3. Materials and Methods
3.1 Chemical compounds

To study the induction of adaptive response in *Drosophila in vivo* test system, nitrosoureas *viz.*, *N*-Ethyl-*N*-nitrosourea (ENU: CAS No. 759-73-9) and *N*-Methyl-*N*-nitrosourea (MNU: CAS No. 684-93-5), well-known potent monofunctional ethylating and methylating agents, obtained from Sigma Chemical Company (USA) were used.

3.1.1 *N*-Ethyl-*N*-nitrosourea

ENU is a potent monofunctional ethylating agent that has been found to be mutagenic in a wide variety of mutagenicity tests system from viruses to mammalian germ cells. It also has been shown to induce tumors in various organs of mammals. ENU has been used only for research purposes. ENU possesses the dual action of ethylation and carbamoylation. The ethyl group can be transferred to nucleophilic sites of cellular constituents, and the carbonyl group can be transferred to an amino group of protein. ENU is able to produce significant levels of alkylation at oxygens, such as the $O^6$ position of guanine and the $O^4$ position of thymine of DNA. The molecular genetic data obtained from ENU-induced mutants on various species suggest that ENU produces mainly GC-AT transitions and, to a small extent, AT-GC, AT-CG, AT-TA, GC-CG and GC-TA base substitutions. This mutation spectrum of ENU is different from that of MNU, which mainly induces GC-AT transitions. ENU is a most potent mutagen in mouse germ cells, especially in stem-cell spermatogonia. It induces intragenic mutations with high frequency in male mouse germ cells. ENU has been established as a model compound for exploring the effects of chemical mutagenesis on mouse germ cells.
ENU was first prepared in 1919 by the reaction of N-ethylurea with nitrous acid (Werner, 1919). Although ENU is available in small quantities for research purposes, no evidence was found that it has been produced in commercially. ENU has been used for the laboratory synthesis of diazoethane. Its mutagenic effect has been studied for promoting the growth of various plants (Ezhakova, 1973; Nikiforova, 1973; Samoshkin and Rodyankov, 1973).

ENU is yellow-pink crystals in appearance having melting point of 103-104°C, soluble in water (approximately 1.3%) and in polar organic solvents; insoluble in non-polar organic solvents. Decomposes to diazomethane in alkaline solutions; stability in aqueous solutions is pH-dependent (20°C) (Druckrey et al., 1967). Sensitive to humidity and light and should be refrigerated for storage. ENU is highly reactive (Garrett et al., 1965; McCalla et al., 1968). Reaction rates with various biologically important nucleophiles have been determined (Veleminsky et al., 1970).
3.1.2 N-Methyl-N-nitrosourea

MNU was first prepared by Bruning in 1889 by the reaction of sodium nitrite with an aqueous solution of methylurea nitrate (Prager et al., 1922). Although MNU is available in small quantities for research purposes, no evidence was found that it has been produced in significant commercial quantities. MNU has commonly been used for the laboratory synthesis of diazomethane, but it has been largely replaced by other reagents such as N-nitroso-N-methyl-para-toluenesulphonamide.

MNU has been studied for use as a cancer chemotherapy agent by a number of investigators. A review was published in connection with a clinical study of its use in combination with cyclophosphamide (Kolaric, 1977). It has also been studied for its mutagenic effects on various plants (Kerkadze et al., 1974; Nikiforova, 1972; Zhuravel and Shlyapunov, 1972).

Chemical formula and Molecular weight

\[ \text{Mol. wt. of MNU is } 103.08 \text{ [g/mol]} \]

The chemical is pale-yellow crystals in appearance with melting point of 124°C, soluble in water (approximately 1.4%) and in polar organic solvents; insoluble in non-polar organic solvents. Decomposes to diazomethane in alkaline
solutions; stability in aqueous solutions is pH-dependent (20°C) (Druckrey et al., 1967). The pure compound is sensitive to humidity and light and should be refrigerated for storage. The compound is highly reactive (Garrett et al., 1965; McCalla et al., 1968). Reaction rates with various biologically important nucleophiles have been measured (Veleminsky et al., 1970).

3.2 Drosophila strains

Two high bioactive strains of Drosophila melanogaster, viz., ORR:mwh/mwh (Plate-I, Fig.1A) and ORR:flr\(^3\)/TM3, Ser (Plate-I, Fig.1B) were used as a \textit{in vivo} test system to get the picture of induction of adaptive response by ENU or MNU.

3.2.1 ORR:mwh/mwh

The \textit{mwh} gene located on the third chromosome, near the tip of the left arm (3-0.3) is a wing cuticle marker which confers multiple trichomes (2-7 processes) per wing blade cell (Plate-III, Fig.1L) instead of the normally unique trichome (Plate-II, Fig.1I). The marker \textit{multiple wing hair (mwh)} in the strain ORR:mwh/mwh (Plate-I, Fig.1A) is a completely, recessive, homozygous viable mutation, kept in a homozygous \textit{mwh} strain (Lindsley and Grell, 1968; Garcia-Bellido and Dapena, 1974; Lindsley and Zimm, 1985; 1992; Frolich and Wurgler, 1989).

3.2.2 ORR:flr\(^3\)/TM3, Ser

The marker \textit{flare}\(^3\) (flr\(^3\)) in the strain ORR:flr\(^3\)/TM3 (Plate-I, Fig.1B) is also a recessive mutation, and it also affects the shape of the wing hairs (produces malformed wing hairs that have the shape of a flare) and is located on the left arm.
of chromosome 3 but in a proximal position (3-38.8). The three known alleles of \textit{flr} that are recessive, zygotic lethals (zygotes homozygous for \textit{flr/flr} are not capable to develop into adult flies) (Garcia Bellido and Dapena, 1974) on the other hand, homozygous cells in the wing imaginal disc are viable and lead to mutant wing cells. Due to the zygotic lethality, \textit{flr} alleles have to be kept in stocks over balancer chromosomes carrying multiple inversions \textit{TM3 (third Multiple 3)} and a dominant marker which is a homozygous lethal \textit{Bd} (\textit{beaded-Serrate}) (Plate-II, Fig.1F) thus prevents crossing over (Lindsley and Zimm, 1992). The homozygous cells, however, are viable in the wing imaginal disc and lead to the formation of mutant wing cells. This allele is responsible for development of serrate wings.

3.3 Methodology

3.3.1 Culturing and crossing of \textit{D.melanogaster}

\textit{D. melanogaster mwh} and \textit{flr}\textsuperscript{3} markers were cultured in culture glass bottles on standard \textit{Drosophila} food (soji, jaggery, agar agar, propionic acid and water cooked) and were kept in the BOD incubator (23±2°C and 65% relative humidity). In the present investigation, virgin \textit{flr}\textsuperscript{3} females collected from optimally fertile parents of ORR:\textit{flr}\textsuperscript{3}/\textit{TM3 Ser} strain were mated with \textit{mwh} males isolated from the ORR:\textit{mwh/mwh} strain. Then the females were allowed to lay eggs (Plate-II, Fig.1C). The parents were then removed and eggs were collected for a period of 8h in culture bottles containing fresh standard \textit{Drosophila} food and allowed to continue normal development. The resulting 2\textsuperscript{nd} instar (48±4h) (Plate-II, Fig. 1D) or 3\textsuperscript{rd} instar (72±4h) larvae (Plate-II, Fig.1D) were collected, washed and randomly allocated to different treatments. All experiments were carried out at 23±2°C and 65% relative humidity.
3.3.2 Preparation of control and chemical media

3.3.2.1 Preparation of control media

Distilled water which was used as a solvent, was mixed with *Drosophila* food in equal quantity and the same was termed as control media for all experiments.

3.3.2.2 Preparation of chemical media

Various concentrations of test chemicals, ENU (viz., 0.1, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4mM) or MNU (viz., 0.01, 0.05, 0.1, 0.5, 1, 1.5 and 2mM) were prepared by mixing with distilled water and was mixed thoroughly with 5ml of freshly prepared *Drosophila* media in plastic vial in equal quantity and was used as chemical media for all experiments.

3.3.3 Exposure technique: Larval Feeding Method

In the present investigation larval feeding method (Vogel, 1977) was employed to administer the chemical. Because larval feeding is supposed to be the easiest route of administration of a chemical substance as the feeding is maximum during the larval stage. When the chemical passes through the gut along with the food, permeates through different parts of the alimentary canal and is transferred to various tissues through the haemolymph hence feeding larvae might well become a useful application procedure for mutagen testing, since it would tend to increase exposure to the mutagen. In the present study, the 2\textsuperscript{nd} instar (48±4h) or 3\textsuperscript{rd} instar (72±4h) *Drosophila* larvae were selected for different experiments.

Variable concentrations of ENU or MNU were thoroughly mixed in equal volume of media to get the said concentrations. Larvae in equal numbers (200 larvae) were placed in vials containing control and ENU or MNU supplemented
media separately and tested for the variable effects. The water medium served as the control. All experiments were conducted at a controlled temperature of 23±2°C and 65% relative humidity.

3.3.4 Parameters used

For the present study, different parameters viz., rate of development, viability, dominant lethal assay and wing mosaic assay were used to get the picture of toxicity, mutagenicity and recombinogenicity of the ENU or MNU and induction of adaptive response to the said test chemicals in 48±4h or 72±4h aged D. melanogaster larvae.

3.3.4.1 Rate of development and viability

In order to get the picture of the rate of development and viability, the 2nd (48±4h) or 3rd (72±4h) instar D. melanogaster larvae trans-heterozygous for mwh and flr^3 were floated out from the food media using 20% NaCl solution (Graf et al., 1984). About 200 larvae per batch were seeded in different plastic vials containing 5ml of standard Drosophila food hydrated with equal quantity of test solution with different concentrations (ENU, ranging from 0.1 to 4mM or MNU, ranging from 0.01 to 2mM). These various concentrations were prepared by dissolving known quantity of ENU or MNU in distilled water. For controls, larvae were placed in vials containing food which is mixed thoroughly with equal quantity of distilled water only. The larvae were fed on this medium for the rest of their development.

The emerged flies were counted and sexed everyday from the day of eclosion of flies to the last day of emergence in each treated group of ENU or MNU along with control. Four replicates were maintained for each concentration of ENU or MNU employed and two independent experiments were assayed. All
experiments were carried out under identical conditions 23±2°C and 65% relative humidity. Results were subjected to statistical analysis to determine the LD₅₀, pattern of emergence, mean developmental time and viability.

### 3.3.4.2 Dominant lethals

The usual method for measuring dominant lethality in *Drosophila* is to treat the male and mate them with untreated virgin females, and calculating the frequency of un-hatched eggs laid by the same female. The trans-heterozygous larvae obtained by crossing virgin *fbr¹* females with *mwh* males were employed for dominant lethal assay. These larvae were treated with various concentrations of test solutions (ENU, ranging from 0.1 to 3.5mM or MNU, ranging from 0.01 to 1.8mM) at 48±4h or 72±4h after hatching, control (distilled water) was included. The emerged flies from these different treatment groups and control group were collected. About 20 males were used for each concentration of ENU or MNU. These males were aged for three days and then mated with 3 days old untreated control virgin females. The mating was carried out in such a way that each vial contains one treated male and two untreated females (1:2). Thus, about 20 vials per concentration of ENU or MNU were used. After mating, the females were allowed to lay eggs for 22h. Then the flies were discarded and number of eggs laid was counted. After 28h, the eggs were counted to find out the number of un-hatched eggs. Un-hatched eggs may be due to late embryonic lethals (un-hatched brown eggs) or early embryonic lethals (un-hatched white eggs or unfertilized eggs). The percentage of un-hatched eggs gives the dominant lethality. Two independent experiments with four replicates for each concentration were assayed and the results were subjected to statistical analysis.
3.3.4.3 SMART: Wing mosaic assay

Wing spot test is one of the options in SMART test, in which the genetic changes were observed through mosaics (spots) on the wings. The somatic mutation and recombination test of *Drosophila* (i.e., somatic wing spot assay) was developed in the 1980s to provide researchers with a rapid and inexpensive assay for genotoxicity in somatic cells of higher eukaryotes (Graf *et al.*, 1984; 1989).

The assay utilizes two loci located on the left arm of chromosome 3 - *mwh* and *flr*³. Both loci influence development of hair growth in each adult wing blade cell. An induced genetic alteration in a developing wing disc cell of a treated larva can result in a clone of cells possessing malformed hairs (i.e. a spot) on the adult wing blade. Larvae exposed to a test substance are allowed to develop into adults, and then their wings are removed and mounted on microscopic slides for scoring at 400X magnification. Three endpoints are distinguished in the assay: (1) small single spots of either *mwh* or *flr*³ phenotype, which consist of just 1-2 cells; (2) large single spots of either *mwh* or *flr*³ phenotype, which consist of 3 or more cells; and (3) twin spots, which consist of adjacent *mwh* and *flr*³ spots. While small and large single spots can arise from a variety of genetic alterations, twin spots result solely from mitotic recombination. The vast majority (90-95%) of spontaneously arising spots on wings of control flies are of the small single type while large single spots and twin spots account for the remaining 5-10% of control spots.

Among the different crosses used so far with the best performance and the best status of validation is the standard one, in which virgin females are collected
from the \( fl^{r^3}/TM3,BD^i \) strain and crossed with \( mwh \) males. The progeny will consist of trans-heterozygous \( mwh \ fl^{r^3}/mwh^+ fl^{r^3} \) and \( mwh \ fl^{r^3}/TM3,BD^i \) larvae. They lead to two types of adults flies among which the inversion heterozygotes are marked with the serrate phenotype (clipped wing margins). Normally, only the wings of the non-serrate flies are analysed. This standard cross leads to a low spontaneous frequency of small mutant spots and a very low frequency of double-haired cells and is only slightly temperature sensitive (Graf, 1986).

To study the genotoxicity, in the standard procedure for the wing spot test, 48±4h or 72±4h old trans-heterozygous larvae were fed with different concentrations of the chemical (ENU, ranging from 0.1 to 3.5mM or MNU, ranging from 0.01 to 1.8mM) for the rest of the larval development. For this chronic feeding, the larvae were put into plastic vials containing 5ml of standard \( Drosophila \) food mixed with different concentrations of said test compounds in equal quantity. For each experiment, control (distilled water) included. Four replicates were maintained for each concentration of ENU or MNU employed and two independent experiments were assayed. For each treatment group a total 800 larvae, 200 in each vial were employed. The larvae were allowed to grow for their rest of life in the same media. Strict control of temperature (23±2°C) and relative humidity (65%) was given attention as it affects the frequencies of spots (Graf, 1986; Katz and Foley, 1993).
3.3.4.3.1 Preparation and microscopic analysis of wings

Flies eclosing from each exposure vial were collected and stored separately in 70% ethanol. Flies (20 males and 20 females) from the trans-heterozygous \((mwh^{+}/+flr^3)\) genotype (non-serrate) were selected (Plate-II, Fig. 1E). The wings of these flies were dissected out in Faure’s solution (30gm gum arabica+20ml glycerol+50gm chloral hydrate+50ml water). The detached wings from 10 males and 10 females were lined up separately on a grease free clean slide (Plate-II, Fig. 1G) and allowed to spread in a fresh drop of Faure’s solution. Such two slides were prepared for each treatment group. Each slide contains 20 wings of males and 20 wings of females. The slides were kept in dust free condition for 24h. This will help to glue the wings firmly to the slides. The glued wings were covered with a clean cover slip. The cover slips were charged with metal cubes. Then slides were allowed to dry up and harden without crumpling of wings. Thus, a total of 80 wings from control as well as treated series were used for scoring. During the microscopic analysis of the wings, the position of the spots is noted according to the sector of the wing. Only the distal wing compartment is scored for clones.

Both the dorsal and ventral surfaces of the wings were analysed under a compound microscope at 400x magnification for the occurrence of any of two categories of spots: single spots \((mwh\text{ or }flr^3\) phenotype) or twin spots \((mwh\text{ clones adjacent to an }flr^3\text{ clone})\). During the microscopic analysis of the wings, the position of the spots is noted according to the sector (A, B, C, D and E) of the wing (Graf et al., 1984) (Plate-II, Fig. 1H). Only the distal wing compartment is scored for clones. This is an area comprising a total of about 24,400 cells. Each
cell of a wing represents a trichome (Dobzonsky, 1929). The different types of spots were small single (Plate-III, Fig. 1J and 1K) with 1-2 affected cells, large single (Plate-III, Fig. 1L) with 3 or more affected cells and twin (Plate-III, Fig. 1M) with both mwh and flr^3 type present adjacent to each other. A spot was classified as mwh spot when the wing cell contains 3 or more hairs in contrast to a single trichome per cell of the wild type wing. The cells with 2 hairs within a clear-cut mwh spot were taken into consideration for the determination of the size of the clone. About 24,400 cells were scored per wing for these different types of spots (Garcia-Bellido et al., 1976). The experiment was repeated twice with four replicates. The experimental data obtained were subjected to statistical analysis to know the genotoxic effects of various concentrations of test solutions.

3.3.5 Study of adaptive response

In the present investigation, to get the picture of the induction of adaptive response to ENU or MNU, different treatment schedules were followed using dominant lethality test and wing mosaic assay in Drosophila in vivo test system.

3.3.5.1. Treatment schedules

Based on the pilot genotoxicity and dose effect relationship studies, the conditioning and challenging doses of ENU and MNU were selected. The doses such as 0.1, 0.25 and 0.5mM for ENU and 0.01, 0.05 and 0.1mM for MNU were selected as conditioning doses and 2.5, 3 and 3.5mM of ENU and 1, 1.4 and 1.8mM of MNU were used as challenging doses.
Treatment schedules adapted for the present study are as follows:

**Control**: The trans-heterozygous larvae fed with 5ml of food thoroughly mixed with five ml of distilled water only.

**Conditioning dose/Low dose (L)**: The trans-heterozygous larvae were fed with media containing 0.1, 0.25 or 0.5mM ENU or 0.01, 0.05 or 0.1mM MNU for 30 minutes. Then these larvae were transferred to fresh media.

**Challenging dose/High dose (H)**: The trans-heterozygous larvae were fed with medium containing 2.5, 3 or 3.5mM of ENU or 1, 1.4 or 1.8mM MNU and allowed to develop in the same media for their rest of life *i.e.*, until their pupation.

**Combined treatment (L-H)**: In this treatment schedule, the trans-heterozygous larvae were fed with conditioning doses of ENU or MNU for 30 minutes. Then they were transferred to fresh medium. After 2, 4 or 6h time lag (TL) the same larvae were transferred to the vials containing challenging doses of ENU.

**ENU (L–TL–H)**

- \([0.1\text{mM (L)} - 2, 4 \text{ or } 6\text{h (TL)} - 2.5, 3 \text{ or } 3.5\text{mM (H)}]\)
- \([0.25\text{mM (L)} - 2, 4 \text{ or } 6\text{h (TL)} - 2.5, 3 \text{ or } 3.5\text{mM (H)}]\)
- \([0.5\text{mM (L)} - 2, 4 \text{ or } 6\text{h (TL)} - 2.5, 3 \text{ or } 3.5\text{mM (H)}]\)

**MNU (L–TL–H)**

- \([0.01\text{mM (L)} - 2, 4 \text{ or } 6\text{h (TL)} - 1, 1.4 \text{ or } 1.8\text{mM (H)}]\)
- \([0.05\text{mM (L)} - 2, 4 \text{ or } 6\text{h (TL)} - 1, 1.4 \text{ or } 1.8\text{mM (H)}]\)
- \([0.1\text{mM (L)} - 2, 4 \text{ or } 6\text{h (TL)} - 1, 1.4 \text{ or } 1.8\text{mM (H)}]\)
3.3.5.2 Study of adaptive response using dominant lethals

To study the induction of adaptive response by monofunctional alkylating agents *viz.*, ENU or MNU, the transheterozygous larvae obtained from crossing of virgin females of *flr*\(^3\) with *mwh* males were used. The larvae were treated with test solutions at 48±4h or 72±4h. The different treatment schedules were employed to understand the adaptive responses.

In each treatment group, 20 males were selected. After three days, they were mated to untreated virgin females (1:2) by placing separately in plastic vials containing *Drosophila* food. The female flies were allowed to lay eggs for 24h then they were discarded. After 24h, the number of hatched eggs and unhatched eggs were counted in each vial. Ratio between hatched and unhatched eggs (brown lethals/white lethals) was analysed. The experiments were repeated twice with four replicates and the results obtained were subjected to statistical analysis.

3.3.5.3 Study of adaptive response using wing mosaic assay

Wing spot assay or wing mosaic assay was performed to study the adaptive response in *in vivo* *D.melanogaster* test system using monofunctional alkylating agents ENU or MNU. The similar conditioning and challenging doses of ENU or MNU that were used to study the induction of adaptive response in case of dominant lethal assay were employed in the wing mosaic assay to demonstrate the occurrence of adaptive response. Furthermore, the same treatment schedules were employed.

Eggs from the cross between virgin females from *flr*\(^3\)/*TM3,Ser* strain and isolated *mwh* male from *mwh*+/+*mwh* strain were collected during 8h periods
on fresh standard *Drosophila* food and the resulting 48±4h or 72±4h aged trans-heterozygous larvae were seeded in plastic vials (50 larvae/vial) with 5 ml *Drosophila* food hydrated with 5 ml of respective test solution. For the control, *Drosophila* food was mixed thoroughly with 5 ml of distilled water only. Various said treatment schedules (conditioning, challenging and combined with time lags) were followed. Flies from the marker trans-heterozygous (*mwh*+/+*flr^3*) genotype which is non-serrate (complete wing) were selected and stored in 70% ethanol. Further, a total of 40 flies (20 males and 20 females) were selected and the wings were detached and mounted using Faure’s solution on slides. The wings were scored at 400X magnification for the presence of clones of cells showing malformed wing hairs which may include single spots (*mwh* or *flr^3*) or twin spots (*mwh* with *flr^3*). Pooled data of two independent experiments along with four replicates were subjected to statistical analysis for the presence of adaptive response.

3.3.6 Data evaluation and statistical analysis

The data from individual experiments were pooled and subjected to statistical analysis.

3.3.6.1 Rate of development and viability

To know the effect of chemicals on the rate of development of 48±4h or 72±4h aged *Drosophila* larvae, dose-response relationship was analyzed. Mean values were computed and were compared by one way analysis of variance (ANOVA) followed by Duncan’s Multiple Range Test (DMRT), a post-hoc test using Statistical Package for Social Sciences (SPSS 17.0 version). Results were judged significant if p<0.05. To study the dose effect relationship (toxicity) of
ENU or MNU, probit analysis was done using SPSS 17.0 version and LD50 for the said chemicals were determined for 48±4h or 72±4h *Drosophila* larvae trans-heterozygous for *mwh* and *flr^3*.

3.3.6.2 Dominant lethal assay

Mean values of control and conditioning or challenging doses furthermore between combined treatments with time lags were calculated and were compared for significance using one way ANOVA followed by DMRT, a post-hoc test using SPSS 17.0 and judged significant if p<0.05.

3.3.6.3 Wing mosaic assay

The data on the frequencies of spots, induced in different treatment schedules of ENU or MNU were subjected to statistical analysis of Frei and Wurgler (1988). Mean values were compared with the control as well as with respective challenging dose by one way ANOVA followed by post hoc test, DMRT using SPSS 17.0 and statistical significance was assessed at p<0.05 level. Further, the wing spot data were evaluated with the computer program SMART (Wurgler, unpublished). For the statistical analysis, the spots were grouped as small single spots of 1 or 2 cells in size (*mwh* or *flr^3*), large single spots of 3 and more cells (*mwh* or *flr^3*), and twin spots with an *mwh* and an *flr^3* area. These spots were evaluated separately. The conditional binomial test of Kastenbaum and Bowman (1970) was used with significance levels $\alpha = \beta = 0.05$ to assess differences between the frequencies of each type of spot in treated and concurrent negative control flies. The formulation of two alternative hypotheses allows distinguishing among the possibilities of a positive, weakly positive, inconclusive or negative result of an experiment (Frei and Wurgler, 1988). In the null
hypothesis ($H_0$), one 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 ($H_A$) postulates a priori that the treatment results in an increased mutation frequency compared to the spontaneous frequency. This alternative hypothesis is rejected if the observed mutation frequency is significantly lower than the postulated increased frequency. Rejection indicates that the treatment did not produce the increase required to consider the compound as mutagenic. If neither of the two hypotheses is rejected, the results are considered inconclusive, as one cannot accept at the same time the two mutually exclusive hypotheses. In the practical application of the decision procedure, one defines a specific alternative hypothesis requiring that the mutation frequency in the treated series is at least $m$ (multiplication factor) times greater than in the control series, which is then used together with the null hypothesis. Since small single spots and total spots have a comparatively high spontaneous frequency, $m$ is fixed at a value of 2 (testing for a doubling of the spontaneous frequency). For the large single spots and the twin spots, which have a low spontaneous frequency, $m = 5$ is used. It may happen in this case that both hypotheses have to be rejected. This would mean that the treatment is weakly mutagenic, but leads to a mutation frequency that is significantly lower than $m$ times the control frequency (Andrade et al., 2004). The frequency of clone formation was calculated, without size correction, by dividing the number of spot-per-wing frequency by the number of cells contained in a wing (24,400) which is the approximate number of cells examined per wing (Alonso-Moraga and Graf, 1989).
Fig. 1A: ORR: *mwh/mwh* strain of *Drosophila melanogaster*

Fig. 1B: ORR: *flr\(^3\)/TM3 Ser* strain of *Drosophila melanogaster*
Fig. 1C: Eggs of *D. melanogaster*

Fig. 1D: 48±4h (middle) and 72±4h (right) larvae of *D. melanogaster (mwh males x flr² females)*

Fig. 1E: Complete wings

Fig. 1F: Serrate wings

Fig. 1G: Slide showing mounted wings

Fig. 1H: Wing showing regions A – E for scoring spots
Fig. 11: Normal trichomes of the wing

Fig. 1J: *mwh* spot with one cell affected (Small single spot)

Fig. 1K: *mwh* spot with two cell affected. (Small single spots)

Fig. 1L: *mwh* spots with more than two cells affected (Large single spots)

Fig. 1M: Twin spots (red line pointing *mwh* and blue line pointing *flr* spot)