been a move against using large numbers of vertebrate animals in screening tests. Unfortunately, it is often difficult to detect genotoxic effects in the more humane experiments, employing only a small number of animals, as it is possible, due to small sample size, to miss effects and it therefore becomes difficult to make conclusions about the mutagenicity of a compound.

At this university the Animal Ethics Committee has set regulations on cage size, type of feed administered, as well as temperature at which experimental animals are kept. Animals are to be kept well nourished in their respective standard diets and disease-free. The establishment of this code of ethics has made experiments involving mammals very expensive. Cancer is a slowly developing disease, and when using mammals, it may be necessary to expose these animals to the compounds for more than two years for cancer to develop. This is not only time consuming but also considered inhumane by

those who take the pain the animals have to suffer into account. With this wave of humane feelings towards the treatment of laboratory animals, most mammalian *in vivo* mutagenicity tests have become very unpopular.

Furthermore, Abelson (1987) stated that many major substances have been labelled carcinogens through the use of questionable evidence. Chemicals are screened for possible carcinogenicity by tests on shorter lived animals such as mice and rats, by subjecting the animals to massive doses of the chemical to be tested, and the levels used vastly exceed those to which humans are likely to be exposed. He added that many of the experiments proving a potential carcinogenicity of a chemical for humans, have been performed on inbred strains of mice that have a natural high incidence of liver tumours. Very often, researchers choose these expensive strains of mice for their experiments because these mice are guaranteed to develop cancer in 90 days.

McConnell (1987) argued that the primary purpose of the tests is to identify chemicals most likely to present a potential hazard to humans. In the case of chemicals, animals are able to detoxify thousands of times faster than humans and to obtain an equivalent dose to the target tissue in such a case would require much more of the chemical in animals. Furthermore, the use of fifty animals per dose group can only detect cancer occuring at a relatively high incidence and therefore the effect in animals needs to be optimized.

Over the past 16 years a long list of man-made chemicals have been labelled potential human carcinogens. A carcinogenic potency data base, set up in the

early 1980s, contains the results of some 4 000 experiments assessing the carcinogenicity of about 1 000 different chemicals in rats and mice (Marx, 1990). More than half of those chemicals were found to cause cancer in at least one species. Ames and Gold (1990) felt, like Abelson (1987), that the large number of positives is essentially an artifact of the way the animal tests are done. The chemicals are usually administered in the maximum tolerated doses (MTDs) which are the highest doses that can be given without causing severe weight loss or other overt signs of toxicity. MTD levels are much higher than the doses to which humans are likely to be exposed but they are used to cut down on the number of animals and thus on the cost required to obtain statistically significant results. Although the MTDs do not cause overt signs of toxicity, they can still have more subtle toxic effects. According to Ames and Gold (1990), this is what accounts for the large number of compounds that test positive for carcinogenicity. They propose that a chemical given at the MTD damages a mammalian organ killing some of the cells and thereby triggering a compensatory increase in cell proliferation to repair the injury. The increased cell proliferation in turn increases the opportunity for mutations that can cause cells to become cancerous. Below the toxic dose of this chemical, carcinogenesis would not be a problem because there would be no increased cell proliferation. Cohen and Ellwein (1990) also found that cell proliferation induced by toxicity played a major role in carcinogenicity. A small number of animals exposed to a potential carcinogen for two years at the MTD may result in an overestimate of risk because when the most sensitive animal species is selected and the highest

tolerated dose is used, there is a potential danger of obtaining false positive results (Vainio and Wilbourn, 1991).

The number of compounds synthesized, as well as the number of natural compounds isolated, is growing steadily and it has therefore become difficult to perform *in vivo,* statistically sound carcinogenicity studies with intact vertebrate animals for every compound. It is inhumane to use these tests for the purpose of hazard identification when the same results can be achieved with the use of lower organisms. There is a need for shorter, cheaper, but nevertheless accurate predictive tests for carcinogenicity. The development of these tests has been dramatic over the last three decades.

1.5 Short-term Tests for Mutagenicity

About 28 years ago, Bruce Ames and his colleagues developed a simple bacterial test (the Ames test, now known as the *Salmonella*/ mammalian microsome assay) for identifying chemical mutagens and used it to show that about 90% of the organic chemical carcinogens tested were mutagens (McCann *et al.*, 1975b; Donahue *et al.*, 1978). The test is used extensively for the preliminary screening of compounds and in 1979, the test was in use in over 2 000 government, industrial and academic laboratories throughout the world and a number of companies have made important economic decisions on the basis of this test (Ames, 1979). The sensitivity of the *Salmonella* test makes it useful for rapidly obtaining information about mutagenic components in complex mixtures such as natural products, air and water pollutants, pyrolized materials, body fluids and the impurities in industrial chemicals.

However, already in 1976, Purchase and colleagues noted that the Ames test missed some potent carcinogens. In 1987, Zeiger surveyed 224 chemicals and reported that the percentage of carcinogens identified as mutagens (the sensitivity of the test) was only 54% and the percentage of non-carcinogens identified as non-mutagens, (the specificity of the test), was only 70%. Tennant and colleagues (1987) tested 73 known carcinogens and non-carcinogens in the Ames test and found that this assay only identified about 45% of the carcinogens.

The test mainly detects chemicals that become electrophiles and attach to DNA. The electrophilic portion of the molecule may be present either because of the particular structure of the chemical or as a result of metabolic activation. The test, however, misses carcinogens that do not form reactive electrophiles. These non-genotoxic carcinogens are thought to operate by a range of other mechanisms, including hormonal changes. Ashby and Tennant (1988) concluded that there is a correlation of around 0.9 between structural analysis (looking at reactive sites on a chemical) and *Salmonella* assay results. They suggest that the two methods, analysis of chemical structure and the *Salmonella*/mammalian microsome assay, when used in combination, will reveal two groups of carcinogens, thos that are genotoxic, and those that cause cancer in other ways.

Several mammalian, short-term *in vivo* tests have been developed. The mouse micronucieus test is an *in vivo* assay used to detect clastogens and spindle poisons. At short intervals, after one or two treatments with a potential

carcinogen, the polychromatophilic erythrocytes in bone marrow are screened for the presence of micronuclei (chromatin fragments).

As previosly mentioned, alteration of DNA is one of the underlying mechanisms of mutagenesis and carcinogenesis. DNA-repair synthesis is a general response to many types of DNA damage at non-toxic concentrations. The hepatocyte primary culture/DNA repair test has been developed as a reliable short-term test for chemical carcinogens. The test detects DNA damage as unscheduled DNA synthesis and the test system has been shown to correspond to carcinogens of a wide variety of structural classes (Mori *et al.*, 1986).

In an evaluation of short-term tests for carcinogens, Purchase (1981) concluded that the *in vivo* mammalian micronucleus, SCE and sperm abnormality tests were not suitable as the initial screening tests for potential carcinogens. Furthermore, the mice still have to be killed at the end of the test. It was then that short-term, *in vivo* tests using lower eukaryotes (such as *Neurospora crassa, Saccharomyces cerevisiae* and *Drosophila melanogaster*) were developed. These tests drastically reduce the number of mammals sacrificed, and at the same time identify the most hazardous carcinogens.

1.6 The Advantages of *Drosophila* for Genotoxicity Testing

Assays with the fruit fly *Drosophila melanogaster* represent a useful intermediate between studies with microorganisms and studies with mammals and man (Würgler and Graf, 1990). This insect is the eukaryote most often used to detect mutations in germ cells. *D. melanogaster* is ideally suited to screen chemical compounds for mutagenic activity because it offers the following advantages: a short generation time of only ten days; low cost of culture media; easy breeding of large numbers of animals and several well-defined and validated test systems measuring different genetic end points in germ cells as well as somatic cells for the detection of genotoxic compounds (Würgler, Sobels and Vogel, 1984). *Drosophila* offers the quickest system for detecting mutation and chromosome aberrations available in animals today (Sobels, 1974).

D. melanogaster was one of the first animals to be intensively studied genetically (Graf et al., 1992b). T.H. Morgan and his students used *Drosophila* to elucidate the mechanisms of Mendelian inheritance and constructed the first linkage maps. In biochemical genetics and more recently in molecular genetics, *Drosophila* has proved useful in every respect (Graf *et al.*, 1992 b).

Mutation research with *Drosophila* began in 1927 when H.J. Muller discovered that X rays induce sex-linked recessive lethals and using a test for these recessive lethals, Auerbach and Robson (1947) published the first unequivocal evidence of a chemical mutagen. The following year, M. Demerec made the

first attempts to test chemical carcinogens for mutagenic activity in *D. melanogaster* (Demerec, 1948). Since that time, many chemicals, including both carcinogens and non-carcinogens, have been tested for mutagenicity in *D. melanogaster*.

Whilst with most test systems only one class of genetic damage can be studied at a time, *Drosophila* permits the assessment of the total spectrum of genetic effects. The wealth of special markers, the ease with which specific tester strains can be constructed for nearly every genetic end point and the possibilities to extend the studies to the molecular level by determining DNA adducts and molecular changes in mutations, make *Drosophila* a powerful tool to study the genotoxic effect of chemicals. Useful inversions and other rearrangements make it possible to test genetic changes ranging from recessive lethal mutations and small deficiencies to translocations (reciprocal, symmetrical exchanges), non-disjunction (of X chromosome or autosomes), mitotic or meiotic recombination and dominant lethal or chromosome loss as an indication of open, unrepaired breaks (Sobels, 1974). Salivary gland polytene chromosomes make cytogenetic analysis possible. Moreover, the small number of chromosomes has the advantage that a test for recessive lethals covers a sizeable section of the total genome.

One class of genetic damage that merits special consideration is constituted by non-disjunction. Non-disjunction presents a serious hazard to man in view of the high frequency of spontaneous abortions and congenital defects associated with the gains or losses of chromosomes. Congenital defects are

also associated with chromosome breakage and rearrangements (such as viable translocations) induced in the germ line. Non-disjunction, chromosome breakage and rearrangements can be studied in *Drosophila.*

Technically, *Drosophila* is a very versatile tester organism. The routes of application: feeding, injection, inhalation (for volatile compounds and gases) and the type of application [chronic or acute (for unstable compounds)] treatments of adults or larvae, can be selected depending on the characteristics of the chemical being studied in order to maximize the dose to the target organ. Internationally accepted standard testing protocols have been developed and are used for routine screening (Graf *et al.*, 1990).

In addition, *Drosophila* possesses a well developed, xenobiotic metabolism capable of activating promutagens to mutagenic/genotoxic products (Würgler, 1983). Wilkinson and Brattsten (1972) found that insect (housefly) microsomes possess a similar degree of metabolic versatility and substrate non-specificity to those from the mammalian liver. Then extensive promutagen testing experiments showed that various indirect carcinogens that are negative or barely positive in other systems, are highly mutagenic in *Drosophila* (Vogel, 1975) and that *Drosophila* adults have a versatile metabolic capacity to activate most classes of promutagens and genotoxic procarcinogens (Vogel and Sobels, 1976; Valencia *et al.*, 1984).

Vogel (1980) observed genetic variation in the enzymatic make up within the species *Drosophila melanogaster*. Both mutagenicity and toxicity data with

mutagens that require activation by cytochrome P450 indicated a higher efficiency of the microsomal metabolism in the insecticide resistant Hikone R strain, when compared with the sensitive strain Berlin K, presumably also constituting the backround for the insecticide resistance. Strains that show resistance to insecticides and other toxic substances suggest the involvement of a multi-function detoxification system more efficient than sensitive strains.

Tsukamoto (1958) discovered the gene responsible for insecticide resistance in Hikone R and named it *RstDDT*. In the same year Kikkawa (1958) found that most DDT resistance in Hikone R was due to a single, dominant region of the second chromosome, but other resistance genes with minor effects were probably also present on th and third chromosomes. In 1961 Kikkawa mapped the *RI* (resistance to insecticides) or Rst(2)DDT resistance gene to 65 cM or map units on the second chromosome.

In 1985, Hällström and Blanck studied the genetic regulation of some P450-dependent enzyme activities in adult *Drosophila* strains having genetically determined high (insecticide resistant strains Oregon R-(R) and Hikone R) or low enzyme activities. These strains were crossed with a marker strain and the metabolism was analysed in microsomes from hybrids carrying different combinations of chromosomes from the strain under test. The authors found that high pNA demethylation, biphenyl-3-hydroxylation (P450 cytochrome-dependent activities) and an increased amount of protein with an apparent MW of 54 KDa after SDS-gel electrophoresis of the microsomes [corresponding to a P450 enzyme (Hallström *et al.*, 1983; Waters and Nix, 1988) in

insecticide resistant strains are inherited as dominant second chromosome traits.

The higher metabolism of vinyl chloride (VC) (Magnusson *et al.*, 1979), increased DMN demethylation (Waters *et al.*, 1983), as well as the more marked toxicity (Hällström *et al.*, 1982) and mutagenicity (Vogel, 1980) of dimethyinitrosamine (DMN) in insecticide resistant strains, indicates a correlation between the genetic regulation of insecticide metabolism and the metabolism of the compounds mentioned. This correlation could be due to one or more changes in a structural gene or to mutation at a regulating locus.

The genes regulating VC metabolism and DMN toxicity have been further localized; like the *Rl* gene mapped by Kikkawa (1961) both are located at 64-66 cM (Hällström *et al.*, 1982). An intragenic localization of pNA demethylation and biphenyl-3-hydroxylation also located the gene coding for these enzyme activities around 65,0 cM on the second chromosome (Hällström, 1985). Hällström and Blanck (1985) suggest that all these characteristics could be determined by the same gene or a cluster of closely linked genes, and that the resistance to insecticides is achieved by a mutation(s) giving a high capacity for cytochrome P450-mediated detoxification of the insecticides. The wide range of metabolic activities affected in the resistant strains suggests that a regulatory gene mutation may be involved (Hällström and Blanck, 1985). The apparently complete dominance of the metabolic pattern in resistant animals could be explained by a mutation in a trans-acting regulatory gene, even if several other explanations are possible (Hällström and

Blanck, 1985). A regulatory gene mutation has also been proposed as the basis of insecticide resistance in the housefly (Plapp, 1984) and a change in a regulatory gene affecting the cytochrome P450 enzyme system is observed in mice (Nebert and Jensen, 1979).

The low capacity for benzo [a] pyrene hydroxylation and 7-ethoxycoumarin deethylation in Hikone R seems to be determined by two genes located close together on the third chromosome. The capacity for biphenyl-4-hydroxylation was shown to be determined by two genes on the second chromosome, one located to the left of the gene *black* (48,0 cM) and the other at 63,0 cM. Hällström's data demonstrate the presence of at least 4 or 5 genes determining cytochrome P450 activity in *D. melanogaster*. According to Hällström (1986) the gene at 65 cM regulates both O and N dealkylations, and aromatic and aliphatic hydroxylations. This further stresses the complexity of the P450 system.

In microsomes prepared from *Drosophila* larvae a nearly four fold higher cytochrome P450 content was observed in the insecticide resistant strains compared with sensitive strains (Hällström *et al.*, 1984). A high P450 content is also correlated with insecticide resistance in the housefly *Musca domestica* (Wilkinson and Brattsten, 1972).

Another molecular mechanism by which enzyme levels can be increased is gene amplification (Waters and Nix, 1988). Gene amplification has been proposed as a mechanism involved in P450-dependent insecticide resistance

(Oppennorth, 1984; Brattsten *et al.*, 1986). Waters and Nix (1988) conclude that further studies using P450-specific gene probes are necessary to establish whether gene amplification is involved in P450 expression in insecticide resistant *Drosophila* strains, and by what mechanism it is regulated.

1.7 Short-term *Drosophila* Tests

Several different short-term mutagenicity tests have been developed in *Drosophila*. The sex linked recessive lethals (SLRL) assay is a test for genetic changes which in hemizygous or homozygous, but not in heterozygous, conditions kill the developing individual at some time between fertilization and the adult stage. Such genetic factors, called recessive lethals, can be induced on all chromosomes, but the detection of factors induced on the X chromosome require only two test generations, whereas those induced on the autosomes require three generations.

The most commonly employed test (Basc or Muller-5) is a variant of the classical *CIB* test developed by Muller in the late 1940's but first published by Spencer and Stern in 1948. Wild type males, characterized by normal, round, red eyes are treated and mated to virgin females homozygous for the *Basc* chromosome (for a description of the *Basc* chromosome see APPENDIX J).

Gene mutations, small deletions as well as certain types of chromosome aberrations can lead to recessive lethality. If a mycotoxin causes deletions and chromosome breakage then this would lead to recessive lethality. Since

most deletions will act as recessive lethals, substances that produce no or few recessive lethals, are not likely to produce many small deletions.

Recessive lethal mutations are much more frequent than viable visible mutations (Auerbach, 1962). For this reason SLRL tests are preferred in routine testing procedures. Another reason for choosing recessive lethals as the genetic change to be scored is that the criterion used to decide whether a mutation is present or not is very objective. The decision is based on observing whether or not a certain Mendelian class is present. If the genetic markers are available, this criterion can be used by different workers throughout the world in the same way.

The SLRL test is very sensitive because the X chromosome represents about 20% of the whole genome. The X chromosome corresponds to about 1 000 bands in salivary gland chromosomes. It has been estimated that some 600-800 genes on the X chromosome can mutate to recessive lethals (Abrahamson *et al.*, 1980). The test is relatively sensitive and detects point mutations as well as genetic changes connected with chromosome breakage. With certain mutagens positive results are obtained at concentrations which do not induce dominant lethals or chromosome aberrations (Vogel, 1975). An important advantage of this test is that a representative part of the *Drosophila* genome is assayed and that, as many loci are tested, sensitivity and specificity differences between loci are averaged out (Vogel, 1981).

In contrast to the SLRL test, the heritable translocations test scores for a very specific type of genetic damage: one particular type of chromosome aberration (Würgler and Graf, 1985). Translocations result from two or more breaks in two or more chromosomes, followed by reunion of the broken ends in such a manner that each newly constructed chromosome has one centromere. Such translocations are transmitted to succeeding generations and may be detected by phenotypic markers on separate chromosomes which, after the rearrangement, appear to be linked ("pseudo-linkage") (Würgler and Graf, 1985). Treatment-induced translocation results in suppression of independent Mendelian assortment as individuals with unbalanced genotypes die during development. The absence of Mendelian classes in the F2 progeny of the treated P1 male is considered an indication that a translocation occurred between the marked chromosomes in the germ line of the treated male.

The advantage of testing for translocations is that, because of their very low rate of spontaneous occurrence, controls become practically superfluous. Moreover, data on the induction of translocations provide a clear indication of the capacity of a chemical to break chromosomes because translocations are exchanges of fragments derived from broken chromosomes. Substances that produce chromosome breaks and then lead to the formation of rearrangements between broken chromosomes (viable balanced translocations) in germ cells carry a concomitant risk of causing exposed women to give birth to congenitally malformed children.

For many years, the test for sex linked recessive lethals (SLRL) was the most commonly used genotoxicity assay with *D. melanogaster*. However, for routine screening the test is relatively expensive and time consuming. Most of these germ cell assay systems (such as the SLRL assay and the heritable translocations test) involve two or more fly generations and are somewhat tedious. Even in a specialized laboratory, the dose finding experiments and the actual test with one repeat and appropriate controls, take at least two months (Würgler, 1983). In addition, the SLRL assay and the heritable translocations test have the disadvantage that for weak mutagens, and non-mutagens, large numbers of chromosomes must be tested. Several carcinogenic polycyclic hydrocarbons and somatic amines have failed to induce SLRL mutations in spermatogenic cells of *Drosophila* or exhibited only very weak genotoxic effects (Vogel, 1988).

For these reasons it was suggested that use be made of somatic cells of *D. melanogaster* for mutagenicity testing (Vogel, 1987). One-generation test systems using somatic cells might improve the detection capacity and versatility of the *Drosophila* assays (Vogel *et al.*, 1985; Würgler and Vogel, 1985)

The correlation between mutagenic and carcinogenic activity has generated much interest in the genotoxic activity of chemicals in somatic cells. Since the essential changes that make cells cancerous seem to be mainly somatic mutations, somatic test systems are an ideal choice for screening compounds for potential carcinogenicity.

As a holometabolic insect undergoing complete metamorphosis, *Drosophila melanogaster* offers unique possibilities to study genotoxic effects in somatic cells. During early development groups of cells (imaginal discs) are set apart. They proliferate mitotically during the three larval instars and, at metamorphosis, they differentiate into adult structures (for example eyes and wings). A mutation occurring in one of these imaginal disc cells during proliferation, will be present in all the descendant cells, and these will form a clone of mutant cells.

Somatic genotoxicity assays are based on the identification of genetic events (mitotic recombination, gene mutation and deletions) induced in mitotically dividing primordial cells of the developing imaginal discs. In individuals heterozygous for visible marker mutations certain mutagenic events can lead to the loss of the wild type (normal) allele opposite the marker mutation, with subsequent expression of the recessive marker allele in an ensuing clone of mutant cells. Such a one generation test system using eye colour markers was first devised by Mollet and Würgler (1974). In their test system mutational events in the primordial cells of the eye led to mosaic spots in the eyes of adult flies. It seemed that polycyclic hydrocarbons and aromatic amines were detectable as mutagens, at least in the eye imaginal discs.

The two main eye somatic tests systems which have been used in the past few years are: the white/white-coral (w/w^{co}) eye mosaic system (Vogel, 1985) and the unstable white-zeste (z-w) eye mosaic system (Fujikawa *et al.*, 1985). Another one-generation somatic system is based on two wing trichome

markers expressed on the wing blade of adult flies (*mwh* and *flr*^{3}). These markers allow the detection of recombinogenic and mutagenic activities of chemicals. This wing spot test is a somatic mutation and recombination test (SMART) and was developed by Graf *et al.*, (1983; 1984; 1989).

Eye tests do not detect small clones whereas single mutant cells are visible in the wing blade. One wing blade contains about 30 800 cells (Garcia-Bellido and Merriam, 1971) and, consequently, many more mutagen-exposed cells can be analysed compared with the eyes. Testing wing mosaicsm is also more accurate and sensitive. It is important to note that different somatic tests assay different genetic events. The *w/w*^{co} and *mwh/flr*^{3} assays detect mitotic recombination, chromosome aberrations (including deletions), aneuploidy and gene mutations. Although with the *z-w* marker configuration small spots can be scored more reliably, this system does not allow detection of twin spots indicative of recombination; this assay only detects point mutations and probably small deletions (Vogel, 1985).

The wing spot test in *Drosophila* is fast and easy to perform for the detection of the mutagenic and recombinogenic activity of chemical compounds. So far, the validation of this somatic test has shown that it is at least as sensitive as the SLRL assay but has the advantage of being quicker (Graf and Würgler, 1986), cheaper and less laborious. From published and unpublished data (Graf *et al.*, 1989; Vogel and Würgler, 1990, respectively), it is estimated that the somatic mutation and recombination tests in *D. melanogaster* have been validated so far with over 330 chemical compounds and complex mixtures

tested. The table of 330 chemicals is at present still confidential, but a condensed version of this table will be published soon (Graf, 1992, personal communication).

Although *Drosophila* possesses a well developed xenobiotic metabolism, there are nevertheless important differences in the ability of this insect to metabolize certain xenobiotics when compared with mammalian systems (Zjilstra *et al.*, 1987). Two classes of procarcinogens, the polycyclic aromatic hydrocarbons and the aromatic amines, are difficult to detect in *Drosophila* assays (Fahmy and Fahmy, 1970; Baars, 1980; Vogel *et al.*, 1983). The low sensitivity of the *Drosophila* assays for aromatic compounds, such as benzo (a) pyrene, exemplifies the problem of the low detection capacity for most polycyclic aromatic hydrocarbons (Vogel *et al.*, 1983; 1988; Graf and Würgler, 1988; Frölich and Würgler, 1990a).

1.8 Improvement of SMART

The first attempts to improve the detection capacity of *Drosophila* assays made use of strains carrying different DNA repair defects. In the early 1980s most emphasis was placed on the study of excision repair defective mutants (Würgler, 1983). Then, recently, in an attempt to improve the overall sensitivity of the test system, a variation of SMART, which makes use of repair defective cells, was investigated (Graf *et al.*, 1990). The reason for introducing repair-defective cells lies in the fact that the complete or partial block of certain repair pathways may lead to increased frequencies of induced mutations. The results obtained with eight chemicals showed, however, that

the introduction of the excision repair defective mutant meiotic 9-L1 (*mei-9*^{L1}) into the wing spot test system does not bring about an enhanced detection of genotoxic compounds (Graf *et al.*, 1990). Furthermore, this version of the wing spot test is more tedious and more subject to variations caused by the properties of the *mei-9* mutant (Graf *et al.*, 1990). Graf and Würgler (1986) also found that the cross between *mwh* females and *flr*^{3} males (repair proficient) was more sensitive in detecting promutagens that require metabolic activation than the cross producing repair deficient progeny. The use of the cross involving the *mei-9* mutant was therefore discontinued for routine screening of compounds with unknown genotoxic activities (Graf *et al.*, 1990). The cross *mwh* females to *flr*^{3} males became the standard cross in SMART assays. It appeared as a possible drawback that these standard tester strains were metabolically uncharacterized (Hällström, 1986). The SMART standard lines have only been characterized indirectly for their metabolic capabilities through genotoxicity testing of model promutagens (Frölich and Würgler, 1990a).

Commonly used tester strains (Berlin K and Canton S) all have a low capacity for several cytochrome P450-dependent reactions, while a substrain of Oregon R (Oregon R Resistant or OR-R) is the only strain with an overall higher metabolic capacity (Hällström, 1986). Hällström (1986) suggested the analysis of the cytochrome P450 system in the standard strains, or a transfer of the genetic markers into the stocks used for the detection of germline mutations. This optimal activity of the activating systems in *Drosophila* screening tests would be an obvious advantage.

The sensitivity and performance of the SMART assay may be improved by using flies that are able to activate the compounds tested more efficiently, especially in the case of compounds which show lower effects in this mutagenicity system, or in borderline cases that may be positive at only one concentration. Weak mutagens may be negative in the standard cross because of toxic effects and it would be desirable to test these compounds at a low concentration (below the critical toxic concentration), but at the same time the concentration should be high enough to cause a detectable mutation rate. The detection capacity of SMART could therefore be optimized by using high bioactivation lines that are more sensitive to the effects of promutagens so that the promutagen may be detected at sub-toxic concentrations.

Frölich and Würgler (1989) attempted to improve the tester strains of the wing spot assay with respect to their metabolic capacity, thereby improving the detectability of promutagens in SMART. Since the genotoxicity assay relies on genetic markers on the third chromosome, the authors decided to introduce, by means of chromosome substitution, genetic determinants for increased metabolic activation capacity, most probably controlled by genes located on the second chromosome, and possibly also by genes located on the first chromosome, into the SMART tester strains. They substituted chromosomes 1 and 2 in the original *mwh* and *flr*^{3} tester strains with chromosomes 1 and 2 from the OR-R strain. This would alter the genetic control of the P450-dependent systems thereby increasing the metabolic capacity of the tester strains and optimizing SMART performance. Frölich and Würgler (1989) showed that they were successful in introducing this genetic condition into

the SMART tester strains. This new set of tester strains, used in the high bioactivation (HB) cross, appears to convey an increased sensitivity to the genotoxic effects of nitrosamines. in assays with the model promutagen diethylnitrosamine (DEN) an increased sensitivity of about 2,5 fold was found for the HB strains. In fact, the use of the HB cross (*ORR;mwh x ORR;flr*^{3}) leads to a significant increase in genotoxicity of a number of promutagens compared with the standard cross (*mwh* x *flr*^{3}*/TM3Ser*) (Frölich, 1989; Frölich and Würgler, 1989). Frölich and Würgler (1990a) undertook a more precise characterization of the metabolic activation systems and found that the HB cross significantly increased the detectability of genotoxic activities of polycyclic aromatic hydrocarbons compared with the standard cross.

Unfortunately, the HB cross and the resulting *ORR/ORR;mwh* +/+ *flr*^{3} individuals present four main disadvantages (Frölich and Würgler, 1989, 1991a; Graf *et al.*, 1991; Graf and van Schaik, 1992) discussed below making the HB cross undesirable for use in the somatic mutation and recombination test. However, Graf and colleagues (1991) suggest several ways of improving the cross including the use of *flr*^{3} as female parents since experience had shown that *flr*^{3} females are more fertile than *mwh* females. The frequencies of spontaneous spots observed with the standard cross using water or solvents, were comparable to those observed previously with the reciprocal standard cross (Alonso Moraga and Graf, 1989). From this it can be concluded that the larvae derived from the reciprocal cross exhibit identical properties in the wing spot test. The reciprocal cross using *flr*^{3} as females is

now employed routinely (Graf and van Schaik, 1992) and this cross has become the new standard cross of the SMART assay.

An additional technical improvement to the SMART assays for all crosses is the collection of larvae according to the method of Magnusson and Ramel (1990) (Graf *et al.*, 1991), instead of the sucrose or saline density separation procedure of Nöthiger (1970). Both larva-collecting methods were used in this research project and the advantages and disadvantages of each technique are discussed and compared.

An unexplained characteristic of OR-R strains is the presence of irregular whorling in the pattern of wing hairs. Adler (1992) suggests that this alteration in the orientation of the trichomes may be due to mutations in genes that disrupt the polarity of the wing hairs. This whorling of wing hairs makes analysis of the wings difficult as the whorling interferes with scoring for mutant spots and may result in inaccuracies, especially with inexperienced scorers. Although the use of OR-R larvae can improve the SMART detection capacity, many researchers prefer not to use this strain due to this undesirable characteristic.

A third disadvantage is a high variability of results from repeated experiments (Graf and van Schaik, 1992). Frölich and Würgler (1991a) recommended that any laboratory using the HB cross should first check the integrity of the stocks. This is best done by checking the sensitivity of the HB cross to urethane or diethylnitrosamine. The authors stress that when doing these

checks it should be kept in mind that for reasons as yet not understood in detail, the quantitative results from repeated experiments might vary up to a factor of two. Therefore, if discrepancies are encountered a repeat of the checks should be performed and results from both experiments should be considered in judging the status of the tester strains. The HB cross is therefore more time consuming and less reliable than the standard cross.

The frequencies of spontaneous spots observed and recorded with the HB cross (ORR homozygous individuals from the cross *ORR;mwh x ORR;flr^3/-TM3Ser)* were consistently higher than the standard cross. One of the reasons for this was the difference in score due to difficulties encountered in those areas of the ORR homozygous wings showing wing hair pattern disturbances (Graf and van Schaik, 1992).

An additional disadvantage of the HB cross is the delay in development of the ORR homozygous individuals compared with the standard cross. When the 3-day-old larvae of these two crosses are collected for treatment, those of the HB cross are visibly smaller than those of the standard cross. Frölich (1989) and Frölich and Würgler (1990a,b) state that the delay is about 12 h when the larvae pupate and approximately one day when the flies eclose. This difference makes a direct comparison of the results obtained with the HB cross with those of the standard cross difficult.

Based on information arising from this study, an Improved High Bioactivation cross for the SMART wing assay was developed (Graf *et al.*, 1991). As

discussed in section 2.2, the genetic determinant(s) responsible for the whorling pattern has been located on chromosome two and is/are thus linked to the gene(s) responsible for the high bioactivation. Since the RI gene which codes for the increase in cytochrome P450 related activities is dominant, and the whorling gene is recessive, the difficulties associated with whorling of the wing hairs can be overcome by using one parent ORR and the other standard.

Graf and van Schaik (1992) used the standard mutagen DEN in two concentrations with each of the following crosses: 1) *flr*^{3} ♀ *x mwh* ♂, 2) *mwh* ♀ *x flr*^{3} ♂, 3) *ORR;flr*^{3} ♀ x *ORR;mwh* ♂, 4) *ORR;mwh* ♀ *x ORRflr*^{3} ♂, 5) *ORR;flr*^{3} ♀ x *ORR;mwh* ♂, 6) *mwh* ♀ *x ORR;flr*^{3} ♂, 7) *flr*^{3} ♀ x *ORR;mwh* ♂, 8) *ORR;mwh* ♀ *x flr*^{3} ♂.

The authors wanted to determine if the hybrids *ORR;flr*^{3} x *mwh* and *flr*^{3} x *ORR;mwh* would show increased sensitivity to the mutagenic effects of DEN without the increased control mutation rate which is probably produced by the whorling disturbance on the wing blades. It was believed that progeny from crosses 7 and 8 would behave in a similar way to progeny from crosses 5 and 6 because progeny should also be heterozygous for the *RI* gene and should also show a high bioactivation capacity. The surprising result was that one hybrid, *ORR;flr*^{3} female x *mwh* male gave the desired result with a sensitivity even better than the ORR x ORR cross; but the other (*flr*^{3} female x *ORR;mwh* male) hybrid was not at all increased in sensitivity and behaved just like the standard cross.

Apparently, the pure lines had never been checked for sensitivity. It seemed then that the *ORR;mwh* line had the gene for whorling but not the gene for resistance to DDT. Although it seemed that the whorling of wing hairs and the high bioactivation are separable phenotypically, it does not mean necessarily that they are not caused by the same gene, but it does seem to show that if they are caused by the same gene, there are different alleles giving different combinations of the two properties. It became necessary to test the *ORR;mwh* stock, together with other stocks, for resistance to DDT and to determine if the *RI* and whorling genes could be separated.

Another interesting result is that the progeny from cross 6 (*mwh* ♀ x *ORR;flr*^{3} ♂) are not as sensitive to the effect of DEN as progeny from the reciprocal cross 5 with *ORR;flr*^{3} as the female parent. Although progeny from crosses 5 and 6 are heterozygous for the *RI* gene it seems that the progeny differ in their bioactivation capacity. This demonstrates that the high constitutive expression of cytochrome P450 seems to show a maternal effect which was also investigated in this research project.

Graf and van Schaik (1992) named the cross *ORR;flr*^{3} females to *mwh* males the Improved high bioactivation (IHB) cross. The authors then presented a series of experiments comparing the standard cross (*flr*^{3} x *mwh*); the HB cross (*ORR;flr*^{3} x *ORR;mwh*) and the IHB cross (*ORR;flr*^{3} x *mwh*) tested with the promutagens diethylnitrosamine (DEN); 7,12-dimethyl benz(a)anthracene (DMBA); N-nitrosopyrrolidine (NNP) and urethane (URE); and the direct acting mutagen ethylnitrosourea (ENU).

Concurrent water controls were included for each cross. The frequency of spontaneously occurring total spots per wing varied for the different crosses. The frequency of spontaneous spots observed in the standard cross is comparable to frequencies observed previously with the reciprocal standard cross (Graf and van Schaik, 1992), and within the usual range (Alonso-Moraga and Graf, 1989). This further indicates that the larvae derived from reciprocal standard crosses exhibit identical properties in the wing spot test. The frequency of spontaneous spots observed with the improved HB cross (giving rise to ORR2 heterozygous individuals) are also in the same range as the standard cross. However, those recorded with the HB cross (giving rise to ORR2 homozygous individuals) are higher than the others. This is probably due to the difficulties encountered in those areas of the ORR homozygous wings showing wing hair pattern disturbances when scoring the wings for mutant spots.

The treatments with these four promutagens led to significantly increased frequencies of spots per wing in all three crosses. However, the frequencies of spots were considerably enhanced when using the HB cross or the IHB cross. When comparing the results obtained with the IHB cross with the results obtained with the HB cross, the frequencies of spots per wing were identical or even higher. It therefore seemed that the hybrid larvae of the IHB cross had a P450-dependent bioactivation capacity equal to or even slightly higher than those of the original HB cross (Graf and van Schaik, 1992).

With 1,0 and 2,5 mM DEN the total spot score per wing was significantly higher in the ORR heterozygous flies than in the ORR (supposedly) homozygous flies. With URE, NNP and DMBA there was no statistically significant difference between the two crosses. Both crosses also show the same type of spot distribution for single and twinspots. However, when compared with the standard cross, both were characterized by the presence of a significant fraction of spots of the larger size classes. It is likely that this is due to the more efficient bioactivation of these compounds in the ORR homozygous and ORR heterozygous larvae leading to an earlier induction of mutation and recombination.

In order to check further the characteristics of the newly developed improved HB cross, the authors also tested it with the direct-acting alkylating agent ENU. The frequency of spots obtained with the standard cross and the improved HB cross were practically the same. This indicates that the characteristics of the larvae of the improved HB cross are similar to those of the standard cross with the exception of the high bioactivation capacity. The same properties were observed by Frölich (1989) for the reciprocal HB cross and the reciprocal standard cross.

There are two main disadvantages in using ORR heterozygous flies. The first is that ORR homozygous flies might have a higher bioactivation capacity than the ORR heterozygotes as the DDT resistance gene might not be completely dominant. Assuming that the *ORR;mwh* stock does not carry the *Rl* gene, both the HB cross and the IHB cross (*ORR;flr*^{3} *x ORR?;mwh* and *ORR;flr*^{3} x

mwh, respectively) would produce *RI* heterozygous progeny. It would be desirable to develop a high bioactivation cross that would give rise to *RI* homozygous progeny to determine if these larvae would show an even higher bioactivation capacity and sensitivity to the effects of promutagens than the heterozygotes. The second is that several events could knock out the wild type whorling gene resulting in the expression of whorling in certain individuals. Mutation events that result in loss of the second chromosome carrying the wild type allele would also result in whorling of the wing hairs. If the mutagen tested in SMART caused chromosome breakage resulting in the section of chromosome two carrying the wild type allele being lost, the whorling gene would then be expressed. Hence the whorling disadvantage still remains although at a considerably lower frequency. The small size of the whorls and their extremely low frequency does not really affect scoring as such and should not lead to inaccuracies. Nevertheless, it would be preferable to eliminate the whorling gene completely from the tester strains, if possible, without eliminating the *RI* gene responsible for the high bioactivation.

1.9 Aims of the Research

The main aim of the research was to investigate the genotoxicity and structure-activity relationships of a variety of mycotoxins using SMART in wings of *D. melanogaster* and to compare some of the results of the SMART test with three other short-term tests including the *Salmonella*/mammalian microsome assay and the *Drosophila* sex-linked recessive lethals and heritable translocations tests. Since some aflatoxins are known to be promutagens, I decided to compare the detecting capacity of the standard tester strains with

the P450 cytochrome-overproducing, Oregon-R (DDT) Resistant (*ORR*) strains to determine if these high bioactivation strains show increased sensitivity to the effects of promutagens during screening procedures. As previously mentioned, a disadvantage of high bioactivation strains is the presence of whorling of trichomes on wing blades. Another aim of the project was to elucidate the genetic nature of this whorling and to eliminate it. The gene responsible for the whorling effect had to be characterized and mapped and then the SMART test was improved by constructing new HB strains with high metabolic capacity and free of the whorling disadvantage. The activity of P450 cytochromes was assayed in these new strains to determine if over-production of this enzyme still occurred. The final aim of the project was to study the nature of the DDT resistance in ORR stocks in the hope of establishing if gene amplication and maternal imprinting were involved.

CHAPTER TWO

2 CHARACTERIZATION AND LOCATION OF THE WHORLING GENE

2.1 INTRODUCTION

The Oregon R Resistant (OR-R) strain from which the SMART high bioactivation lines were constructed, was originally derived from an Oregon R line by D.J. Merrell (Minneapolis, MN, U.S.A) who did recurrent selection for resistance to the insecticide DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane] from 1952 to 1978 (Hällström *et al.*, 1984). The resistant line was then sent to Inger Hällström (University of Stockholm, Sweden) in 1978 and transferred to the Institute of Toxicology, Schwerzenbach, Switzerland, in 1985. The line was eventually sent to the University of the Witwatersrand, South Africa, in 1991 by Merrell's former student, P. Barnes (Connecticut, U.S.A.). The line has been exposed to increasing levels of DDT for 40 years and some 600 generations. The line carries the *Rst(2)DDT*, formerly known as the *Rl* (resistance to insecticides) gene located at map position 65,0 cM on the long arm of chromosome two (Kikkawa, 1961) (also believed to lie in section 2-64,0-66,0) and is characterized by an inherited, constitutively high capacity for metabolic activation (Hällström, 1986) as the Rst(2)DDT gene is also responsible for the high constitutive expression of cytochrome P450-dependent activities typical of this line (Hällström and Blanck, 1985). For reasons of

convenience, the former abbreviation of the resistance gene (*RI*) will be used in this chapter.

When scoring wings from the high bioactivation cross, it was observed that the pattern of wing hairs was disturbed in certain areas. The phenomenon, referred to as whorling, interferes with analysis of the wings for mutant spots and it would be desirable to eliminate this disadvantage from the high bioactivation lines to once again encourage their use in research. The *OR-R* stock was obtained from P. Barnes to examine if whorling of the wing hairs was present in these flies.

2.2 MATERIALS AND METHODS

2.2.1 Temperature Effect and Differences between Males and Females

Wings from several *D. melanogaster* stocks were examined for the presence of whorling but it was observed only in ORR flies. The irregular whorling of wing hairs is either a spiralling arrangement of the trichomes (as seen in the proximal part of sectors C and D, and, sometimes, in a restricted area of field E) or as a disarrangement of the wing hairs along the veins that delineate sectors D and E of the wing blade. Fig. 2.2.1 shows the different areas and fields where whorling of trichomes most often occurs on the wing blade and is further illustrated in Fig 2.2.2.

Whorling occurs on wings of practically all *ORR* flies, although there seems to be a difference between males and females. It also appears to be sensitive to temperature. To investigate these factors, flies from the Oregon R (R) stock were allowed to lay eggs in well yeasted culture bottles for twenty four hours and then discarded. Eggs were allowed to develop at three different temperatures by placing the culture bottles in incubators at 18°C, 25°C and 29°C for ten days.

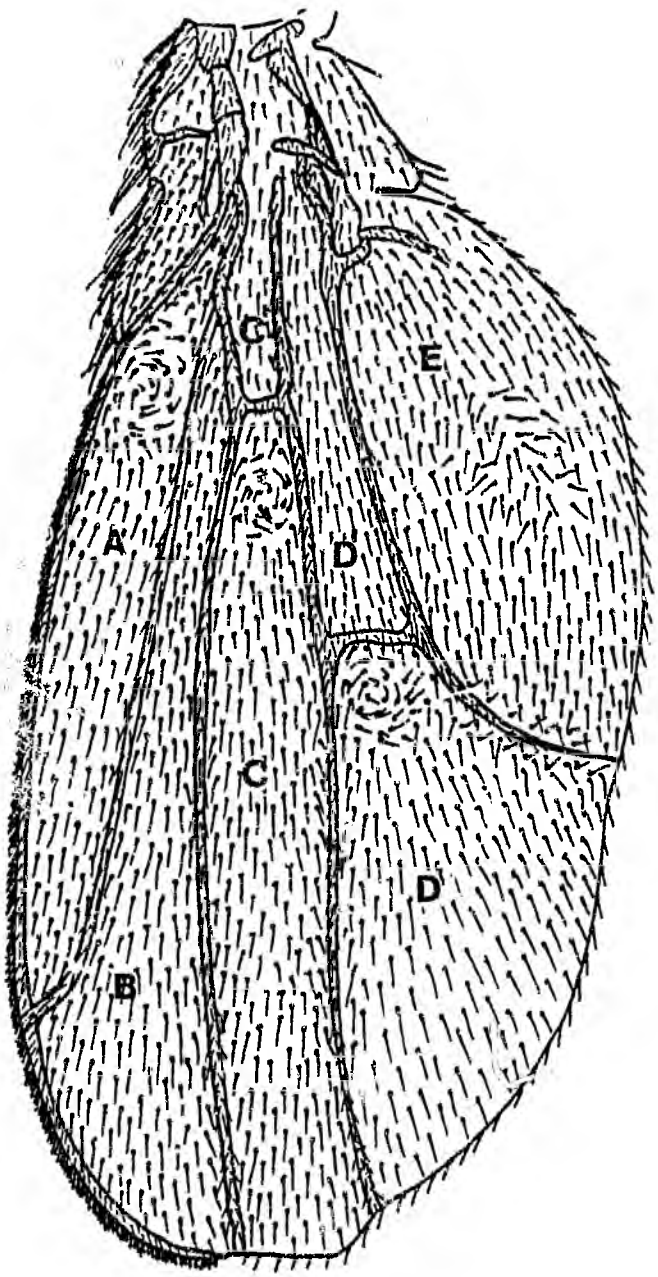
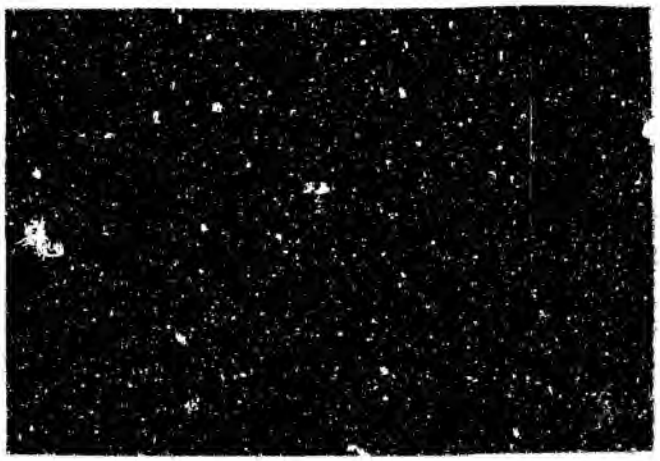
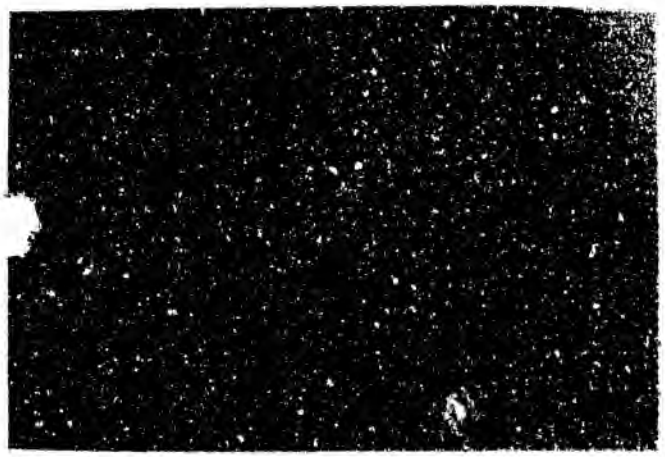


Figure 2.2.1 **Diagramatic representation of wing blade of *Drosophila melanogaster* showing different sectors where the whorling of the trichomes most often occurs.**



a)



b)



c)

Figure 2.2.2 Whorling patterns on wing blades of *OR-R* females raised at 18°C (magnification 100x). Spiralling effect in sectors D (a) and E (b). c) Enlarged photograph showing the normal pattern of trichomes on a wing blade of an *OR-C* female raised at 18°C.

d)

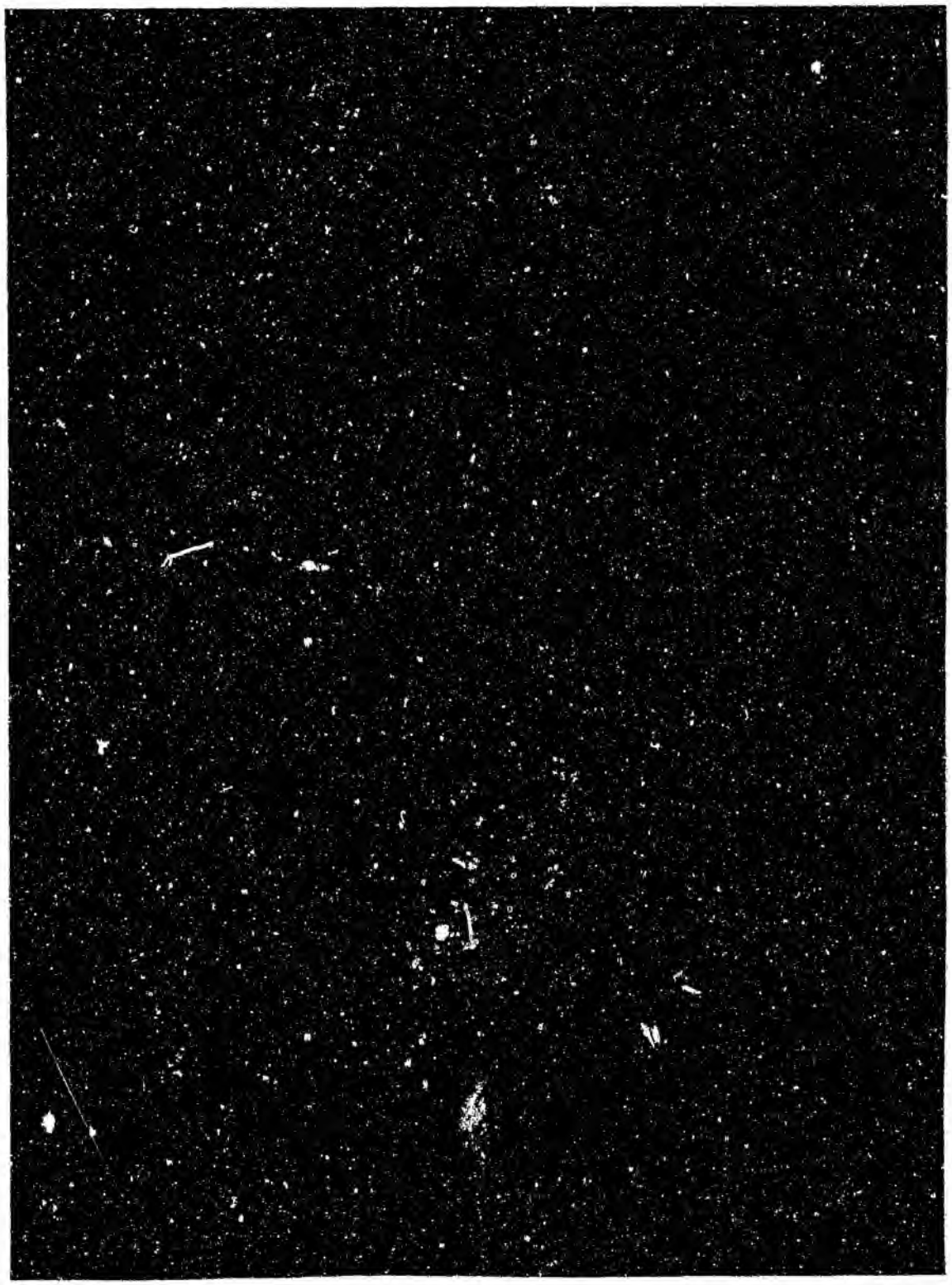


Figure 2.2.2 d) enlarged photograph showing paisley in sectors D, C and D/E on wing blade of F1 female from the original HB cross: *ORR;flr*^{3} x *ORR;mwh*. Arrows point to areas where scoring of the wing would be complicated by the presence of two hair cells which are due to the whorling pattern.

When the adult flies hatched both wings of ten females and ten males for each incubation temperature were mounted on slides and the number of whorls per wing scored. The average number of whorls per wing was calculated for females and listed in Table 2.2.1, together with the sectors where the whorling was most often observed. Due to the apparent differential expression of the trait in this experiment, most subsequent crosses were carried out at 18°C and 25°C and wings of female flies were scored.

TABLE 2.2.1 TEMPERATURE AND SEX EFFECTS ON THE EXPRESSION OF THE PAISLEY GENE

	TEMPERATURE		
SEX	18°C	25°C	29°C
FEMALE average number of whorls per wing	3,8	1,9	0,4
sector	A, D & E	C & D	D & C'
size of whorls number of cells	large > 200	medium ca. 100	small 20-40
MALE average number of whorls per wing	2,1	0,6	0,1
sector	A, D & E	C & D	D & C'
size of whorls number of cells	medium ca. 100	small 20-40	very small < 20

2.2.2 Dominance Relations

Data from the cross *OR-R* females x *OR-C* (wild type) males and the reciprocal cross at 25°C and 18°C, indicated that whorling of the wing hairs is a recessive trait as it was not present on wings of females or males in the F1 progeny of either cross. Since the whorling patterns resemble the classical paisley design printed on fabrics, this name was adopted for the trait and the putative gene abbreviated to *ply*.

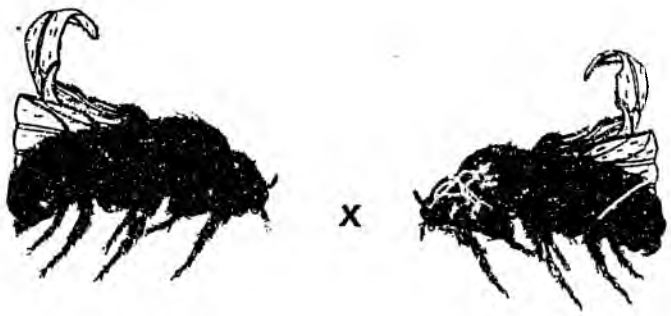
2.2.3 Construction of *Basc;Cy/Pm;Sb/Ser* Marker Line

In order to study the genetics of paisley wings further, flies with first, second and third chromosome markers were constructed. Virgin females from the stock *Basc;Cy/Pm* were crossed to males from the stock *Cy/Pm;Sb/Ser* (see Figure 2.2.3), where the markers and balancer chromosomes employed are described in APPENDIX J. All F1 individuals were *Cy* +/+ *Pm*. Females with kidney shaped plum eyes, curly wings and stubble bristles were selected and crossed with non-stubble males. The margins of curly wings often break in adult flies and hence it is difficult to distinguish curly serrate from curly files. Since males and females in the F1 had either stubble bristles or serrate wings, but never both, F1 males with normal bristles had to carry the *Ser* gene on chromosome 3.

The *Basc/Basc;Cy* +/+ *Pm;Sb* +/+ *Ser* and *Basc/Y;Cy* +/+ *Pm;Sb* +/+ *Ser* individuals were selected from among the F2 progeny on the basis of: extreme Bar eyes [this would mean that the female and male was homozygous and hemizygous for the *Basc* chromosome, respectively; all individuals in the F2

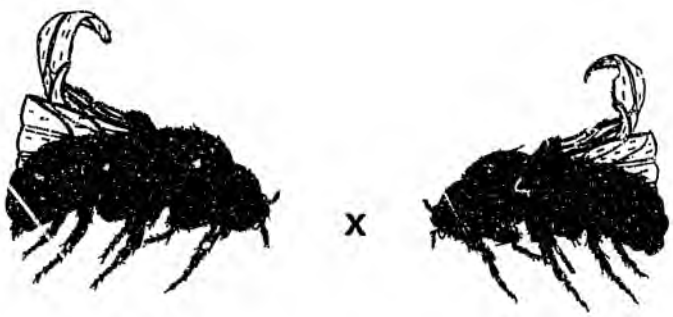
generation were also *Cy* +/+ *Pm*, so flies selected had curly wings and carried the *Pm* gene, which in combination with the apricot gene in *Basc* gives dark orange eye colour; stubble bristles (short and thick bristles on the thorax); since the individuals have curly wings it was necessary to select for serrate wings when the flies just hatched and the margins of the curly wings had not yet broken through wear and tear. The males and females selected from the F2 were mated and used to set up a pure breeding *Basc;Cy/Pm; Sb/Ser* stock.

P1



Basc/Basc;Cy +/+ Pm; +/+ ***+/Y; Cy +/+Pm; Sb +/+ Ser***

F1/P2



Basc/+;Cy +/+ Pm;Sb +/+ ***Basc/Y;Cy +/+ Pm;Ser +/+***

F2/P3



Basc/Basc;Cy/Pm;Sb/Ser ***Basc/Y;Cy/Pm;Sb/Ser***

Figure 2.2.3 Construction of ***Basc; Cy/Pm; Sb/Ser*** marker strain carrying markers on first, second and third chromosomes.

2.2.4 Linkage Group Determination of *ply*

A series of crosses was conducted in an attempt to determine the linkage group of the putative gene responsible for paisley wings. This was done by crossing the *OR-R* stock to the marker line *Basc; Cy/Pm; Sb/Ser*. Chromosome four was uncontrolled. *OR-R* females were crossed to *Basc;Cy/Pm;-Sb/Ser* males at 25°C and 18°C together with the reciprocal cross. Segregation results from these crosses indicated that the paisley wing is due to a single gene located on the second chromosome.

2.2.5 Determination of the Location of *ply* on the Second Chromosome

Further crosses were then carried out to determine the location of the *ply* gene on the second chromosome. The *CR-R* stock, carrying the *RI* and *ply* genes was crossed to stocks carrying marker genes with known locations on the second chromosome.

The location of the vestigial gene is 2-67,0, i.e., 1 to 3 map units removed from *RI* (2-64-66) in *OR-R*. As the *RI* and *vg* loci are closely linked, very little recombination would be expected to occur between these two genes. If the *ply* and RI genes were also closely linked, or one and the same gene, then no paisley-free progeny should be found in the crosses between paisley and vestigial flies described below.

Virgin females from the *OR-R* stock were crossed to *vg* males at 25°C. F1 heterozygous females were then crossed to *Cy/Pm* males. This cross could not be carried out in the reverse direction as no crossing over occurs in males

and crossing over between *vg* and *ply* was to be used to determine the map distance between the two genes and to separate the resistance and paisley genes if possible. Progeny from this cross should all have one chromosome from the female parent (one of the following eight possibilities assuming *ply* to the right of *vg*):

the non-crossovers	a) *Rl + ply*
	b) *+ vg +*
the single crossovers	c) *Rl + +*
	d) *+ vg ply*
	e) *Rl vg +*
	f) *+ + ply*
the double crossovers	g) *Rl vg ply*
	h) *+ + +*

and one balancer second chromosome from the male parent (carrying either the *Cy* or *Pm* marker).

In the F2 progeny, individuals carrying non-crossover second chromosomes from their mothers were not distinguishable from those carrying crossover chromosomes. It was therefore necessary to select large numbers of males from the progeny to have a reasonable chance of recovering crossovers if the genes were closely linked.

Two hundred males were selected from the F2 progeny and each was crossed individually in a vial with two *Cy/Pm* virgin females at 25°C. The parents were discarded after six days. When the F3 hatched, the *Cy/Pm* individuals were discarded, and the remaining Plum or Curly flies were transferred to a fresh vial and incubated at 18°C. They were allowed to intercross and lay eggs, and then after about ten days, this parental (F3/P4) generation was discarded and trays returned to the 18°C incubator (Figure 2.2.4).

The F4 progeny included some individuals that were homozygous for one of the eight possible chromosomes produced by the heterozygous females (*ORR1/+; RI + ply/+ vg +; ORR3/+; ORR4/+*) chosen for the F1/P2 parent in the P2 generation. In the F4, about half of the vials contained individuals homozygous for the vestigial gene and these vials were discarded.

Vials with no vestigial flies contained either individuals homozygous for second chromosomes *RI+ ply/RI+ ply* and + + *ply/+ + ply* [these flies have normal wild type eyes and wings but show whorling of the wing hairs since they are homozygous for *ply*]; or individuals homozygous for *RI+ +/RI+ +* and + + +/+ + + second chromosomes [these flies also have normal wild type eyes and wings but they are paisley-free]. These four types of flies could obviously not be distinguished under the stereo microscope, so it was necessary to analyse the wings of these flies under a compound microscope for the presence of paisley, having raised the flies at 18°C to obtain maximum expression of the trait.

P1



X



♀ ***Rl + ply/Rl + ply*** ♂ ***+ vg +/+ vg +***

F1/P2



X



♀ ***Rl + ply/+ vg +*** ♂ ***Cy +/+ Pm***

F2/P3



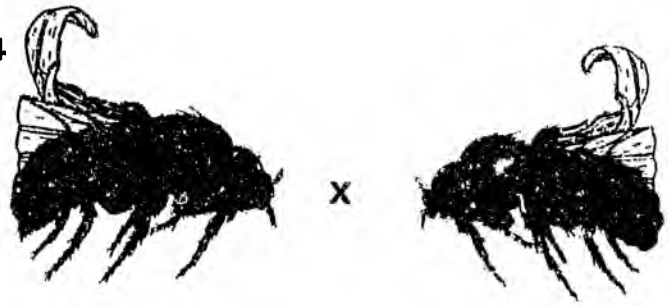
X



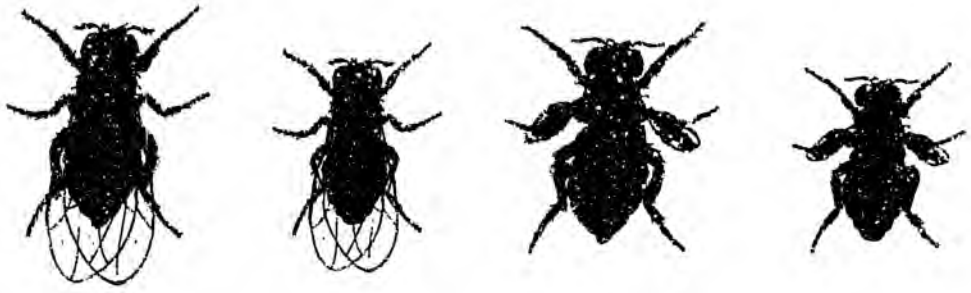
♀ ***Cy +/+ Pm***

♂ ***Cy +/Rl + ply***
Cy +/+ vg +
Cy +/Rl + +
Cy +/+ vg ply
Cy +/Rl vg +
Cy +/+ vg +
Cy +/Rl vg ply
Cy +/+ + +

F3/P4



♀		
Cy +/Rl + ply		*Cy +/Rl + ply*
Cy +/+ vg +		*Cy +/+ vg +*
Cy +/Rl + +		*Cy +/Rl + +*
Cy +/+ vg ply		*Cy +/+ vg ply*
Cy +/Rl vg +		*Cy +/Rl vg +*
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Cy +/+ + +		*Cy +/+ + +*

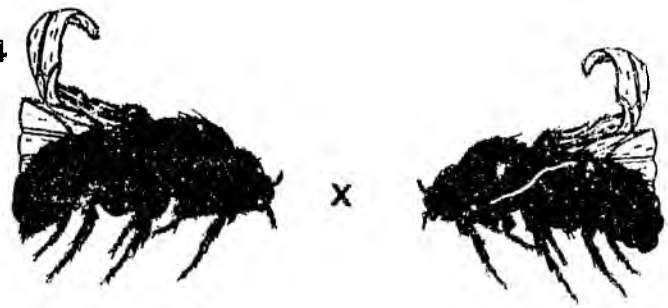


Rl+ +/Rl + +, Rl + ply/Rl+ ply, Rl vg +/Rl vg +, Rl vg ply/Rl vg ply,

+ + +/ + + +, + + ply/+ + ply, + vg +/+ vg +, + vg ply/+ vg ply

Figure 2.2.4 Mating scheme to determine the map distance between *vg* and *ply*. Only the second chromosome constitutions are shown. P1 and P2 were mass matings, P3 and P4 were individual matings. In F2/P3 and F3/P4 only *Cy* individuals are shown; the other half of the progeny would be *Pm*. In F3 all *Cy/Pm* individuals were discarded and the rest of the F3 generation was transferred to a fresh vial. P1, P2 and P3 crosses were carried out at 25°C while P4 was carried out at 18°C. In F4 only the genotypes of non-*Cy* and non-*Pm* individuals are shown.

F3/P4



♀ *Cy +/Rl + ply*
Cy +/+ vg +
Cy +/Rl + +
Cy +/+ vg ply
Cy +/Rl vg +
Cy +/+ + ply
Cy +/Rl vg ply
Cy +/+ + +

Cy +/Rl + ply
Cy +/+ vg +
Cy +/Rl + +
Cy +/+ vg ply
Cy +/Rl vg +
Cy +/+ + ply
Cy +/Rl vg ply
Cy +/+ + +



Rl + +/Rl + +, Rl + ply/Rl + ply, Rl vg +/Rl vg +, Rl vg ply/Rl vg ply,

+ + +/+ + +, + + ply/+ + ply, + vg +/+ vg +, + vg ply/+ vg ply

Figure 2.2.4 Mating scheme to determine the map distance between *vg* and *ply*. Only the second chromosome constitutions are shown. P1 and P2 were mass matings, P3 and P4 were individual matings. In F2/P3 and F3/P4 only *Cy* individuals are shown; the other half of the progeny would be *Pm*. In F3 all *Cy/Pm* individuals were discarded and the rest of the F3 generation was transferred to a fresh vial. P1, P2 and P3 crosses were carried out at 25°C while P4 was carried out at 18°C. In F4 only the genotypes of non-*Cy* and non-*Pm* individuals are shown.

Since all individuals with red eyes and straight wings in the same vial were genetically identical, the wings from one female from each numbered vial with no vestigial flies were mounted on a slide. These preparations were not made permanent and were scanned quickly at low power (100x) for the presence of paisley. This method made it possible to trace back paisley-free flies to the correct vial where all flies would also be paisley-free. Individuals from all the paisley-free vials were treated with DDT (see section 3.2.1) to identify those that were homozygous for the RI gene. Vials containing the most DDT resistant, paisley-free individuals were set aside and used to start the DDT resistant, paisley-free stock for use in subsequent crosses with *Basc; Cy/Pm; Sb/Ser* individuals (sections 2.2.6-2.2.9).

An estimate of map distance between *vg* and *ply* was calculated by counting the number of vials with paisley-free individuals and dividing by the total number of vials with no vestigial flies, i.e.,

$$\text{Map distance (cM)} = \frac{\text{number of paisley-free vials}}{\text{Total number of vials with no vestigial flies}} \times 100$$

In separate determinations of the map distance between *vg* and *ply*, the number of map units was calculated to be 23 (Table 2.2.2). This could mean that *ply* was 23 map units to the left (at position 2-44,0) or 23 map units to the right (at position 2-90,0) of *vg*. Hence, it became necessary to cross *OR-R* females to a stock carrying a different second chromosome marker. The

gene *pr* (purple eyes) is located at position 54,5 on the second chromosome and it was decided that this was the best marker to use because it would also serve to double check the map distance calculated using *vg*. If *ply* is at position 2-44,0 then, when using *pr* as the marker gene, the amount of recombination between *pr* and *ply* would be 10,5% and if *ply* is at position 2-90,0, then the expected recombination between the two genes would be 35,5%. If, when using 200 vials, very few or no paisley-free individuals occurred, this would mean that the paisley gene was near *pr* and therefore to the left of *vg*.

A similar procedure to that used in determining the distance between *vg* and *ply* was repeated with *pr* in order to determine the distance between *pr* and *ply*. In separate determinations of the map distance between *pr* and *ply*, the number of map units was calculated to be 35,1 (Table 2.2.3). Hence, on this evidence, *vg* is located 23 map units to the left of *ply* and this places *ply* at approximately 2-89,6 or 2-90 and about 24-26 map units removed from the *Rl* gene as follows:

54,5 65,0 67,0 <-----23-----> 89,6-90

pr *Rl* *vg* *ply*

<----------35,1----------->

TABLE 2.2.2 MAP DISTANCE BETWEEN *vg* AND *ply*

N° of non-*vg* vials	N° of paisley-free vials	Map Distance (cM)	Sex of individuals sampled
20	2	10	Female
23	6	26	Female
23	7	30	Female
9	2	22	Female
109	25	23	Female
TOTAL: 184*	42	23	Female
20	4	20	Male
9	2	22	Male
15	4	27	Male
TOTAL: 44	10	23	Male

* Results from two repeat experiments. Out of 400 vials, 184 (46%) were non-vestigial and 216 (54%) contained vestigial individuals. Out of 42 paisley-free vials, one vial was DDT sensitive so almost 98% of the vials were DDT resistant.

TABLE 2.2.3 MAP DISTANCE BETWEEN *pr* AND ply

N° of non-*pr* vials	N° of paisley-free vials	Map Distance (cM)	Sex of individuals sampled
55	19	34,5	Female
76	27	35,5	Female
TOTAL: 131*	46	35,1	Female

* Results from two repeat experiments. Out of 382 vials, 131 (34%) were non-purple and 251 (66%) contained purple eyed individuals.

2.2.6 Incorporation of First and Third Chromosome Markers into Paisley-free *OR-R* Individuals

The vials containing paisley-free, DDT-resistant individuals, set aside at the end of the crosses to determine the distance between the paisley and vestigial genes (section 2.2.5), were used in these crosses (Fig. 2.2.5). These paisley-free individuals were believed to carry second chromosome *Rl + +/Rl + +* and different combinations of the first, third and fourth chromosomes.

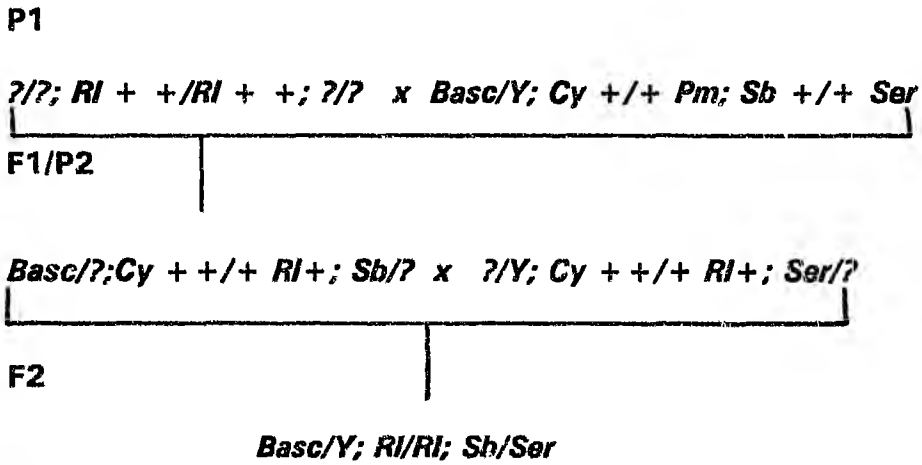


Figure 2.2.5 Mating scheme used for the construction of a chromosome substitution strain carrying chromosomes 1 and 3 from the marker strain *Basc;Cy/Pm;Sb/Ser* and chromosome 2 from the paisley-free, DDT-resistant stock. All crosses were mass matings. The males selected from F2 for subsequent crosses were also DDT-resistant and whorlfree.

2.2.7 Incorporation of First chromosome *ORR1* and Third Chromosome *flr*^{3}*/TM3Ser* into *Basc;Cy/Pm;Sb/Ser* Marker Strain

These crosses were carried out in order to substitute the first chromosome *ORR1* (believed to carry DDT resistance genes as well) from *OR-R* and the

third chromosome carrying the *flr*^{3} marker from the *flr*^{3}*/TM3Ser* tester strain into the paisley-free, DDT resistant individuals (see Fig. 2.2.6).

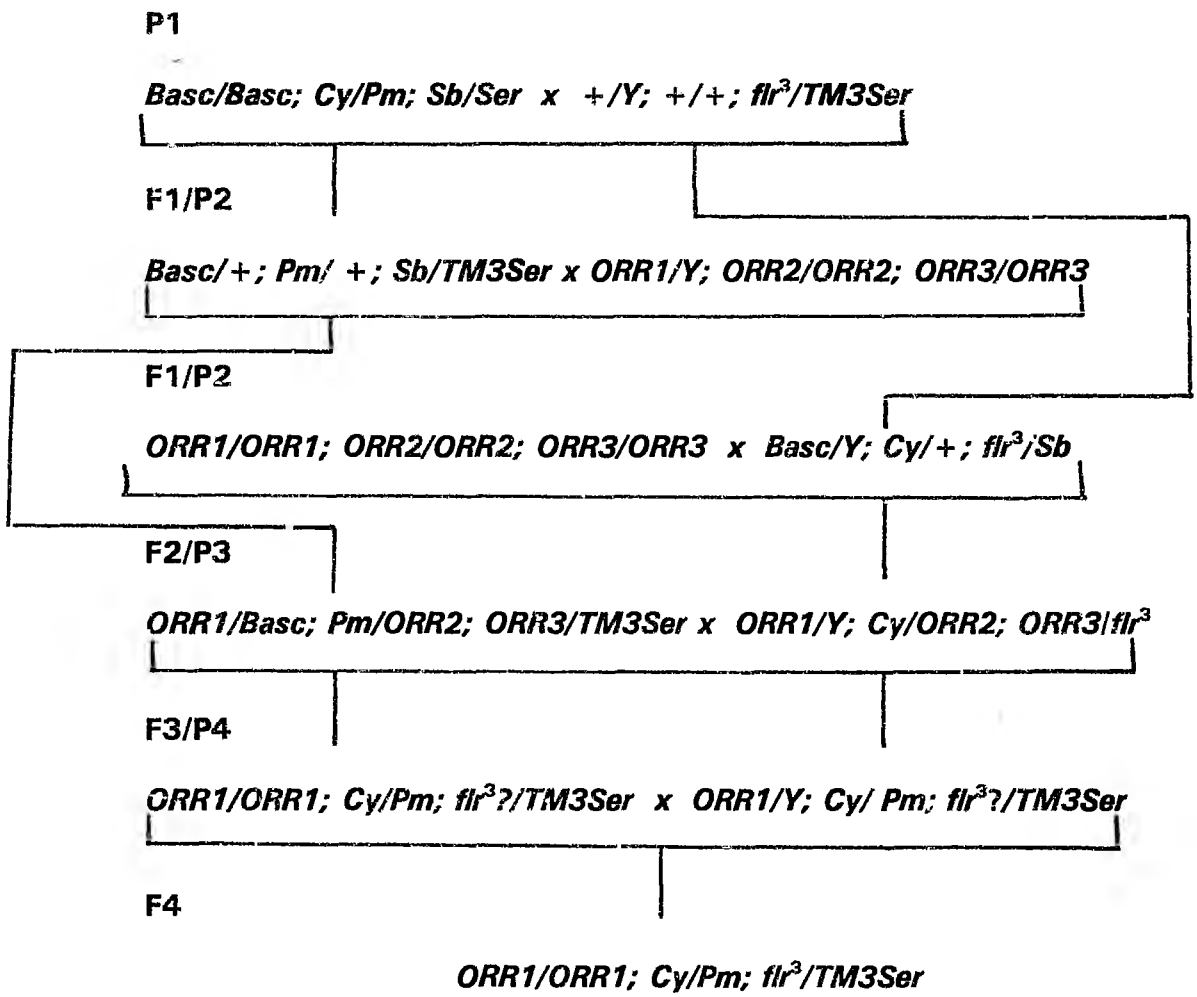


Figure 2.2.6 **Mating schemes used for the construction of a chromosome substitution strain carrying chromosome 1 from the *OR-R* strain (ORR1) and the *flr*^{3}*/TM3Ser* third chromosome from the flare^3 stock. In order to obtain the flies needed in the F2, two different crosses were used in the F1 generation in parallel. All crosses were mass matings with the exception of individual matings that led from generation F3 to generation F4. In F4, females from vials containing only Serrate progeny [where non-Serrate progeny must have been homozygous for *flr*^{3} (a zygotic recessive lethal) did not survive] were collected for subsequent crosses.**

2.2.8 Construction of Paisley-free *ORR;flr*^{3}*/TM3Ser* Tester Strain

The male and female parents used in the P1 of this cross were obtained from crosses outlined in Figures 2.2.5 and 2.2.6, respectively.

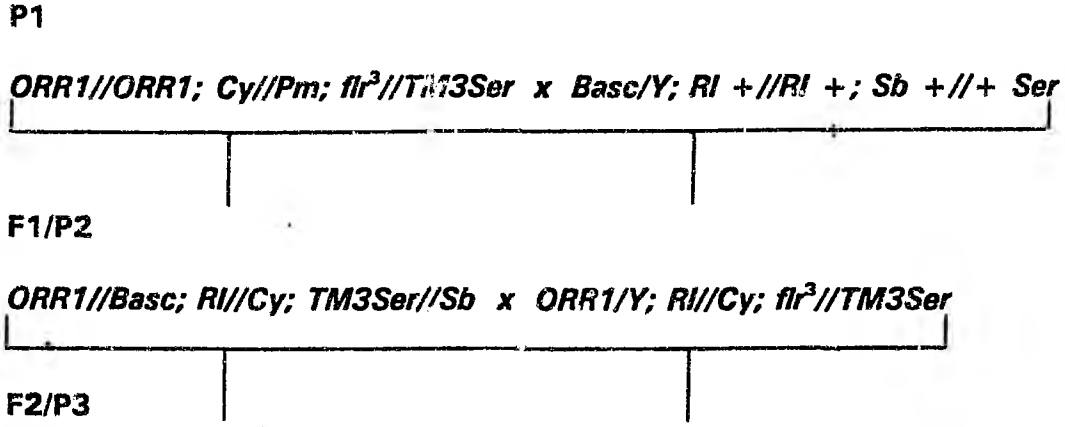


Figure 2.2.7 Mating scheme used for the construction of a paisley-free *ORR;flr*^{3}/TM3Ser tester strain carrying chromosome 1 from the *OR-R* strain, the *RI* gene but not the paisley gene on chromosome 2 and the *flr*^{3}*/TM3Ser* third chromosome from the flare^3 stock. All crosses were mass matings.

2.2.9 Construction of paisley-free *ORR;mwh* Tester Strain

The females used in the P1 of this cross were F1 females from the P1 cross (with the same genotype as the F1/P2 females) outlined in Figure 2.2.7 and the males were from the stock *Basc; Cy/Pm; mwh* constructed by N. van Schaik in Schwerzenbach in 1986.

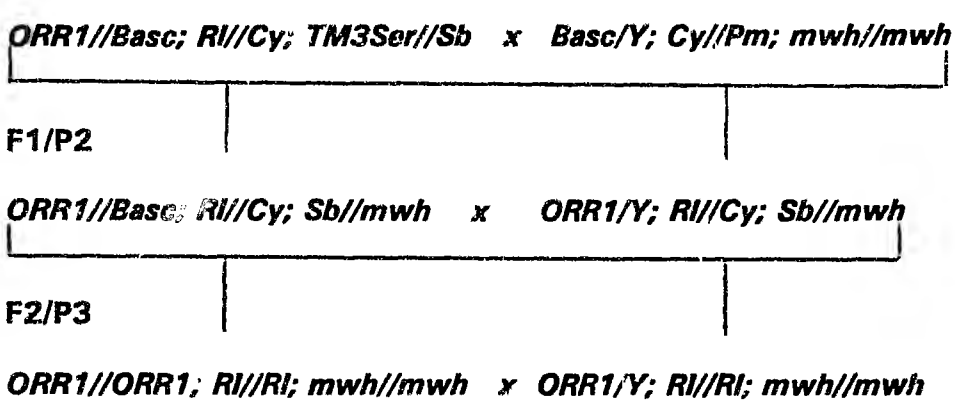


Figure 2.2.8 Mating scheme used for the construction of a paisley-free *ORR;mwh* tester strain carrying chromosome 1 from the *OR-R* strain, the *RI* gene but not the paisley gene on chromosome 2 and the *mwh* third chromosome from the *Basc;Cy/Pm;mwh* stock. All crosses were mass matings. The presence of *mwh* was ensured by inspection of aristae.

These new paisley-free *ORR;flr*^{3} and *ORR;mwh* stocks were then tested for DDT resistance and used in the SMART assay to determine if these new stocks possess higher metabolic capacity to activate promutagens than the SMART standard tester strains and original and improved high bioactivation strains (see chapters 3 and 9). The P450 cytochrome content and activity in these new HB strains was assayed in biochemical assays in chapter 5.

CHAPTER THREE

3 TESTS OF DDT RESISTANCE

3.1 INTRODUCTION

DDT or 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane, is a polychlorinated, non-degradable pesticide. It is insoluble in water but soluble in acetone and 95% ethanol. Since 95% ethanol is toxic to flies, 5% Tween 80 and 5% ethanol was used as the solvent in these experiments. The structure of DDT and site of oxidative attack catalyzed by *Drosophila* microsomes (the P450 cytochrome-NADPH system) appear in Figure 3.1.1.

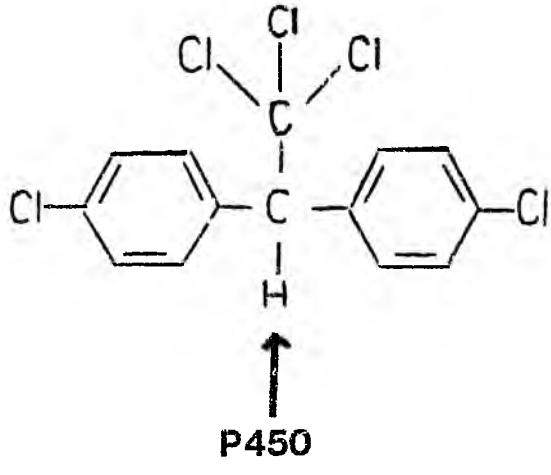


Figure 3.1.1 Structure of DDT ($C_{14}H_9O_{15}$, MW 354,50) and site of oxidative attack catalyzed by P450 cytochromes (Adapted from Casida, 1969).

3.1.1 Resistance Alleles

In *Drosophila*, resistance to DDT has been found to be due to a naturally occuring allele: *Rst(2)DDT* (Tsukamoto, 1958) or *Rl* at position 2-65 (Kikkawa, 1961). However, Dapkus and Merrell (1977) studied the genetic basis of DDT resistance in a population of *Drosophila* that had been continually selected for DDT resistance from 1952 to 1977 (see method for maintaining stocks on DDT in Appendix E) and had achieved a very high level of resistance. The genetic basis of resistance was studied by means of standard chromosome substitution. The analysis of the data showed that resistance was multifactorial with each of the three major chromosomes involved. Although all three chromosomes have significant effects on resistance, the second and third chromosomes are the most important sources of variation.

In general, DDT resistance was found to involve genes of intermediate dominance, producing partially resistant heterozygotes. Both the second and third chromosomes had major effects on resistance, while the first chromosome showed a much smaller, but statistically significant dominant effect. The resistance genes on the second chromosome also had a considerably greater effect than those on the third chromosome (Dapkus and Merrell, 1977).

3.1.2 Maternal Imprinting

According to Tsukamoto (1958) resistance to DDT shows a maternal effect and maternal transmission of DDT resistance causes a specific dominant gain of resistant phenotype in both male and female progeny. There are at least

two general models that can adequately explain maternal transmission effects: 1) Inclusion of a non-nuclear or non-genetic substance (cytochrome P450 enzymes) in the egg; this is referred to as a maternal effect. 2) A differential conditioning of the maternal and/or paternal genome during gametogenesis; this is referred to as maternal/paternal imprinting.

Inclusion of some non-genetic substance in the egg is not likely because, although this might increase resistance in larval stages, the effect would be expected to "wear off" in the adult once it is able to produce its own level of P450 cytochromes. A maternal effect, however, might explain the fact that larvae are more resistant than adults as observed by Tsukamoto in 1958. Perhaps a level of cytochrome P450 present in the egg cytoplasm confers increased resistance to the larvae which, in addition, synthesize their own level of P450 cytochromes.

The more plausible explanation is a differential conditioning of the genomes during gametogenesis. The responsible factor in DDT resistance is perhaps conditioned to function differently during embryogenesis by events occurring during oogenesis but not spermatogenesis. The differentially conditioned RI resistance factor would then act postzygotically to cause enhanced resistance in progeny. This may represent a case of maternal imprinting since the increased or enhanced resistant phenotype will occur only if the *RI* gene is maternally transmitted.

This type of genetic transmission proposed for DDT resistance is similar to gamete conditioning reported in mice where the paternal and maternal genomes appear to have different functions during early development (McGrath and Solter, 1984). Often, the underlying mechanism involves differential DNA methylation during gametogenesis. It is generally held, however, that *D.melanogaster*, unlike other eukaryotes, lacks methylated cytosines (Razin and Riggs, 1980). Thus the underlying mechanism for imprinting may be quite different from that reported for mice.

In a review article by Paro (1990) a mechanism is described for imprinting a determined state into the chromatin of *Drosophila*. The author suggests a plausible model to explain the transmission of determined states of homeotic genes. The chromatin of early embryos is potentially in a "neutral" state, allowing initiation to activate homeotic gene transmission in a position specific manner. Activation converts the chromatin surrounding the gene into an "open" conformation. In regions where a particular homeotic gene was not activated, or was actively repressed, the chromatin becomes "closed". Factors that contain sequence-specific-DNA-binding motifs, interact with the *cis*-regulatory elements in the closed chromatin conformation and function as nucleation signals. These start a compaction of the chromatin. This mechanism would imply that particular termination signals prevent spreading into neighbouring genes and regulatory regions. Once heterochromatic regions are formed, transcription is permanently silenced and the repressed state can be clonally inherited through development.

3.2 MATERIALS AND METHODS

3.2.1 Protocol to Determine the Lethal DDT Concentration of the Different Stocks

To determine the lethal or critical concentration necessary to kill sensitive strains, a range of 0,5 to 0,0005 mg DDT per ml of medium was chosen. Three day old adults (50 females and 50 males) from fourteen different stocks (see Table 3.3.1) were starved for two hours and exposed to the various DDT concentrations for 24, 36, 48 and 72 hours and it was observed that the 24 hour exposure gave the most meaningful results as only resistant stocks were able to survive on 0,5 mg DDT/ml of medium for 24 hours or more. Hence all subsequent tests of DDT resistance were carried out at 24 hour exposures. One hundred 72-hour old larvae from the *OR-C* and *OR-R* stocks were also exposed to DDT for 24 hours and incubated until the adult flies hatched. The live flies at each concentration were harvested and placed in 70% ethanol. The number of surviving flies at each concentration was then counted. To ensure that only surviving flies and not dead flies were harvested, surviving flies were allowed to crawl into empty vials held above the treatment vials and these flies were then anaesthetized and placed in 70% ethanol. The procedure was repeated on separate days.

3.2.2 Investigation of Maternal Imprinting

The following crosses were made in fresh culture bottles at 25 °C:

1) *flr*^{3} ♀ x *mwh* ♂

2) *mwh* ♀ x *flr*^{3} ♂

3) *ORR;flr*^{3} ♀ x *ORR;mwh* ♂ *(original)*

4) *ORR;mwh* ♀ x *ORRflr*^{3} ♂ (original)

5) *ORR;flr*^{3} (original)♀ x *mwh* ♂

6) *mwh* ♀ x *ORR;flr*^{3} (original)♂

7) *flr*^{3} ♀ x *ORR;mwh* (original)♂

8) *ORR;mwh (original)*♀ x *flr*^{3} ♂

9) *ORR;flr*^{3} (new)♀ x *ORR;mwh* (new)♂

10) *ORR;mwh* (new)♀ x *ORR;flr*^{3} (new)♂

11) *ORR;flr*^{3} (new)♀ x *mwh* ♂

12) *mwh* ♀ x *ORR;flr*^{3} (new)♂

13) *flr*^{3} ♀ x *ORR;mwh* (new)♂

14) *ORR;mwh (new)*♀ x *flr*^{3} ♂

In order to determine if the standard lines carry any endogenous resistance alleles, it was necessary to compare their DDT-sensitivity to that of a stock with different first and second chromosomes but the same third chromosome. The *πmwh* strain was constructed by N van Schaik in 1985. The first and second chromosomes of this strain were derived from *π2(P)* a strong P strain, and so have a number of P elements present. Chromosomes three and Y are derived from the standard *mwh* line. Chromosome four is uncontrolled. The *π*mwh line was crossed to *flr*^{3} and *ORR;flr*^{3} stocks in both directions. If resistance genes are located on chromosomes one or two of *mwh*, then both male and female progeny from the cross *ORR;flr*^{3} (old) x *πmwh* (and the reciprocal

cross) would be more sensitive to DDT than progeny from cross *ORR:flr*^{3} (old) x *mwh* (and the reciprocal cross).

After six days the parents were discarded and all progeny that hatched on day eleven were collected and transferred to a fresh bottle. After three days, adult males were separated from adult females and starved for two hours. Fifty 3-4 day old adult females and adult males were treated with DDT (0,25 mg DDT/ ml of medium) for 24 hours as described in section 3.2.1. The surviving flies were then placed in 70% ethanol and counted. The experiment was repeated on separate days.

In addition, virgin females from the marker line *Basc; Cy/Pm; Sb/Ser* stock were crossed with males from the original *ORR;flr*^{3}*/TM3Ser* stock at 25°C. Virgin females with kidney-shaped, red eyes, stubble bristles and curly, serrate wings [*Basc/ORR1; Cy/Rl; Sb/Ser]* were crossed with males with narrow, bar apricot eyes, stubble bristles and curly, serrate wings [*Basc/Y; Cy/Rl; Sb/Ser*] to set up the P2 generation. Male and virgin female flies with narrow, bar apricot eyes, stubble bristles and straight, serrate wings [*Basc/Basc; Rl/Rl; Sb/Ser* and *Basc/Y; Rl/Rl; Sb/Ser*] were then selected from the F2 generation. These male and female flies were crossed and larvae and adults from this stock were then tested for DDT resistance with *Basc; Cy/Pm; Sb/Ser* as a control.

Virgin females from the stock *Basc; Rl/Rl; Sb/Ser* were then crossed with males from the marker line *Basc; Cy/Pm; Sb/Ser* and the reciprocal cross was

also carried out, simultaneously. One hundred 72-hour old larvae (not sexed) from these crosses were exposed to 0,25 mg/ml DDT in plastic tubes with nylon gauze at one end so that the larvae fed on a DDT-cellulose (0,3 g cellulose plus 1 ml DDT solution) suspension for twenty four hours. When the adult flies hatched, survivors were harvested and counted. In addition, fifty adult females and fifty males (3-4 days old) from the F1 progeny of the two reciprocal crosses were allowed to feed on 0,25 mg/ml solution of DDT and after twenty four hours, the survivors were harvested and counted.

The reciprocal of the P1 cross described above [original *ORR;flr*^{3}*/TM3Ser* females x *Basc; Cy/Pm; Sb/Ser* males] was also carried out at 25°C. After ten days, F1 females with kidney-shaped red eyes, curly, serrate wings and stubble bristles [*ORR1/Basc; Rl/Cy; Ser/Sb*] were crossed with males with normal plum eyes, stubble bristles and straight serrate wings [*ORR1/Y; Rl/Pm; Sb/Ser*]. Progeny from this P2 cross included: male and female flies that had plum eyes, stubble bristles and curly, serrate wings [*ORR1/ORR1; Cy/Pm; Sb/Ser* and *ORR1/Y; Cy/Pm; Sb/Ser*] that were homozygous for genes on the X chromosome; as well as female flies that had kidney-shaped, plum eyes, stubble bristles and curly, serrate wings [*Basc/ORR1; Cy/Pm; Sb/Ser*] that were heterozygous for genes on the X chromosome. These flies were also tested for DDT resistance.

3.3 RESULTS

3.3.1 Sensitivity and Resistance of the Different Stocks

The term sensitive refers to stocks not able to survive on medium containing 0,5 mg DDT/ml for twenty four hours while the term resistant refers to stocks able to survive on this medium. The counts of two replications were averaged and are presented in Table 3.3.1. The standard tester strains *mwh* and *flr*^{3}, the *mmwh* and *Oregon R* (OR-C) stocks and the newly constructed marker line *Basc; Cy/Pm;Sb/Ser* were all sensitive to the toxic effects of the insecticide DDT. Overall, the survival of the *flr*^{3} stock was always greater than the survival of the *mwh* stock. The most resistant stock was the *OR-R* stock supplied by Dr Barnes from the U.S.A. The original *ORR;flr*^{3}*/TM3Ser* strain also showed some resistance as did the *Basc; RI/RI; Sb/Ser* stock, the paisley line and some of the paisley-free lines (obtained from the mapping of the paisley gene crosses see Table 3.3.1 and section 2.2.5) probably due to the presence of the *RI* gene on chromosome two. The most DDT resistant, paisley-free stock was then used to construct the new *ORR;flr*^{3} and *ORR;mwh* stocks (section 2.2.6-9) and these new strains were also resistant to the effects of DDT. The slightly increased resistance of *RI* homozygotes compared with *RI* heterozygotes (Plum eyes phenotype) indicates that the *RI* gene may not be completely dominant at the molecular level but chi-square analysis indicated that the increase was not statistically significant at the 5% level. Overall, males appeared to be more resistant than females to the toxic effects of DDT suggesting sex differences in the metabolism and detoxification of this insecticide. For some stocks, the number of male and

female survivors at the highest concentration are recorded in Table 3.3.1. The activity of the DDT-detoxifying P450 enzymes in males appeared to be about 2-3 times higher than in females. Chi-square analysis indicated that this difference was statistically significant at the 5% level. Larvae also appeared to be more resistant than adults but the increase in survival of larvae compared with adults was only statistically significant in the case of the *Basc; RI/RI; Sb/Ser stock*.

The slight DDT resistance exhibited by *ORR1* homozygotes [*ORR1/ORR1; Cy/Pm; Sb/Ser* and *ORR1/Y; Cy/Pm; Sb/Ser*] but not *ORR1* heterozygotes, seems to indicate the presence of a recessive resistance allele on the X chromosome that contributes to resistance at the lower concentrations. A somewhat surprising result was the lack of resistance of the original *ORR;mwh* stock suggesting that this stock carries the paisley gene but not the *RI* gene. Hence the original *ORR?;mwh* strain may not be *ORR*. The increased resistance of this stock compared to other sensitive stocks at the lower concentrations seems to indicate the presence of a resistance allele on the first chromosome.

TABLE 3.3.1 PERCENTAGE SURVIVAL OF FLIES TREATED WITH DDT FOR 24 h (COUNTS AVERAGED OVER TWO REPLICATIONS)[1].

STOCK	DDT CONCENTRATION (mg/ml)								
	0,5	0,25	0,1	0,05	0,01	0,005	0,001	0,0005	0,00
ORR;flr[3] (old)	♂17% ♀ 8	54%	100%	100%	100%	100%	100%	100%	100%
ORR;mwh (old)	0%	2%	45%	84%	100%	100%	100%	100%	100%
OR-C (adults)	0%	0%	29%	71%	97%	100%	100%	100%	100%
OR-C (larvae)	0%	0%	33%	84%	100%	100%	100%	100%	100%
OR-R (adults)	♂57% ♀25%	100%	100%	100%	100%	100%	100%	100%	100%
OR-R (larvae)	96%	100%	100%	100%	100%	100%	100%	100%	100%
Basc;Cy/Pm; Sb/Ser (A)	0%	0%	22%	80%	100%	100%	100%	100%	100%
Basc;Cy/Pm; Sb/Ser (L)	0%	0%	40%	85%	100%	100%	100%	100%	100%
Basc;RI; Sb/Ser (A)	♂13% ♀ 8%	45%	93%	100%	100%	100%	100%	100%	100%
Basc;RI; Sb/Ser (L)	37%	92%	100%	100%	100%	100%	100%	100%	100%
ORR1/Y;Cy/ Pm;Sb/Ser ♂[2]	0%	6%	44%	75%	100%	100%	100%	100%	100%
ORR1/ORR1; Cy/Pm;Sb/ Ser ♀[3]	0%	8%	51%	80%	100%	100%	100%	100%	100%
ORR1/+;Cy/ Pm;Sb/Ser ♀[4]	0%	0%	31%	67%	100%	100%	100%	100%	100%
mwh	0%	0%	27%	75%	90%	100%	100%	100%	100%
flr[3]	0%	0%	36%	81%	100%	100%	100%	100%	100%
New pais-ley-free	23%	48%	90%	100%	100%	100%	100%	100%	100%
New (Pm)	18%	43%	84%	100%	100%	100%	100%	100%	100%
New (Cy/Pm)	0%	0%	25%	68%	92%	100%	100%	100%	100%
paisley*	24%	47%	92%	100%	100%	100%	100%	100%	100%
New ORR;flr[3]	♂19% ♀ 5%	53%	100%	100%	100%	100%	100%	100%	100%
New ORR;mwh	♂14% ♀ 6%	35%	100%	100%	100%	100%	100%	100%	100%

***This stock presumably carries second chromosome *RI ply//RI ply* with first, third and fourth chromosomes uncontrolled.**

A = adults and L = larvae

1) 50 ♀ and 50 ♂ treated in each experiment except where otherwise stated: 2) 50 males treated; 3) 50 females treated; 4) fifty females treated

TABLE 3.3.2 FINAL RESULTS OF DDT TREATMENT (0,25 mg/ml) FROM THREE INDEPENDENT EXPERIMENTS, 2-3 DAY OLD ADULTS EXPOSED FOR 24 HOURS

CROSS	SEX	N° OF SURVIVORS (150 treated)
flr^3 x *mwh*	♀	0
	♂	0
	♀ + ♂	0
mwh x *flr*^{3}	♀	0
	♂	0
	♀ + ♂	0
flr^3 x *πmwh*	♀	0
	♂	0
	♀ + ♂	0
πmwh x *flr*^{3}	♀	0
	♂	0
	♀ + ♂	0
ORR;flr^3 x *mwh* (old)	♀	29
	♂	75
	♀ + ♂	104
mwh x *ORR;flr*^{3} (old)	♀	17
	♂	14
	♀ + ♂	31
ORR;flr^3 x *πmwh* (old)	♀	45
	♂	90
	♀ + ♂	135
πmwh x *ORR;flr*^{3} (old)	♀	30
	♂	23
	♀ + ♂	53
ORR;flr^3 x *ORR;mwh* (old lines)	♀	53
	♂	66
	♀ + ♂	119
ORR;mwh x *ORR;flr*^{3} (old lines)	♀	21
	♂	24
	♀ + ♂	45
ORR;mwh x *flr*^{3} (old)	♀	2
	♂	9
	♀ + ♂	11
flr^3 x *ORR;mwh* (old)	♀	2
	♂	0
	♀ + ♂	2
ORR;flr^3 x *ORR;mwh* (new lines)	♀	34
	♂	66
	♀ + ♂	100
ORR;mwh x *ORR;flr*^{3} (new lines)	♀	40
	♂	48
	♀ + ♂	88

ORR;flr³ x mwh (new)	♀	25
	♂	58
	♀ + ♂	83
mwh x ORR;flr³ (new)	♀	15
	♂	12
	♀ + ♂	27
ORR;mwh x flr³ (new)	♀	20
	♂	45
	♀ + ♂	[illegible]
flr³ x ORR;mwh (new)	♀	12
	♂	9
	♀ + ♂	21
Basc;RI;Sb/Ser x Basc;Cy/Pm;Sb/Ser (adults)	♀	23
	♂	71
	♀ + ♂	94
Basc;Cy/Pm;Sb/Ser x Basc;RI;Sb/Ser (adults)	♀	0
	♂	15
	♀ + ♂	15
Basc;RI;Sb/Ser x Basc;Cy/Pm;Sb/Ser (larvae)	♀ + ♂	112*
Basc;Cy/Pm;Sb/Ser x Basc;RI;Sb/Ser (larvae)	♀ + ♂	29*

*** 300 larvae treated (100 larvae x 3 experiments)**

3.3.2 Evidence for Maternal Imprinting

The counts averaged over repeats are presented in Table 3.3.2. Chi-square analysis of the number of survivors indicated that maternal transmission of the *RI* gene caused a statistically significant increase in survival of both male and female adult progeny (with male progeny usually more resistant than female progeny) from crosses: OR*R;flr*^{3} x *mwh* (both original and new *OR-R* stocks); *ORR;mwh* (new) x *flr*^{3}; *ORR;flr*^{3} (original) x *pimwh*; and an increase in survival of larval and both male and female adult progeny from cross *Basc; RI/RI; Sb/Ser* x *Basc;Cy/Pm;Sb/Ser*, where the female parents were DDT resistant, but not from the reciprocal crosses where the male parent was resistant. These results seem to suggest that maternal imprinting may be involved in DDT resistance. As observed in section 3.3.1, the difference in survival of male and female progeny from crosses involving resistant parents, was statistically significant at the 5% level.

The statistically significant increased survival of both male and female progeny from cross *ORR;flr*^{3} x *ORR;mwh* (both parents from the original *ORR* line) compared with the reciprocal cross where the female parent was *ORR;mwh* and the male parent *ORR;flr*^{3} provided further evidence that the original *ORR;mwh* stock did not carry the *RI* gene. The original *ORR;mwh* stock carried some resistance genes, however, because the survival of progeny from the cross *ORR;mwh* x *flr*^{3} (and the reciprocal cross) is greater than that from cross *mwh* x *flr*^{3} (and the reciprocal cross).

3.4 DISCUSSION

3.4.1 The Use of the *mmwh* Stock

The results showed that both *mmwh* and *mwh* were sensistive to the effects of DDT and that there were no resistance alleles along the X, second and third chromosomes of *mwh* flies. Hence, any resistance exhibited by *ORR;mwh* stocks must have been due to the presence of the *ORR1* and *ORR2* chromosomes from the *OR-R* stock which were introduced into the standard tester strain *mwh* during the construction of high bioactivation tester strains. Although it seemed that progeny from the cross *ORR;flr*^{3} ♀ to *mmwh* ♂ was more resistant than progeny from the cross *ORR;flr*^{3} ♀ x *mwh* ♂, this could have been due to chance because progeny from the cross *flr*^{3} x *mmwh* (and the reciprocal cross) did not show increased resistance when compared with progeny from the cross *flr*^{3} x *mwh* (and the reciprocal cross).

3.4.2 Lethal Concentrations of Sensitive and Resistant Stocks

In 1958, Tsukamoto reported that *RI* homozygotes and heterozygotes can resist large concentrations of DDT in the food medium on which oviposition, larval development, emergence and copulation take place (the LD50 for resistant strains in 1958 was 4 000 μg/ml) whereas susceptible strains (such as Canton S) could not survive on medium containing 50-100 μg DDT/ml. According to Zjilstra and colleagues (1984), the survival of flies after a three day period on DDT containing food (0,5 mg/ml) was 100% for DDT resistant strains and zero for wild type sensitive strains. This was not observed in

these experiments as the survival of *OR-R* flies treated with 0,5 mg/ml DDT for 24 hours was 82% although for larvae it was 96%.

In these experiments the lethal concentration refers to the critical concentration below which all flies survive and above which lethality occurs. The lethal concentration of the most resistant stock, OR-R, was less than 0,5 but greater than 0,25 mg DDT per ml of medium (Table 3.3.1) as at 0,25 mg/ml, survival of flies was already 100%. For *ORR;flr*^{3} (original and new HB tester strain), the new *ORR;mwh* as well as the *Basc;RI/RI;Sb/Ser* line, the lethal concentration was between 0,25 and 0,1 mg DDT/ml of medium and 0,1-0,05 mg DDT/ml, respectively. The results show that only stocks with the RI gene on chromosome two were able to survive on medium containing 0,5 mg of DDT per ml for 24 hours. The enhanced resistance of OR-R seems to indicate that this stock carried extra resistance alleles, possibly on chromosome three. Since the original and new *ORR;flr*^{3} stock and the new *ORR;mwh* stock carried the first and second chromosome (ORR1 and ORR2) from the *OR-R* line while *Basc;RI/RI;Sb/Ser* carried only ORR2, it seems that the first chromosome contributed to the slightly higher resistance of the former three strains compared with the latter strain, especially at the lower concentrations but this increase in resistance was not statistically significant. The statistically significant increased survival of *ORR1/ORR1* homozygotes compared with *ORR1/+* heterozygotes suggests that the resistance gene on chromosome one may be recessive. The presence or absence of whorls [see Table 3.3.1 New (paisley-free) and New paisley] did not seem to affect DDT resistance. The reduced survival of *RI* heterozygotes, (see Table 3.3.1 New (*Pm*)), although very slight,

suggests that dominance of the *Rl* gene may not be complete, with *Rl* homozygotes being slightly more resistant than *Rl* heterozygotes but this increase in resistance was also not statistically significant.

The lethal concentration of sensitive strains was in the 0,05-0,01 mg/ml range as the survival of flies decreased from 100% at concentrations higher than 0,01 mg/ml. Although the original *ORR?;mwh* HB tester strain could not survive the highest (0,5mg/ml) DDT treatment, the lethal concentration of this stock was slightly higher than that of the sensitive stocks, perhaps because this strain carried resistance alleles on the first chromosome (ORR1) but not on the second chromosome.

The difference in resistance between male and female progeny in Table 3.3.2 seems to indicate the presence of a resistance gene on the X chromosome of the new and original *ORR;flr*^{3} and *ORR;mwh* stocks. If the resistance gene on the X chromosome was recessive, then male progeny from a cross such as *ORR;flr*^{3} (DDT resistant) females with *mwh* (DDT sensitive) males, would be expected to be more resistant than female progeny; but when the female parent is sensitive and the male parent resistant [such as in the cross *mwh* x *ORR;flr*^{3}] both male and female progeny should show the same level of resistance. This was observed in these experiments. The problem, however, is that there was also a difference in resistance between male and female progeny from the cross *Basc//Basc; Rl//Rl; Sb +//+ Ser x Basc//Y; Cy +//+ Pm; Sb +//+ Ser* where the first and third chromosomes had been replaced by balancer chromosomes. Since the stock *Basc; Cy/Pm; Sb/Ser* was

sensitive to DDT (Table 3.3.1), any resistance contributed by the *Basc//Basc; RI//RI; Sb +//+ Ser* stock must have been due to the *RI* gene on chromosome two. The difference between male and female progeny was probably due to differences in expression of the *RI* gene in males and females (see also section 3.3.1).

3.4.3 A Model Describing the Maternal Transmission of DDT Resistance

A protein product (or regulatory protein) must initiate gene expression by binding to the regulatory site of the *RI* gene. The chromatin is switched into an "open" conformation and transcription (transcription initiation complex) is activated. Gene products keep the chromatin in the "open" conformation, allowing autoregulatory mechanisms as well as general transcription factors to sustain gene expression after the cessation of early gene action. The chromosomal genomic imprinting might be present in the gene coding for the regulatory protein of the *RI* gene, or in the amplified *RI* gene itself.

It seems likely that the regulatory protein that initiates gene expression is not present in sperm so that in sperm from a DDT resistant male the *RI* gene, which might even be amplified, remains repressed, or some copies remain repressed so that P450 cytochromes are present in a lower concentration than in females. It is difficult to explain, however, why some copies of the gene would be selectively repressed, and hence a better explanation may be that all copies of the amplified gene are partially repressed.

Another possibility is that the regulatory protein of the amplified *RI* gene must also be amplified in order to be present in sufficient quantities to regulate the expression of the resistance gene. Perhaps the gene coding for this regulatory protein is only amplified in females, so that in males this regulatory protein is present in very low concentrations and some copies of the resistance gene are not transcribed. Since both male and female adults are resistant to DDT, all copies of the amplified *RI* gene must be active in adult fat bodies, but in sperm, however, some copies of the gene are repressed, so that the amplified gene is inherited mainly as heterochromatin although some copies are active.

In male germ cells after meiosis, sequence specific DNA binding factors may bind to the silent *cis* regulatory regions of the *RI* gene so that transcription is not initiated. The bound factors can act as signals for compacting the chromatin into a heterochromatin-like conformation. Termination signals prevent the effect from invading neighbouring genes/regulatory regions. Transcription of some copies of the amplified gene is thus prevented and the repressed state is clonally inherited by being locked into the structure of the chromatin but in this case the repressed state would probably be semi-permanent as in the zygote from the cross resistant female to resistant male the chromatin is somehow switched into an open conformation and transcription of the repressed copies of the gene is activated again. Some factor present in the egg somehow cancels the effect of the sequence specific DNA binding factors so that all copies of the amplified gene are transcribed in the zygote. In addition, gene products or regulatory proteins encoded by the

maternal genome may compensate for the lack of this protein in sperm so that the chromatin is kept in an open conformation.

When a resistant female is crossed with a sensitive male, a high concentration of the regulatory protein would be present in the heterozygous progeny so all copies of the dominant *Rl* gene would be active resulting in progeny that are almost as resistant as the female parent. When, however, a sensitive female is crossed with a resistant male, the heterozygous progeny are not as resistant as the male parent as not all copies of the *Rl* gene would be expressed and some would remain partially repressed.

3.4.4 The Relationship Between DDT Resistance and Bioactivation Capacity

Graf and van Schaik (1992) presented the results of a series of experiments which compared larvae from the standard cross (in both directions), the HB cross (in both directions) and the improved HB cross (in both directions) tested with the promutagen DEN (diethylnitrosamine) at 1,0 mM and 2,5 mM concentrations. The crosses are numbered for convenience: 1) *flr*^{3} ♀ *x mwh* ♂, 2) *mwh* ♀ *x flr*^{3} ♂. 3) *ORR;flr*^{3} ♀ x *ORR;mwh* ♂, 4) *ORR;mwh* ♀ *x ORRflr*^{3} ♂, 5) *ORR;flr*^{3} ♀ x *ORR;mwh* ♂, 6) *mwh* ♀ *x ORR;flr*^{3} ♂, 7) *flr*^{3} ♀ x *ORR;mwh* ♂, 8) *ORR;mwh* ♀ *x flr*^{3} ♂.

With 1 mM and 2,5 mM DEN the total spots per wing were significantly higher in the ORR heterozygous individuals (from cross 5) than in the ORR supposedly homozygous ones (from cross 3). However, in cross 6 (the reciprocal of the improved HB cross) the frequency of spots per wing was

higher than in the standard crosses (1 and 2), but not as high as with cross 5. This could be evidence for a maternal effect or maternal imprinting.

The progeny from the cross *ORR;flr*^{3} virgin females to *mwh* males would be heterozygous for the dominant *RI* gene on chromosome two and should exhibit high bioactivation capacity. The progeny from the reciprocal cross *mwh x ORR;flr*^{3} would also be heterozygous for the resistance gene, and yet they are not as sensitive to the effect of DEN as the progeny from cross 5 with *ORR;flr*^{3} as the female parent. This demonstrates that the high constitutive expression of cytochrome P450 also seems to show a maternal effect or maternal imprinting.

As previously mentioned, the *RI* gene responsible for the DDT resistance is also responsible for the high constitutive expression of cytochrome P450 enzymes responsible for the high bioactivation of promutagens This *RI* gene is a dominant gene and, as has already been mentioned, the increased resistance to DDT is maternally transmitted in that larval and adult progeny of resistant females crossed with a sensitive male are more resistant than those of the reciprocal cross. The high constitutive expression of cytochrome P450 should also show a maternal effect and the results obtained with the DEN experiments by Graf and van Schaik (1992) are in accordance with this theory. Resistance to DDT and bioactivation capacity of progeny from the various crosses are compared in Table 3.4.1. The crosses are listed in an increasing order of bioactivation and resistance and it is clear that these two traits are positively correlated. Graf and van Schaik (1992) also provide

indirect evidence for the absence of the *Ri* gene in the original *ORR;mwh* stock. The authors report that the hybrids from crosses 7 and 8 are not increased in sensitivity and behave just like the standard cross.

The fact that differences in survival of progeny from the SMART new HB cross *ORR;flr*^{3} x *ORR;mwh* was not statistically significant from survival of progeny from the original HB cross *ORR;flr*^{3} x *ORR?;mwh*, *ORR;flr*^{3} (old and new) x *mwh* (improved HB cross), and *ORR;flr*^{3} (old) x *mmwh* cross in these tests of DDT resistance, suggests that most of the resistance in the offspring is contributed by the female parent of the cross. Hence, progeny from the original HB cross, the improved HB cross and the new HB cross would be expected to exhibit similar bioactivatic acity. These results also suggest that the *RI* gene may be completely dominant as the *RI* heterozygous and homozygous progeny showed similar survival rates after DDT exposure as long as resistance was maternally transmitted to *RI* heterozygotes.

TABLE 3.4.1 CORRELATION BETWEEN DDT RESISTANCE AND BIOACTIVATION CAPACITY

INCREASING ORDER OF DDT RESISTANCE (0,5mg DDT/ml INSTANT MEDIUM; 24H TREATMENT)	INCREASING ORDER OF BIOACTIVATION (2,5 mM DEN; 48H CHRONIC TREATMENT
1) *flr*^{3} x *mwh*	1) *flr*^{3} x *mwh*
2) *mwh* x *flr*^{3}	2) *flr*^{3} x *ORR;mwh*
3) *flr*^{3} x *ORR;mwh*	3) *mwh* x *flr*^{3}
4) *ORR;mwh* x *flr*^{3}	4) *ORR;mwh* x *flr*^{3}
5) *mwh* x *ORR;flr*^{3}	5) *mwh* x *ORR;flr*^{3}
6) *ORR;mwh* x *ORR;flr*^{3}	6) *ORR;flr*^{3} x *ORR;mwh*
7) *ORR;flr*^{3} x *mwh*	7) *ORR;mwh* x *ORR;flr*^{3}
8) *ORR;flr*^{3} x *ORR;mwh*	8) *ORR;flr*^{3} x *mwh*

All *ORR* stocks are original HB stocks constructed by Frölich and Würgler (1989).

CHAPTER FOUR

4 CYTOGENETIC ANALYSIS OF SALIVARY GLAND CHROMOSOMES

4.1 INTRODUCTION

Until recently, very little information was available on the mechanisms of insecticide resistance at the genetic and molecular levels. The biochemical basis of insecticide resistance has been identified in many species, but generally, the molecular events responsible are poorly understood. An increase in insecticide degrading activity is a common resistance mechanism, but in most cases it is not known whether this arises from mutant enzymes or from the increased production of an enzyme already present in susceptible insects. Many genes responsible for physiological and enzymological changes in insecticide resistant insects have been identified and mapped. Hypotheses concerning genomic changes include structural changes as well as over-production of the target molecule of the insecticide, or overproduction of the enzyme that detoxifies the insecticide.

In the mosquito *Culex quinquefasciatus*, and in many other species of arthropods, resistance to temephos, an organophosphate (OP) insecticide, arises because one or several detoxifying esterases have become highly active and thus prevent the toxicant from reaching its target at lethal concentrations. One of these enzymes (esterase B1) was purified from a Californian strain

Tem-R of the mosquito and it was estimated that esterase B1 is 500 fold more abundant in OP-resistant *Tem-R* than in susceptible mosquitoes (Towbin *et al.*, 1979). Mouchés and colleagues (1986) showed that the overproduction of this detoxifying esterase in *C. quinquefasciatus* is the result of gene amplification. Adults of the *Tem-R* strain were found to possess at least 250 times more copies of the esterase gene than adults of the susceptible strain.

Then an association between resistance to temephos, quantitative increase in the activity of a detoxifying esterase and specific DNA sequence amplification was described in the housefly *Musca domestica*. The resistant strain of the housefly produces much more glutathione-S-transferase, and possibly an esterase, than the susceptible strain (Hyrien and Buttin, 1986).

It has been established that OP resistance in peach-potato aphids, *Myzus persicae Sulz.*, results from the increased synthesis of an esterase (E4) (Field *et al.*, 1988). In 1988 Field and colleagues reported the isolation of two E4cDNA clones and their use in studying the genetic basis of the increased esterase production responsible for resistance. Their work established that insecticide resistance in *M. persicae* is accompanied by amplification of the esterase structural gene. Hybridization of the cDNA clones to genomic Southern blots showed that only some of the esterase-related restriction fragments are amplified. Multiple esterase gene copies appeared grouped together in tandem arrays, rather than scattered throughout the genome.

It is plausible that gene amplification is also involved in P450 cytochrome expression and responsible for resistance to the insecticide DDT in *OR-R* strains of *D. melanogaster*. If gene amplification is involved, then in a cross between DDT resistant and sensitive individuals, where the offspring would be expected to be heterozygous for the *RI* gene, then the resistant parent would have a section of the second chromosome where the *RI* gene is located (at 2-64-66 map units) that is amplified or longer than that of the DDT sensitive parent which carries the RI^+ gene. If long enough, this would result in a short region of asynapsis, or in a loop being observed in the second chromosome of F1 larvae in the 64-66 section, as the larvae are heterozygous for the *RI* gene. Since in *Drosophila* homologous chromosomes are paired in somatic tissue, these loops should be observable in salivary gland chromosomes. If, however, gene amplification is not responsible for insecticide resistance, then loops would not be observed in section 64-66 of the second chromosome in F1 larvae, as both *RI* and RI^+ genes would then be the same length. Resistance might then be a consequence of: i) point mutation, ii) amplification of the *RI* gene at a different chromosomal site, or iii) extra-chromosomal amplification of the *RI* gene. Since the *RI* gene seems stable, the third possibility is unlikely. Another possibility is that the amplification of the *RI* gene, if it does occur, is too small to be cytologically visible.

4.2 MATERIALS AND METHODS

4.2.1 Drosophila Crosses

The following crosses were made at 18°C in well yeasted culture bottles containing fresh *Drosophila* medium. For the preparation of large salivary gland chromosomes larvae should be grown at low temperatures (Graf *et al.*, 1992 b), below 18°C, and that is the reason for incubating stocks at 18°C in these cytology studies. Twenty males and twenty females were used to start up the cultures in each of the following crosses.

1) *flr*^{3} ♀ x *mwh* ♂
2) *mwh* ♀ x *flr*^{3} ♂
3) *ORR;flr*^{3} ♀ x *ORR;mwh* ♂ (original)
4) *ORR;mwh* ♀ x *ORRflr*^{3} ♂ (original)
5) *ORR;flr*^{3} ♀ x *mwh* ♂ (original)
6) *mwh* ♀ x *ORR;flr*^{3} ♂ (original)
7) *flr*^{3} ♀ x *ORR;mwh* ♂ (original)
8) *ORR;mwh* ♀ x *flr*^{3} ♂ (original)
9) *ORR;flr*^{3} ♀ x *ORR;mwh* (new)
10) *ORR;mwh* ♀ x *ORR;flr*^{3} ♂ (new)
11) *ORR;flr*^{3} ♀ x *mwh* ♂ (new)
12) *mwh* ♀ x *ORR;flr*^{3} ♂ (new)
13) *flr*^{3} ♀ x *ORR;mwh* ♂ (new)
14) *ORR;mwh* ♀ x *flr*^{3} ♂ (new)

15) *OR-R* ♀ x *OR-C* ♂

16) *OR-C* ♀ x *OR-R* ♂

In addition, cultures of stocks *OR-R* and *OR-C* were set up. After three days, the parental generation was discarded. About five to seven days after egg-laying, many large third instar larvae were present in these uncrowded cultures, and these could be used for the polytene studies. Third instar larvae were taken during the wandering phase when the larvae move off the surface of the food and crawl up the sides of the culture bottles.

4.2.2 Procedure for Removing Larval Salivary Glands (Adapted from Atherton and Gall, 1972)

A late third instar larva was removed from the side of the culture bottle using a clean dissecting needle immersed in isotonic insect Ringer's (see APPENDIX H for recipe) so that the larva did not drop off the needle. The larva was washed in a little Ringer's solution in a well slide and then placed in a drop of Ringer's on a clean slide under a low power dissecting microscope. Larvae should not be left in Ringer's solution for too long as the osmotic shock to the larvae may cause salivary glands to shrink. The light source was adjusted to give bright bottom light shining through the clear stage plate. A white tile should not be used as this makes it more difficult to see the salivary glands.

The posterior end of the larva was held with a needle and the tip of a second needle was pressed down on the head immediately behind the mouthparts. The head was pulled gently but firmly so that it came cleanly away from the body with the paired salivary glands attached to it by the salivary ducts

(please see Figure 4.2.1). If the glands did not come away with the head, then the body was gently squeezed from posterior to anterior ends to expel them. Care was taken not to touch the glands; the glands were handled by holding the ducts or the fat bodies as they are very fragile.

The glands were gently teased away from the rest of the viscera and then most of the adhering, non-glandular tissue (including the mouthparts) was carefully removed. The best preparations are made from glands with the least amount of fat body attached, but it was essential not to risk damaging the glands by attempting to clear away all the fat body.

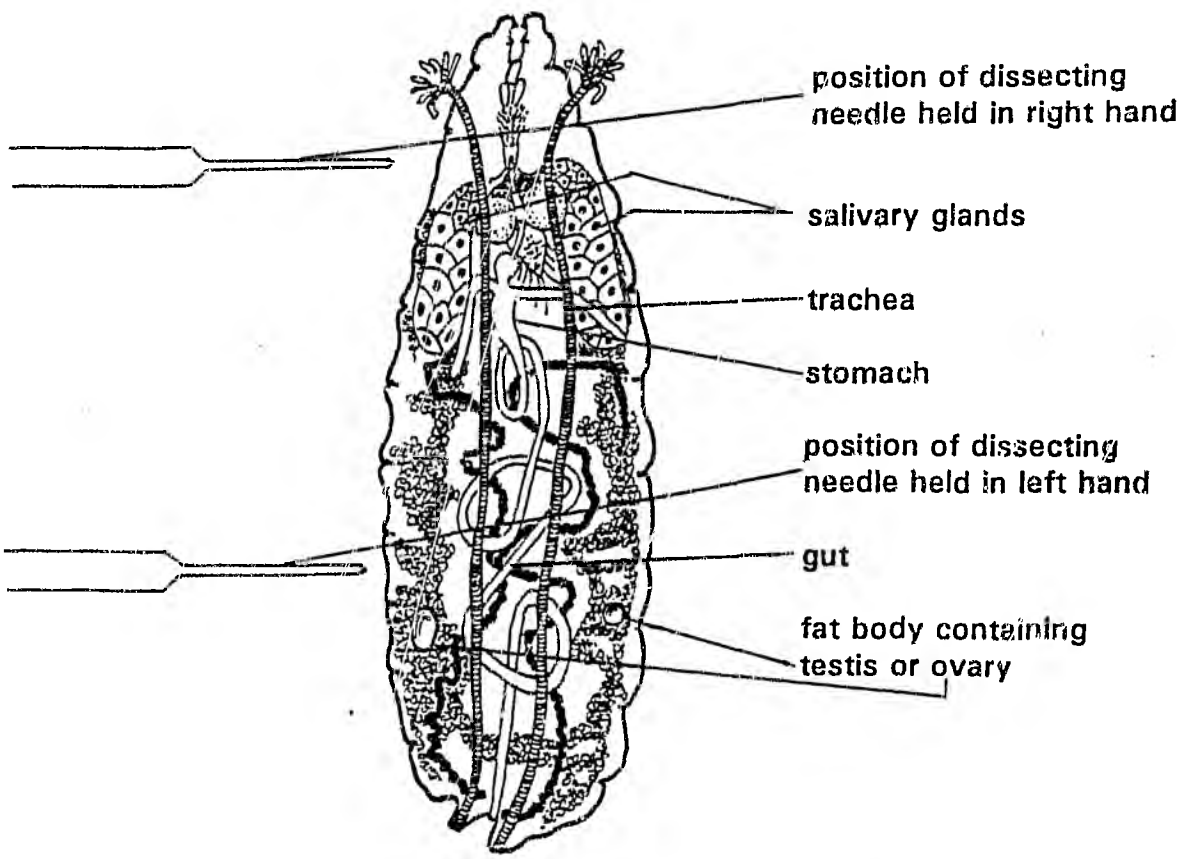


Figure 4.2.1 Longitudinal section through dorsal view of third instar larva of *Drosophila melanogaster* showing position of the salivary glands and how larva is held with two dissecting needles. Magnification 95x. After Darlington and La Cour (1969) and Graf *et al.* (1992b).

4.2.3 The Squash Technique

Once the glands were as clean as possible, they were transferred to a drop of propio-carmine (please see preparation in APPENDIX H) on a clean slide. If the slides or coverslips are dirty then there is no possibility of producing a well spread, flat chromosome preparation. Any hard, foreign material left on the slide or coverslip will prevent effective squashing of cells and chromosomes (McGregor, 1983).

The glands were examined under the dissecting microscope and the two lobes of the gland were adjusted if they were overlapping or unsubmerged. The drop of stain was tapped gently three times with the tip of an unplated steel dissecting needle until the carmine just started to change colour becoming darker. The iron propionate formed by the action of the propionic acid on the needle acts as a mordant for the carmine, but the needle should be removed just as the carmine changes colour, because too much iron precipitates the carmine. The slide was then gently heated over a spirit flame, monitoring that it did not get too hot by testing the temperature on the back of the hand. Gentle heating of the slide over a spirit flame evaporates residue stain, flattens the cells, sticks them to the slide and spreads the chromosomes (M°Clintock, 1929), but if this step is exceeded, then the chromosomes will rapidly degenerate into a non-refractile mass.

The glands were left in the stain for ten minutes on a flat level surface to ensure that the preparation did not dry up if the drop moved. An acid-washed coverslip (see APPENDIX H) was then placed over the glands, avoiding air

bubbles. The slide was placed, coverslip up, between a filter paper disc that had been folded in half and the excess propio-carmine around the coverslip was gently blotted away. The coverslip was then held firmly in position with the index finger of one hand and tapped sharply ten times with the eraser end of a pencil. Care was taken not to tap too much as, although this breaks the nuclear membrane and causes chromosomes to spread, it may also eventually break the chromosomes into fragments. Similarly, care was taken not to move the coverslip sideways during the squashing procedure because this causes distortion of the chromosomes and chromosome breakage. Thumb pressure was applied gently but firmly to the coverslip through the underlying filter paper. The preparation was then checked under a compound microscope (100 times magnification) to ensure that the chromosomes were well spread out and to decide if more tapping or squashing was needed. If necessary, the coverslip was tapped a few more times until the desired degree of spreading was achieved, or squashed more if the preparation was not flat. It rarely, if ever, occurs that every chromosome set is well spread. It is better to stop tapping when a few sets of chromosomes are well spread than to spoil these by trying to spread all the nuclei to the same quality. An ideal preparation will not only have well spread chromosome arms, but the chromosomes themselves will be flat and in focus in a single plane. The coverslip was sealed with wax and made permanent by means of the dry-ice quick-freeze method of Conger and Fairchild (1953).

4.2.4 Permanent Preparations

The slide was placed on a flat block of dry-ice and left for ten minutes. A covered dry-ice container was used to avoid condensation and ice forming on the surface of the slide. The slides were removed from the dry ice and immediately the coverslips were flicked off by prising a single edge razor blade under one corner. This had to be done in one swift movement making sure that the coverslip did not fall back on to the preparation. The slide was plunged directly (before thawing) into a clean Coplin jar containing 96% ethanol and left for five minutes. The slide was then passed through two changes of absolute (100%) ethanol (five minutes in each).

A small drop of Euparal was placed on a clean coverslip. The slide was removed from the last 100% ethanol wash and, leaving an excess of ethanol on the slide, the slide was lowered on the drop of Euparal and mounted. The slide was then allowed to dry for five days on a warming plate.

This procedure was repeated for each cross. After five days the slides were observed under high power magnification, first under 400 times and then 1000 times magnification under oil immersion. Using a reference map of the *D. melanogaster* salivary gland chromosomes, the chromosome ends were identified according to their banding patterns. Since the RI gene is located between 64,0 and 66,0 map units it was expected to lie approximately 2/3 (67%) of the length along the long arm or right arm of chromosome two (2R). Selected squashes were then photographed and the film was developed in the darkroom.

4.3 RESULTS

In salivary glands of *OR-C* stock larvae and in offspring from the crosses *mwh* x *flr*^{3} and *flr*^{3} x *mwh*, homozygous for the *RI*$^+$ gene, as well as in salivary glands of the *OR-R* stock larvae, homozygous for the *RI* gene, it was easy to follow 2R and the X chromosomes along their entire length and no loops or regions of asynapsis were observed (Figures 4.3.1 a and 4.3.2 a). However, in RI heterozygous progeny from crosses between DDT resistant and sensitive parents, it was often difficult to follow 2R and the X chromosomes along their lengths, due to excessive coiling of the chromosomes, although this may have been purely coincidental.

In progeny from a few crosses, regions of asynapsis were observed along 2R and the X chromosome (Figures 4.3.1 c, d, e and 4.3.2 c, d and e) and the frequency at which asynapsis occurred, is listed in Table 4.3.1. Although these regions of asynapsis were not observed in *RI*$^+$ and *RI* homozygotes, it was not enough evidence to conclude that these regions of asynapsis only occur in RI heterozygotes. Several hundreds of slides would have to be observed to conclude that regions of asynapsis do not occur in *RI*$^+$ and *RI* homozygotes. The lengths of the 2R and X asynaptic regions were measured in three different photographs (Figures 4.3.1 and 4.3.2 c, d and e) to determine if the same area of the second chromosomes was involved in all these cases. A slide of these photographs was projected onto a screen and the distance from the tip of 2R (or X) to the chromocentre was measured using a string. This was distance z. The region of asynapsis was distance y

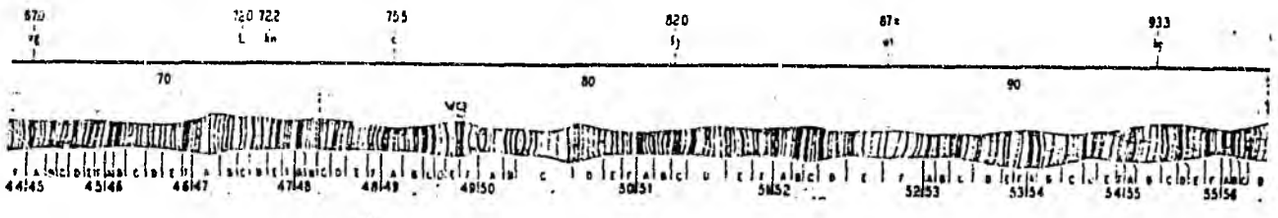
and the distance from the tip of 2R (or X) to the region of asynapsis was x. The ratios were calculated in order to compare the proportions of the three asynaptic regions in the different photographs. These appear in Table 4.3.2.

Regions of asynapsis observed along the X chromosome, as seen in Table 4.3.1, occurred less frequently than on 2R. This could be because, theoretically, half of the larvae observed would have been male and the other half female, and male larvae would not have been heterozygous for genes on the X and hence would not have exhibited any regions of asynapsis along the X chromosome. Larvae were not sexed before use in these cytogenetic experiments.

The pattern of bands in the photographs was carefully studied and with the aid of the reference map it was possible to conclude that the asynaptic region along 2R extended from bands 47C/D to about 54A/B. It seemed that there were two non-homologous regions where the bands did not match in the asynaptic region: between bands 50/51 and 52D and between bands 52E and 53C and the two homologues differed in length due to the presence of extra bands in these non-homologous regions. The region of asynapsis in the X chromosome started around band 11B and ended around 14B in some cases, and extended from bands 12/13 to 14/15 in other cases. The bands apparently failed to coincide between 12/13 and 13C, 13E and 14A and 14/13 to 14/15.



a)



b)

Figure 4.3.1 Salivary gland chromosomes of *Drosophila melanogaster*. Regions of asynapsis observed near the right arm of chromosome 2 (2R). Magnification 400x. a) Normal 2R observed in *RI* homozygotes (*OR-R*). b) Section of 2R from reference map showing position 64,0-66,0.



c)



d)

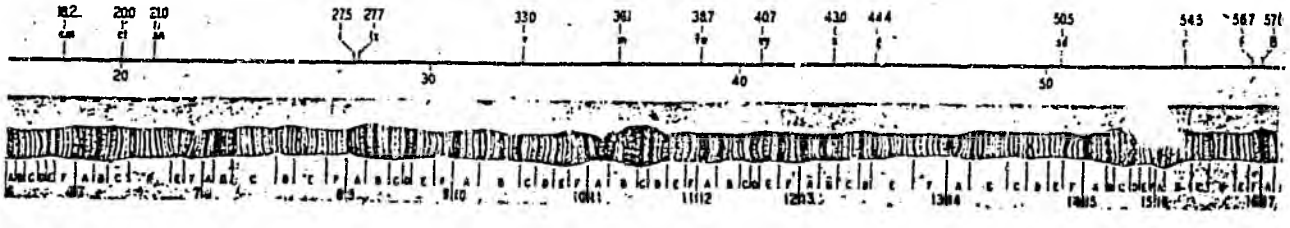


e)

Figure 4.3.1 c,d,e) Different regions of asynapsis observed in *RI* heterozygotes from crosses *OR-R x OR-C; ORR;flr*^{3} (old) x *mwh; mwh x ORR;flr*^{3} (old). In each case the section of the photograph where the region of asynapsis appears was enlarged to determine if the banding pattern of the two homologues corresponded.



a)



b)

Figure 4.3.2 Salivary gland chromosomes of *Drosophila melanogaster*. Regions of asynapsis observed along the X chromosome. Magnification 400x. a) Normal X chromosome observed in resistance homozygotes (*OR-R*). b) Section of X chromosome from reference map showing position 12-15.



c)



d)



e)

Figure 4.3.2 c, d, e) Different regions of asynapsis observed in resistance heterozygotes *(OR-R x OR-C; ORR;flr*^{3} (old) x *mwh* and *flr*^{3} x *ORR;mwh*. In each case, the section of the photograph, where the region of asynapsis appears, was enlarged to determine if the banding pattern of the two homologues corresponded.

TABLE 4.3.1 FREQUENCY OF ASYNAPTIC REGIONS

CROSS	CYTOGENETIC OBSERVATION	FREQUENCY[a]
STANDARD		
flr³ x mwh		0/5 0%
mwh x flr³		0/5 0%
ORIGINAL HB		
ORR;flr³ x ORR;mwh	region of asynapsis on 2R	1/3 33%
ORR;mwh x ORR;flr³		0/4 0%
ORR;flr³ x mwh *(Improved HB)*	region of asynapsis on 2R region of asynapsis on X	2/10 20% 1/10 10%
mwh x ORR;flr³		0/6 0%
flr³ x ORR;mwh	region of asynapsis on X	1/8 12,5%
ORR;mwh x flr³		0/6 0%
NEW HB		
ORR;flr³ x ORR;mwh		0/5 0%
ORR;mwh x ORR;flr³		0/5 0%
ORR;flr³ x mwh	region of asynapsis on X	1/5 20%
mwh x ORR;flr³	region of asynapsis on 2R[c]	2/5 40%
flr³ x ORR;mwh		0/5 0%
ORR;mwh x flr³	region of asynapsis on X	1/5 20%
USA STRAINS*		
OR-R		0/6 0%
OR-C		0/6 0%
OR-R x OR-C	region of asynapsis on 2R[b] region of asynapsis on X[b]	2/6 33% 1/6 17%
OR-C x OR-R		0/6 0%

* Sent to me by Dr Barnes in 1991.

[a] Number of asynaptic regions/number of slides (or individuals) examined. Each slide corresponded to a pair of salivary glands from the same individual. The asynaptic regions were observed in chromosomes from different individuals except in the case of [b] where the regions of asynapsis were observed on chromosomes X and 2R in the same nucleus of the same salivary gland of one individual and [c] where the regions of asynapsis on 2R were observed in two nuclei of the same salivary gland.

TABLE 4.3.2 COMPARISON OF THREE ASYNAPTIC REGIONS IN 2R AND X CHROMOSOMES

PHOTOGRAPH	DISTANCE (cm)	RATIO	PROPORTION
2R CHROMOSOME 4.3.1 c	x= 3,0 y= 3,0 z= 13,0	x/y= 1,0 x/z= 0,23 y/z= 0,26 x + y/z= 0,46	100% 23% 26% 46%
  4.3.1 d	x= 3,8 y= 2,6 z= 11,5	x/y= 1,46 x/z= 0,33 y/z= 0,23 x + y/z= 0,56	146% 33% 23% 56%
  4.3.1 e	x= 3,8 y= 2,5 z= 9,8	x/y= 1,33 x/z= 0,39 y/z= 0,26 x + y/z= 0,64	133% 39% 26% 64%
X CHROMOSOME 4.3.2 c	x= 13,5 y= 3,0 z= 21,0	x/y= 4,5 x/z= 0,64 y/z= 0,14 x + y/z= 0,79	450% 64% 14% 79%
  4.3.2 d	x= 5,5 y= 1,2 z= 10,0	x/y= 4,58 x/z= 0,55 y/z= 0,12 x + y/z= 0,67	458% 55% 12% 67%
  4.3.2 e	x= 10,0 y= 2,7 z= 21,5	x/y= 3,7 x/z= 0,47 y/z= 0,13 x + y/z= 0,5	370% 47% 13% 59%

The presence of asynaptic regions in F1 progeny from the original HB cross *ORR;flr*^{3} x *ORR;mwh* suggests that the F1 progeny is *RI* heterozygous and provides indirect evidence that the original *ORR;mwh* strain does not carry the *RI* gene.

Inversion loops were observed near 3L and 3R in larvae from crosses involving one parent *flr*^{3}*/TM3 Ser*. These inversion loops were identified as the TM3 inversion (Fig. 4.3.3) and were observed in more or less 20% of the cases although expected in 50%.



Figure 4.3.3 Salivary gland chromosomes of *D. melanogaster*. Loops indicating the presence of the TM3 inversion in F1 heterozygous larva from the cross *ORR;flr*^{3} x *mwh*.

4.4 DISCUSSION

In *D. melanogaster* the genetic map is well characterized and it has been possible to localize gene sequences to many polytene bands. The cytology of the vestigial gene (2-67.0) is between bands 49D2-E1 (Lindsley and Zimm, 1990) and this region of chromosome two was identified to determine if the *Rst(II) DDT* gene (2-65,0) was amplified. As can be seen in Table 4.3.1, asynaptic regions along 2R were observed in 20-40% (average 32%) of *R/* heterozygotes while asynaptic regions along the X chromosome occurred less frequently, in about 10-20% (average 16%) of heterozygotes. In the three [illegible] regions studied, the region of asynapsis on chromosome 2 was [illegible] more or less the same section of the chromosome, between bands 47C1 and 51 A/B and this region would be adjacent to the vestigial gene. This suggests that the region of asynapsis along 2R occurs in the same region where the *Rst(II)DDT* gene has been mapped. There are sections within the asynaptic region where the bands do not coincide. As can be seen in Figures 4.3.1 c, d and e, some bands appear to be missing in one of the homologues and the two homologues in the asynaptic region differ in length. Even though the length of the chromosome varies according to the amount of thumb pressure applied during the squashing procedure, comparison of the three asynaptic regions in Table 4.3.2 indicates that the size of the asynaptic region and the ratio of length of region of asynapsis to length from the tip of the chromosome to the chromocentre is approximately the same in all three cases. These findings seem to agree with the hypothesis that resistance to the insecticide DDT is due to amplification of the resistance gene at position 2-

65,0, giving rise to over-expression of P450 cytochromes involved in the oxidative attack and detoxification of this insecticide. The presence of asynaptic regions along the X chromosome, suggests that gene amplication of a resistance gene(s) on the first chromosome may also occur.

Even though regions of asynapsis were observed in certain regions near the 2R end of chromosome 2, molecular evidence is required. It is necessary to isolate cDNA clones of the *Rst(II)DDT* gene and to study hybridization of these cDNA clones to OR-R genomic Southern blots to determine if DDT resistance does in fact result from amplification of this gene, before any definite conclusions can be made.

CHAPTER FIVE

5 BIOCHEMICAL ASSAYS FOR P450 CYTOCHROME ACTIVITY

5.1 INTRODUCTION

Many chemical mutagens to which the human population is exposed, are not mutagenic *per se*. interaction with DNA usually requires some reactivity of the compounds. Natural exposure to directly active compounds is generally low, as these compounds have a good chance of reacting with alternative nucleophiles, such as water and amino acids in food, before they enter the human body. Therefore, most mutagenic and carcinogenic compounds are chemically rather inert (Zjilstra *et al.*, 1987) but are metabolized in the body to their ultimate mutagenic and carcinogenic forms.

In mammals the major organ responsible for the metabolism of xenobiotic compounds (foreign drugs and chemicals) is the liver, but other organs and tissues such as the lungs, mucous membranes and skin, are also capable of metabolizing foreign chemicals. The function of xenobiotic metabolism is to convert lipid-soluble, non-polar, non-excretable forms of chemicals to water soluble, polar forms that are excretable in bile and urine. Lipophilic compounds are extensively metabolized through oxidizing enzymes that introduce hydroxyl groups in the molecule, either through direct insertion of an oxygen atom in a C-H bond, or through epoxidation of an unsaturated bond followed by spontaneous or enzymic hydrolysis. Oxidation of these compounds results

in metabolites with a higher solubility in water which can therefore be excreted more easily. Hydroxylation of lipophilic compounds is usually performed by the cytochrome P450 system. P450 cytochrome functions as the terminal oxidase in the mixed function mono-oxygenase system. In cytochrome P450, the 450 refers to the position of the intense Soret band of the carbon monoxide difference spectrum (Metzler, 1977).

These enzymes metabolize endogenous chemicals and process foreign chemicals and natural products (Guengerich, 1991). The overall oxygenation reactions include hydroxylation at carbon atoms and the heteroatoms nitrogen and sulphur, dealkylations of amines and ethers, desulphuration and epoxidation. The different P450 enzymes should not be considered invidually as being strictly demethylases or oxidases; the reaction effected is a function of the fit of the substrate with the apoprotein. A single P450 protein can catalyze different types of reactions, depending on the substrate presented (Guengerich, 1991).

In 1980, Baars obtained enzymatically active microsomal preparations from *D.melanogaster* and began studies of its polysubstrate mono-oxygenase systems. Several types of cytochrome P450 were identified in whole body homogenates from a variety of *Drosophila* strains. Microsomal fractions showed enzymatic activities such as aryl hydrocarbon hydroxylation, paranitroanisole (pNA) demethylation, aldrin epoxidation, UDP-glucuronyl-transferation and were able to perform the epoxide hydrolase catalyzed

hydration of styrene oxide. The post mitochondrial supernatant contained glutathione-S-transferase and phosphotransferase activity (Baars, 1980).

Metabolic differences between larvae and adult flies were sometimes observed. In 1983, Hällström, Blanck and Atuma compared the activities of several drug metabolizing enzymes in microsomes from larval and adult *Drosophila*. The cytochrome P450 content and the benzo(a)pyrene (BP) hydroxylation, pNA demethylation and 3- and 4-hydroxylation of biphenyl were four to twenty fold higher in microsomes from adult flies, while 7-ethoxycoumarin deethylase activity and cytochrome c reductase activity were similar in the two stages. Hence, the results demonstrate a much higher P450 content and a higher capacity for most P450-mediated reactions in adult flies when compared with larvae (Hällström *et al.*, 1983). Hällström and Gräfström (1981) also found that levels of cytochrome P450 content were much lower in larvae than in adults. In addition, Frölich and Würgler (1991b) reported that cytochrome P450 content in microsomal fractions from larval was lower than that from adult progeny of both the standard and high bioactivation [DDT resistant] cross of SMART.

Hällström and colleagues (1983) concluded that the higher metabolic capacity of most cytochrome P450-dependent enzyme activities in adult flies indicates that treatment of adult flies should result in a higher sensitivity to detect indirect mutagens and carcinogens than larval treatment. Based on these studies, differential responses to procarcinogens could be expected with treatments of larvae and adults (Hällström *et al.*, 1983).

However, Tsukamoto (1958) stated that larvae were more resistant than adults to the toxic effects of DDT and this was also observed in these experiments (see section 3.3). Then, in 1984, Hällström and colleagues found that metabolic differences were more marked in larvae indicating that the selection pressure of the insecticides is acting preferentially on the larval stages. In 1991b, Frölich and Würgler found an increased aldrin epoxidase activity in microsomes of larvae compared with adults of both the standard and HB crosses. Therefore, treatment of larvae may also represent a sensitive system for the detection of promutagens. The P450 content and activity of larva and adult microsomes was also compared in this study.

Pre-treatment with inducers may increase several enzyme activities leading to higher sensitivity to some classes of indirect mutagens so Hällström and colleagues (1983) compared the inducing capacities of phenobarbital (PB), aroclor 1254 and beta naphthoflavone and found that in *Drosophila* the cytochrome P450 enzyme system is inducible and phenobarbital is the best inducer of the activities studied. The following year, genetic variation between the strains was observed after PB treatment in the content of P450 and in the various enzyme activities, varying from non-responsiveness to a 4-5 fold increase. Again, aroclor 1254 (PCB) was less efficient in enhancing the activities (Hällström *et al.*, 1984).

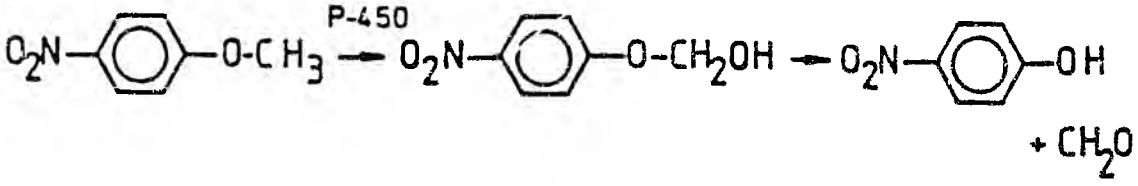
In the *Salmonella*/mammalian microsome assay, the rats from which the liver S9 fraction is prepared are routinely pretreated with polychlorinated biphenyls (PCB aroclor 1254). This pretreatment can not be used in *Drosophila* tests

because of the weak inducing potency of PCB in adult flies when compared with the potency in rat liver. Furthermore, according to Hällström and colleagues (1983) enzyme induction is not a general way of increasing the detection capacity and sensitivity of mutagenicity tests with *Drosophila* as the test organism. So far, enzyme induction has been of only limited value in mutagenicity testing in *Drosophila*, often decreasing mutagenicity rather than increasing it (Zjilstra *et al.*, 1984; Frölich and Würgler, 1990a). Enzyme induction by PB was investigated in this study to determine if pre-treatment with inducers leads to an increase in P450 content and activity.

In addition to metabolic differences between larvae and adult flies, distinct strain differences have also been observed (Zjisltra *et al.*, 1984). In a study by Hällström and colleagues (1984), seven *Drosophila* strains were compared by means of several enzymatic activities in subcellular fractions. The study covered four wild type strains of different geographic origin, two DDT resistant strains [Hikone R and Oregon R (R) (OR-R)] and an AFB1 tolerant strain. A marked genetic variation in the capacity to perform xenobiotic metabolism was observed in microsomal fractions from the seven strains. The two insecticide resistant strains Hikone R and OR-R differed marked'y when compared to sensitive strains by having a 3-17 fold higher pNA demethylase activity and biphenyl-3-hydroxylase activity and a nearly four fold higher cytochrome P450 content was observed in the insecticide resistant strains compared with sensitive strains (Hällström *et al.*, 1984). Although Zjilstra and colleagues (1984) also found an increase (32-fold) in pNA demethylase activity in insecticide resistant compared with insecticide sensitive adults the

P450 content was about the same in insecticide resistant and sensitive strains.

pNitroanisole is often used as the model substrate in the *in vitro* study of metabolic enzyme activities. The methyl group of pNA can be hydroxylated by P450 cytochromes. This results in an unstable hydroxymethyl group which decomposes to form formaldehyde and the highly coloured p-nitrophenol, which is easily detected spectrophotometrically (see Figure 5.1).



hydroxylation of methyl group by P450 cytochromes

decomposition

p-nitroanisole

unstable intermediate with hydroxymethyl group

p-nitrophenol + formaldehyde

Figure 5.1 pNitroanisole demethylation reaction

In 1991, Frölich and Würgler isolated progeny larvae from both the standard and original HB crosses and of the Oregon R-R strain and examined these for aldrin epoxidation activity and cytochrome P450 content *in vitro*. They found that the larval microsomes of the original HB cross showed a significantly increased activity for this monooxygenase mediated reaction in comparison with the standard cross and similar activity to microsomes of the *OR-R* strain. The authors concluded that the introduction of chromosomes one and two from the *OR-R* strain into the SMART tester strains significantly improved the metabolic capacity of these strains with respect to the epoxidation of aldrin. However, their results may be interpreted as indicating only that the offspring from the HB cross had a significantly improved bioactivation capacity compared with progeny from the standard cross, but, since the *RI* gene responsible for the high bioactivation capacity in *ORR* strains is a dominant gene, it was not possible to conclude from their results that both parents of the HB cross exhibit improved metabolic capacity as the progeny could be heterozygous for the *RI* gene and still show high bioactivation capacity. This was the motivation for examining the P450 cytochrome-dependent pNA demethylase activity and P450 content in tester strain adults for direct evidence that the newly constructed HB tester stocks possess biochemically detectable improvement in their metabolic systems.

5.2 MATERIALS AND METHODS

5.2.1 Induction of P450 cytochromes

This procedure was based on the recommendation of Zijlstra and colleagues (1984). One day old adults from the original *ORR;flr*^{3} and *ORR;mwh*, the new *ORR;flr*^{3} and *ORR;mwh*, *OR-R* and *OR-C*, and standard *flr*^{3} and *mwh* stocks were transferred to fresh medium bottles containing 3 g of phenobarbital per ml of medium for three days for the induced group, and to ordinary fresh medium bottles for the control group. The phenobarbital medium bottles were prepared by dissoving 3 g sodium phenobarbital in 10 ml water and adding it to one litre of liquid, warm (50°C) food, giving a 12 mM concentration.

5.2.2 Isolation of Microsomes

The entire procedure was performed at 4°C. Approximately 3 g of flies (± 3000 ♂ and ♀) were anaesthetized with CO_2 and placed in an ice cold beaker. Microsomes were prepared according to the procedures of Hällström *et al.* (1983) and Frölich and Würgler (1991b). The adult flies from the stocks in 5.2.1 and third instar larvae from the new HB cross (*ORR;flr*^{3} x *ORR;mwh*) and the standard cross (*flr*^{3} x *mwh*) [uninduced] were homogenized in ice cold 0,2 M phosphate buffer (pH 7,5; 0,1 g flies/ml; 0,25 g larvae/ml) using a pestle and mortar and the adult fly homogenate was filtered through nylon gauze in a separating funnel to remove legs, wings and other debris. The adult filtrate and larval homogenate were homogenized in a motor driven Virtis homogenizer. Care was taken to homogenize at low speed and discontinuosly

at 5 second intervals so that the temperature of the sample did not rise above 4°C due to the heat released by the rotating blades.

The new larval homogenate was centrifuged at 600 x g for two minutes followed by 15 000 x g for ten minutes. The new adult homogenate was first filtered through nylon gauze and then centrifuged at 15 000 x g for ten minutes. The adult and larval supernatants were layered over 0,3 M sucrose buffer and centrifuged at 115 000 x g for 60 minutes. The supernatant was discarded, the walls of the centrifugation vials were cleaned and the remaining pellets were resuspended in buffer and frozen at -70°C.

5.2.3 Protein Determination

The protein concentration in the microsomal preparation was determined by the method of Lowry *et al.*, (1951) using BSA protein standards in a concentration range of 0 to 1,6 mg/ml (see section 6.2.6).

5.2.4 Determination of the P450 Cytochrome Content

The microsomal preparation was diluted to a final concentration of 2 mg of protein per ml in 0,2 M phosphate buffer (pH 7,5). The cytochrome P450 content was measured using the method of Omura and Sato (1964 a). The CO difference spectrum was first recorded in a NADPH-reduced suspension which gave a clear P450 spectrum and then in a sodium dithionite reduced suspension (Zijlstra *et al.*, 1984). The cytochrome P450 concentration per mg protein was calculated using the Beer-Lambert Law (Williams and Wilson,

1976) assuming a molar extinction coefficient of 91 $cm^{-1}mM^{-1}$ (450 nm-490nm) in sodium dithionite reduced samples (Omura and Sato, 1964 b).

5.2.5 p Nitroanisole Demethylase Assay

This assay was performed according to the procedure described by Zijlstra and colleagues (1984). The optical density at 400 nm was recorded versus a reference wavelength of 500 nm. Two hundred microlitres of microsomes were added to 3 ml 2 mM pNA in buffer and warmed to 25°C. Three millilitres of the mixture were transferred to the cell and after optical equilibration, the reaction was started by adding 50 μl 2 mM NADPH. Values for pNA demethylation were measured at a concentration of 1,84 mM pNA for the first three minutes of enzyme action, at which the activity was linear.

5.3 RESULTS

5.3.1 Cytochrome P450 Content

Overall, the P450 content in the induced group was twice that in the uninduced or control group. The highest P450 content in adult microsomes was found in the *OR-R* microsomes. The P450 content in the new and old *ORR;flr*^{3} and new *ORR;mwh* microsomal suspension was significantly increased over that of the standard tester strains *flr*^{3} and *mwh* for both the induced and control group. The P450 content in the new *ORR;mwh*, however, was not significantly increased over that of the *OR-C* microsomes in both the induced and control group and similarly the P450 content of the original *ORR;flr*^{3} microsomes was not significantly increased over that of the *OR-C* microsomes in the induced group. The original *ORR;mwh* showed a similar P450 content to the standard tester strains and *OR-C* strain in both the induced and control group. The P450 content in third instar progeny larvae from the standard cross and new HB cross was in both cases higher than the P450 content in microsomes of the adult parents of the respective crosses. The P450 content in uninduced larval microsomes from the new HB cross was about twice that in larval microsomal suspensions from the standard cross, and similar to the P450 content of uninduced *OR-R* adult microsomes.

TABLE 5.3.1 CYTOCHROME P450 CONTENT OF DIFFERENT STRAINS

SOURCE OF MICROSOMES	CYTOCHROME P450 CONTENT (nmol/mg protein)	
	CONTROL GROUP	INDUCED GROUP
ORR;flr^3 (original)	0,134	0,235
ORR;mwh (original)	0,093	0,201
ORR;flr^3 (new)	0,157	0,280
ORR;mwh (new)	0,120	0,255
New HB larvae	0,186	-
OR-R	0,161	0,308
OR-C	0,102	0,229
flr^3	0,070	0,105
mwh	0,025	0,082
Std larvae	0,094	-

In this table, the F- ratio for heterogeneity between strains was highly significant (P = 0.0034) and for between groups it was P = 0.0001.

5.3.2 p Nitroanisole Demethylase Assay

pNA demethylase activity was detected in microsomal fractions from all stocks and the activity in the induced group was, on average, 2-3 times higher than the activity in the control group for all stocks. pNA demethylase activity was similar in the *ORR;flr*^{3} (new and original), *ORR;mwh* (new) and *OR-R* stocks and the activity in these stocks was 3-5 times higher (in the control group) and 10-13 times higher (in the induced group) than the activity in the standard tester strains and original *ORR;mwh* strain. The activity in the *OR-R* was 8 and 10,5 fold higher than the activity in the OR-C microsomes in the induced and control microsomes, respectively (see Table 5.3.2). The pNA demethylase activity in larval progeny from the new HB cross and standard

cross was in both cases higher than the activity in microsomal fractions of the adult parents of the respective crosses. The pNA demethylase activity in control larval microsomes from the new HB cross was about 5 times that in larval microsomal fractions from the standard cross.

TABLE 5.3.2 pNA DEMETHYLASE ACTIVITY

SOURCES OF MICROSOMES	pNA DEMETHYLASE ACTIVITY (nmol p-nitrophenol formed/mg microsomal protein/minute)	
	INDUCED	CONTROL
ORR;flr^3 (old)	1,36	0,246
ORR;mwh (old)	0,20	0,130
ORR;flr^3 (new)	1,41	0,441
ORR;mwh (new)	1,39	0,392
New HB larvae	-	0,547
OR-R	1,59	0,769
OR-C	0,21	0,073
flr^3	0,14	0,097
mwh	0,10	0,08
Std larvae	-	0,111

In this table heterogeneity between strains was less marked (P=0.0621) and between groups it was significant (P=0.0200).

5.4 DISCUSSION

The increased sensitivity of the original, improved and new high bioactivation crosses to promutagens provides only indirect evidence that the substitution of chromosomes one and two from the DDT resistant *OR-R* strain for chromosomes one and two from the standard tester strains, results in an increased activity of cytochrome P450-dependent enzyme activities. These biochemical studies confirm directly the increased metabolic capacity of the high bioactivation strains.

Although the P450 cytochrome content in adult flies from original and new *ORR;flr*^{3} and new *ORR;mwh* stocks was significantly higher than that in the standard tester strains, the cytochrome P450 contents in adult flies from the *OR-C* stock (DDT sensitive) and the old *ORR;flr*^{3} and new *ORR;mwh* (DDT resistant) high bioactivation strains were similar. Zijistra and colleagues (1984) and Frölich and Würgler (1991b) report similar findings with measurements of P450 content in DDT resistant and sensitive stocks. It may be that this technique is not sensitive enough to detect slight differences (nmol amounts) in P450 content. Since quantitative measurements of cytochrome P450 protein may not directly correlate with the metabolic importance of the related enzyme activity, biologically and functionally important differences must be verified by measurements of enzyme activities (Frölich and Würgler, 1991b) such as the P450 cytochrome dependent pNA demethylase activity.

The pNA demethylation activity was significantly higher in the high bioactivation strains than in the standard tester strains. The observation that the original *ORR;flr*^{3} and new *ORR;flr*^{3} and *ORR;mwh* strains show similar pNA demethylase activity to the *OR-R* strain, provides evidence that the increase of this particular monooxygenase function in these strains is dependent on the chromosome substitution and that the newly constructed HB strains and original *ORR;flr*^{3} strain carry the *RI* gene responsible for high P450 cytochrome activity from *OR-R*. Further evidence that the original *ORR;mwh* strain does not carry the *RI* gene comes from the finding that pNA demethylase activity in this strain is similar to that in the standard strains. These results indicate that progeny from the original HB cross would be *RI* heterozygous. Out of the four SMART crosses: the standard cross *flr*^{3} x *mwh*; the original HB cross *ORR;flr*^{3} x *ORR?;mwh*; the improved HB cross *ORR;flr*^{3} x *mwh* and the new HB cross *ORR;flr*^{3} x *ORR;mwh*, the new HB cross is the only cross that produces *RI* homozygous progeny.

Quantitatively, the P450 cytochrome activity differences between the standard and high bioactivation crosses were more pronounced than the cytochrome P450 content differences between strains. The cytochrome P450 enzyme system is inducible and the higher P450 content and increased pNA demethylase activity in the induced group compared with the control group is evidence for this. Hällström and Blanck (1985) reported that the insecticide resistant mutant was not inducible and therefore characterized it as a constitutive mutation. The present results are not in agreement with this, but receive support from the study of Zjilstra *et al.* (1984) of the strain 91-R

which is most probably identical to the *OR-R* stock used here (Frölich, 1989).

When performing SMART, however, it would not be advantageous to induce P450 cytochrome activities in the parents of the cross as it is the trans-heterozygous, third instar larvae from this cross that are treated in the test. It may be plausible, though, to induce the system in larvae from the cross by mixing phenobarbital with the yeast in egg-laying bottles (see APPENDIX E). Both the new high bioactivation strains and third instar progeny larvae of the new HB cross showed more efficient metabolic capacity than the standard tester strains and third instar larvae of the standard cross. The results seemed to indicate that the P450 content and pNA demethylase activity in larvae may be higher than in adults as the P450 content and pNA demethylase activity was higher in third instar larval progeny from the new HB and standard crosses than in the adult parents of the respective crosses This provides evidence that, at least in these experiments, larvae represent the more sensitive system for the detection of promutagens as, in these assays, larvae showed a higher P450 cytochrome content and higher metabolic capacity for the P450 cytochrome-dependent pNA demethylase reaction, than adults.

CHAPTER SIX

6 THE *SALMONELLA*/MAMMALIAN MICROSOME ASSAY

6.1 INTRODUCTION

The *Salmonella*/mammalian microsome assay is based on reversion of base pair substitution or frameshift mutations leading from histidine auxotrophy to histidine prototrophy. The mutagenic ability of a compound is estimated by incubating an indicator strain of *Salmonella* (having a mutation at the *his* locus) with the compound. Essential parts of the mammalian xenobiotic metabolism are included by addition of liver homogenates (the 9 000 x g or supernatal fraction, S9) of mammalian liver microsomes and a NADPH-generating system to metabolize promutagens. Bacteria that form colonies on histidine-free medium arise from revertants at the *his* locus induced by the mutagen, which in turn could have been activated by the enzymes present in the liver extract.

6.2 MATERIALS AND METHODS

6.2.1 THE STANDARD TESTER STRAINS

A set of histidine-requiring *Salmonella typhimurium* strains was used for mutagenicity testing. Each tester strain contains a different type of mutation in the histidine operon (Table 6.2.1). Besides the histidine mutation, the standard tester strains contain mutations that greatly influence their ability to detect mutagens. One mutation, deep rough (*rfa*), causes partial loss of the lipopolysaccharide layer that coats the surface of the bacteria and increases permeability to large molecules that do not penetrate the normal cell wall (Ames *et al.*, 1973). The other mutation (*uvrB*) is a deletion of a gene involved in the DNA excision repair system, which results in increased sensitivity in detecting many mutagens (Ames, 1971). For technical reasons, the deletion excising the *uvrB* gene extends through the *bio* gene and, consequently, these bacteria also require biotin for growth.

The standard tester strains TA97, TA98, TA100 and TA102 contain the R-factor plasmid pKM101. In both *Escherichia coli* and *S. typhimurium*, pKM101 increases chemical and spontaneous mutagenesis by enhancing an error prone DNA repair system that is normally present in these organisms. TA102 also contains the multicopy plasmid pAQ1 which carries the *his G428* mutation and a tetracycline resistance gene (Maron and Ames, 1983). All the reagents, glassware, petri dishes and utensils used, were sterile. All growth media and reagents were prepared according to the methods in APPENDIX A.

The sources of the tester strains and all chemicals used in these experiments are listed in APPENDIX J.

TABLE 6.2.1 GENOTYPES OF THE TESTER STRAINS USED FOR MUTAGENESIS

	Tester strains			
	TA97	**TA98**	**TA100**	**TA102**
histidine mutation	*his D6610*	*his D3052*	*his G46*	*his G428 (pAQ1)*
histidine requirement	+	+	+	+
Lipopolysaccharide	*rfa*	*rfa*	*rfa*	*rfa*
Repair	Δ *uvrB*	Δ *uvrB*	Δ *uvrB*	+
R-factor	+R	+R	+R	+R
pAQ1	-	-	-	+

Δ = deletion through *uvrB* also includes the *bio* gene
***rfa* = deep rough**
+R= R factor present
All strains were originally derived from *S.typhimurium LT2*.
Adapted from Maron and Ames (1983).
Wild type genes are indicated by a +

6.2.2 PROCEDURE FOR GROWING CULTURES

Tester strains were grown in nutrient broth to a density of $1 - 2 \times 10^9$ cells per millilitre according to the procedure of Maron and Ames (1983). The freshly inoculated broth cultures were placed in a 37°C gyrotory incubator

connected to a timer programmed to turn on automatically ten hours before the cultures were to be used.

6.2.3 CONFIRMATION OF GENOTYPES OF TESTER STRAINS

The tester strain genotypes were confirmed as follows after receiving the cultures and before a new set of frozen permanents was prepared. The results of confirming of genotypes are summarized in Table 6.2.2.

6.2.3.1 *rfa* Mutation

Strains having the deep rough (*rfa*) character were tested for crystal violet sensitivity. The *rfa* mutation permits large crystal violet molecules to enter and kill the bacteria. Wild type strains are not inhibited because the crystal violet cannot penetrate the cell.

The crystal violet diffused from the filter paper disc (placed in the centre of the seeded plate) into the surrounding agar. As molecules of crystal violet entered bacterial cells, these cells were killed creating a clear zone of inhibition. The 16 mm zone of inhibition was observed after 48 hours for all four strains.

6.2.3.2 *uvrB* Mutation

The presence of the *uvrB* mutation was confirmed by demonstrating UV sensitivity in strains that contained this mutation. For TA97, TA98, and TA100, growth was observed only on the half of the nutrient agar plate which

had been covered by aluminium foil and not exposed to UV light. An exact line of growth divided the exposed half from the protected half of the plate. The half of the seeded plate which had been irradiated with UV light was clear. This indicated the presence of the *uvrB* mutation in these strains as these bacteria have the gene coding for the DNA excision repair system deleted and are sensitive to the effects of UV light. TA102, on the other hand, does not carry the uvrB mutation, so this strain is able to repair the damage to DNA caused by UV light and growth was observed all over TA102 seeded plates.

6.2.3.3 R-factor

The R-factor strains (TA97, TA98, TA100 and TA102) were tested for the presence of the ampicillin resistance factor. The pKM101 plasmid is quite unstable and can be lost from the bacteria (McCann *et al.*, 1975b). Ampicillin resistance is a convenient marker that makes it possible to test for the presence of the plasmid, but it does not affect the increased sensitivity of the R-factor strains to reversion by mutagens. Growth was observed on ampicillin plates indicating that the plasmid was present in all four strains.

6.2.3.4 pAQ1 Plasmid

The pAQ1 strain (TA102) was tested for both ampicillin and tetracycline resistance on ampicillin/tetracycline plates. Growth on tetracycline/ampicillin plates was observed only on the four TA102 plates. All other plates were clear. This indicated that only TA102 carried the pAQ1 plasmid as only TA102 was resistant to both ampicillin and tetracycline.

6.2.3.5 Histidine Requirement

Fresh overnight culture of each tester strain was added to molten top agar in a test tube and then poured on minimal medium plates which did not contain histidine. None of the strains formed colonies on minimal medium plates. This indicated that all four strains have the His$^-$ character and require histidine for growth. Biotin is also required by all the standard tester strains (except TA102) because of the *uvrB* deletion which extends through the *bio* gene.

TABLE 6.2.2 CONFIRMATION OF GENOTYPES

PROPERTY TESTED FOR	TA97	TA98	TA100	TA102
Growth on minimal medium	-	-	-	-
Growth on ampicillin plates	+	+	+	+
Growth on tet/amp plates	-	-	-	+
Zone of inhibition around disc	+	+	+	+
Growth on UV irradiated half of plate	-	-	-	+

(+)= present and (-)= absent

6.2.4 STORAGE OF THE TESTER STRAINS

Frozen permanent duplicates of the tester strains were prepared from fresh overnight cultures to which DMSO was added as a cryoprotective agent and stored at -70°C.

6.2.5 MAMMALIAN LIVER S9

6.2.5.1 Induction of rat liver enzymes

S9 fraction was purchased from the CSIR where it is prepared according to the procedure in APPENDIX B from rats induced with a polychiorinated biphenyl (PCB) mixture, Aroclor 1254.

6.2.5.2 Components of the S9 Mix

The optimum concentrations of the components of the S9 mix were determined in a series of experiments by Ames and colleagues in 1973. Based on the findings of Maron and Ames (1983) and the recommendation of Miss Genthe at the CSIR, a high S9 mix was prepared with a concentration of 50 μl of S9 fraction per plate. The components of the S9 mix were 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, 100 mM sodium phosphate (pH 7.4) and S9 in a high concentration of 0,1 ml of S9 per millilitre of mix. The S9 mix was freshly prepared for each mutagenicity assay and kept on ice.

6.2.6 PROTEIN DETERMINATION

(Following the procedure of Lowry and colleagues, 1951).

6.2.6.1 The standard:

A stock solution of bovine serum albumin (BSA) [concentration 2 000 μg-BSA/1 000 μl distilled water] was prepared and the following dilutions were

made in labelled test tubes: 200 µg, 400 µg, 800 µg and 1 600 µg BSA/ml of water.

6.2.6.2 The blank:

The blank contained all the ingredients of the S9 mix except for the S9 fraction and NADP. One millilitre of the blank was then added to a test tube.

6.2.6.3 The test sample:

(i) 50 µl S9 mix was added to 950 µl of water in a test tube.

(ii) 50 µl S9 fraction was added to 950 µl of water.

(iii) 10 µl S9 fraction was added to 990 µl of water.

Therefore, each test tube contained 1 ml sample. A 5 ml aliquot of reagent A was added to 1 ml of sample in each tube and after mixing, the tubes were allowed to stand for 20 minutes. Then, 0,5 ml of reagent B (folin) was added and, after mixing, the samples were allowed to stand for 30 minutes. The reagents were prepared according to the recipes in APPENDIX C.

The absorbance at 750 nm was read 30 minutes after adding the folin solution before any precipitation occurred. The absorbance values are shown in Table 6.2.3. A standard curve was plotted and then the protein concentration in the S9 mix used in the mutagenicity assay was determined from the graph and the protein concentration in crude S9 calculated.

(i) From the graph (Figure 6.2.1) the concentration of protein in the S9 mix was approximately 145 μg/50 μl. As 2 ml of S9 fraction was added per 25 ml S9 mix, 36,25 mg of protein was present per millilitre S9 fraction [25ml/2ml x 145μg/50μl = 36,25 mg of protein].

(ii) The spectrophotometer read the absorbance of 50 μl pure S9/950 μl water as 1,341 and the computer calculated the concentration from the standard curve to be 1 934,7 μg/50 μl or 38,69 mg of protein per millilitre of crude S9.

(iii) Absorbance was 0,520 and the concentration was given as 370 μg/10 μl. The protein concentration per millilitre was therefore 37 mg.

One millilitre of S9 fraction contains microsomes from about 250 mg of wet liver. In 1983, Maron and Ames used the procedure of Lowry and colleagues (1951) and found that the protein concentration of their S9 fraction was 40 mg of protein per ml of fraction and the authors stated that the concentration is constant from one S9 fraction batch to the next. The results indicate that the S9 fraction used in these experiments contained liver enzymes in a similar concentration (36-38 mg/ml) to that used by other researchers.

TABLE 6.2.3 RESULTS OF PROTEIN DETERMINATION

Concentration (µg/ml)	Absorbance	(- blank)	Average
0[illegible] (blank)	0,082		
200,00	[illegible] 0,325	0,250 0,246	0,248
400,00	0,590 0,598	0,508 0,466	0,487
800,00	0,727 0,708	0,645 0,626	0,636
1 600,00	1,111 1,016	1,029 0,934	0,9815

6.2.7 THE MUTAGENICITY TEST OR PLATE INCORPORATION TEST

The plate incorporation test consists of combining the test compound, the bacterial tester strain and S9 mix in soft agar which is poured onto a minimal agar plate; positive and negative controls are also included in each assay. After incubation at 37°C for 48 hours, revertant colonies are counted.

Duplicate plates were poured for each dose of mutagen. The decision to employ strains TA100 and TA98 to test aflatoxins was based on the recommendations by McCann and colleagues (1975b) as the authors found that these strains were most sensitive to the effects of aflatoxin. The aflatoxins were tested with and without S9 mix and the S9 mix was also plated on its own, without inoculating the plate with bacteria.

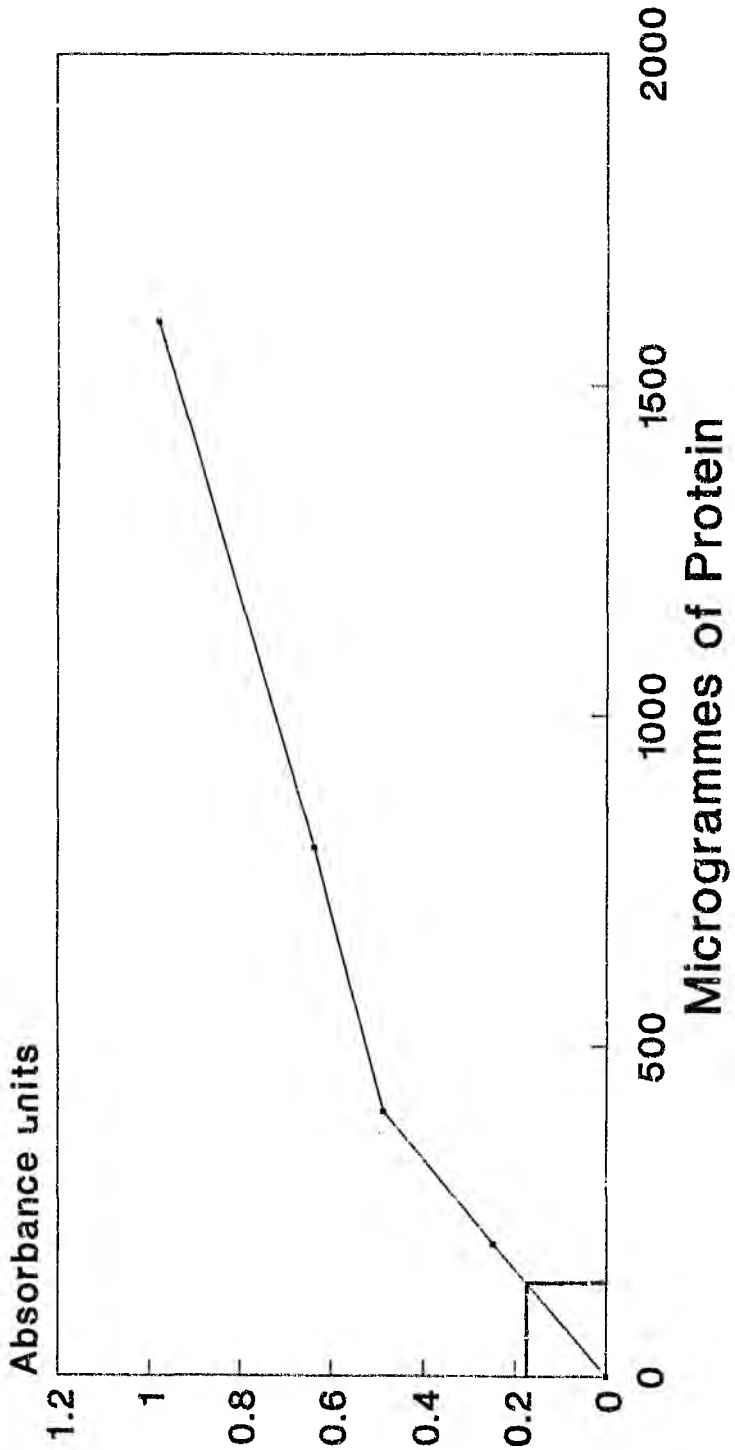


Figure 6.2.1 Graph of absorbance versus concentration

Negative controls, containing the bacteria, S9 mix and solvent (DMSO) were included to establish the number of spontaneous revertants for each of the tester strains. Sodium azide (1,5 μg per plate) without S9 mix was used as the positive control for TA100. Positive controls, containing 10 μg 2-aminofluorene (2-AF) per plate as the standard diagnostic mutagen, as well as S9 mix, were also included to confirm the reversion properties and specificity of each strain as well as the efficacy of the S9 mix.

6.3 RESULTS OF MUTAGENICITY TEST

The bacterial background lawn resulting from the trace of histidine added to the top agar was examined under a dissecting microscope on control and test plates. If massive cell death had occurred on the test plates, the background lawn was sparse compared with control plates. The observations made were recorded in Table 6.3.1.

The number of spontaneous revertants for each strain was calculated from plates containing only the tester strain. Negative controls indicated the number of spontaneous revertant colonies growing on plates to which the tester strain, together with solvent and S9 mix, had been added. The number of spontaneous revertants with and without S9 mix are recorded in Table 6.3.2.

TABLE 6.3.1 BACKGROUND LAWN EXAMINATION

Mutagen	Amount (in μg)	Presence of background lawn Dense growth	Cell death Medium growth	Massive cell death Very sparse growth	Toxicity
Control	0	+			
AFB_1	0,01	+			not toxic
	0,1	+			not toxic
	0,5	+			not toxic
	1,0		+		slightly toxic
AFG_1	0,01	+			not toxic
	0,1	+			not toxic
	0,5	+			not toxic
	1,0		+		slightly toxic

The number of colonies on positive control plates was calculated by counting the number of colonies in 1 cm squares in different sections of the plate, averaging the number of colonies per cm^2 and then multiplying by the area of the plate. This was repeated for the four plates. The number of revertants on positive control plates appear in Table 6.3.3.

Finally, the number of colonies growing on plates inoculated with aflatoxin B_1 and aflatoxin G_1 at the four different concentrations was counted. The number of revertants shown in Table 6.3.4 is the average number per plate after the duplicate plates were examined. Figures 6.3.1 and 6.3.2 represent dose response curves for aflatoxin B_1 and G_1 respectively. Aflatoxin B1 (0,5 μg + S9) and AFG1 (0,5 μg) TA100 plates were photographed together with

AFB1 (0,5 μg without S9) and negative controls (with S9 fraction) (Figures 6.3.3 a-d).

TABLE 6.3.2 SPONTANEOUS REVERSION

	Number of colonies observed on plates			
	TA97	TA98	TA100	TA102
Spontaneous (no S9)	93 98 85 79	33 34 29 43	121 125 137 139	226 274 307 277
Total	355	139	522	1 084
Mean	88,75	34.75	130,5	271
Spontaneous (S9) (DMSO)		37 43 42 54	151 153 148 156	
Total		176	608	
Mean		44	152	
S9 mix on its own (contamination cheok)		0	1	

TABLE 6.3.3 REVERTANTS PER PLATE ON POSITIVE CONTROL PLATES

Mutagen	Amount (in µg)	S9	Number of colonies observed on plates			
			TA97	TA98	TA100	TA102
Sodium azide	1,5	-	148 164 160	48 44 45 50	3 048,7 1 985,2 2 339,7 3 261,4	461 472 477 454
Total			600	187	10 635,0	1 864
Mean			150	46,75	2 658,75	466
-Spontaneous (no S9)			61	12	2 528,25	195
2-aminofluorene	10	+		4 112,2 4 395,2 4 185,1 4 324,9	3 615,9 2 268,8 2 119,6 2 899,5	
Total				17 016	12 903,8	
Mean				4 254	3 225,95	
-Spontaneous (+ S9)				4 210	3 109,4	
2-aminofluorene	10	-		35 36 49 43	174 140 151 163	
Total				183	628	
Mean				40,75	157	
-Spontaneous (no S9)				8	27	

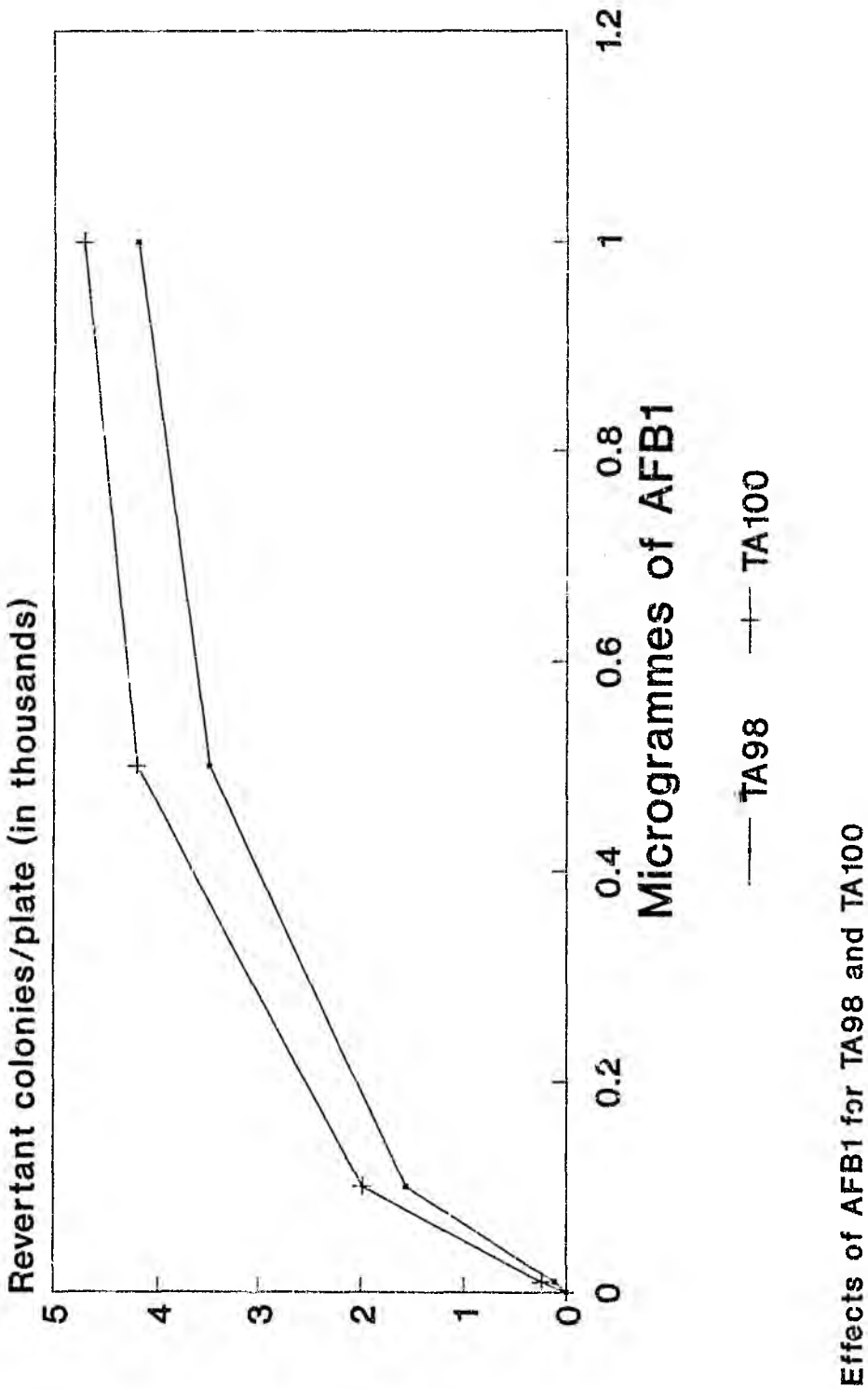


Figure 6.3.1 Dose-response curve for Aflatoxin B1

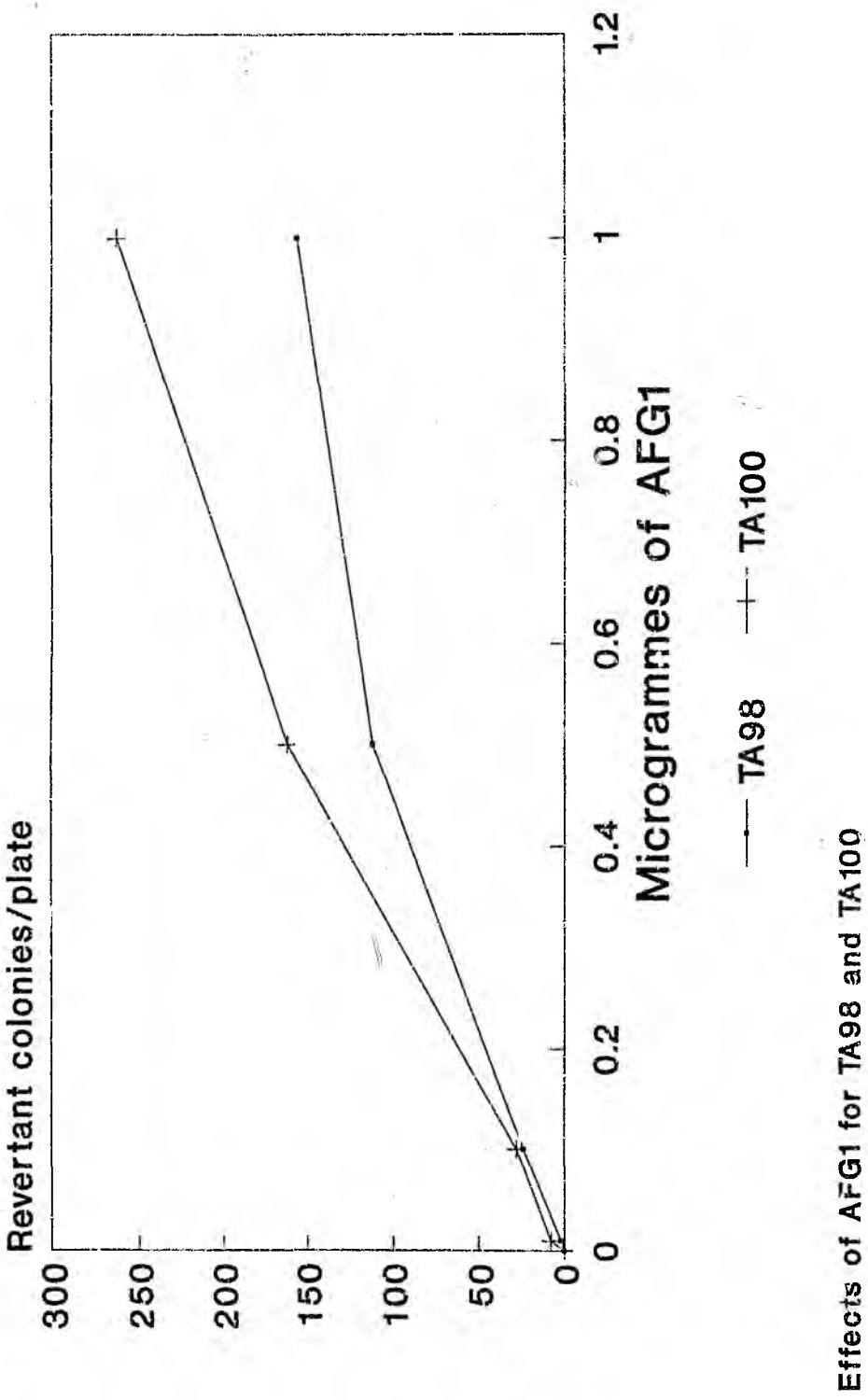
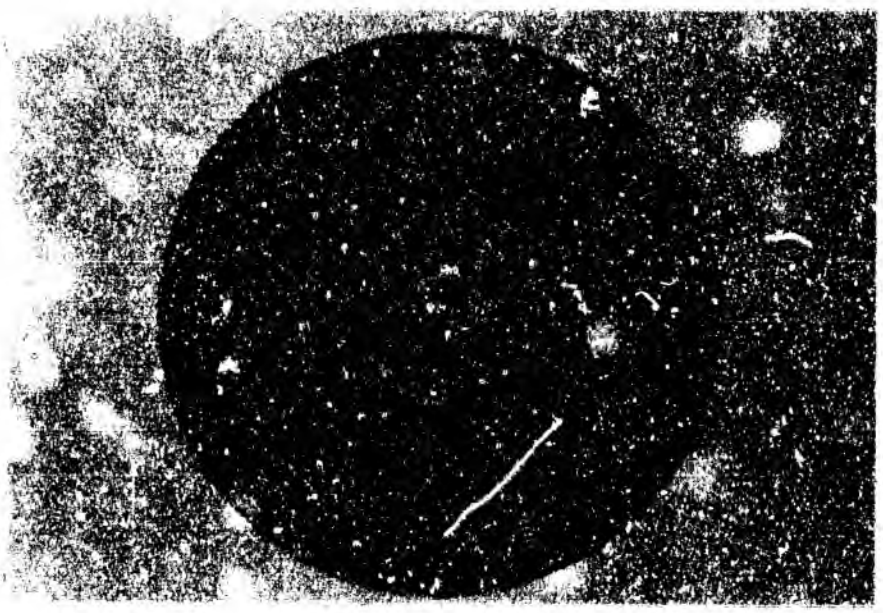
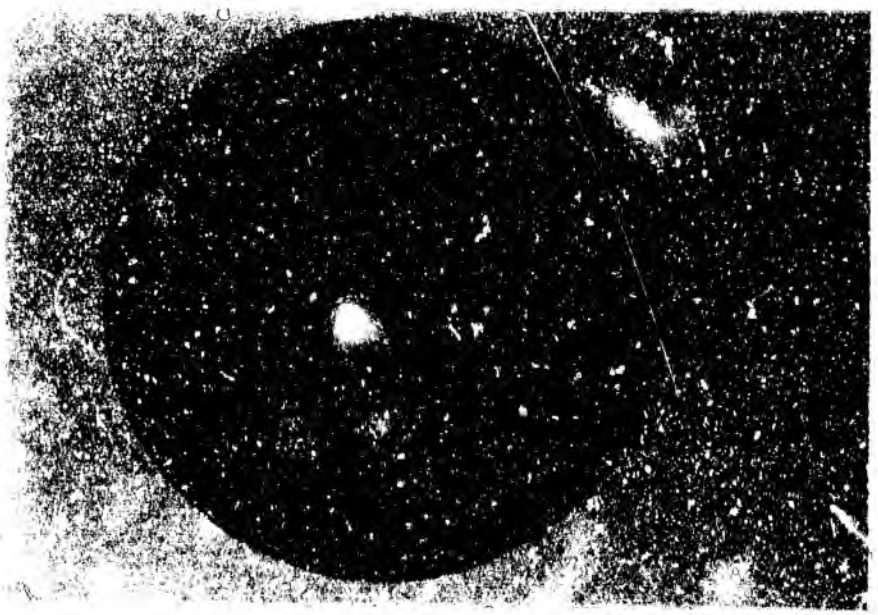


Figure C.3.2 Dose-response curve for Aflatoxin G1

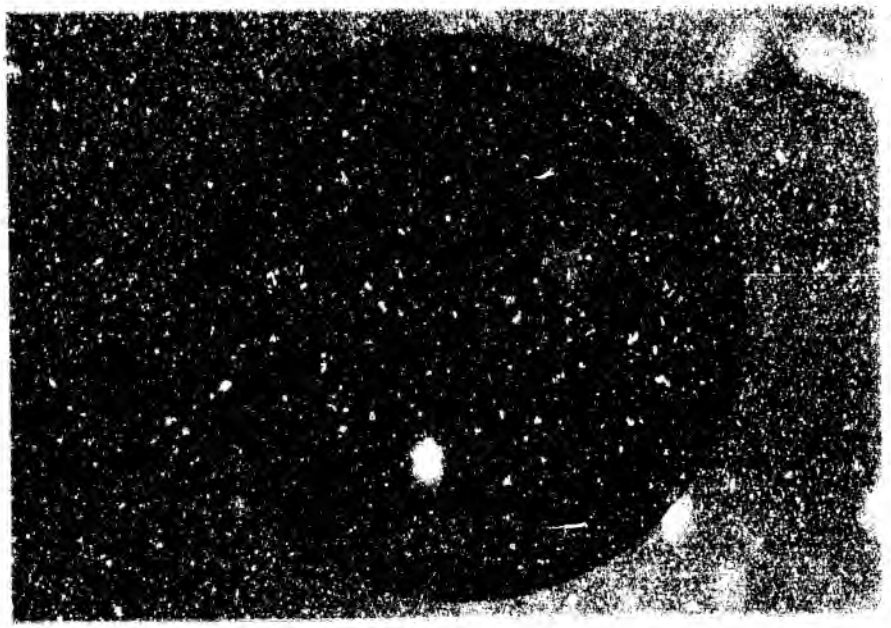


[a]



[b]

Figure 6.3.3 Photographs of TA100 revertant colonies on plates inoculated with AFB1 (0,5 μg + S9 mix) [a] and AFB1 (0,5 μg without S9 mix) [b].



[c]



[d]

Figure 6.3.3 AFG1 (0,5 μg + S9 mix) [c], negative control (with S9 mix) [d].

TABLE 6.3.4 REVERSION OF TA100 AND TA98 TESTER STRAINS WITH AFLATOXINS

Mutagen	Amount (in μg)	S9	Number of corrected colonies * and Statistical Diagnoses[a]	
			TA98	TA100
AFG_1	0,01	y	2 -	8 -
	0,1	y	24 -	28 -
	0,5	y	112 +	162 +
	1,0	y	156 +	263 +
	0,5	n	0	1
AFB_1	0,01	y	117 +	240 +
	0,1	y	1 563 +	1 982 +
	0,5	y	3 477 +	4 201 +
	1,0	y	4 198 +	4 724 +
	0,5	n	3	5

* Control values (number of spontaneous revertants) have been subtracted. Average results from duplicate plates.

a) Statistical diagnoses according to Chu and colleagues (1981) using the modified 2-fold increase rule where (+) indicates that the difference between the number of revertants on aflatoxin and control plates was statistically significant and (-) indicates that the difference was not significant.

y = yes (S9 added)

n = no (S9 not added)

6.4 DISCUSSION

6.4.1 Toxicity Effects

For most mutagens tested, there is a range that produces a linear dose-response curve. The number of revertants per plate reported for a mutagen should be taken from this region of the curve. Most mutagens are toxic to the bacteria at a certain concentration. In the toxic range there is a decrease in the number of revertants per plate. If a compound is tested at only one concentration which happens to be in the descending portion of the curve, the results would be misleading as to the quantitative mutagenic activity of this compound. In Figure 6.3.1 it is quite clear that, in the case of AFB1, the range that produces a linear dose response curve is between 0,01 μg and a concentration slightly less than 0,5 μg. At doses higher than 0,5 μg, the toxic range, there is a levelling off or decrease in the number of revertants per plate. The toxicity of the mycotoxins was also demonstrated by observing the backround lawn of bacteria to determine the extent of cell death (see Table 6.3.1).

6.4.2 The Control Values

Control values were presented both for the number of revertant colonies on plates with compound and no S9 mix and for the number of colonies on plates with S9 and no mutagen. The control values were low in all cases, indicating that the S9 mix was sterile and did not significantly enhance the spontaneous mutation rate characteristic of each tester strain, and that neither of the aflatoxins tested were mutagenic before activation.

Spontaneous reversion of the tester strains to histidine independence was measured in a trial mutagenicity experiment and was expressed as the number of spontaneous revertants per plate. The revertant colonies are clearly visible in a background having auxotrophic bacteria. Each tester strain reverts spontaneously at a frequency that is characteristic of the strain. The number of spontaneous revertants varies from one experiment to another and from one plate to another, therefore, four spontaneous mutation control plates were included for each strain in the mutagenicity assays. The values may also differ slightly on plates with S9 (Maron and Ames, 1983). The negative control values obtained in these experiments were well within the expected spontaneous range reported by Maron and Ames (1983).

The characteristic reversion pattern of the standard tester strains to some diagnostic mutagens are also reported in the Maron and Ames (1983) article. As the aflatoxins require metabolic activation, it was necessary to include positive controls containing 2-AF to confirm that the S9 was active. Sodium azide (1,5μg per plate) without S9 mix was used as the positive control for TA100.

In these experiments, the average number of revertants per plate on positive control (1,5μg sodium azide) plates for TA100 was close to the value reported in the Maron and Ames article for this diagnostic mutagen (see Table 6.3.3) indicating that TA100 was reverting normally without S9 mix. The positive control values for 2-AF with S9 were also similar to those reported by the authors for TA100 but slightly lower for TA98, possibly due to experimental

error in counting the colonies as the number of revertants per plate was estimated by counting the number of revertants in different sectors of the petridish and then multiplying by the total area of the plate. Nevertheless, this similarity indicates that the S9 mix was efficient in activating the diagnostic mutagens and would therefore also efficiently activate the aflatoxins. Furthermore, the tester strains were reverting in their characteristic manner and would behave in a similar fashion with the aflatoxins.

6.4.3 Previous studies with aflatoxin B1 and aflatoxin G1

Aflatoxin B1, aflatoxin G1 and most other well known mycotoxins have already been tested in the *Salmonella*/mammalian microsome assay. The reason for retesting the two aflatoxins in this bacterial assay was mainly for verification purposes in order to compare the performance of this test with that of SMART. In 1975 McCann and colleagues tested 300 chemicals in this test (McCann *et al.*, 1975a). For mutagenic compounds the data reported was from the linear region of dose-response curves. Under the list of fungal toxins and antibiotics, AFB1 was tested on strains TA100, TA98 and TA1538 and was mutagenic in all three strains. The data given come from the test with strain TA100. The number of revertant colonies on a petriplate per microgram of AFB1 incorporated in the plate, was 2260 revertants per 0,1μg tested after spontaneous revertant colonies (140 for TA 100) had been subtracted. They then calculated the number of revertants per nmol from the revertants per plate and the molecular weight. The value 7057 for AFB1 represents the mutagenic potency of the particular chemical in reverting the strain. In another study by McCann and colleagues in the same year, the

number of revertant colonies per plate was 1940 per 0,1μg in TA98 (McCann *et al.*, 1975b). In the case of AFG1, the data given come from the test with strain TA98. The number of revertant colonies per plate was 142 per 0,4μg of AFG1 after the control value (40 for TA98) had been subtracted and the number of revertants per nmol was 116 (McCann *et al.*, 1975a). As can be seen in Table 6.3.4, the figures reported in previous studies are similar to those obtained in these experiments. The results obtained in these experiments can be compared with those obtained in previous studies because the same tester strains TA100 and TA98 were used and the aflatoxins (B1 and G1) were also activated by S9 liver homogenate from aroclor induced rats. Similar control values were also observed in these experiments, indicating that the spontaneous mutation rates were well within the ranges characteristic of each tester strain. Hence the *Salmonella*/ mammalian microsome assay was carried out under conditions similar to those by McCann and colleagues in 1975 and, since these results are comparable to those previously reported by the authors, the strains used reverted in their characteristic and specific pattern in the presence of the aflatoxins, and the S9 mix prepared was effective in activating these promutagens in vitro. It can be seen from Table 6.3.4 that after activation both AFB1 and AFG1 revert the frameshift mutant TA98 and the base pair substitution mutant TA100. The aflatoxin data were evaluated according to the criteria set by Chu *et al.* (1981). In the case of AFG1, positive results were obtained only for concentrations higher than 0,5 μg per plate while AFB1 was positive at all concentrations tested.

CHAPTER SEVEN

7 THE SEX-LINKED RECESSIVE LETHALS ASSAY

7.1 INTRODUCTION

All tests for SLRL rely on the fact that among the progeny of a female carrying a recessive lethal on one of her X chromosomes (heterozygous for a recessive lethal) half of the sons will die. Consequently, progeny of such females will contain half as many males as females. By providing suitable genetic markers, the class of males carrying the X chromosome of the treated grandfather can easily be determined. If a lethal was induced, this class will be missing and this is easily scored. Another pre-requisite for this assay system is the prevention of crossing over in the females heterozygous for the lethal-bearing chromosome, because transfer of the lethal from the paternal to the maternal X chromosome by genetic recombination would restore viability of the chromosome under test and thus lead to the incorrect result that males receiving this X chromosome survive.

In these tests, wildtype males are used for treatment and mated to females homozygous for the *Basc* chromosome. The *Basc* chromosome carries the genetic markers *Bar* and *white-apricot*. *Bar* produces a narrow eye shape in homozygous or hemizygous conditions 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. It is essential for the procedure

that the X chromosomes be inherited as units (Würgler, 1983). In addition to these visible markers, the *Basc* chromosome contains a combination of two *scute* inversions: sc^{S1} and sc^{8}, covering the entire X chromosome, and a smaller inner inversion, *In-S* (see APPENDIX J for a precise genetic description of the *Basc* chromosome). The two overlapping inversions lead to the elimination of crossover products and consequently, the two X chromosomes in the *Basc* heterozygous F1 females do not exchange information during meiosis.

7.2 MATERIALS AND METHODS

7.2.1 Treatment of Males

For general screening purposes, males should be used for chemical treatment since females are more easily sterilized by chemicals and have, so far, proved more refractory to the induction of genetic changes (Clark and Sobels, unpublished in Würgler *et al.*, 1977). An advantage is that, if males are used for treatment, the treated X chromosome does not contain pre-existing lethals with the exception of those induced in germ cells during treatment or those arising spontaneously during development of that particular male fly (Würgler *et al.*, 1977).

A culture of the Oregon R stock was started and 10 days later the flies were transferred to fresh bottles. All flies that hatched on the eleventh day were collected and placed in a fresh bottle and allowed to age for two days. Similarly, on the twelfth day, flies were collected and aged for the repeat

experiment. The day before the treatment was carried out, fifty males were separated from females. This procedure is recommended for the SLRL test because, by mating with the females in the same bottles, the first sperm batches- which show an increased rate of spontaneous lethals (Graf, 1972)- will not be included in the experiment. On the day of the treatment an AFG1 [0,2mM concentration in 5% (w/v) sucrose] solution was fed to adult male flies. Solutions were made in 5% sucrose so that they were reasonably palatable to flies (Würgler and Graf, 1985). All procedures involving this mycotoxin were carried out following the guidelines set by the NIH (USA). In order to ensure immediate uptake, the male flies to be treated were starved for two hours before the feeding began.

The drug administration procedure published by Vogel and Luërs (1974) was employed. Fifty 2-3 day old males from the Oregon R stock (for each treatment), were placed in a 30 ml glass filter (1D3 of Schott, Mainz, G.F.R.) and the filter (or sintered glass crucible) was closed with a foam rubber stopper. The crucible was placed in a 100 ml beaker which contained 20 ml test solution. The mycotoxin solution was almost in contact with the lower part of the filter plate. A wick of cellulose wadding ensured contact between the AFG1 solution and the plate. A cotton wool ring around the upper part of the filter prevented water evaporation. The male flies were exposed to either AFG1 or solvent (control) for twenty four hours. This procedure was repeated on a separate day.

7.2.2 P1 Mating Schedule or Rearing of the F1 Generation (Figure 7.2.1)

Following the AFG1 treatment, 60 of the 67 surviving male flies (for the two repeats) were mated individually in single vials to three virgin *Basc* females. In these assays, where the spontaneous mutation rate was determined in the same experiment in an appropriate control group, about equal mumbers of chromosomes were tested in the solvent control and AFG1-treated groups as this gives the optimal power to test the statistical signlficance between the two groups (Würgler and Graf, 1985). The culture vials were numbered and records were kept so that at the end of the repeat experiments the origin of every tested X chromosome could be traced back to the individual male used initially. After two days the parental flies were anaesthetized and males and females were separated. The females of the first mating were placed in a new vial, so as to increase the number of progeny. This vial was attached to the first vial and these two vials then constituted the first brood. Every parental male was given three fresh virgin *Basc* females in order to raise the second brood. This procedure was repeated two days later to produce the third brood.

In order to avoid admixture of the parents used to start the culture with progeny, the parental flies were eliminated six days after starting the culture. Twelve days after the first culture had been started, the F1 progeny was shaken over to fresh vials supplemented with extra yeast and aged for three days so as to promote rapid maturation of the ovaries of the F1 females.

7.2.3 The Test Matings for the F2 Generation (Figure 7.2.1)

When sufficient progeny had been collected and aged, the flies were anaesthetized and females separated from males. The F1 females were carefully inspected to make sure that no Bar apricot flies were included. Such flies would have indicated either that the P1 females were non-virgin or that they had not been discarded. The F1 females with kidney-shaped, red eyes were then individually mated to two *Basc* males (their brothers). Each F1 female represented one paternal X chromosome treated in the male gametes. Twenty F1 females were mated per treated P1 male. The vials were labelled to enable identification of the individual P1 male from which the progeny was derived. Vials were maintained at 25°C and after fourteen days (allowing males with semi-lethals, characterized by longer development, to hatch) the F2 progeny was scored.

P1



X



Basc//Basc
Bar apricot eyed virgin female

/Y
Normal red eyed AFG1 treated male

F1/P2 If lethal occurred:



X



Basc//
Kidney-shaped, red eyed female

Basc/Y
Bar apricot eyed male

F2





Basc//
Kidney-shaped, red eyed female

Basc//Basc
Bar apricot eyed female

DIES





/Y
normal red eyed male

Basc/
Bar apricot eyed male

Figure 7.2.1 The sex-linked recessive lethal test mating schedule

P1



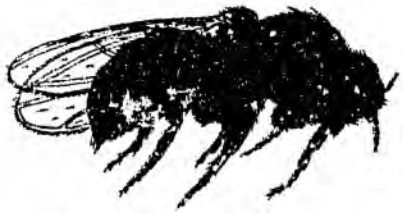
X



Basc//Basc
Bar apricot eyed virgin female

/Y
Normal red eyed AFG1 treated male

F1/P2 If lethal occurred:



X



Basc//
Kidney-shaped, red eyed female

Basc/Y
Bar apricot eyed male

F2





Basc//
Kidney-shaped, red eyed female

Basc//Basc
Bar apricot eyed female

DIES





/Y
normal red eyed male

Basc/Y
Bar apricot eyed male

Figure 7.2.1 The sex-linked recessive lethals test mating schedule

7.2.4 Scoring of the F2 Generation

The F2 progeny was inspected for the occurrence of sex linked recessive lethals by simply looking for the presence or absence of wild type males with red, round eyes. If these appeared to be missing from a culture vial containing at least twenty flies, it was concluded that the treated male gamete contained a recessive lethal (with $P < 0,05$). The presence of at least two wild type males was used as a criterion for a non-lethal culture.

7.3 RESULTS

The total number of F1/P2 cultures set up for the three different broods in the treated and control series for the repeat experiments is recorded in Tables 7.3.1 and 7.3.2. The number of sterile cultures was subtracted from the total number of cultures set up, and the total number of treated and control X chromosomes was calculated for each brood. These figures and the number of cultures containing recessive lethals were then recorded and used to determine the frequency of lethals as shown in Tables 7.3.1 and 7.3.2. The spontaneous mutation rate observed in these experiments was well within the expected range (Wallace, 1970; Graf, 1972; Gocke *et al.*, 1982; Woodruff *et al.*, 1983 and Ashburner, 1989).

After the sterile cultures had been eliminated, the experimental result consisted of the following numerical values:

Nc = number of tested X chromosomes in the control group

Mc = number of lethals found in the control group

Nt = number of tested X chromosomes in the treated group

Mt = number of lethals found in the treated group

7.3.1 Statistical Analysis

It was necessary to determine if the rate pt = Mt/Nt found in the treated group was significantly greater than the rate pc = Mc/Nc found in the control group. The Kastenbaum-Bowman significance test (Kastenbaum and Bowman, 1970) was then applied as described in Würgler and colleagues (1975).

According to the table "Sample Sizes Based on the Kastenbaum-Bowman Test" (Würgler *et al.*, 1977), the smallest sample size which is needed to detect a significant difference between pt% 1,00-1,81 and pc% 0,17-0,26 is between 1100 and 1300 treated X chromosomes. Hence the number of X chromosomes tested in these experiments (3302) for all broods with one repeat, was actually larger than the recommended range.

TOTAL TREATED X CHROMOSOMES IN ALL BROODS

%pt = 0,999% or 1,00% %pc = 0,23%

M = Mt + Mc	K =	Nt/(Nt + Nc)
= 33 + 8	=	3302/(3302 + 3500)
= 41	=	0,485 or 0,49

From the tables, with K=0,49 and M=41 the Mt value is 28. This value is less than the value Mt= 33. Hence, it was concluded that the difference between pt and pc was significant at the 5% level.

TABLE 7.3.1 FINAL SCORE OF THE SEX-LINKED RECESSIVE LETHALS ASSAY CONTROL SERIES, 2-3 DAY OLD MALES TREATED WITH SOLVENT FOR 24 HOURS

	TOTAL N° OF CULTURES SET UP	N° OF STERILE CULTURES	N° OF TREATED X CHROM.	N° OF CULTURES CONTAINING RECESIVE LETHALS	FREQ. OF LETHALS (%)	STAGE
Days 1-2	600	6	594	2	0,34	MATURE SPERM
Days 3-4	600	2	598	1	0,17	L. SPERMATIDS
Days 5-6	580	7	573	2	0,35	E. SPERMATIDS
REPEAT						
Days 1-2	600	6	594	0	0,00	MATURE SPERM
Days 3-4	580	10	570	2	0,35	L. SPERMATIDS
Days 5-6	580	9	571	1	0,18	E. SPERMATIDS
TOTAL						
Days 1-2	1 200	12	1 188	2	0,17	MATURE SPERM
Days 3-4	1 180	12	1 168	3	0,26	L. SPERMATIDS
Days 5-6	1 160	16	1 144	3	0,26	E. SPERMATIDS
All broods	3 540	40	3 500	8	0,23	ALL STAGES

CHROM. = Chromosomes
FREQ. = Frequency
(L.) = late
(E.) = early

TABLE 7.3.2 FINAL SCORE OF THE SEX-LINKED RECESSIVE LETHALS ASSAY AFTER TREATMENT OF 2-3 DAY OLD MALES WITH AFG1 (0,2 mM) FOR 24 HOURS

BROOD	TOTAL N° OF CULT. SET UP	N° OF STERILE CULT.	N° OF TREATED X CHROM.	N° OF CULT. CONTAINING RECESSIVE LETHALS	FREQ. OF SLRL (%)	S.D	STAGE
Days 1-2	600	20	580	2	0,35	?	MATURE SPERM
Days 3-4	560	9	551	5	0,90	?	L. SPERMATIDS
Days 5-6	540	14	526	10	1,90	-	E. SPERMATIDS
REPEAT							
Days 1-2	600	12	588	3	0,50	?	MATURE SPERM
Days 3-4	540	8	532	4	0,75	?	L. SPERMATIDS
Days 5-6	540	15	525	9	1,71	??	E. SPERMATIDS
TOTAL							
Days 1-2	1 200	32	1 168	5	0,43	-	MATURE SPERM
Days 3-4	1 100	17	1 083	9	0,83	-	L. SPERMATIDS
Days 5-6	1 080	29	1 051	19	1,81	+	E. SPERMATIDS
All broods	3 380	78	3 302	33	1,00	+	ALL STAGES

Statistical diagnoses according to Würgler *et al.* (1975) using Kastenbaum-Bowman (1970) tables.
(-) = negative (the difference between %pc and %pt is not significant)
(+) = positive (the difference between %pc and %pt is significant)
(?) = a dash appeared in the tables (inconclusive)
(??) = the same value appeared in the table, it was not < or > but =
CHROM. = Chromosomes
FREQ. = Frequency
S.D. = Statistical Diagnoses
CULT. = Cultures
SLRL = Sex-linked recessive lethals
(L.) = late
(E.) = early
Probability levels: $\alpha = \beta = 0,05$

7.4 DISCUSSION

Failure to recover damage from mutagens may result not only from an inappropriate mutation assay but also from a pronounced stage specificity (Purdom *et al.*, 1968; Vogel and Leigh, 1975). The application of the brooding technique safeguards against the possibility that chemicals with more pronounced effects in earlier stages of spermatogenesis are not erroneously dismissed as false negatives.

It was Muller who noted that early spermatids were among the most sensitive cells to radiation and to chemical mutagens (Purdom *et al.*, 1968). Early spermatids and spermatocytes also appear to be highly sensitive to the effects of indirect mutagens (Vogel, 1975). This can also be seen in the results of Lamb and Lilly (1971) with AFB1. They found the greatest increase in second chromosome recessive lethals in the third brood corresponding to early spermatids. The most plausible explanation for this response with promutagens seems to be that metabolism (bioactivation) is restricted to certain stages of spermatogenesis. This idea is supported by the apparent relationship between peak mutagenic activity and stage of development of the endoplasmic reticulum (ER) (location of P450 type of enzymes) (Vogel, 1975).

Electron micrographs by Tates (1971) demonstrated the presence of a well developed ER in metabolically active germ cells such as spermatids, spermatocytes and spermatogonia, but not in sperm (in Vogel, 1975). Hence the

activation of indirect mutagens can take place in metabolically active germ cells because of the presence of a well developed ER and the P450 cytochrome enzyme system.

The pattern shown by AFG1 seems to indicate the brood pattern sensitivity characteristic of some promutagens forming short-lived active metabolites. Mutagenicity was low in metabolically inactive sperm, while highest activity was found in early spermatids.

CHAPTER EIGHT

8 THE HERITABLE TRANSLOCATIONS TEST

8.1 INTRODUCTION

Simple reciprocal translocations are rare spontaneous events, but, since they can result from only two chromosome breaks, they are the frequent products of mutagenesis with ionizing radiation or treatment with other mutagens that break chromosomes effectively (Ashburner, 1989). Translocation tests are sometimes used in screens for mutagens and they are suitable to detect clastogens (Würgler and Graf, 1985). For this reason, there are considerable data on the frequency of their spontaneous occurrence in standard laboratory stocks.

The presence of markers on the second and third chromosomes in the *bw; st* p^p stocks allows testing for the induction of heritable reciprocal translocations. These marker genes are brown (*bw*) on the second chromosome, producing a phenotype characterized by light brown eyes, because the development of red eye pigment is defective. The third chromosome carries the gene scarlet (*st*) resulting in bright red eyes, since the formation of the brown eye pigment is defective. In addition, the pink allele of the gene peach (p^p) has been incorporated. In combination with p^p the scarlet eye colour is changed to light orange, and this facilitates the scoring of the F2 progeny for the presence or absence of a translocation. The combination of homozygous *bw* and

homozygous *st* in the same fly results in a white-eyed phenotype, because the formation of both pigments is defective.

Males with the wild type eye colour genes on the second and third chromosomes are treated with the test solution. These males are then crossed with *bw; st* p^p females, which have white eye colour, and F1 progeny from this cross are heterozygous for the genes *bw* on their second and *st* p^p on their third chromosomes. Since crossing over only occurs in *Drosophila* females, F1 males are used to generate the F2 generation. This keeps the treated wild type paternal chromosome separate from the untreated marked maternal chromosome. These males are then crossed individually to females of the maternal genotype (*bw; st* p^p).

This test relies on independent assortment of the chromosomes at melosis, an individual heterozygous for two genes located on different chromosomes will produce four different types of gametes. These can be visualized by back crossing such a "double" heterozygote to flies homozygous for the marker genes under consideration. The scheme for detecting translocations using the brown-scarlet-pink-peach system is shown in Figure 8.2.1.

In the absence of a II-III translocation, the F2 progeny will contain four different phenotypes with red, white, orange and brown eyes, respectively. However, if the P2 male is heterozygous for a translocation, two unbalanced genotypes will be formed. The zygotes receiving the second chromosome with a translocated segment of the third attached to it and a *st* p^p chromo-

some are unbalanced. Instead of a complete chromosome set, they carry a deficiency for a part of their second and a duplication for a part of their third chromosomes. Similarly, genotypes receiving the third chromosome with a translocated segment of the second attached to it, and the second chromosome with *bw* are not viable, because of a deficiency for part of the third and a duplication for part of the second chromosomes. As a result, individuals with these unbalanced genotypes die during development, and instead of four Mendelian phenotypic classes with red, white, orange and brown eyes, only two types of flies with red and white eyes, respectively, are observed. Translocation thus results in failure of independent Mendelian assortment with genes on the second and third chromosomes of a II-III translocation heterozygote segregating together.

8.2 MATERIALS AND METHODS

8.2.1 Treatment of Males

A culture of the Oregon R stock was started and six days later these parental flies were discarded. All progeny that hatched by day eleven was transferred to a clean bottle and allowed to age for two days. Males were separated from females the day before the treatment was carried out. Fifty 2-3 day old males were starved in a glass vial for 2 hours before the treatment began. The procedure followed was identical to that used in the sex-linked recessive lethals assay (see section 7.2.1). Aflatoxin G1 concentrations of 0,2 mM and 0 (solvent control) in 5% sucrose were again used in repeat experiments.

8.2.2 P1 Mating Schedule (Figure 8.2.1)

A total of 64 flies out of 100 male flies exposed in the two repeats survived the AFG1 treatment. Each surviving male was mated individually in single vials to three *bw; st* p^p virgin females, to set up the P1 generation. The brood pattern procedure was identical to that described for the SLRL test. After two days (on the third day), the parental flies in each vial were anaesthetized and males and females were separated. The females of the first mating were placed in the same vial. This vial then constituted the first brood (roug. corresponding to treated mature sperm). Every parental male was given three fresh virgin females (*bw; st* p^p) in order to produce the second brood. The procedure was repeated to set up the third brood. The F1 is thus obtained from pair matings which provide information on genetic effects in germ cells of individual males. Within the progeny of the first generation cross, each F1

male represents one gamete of the treated father. After six days, parental flies were removed from vials.

8.2.3 P2 Mating Schedule (Figure 8.2.1)

The second test generation was obtained by crossing the double heterozygous F1 males to homozygous virgin *bw; st* p^p females. Since each F1/P2 male represented one treated gamete of the father, the F1/P2 males were mated individually in single vials to three white eyed females. Twenty F1 males from each P1 generation vial were mated to two well fed virgin females to ensure sufficient progeny. The test matings for this test therefore required large scale collections of virgin females from the *bw; st* p^p stock. Since about 1 800 gametes of the treated P1 males were tested, at least 3 600 virgin females were collected. As the experiment was repeated and the same number of gametes were tested in the controls, this meant that at least 7 500-8 000 (for the treatment series) and the same number for the control series (making a total of about 15 000) virgin females had to be collected to allow for some loss through the ageing period. In addition, more than 1 100 virgin females were collected to set up the P1 generation. Virgin females were collected every day, at 8 am, 12 noon and 4 pm for three weeks. Extra cultures were set up to compensate for the considerable sterility in the test matings. With the second test generation, the parental flies did not have to be discarded from the vials because the genotypes of the P2 flies were identical to those present in the progeny.

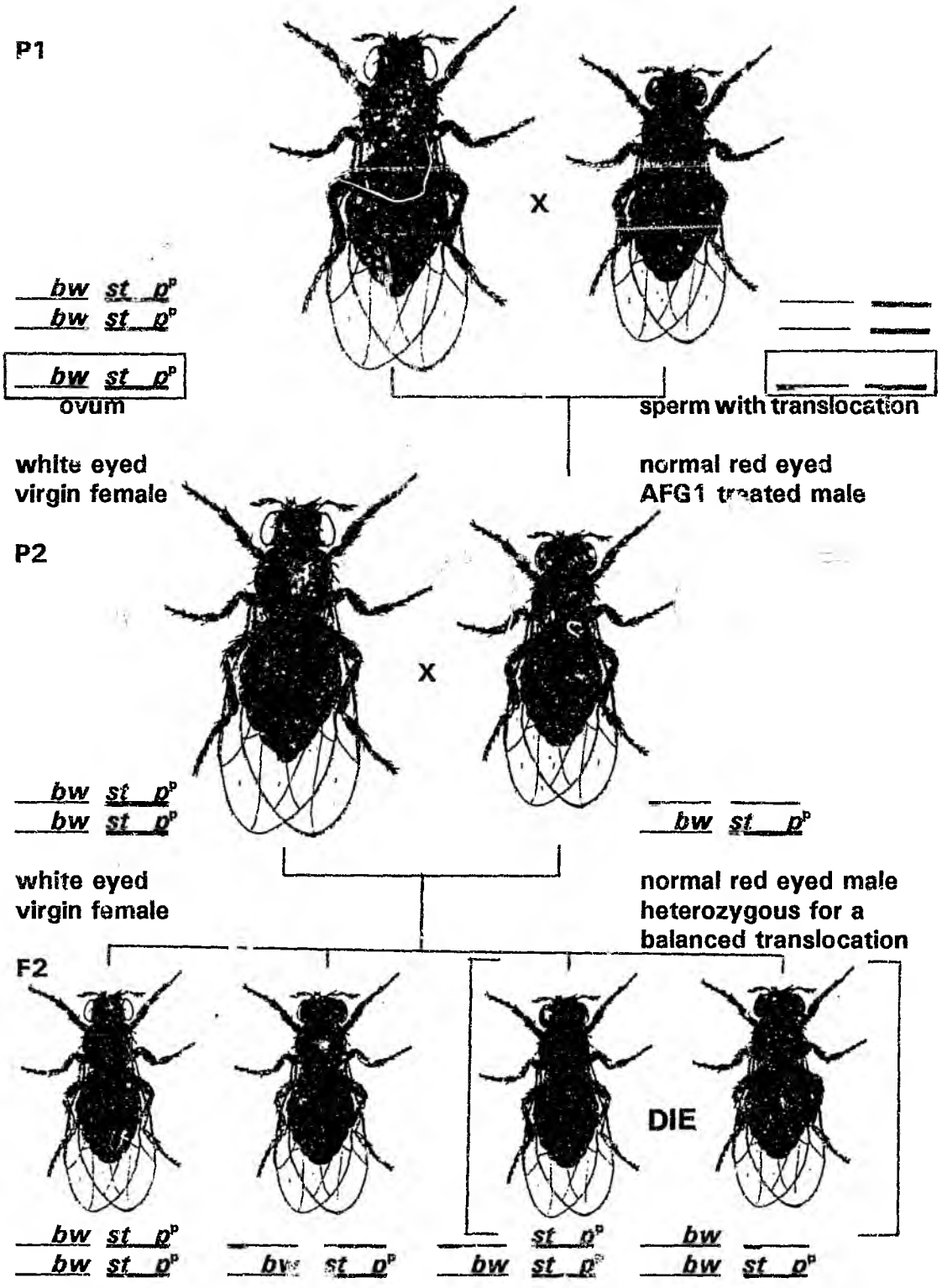


Figure 8.2.1 The heritable translocation test mating schedule

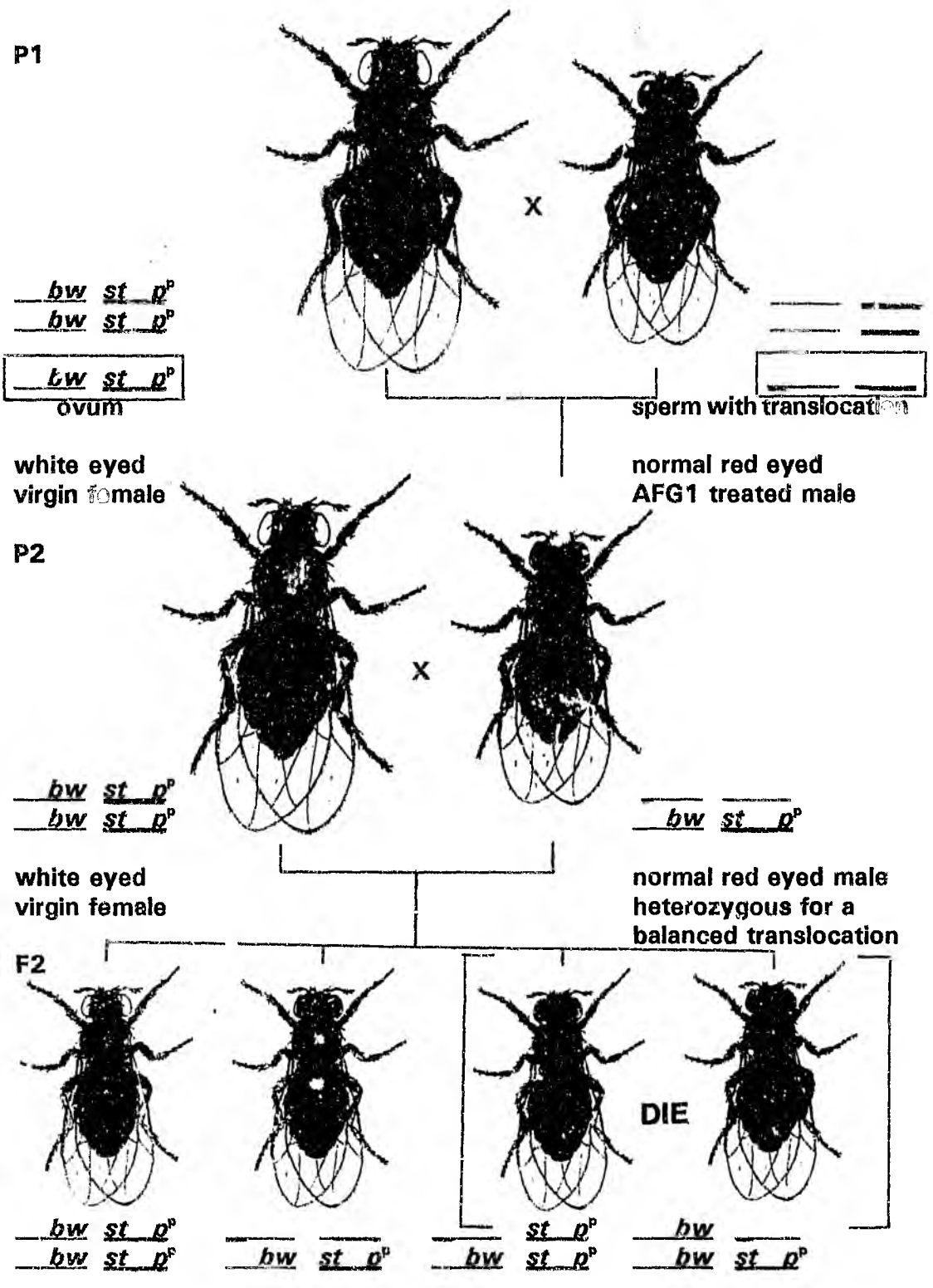


Figure 8.2.1 The heritable translocation test mating schedule

8.2.4 Scoring of the F2 Progeny

Scoring of the F2 progeny was simple and relied on the presence or absence of flies (males and females) with brown or orange eyes. Any vial with more than 20 flies, which only contained red and white eyed progeny, and no brown or orange eyed progeny, was marked. Absence of these two Mendelian classes in the F2 progeny of the treated males indicated that a II-III translocation had occurred in the gamete of the original P1 male exposed to AFG1. The marked vials in each brood were counted. The absence of these classes can be considered as an indication that the F1/P2 male had been heterozygous for a II-III translocation

8.3 RESULTS

The total numbers of F1/P2 cultures set up for the three different broods in both the treated and control series were recorded. The number of sterile cultures was subtracted from the total number of cultures set up, and the total number of treated and control gametes was calculated for each brood. The number of cultures containing translocations were recorded and used to determine the frequency of translocations as shown in Tables 8.3.1 and 8.3.2.

TABLE 8.3.1 FINAL SCORE OF THE HERITABLE TRANSLOCATIONS TEST CONTROL SERIES, 2-3 DAY OLD MALES TREATED WITH SOLVENT FOR 24 HOURS

BROOD	TOTAL N°OF CULTURES SET UP	N° OF STERILE CULTURES	TOTAL GAMETES TESTED	N° OF T*	%	STAGE
Days 1-2	620	48	572	0	0,00	MATURE SPERM
Days 3-4	620	51	569	0	0,00	L. SPERMATIDS
Days 5-6	620	32	588	0	0,00	E. SPERMATIDS
REPEAT						
Days 1-2	640	75	565	0	0,00	MATURE SPERM
Days 3-4	620	35	585	0	0,00	L. SPERMATIDS
Days 5-6	580	36	544	0	0,00	E. SPERMATIDS
TOTAL						
Days 1-2	1 260	123	1 137	0	0,00	MATURE SPERM
Days 3-4	1 240	86	1 154	0	0,00	L. SPERMATIDS
Days 5-6	1 200	68	1 132	0	0,00	E. SPERMATIDS
All broods	3 700	277	3 423	0	0,00	ALL STAGES

T = translocations
T* = number of cultures containing translocations
(L.) = late
(E.) = early

TABLE 8.3.2 FINAL SCORE OF THE HERITABLE TRANSLOCATIONS TEST AFTER TREATMENT OF 2-3 DAY OLD MALES WITH AFG1 (0,2 mM) FOR 24 HOURS

BROOD	TOTAL N°OF CULTURES SET UP	N° OF STERILE CULTURES	TOTAL GAMETES TESTED	N° OF T*	% T	S.D	STAGE
Days 1-2	620	40	580	0	0,00	-	MATURE SPERM
Days 3-4	580	43	537	1	0,19	?	L. SPERMATIDS
Days 5-6	580	37	543	6	1,10	?	E. SPERMATIDS
REPEAT							
Days 1-2	640	35	605	0	0,00	-	MATURE SPERM
Days 3-4	640	41	599	0	0,00	-	L. SPERMATIDS
Days 5-6	620	35	585	8	1,37	??	E. SPERMATIDS
TOTAL							
Days 1-2	1 260	75	1 185	0	0,00	-	MATURE SPERM
Days 3-4	1 220	84	1 136	1	0,09	?	L. SPERMATIDS
Days 5-6	1 200	72	1 128	14	1,24	+	E. SPERMATIDS
All broods	3 680	231	3 449	15	0,43	+	ALL STAGES

Statistical diagnoses according to Würgler *et al.* (1975) using Kastenbaum-Bowman (1970) tables.
(-) = negative (the difference between %pc and %pt is not significant)
(+) = positive (the difference between %pc and %pt is significant)
(?) = inconclusive (a dash appeared in the tables)
(??) = the same value appeared in the table, it was not < or > but =
Probability levels: $\alpha = \beta = 0,05$
(L.) = late
(E.) = early
T = translocations
S.D = Statistical Diagnoses
T* = number of cultures containing translocations

8.3.1 Statistical Analysis

Again, it was necessary to determine if the rate pt = Mt/Nt found in the treated group was significantly greater than the rate pc = Mc/Nc found in the control group. The Kastenbaum-Bowman significance test (Kastenbaum and Bowman, 1970, as described in Würgler *et al.*, 1975), was then applied.

According to the table "Sample Sizes Based on the Kastenbaum-Bowman Test" (Würgler *et al.*, 1977), the smallest sample size which is needed to detect a significant difference between pt% 0,43-1,24 and pc% 0,00 is between 800 and 2 000. Hence, the number of gametes tested in these experiments (3 449) for all broods with one repeat is actually larger than the recommended range.

After the sterile cultures had been eliminated, the experimental result consisted of the following numerical values:

Nc = number of tested gametes in the control group

Mc = number of translocations in the control group

Nt = number of tested gametes in the treated group

Mt = number of translocations in the treated group

TOTAL TREATED AND CONTROL GAMETES IN ALL BROODS

%pt = 0,43% %pc = 0,00%

M =	Mt + Mc	K =	Nt/(Nt + Nc)
=	14 + 1	=	3449/(3449 + 3423)
=	15	=	0,50

In the tables, in the column with the heading K = 0,50 in the line M = 15, a value of 13 was found. This value is less than the value Mt = 14. Hence, it was concluded that the difference between pt and pc was significant. The probability level for significance was 5%.

8.4 DISCUSSION

In many laboratories, translocation tests are run without controls. This is justified because the spontaneous frequency of translocations between the second and third chromosome is very low. Based on the pooled data from the literature it is estimated that the freqency in the tester strains is 0,01% (11/116 981) (Würgler *et al.*, 1983). In these experiments, however, the concurrent control was 0,00% (0/3 423) and below this expected range but the difference between these two control rates is not statistically significant. The results show that aflatoxin G1 induced translocations in *Drosophila* probably because of induced chromosome breakage. Mutagenic activity of this promutagen was highest in the third brood corresponding to metabolically active early spermatids (see section 7.4).

CHAPTER NINE

9 THE SMART TEST

9.1 INTRODUCTION

Many organs present in adult flies are not present in larvae; however, the cells that will develop into the adult organs are pre-determined in the larvae. The cells that will divide to form the wings are in the wing primordia or wing imaginal discs so that defined adult structures such as the eye or the wing are the products of cell lines or clones of cells descending from imaginal disc cells of the larval and pupal stages. During these stages, the wing primordia go through approximately twelve rounds of cell division, beginning with 10-30 cells after embryogenesis, and ending up with about 30 800 cells (Garcia-Bellido and Merriam, 1971) in two layers of 15 400 cells each, when cell division ceases at the onset of metamorphosis (Posthlewait, 1978). The number of individual cell divisions taking place during the development of the fly is equal to the number of cells finally present in the wing, estimated to be about 24 000 for the blade of the wing.

The mitotically dividing somatic cells in the imaginal discs of developing larvae can be exposed to mutagens by feeding, injection or gas exposure of larvae. The route of administration chosen depends on the chemical properties of the compound, its palatability and the aim of the experiment.

In appropriately marked animals, genetic change induced in imaginal disc cells will give rise to genetically marked clones. If easily recognizable phenotypes are used, the mutant clones can easily be detected by inspection of the external appearance of the surviving flies. In individuals transheterozygous for two recessive visible marker mutations on the same chromosome arm, certain mutagenic events can lead to the loss of the dominant wild type allele, followed by subsequent expression of the recessive marker allele in a clone of ensuing cells. In these experiments, the cells of the larval wing imaginal discs were trans-heterozygous for two recessive markers located on the left arm of chromosome three (3L). Multiple wing hairs (*mwh*) is at 0,0 cM and flare^3 (*flr*^{3}) at 39,0 cM while the centromere is located at 47,7 (Lindsley and Zimm, 1985).

A mitotic recombination event between two non-sister chromatids may occur during cell division. *Drosophila* is unusual in that homologous chromosomes are paired in somatic cells so that the two third chromosomes lie next to each other. In preparation for mitosis during larval development, the chromosomes replicate and each consists of two identical sister chromatids. In normal segregation, the two centromeres of each pair separate, so that the two replicas, or sister chromatids, move to opposite poles during mitosis. Mitotic recombination does not normally occur, but if chromosome breakage occurs in the two replicas followed by rejoining, the effect is a crossover event between two of the replicas of the two different homologous chromosomes.

If the mitotic crossing over event occurs between the centromere and the *flr*^{3} locus in the control or mycotoxin-exposed transheterozygote, followed by X-type segregation, so that each daughter cell receives one recombined and one non-recombined chromosome (Becker, 1976), the *flr*^{3} marker becomes homozygous in one daughter cell and *mwh* in the adjacent daughter cell. A twin spot is formed, each spot or patch of tissue being derived from division of the two original cells in which the markers became homozygous. Mitotic recombination is also one of the several mechanisms leading to the appearance of single spots. A recombination event between *mwh* and *flr*^{3} loci may result in a single *mwh* spot. If both types of recombination events, one between *flr*^{3} and the contromere, and the second between *mwh* and *flr*^{3}, take place within the same cell, a *flr*^{3} single spot may result, but this event is relatively rare. A single spot may also result if in a growing twin spot one of the two homozygous marked subclones is selected against, with extinction of the one and persistence of the other, or if one of the cell lineages in the original twin spot is damaged (Szabad *et al.*, 1983).

Single spots can have a number of genetic causes. A gene mutation or gene conversion in the wild type allele can lead to loss of function of the dominant allele of either marker; deletions (small or large) and breakage of the chromosome segment involving the wild type allele leading to the loss of this dominant allele when the cell divides (if the broken segment does not have a centromere), can result in a single spot. Non-disjunctional or other loss of the chromosome carrying the wild type allele represents another possibility that may lead to single spots. Non-disjunction seems to be detected by the wing

spot test, as vinblastine, a spindle poison interfering with tubulin assembly, induces single spots but not twin spots (Graf *et al.*, 1984).

The various types of spots differ in frequency of occurrence with single spots expressing the *mwh* phenotype being the most frequent, twin spots being less frequent and single spots with the *flr*^{3} phenotype being the rarest. There are several reasons for this observation. Twin spots are only the result of mitotic recombination between the centromere and *flr*^{3} locus which is a relatively rare spontaneous event, while the appearance of single spots may be caused by somatic recombination as well as by other genetic causes as mentioned above. Untwinned *flr*^{3} single spots are less numerous than untwinned *mwh* single spots because the *flr*^{3} mutation affects development and is homozygous lethal in the zygote, so if a mutation from normal to *flr*^{3} should occur early in development in a somatic cell, it may be lethal, and hence large *flr*^{3} spots will be unlikely. One cell single *flr*^{3} spots are not found, and two or more cells must be affected in order to show the *flr*^{3} phenotype. According to Szabad *et al.* (1983), the *flr*^{3} allele is not fully expressed in clones smaller than about four cells while one cell *mwh* spots are frequent. Furthermore, *mwh* is near the end of the third chromosome while *flr*^{3} is nearer the centromere. Interstitial deletions in a relatively short region of chromosome 3 (about 8 map units) would result in a *flr*^{3} spot while any break ocurring in 3L, from the tip of the left arm to the centromere (about 48 map units) would result in a *mwh* spot, so that *mwh* spots due to deletion would be about 6 times more frequent than *flr*^{3} spots. As most mutations leading to genetically marked clones are due to chromosome breaks, *mwh* spots are more likely because

many breaks leading to the loss of the tip of the chromosome and *mwh*$^+$ allele can occur and be tolerated, while very large sections of the third chromosome would have to be lost to result in a single *flr*^{3} spot and if such a large segment of the chromosome is deleted, then perhaps the consequent deficiencies would make the cell inviable so that it will not divide to form a clone. Therefore, single *flr*^{3} spots as a result of chromosome breakage are probably also unlikely. Another reason why *flr*^{3} single spots are less numerous is that two recombination events, one between the *flr*^{3} locus and the centromere and a second between *mwh* and *flr*^{3} loci, must take place in the same cell to form a single *flr*^{3} spot. This double crossing over event is rare, as the probability that chromosome breaks at these two positions will occur in the same cell, is low. On the other hand, a single recombination event anywhere between *mwh* and *flr*^{3} (a relatively long segment of approximately 39 map units, results in a *mwh* single spot. Furthermore, according to Szabad *et al.* (1983), a single *mwh* spot can appear if *flr*^{3} is not phenotypically expressed in a twin spot.

9.2 MATERIALS AND METHODS

9.2.1 Chemicals and Mycotoxins

The mycotoxins and other chemicals were obtained from the sources indicated in APPENDIX J. All fungal metabolites were dissolved in 5% ethanol and 5% Tween 80 as they are insoluble in water. AFB1 was also dissolved in 2% DMSO in oder to compare the efficiency of these two solvents. The structural formulas and producing organisms of the mycotoxins are shown in Table 1.1 (section 1.3 of the INTRODUCTION).

9.2.2 Concentrations and Protocols

Stock solutions of the mycotoxins (0,5 mM concentrations) were prepared as shown on the protocol sheets. Where amounts of test substances available permitted, concentrations of 0,2 mM; 0,1mM; 0,01mM and 0,001mM were prepared. This concentration range was selected in accordance with the results published for AFB1 by Graf *et al.* (1984). Chronic treatments of AFG1, AFM1, VRCA, CYTB and chronic and acute treatments of OCHA were carried out at the Institute of Toxicology in Schwerzenbach (Switzerland) where ultrasound was also used to dissolve these compounds. Chronic treatments of the rest of the mycotoxins were carried out at the University of the Witwatersrand (Johannesburg). Ochratoxin A and aflatoxin G1 were also restested in Johannesburg using the Improved High Bioactivation (IHB) and new HB crosses. The mycotoxins were used according to the guidelines for the laboratory use of chemical carcinogens set by the National Institute of Health (U.S.A) (1981).

9.2.3 *Drosophila* Crosses

9.2.3.1 The standard cross

The sources of all stocks used are listed in APPENDIX 3. All stocks were checked for the correct phenotype prior to use. For the standard cross, virgin females from the stock *flr*^{3}*/TM3Ser* were collected and crossed with males from the stock *mwh* in fresh bottles and allowed to age for 2-3 days. The use of the strain carrying the *flr*^{3} marker as the female parent assures good fertility and this is the reason for using the *flr*^{3} line as the female parent in all SMART crosses. All mycotoxins were tested using the standard cross. See section 9.2.8 for a description of the markers.

9.2.3.2 The high bioactivation (HB) cross

For the HB cross, *ORR;flr*^{3}*/TM3Ser* virgin females were crossed with *ORR?;mwh* males. This is the reciprocal of the cross described by Frölich and Würgler (1989) and, once again, use of the reciprocal cross facilitated collection of ample numbers of larvae. This cross was used in Schwerzenbach to test cytochalasin B, verrucarin A, aflatoxin M1 and G1.

9.2.3.3 The Improved HB cross

The other mycotoxins were tested using the Improved HB cross. For this cross, virgin females from the stock *ORR;flr*^{3}*/TM3Ser* were crossed to males from the stock *mwh*.

9.2.3.4 The New HB cross

For the New high bioactivation cross, virgin females from the newly constructed stocks *ORR;flr*^{3}*/TM3Ser* were crossed to males from the newly constructed *ORR;mwh* stock (refer to section 2.2 for detailed steps of the construction of these stocks). Aflatoxins G1 and G2 and ochratoxin A and cytochalasin B were retested using this cross. The mycotoxins sterigmatocystin and zearalenone were tested in the standard cross and the new HB cross.

After a three day maturation period, parental flies (which should be optimally fertile) from the various crosses were placed in fresh, well yeasted bottles (for the set of mycotoxins tested before July 1991) and in the new egg-laying bottles (see APPENDIX E) (for mycotoxins tested after July 1991) for an egg collection period of eight hours at 25°C.

Larvae transheterozygous for *mwh* and *flr*^{3}, obtained from these crosses, constituted half of the larval progeny; the other half consisted of *TM3Ser* +/+ *mwh* heterozygotes. The TM3 (Third multiple 3) chromosome is a balancer chromosome (see APPENDIX J) that acts as a crossover suppressor by eliminating the products of crossing over between the balancer chromosome and the other third chromosome. The *flr*^{3} mutant is homozygous lethal, although flies possessing the flare phenotype in sectors of somatic cells are viable (Garcia-Bellido and Dapena, 1974) so the mutation must be kept in the heterozygous state by the use of the balancer chromosome TM3. A dominant marker, *Ser* (Serrate), is used to identify the balancer chromosome. This

balancer chromosome is homozygous lethal, as the break points of the inversions disrupt essential genes, and hence *flr*^{3} can be kept in the heterozygous state. The dominant marker *Ser* is also homozygous lethal. In the heterozygous form, *Ser* results in wings missing p[illegible] of the margin. The two types of larvae from the cross are not distinguishable. However, after metamorphosis, *mwh* +/+ *flr*^{3} flies with wild type wing margins, were distinguished from their sibs *mwh* +/+ *TM3Ser* which have the serrated wing phenotype. Similarly, 50% of the progeny from the HB, Improved HB and New HB crosses was of the desired genotype (*ORR/?;mwh* +/+ *flr*^{3}, *ORR/+; mwh* +/+ *flr*^{3} and *ORR/ORR; mwh* +/+ *flr*^{3}, respectively). Although normal wings were mounted in most cases, serrate wings were mounted in the case of genotoxic mycotoxins to determine the proportion of spots due to somatic recombination.

9.2.4 Treatments

Studies performed by Graf *et al.* (1984) with a large variety of mutagens indicated that acute exposure allowed the detection of genotoxic activity but for general screening purposes, chronic treatments yield better results than acute treatments (Würgler *et al.*, 1985) because they allow the time needed to induce the metabolic processes that may be required to render a compound mutagenically active. For these experiments a chronic, 48 hour exposure of 72 ± 4 hour old larvae was chosen because 72 hour old larvae are fairly large and relatively easy to handle. Older larvae would have been larger but have the disadvantage that they do not develop easily recognizable wing spots as the size of the spots depends on the time of the mutation event. Most of the

cell division occurs during larval development and the earlier the mutation occurs, the larger the spot.

Third instar larvae were washed from the bottles according to the density separation procedure of Nöthiger (1970), except that the 20% sucrose solution was replaced by a 30% NaCl solution. The larvae were then collected in a nylon gauze strainer. The problem with this technique is that not all the larvae that have burrowed into the medium are washed out with the saline solution. Also, medium granules often clog the tap of the separating funnel prolonging the stay of the larvae in the saline thereby increasing the duration of the osmotic shock. In addition, the larvae themselves can clog the tap and many larvae are killed when trying to unclog the funnel.

With the new egg-laying medium and larva collection method of Magnusson and Ramel (1990), larvae were washed out of the yeast-agar bottles with tap water and collected in a fine mesh stainless steel strainer. This eliminates the use of a separating funnel and of the sodium chloride (or sucrose) solution, preventing an osmotic shock to the larvae. The method is less time consuming and optimizes the number of larvae collected. The thick layer of yeast completely covering the surface of the agar (APPENDIX E), provides an excellent medium for egg-laying as *Drosophila* flies prefer to lay eggs on uneven surfaces in nature and the yeast provides many "hills" and "valleys" compared with the relatively smooth surface of ordinary medium. Omitting sucrose from the agar prevents the larvae that hatch from the eggs, from

burrowing into the agar medium and hence the larvae remain in the yeast where they are easily washed out with water onto a stainless steel mesh. This technique was used in the second half of the experiments.

Groups of about 100 larvae were placed in labelled culture vials containing 1,5 g instant medium and 5 ml of the mycotoxin solution at the different concentrations. A small piece of filter paper was added to the vials to regulate the humidity, and the vials were then closed with foam rubber stoppers. The cultures were kept in an incubator at 25°C until the adult flies hatched. Each experiment was repeated on a separate day as recorded on protocol sheets. Concurrent water and solvent controls were included in each test. Controls detect events that occur during normal development that result in the markers becoming homzygous and are essential to calculate the spontaneous mutation rate. Controls may be pooled over a small series of experiments. Positive controls are not carried out routinely. It usually suffices to ensure that the test is performed correctly by checking the negative controls. In Schwerzenbach, however, positive controls were also included in the test: chromium oxide (10 mM) and AFG1 at different concentrations.

Where availability of the compounds was limiting, miniature treatments were used. For these, an exact number of larvae is treated in miniature vials as indicated in AFM1 protocol sheets. This method also allows an accurate estimate of toxicity to be made, as the number of surviving flies can be counted and used to calculate the percentage mortality.

9.2.5 Toxicity tests or Miniature treatments

A miniature treatment at 0,5 mM concentration was included for each mycotoxin to estimate the relative toxicities of these compounds in the standard cross. Exactly 100 larvae of similar size (and therefore age) were counted under a dissecting microscope, and these larvae were then placed in miniature vials with 0,28 g of instant medium and 1 ml of mycotoxin solution per 100 larvae, instead of 5 ml of solution as in the standard vials. Reduced survival due to the high toxicity of the treatment as well as delayed hatching effects were recorded on the protocol sheets upon inspection of the treatment vials during harvesting.

9.2.6 Preparation of Wings

With these chronic treatments, the treated individuals remained in the treatment vials until the emergence of the surviving adult flies. Adult flies were harvested 1-2 days after eclosion. They were anaesthetized (using ether) and placed in 70% ethanol for at least three days. For miniature treatments, the number of surviving flies was counted. The wings were removed and mounted in Faure's solution (APPENDIX D).

9.2.7 Analysis of Wings

The wings were scored under a compound microscope at 400x magnification. Both the dorsal and ventral surfaces of the wings were screened by constantly focusing up and down. Wings are scored by sections delineated by veins and cross-veins and each sector is designated by a letter (see Figure 2.2.1). Wings were numbered and single and twin spots on each wing were recorded

on the score sheets. The position of the spots was noted according to the sector of the wing where the spot appeared; the size of each spot, determined by counting the number of cells affected, as well as the type of spot, whether it was a single *mwh* or *flr*^{3} spot or a twin spot, were also determined. All slides, score sheets and protocol sheets are filed at this university and are available for reference.

9.2.8 Classification of Spots

Each cell on the surface of the wing normally forms one long, thin trichome in wild type flies. The multiple wing hairs mutation, when homozygous, causes the single long hair to be replaced by a group of small hairs. If a wing cell contained three or more small hairs instead of one hair per cell, it was classified as *mwh*. Cells with two hairs, which appear frequently in the D sector of *mwh* +/+ *flr*^{3} wings, seem to be formed independently of the *mwh* mutation. Two hairs may form on normal cells that do not carry the *mwh* mutation if the temperature rises. Graf *et al.* (1984) excluded all single cells with only two hairs from the *mwh* single spot counts. If, however, a cell with two hairs appeared within a *mwh* spot, or at its margin, it was included in the determination of the size of the clone. The *flr*^{3} mutation leads to abnormal hair development. One cell *flr*^{3} spots have not been observed and it seems that *flr*^{3} spots of less than two cells are not expressed, whereas single cell *mwh* spots are common. In large spots *flr*^{3} expression ranges from pointed, shortened and thickened hairs to amorphic extrusions of chitinous material (Graf *et al.*, 1984). Electron micrographs (see APPENDIX F) have been included to illustrate the difference between a normal trichome (Fig 9.2.1a),

the *mwh* mutation (Fig 9.2.1b) and different manifestations of the *flr*^{3} mutation (Fig 9.2.1c); photographs of *mwh* and *flr*^{3} spots are shown in Figures 9.2.2a-b and twin spots in Figure 9.2.2c. A problem leading to inaccuracies in scoring is classifying split clones. Marked clones usually appear as contiguous spots, but sometimes the spots are split into two or more cell groups. The different parts of the split spots can be separated by one or several rows of normal cells. These clones are thought to be due to partial separation of the cells of a clone during development owing to internal tissue pressure or independent cell movements (Garcia-Bellido and Merriam, 1971). To ensure standardization in scoring, spots separated by more than two rows of normal cells are scored as two different spots while two spots separated by one or two rows of cells are scored as one spot.

For the evaluation of the data, the spots were grouped into three categories: small single spots (one or two cells affected), large single spots (three or more cells affected) and twin spots. The frequencies of small and large singles, as well as twin spots in the treated series could then be tested against control values. The three categories were combined to calculate total spots per wing. Spots were classified according to the following size classes, numbered in increasing order beginning with 1: 1, 2, 3, 3-4, 5-8, 9-16, 1-32 (as seen in Figure 9.3.11). The mean size class rather than the mean number of cells in spots was calculated as a measure of spot size so that the few very large spots among the many relatively small ones would not bias the calculation of the mean spot size.

The differentiation of small single spots from large single spots quite conveniently separates the target cell populations exposed to the mycotoxin after feeding, in cases where the mycotoxin remains in the tissues. In the water and solvent controls, small *mwh* spots predominate. By dividing total single spots into three groups, it was possible to test the frequency of large single spots in the mycotoxin treated series against the low control values to determine if the treatment induced large spots. A mycotoxin may induce large spots but not small single spots, and if the single spots are not differentiated the overall diagnosis may be negative. This demonstrates that a differentiation and separate statistical analysis of each type of spot gives more and better information than the overall diagnosis based on the total number of spots. Another reason for evaluating small single spots separately is that it is likely that genetic deficiencies due to chromosome aberrations, which uncover the *mwh* mutation for instance, lead mostly to small clones (Graf *et al.*, 1984). Independently of the time of initiation these clones remain small as they appear to proliferate slowly, if at all. Hence, a compound that induces small spots rather than large spots can be identified and such a compound would be expected to cause genetic change mainly through chromosome aberrations.

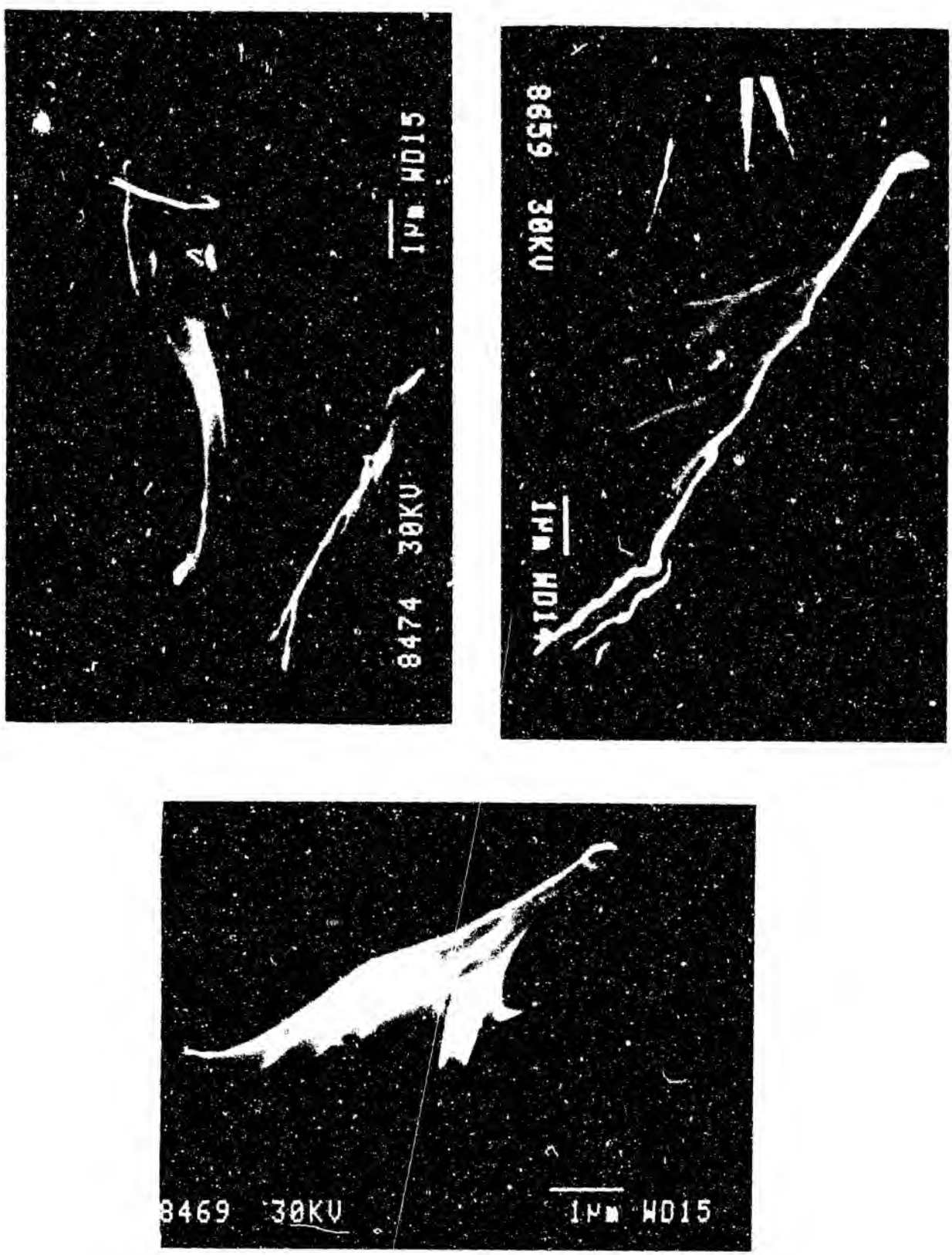


a



b

Figure 9.2.1 **Scanning electron micrographs of wing blade trichomes a) normal trichome b) *mwh* trichome**

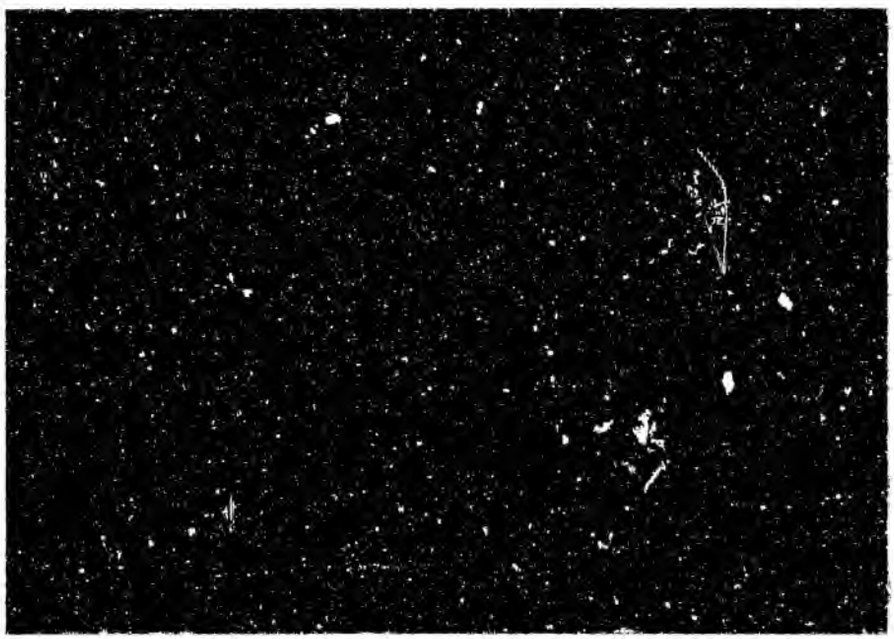


c

Figure 9.2.1 c) different manifestations of the *flr*^{3} trichome.



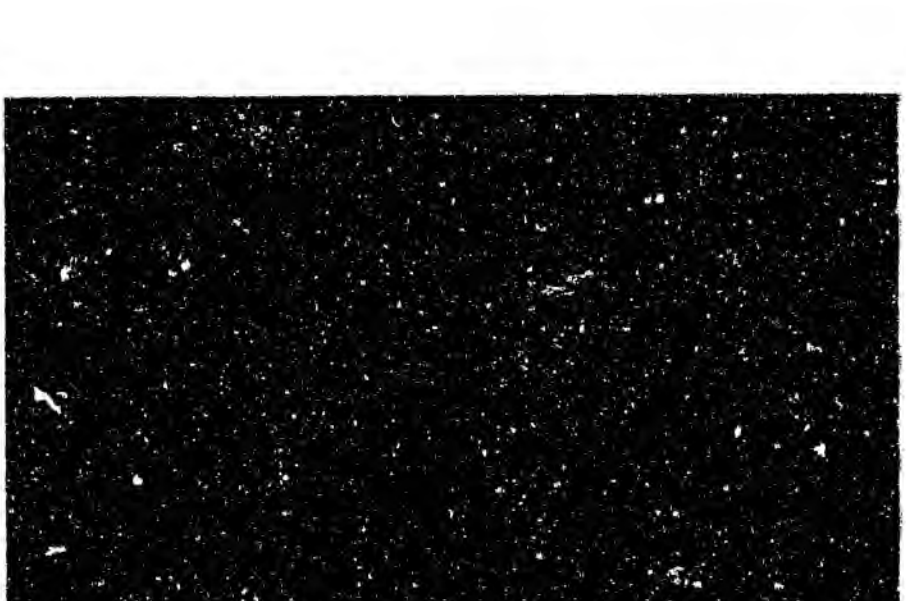
a



b

Figure 9.2.2 Photographs of a) a multicelled *mwh* spot against a normal backround, b) a multicelled *flr*^{3} spot against a normal backround.





c

Figure 9.2.2 **c) multicelled twinspots against normal backrounds.**

9.2.9 Exposure-Effect Curves

The concentration of the mycotoxin solution could not be considered the dose. In feeding, the volume of liquid ingested varies with the route of administration and it is only the amount of mycotoxin reaching the target cells which represents the actual dose. This dose can only be determined by chemical analysis. The chronic feeding period was 48 hours in most cases and exposure was simply expressed by the mM concentration. Exposure was plotted against the number of spots per wing and exposure-effect curves for the different mycotoxin treatments are shown in Figures 9.3.1-9.3.10.

9.2.10 Statistical Analysis

In these experiments, the mutagenicity of the mycotoxins was assessed by comparing the frequency of the three types of spots in the treatment series with the frequency of these spots in the control series. According to Selby and Olson (1981), the formulation of two alternative hypotheses allows differentiation among the possibilities of a positive, inconclusive or negative result of an experiment. The null hypothesis (Ho) assumes that there is no difference in the mutation frequency between the control and treated series. Rejection of the null hypothesis indicates that the treatment resulted in a statistically increased mutation frequency. The alternative hypothesis (Ha) postulates *a priori* that the treatment resulted in an increased mutation frequency that is m times the spontaneous frequency. This alternative hypothesis was rejected if the observed mutation frequency was significantly lower than the expected increased frequency. Rejection indicates that the treatment did not produce the increase required to consider the treatment as

mutagenic. If neither of the two hypotheses are rejected, the results are considered inconclusive as the two mutually exclusive hypotheses cannot be accepted at the same time. If both hypotheses are rejected, the treatment is considered weakly mutagenic. Each hypothesis was tested at the 5% level of significance ($\alpha = \beta = 0{,}05$).

In the practical application of the decision procedure, a specific alternative hypothesis is defined requiring that the mutation frequency in the treated series be m times that in the control series. As small single spots and total single spots have high spontaneous frequencies, the multiplication factor m was fixed at a value of two ($m = 2$). For the rarer large single and twin spots, m was fixed at a value of five ($m = 5$). The statistical diagnoses were performed according to Frei and Würgler (1988). The wing spot data were evaluated with the computer program SMART (Würgler, unpublished) by Dr Graf at the Institute of Toxicology, Swiss Federal Institute of Technology and University of Zürich, Schwerzenbach, Switzerland. For the calculations, the Kastenbaum-Bowman (1970) test was used with $P = 0{,}05$.

Based on the number of wings analyzed, the number of *mwh* clones and the number of cells scored in each wing (about 24 400), it is possible to calculate the clone formation frequency per cell cycle per 10^5 cells. As growth in the imaginal wing disc is approximately exponential, the target cell population may be expected to double at each round of cell division (Frei *et al.*, 1992). On the other hand, clone size reflects time of induction and stage of development. For chronic exposure, the expected clone size distribution in the ideal situation

therefore corresponds to a geometric series with frequencies decreasing by a factor of two as clone size (cells) increases by a factor of two (Frei *et al.*, 1992).

Thus, according to the number of *mwh* cells they contained, clones were classified into size classes (i) delimited by powers 2^{i-1}. For continuous exposure in the ideal case, the mean clone size class is i = 2 and the mean clone size is 2^{i-1} = 2 cells (geometric mean). In practice, the clones may be smaller or larger than theoretically expected (Frei *et al.*, 1992). It was not necessary to make clone size corrections, because these were chronic treatments with stable compounds (Frei and Würgler, 1988; van Schaik and Graf, 1991; Frei *et al.*, 1992).

9.3 RESULTS

9.3.1 Toxicity and Miniature Treatments

After treatment with certain mycotoxins at high concentrations dead, "black", larvae and pupae were observed on the walls of the culture vials after treatment. Flies also died after hatching and hence care was taken during harvesting to collect only surviving flies. It was easy to distinguish flies that were dead before they were placed in the ethanol because they had a shrivelled appearance. The mycotoxins led to an overall delayed hatching at the 0,5 and 0,2 mM concentrations, with flies hatching on days 12-14 rather than on day 10 as in the controls. Exactly 100 larvae were treated in the miniature vials and hence the toxicity of the mycotoxins could be compared in a quantitative manner as seen in Table 9.3.1. All the mycotoxins were toxic to *Drosophila* larvae at 0,5 mM, but to different extents: the aflatoxins were the most toxic compounds with AFB1 (0,5 mM) treatments resulting in 96% mortality; ochratoxins were also potently toxic to larvae; roridin A, verrucarin A and the fumonisins were about equally toxic and, as can be seen in Table 9.3.1 the cytochalasins were the least toxic compounds. In the controls all larvae survived indicating that the solvent 5% tween 80 + 5% ethanol was not toxic to larvae. The controls indicated that the larvae of the different crosses were viable and developed normally, that the medium and solvent did not have a toxic effect on the larvae and, most importantly, that the conditions of treatment and exposure were standard and optimal.

Serrate flies usually hatch before flies with normal wings and if flies are harvested early, it may seem that more serrate flies are present in vials. In these experiments, flies were harvested late to ensure that the majority of flies had hatched taking into account the delayed hatching effects of most mycotoxins. The diference between the number of flies with serrate wings and normal wings was not statistically significant. With the aflatoxins, the number of male and female survivors was recorded and the data indicated a statistically significant difference in the survival of male and female flies with male flies about 2-4 times more resistant to the toxic effects of the aflatoxins.

9.3.2 Control Frequencies

The compounds were usually tested in the standard cross and a high bioactivation cross simultaneously to obtain quantitative data which allowed for comparative analysis. The data collected are compiled in Tables 9.3.2-9.3.6. Two large concurrent negative control series were performed with water and 5% Tween 80 and 5% ethanol and these control rates were pooled in the two different laboratories and with the four different crosses. The controls from these experiments indicated a spontaneous, total spot per wing rate of 0,13 (water control) and 0,20 (5% ethanol + 5% tween 80) solvent control) for the Schwerzenbach standard cross (Table 9.3.2) while the Johannesburg standard cross had a lower spontaneous mutation rate of 0,15 (water) and 0,14 (5% tween 80 + 5% solvent) (Table 9.3.4). The Johannesburg 2% DMSO solvent control rate was very low (0,05) compared to the rate observed in 1989 (Honours Dissertation) (see Table 9.3.7) and this was probably due to small sample size as only 40 wings were scored.

TABLE 9.3.1 TOXICITY RESULTS

MYCOTOXINS	CROSS	CONCENTRATION (mM)	NUMBER OF SURVIVORS (100 larvae treated)
SOLVENT CONTROL	STD	0,5	21 n ♀ + 26 n ♂ 30 s ♀ + 23 s ♂
	HB	0,5	100
	IHB	0,5	100
	NHB	0,5	100
AFB1	STD	0,5	2 s ♂ + 2 n ♂
AFB2	STD	0,5	33
AFG1	STD	0,5	8 n ♂ + 9 s ♂ 3 n ♀ + 4 s ♀
	HB	0,5	6 n ♂ + 2 s ♂
	IHB	0,5	5 n ♂ + 4 s ♂ 3 n ♀ + 3 s ♀
	NHB	0,5	3 n ♂ + 5 s ♂ 1 n ♀ + 0 s ♀
AFG2	STD	0,5	42
AFM1	HB	0,05	40
	STD	0,1	22
CYTB	STD	0,5	87
CYTH	STD	0,5	48
CYTJ	STD	0,5	51
DEOX	STD	0,5	42
DIAC	STD	0,5	25
FB1	STD	0,5	18
FB2	STD	0,5	18
OCHA	STD	0,5	6
RORA	STD	0,5	18
STM	STD	0,5	42
VRCA	STD	0,5	16
	STD	0,1	80
ZEA	STD	0,5	62

n= individuals with normal wings and s= individuals with serrate wings

The solvent control rate of the Johannesburg improved high bioactivation cross (0,14) was the same as that of the Johannesburg standard cross (Table 9.3.5) while the spontaneous mutation frequency of the Johannesburg new HB cross was 0,22 (water) and 0,25 (solvent) (Table 9.3.6). The highest spontaneous mutation rate (0,27) was observed with the Schwerzenbach original HB cross (Table 9.3.3).

Aflatoxin B1 was also tested using 2% DMSO as the solvent at a concentration of 0,5 mM. Since the overall spontaneous mutation rate observed with water controls was not significantly different from the rate observed with the solvent controls, it was concluded that 2% DMSO and 5% ethanol + 5% tween 80 were not genotoxic to larvae.

9.3.3 The Standard Cross

Only aflatoxin G1 gave clearly positive results at all concentrations in the standard cross. Aflatoxin M1 was positive at concentrations higher than 0,01 and with AFB1 and sterigmatocystin, the frequencies of total spots per wing were significantly increased over the control from 0,1 mM and higher, whereas with AFB2 and AFG2 this was the case at only 0,5 mM in the standard cross. All other, non-aflatoxin mycotoxins tested were non-genotoxic and gave clearly negative results with the standard cross. With cytochalasin B at 0,1 mM concentration, cytochalasin H at 0,001 mM concentration (48-hour chronic treatments) and ochratoxin A at 0,1 and 0,2 mM (four hour acute treatments), the frequencies of twin spots per wing were

significantly increased over the control but these were probably false positive results (Tables 9.3.2 and 9.3.4).

Spot-size distributions for single and twin spots were plotted against spot density distributions (Figures 9.3.11-9.3.13) to visualize the data, to compare the results of the standard cross and high bioactivation crosses and to compare the frequency of spots on marker transheterozygous and balancer heterozygous wings. It can be seen from Figure 9.3.11-9.3.13 that aflatoxins G1, B1 and M1 were recombinogenic (see also discussion section 9.4.3 normal transheterozygous versus balancer heterozygous wings). When considering the three categories of spots separately, it was obvious that for AFG1 and AFM1 they were all significantly increased at the higher concentrations. With AFB1 the situation was less clear, only the small single and large single spots were significantly increased whereas the twin spots were inconclusive at the highest concentration (0,5 mM). It was assumed that this was due mainly to the rather small number of wings analyzed at the higher concentrations (only ten wings for 0,5 mM AFB1). Due to the greater toxicity of this compound (refer to Table 9.3.1), the number of wings obtained was lower than that obtained at the same concentrations with the other aflatoxins. For this reason the data did not allow a conclusion to be reached about the possible recombinogenic activity of AFB1 at 0,5 mM. At the lower AFB1 concentration (0,1 mM), however, all three spot categories were increased so AFB1 was recombinogenic at this high, but nevertheless less toxic, concentration. AFB1 (0,5 mM) was also tested using 2% DMSO as the solvent and it was observed that the frequency of spots per wing (for all categories) was

significantly reduced (the reduction was about 11 fold) on 2% DMSO wings compared with 5% ethanol + 5% tween 80 wings (see Tables 9.3.4 and 9.3.7).

The decrease in spot frequency at the higher, toxic concentrations is clearly evident in the exposure-effect curves, where exposure (expressed simply as the mM concentration) was plotted against the number of total spots per wing. The exposure-effect curves for the aflatoxins and other positive and negative compounds, together with the pooled solvent control, are shown in Figures 9.3.1-9.3.10. A drop in spot frequency at the highest AFG1 concentration tested was clearly evident in the corresponding exposure-effect curve (Fig. 9.3.3 a) This was probably due to the pronounced toxicity of this compound at this concentration. The aflatoxins were arranged in decreasing order of genotoxicity as AFM1 > AFB1 > AFG1 > AFB2 > AFG2 according to the spot frequency data (total spots per wing per mM at the relatively non-toxic concentration 0,1 mM).

The frequency of spots per wing for the five aflatoxins was compared in female and male wings but any differences observed were not statistically significant. This indicates that the bioactivation capacity of male and female flies was probably the same. Male wings are usually smaller than female wings and if this difference in size was due to fewer cells on male wings, then perhaps fewer spots would be observed on male wings than on female wings and since the number of spots per wing was about the same in the two sexes, this would indicate that males have a higher bioactivation capacity than

female flies. Graf and Frei (personal communications), however, believe that the number of wing cells (about 24 000), is the same in males and females but in males the cells are smaller so that the wings appear smaller. Hence male and female *Drosophila* showed equal capacity to activate promutagenic mycotoxins.

9.3.4 The High Bioactivation and Improved High Bioactivation Crosses

Four mycotoxins (AFG1, AFM1, cytochalasin B and verrucarin A) were tested in the HB cross, twelve mycotoxins were tested in the IHB cross (AFG1 was tested in both the HB and IHB crosses). The results demonstrated that the genotoxic activity of AFM1, AFG1 and AFB1 (rather potent mutagens) was readily detectable even in the standard cross. However, the frequencies of total spots were considerably enhanced when using the HB cross or the IHB cross. This was most pronounced with AFG1 (0,001 mM) for the HB cross where the increase of total spots over the the standard cross was close to ten fold (Tables 9.3.3 and 9.3.5).

When comparing the results of the treated series obtained with the IHB cross with those obtained with the HB cross, with 0,001 mM AFG1 the frequencies of spots per wing were lower in the *ply* heterozygous than in the *ply* homozygous ones. This could either have been due to a slightly higher bioactivation capacity contributed by the *ORR;mwh* parent, or due to inaccuracies in scoring wings with whorling of the trichomes.

The two borderline cases, AFB2 and AFG2, which were only slightly genotoxic in the standard cross and then only for large single, twin and total spots at the highest concentration, and inconclusive for the small single spots, were detected as clearly genotoxic in the IHB cross. With the IHB cross, the frequencies of all categories of spots were significantly increased over the control at the higher concentrations (0,2 mM and 0,5 mM) of AFB2 indicating not only the genotoxicity of this compound but also the recombinogenic activity, whereas the frequencies of twin spots were inconclusive at all concentrations in the standard cross. Similarly, AFG2 has recombinogenic and genotoxic activity at 0,2 mM and 0,5 mM concentrations in the IHB cross while in the standard cross the frequencies of twin spots were inconclusive at all concentrations except at the lowest concentration where the result was negative for twin spots.

By comparing the control corrected frequencies of clone formation per 10^5 cells with 0,001 mM AFG1 these values were: 7,9 for the HB cross, 1,2 for the IHB cross and 0,5 for the standard cross, and an increase of about 2,5 fold for the IHB and about 16 fold for the HB crosses was calculated. With 0,2 mM AFB2 these values were 1,8 for the IHB and 0,4 for the standard cross and with 0,2 mM AFG2 these values were 1,7 for the IHB cross and 0,3 for the standard cross, an increase of 4-5 and 5-6 fold, respectively. With 0,01 mM AFB1 these values were 1,8 for the IHB and 0,3 for the standard, corresponding to a 6 fold increase over the standard cross. Hence with AFB1, AFB2 and AFG2 the increase was of the same order of magnitude.

The other non-aflatoxin mycotoxins, which were non-genotoxic in the standard cross, were also non-genotoxic in the IHB and HB crosses. The frequency of large single spots, however, was significantly increased at the highest (0,5 mM) concentration of ochratoxin A in the IHB cross. Similarly, the frequency of small single and total spots was significantly increased with 0,01 mM cytochalasin B. These two compounds had also shown positive results at one concentration in the standard cross and therefore these two mycotoxins were retested with the new HB cross, where both parents are DDT resistant but whorl-free, and progeny larvae are homozygous for the *RI* gene, to gain more conclusive evidence about their genotoxicity.

The results for some non-aflatoxin mycotoxins were negative for some mycotoxin concentrations and inconclusive for the rest. Inconclusive results were probably due to the small number of wings analyzed in the high bioactivation crosses, because of the reduced survival of progeny from high bioactivation crosses compared with progeny from the standard cross (Table 9.3.1).

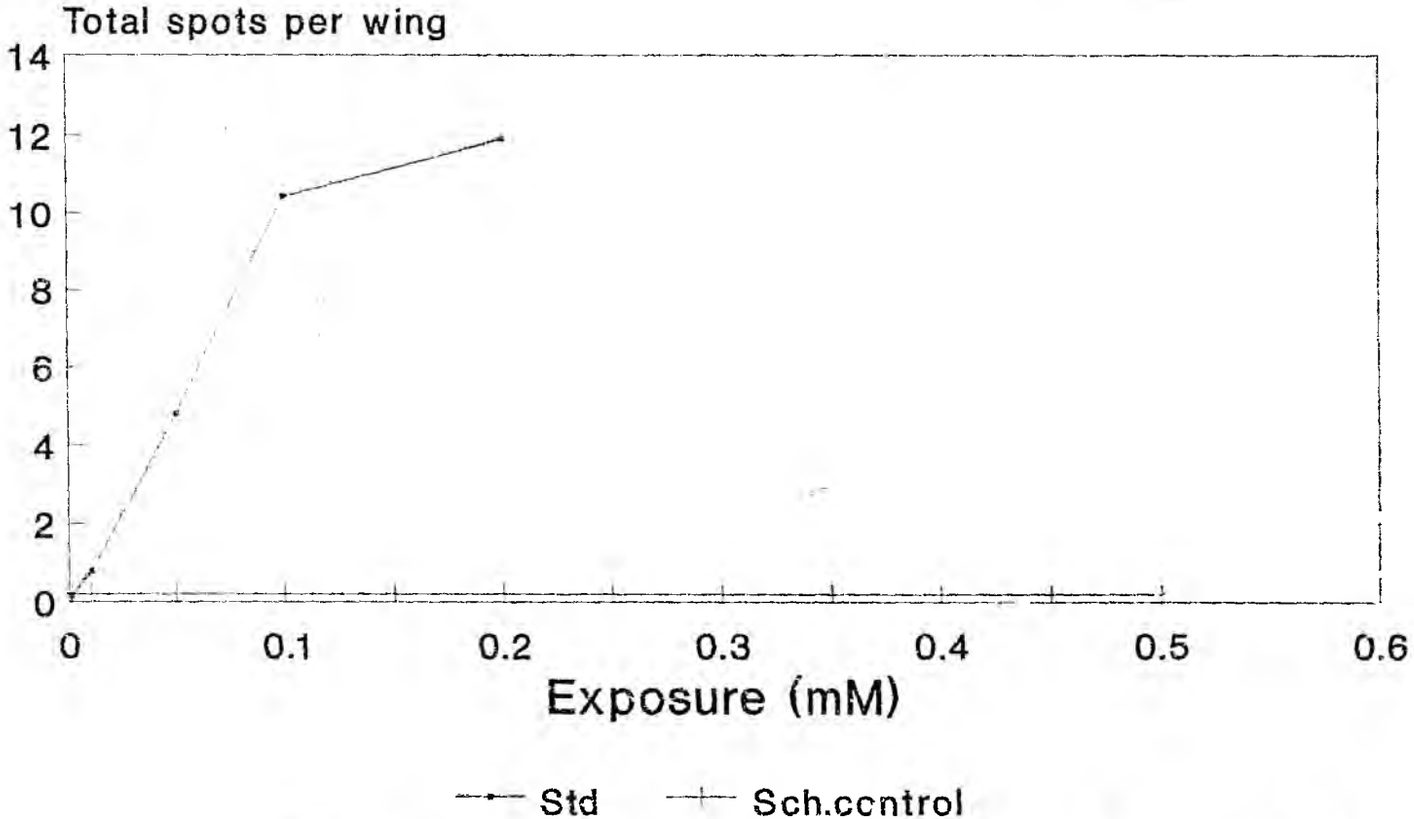
9.3.5 The New High Bioactivation Cross

Six mycotoxins were tested using the new HB cross: AFG1 at only one concentration (0,01 mM); the borderline promutagens AFG2 and sterigmatocystin at five concentrations; cytochalasin B and ochratoxin A at only one concentration (0,1 mM) and zearalenone at all five concentrations. The new HB cross was more sensitive than the standard and IHB crosses to the genotoxic effects of promutagens AFG1, AFG2 and sterigmatocystin and the frequencies of total spots were significantly increased over the controls even

at the lower concentrations of the borderline promutagens sterigmatocystin and AFG2. The frequency of total spots recorded with the new HB cross for AFG2 (0,5 mM) was significantly higher than the frequencies of total spots recorded with the standard and improved HB crosses at the same concentration. Cytochalasin B and ochratoxin A, however, were also negative in this cross and this indicates that the random positive results obtained in the standard and IHB crosses were probably false positives. Zearalenone, which was negative at all concentrations in the standard cross, was positive at the two higher concentrations in the new HB cross.

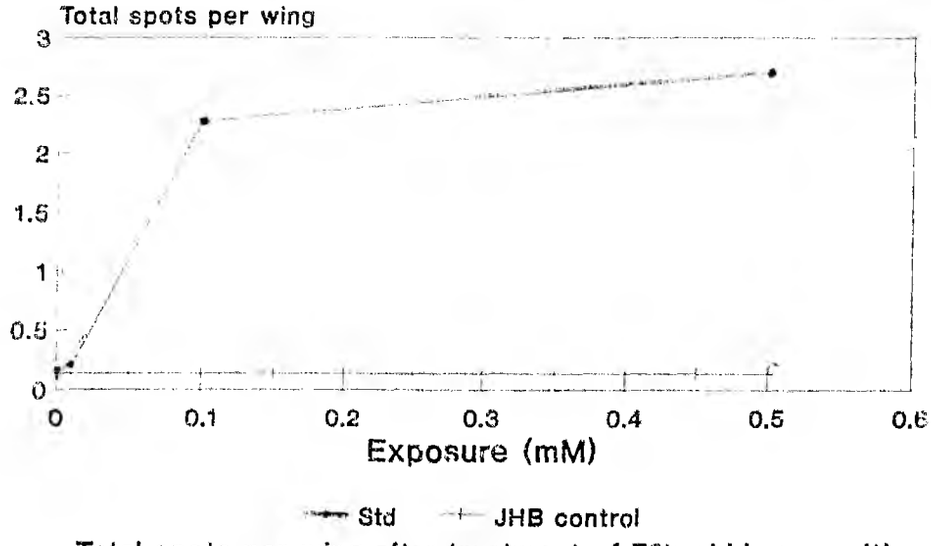
The similarity of the results recorded with the new HB, the improved HB and original HB crosses was evident from the spot-size distributions registered with AFG1. The new HB, improved HB and original HB crosses showed similar size distributions for single and twin spots (see Figures 9.3.11 b, c and d) and, when compared with the standard cross (Fig. 9.3.11 a), the HB crosses were characterized by the presence of a significant fraction of spots of the larger size classes. It is likely that this is due to the more efficient bioactivation of these compounds in the ORR homozygous and ORR heterozygous larvae leading to an earlier induction of mutation and recombination. Comparison of spot frequencies in marker transheterozygous and balancer heterozygous wings indicated that AFG1 was also recombinogenic in the new HB cross (Figure 9.3.11 d) and that the reduction in spot frequency in balancer heterozygous wings compared with marker transheterozygous wings was of the same order of magnitude in the standard, original HB, improved HB and new HB crosses (Figure 9.3.11 a-d).

Figure 9.3.1
AFM1 exposure-effect curve



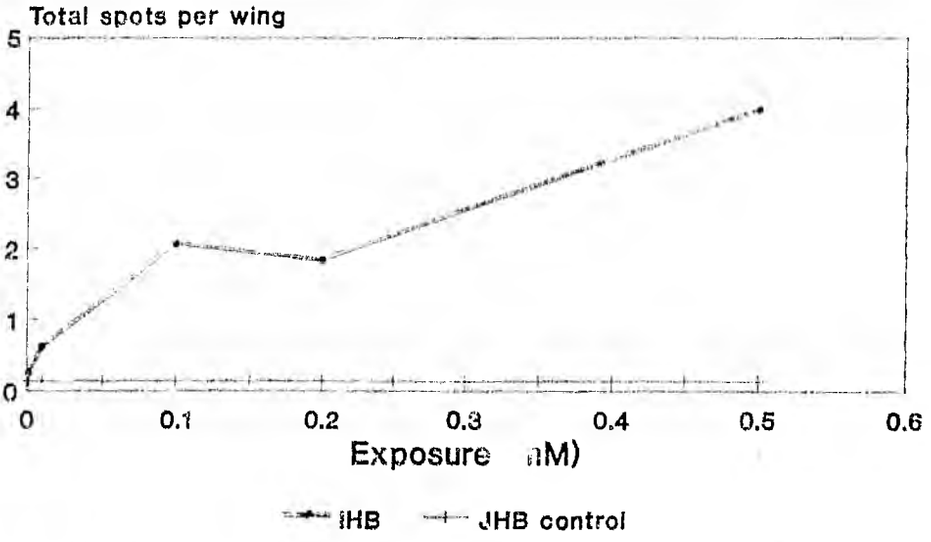
Total spots per wing after treatment of 72h old larvae with varying concentrations of AFM1. Standard cross wings (Std)

Figure 9.3.2 a
AFB1 exposure-effect curve



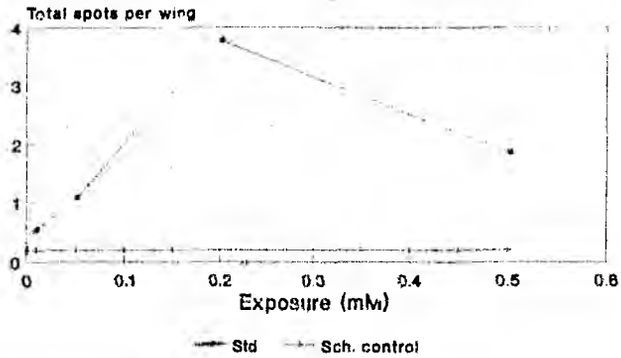
Total spots per wing after treatment of 72h old larvae with varying concentrations of AFB1. Standard cross wings (Std)

Figure 9.3.2 b
AFB1 exposure-effect curve



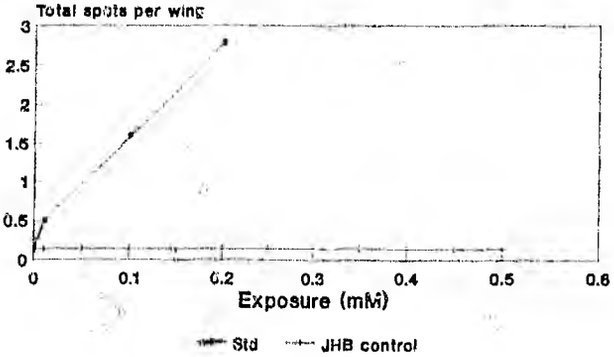
Total spots per wing after treatment of 72h old larvae with varying concentrations of AFB1. IHB cross wings.

Figure 9.3.3 a
AFG1 exposure-effect curve



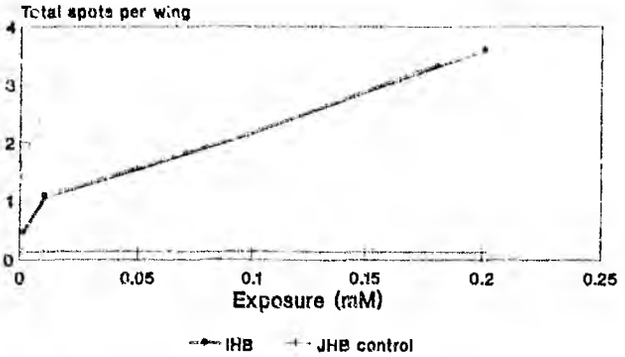
Total spots per wing after treatment of 72h old larvae with varying concentrations of AFG1. Standard cross wings (Std)

Figure 9.3.3 b
AFG1 exposure-effect curve



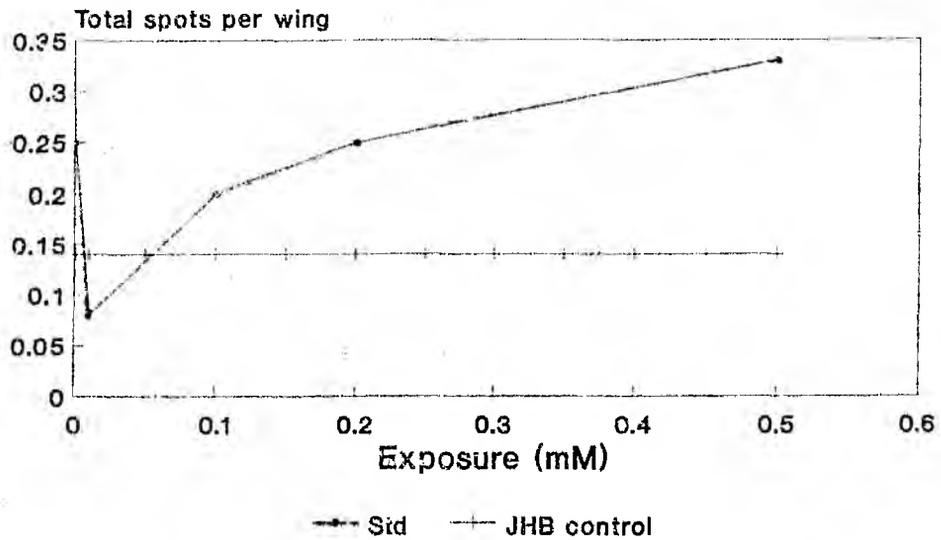
Total spots per wing after treatment of 72h old larvae with varying concentrations of AFG1. Standard cross wings (Std)

Figure 9.3.3 c
AFG1 exposure-effect curve



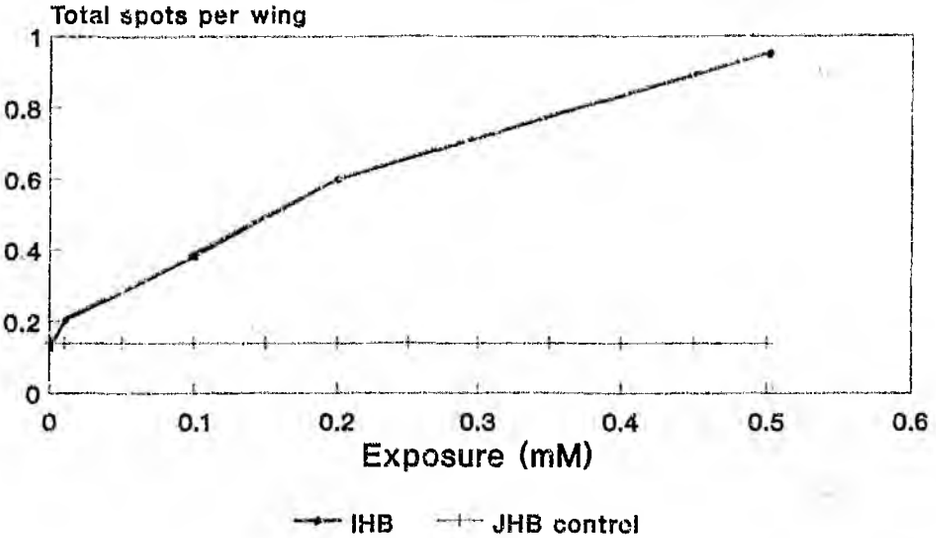
Total spots per wing after treatment of 72h old larvae with varying concentrations of AFG1. IHB cross wings.

Figure 9.3.4 a
AFB2 exposure-effect curve



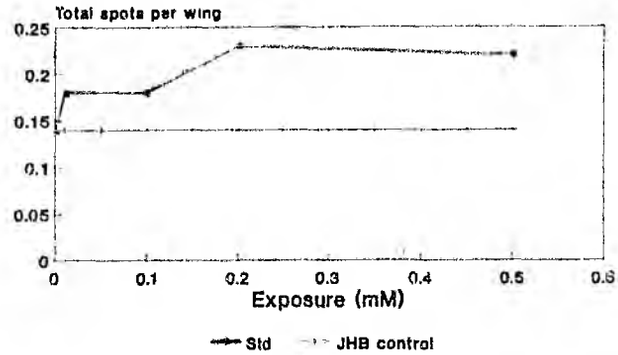
Total spots per wing after treatment of 72h old larvae with varying concentrations of AFB2. Standard cross wings (Std)

Figure 9.3.4 b
AFB2 exposure-effect curve



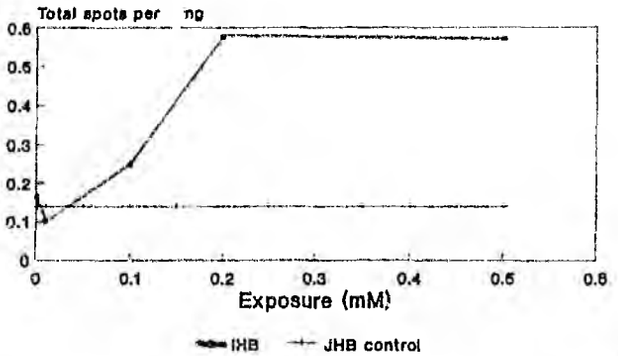
Total spots per wing after treatment of 72h old larvae with varying concentrations of AFB2. IHB cross wings.

Figure 9.3.5 a
AFG2 exposure-effect curve



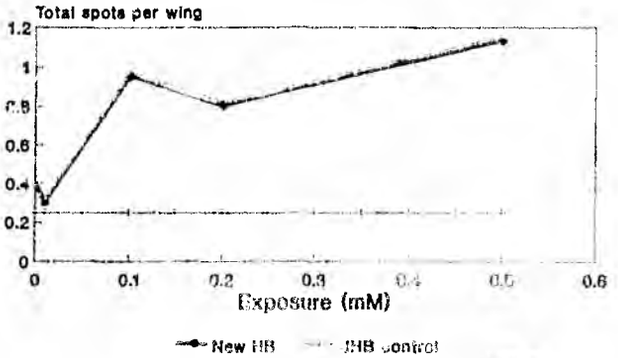
Total spots per wing after treatment of 72h old larvae with varying concentrations of AFG2. Standard cross wings (Std)

Figure 9.3.5 b
AFG2 exposure-effect curve



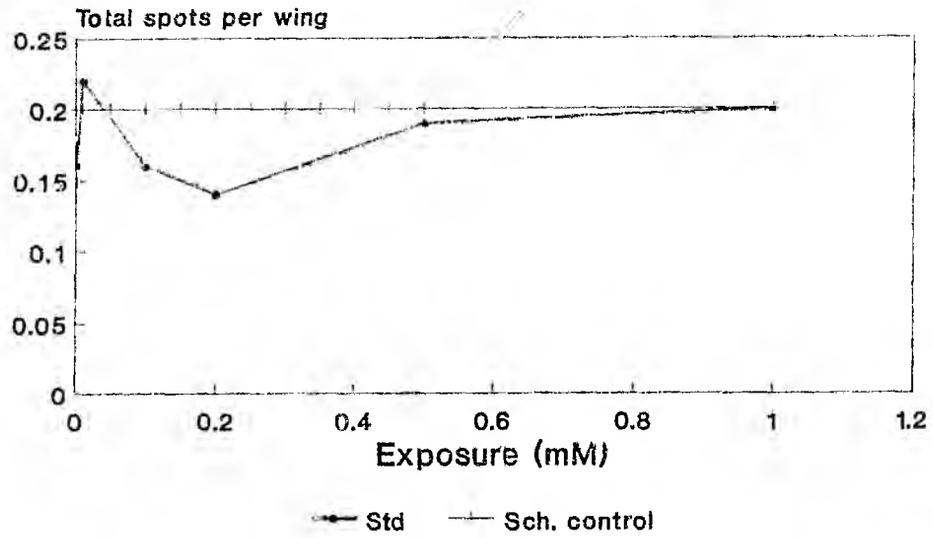
Total spots per wing after treatment of 72h old larvae with varying concentrations of AFG2. IHB cross wings.

Figure 9.3.5 c
AFG2 exposure-effect curve



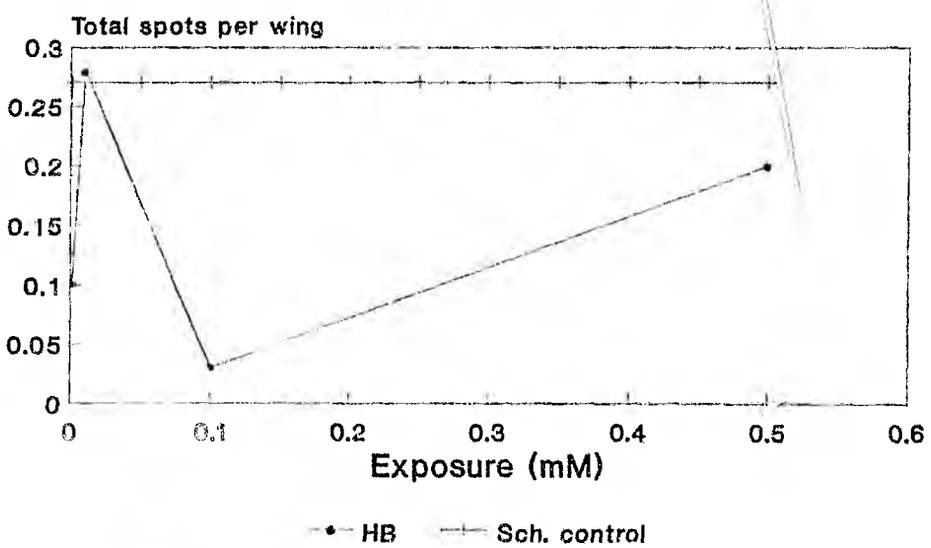
Total spots per wing after treatment of 72h old larvae with varying concentrations of AFG2. New IHB cross wings.

Figure 9.3.6 a
CYTB exposure-effect curve



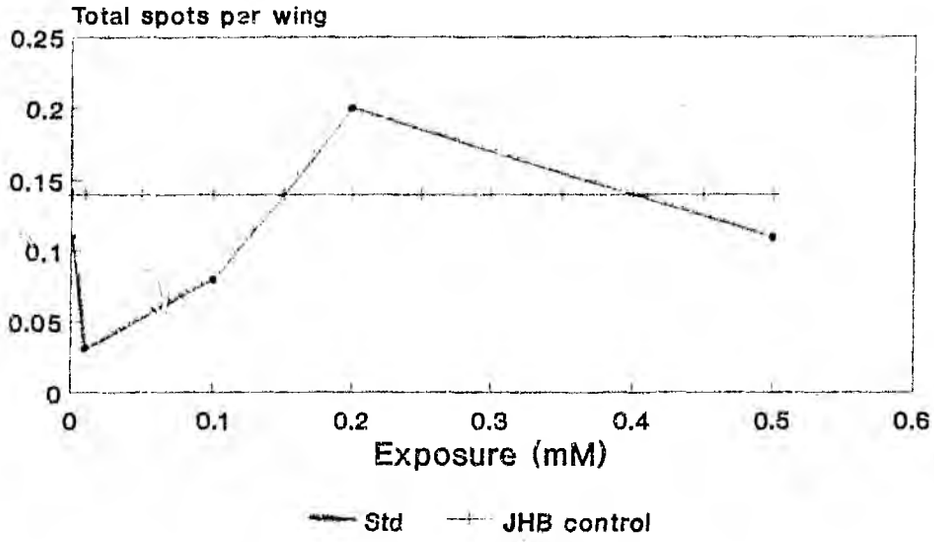
Total spots per wing after treatment of 72h old larvae with varying ooncentrations of CYTB. Standard cross wings (Std)

Figure 9.3.6 b
CYTB exposure-effect curve



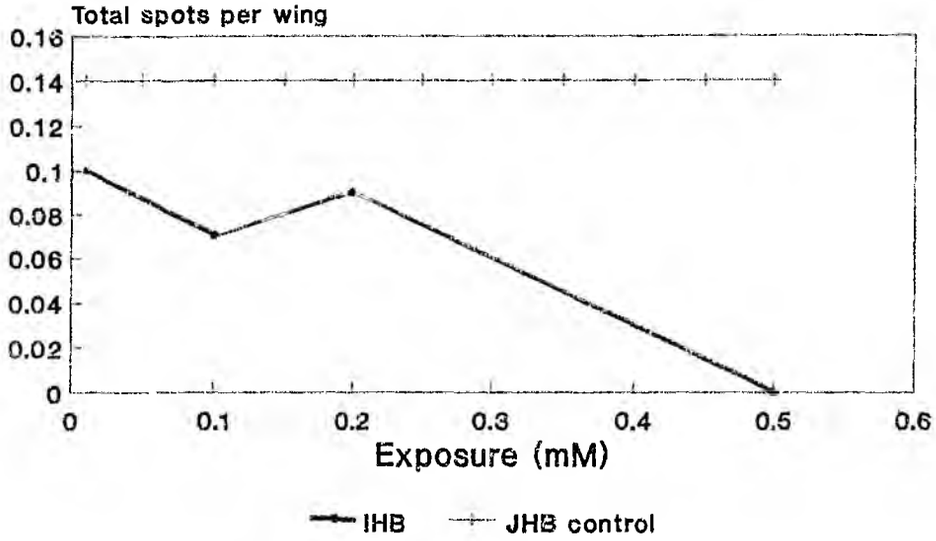
Total spots per wing after treatment of 72h old larvae with varying concentrations of CYTB. HB cross wings.

Figure 9.3.7 a
DIAC exposure-effect curve



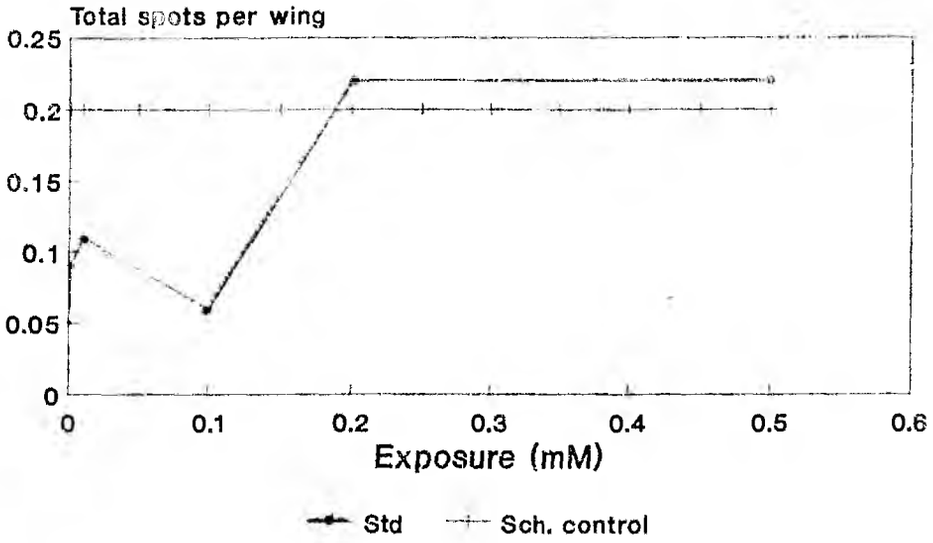
Total spots per wing after treatment of 72h old larvae with varying concentrations of DIAC. Standard cross wings (Std)

Figure 9.3.7 b
DIAC exposure-effect curve



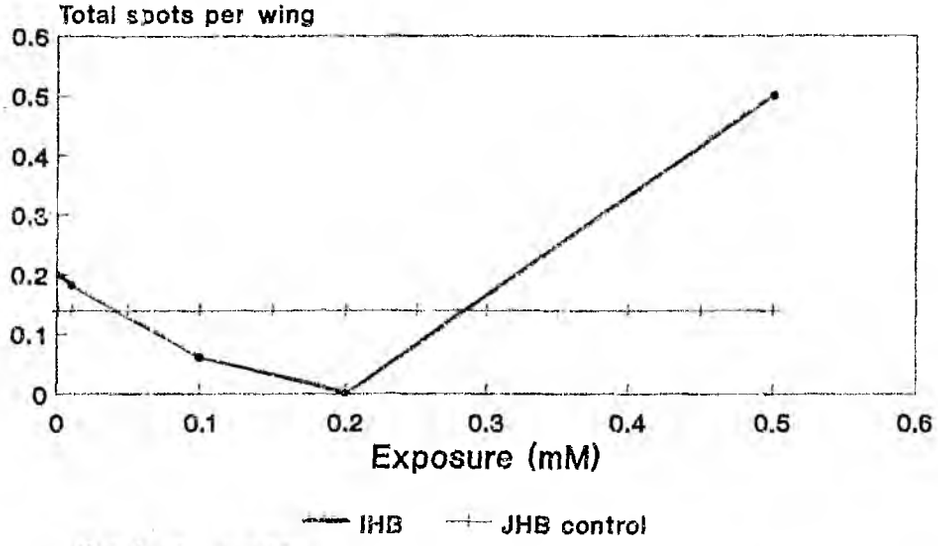
Total spots per wing after treatment of 72h old larvae with varying concentrations of DIAC. IHB cross wings.

Figure 9.3.8 a
OCHA exposure-effect curve



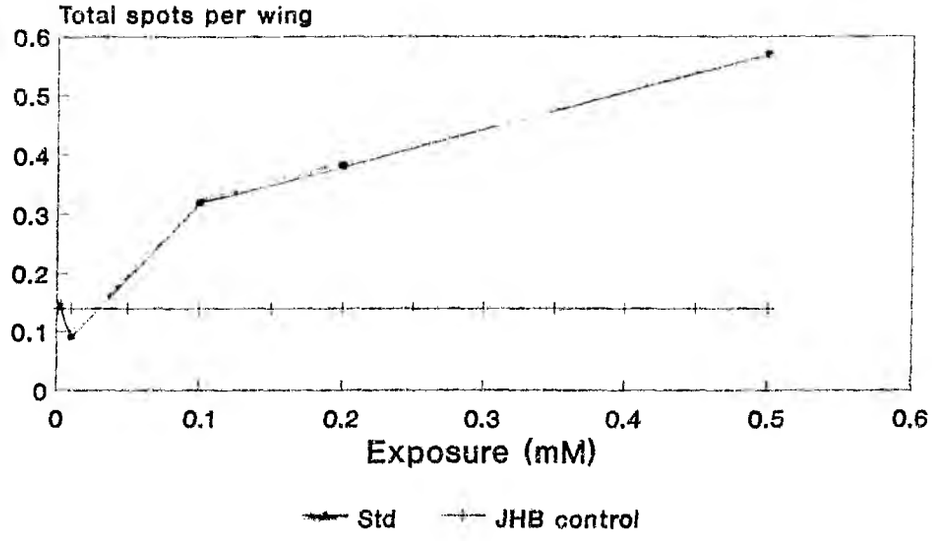
Total spots per wing after treatment of 72h old larvae with varying concentrations of OCHA. Standard cross wings (Std)

Figure 9.3.8 b
OCHA exposure-effect curve



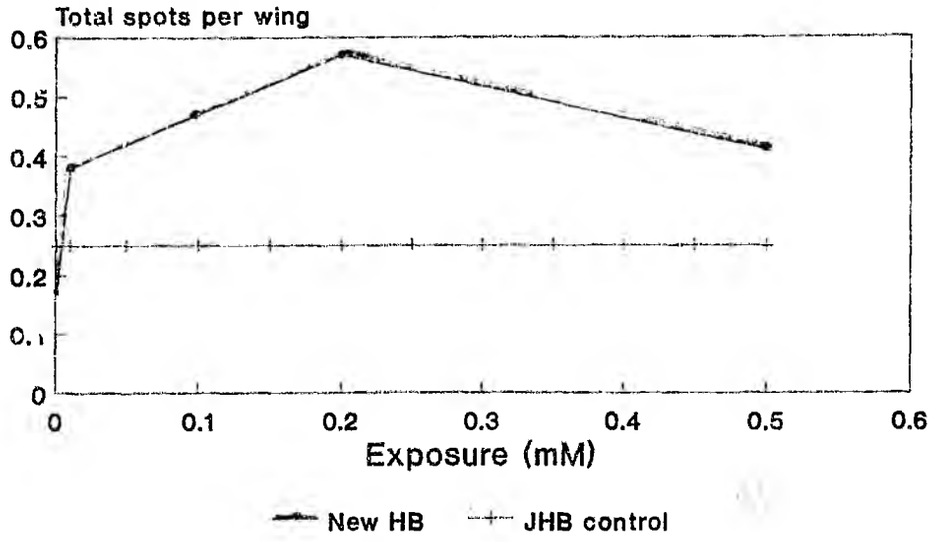
Total spots per wing after treatment of 72h old larvae with varying concentrations of OCHA. IHB cross wings.

Figure 9.3.9 a
STM exposure-effect curve



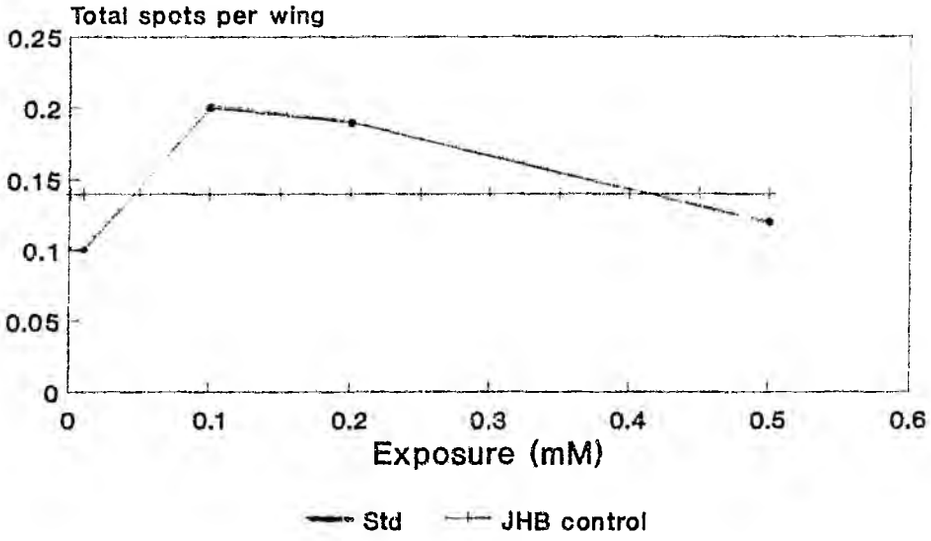
Total spots per wing after treatment of 72h old larvae with varying concentrations of STM. Standard cross wings (Std)

Figure 9.3.9 b
STM exposure-effect curve



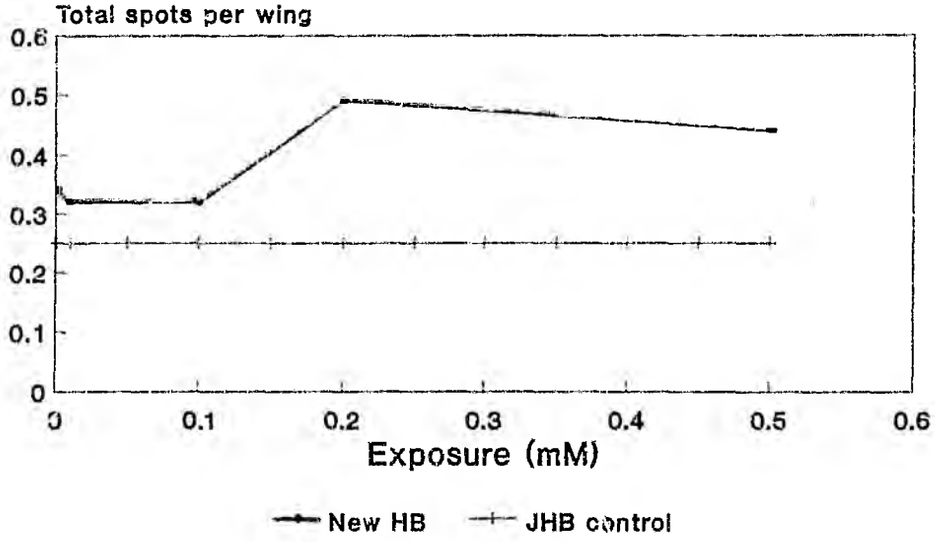
Total spots per wing after treatment of 72h old larvae with varying concentrations of STM. New HB cross wings

Figure 9.3.10 a
ZEA exposure-effect curve

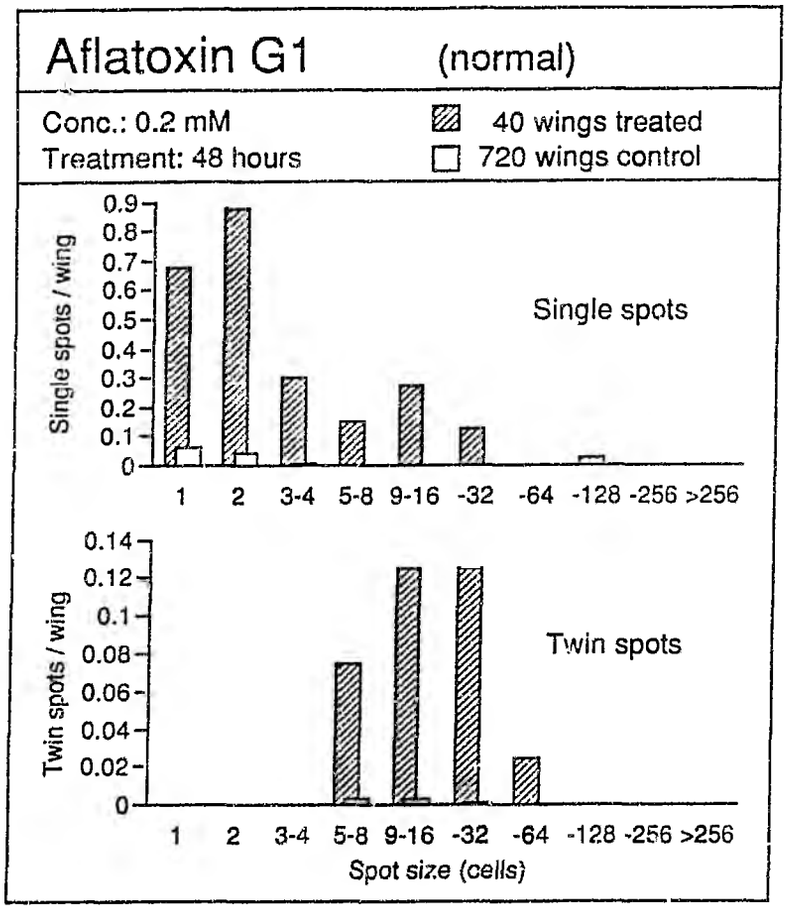


Total spots per wing after treatment of 72h old larvae with varying concentrations of ZEA. Standard cross wings (Std)

Figure 9.3.10 b
ZEA exposure-effect curve



Total spots per wing after treatment of 72h old larvae with varying concentrations of ZEA. New HB cross wings



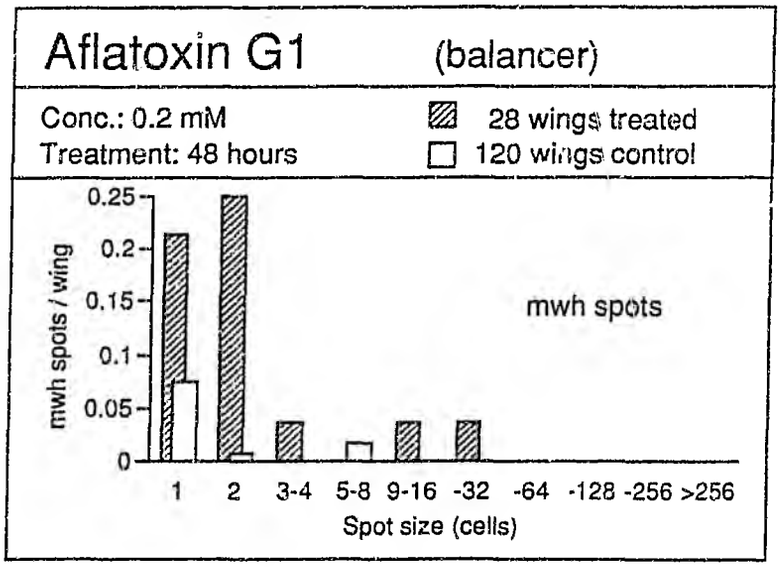
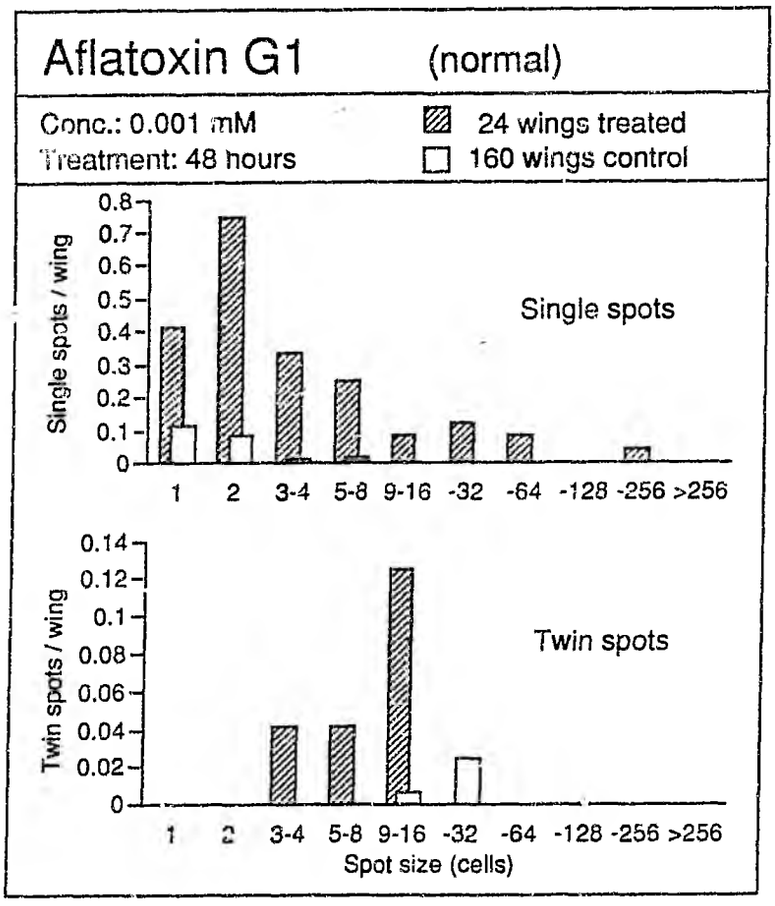


Figure 9.3.11 a **Spot size distributions for single and twin spots in the marker transheterozygous (normal) wings and for *mwh* spots in the balancer heterozygous (balancer) wings of the (JHB) standard cross obtained with 0,2 mM AFG1.**



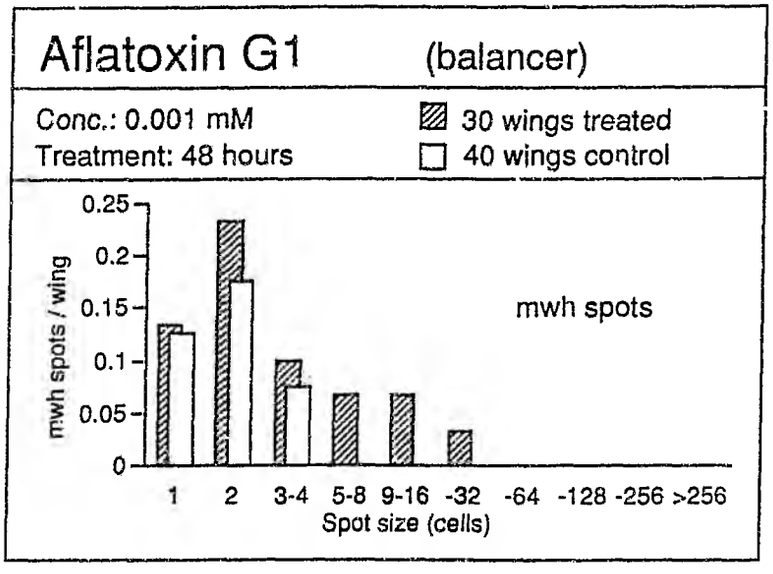
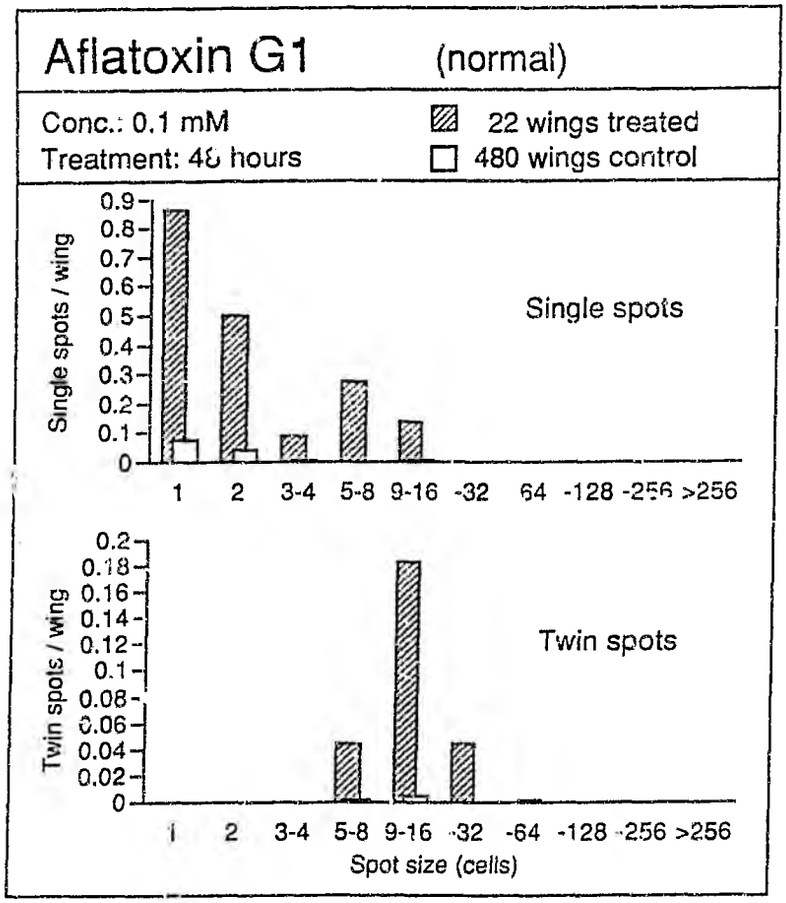


Figure 9.3.11 b **Spot size distributions for single and twin spots in the marker transheterozygous (normal) wings and for *mwh* spots in the balancer heterozygous (balancer) wings of the (Sch.) HB cross obtained with 0,001 mM AFG1.**



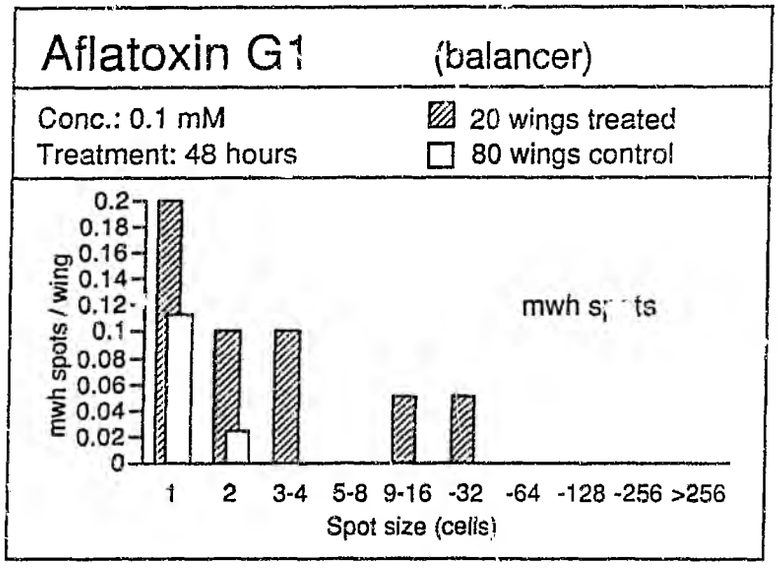
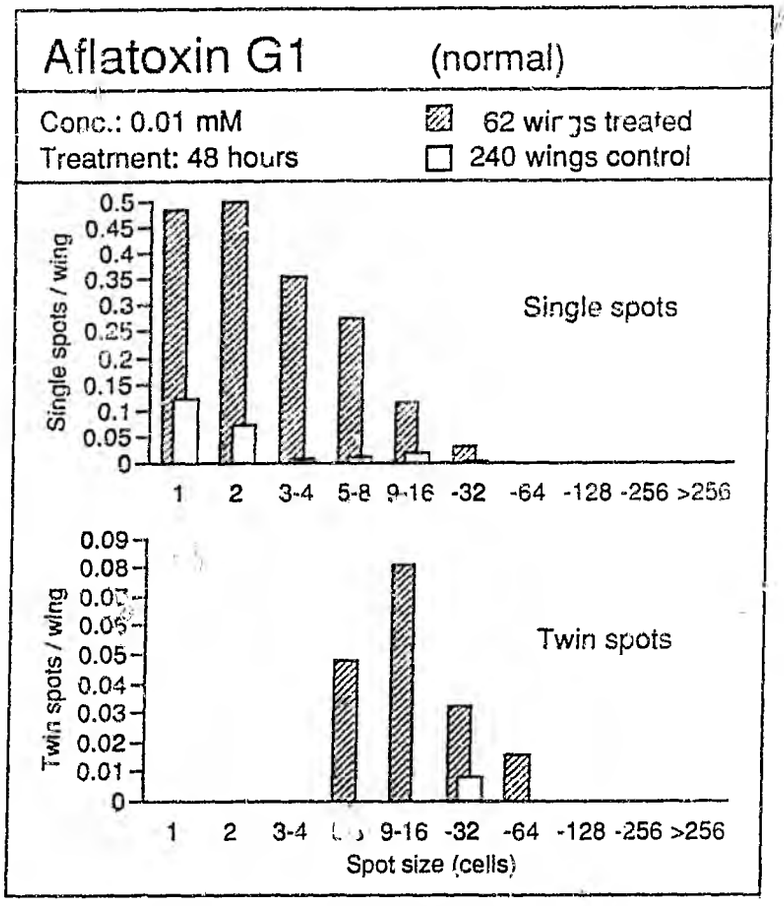


Figure 9.3.11 c **Spot size distributions for single and twin spots in the marker transheterozygous (normal) wings and for *mwh* spots in the balancer heterozygous (balancer) wings of the (JHB) improved HB cross obtained with 0,1 mM AFG1.**



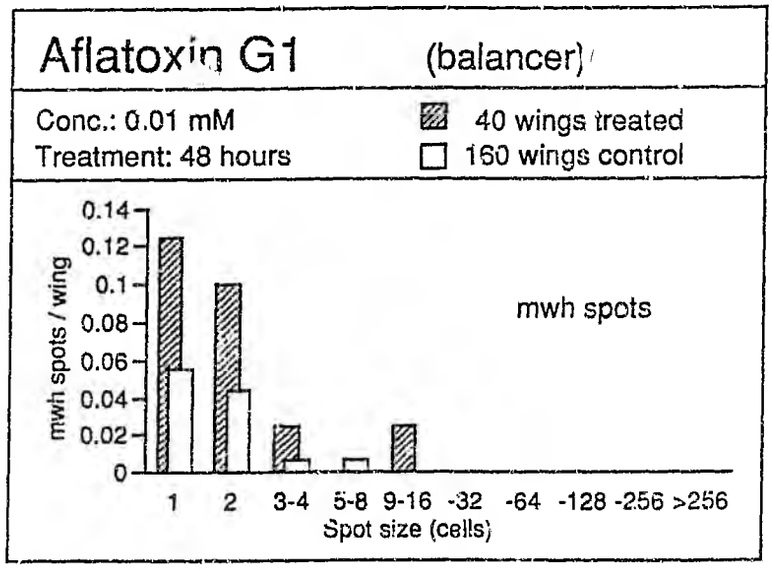
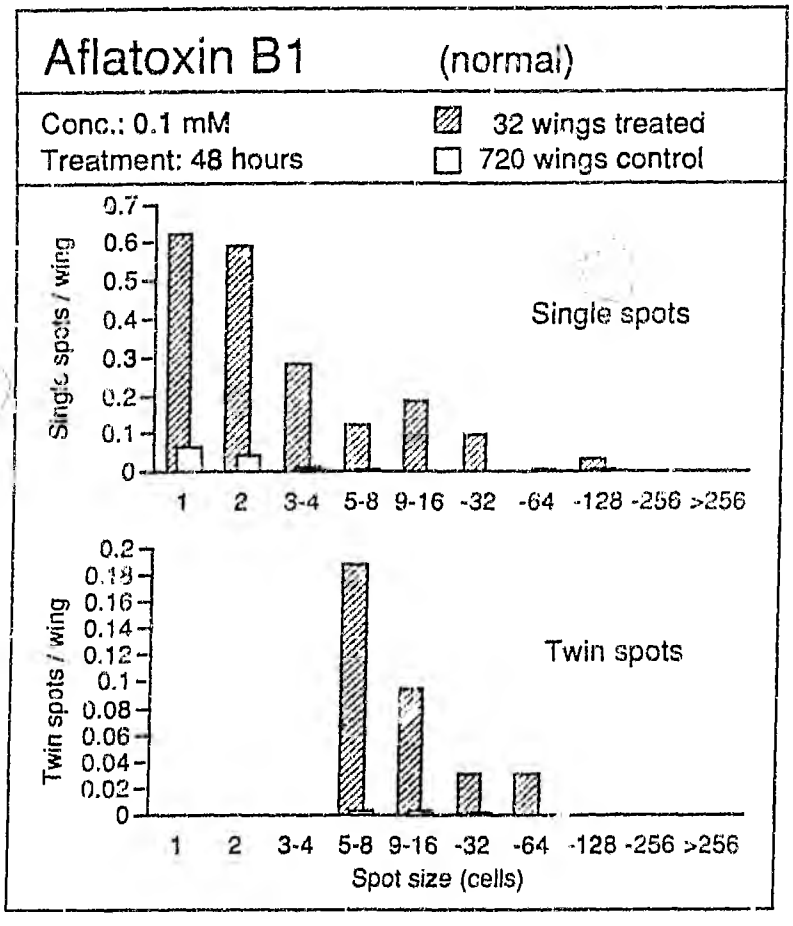


Figure 9.3.11 d **Spot size distributions for single and twin spots in the marker transheterozygous (normal) wings and for *mwh* spots in the balancer heterozygous (balancer) wings of the (5HB) new HB cross obtained with 0,01 mM AFG1.**



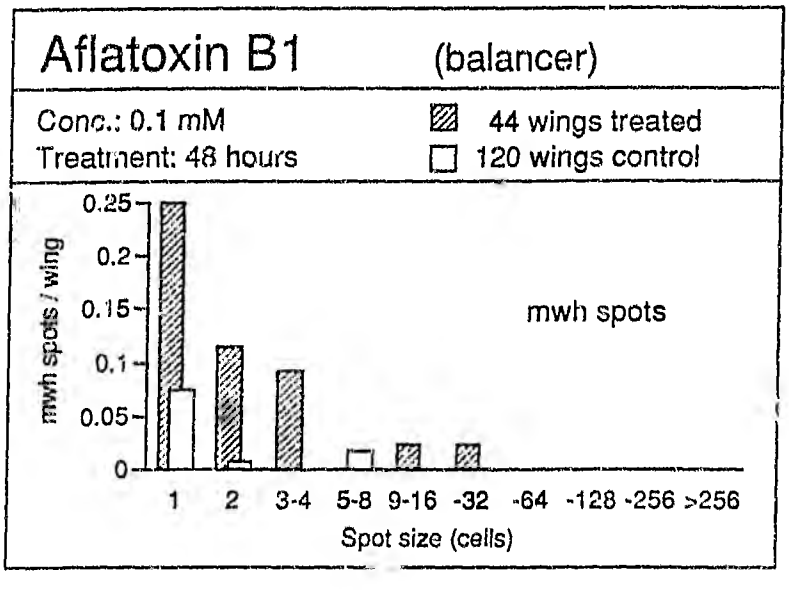
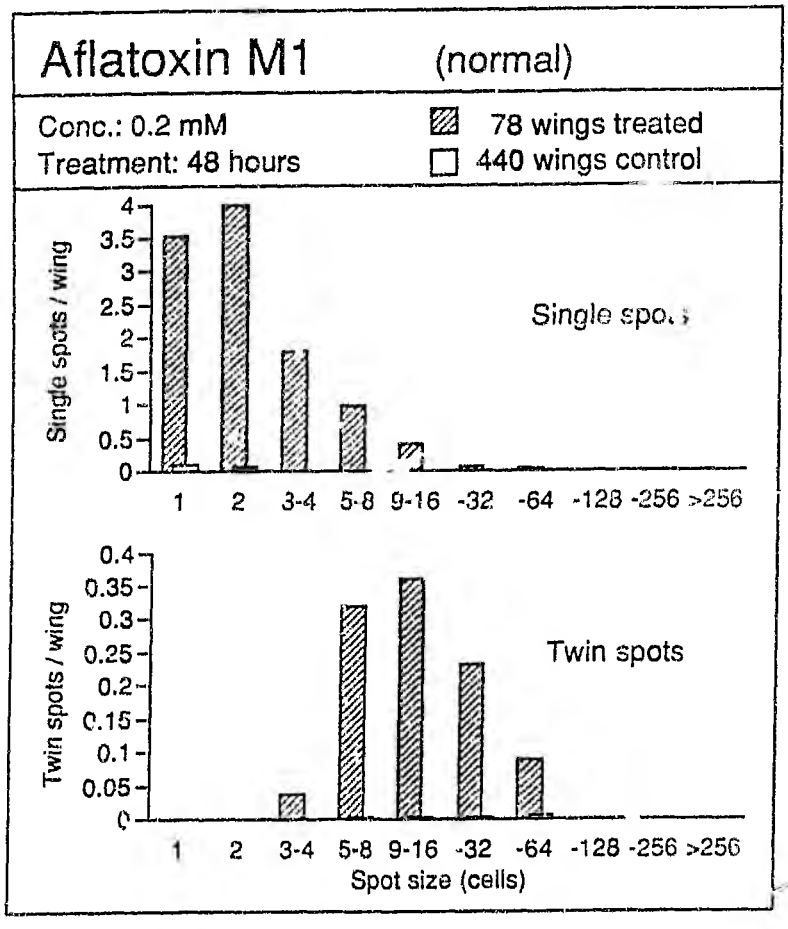


Figure 9.3.12 **Spot size distributions for single and twin spots in the marker transheterozygous (normal) wings and for *mwh* spots in the balancer heterozygous (balancer) wings of the (JHB) standard cross obtained with 0,1 mM AFB1.**



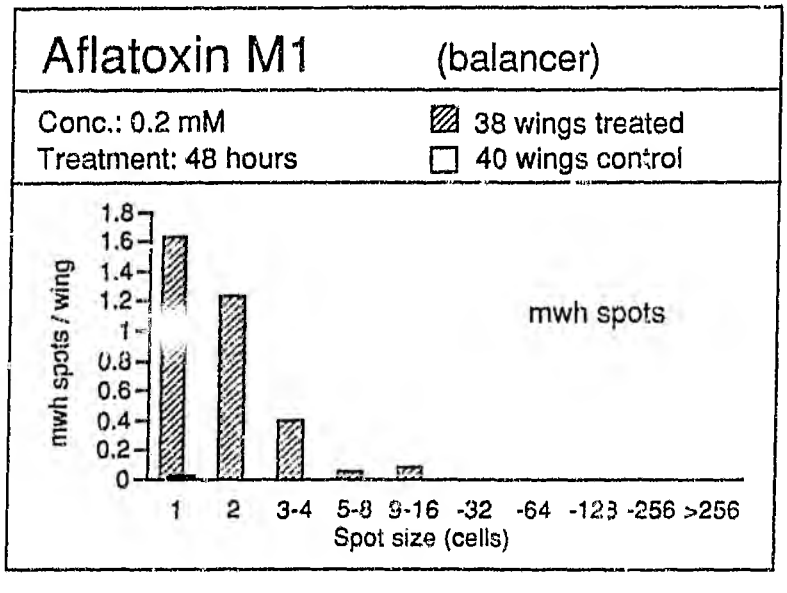


Figure 9.3.13 **Spot size distributions for single and twin spots in the marker transheterozygous (normal) wings and for *mwh* spots in the balancer heterozygous (balancer) wings of the (Sch.) standard cross obtained with 0,2 mM AFM1.**

TABLE 9.3.2 SUMMARY OF THE RESULTS OBTAINED IN THE DROSOPHILA WING SPOT TEST WITH DIFFERENT MYCOTOXINS USING THE SCHWERZENBACH STANDARD CROSS

MARKER TRANSHETEROZYGOUS WINGS

Compound / Conc. (mM)	Number of wings	Spots per Wing (Number of Spots) Stat. Diagn. • Small single spots (1–2 cells) m = 2.00	Large single spots (>2 cells) m = 5.00	Twin spots m = 5.00	Total spots m = 2.00	Spots with mwh clone	Mean clone size class	Frequency of clone formation per 10(5) cells observed	Frequency of clone formation per 10(5) cells control corrected
Control (water)									
0	240	0.10 (23)	0.03 (6)	0.01 (2)	0.13 (31)	30	0.00	0.0	
Control (5% Tween-80 + 5% ethanol)									
0	440	0.15 (68)	0.04 (16)	0.01 (5)	0.20 (89)	89	2.13	0.8	
Aflatoxin G1									
0.001	40	0.35 (14)+	0.05 (2)–	0.05 (2)I	0.45 (18)+	18	1.94	1.8	1.0
0.01	40	0.38 (15)+	0.15 (6)+	0.03 (1)I	0.55 (22)+	21	2.38	2.2	1.3
0.05	40	0.70 (28)+	0.28 (11)+	0.10 (4)+	1.08 (43)+	43	2.33	4.4	3.6
0.1	40	1.08 (43)+	0.82 (33)+	0.15 (6)+	2.05 (82)+	81	2.59	8.3	7.5
0.2	40	2.15 (86)+	1.27 (51)+	0.35 (14)+	3.78 (151)+	144	2.35	14.8	13.9
0.5	40	1.10 (44)+	0.68 (27)+	0.10 (4)+	1.88 (75)+	72	2.58	7.4	6.6
Aflatoxin M1									
0.001	52	0.13 (7)–	0.00 (0)–	0.02 (1)I	0.15 (8)–	8	1.75	0.6	–0.2
0.01	80	0.40 (32)+	0.26 (21)+	0.10 (8)+	0.78 (61)+	60	2.67	3.1	2.2
0.05	32	2.56 (82)+	1.72 (55)+	0.50 (16)+	4.78 (153)+	148	2.72	19.0	18.1
0.1	40	6.13 (245)+	3.15 (126)+	1.15 (46)+	10.43 (417)+	404	2.42	41.4	40.6
0.2	78	7.54 (588)+	3.32 (259)+	1.04 (81)+	11.90 (928)+	879	2.27	46.2	45.4
Cytochalasin B									
0.001	80	0.10 (8)–	0.05 (4)–	0.01 (1)I	0.16 (13)–	13	2.92	0.7	–0.2
0.01	80	0.19 (15)–	0.04 (3)–	0.00 (0)–	0.22 (18)–	18	1.94	0.9	0.1
0.1	80	0.08 (6)–	0.04 (3)–	0.05 (4)+	0.16 (13)–	13	2.92	0.7	–0.2
0.2	80	0.09 (7)–	0.04 (3)–	0.01 (1)I	0.14 (11)–	11	3.36	0.6	–0.3
0.5	80	0.17 (14)–	0.00 (0)–	0.01 (1)I	0.19 (15)–	15	1.53	0.8	–0.1
1	40	0.17 (7)I	0.00 (0)–	0.03 (1)I	0.20 (8)–	8	1.75	0.8	0.0
Ochratoxin A									
0.0001	40	0.03 (1)–	0.03 (1)–	0.00 (0)I	0.05 (2)–	2	3.00	0.2	–0.6
0.001	80	0.08 (6)–	0.00 (0)–	0.01 (1)I	0.09 (7)–	7	2.00	0.4	–0.5
0.01	80	0.09 (7)–	0.01 (1)–	0.01 (1)I	0.11 (9)–	9	2.33	0.5	–0.4
0.1	80	0.06 (5)–	0.00 (0)–	0.00 (0)–	0.06 (5)–	5	1.40	0.3	–0.6
0.2	120	0.17 (20)–	0.04 (5)–	0.02 (2)I	0.22 (27)–	27	2.07	0.9	0.1
0.5	78	0.18 (14)–	0.04 (3)–	0.00 (0)–	0.22 (17)–	17	2.12	0.9	0.1
Ochratoxin A (4 h acute feeding)									
0.01	80	0.06 (5)–	0.03 (2)–	0.04 (3)I	0.13 (10)–	10	3.20	0.5	–0.3
0.1	80	0.03 (2)–	0.01 (1)–	0.05 (4)+	0.09 (7)–	7	4.43	0.4	–0.5
0.2	80	0.10 (8)–	0.03 (2)–	0.05 (4)+	0.17 (14)–	13	2.85	0.7	–0.2
0.5	80	0.10 (8)–	0.00 (0)–	0.01 (1)I	0.11 (9)–	9	1.44	0.5	–0.4
Verrucarin A									
0.001	80	0.13 (10)–	0.04 (3)–	0.03 (2)I	0.19 (15)–	14	2.29	0.7	–0.1
0.01	80	0.08 (6)–	0.05 (4)–	0.00 (0)–	0.13 (10)–	9	2.22	0.5	–0.4
0.1	120	0.09 (11)–	0.03 (4)–	0.01 (1)I	0.13 (16)–	16	2.50	0.5	–0.3
0.2	80	0.17 (14)–	0.03 (2)–	0.03 (2)I	0.22 (18)–	18	2.11	0.9	0.1
0.5	160	0.19 (30)–	0.01 (2)–	0.01 (2)I	0.21 (34)–	33	1.87	0.8	0.0

TABLE 9.3.2 CONT.

BALANCER HETEROZYGOUS WINGS

Compound	Conc. (mM)	Number of wings	Spots per Wing (Number of Spots) Stat. Diagn. * Small single spots (1-2 cells) m = 2.00	Large single spots (>2 cells) m = 5.00	Twin spots m = 5.00	Total spots m = 2.00	Spots with mwh clone	Mean clone size class	Frequency of clone formation per 10(5) cells observed	Frequency of clone formation per 10(5) cells control corrected
Control	0	40	(5% Tween-80 + 5% ethanol) 0.03 (1)	0.00 (0)	0.00 (0)	0.03 (1)	1	1.00	0.1	
Aflatoxin M1	0.2	38	2.87 (109)+	0.53 (20)+	0.00 (0)i	3.39 (129)+	129	1.74	13.9	13.8

* Statistical Diagnoses according to Frei and Würgler (1988) for comparisons with corresponding control: (+)= positive; (-)= negative; (i)= inconclusive. m= multiplication factor. Kastenbaum-Bowman (1970) tests one-sided. Probability levels: α= ß= 0.05. Frequency of clone formation: *mwh* clones/wings/24 400 cells (without size correction).

TABLE 9.3.3 SUMMARY OF THE RESULTS OBTAINED IN THE DROSOPHILA WING SPOT TEST WITH DIFFERENT MYCOTOXINS USING THE SCHWERZENBACH ORIGINAL HIGH BIOACTIVATION CROSS

MARKER TRANSHETEROZYGOUS WINGS

Compound Conc. (mM)	Number of wings	Spots per Wing (Number of Spots) Stat. Diagn. * Small single spots (1–2 cells) m = 2.00	Large single spots (>2 cells) m = 5.00	Twin spots m = 5.00	Total spots m = 2.00	Spots with mwh clone	Mean clone size class	Frequency of clone formation per 10(5) cells observed	Frequency of clone formation per 10(5) cells control corrected
Control (water)									
0	120	0.13 (15)	0.04 (5)	0.00 (0)	0.17 (20)	20	0.00	0.0	
Control (5% Tween-80 + 5% ethanol)									
0	160	0.21 (33)	0.03 (5)	0.03 (5)	0.27 (43)	43	2.07	1.1	
Aflatoxin G1									
0.001	24	1.17 (28)+	0.92 (22)+	0.21 (5)+	2.29 (55)+	50	2.84	8.5	7.4
Aflatoxin M1									
0.01	30	0.40 (12)+	0.40 (12)+	0.17 (5)+	0.97 (29)+	28	3.18	3.8	2.7
Cytochalasin B									
0.001	40	0.10 (4)–	0.00 (0)–	0.00 (0)–	0.10 (4)–	4	2.00	0.4	–0.7
0.01	40	0.22 (9)i	0.05 (2)i	0.00 (0)–	0.28 (11)–	9	1.67	0.9	–0.2
0.1	40	0.03 (1)–	0.00 (0)–	0.00 (0)–	0.03 (1)–	1	2.00	0.1	–1.0
0.5	40	0.13 (5)–	0.08 (3)i	0.00 (0)–	0.20 (8)–	8	2.63	0.8	–0.3
Verrucarin A									
0.001	40	0.10 (4)–	0.03 (1)i	0.03 (1)i	0.15 (6)–	6	2.17	0.6	–0.5
0.1	40	0.10 (4)–	0.05 (2)i	0.03 (1)i	0.17 (7)–	7	3.86	0.7	–0.4

BALANCER HETEROZYGOUS WINGS

Control (water)									
0	40	0.10 (4)	0.05 (2)	0.00 (0)	0.15 (6)	6	0.00	0.0	
Control (5% Tween-80 + 5% ethanol)									
0	40	0.30 (12)	0.08 (3)	0.00 (0)	0.38 (15)	15	1.87	1.5	
Aflatoxin G1									
0.001	30	0.37 (11)i	0.27 (8)+	0.00 (0)i	0.63 (19)i	19	2.68	2.6	1.1

* Statistical Diagnoses according to Frei & Würgler (1988) for comparisons with corresponding control: (+) = positive; (–) = negative; (i) = inconclusive. m = multiplication factor. Kastenbaum-Bowman (1970) tests one-sided. Probability levels: α = ß = 0.05. Frequency of clone formation: *mwh* clones/wings/24 400 cells (without size correction).

TABLE 9.3.4 SUMMARY OF THE RESULTS OBTAINED IN THE DROSOPHILA WING SPOT TEST WITH DIFFERENT MYCOTOXINS USING THE JOHANNESBURG STANDARD CROSS

MARKER TRANSHETEROZYGOUS WINGS

Compound	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)	Large single spots (>2 cells)	Twin spots	Total spots				
Conc. (mM)		m = 2.00	m = 5.00	m = 5.00	m = 2.00			observed	control corrected
DMSO SOLVENT									
Control (2% DMSO)									
0	40	0.03 (1)	0.00 (0)	0.03 (1)	0.05 (2)	2	2.50	0.2	
Aflatoxin B1									
0.5	6	0.25 (2)!	0.00 (0)!	0.00 (0)!	0.25 (2)!	2	2.00	1.6	0.8
5% ETHANOL + 5% TWEEN 80 SOLVENT									
Control (water)									
0	640	0.11 (73)	0.03 (21)	0.00 (3)	0.15 (97)	97	0.00	0.0	
Control (5% Tween-80 + 5% ethanol)									
0	720	0.11 (77)	0.03 (20)	0.01 (5)	0.14 (102)	102	2.25	0.6	
Aflatoxin B1									
0.001	80	0.14 (11)!	0.04 (3)-	0.00 (0)!	0.17 (14)!	14	2.21	0.7	0.1
0.01	80	0.17 (14)!	0.03 (2)-	0.01 (1)!	0.21 (17)!	17	1.59	0.9	0.3
0.1	32	1.22 (39)+	0.72 (23)+	0.34 (11)+	2.28 (73)+	71	2.65	2.1	8.5
0.5	10	1.90 (19)+	0.80 (8)+	0.00 (0)!	2.70 (27)+	27	2.19	11.1	10.5
Aflatoxin B2									
0.001	40	0.25 (10)+	0.00 (0)-	0.00 (0)!	0.25 (10)!	10	1.50	1.0	0.4
0.01	40	0.08 (3)-	0.00 (0)-	0.00 (0)!	0.08 (3)-	3	1.00	0.3	-0.3
0.1	40	0.20 (8)!	0.00 (0)-	0.00 (0)!	0.20 (8)!	8	1.25	0.8	0.2
0.2	40	0.20 (8)!	0.03 (1)-	0.03 (1)!	0.25 (10)!	10	2.40	1.0	0.4
0.5	110	0.16 (18)!	0.12 (13)+	0.05 (5)+	0.33 (36)+	36	2.97	1.3	0.8
Aflatoxin G1									
0.001	80	0.14 (11)!	0.04 (3)-	0.00 (0)!	0.17 (14)!	14	1.50	0.7	0.1
0.01	80	0.31 (25)+	0.13 (10)+	0.08 (6)+	0.51 (41)+	41	2.76	2.1	1.5
0.1	40	0.98 (39)+	0.47 (19)+	0.13 (5)+	1.58 (63)+	60	2.28	6.2	5.6
0.2	40	1.55 (62)+	0.88 (35)+	0.35 (14)+	2.78 (111)+	105	2.69	10.8	10.2
Aflatoxin G2									
0.001	80	0.13 (10)!	0.01 (1)-	0.00 (0)!	0.14 (11)-	11	1.45	0.6	0.0
0.01	116	0.16 (18)!	0.01 (1)-	0.02 (2)!	0.18 (21)-	21	2.05	0.7	0.2
0.1	102	0.10 (10)-	0.05 (5)-	0.03 (3)!	0.18 (18)-	18	2.39	0.7	0.1
0.2	94	0.16 (15)!	0.05 (5)-	0.02 (2)!	0.23 (22)+	21	2.19	0.9	0.3
0.5	160	0.11 (17)-	0.08 (13)+	0.04 (6)+	0.22 (36)+	36	3.53	0.9	0.3
Cytochalasin H									
0.001	80	0.11 (9)-	0.01 (1)-	0.04 (3)+	0.16 (13)-	13	2.62	0.7	0.1
0.01	80	0.10 (8)-	0.00 (0)-	0.00 (0)!	0.10 (8)-	8	1.25	0.4	-0.2
0.1	80	0.06 (5)-	0.00 (0)-	0.00 (0)!	0.06 (5)-	5	1.40	0.3	-0.3
0.2	80	0.08 (6)-	0.00 (0)-	0.00 (0)!	0.08 (6)-	6	1.00	0.3	-0.3
0.5	92	0.11 (10)-	0.02 (2)-	0.01 (1)!	0.14 (13)-	13	1.92	0.6	0.0

TABLE 9.3.4 CONT.

MARKER TRANSHETEROZYGOUS WINGS

Compound / Conc. (mM)	Number of wings	Spots per Wing (Number of Spots) Stat. Diagn. * Small single spots (1–2 cells) m = 2.00		Large single spots (>2 cells) m = 5.00		Twin spots m = 5.00		Total spots m = 2.00		Spots with mwh clone	Mean clone size class	Frequency of clone formation per 10(5) cells: observed	Frequency of clone formation per 10(5) cells: control corrected
Cytochalasin J													
0.001	34	0.06	2)–	0.06	2)I	0.00	1)I	0.15	5)I	5	3.00	0.6	0.0
0.01	24	0.17	4)I	0.00	0)–	0.00	0)I	0.17	4)I	4	1.25	0.7	0.1
0.1	24	0.17	4)I	0.00	0)–	0.04	1)I	0.21	5)I	5	2.00	0.9	0.3
0.2	46	0.13	5)I	0.00	0)–	0.03	1)I	0.15	6)I	6	1.17	0.6	0.0
0.5	80	0.13	10)I	0.01	1)–	0.03	2)I	0.16	13)–	12	2.00	0.6	0.0
Deoxynivalenol													
0.001	80	0.08	6)–	0.03	2)–	0.01	1)I	0.11	9)–	9	3.44	0.5	-0.1
0.01	58	0.10	6)–	0.02	1)–	0.00	0)I	0.12	7)–	7	2.00	0.5	-0.1
0.1	80	0.06	5)–	0.00	0)–	0.03	2)I	0.09	7)–	7	2.29	0.4	-0.2
0.2	78	0.08	6)–	0.03	2)–	0.00	0)I	0.10	8)–	7	1.71	0.4	-0.2
0.5	108	0.06	7)–	0.00	0)–	0.02	2)I	0.08	9)–	9	2.00	0.3	-0.3
Diacetoxyscirpenol													
0.001	120	0.08	10)–	0.03	3)–	0.00	0)–	0.11	13)–	13	2.54	0.4	-0.1
0.01	66	0.02	1)–	0.00	0)–	0.02	1)I	0.03	2)–	2	4.00	0.1	-0.5
0.1	72	0.03	2)–	0.04	3)–	0.01	1)I	0.08	6)–	6	4.50	0.3	-0.2
0.2	74	0.14	10)I	0.05	4)I	0.01	1)I	0.20	15)I	15	2.33	0.8	0.3
0.5	54	0.06	3)–	0.06	3)I	0.00	0)I	0.11	6)–	6	3.50	0.5	-0.1
Fumonisin B1													
0.001	40	0.13	5)I	0.00	0)–	0.00	0)I	0.13	5)–	5	1.40	0.5	-0.1
0.01	24	0.17	4)I	0.08	2)I	0.04	1)I	0.29	7)I	7	2.57	1.2	0.6
0.1	16	0.13	2)I	0.00	0)I	0.00	0)I	0.13	2)I	2	2.00	0.5	-0.1
0.2	18	0.11	2)I	0.06	1)I	0.00	0)I	0.17	3)I	3	2.33	0.7	0.1
0.5	36	0.11	4)I	0.00	0)–	0.00	0)I	0.11	4)–	4	1.25	0.5	-0.1
Fumonisin B2													
0.001	32	0.22	7)I	0.00	0)–	0.00	0)I	0.22	7)I	7	1.57	0.9	0.3
0.01	32	0.19	6)I	0.06	2)I	0.00	0)I	0.25	8)I	8	2.38	1.0	0.4
0.1	30	0.13	4)I	0.00	0)–	0.00	0)I	0.13	4)I	4	1.50	0.5	0.0
0.2	46	0.07	3)–	0.02	1)–	0.02	1)I	0.11	5)–	5	2.20	0.4	-0.1
0.5	46	0.13	6)I	0.02	1)–	0.04	2)I	0.20	9)I	9	2.44	0.8	0.2
Roridin A													
0.001	60	0.10	6)–	0.02	1)–	0.03	2)I	0.15	9)–	9	2.56	0.6	0.0
0.01	60	0.01	1)–	0.01	1)–	0.00	0)I	0.03	2)–	2	2.50	0.1	-0.5
0.1	16	0.00	0)–	0.00	0)I	0.00	0)I	0.00	0)–	0	0.00	0.0	-0.6
0.5	12	0.08	1)	0.00	0)I	0.00	0)I	0.08	1)I	1	1.00	0.3	-0.2
Sterigmatocystin													
0.001	80	0.14	11)I	0.01	1)–	0.00	0)I	0.15	12)–	12	1.50	0.6	0.0
0.01	80	0.05	4)–	0.03	2)–	0.01	1)I	0.09	7)–	7	2.43	0.4	-0.2
0.1	40	0.17	7)I	0.08	3)I	0.08	3)+	0.32	13)+	13	2.54	1.3	0.8
0.2	80	0.24	19)+	0.13	10)+	0.01	1)I	0.38	30)+	30	2.47	1.5	1.0
0.5	76	0.20	15)+	0.22	17)+	0.14	11)+	0.57	43)+	41	3.17	2.2	1.6
Zearalenone													
0.001	40	0.05	2)–	0.03	1)–	0.03	1)I	0.10	4)–	4	3.00	0.4	-0.2
0.01	40	0.10	4)I	0.00	0)–	0.00	0)I	0.10	4)–	4	1.25	0.3	-0.2
0.1	40	0.17	7)I	0.00	0)–	0.03	1)I	0.20	8)I	8	1.88	0.8	0.2
0.2	32	0.16	5)I	0.03	1)I	0.00	0)I	0.19	6)I	6	2.00	0.8	0.2
0.5	60	0.08	5)–	0.02	1)–	0.02	1)I	0.12	7)–	7	2.80	0.5	-0.1

TABLE 9.3.4 CONT.

BALANCER HETEROZYGOUS WINGS

Compound	Conc. (mM)	Number of wings	Spots per Wing (Number of Spots) Stat. Diagn. * Small single spots (1-2 cells) m = 2.00	Large single spots (>2 cells) m = 5.00	Twin spots m = 5.00	Total spots m = 2.00	Spots with mwh clone	Mean clone size class	Frequency of clone formation per 10(5) cells observed	Frequency of clone formation per 10(5) cells control corrected
Control (water)	0	40	0.10 (4)	0.00 (0)	0.00 (0)	0.10 (4)	4	0.00	0.0	
Control (5% Tween-80 + 5% ethanol)	0	120	0.08 (10)	0.02 (2)	0.00 (0)	0.10 (12)	12	1.58	0.4	
Aflatoxin B1	0.1	44	0.36 (16)+	0.14 (6)+	0.00 (0)i	0.50 (22)+	22	2.00	2.1	1.6
	0.5	4	1.50 (6)+	0.25 (1)i	0.00 (0)i	1.75 (7)+	7	1.43	7.2	6.8
Aflatoxin B2	0.2	40	0.10 (4)i	0.03 (1)i	0.00 (0)i	0.13 (5)i	5	2.20	0.5	0.1
Aflatoxin G1	0.2	26	0.46 (13)+	0.11 (3)+	0.00 (0)i	0.57 (16)+	16	2.13	2.3	1.9
Aflatoxin G2	0.5	79	0.30 (3)i	0.00 (0)i	0.00 (0)i	0.30 (3)i	3	1.00	1.2	0.8
Sterigmatocystin	0.2	40	0.10 (4)i	0.00 (0)i	0.00 (0)i	0.10 (4)i	4	1.75	0.4	0.0

* Statistical Diagnoses according to Frei and Würgler (1988) for comparisons with corresponding control: (+) = positive; (-) = negative; (i) = inconclusive. m = multiplication factor. Kastenbaum-Bowman (1970) tests one-sided. Probability levels: α = ß = 0.05. Frequency of clone formation: *mwh* clones/wings/24 400 cells (without size correction).

TABLE 9.3.5 SUMMARY OF THE RESULTS OBTAINED IN THE DROSOPHILA WING SPOT TEST WITH DIFFERENT MYCOTOXINS USING THE JOHANNESBURG IMPROVED HIGH BIOACTIVATION CROSS

MARKER TRANSHETEROZYGOUS WINGS

Compound / Conc. (mM)	Number of wings	Spots per Wing (Number of Spots) Stat. Diagn. * Small single spots (1–2 cells) m = 2.00	Large single spots (>2 cells) m = 5.00	Twin spots m = 5.46	Total spots m = 2.00	Spots with mwh clone	Mean clone size class	Frequency of clone formation per 10(5) cells observed	Frequency of clone formation per 10(5) cells control corrected
Control (water)									
0	480	0.14 (67)	0.01 (7)	0.00 (2)	0.16 (76)	76	0.00	0.9	
Control (5% Tween-80 + 5% ethanol)									
0	480	0.11 (55)	0.02 (9)	0.01 (4)	0.14 (68)	67	1.91	0.8	
Aflatoxin B1									
0.001	52	0.21 (11)i	0.04 (2)i	0.02 (1)i	0.27 (14)+	14	2.36	1.1	0.5
0.01	10	0.60 (6)+	0.00 (0)i	0.00 (0)i	0.60 (6)+	6	1.33	2.5	1.9
0.1	18	1.11 (20)+	0.39 (7)+	0.56 (10)+	2.06 (37)+	36	3.11	8.2	7.6
0.2	6	1.33 (8)+	0.17 (1)i	0.33 (2)+	1.83 (11)+	10	2.20	6.8	6.3
0.5	2	2.00 (4)+	1.00 (2)+	1.00 (2)+	4.00 (8)+	8	2.63	16.4	15.8
Aflatoxin B2									
0.001	40	0.05 (2)-	0.08 (3)i	0.00 (0)i	0.13 (5)-	5	4.20	0.5	-0.1
0.01	40	0.15 (6)i	0.05 (2)i	0.00 (0)i	0.20 (8)i	8	2.13	0.8	0.2
0.1	40	0.25 (10)+	0.10 (4)+	0.03 (1)i	0.38 (15)+	15	2.27	1.5	1.0
0.2	40	0.38 (15)+	0.15 (6)+	0.08 (3)+	0.60 (24)+	24	3.04	2.5	1.9
0.5	40	0.47 (19)+	0.28 (11)+	0.20 (8)+	0.95 (38)+	38	2.76	3.9	3.3
Aflatoxin G1									
0.001	34	0.41 (14)+	0.03 (1)i	0.00 (0)i	0.44 (15)+	15	1.67	1.8	1.2
0.01	38	0.68 (26)+	0.24 (9)+	0.13 (5)+	1.05 (40)+	37	2.41	4.0	3.4
0.1	22	1.36 (30)+	0.50 (11)+	0.27 (6)+	2.14 (47)+	45	2.27	8.4	7.8
0.2	10	2.40 (24)+	0.70 (7)+	0.50 (5)+	3.60 (36)+	35	2.14	14.4	13.8
Aflatoxin G2									
0.001	40	0.13 (5)i	0.05 (2)i	0.00 (0)i	0.17 (7)i	7	2.43	0.7	0.1
0.01	40	0.08 (3)-	0.00 (0)-	0.03 (1)i	0.10 (4)-	4	2.00	0.4	-0.2
0.1	40	0.22 (9)i	0.00 (0)-	0.03 (1)i	0.25 (10)i	10	1.60	1.0	0.5
0.2	38	0.47 (18)+	0.03 (1)i	0.08 (3)+	0.58 (22)+	22	2.00	2.4	1.8
0.5	40	0.22 (9)i	0.30 (12)+	0.05 (2)i	0.57 (23)+	23	3.39	2.4	1.8
Cytochalasin H									
0.001	40	0.13 (5)i	0.00 (0)-	0.03 (1)i	0.15 (6)i	6	2.00	0.6	0.0
0.01	60	0.10 (6)-	0.05 (3)i	0.00 (0)i	0.15 (9)-	9	3.00	0.6	0.0
0.1	34	0.03 (1)-	0.00 (0)i	0.03 (1)i	0.06 (2)-	2	3.00	0.2	-0.3
0.2	40	0.00 (0)-	0.03 (1)i	0.00 (0)i	0.03 (1)-	1	5.00	0.1	-0.5
0.5	25	0.04 (1)-	0.00 (0)i	0.00 (0)i	0.04 (1)-	1	1.00	0.2	-0.4
Cytochalasin J									
0.001	40	0.05 (2)-	0.03 (1)i	0.00 (0)i	0.08 (3)-	3	3.00	0.3	-0.3
0.01	32	0.06 (2)-	0.03 (1)i	0.03 (1)i	0.13 (4)i	3	2.33	0.4	-0.2
0.1	40	0.17 (7)i	0.00 (0)-	0.00 (0)i	0.17 (7)i	7	1.00	0.7	0.1
0.2	40	0.15 (6)i	0.03 (1)i	0.00 (0)i	0.17 (7)i	7	1.57	0.7	0.1
0.5	36	0.08 (3)-	0.05 (2)i	0.00 (0)i	0.14 (5)i	5	2.40	0.6	0.0
Deoxynivalenol									
0.01	10	0.20 (2)i	0.00 (0)i	0.00 (0)i	0.20 (2)i	2	2.00	0.8	0.2
0.2	80	0.08 (6)-	0.03 (2)i	0.04 (3)i	0.14 (11)-	11	3.09	0.6	0.0
0.5	8	0.25 (2)i	0.00 (0)i	0.00 (0)i	0.25 (2)i	2	1.00	1.0	0.5
Diacetoxyscirpenol									
0.01	20	0.10 (2)i	0.00 (0)i	0.00 (0)i	0.10 (2)i	2	1.50	0.4	-0.2
0.1	30	0.07 (2)-	0.00 (0)i	0.00 (0)i	0.07 (2)-	2	2.00	0.3	-0.3
0.2	22	0.09 (2)i	0.00 (0)i	0.00 (0)i	0.09 (2)i	2	1.50	0.4	-0.2
0.5	16	0.00 (0)-	0.00 (0)i	0.00 (0)i	0.00 (0)-	0	0.00	0.0	-0.6

TABLE 9.3.5 CONT.

Compound / Conc. (mM)	Number of wings	Spots per Wing (Number of Spots) Stat. Diagn. * Small single spots (1-2 cells) m = 2.00	Large single spots (>2 cells) m = 5.00	Twin spots m = 5.00	Total spots m = 2.00	Spots with mwh clone	Mean clone size class	Frequency of clone formation per 10(5) cells observed	Frequency of clone formation per 10(5) cells control corrected
Fumonisin B1									
0.001	40	0.15 (6)I	0.03 (1)I	0.05 (2)I	0.22 (9)I	9	2.33	0.9	0.4
0.01	26	0.15 (4)I	0.08 (2)I	0.04 (1)I	0.27 (7)I	7	2.57	1.1	0.5
0.1	20	0.20 (4)I	0.00 (0)I	0.00 (0)I	0.20 (4)I	4	2.00	0.8	0.2
0.2	10	0.30 (3)I	0.00 (0)I	0.00 (0)I	0.30 (3)I	3	1.33	1.2	0.7
0.5	12	0.17 (2)I	0.00 (0)I	0.00 (0)I	0.17 (2)I	2	1.00	0.7	0.1
Fumonisin B2									
0.001	70	0.14 (10)I	0.03 (2)I	0.01 (1)I	0.19 (13)I	13	2.23	0.8	0.2
0.01	80	0.09 (7)-	0.01 (1)-	0.03 (2)I	0.13 (10)-	9	1.78	0.5	-0.1
0.1	66	0.12 (8)I	0.00 (0)-	0.00 (0)I	0.12 (8)-	8	1.13	0.5	-0.1
0.2	40	0.03 (1)-	0.00 (0)-	0.03 (1)I	0.05 (2)-	2	2.00	0.2	-0.4
0.5	56	0.11 (6)-	0.00 (0)-	0.00 (0)I	0.11 (6)-	6	1.67	0.4	-0.1
Ochratoxin A									
0.001	80	0.15 (12)I	0.04 (3)I	0.01 (1)I	0.20 (16)I	16	2.13	0.8	0.2
0.01	72	0.13 (9)I	0.03 (2)I	0.03 (2)I	0.18 (13)I	13	2.00	0.7	0.2
0.1	16	0.06 (1)I	0.00 (0)I	0.00 (0)I	0.06 (1)I	1	1.00	0.3	-0.3
0.2	2	0.00 (0)I	0.00 (0)I	0.00 (0)I	0.00 (0)I	0	0.00	0.0	-0.6
0.5	2	0.00 (0)I	0.50 (1)+	0.00 (0)I	0.50 (1)I	1	4.00	2.1	1.5
Roridin A									
0.01	42	0.02 (1)-	0.00 (0)-	0.00 (0)I	0.02 (1)-	1	1.00	0.1	-0.5
0.1	22	0.09 (2)I	0.00 (0)I	0.00 (0)I	0.09 (2)I	2	1.00	0.4	-0.2
0.2	22	0.09 (2)I	0.09 (2)I	0.00 (0)I	0.18 (4)I	4	2.75	0.7	0.2
0.5	22	0.05 (1)-	0.00 (0)I	0.00 (0)I	0.05 (1)-	1	1.00	0.2	-0.4

BALANCER HETEROZYGOUS WINGS

Control (Water)									
0	80	0.08 (6)	0.00 (0)	0.00 (0)	0.08 (6)	6	0.00	0.0	
Control (5% Tween-80 + 5% ethanol)									
0	80	0.14 (11)	0.00 (0)	0.00 (0)	0.14 (11)	11	1.18	0.8	
Aflatoxin B1									
0.01	16	0.31 (5)I	0.00 (0)I	0.00 (0)I	0.31 (5)I	5	1.40	1.3	0.7
0.1	8	0.38 (3)I	0.13 (1)I	0.00 (0)I	0.50 (4)+	4	2.25	2.1	1.5
0.2	2	0.50 (1)I	0.00 (0)I	0.00 (0)I	0.50 (1)I	1	1.00	2.1	1.5
Aflatoxin G1									
0.1	20	0.30 (6)I	0.20 (4)+	0.00 (0)I	0.50 (10)+	10	2.50	2.1	1.5

* Statistical Diagnoses according to Frei and Würgler (1988) for comparisons with corresponding control: (+)= positive; (-)= negative; (I)= inconclusive. m= multiplication factor. Kastenbaum-Bowman (1970) tests one-sided. Probability levels: α= ß= 0.05. Frequency of clone formation: *mwh* clones/wings/24 400 cells (without size correction).

TABLE 9.3.6 SUMMARY OF THE RESULTS OBTAINED IN THE DROSOPHILA WING SPOT TEST WITH DIFFERENT MYCOTOXINS USING THE JOHANNESBURG NEW HIGH BIOACTIVATION CROSS

MARKER TRANSHETEROZYGOUS WINGS

Compound / Conc. (mM)	Number of wings	Spots per Wing (Number of Spots) Stat. Diagn. * Small single spots (1-2 cells) m = 2.00	Large single spots (>2 cells) m = 5.00	Twin spots m = 5.00	Total spots m = 2.00	Spots with mwh clone	Mean clone size clone	Frequency of clone formation per 10(5) cells observed	Frequency of clone formation per 10(5) cells control corrected
Control (wa)									
0	160	0.16 (26)	0.05 (8)	0.01 (2)	0.22 (36)	35	0.00	0.0	
Control (5% Tween-80 + 5% ethanol)									
0	240	0.19 (46)	0.05 (11)	0.01 (2)	0.25 (59)	58	2.00	1.0	
Aflatoxin G1									
0.01	62	0.98 (61)+	0.77 (48)+	0.18 (11)+	1.94 (120)+	108	2.47	7.1	6.2
Aflatoxin G2									
0.001	52	0.38 (20)+	0.02 (1)-	0.00 (0)i	0.40 (21)+	21	1.62	1.7	0.7
0.01	80	0.22 (18)-	0.04 (3)-	0.04 (3)i	0.30 (24)-	23	1.87	1.2	0.2
0.1	80	0.69 (55)+	0.20 (16)+	0.06 (5)+	0.95 (76)+	74	2.01	3.8	2.8
0.2	56	0.54 (30)+	0.20 (11)+	0.07 (4)+	0.80 (45)+	44	2.32	3.2	2.2
0.5	56	0.73 (41)+	0.32 (18)+	0.07 (4)+	1.13 (63)+	57	2.12	4.2	3.2
Cytochalasin B									
0.1	80	0.19 (15)-	0.00 (0)-	0.01 (1)i	0.20 (16)-	16	1.63	0.8	-0.2
Ochratoxin A									
0.1	80	0.17 (14)-	0.05 (4)-	0.00 (0)i	0.22 (18)-	18	1.78	0.9	-0.1
Sterigmatocystin									
0.001	70	0.14 (10)-	0.01 (1)-	0.01 (1)i	0.17 (12)-	12	2.00	0.7	-0.3
0.01	40	0.30 (12)i	0.09 (3)i	0.00 (0)i	0.38 (15)i	14	2.07	1.4	0.4
0.1	40	0.32 (13)i	0.10 (4)i	0.00 (0)i	0.47 (19)+	18	2.11	1.3	0.9
0.2	40	0.47 (19)+	0.10 (4)i	0.00 (0)i	0.57 (23)+	23	1.87	2.4	1.4
0.5	22	0.41 (9)+	0.00 (0)-	0.00 (0)i	0.41 (9)i	9	1.22	1.7	0.7
Zearalenone									
0.001	80	0.22 (18)-	0.09 (7)-	0.03 (2)i	0.34 (27)i	26	2.46	1.3	0.3
0.01	80	0.26 (21)i	0.05 (4)-	0.01 (1)i	0.32 (26)-	26	1.92	1.3	0.3
0.1	80	0.28 (22)i	0.04 (3)-	0.01 (1)i	0.32 (26)-	26	1.85	1.3	0.3
0.2	80	0.36 (29)+	0.10 (8)i	0.03 (2)i	0.49 (39)+	38	2.05	1.9	1.0
0.5	32	0.34 (11)i	0.09 (3)i	0.00 (0)i	0.44 (14)+	14	2.21	1.8	0.8

TABLE 9.3.6 CONT.

BALANCER HETEROZYGOUS WINGS

Compound	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)	Large single spots (>2 cells)	Twin spots	Total spots				
Conc. (mM)		m = 2.00	m = 5.00	m = 5.00	m = 2.00			observed	control corrected
Control (5% Tween-80 + 5% ethanol)									
0	160	0.10 (16)	0.01 (2)	0.00 (0)	0.11 (18)	18	1.67	0.5	
Aflatoxin G1									
0.01	40	0.22 (9)+	0.05 (2)i	0.00 (0)i	0.28 (11)+	11	1.91	1.1	0.7
Aflatoxin G2									
0.2	40	0.35 (14)+	0.05 (2)i	0.00 (0)i	0.40 (16)+	16	1.88	1.6	1.2
0.5	40	0.30 (12)+	0.08 (3)i	0.00 (0)i	0.38 (15)+	15	1.67	1.5	1.1
Sterigmatocystin									
0.2	40	0.20 (8)i	0.03 (1)i	0.00 (0)i	0.22 (9)i	9	1.67	0.9	0.5
Zearalenone									
0.2	40	0.05 (2)-	0.00 (0)i	0.00 (0)i	0.05 (2)-	2	1.50	0.2	-0.3

* Statistical Diagnoses according to Frei and Würgler (1988) for comparisons with corresponding controls: (+)= positive; (-)= negative; (i)= inconclusive. m= multiplication factor. Kastenbaum-Bowman (1970) tests one-sided. Probability levels: α= ß= 0.05. Frequency of clone formation: *mwh* clones/wings/24 400 cells (without size correction).

TABLE 9.3.7 COMPARISON OF TWO DIFFERENT SOLVENTS USED IN SMART STANDARD CROSS ASSAYS

MYCOTOXIN EXPOSURE	TREATMENT DURATION	TOTAL SPOTS PER WING	
		2% DMSO	5% TWEEN 80 + 5% ETOH
AFB1 (0,5 mM)	48 h	0,25	2,70
AFB1 (0,5 mM)	48 h	0,20[c]	10,50[c]
AFG1 (0,2 mM)	4 h	0,68*	
AFG1 (0,2 mM)	4 h	1,70[c]*	
AFG1 (0,2 mM)	48 h		2,78
AFG1 (0,2 mM)	48 h		10,20[c]
STM (0,5 mM)	4 h	0,39*	
STM (0,5 mM)	4 h	0,30[c]*	
STM (0,5 mM)	48 h		0,57
STM (0,5 mM)	48 h		1,60[c]
SOLVENT CONTROL	4 h	0,33*	0,13
SOLVENT CONTROL	48 h	0,05	0,14

* Data from Honours Dissertation (1989)
c Control corrected frequencies
ETOH = ethanol

9.4 DISCUSSION

9.4.1 Control Frequencies

In these experiments, variation in control frequencies was observed among the four different *Drosophila* crosses used, between the two laboratories where SMART was performed and among the different solvents tested. This variation in control rates in the Johannesburg and Schwerzenbach laboratories observed with the same solvent in the standard cross may have been due to differences in the laboratory methods (instant media used, buffering capacity of the media, pH of the food, temperature of incubation) or to differences in the genetic backround of the *flr*^{3} and *mwh* stocks used (Frei *et al.*, 1992). Clements and colleagues (1988) and Frei and colleagues (1992) also reported variations in spontaneous spot frequency with different SMART stocks used. It seems that the Johannesburg IHB and standard SMART crosses have a slightly lower control rate than the SMART stocks used by other researchers in Schwerzenbach (Graf *et al.*, 1984; Alonso Moraga and Graf, 1989; van Schaik and Graf, 1991; Graf and van Schaik, 1992). It was interesting to note that the newly contructed HB stocks (with whorl-free wings), have a higher control rate than the Johannesburg standard and IHB crosses that is similar although still slightly lower than, the usual control rate. Areas with whorling of the wing hairs were usually not included when scoring HB cross (Schwerzenbach) wings and the HB control rate was similar to the standard Schwerzenbach control rate observed in these experiments. The HB (Schwerzenbach) control rate for serrate wings, however, was very high and this may have been due to small sample size or to the whorling disadvantage.

Different researchers score differently and may apply different scoring criteria with HB wings due to the difficulties encountered in those areas of the *ORR* homozygous wings showing wing hair pattern disturbances, and this explains the usual inconsistency in spontaneous spot frequency reported for the HB cross.

9.4.2 Toxic Effects of the Mycotoxins and Shape of Exposure-effect Curves

Toxicity was probably unrelated to genotoxicity as many non-genotoxic compounds, such as verrucarin A, were still highly toxic to larvae. Corrosive compounds such as chromium (IV) oxide, which was used as a positive control in some SMART assays may "burn" the skin of larvae so that the larvae die without having eaten the compound. Some toxic compounds affect the nervous system at a critical concentration, thereby killing the organism. Larvae had to be exposed to a critical (lethal) concentration of the mycotoxin to be killed. Apart from killing the larvae mycotoxins also retarded development and delayed hatching effects were observed in these experiments.

Toxicity tests with dimethyl nitrosamine (DMN) showed that the toxic effect of DMN was higher in the insecticide resistant Hikone R strain than in sensitive strains (Vogel, 1980), suggesting that toxicity effects of this promutagen in the HB crosses would also be nigher than in the standard cross and this was observed by Frölich and Würgler (1989). The higher toxicity of the aflatoxins (promutagens) was also observed in these experiments with the

original HB, improved HB and new HB crosses being more sensitive to the toxic effects of the treatment.

It was observed in Figures 9.3.1-9.3.10, that exposure-effect curves for genotoxic mycotoxins show a levelling off at the highest exposure. This decrease in the number of spots per wing at the highest exposure level was more pronounced with the high bioactivation crosses than with the standard cross. There are two possible reasons for this shape of exposure-effect curves. The shape of the curve may be influenced by toxic effects of genotoxic mycotoxins on larvae. At the higher concentrations, there are complications with the extremely reduced survival. At a concentration of 0,5 mM, flies were usually very small. *Drosophila* is a cold blooded animal and hence larvae can pupate and hatch into very small flies without having eaten much of the medium. The few surviving individuals are often those that were not treated with the average dose per individual but rather avoided the genotoxic compound and therefore survived (with fewer spots than expected .

Vogel and Leigh (1975) also found the levelling off of exposure-effect curves at the highest exposure in the case of spermatids. Frölich and Würgler (1989) reported similar findings with diethyl nitrosamine in the SMART assay and they suggested the second possible reason. The shape of the curve was interpreted to indicate a partial saturation of the metabolic capacity at high exposure doses. Although this saturation occurred in both the standard and HB crosses, the plateau appeared at a higher level of genotoxic effects than in the standard cross. This in agreement with the assumption that the P450-

dependent metabolism is more effective in the chromosome substitution larvae than in the standard larvae (Frölich and Würgler, 1989). With the new HB cross, exposure-effect curves were not always linear (see Figures 9.3.5 c; 9.3.9 b and 9.3.10 b). This may have been due to the enhanced toxicity effects of the mycotoxins in the *RI* homozygous progeny of the new HB cross, or to the saturation of the metabolic capacity. Since the bioactivation capacity was more efficient in this cross, genotoxicity (measured by number of spots per wing) was significantly higher at the lower concentrations in new HB cross wings than in wings from the other crosses, but at the higher concentrations, the metabolic capacity became saturated so that more or less the same number of spots were recorded per wing at 0,1-0,5mM and the increase in number of spots per wing (genotoxicity) was no longer directly proportional to the exposure level.

9.4.3 Marker Transheterozygous versus Balancer Heterozygous Wings

The observation of twin spots after X-ray and AFB1 treatment clearly indicates that mitotic recombination between *flr*^{3} and the centromere occurs in wing disc cells (Graf *et al.*, 1984). As has already been mentioned in section 9.1, recombination events may result in *mwh* and *flr*^{3} single spots. It was shown by Merriam and Garcia-Bellido (1972) that the frequency of X-ray induced *mwh* spots is reduced in larvae that are inversion heterozygous for the third chromosome. Therefore, it is expected that those mutagens that lead to spots predominantly by recombination events would do so in the absence of inversion-heterozygosity. On the other hand, mutagens acting

through mechanisms independent of recombination are expected to lead to the same *mwh* frequency in normal larvae as in inversion heterozygous larvae.

In the TM3 balancer heterozygous wings, all mitotic recombination events are eliminated due to the multiple inversions present, so that all the *mwh* single spots recovered are produced by non-crossover type mutational events exclusively. To determine the fraction of all aflatoxin-induced spots that were due to induced mitotic recombination, the frequencies of *mwh* spots observed on the marker transheterozygous wings were compared with those recorded on the TM3 balancer heterozygous, Serrate, ones, where all recombinational [illegible] are eliminated. The data obtained with the marker transheterozygous [illegible] standard cross proved the high genotoxic activity of the [illegible] importantly, however, the analysis of the balancer heterozygous wings demonstrates a drastic reduction in the frequency of induced spots. This is shown in Figures 9.3.11 a and 9.3.13 where the spot size distribution for single spots obtained with both types of wings of the standard cross after treatment with 0,2mM AFG1 and AFM1, respectively, are given. From the reduced frequency of *mwh* spots on the balancer heterozygous wings, it can be concluded that the majority of the spots induced on the normal wings must have been due to recombinational events. The fraction of the spots on the normal wings which is not due to mitotic recombination can be calculated by dividing the frequency of *mwh* spots on the balancer heterozygous wings, by that on the marker transheterozygous wings. The value for 0,2 mM AFG1 was (0,18). In other words, 82% of all the spots induced by AFM1 were due to mitotic recombination 100 - (0,18 x 100) = 82%. These

values were also calculated for 0,1 mM AFB1 (77%) and 0,2mM AFM1 (70%). Since mitotic or somatic recombination is associated with chromosome breakage, these percentages also represent the fraction of the spots on the normal wings that were due to chromosome breakage. Hence the results indicate that the aflatoxins had chromosome-breaking ability. The control frequencies were also lower on balancer heterozygous wings than on marker transheterozygous ones and the reduction was about 28% in the standard cross.

The reduction in the frequency of *mwh* spots observed on the balancer heterozygous wings as compared with the marker transheterozygous ones is of the same order of magnitude in the HB, IHB and new HB crosses as in the standard cross. The reduction is 70% (0,001 mM AFG1) in the HB cross, 75% (0,1 mM AFG1) in the IHB cross and 84% (0,01 mM AFG1) in the new HB cross; and for 0,1 mM AFB1 it is 75% in the IHB cross. This proves again the high recombinogenic activity of the aflatoxins.

The reactive products of aflatoxin activation induce various types of lesions in the chromosomes of the imaginal disc cells and can give rise to mutations or to mitotic recombinations. It is evident that the wing spot test is able to pick up mitotic recombination much more efficiently than mutation. In fact, the possibility to score for point mutations is limited to the two genes *mwh* and *flr*^{3} whereas practically the whole left arm of chromosome three is available for the scoring of mitotic recombination (Graf *et al.*, 1992a). The SMART test therefore gives clearer evidence of chromosome breakage than

the heritable translocations test. In conclusion the results show that in somatic cells of *D. melanogaster* the high genotoxic activity of aflatoxin is 70-84% due to recombinogenic activity which is influenced by cytochrome P450-dependent xenobiotic metabolism.

Similar calculations indicated that sterigmatocystin was also genotoxic and recombinogenic in somatic cells of *Drosophila* and the genotoxicity of sterigmatocystin was 74% and 61%, for the standard and new HB crosses, respectively, due to recombinogenic activity. The highest reduction in frequency of *mwh* spots on balancer heterozygous wings compared with marker transheterozygous, was found with zearalenone, where 90% of the genotoxic activity of this compound was due to recombinogenic activity.

9.4.4 The New High Bioactivation Cross

Aflatoxin G2, which was negative in the *Salmonella*/mammalian microsome assay, was detected positive for total spots at the highest (0,5 mM) concentration in the SMART standard cross, at 0,2 mM and 0,5 mM concentrations in the improved HB cross and at high and low concentrations (even as low as 0,001 mM) in the new HB cross. With sterigmatocystin (0,1 and 0,2 mM) and aflatoxin G1 (0,01 mM), the total spots per wing were higher in the new HB cross than in the improved HB cross. Furthermore, zearalenone, which was negative in the standard cross, showed positive results at the higher concentrations in the new HB cross. This increased sensitivity of the new HB cross for promutagens indicates that the larvae of the new HB cross showed P450 dependent bioactivation capacity slightly

higher than that of the improved HB cross and, since the bioactivation capacity of the improved HB cross is equal to or even slightly higher than the original HB cross (Graf and van Schaik, 1992), the new HB cross bioactivation capacity is probably also better than that of the original HB cross.

Larvae of the new HB cross developed normally and the spontaneous mutation rate observed with the new HB cross was within the usual range (Graf *et al.*, 1984; Alonso Moraga and Graf, 1989; van Schaik and Graf, 1991; Graf and van Schaik, 1992). Spot size distributions observed with the new HB cross were similar to those observed with the improved HB cross and all HB crosses used in these experiments were characterized by the presence of spots of the larger size classes probably due to the earlier induction of mutation and recombination in high bioactivation larvae. As discussed in section 9.4.3, the reduction in the frequency of *mwh* spots observed on the balancer-heterozygous wings as compared with the marker-transheterozygous ones is of the same order of magnitude in the new HB, improved HB and original HB crosses as in the standard cross. The advantage of this cross was that wings of new HB cross flies had a normal, undisturbed wing hair pattern and could be scored using the same criteria as with the standard cross, while whorling of the wing hairs was observed often on original HB cross wings and sometimes on improved HB cross wings. This, together with the high bioactivation capacity and increased sensitivity of this cross for borderline promutagens, could make this new HB cross a desirable cross to use in SMART assays.

9.4.5 Comparison of the Different Solvents

Aflatoxin B1 was tested using 2% DMSO in water and 5% Tween 80 + 5% ethanol in order to compare the efficiency of these two solvents. The combination of ethanol and Tween 80 (5% ethanol + 5% tween 80) was found to be the better solvent, not only because the mycotoxins went into solution more easily in this solvent than in DMSO, but also because the frequency of spots per wing (for all categories) was significantly reduced on 2% DMSO wings compared with 5% ethanol + 5% tween 80 wings (see Table 9.3.7). The frequency of total spots per wing was 11 fold greater (53 fold greater for control corrected frequencies) on wings of flies exposed to 0,5 mM AFB1 in 5% ethanol + 5% tween 80 than on wings of flies exposed to the same mycotoxin concentration in 2% DMSO. In Table 9.3.7, when the data from the honours dissertation was compared with the results from these experiments, the reduction in total spot frequency on 2% DMSO wings was about 5 fold (8 fold control corrected) for AFG1 and 2 fold (5 fold control corrected) for sterigmatocystin. This decrease in spot frequency may have been due to the effect of the 2% DMSO solvent on metabolic activities, or to differences between acute and chronic treatments.

Studies have shown that DMSO can have unexpected side effects. DMSO may interfere with the enzyme activities necessary for promutagen conversion (Younes *et al.*, 1979) or with enzyme induction (Magnusson *et al.*, 1979). Using ethanol as the solvent is advantageous because the flies possess the enzyme alcohol dehydrogenase in large quantities and can readily metabolize ethanol.

9.4.6 STRUCTURE-ACTIVITY RELATIONSHIPS OF THE MYCOTOXINS

9.4.6.1 AFLATOXINS AND STERIGMATOCYSTIN

The genotoxicity of the aflatoxins depends on metabolism by the microsomal mixed function, monooxygenase system. AFB1 undergoes oxidative hydroxylation at the tertiary carbon of the bisfuran ring system, and epoxidation to form AFM1 and the 2,3-oxide of AFB1, respectively (see Figure 9.4.6.1). The putative 2,3-oxide or aflatoxin-epoxide is generally accepted as the active electrophilic form of the aflatoxins (Swenson *et al.*, 1974). The activation of AFB1 and AFG1 and the formation of their epoxides is mediated by a specific microsomal and nuclear cytochrome P450-associated monooxygenase (Hsieh, 1987), whereas AFM1 is formed by a specific cytochrome P448-associated monooxygenase (Raina *et al.*, 1983). The positive results obtained with AFM1 in these experiments suggest that, if this is the case, then *Drosophila* must possess a cytochrome with similar function.

Results of experiments by Wogan and colleagues (1971) revealed several characteristics of the aflatoxin molecular configuration that are important to its biological activity. It is now well established that the chemical site implicated as responsible for the biological activities of aflatoxins and related compounds is the C2-C3 double bond in the dihydrofuran moiety of these molecules. Reduction of this bond yielding 2,3-dihydro AFB1 (or AFB2) results in a 200-500 fold decrease in mutagenicity and about 150-fold

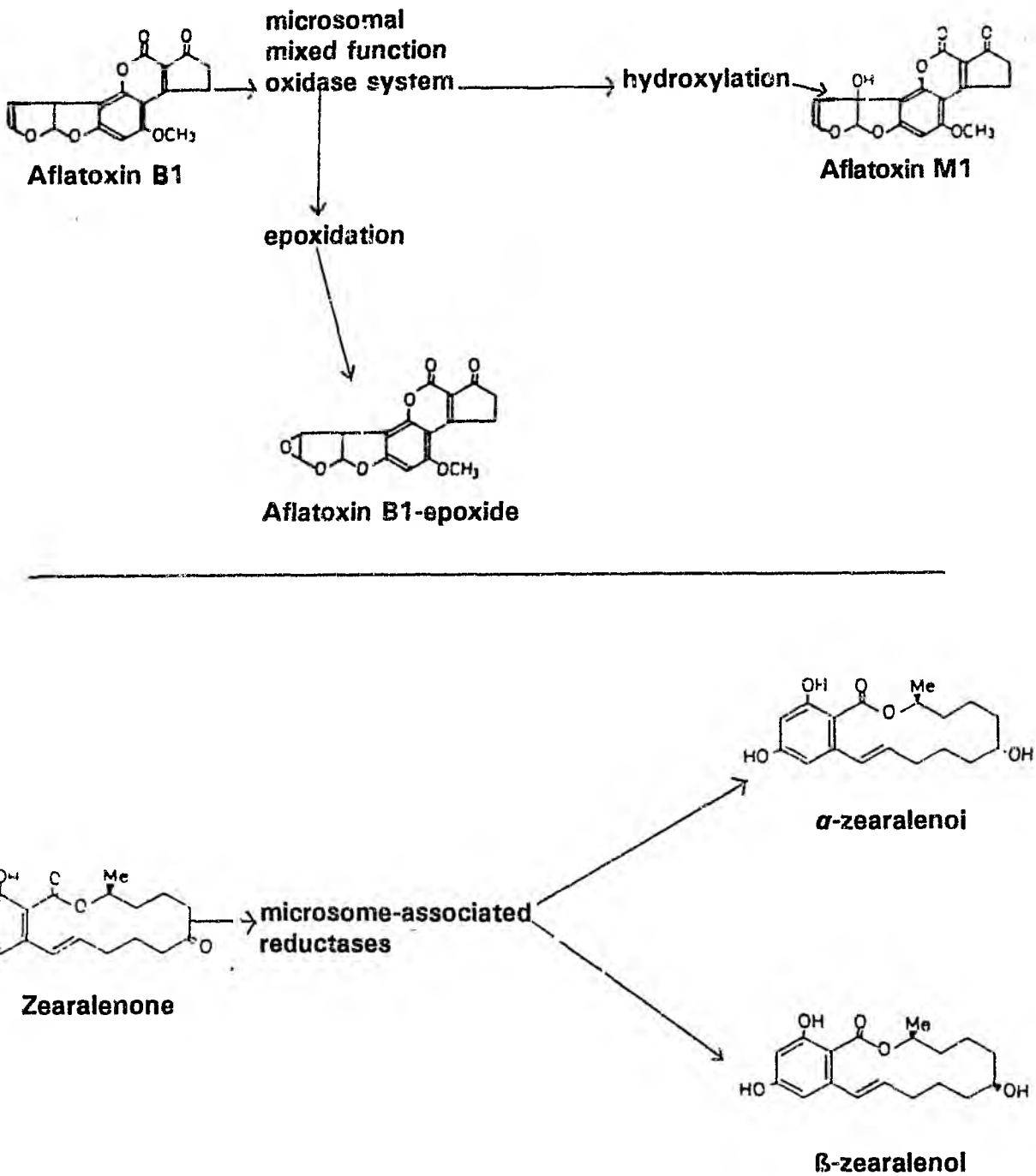


Figure 9.4.6.1 Aflatoxin B1 and zearalenone and their primary metabolites

decrease in carcinogenicity. The same is true for AFG1 and its 2,3-dihydro form, AFG2.

The OCH_3 group attached to the coumarin nucleus is a structure that would lead to a chemical being classed as a potential mutagen (Hay, 1988) and this electrophilic structure is present in all the aflatoxins. It should be noted that the 2,3 double bond in the bisfuran ring system, required for epoxide formation and which is sufficient to elicit mutagenicity is preserved in the major aflatoxins (AFB1, AFG1 and AFM1), their metabolites and even their precursor sterigmatocystin (the presence of this bond has been highlighted in the mycotoxin structures in Figure 9.4.6.1). Yet their mutagenic and carcinogenic potencies are reduced from that of AFB1. Apparently, the substituted coumarin moiety of the AFB1 molecule is the optimal structure for its toxicity and genotoxicity, and any modification at this moiety results in a reduction in potency. The *S. typhimurium* data of Wong *et al.* (1977) provide evidence for this by showing that sterigmatocystin and other intermediates in the aflatoxin biosynthetic pathway were less mutagenic than AFB1. Changes in this (AFB1) optimal structure probably alter the affinity of the toxin to the receptor molecule (DNA, RNA or protein). Structure differences in the moieties attached to the bisfuran ring (xanthone instead of coumarin in sterigmatocystin) or the addition of the second lactone ring to AFB1 (as in AFG1), alters the affinity of the 2,3-oxide (formed by the activation of these molecules) for DNA and this explains why a lower mutagenic and carcinogenic potency is observed with AFG1 and sterigmatocystin. Hence, carcinogenicity,

genotoxicity and DNA-binding capacity of AFB1 far surpass that of the other metabolites containing a C2-C3 unsaturated bond.

Aflatoxins G1 and B1 were active in every system in which they were tested and in all systems AFB1 was more potent than AFG1. AFG1 differs from AFB1 by containing one additional oxygen, giving a double lactone configuration and the addition of the second lactone ring to AFB1 reduces biological activity by a factor of two (Wogan *et al.*, 1971). The toxicity and genotoxicity data of Wogan and colleagues (1971) indicated a structure-activity series with decreasing potency in the order aflatoxin B1 > G1 > B2 > G2 which is identical to that observed in these studies (section 9.3.3). On account of the sterigmatocystin and IHB data obtained in these experiments, AFM1 > > AFB1 ≈ AFG1 > > STM > AFB2 > AFG2 gives a better overview over the actual relationships. An interesting result obtained in these experiments was the very high mutagenic activity of AFM1, with AFM1 > > AFB1 in the standard cross. In other test systems, such as the *Salmonella*/mammalian microsome assay and with respect to carcinogenicity the relationship is the other way around with AFB1 always more active than AFM1 and other aflatoxins (Wong and Hsieh, 1976). The high mutagenic potency of AFM1 in these SMART tests remains uexplained, hut it should be taken into account that since just 1 mg of AFM1 was available, the experiment was not repeated.

Most of the carcinogenic and genotoxic activities of the aflatoxins and related compounds, have been observed with metabolically activated aflatoxins able to bind in a covalent irreversible manner to macromolecules with specific

preference to DNA. The intermediate compound in the metabolic activation of aflatoxins is the 2,3-oxide derivative or aflatoxin epoxide. Structural analysis of AFB1-DNA, AFG1-DNA, AFM1-DNA and sterigmatocystin-DNA adducts revealed that the N7 atom of guanine in DNA and C2 atom of the dihydrofuran moiety of these mycotoxins were responsible for the principal adducts formed *in vivo* and *in vitro* (Essigman *et al.*, 1977; Essigman *et al.*, 1979). Oxidation at the C2-C3 site renders the C2 atom of these compounds electron deficient and able to accept electrons, thus enabling an effective electrophilic attack at the proper, sterically available N7 atom of guanine (Essigman *et al.*, 1979). The N7 atom of guanine is a prime target for many other mutagens and carcinogens due to this steric availability. Substitution at this site introduces a positive charge in the imadozole ring where the adduct is bound to DNA and renders the glycosidic bond labile to acid hydrolysis leading to depurination at this site. It has been suggested that breakage of the phosphodiester at the site of modification of guanines by aflatoxins may lead to the elimination of the modified purine thus causing deletion. Invariably, the major aflatoxin-DNA adduct found in each *in vivo* or *in vitro* system is 2,3-dihydro-2-(N7-guanyl)-3-hydroxyaflatoxin. It was found in the DNA of rat liver and kidney, and mouse liver and kidney (Croy *et al.*, 1978).

A strong positive correlation exists between carcinogenicity and the extent of covalent binding to DNA among the various aflatoxins, their metabolites and precursors. Differential covalent binding of aflatoxins has been observed in appropriate organs and species and the main reason for organ and species

specificity seems to reside in differential oxidative metabolism in the various animals.

Aflatoxins B1, M1 and G1 are positive in most short-term mutagenicity tests (see Introduction Tables 1.4 and 1.5) and these mycotoxins were detected positive in these SMART tests even in the standard cross. Aflatoxin G2, which was negative in the *Salmonella*/mammalian microsome assay (McCann *et al.*, 1975a) was positive in SMART crosses and, in the new HB cross, the frequency of AFG2 induced spots was significantly increased over the control frequency even at the low concentrations. Sterigmatocystin and all five aflatoxins tested were genotoxic and recombinogenic in somatic cells of *Drosophila* and hence *Drosophila* possesses the specific cytochrome P450 enzymes to catalyse the epoxidation and activation of these compounds.

9.4.6.2 OCHRATOXIN A

The ochratoxins constitute a group of closely related derivatives of isocoumarin linked to phenylalanine and are classified according to biosynthetic origin as pentaketides within the group of polyketides (Turner, 1971). In *In vitro* experiments with pig, human and rat liver microsomes, both (4S) and (4R)-4-hydroxy-ochratoxin A were produced in a hydroxylation process involving cytochrome P450 (Stormer and Pedersen, 1980; Stormer *et al.*, 1981). Since 4-hydroxy-ochratoxin A is non-toxic to rats (Hutchinson and Steyn, 1971), it was concluded that the microsomal hydroxylation most likely represents a detoxification reaction.

Ochratoxin A is an inhibitor of tRNA synthetase and protein synthesis in several microorganims as well as in rat hepatoma cells (Konrad and Röschenthaler, 1977). The kidney is the target organ for the toxic effects of ochratoxin, but changes in the liver have also been noted. Balkan endemic nephropathy is a kidney disease observed in rural populations in Bulgaria, Romania and Yugoslavia. A high incidence of urinary tract tumours has been found to be correlated with the incidence of Balkan nephropathy. There are also striking similarities between Balkan endemic nephropathy and ochratoxin A-induced porcine nephropathy, suggesting common causal relationships (Krogh, 1987). A correlation between exposure to ochratoxin A and a high prevalence of human nephropathy was found by Krogh and colleagues in 1977.

Kanizawa and Suzuki (1978) published a study which indicated that ochratoxin A is a hepatic and renal carcinogen in rodents. These findings were confirmed in subsequent sudies by Kanizawa (1984), Ueno (1984), Bendele and colleagues (1985) and Boorman (1988). Although ochratoxin A is also teratogenic (Hayes *et al.*, 1974), it was not mutagenic in the *rec* assay measuring DNA damage (Ueno and Kubota, 1976), nor the *Salmonella*/-mammalian microsome assay (Kuczuc *et al.*, 1978; Würgler *et al.*, 1991). In addition, ochratoxin A did not induce mutations in C3H mouse mammary carcinoma cells (Umeda *et al.*, 1977).

Ochratoxin A was also negative in the *Drosophila* wing spot test in all three crosses: standard (both acute and chronic treatments), improved high

bioactivation and new high bioactivation crosses. This suggests that ochratoxin A is probably non-genotoxic. If this compound is a vertebrate genotoxic carcinogen then failure of *Drosophila* to detect this compound may be due to differences between vertebrate and insect metabolism. If, however, ochratoxin A is a non-genotoxic carcinogen, then it may be causing cancer in other ways.

9.4.6.3 ZEARALENONE

Zearalenone, an oestrogen, exhibits its physiological activity by first binding with cytosolic receptor protein and then by translocation of the oestrogen receptor complex into the nuclei, in a similar fashion to the action of steroid hormones. It possesses a high affinity for cytoplasmic oestrogen receptors (Tashiro *et al.*, 1980). In rats, zearalenone is carried into the brain and binds with oestrogen receptors of the hypothalamus and hypophysis (Hsieh, 1987). Zearalenone is a phenolic resorcyclic acid lactone. It is classified as a nonaketide within the group of polyketides. Zearalenone can be stereospecifically reduced by different forms of reductases to α and ß-zearalenol (Figure 9.4.6.1). The α-zearalenol is ten times more oestrogenic than the parent compound and appears to be the active form of zearalenone (Hsieh, 1987). The zearalenone reductases are mostly microsome-associated (Hsieh, 1987).

Zearalenone has been found to be carcinogenic (Ames *et al.*, 1990), it has been associated with stillbirths in animals (Hsieh, 1987) and it has produced a dose-dependent increase in the incidence of skeletal anomalies in rat embryos (Hsieh, 1987). The few reported studies on the mutagenicity of this

compound have been negative or if positive, the data have been unconvincing (Wehner *et al.*, 1978; Hsieh, 1987; Kuczuc *et al.*, 1978). In these experiments, zearalenone was negative in the standard cross but positive at the higher concentrations in the new HB cross. This indicated that zearalenone was probably genotoxic and recombinogenic in somatic cells of *Drosophila* but more evidence is required before we can conclude genotoxicity in mammals as the new HB cross has only been validated with a small number of chemicals.

9.4.6.4 TRICHOTHECENES

The trichothecene complex of antibiotics is a group of chemically related compounds that possess the tetracyclic 12,13-epoxy-trichothecene skeleton of the sesquiterpenoids. These compounds are best known for their role as mycotoxins, posing a serious threat to human and animal health. Verrucarin A is the most potently toxic trichothecene. The toxic potency of the trichothecenes depends on the chemical features of side chains in the molecules. The type D macrocyclic trichothecenes such as verrucarin A and roridin A, containing a macrocyclic ring between C14 and C15 with two ester linkages are the most potent (Ueno, 1987).

The trichothecene mycotoxins inhibit protein synthesis selectively in eukaryotic cells through breakdown of the polyribosomal profile in the exposed cells. Depending upon the sites of toxic action, the trichothecenes fall into two classes: the initiation inhibitors (I) which act on the initiation step of translation and the elongation-termination inhibitors (ET) (Wei and McLaughlin,

1974). Verrucarin A is the most potent I type inhibitor while diacetoxyscirpenol is the least potent. Deoxynivalenol is an ET type inhibitor. The primary targets are the 60S ribosome subunits and the peptidyl transferase enzyme, and the toxic action results from binding of the trichothecenes to this enzyme (Cannon *et al.*, 1976).

This biochemical feature of the trichothecenes to inhibit protein synthesis explains why they selectively attack the haematopoeitic tissues and cause cellular damage. The functional damage in these tissues leads to a decrease in white blood cells in exposed animals (Ueno, 1987). Verrucarin A is potently cytotoxic and this is due to the optimal structure of VRCA for binding to the target enzyme. As this structure changes in the other trichothecenes, so does the affinity for the enzyme resulting in decreases in the potency of the toxic action.

The carcinogenicity and genotoxicity of the trichothecenes are of great concern. Genotoxicity tests with *in vitro* assays, the *Salmonella*/mammalian microsome assay and mammalian cell cultures, revealed no genotoxic activity of trichothecenes (Ueno and Kubota, 1976; Ueno *et al.*, 1978; Ueno, 1987). Watanabe and colleagues (1982) also failed to demonstrate the promoting activity of VRCA and RORA. None of the trichothecenes tested showed genotoxic activity in SMART although they did show toxic effects.

9.4.6.5 FUMONISINS

In 1988, Gelderblom and colleagues isolated two novel mycotoxins with cancer promoting activities from cultures of *Fusarium moniliforme*. The two pure compounds were chemically chracterized and given the names fumonisin B1 and B2. FB1 proved to be acutely toxic to rats and high doses caused death within three days. Their results also implied that FB1 is a carcinogen.

In the south eastern Transkei, the rate of human oesophageal cancer is high, while the rate in the northern region is relatively low. Maize is the dietary staple in both areas. Several toxigenic *Fusarium* strains were isolated from maize kernels and two *Fusarium* mycotoxins deoxynivalenol and zearalenone were detected in infected maize from both areas, although the level of contamination with mycotoxins was considerably higher in the high incidence area of oesophageal cancer than in the low incidence area (Ueno, 1987). It is now believed that the rate of oesophageal cancer may be related to contamination by the novel mycotoxins FB1 and FB2.

Although the presence of the NH_2 structural unit may lead to FB1 being classed as a potential carcinogen [according to Hay (1988) this is one of the units which may react with DNA], no evidence of genotoxicity of FB1 and FB2 was found in the SMART test in these experiments.

9.4.6.6 CYTOCHALASINS

Most of the effects caused by cytochalasins can be attributed to the interaction between these mycotoxins and the target proteins actin or tubulin (Yahara *et al.*, 1982). Cytochalasin B inhibits gelation of actin filaments (Hartwig and Stossel, 1979). Low levels of CYTB inhibit both the rate of actin polymerization and the interaction of actin fialments in solution (MacClean-Fletcher and Pollard, 1980). All cytochalasins inhibit cytoplasmic cleavage in mammalian cell cultures, but differ in the intensity of their action. In the presence of cytochalasin B normal mitosis occurs in mammalian cells, but cytokinesis (the cleavage of the cell plasma) is blocked (Carter, 1967). Prolonged contact with CYTB leads to the formation of large multinucleate cells, but instead of doubling the number of nuclei per cell, each mitosis gives only one nucleus. High concentrations, required for the inhibition of cytokinesis, causes ejection of the cell nucleus. These processes are thought to account for the toxicity of the compound.

Sekita and colleagues (1977) reported on the structure-activity relationship of CYTB. As a whole, the core of the macrocyclic ring and the hydrophobic region from C15 to C16 are assumed to be essential for the toxic effects. This structure may allow CYTB to interact with actin. Furthermore, the aromatic ring, the units over C16 of the macrocyclic ring including the conjugated enedione system and the physical properties of the compounds, such as the lipophilicity, presumably influence their relative effectiveness.

The results of these experiments demonstrated that CYTB, CYTH and CYTJ were toxic in both lines at the higher concentrations, although by comparing the number of survivors at the same concentration, it can be seen that the cytochalasins were the least toxic of the mycotoxins tested. The toxic effects decreased with decreasing concentration and 2-day delayed hatching effects were observed. The results showed that the cytochalasins possess no genotoxic activity in the SMART test. Although cytochalasin B was positive at one concentration (0,1 mM for twin spots) in the standard cross, this was probably a false positive as the compound was negative at the same concentration in the original and new HB crosses.

CHAPTER TEN

10 COMPARISON OF THE PERFORMANCE OF THE FOUR SHORT-TERM TESTS

10.1 INTRODUCTION

According to Zimmerman (1985) mutagenic hazards cannot be reliably detected on a single test basis because a single assay may not detect mutagens with differing genetic effects, and he recommends a test battery approach to monitor mutagenic/carcinogenic hazards. The test battery should cover the full spectrum of genetic alterations that can occur in humans. Adding more tests to a screening program, however, such as performing SMART together with the sex-linked recessive lethals assay, the heritable translocations test and the *Salmonella*/mammalian microsome assay, means additional expenses for investment in equipment, laboratory personnel and testing experience. A balance between the cost of a complete test battery and the potential increase in information gained, must be struck (Zimmerman and Taylor-Mayer, 1985). The best mutation assays to use in the battery system must be carefully chosen. Each assay used in these experiments was assessed as to cost and time involved in performing the assay and for reproducibility of results. The four tests were compared and advantages and disadvantages were mentioned.

10.2 ADVANTAGES AND DISADVANTAGES OF THE *SALMONELLA*/MAMMALIAN MICROSOME ASSAY

A bacterial mutagenicity test system has many practical and theoretical advantages, among which are the small genome (approximately 4 x 10^6 base pairs) and large numbers of organisms exposed (10^9 per plate) (Ames *et al.*, 1973). The results of testing a compound can be scored in two days and only very small amounts of the test compound are needed (Ames *et al.*, 1973).

The principal advantage of reversion assays, relative to forward mutation assays is the well defined nature of the induced mutation: while a great variety of forward mutations can cause loss of function of an active gene product, only a few specific reverse mutations can restore function to an inactive one. The nucleotide sequences of the frameshift (*hisD3052,* hisD6610) and base substitution (*hisG428, his*G46) mutants have been determined (Hartman *et al.*, 1986).

The problems are that a negative result does not necessarily mean that a compound is not a mutagen. Although rat liver homogenates are effective in activating carcinogens, it is not the same as an *in vivo* system as loss of activity can occur. Carcinogens that cause mutation indirectly by inhibiting repair may not be detected. The assay can also be very costly and time consuming as the time taken to prepare all the media and solutions, confirm the genotypes of all the stocks and perform the mutagenicity test with two repeats was about two months.

10.3 ADVANTAGES OF *DROSOPHILA* ASSAYS

The advantage of *Drosophila* assays is that they employ a lower eukaryote as the tester organism, while the *Salmonella*/mammalian microsome assay employs a unicellular, prokaryotic organism, lacking a distinct membrane bound nucleus and having its genetic material in the form of a single DNA strand. *Drosophila*, on the other hand, has a similar cellular and chromosomal organization as mammals and therefore detects chromosome aberration, somatic recombination and non-disjunction. *Drosophila* is also capable of activating promutagens *in vivo* to their ultimately mutagenic form because this insect possesses a well developed P450 cytochrome-dependent metabolic system similar to humans. In the *in vitro Salmonella*/mammalian microsome assay, however, the essential enzymes of xenoblotic metabolism must be added in the form of a liver S9 fraction, and loss of monooxygenase function can occur if, for example, the enzymes denature. Other disadvantages of the bacteria *in vitro* system arise from under-representation of detoxifying activities, from secondary metabolism, or from binding of the active metabolites to proteins within the S9 liver fraction (Würgler, 1983). In addition to the differences between *in vitro* and *in vivo* metabolism of promutagens, the bacterial assay is also undesirable because mammals have to be sacrificed in order to obtain the S9 fraction. *Drosophila* assays are cheap and easy to perform and for the SMART assay results can be obtained in under two weeks.

10.3.1 ADVANTAGES AND DISADVANTAGES OF THE SEX LINKED RECESSIVE LETHALS ASSAY

An important advantage of this test is that a representative part of the *Drosophila* genome is assayed and that, as many loci are tested, sensitivity and specificity differences between loci are averaged out (Würgler, 1983). The SLRL test detects mutagens with and without chromosome breaking ability. Mutagens like diethylnitrosamine (DEN) lead to practically no recoverable translocations at doses which are extremely efficient in inducing high frequencies of SLRL. In addition, the SLRL assay records point mutations (base pair substitutions, frameshifts, small deletions and small and large aberration-type mutations which exhibit a recessive lethal effect (Würgler, 1980). Because the mutagenic risk for germ cells can be detected, this *Drosophila* test is indicated with compounds which can pose a risk for germ cells in people of the reproductive age. Another advantage is that the test is simple to perform and scoring for sex linked recessive lethals is very objective. The test relies on the disappearance of a whole Mendelian class (half of the F2 males, characterized by round, red eyes). Since this is a criterion on which different observers cannot disagree, personal bias is reduced to a minimum in the scoring procedure, and this is one of the great advantages of the test for SLRL. In the SMART test, different individuals may differ in the scoring of a slide for mutant spots, although this does not usually affect the statistical evaluation. The main disadvantage of the SLRL assay is that it is time consuming and tedious as thousands of vials must be prepared in order to test large numbers of chromosomes.

10.3.2 ADVANTAGES AND DISADVANTAGES OF THE HERITABLE TRANSLOCATIONS TEST

Data on the induction of translocations provide a clear indication of the capacity of the chemical under test to break chromosomes. Another advantage of the test is that the spontaneous occurrence rate of translocations is very low so negative controls are not really necessary; this decreases the labour and cost of the test considerably. Furthermore, scoring of the F2 progeny for the presence of translocations is simple and objective as it relies on the presence or absence of Mendelian classes with clearly distinguishable phenotypes. This test, however, is not well validated as very few chemicals have been tested in the heritable translocations test. The main disadvantage of the test is that it involves large scale collections of thousands of virgins and the preparation of thousands of culture vials which make the test time consuming, costly and undesirable to use for the preliminary screening of potential mutagens.

10.3.3 THE SMART ASSAY

10.3.3.1 The Present Status of Validation of SMART

A pilot study on the evaluation of the wing system for the detection of chemical mutagens was completed by Graf and colleagues in 1983. This study showed that chemical mutagens, such as simple alkylating agents, promutagens leading to alkylating species or to large DNA adducts and intercalating agents were recognized as mutagenic compounds. A wide range