A GENETICAL AND CYTOLOGICAL STUDY OF CHEMICALLY
INDUCED REARRANGEMENTS IN THE IMMATURE
GERM CELLS OF DROSOPHILA MELANOGASTER.

by

William A. F. Watson, B. Sc.

Thesis presented for the degree of Doctor of
Philosophy of the University of Edinburgh in
the Faculty of Science.

## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td></td>
</tr>
<tr>
<td>I. Stocks</td>
<td>25</td>
</tr>
<tr>
<td>II. Treatment</td>
<td>27</td>
</tr>
<tr>
<td>III. Breeding techniques</td>
<td>28</td>
</tr>
<tr>
<td>IV. Genetical tests</td>
<td>30</td>
</tr>
<tr>
<td>V. List of chemical mutagens used</td>
<td>35</td>
</tr>
<tr>
<td>VI. Cytological techniques</td>
<td>41</td>
</tr>
<tr>
<td>RESULTS</td>
<td>43</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>74</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>94</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>97</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>98</td>
</tr>
</tbody>
</table>
INTRODUCTION

Since the discovery in 1946 by Auerbach and Robson of the mutagenic properties of mustard gas, many other chemicals have been tested and also found to be mutagenic. From the results obtained in numerous experiments, it is clear that great variations exist between them. They differ in the degree of their mutagenic activity, in the stage of germ cell development in which they show their greatest effect, and in their toxicity, as well as other differences. It is the purpose of this investigation to use such chemicals in order to throw some light on two problems. Firstly, why chromosome rearrangements are so scarce in pre-meiotic germ cells following irradiation, and secondly, how the structure of a mutagenic chemical can effect the nature of the genetic changes it produces. As explained below, the two problems are investigated simultaneously, by using different types of chemical known to act on both pre- and post-meiotic germ cell stages.

In order to clarify the problems, they are dealt with separately in this introduction.

PART 1

The problem of whether translocations can be induced in pre-meiotic germ cells is one which has not yet been clearly elucidated. It is of both theoretical and practical interest to find out if such structural changes in the chromosome are found at this time and carried through to the next generation, and in view of the present interest in the effects of radiation, the practical aspect is more important for, as explained below,
the presence of translocations in early germ cells can result in inherited semi-sterility.

Because of the low numbers of translocations obtained in most experiments, and the difficulty of ascertaining whether they had arisen in pre-meiotic stages, conflicting results have been obtained. Before considering these results, it is necessary to set out the events leading to the formation of a translocation, as well as the theoretical arguments against their production in pre-meiotic cells.

In order for a translocation to be formed, two breaks must occur in different, non-homologous chromosomes. This is the "Two Break" hypothesis now widely accepted. If a fragment of one of these chromosomes unites by its broken end with a fragment of the other, the resulting chromosome configuration is called a translocation. It is probable that in many cases the other two fragments fail to make contact with each other. If these uncombined fragments should later heal or their mother or daughter chromatids unite with each other to form acentric or dicentric chromosomes (i.e., with none or two centromeres respectively), the descendants would be aneuploid, providing they had survived bridge formation. If this takes place in germ cells, which is the case considered here, it is very unlikely that the translocation would survive to the next generation.

When the broken ends of the other two fragments likewise form a union, a reciprocal translocation is formed. It is a matter of choice whether the centric fragment of one chromosome joins with the centric fragment of the other, so as to form a dicentric chromosome, while the other two fragments join to form an acentric chromosome, or each centric fragment joins on to the acentric fragment of the other chromosome. In
the first case, an aneucentric configuration is formed, which eventually leads to the loss of all parts concerned for, in the ensuing cell division, the acentric chromosome fails to be drawn to the poles, while the dicentric one, pulled both ways by its two centromeres, tends to form a bridge which, if pulled by the tension, repeats the process of dicentric formation, and another bridge results at the next division. This continues until the chromosome is lost. On the other hand, the second case results in the formation of a eucentric configuration, and two monocentric chromosomes are formed, both of which are transported in a regular manner at mitosis.

Muller (1954) proposed the following hypothesis to explain the absence of translocations induced by X-rays in cells which are mostly in interphase, which include spermatogonia. If a cell in interphase is irradiated, it is very probable that any breaks produced will join in the same configuration as before, since the chromosomes are very much extended and dispersed, undergoing small movements. Thus a broken end will, by its Brownian movement, come into contact with the other broken end derived from the same break, long before it has a chance to meet an end derived from a different break. Even when it does fail to make a restitutinal contact, it is not likely to meet with any of the other broken ends at all, until each of the pieces has replicated to give two chromatid fragments in preparation for the next mitosis. Thus the adjacent homologous ends of each pair of identical twin chromatid pieces touch and rejoin together forming an acentric and a dicentric isochromosome, which are usually eliminated as explained above. Thus irradiation during interphase gives few structural changes, those obtained being mainly chromatid bridges and lagging acentric fragments.
If irradiation is carried out during some stage of mitosis, other factors come into operation which hinder restitution, and favour the union of ends derived from different breaks, giving rise to structural changes. During mitosis the chromosomes are tightly spiralised and condensed, and after treatment no breaks can be seen, but when they appear in the next mitosis, various types of structural change are found. This points to the fact that when the chromosomes are in the condensed state, they are unable to fall apart into fragments, although they have been broken by radiation. The work of Wolff & Luippold (1955) and Wolff (1960) showed that the rejoining of breaks was not a passive process, but an active metabolic one which required energy, as it was inhibited by all the various inhibitors of protein synthesis. Thus during mitosis, the breaks cannot rejoin, as the chromosomes are not in a state of active metabolism. Then at a later stage, probably late telophase, the breaks tend to fall apart before rejoining can take place. As the chromosomes at this stage begin to become unspiralised and extended, their parts must undergo much more movement relative to one another than before. Thus when the broken ends regain their ability to rejoin, they are much less likely to rejoin with the other end from which they had broken off. Conversely they are more likely to join with ends derived from other breaks, and structural changes result. Muller based his arguments on the effects of radiation on spermatozoa, having shown that breaks arising in spermatozoan chromosomes were retained as such, without restitution, until after fertilisation.

It thus seems probable that the formation of translocations in
spermatogonia is more likely to occur during mitosis. It has also been suggested that the majority of translocations induced in pre-meiotic stages would be eliminated by aneuploid segregation during meiosis. Glass (1955), basing his argument on translocations induced in mature oocytes of *Drosophila*, estimated that at meiosis, there is an exchange giving rise to half aneuploid and half orthoploid combinations (see Figure 1). The aneuploid combinations would be eliminated, and only half the orthoploid combinations would carry the translocation as it is heterozygous. However it should be remembered that oocytes and spermatogonia are diploid, and a broken chromosome has twice the chance of joining with another in a diploid cell than in a haploid one, so that effectively more translocations are produced. But the problem still remains, for meiotic elimination is not sufficient to account for the low numbers of translocations obtained in most experiments.

The following results have been obtained using radiation as a mutagen.

*Drosophila melanogaster*

Catsch & Radu (1943) using a dose of 4000 R, tested for translocations involving chromosomes II and III. Irradiated males were kept for 31 days after treatment and were mated to fresh females every five days. No mention is made of the age of the males at the time of treatment or the number of females used per brood, and in view of the later work determining the age of sampled gametes (see below) their work is open to question as to whether the later broods represent spermatogonia at the time of treatment. Their results were as follows:
<table>
<thead>
<tr>
<th>Age of gametes (in days after irradiation)</th>
<th>No. sperms tested</th>
<th>No. translocations</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2559</td>
<td>306</td>
<td>11.59</td>
</tr>
<tr>
<td>2-7</td>
<td>1483</td>
<td>92</td>
<td>6.21</td>
</tr>
<tr>
<td>8-13</td>
<td>1133</td>
<td>78</td>
<td>6.89</td>
</tr>
<tr>
<td>14-19</td>
<td>1532</td>
<td>21</td>
<td>1.37</td>
</tr>
<tr>
<td>20-25</td>
<td>1028</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>26-31</td>
<td>629</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Ward & Alexander (1957) found no chromosome aberrations detectable cytologically in spermatogonia which had been treated in the larval stage with 900 R X-rays.

Bateman & Chandley (1959) using a dose of 1000 R, found frequency of 0.8% translocations in what might be interpreted as spermatogonia. They determined the age of their sampled gametes by induced crossing-over (see below).

Alexander (1960), in a cytological examination, found no translocations in variants produced by 900 R in spermatogonia.

Savhagen (1960) found 0.4% translocations in sperm tested 14 days after treatment with 1100 R, and 0.43% when the dose was fractionated into two 550 R doses given 1 hour apart. This result indicated that 1 hour was not sufficient time to allow rejoining of chromosome breaks giving rise to translocations.

Traut (1960), using a dose of 3000 R, found that gametes sampled 9-11 days after treatment gave a translocation frequency of 2.9%, but did not take his result as representative of spermatogonial stages.
McCarthy & Nafei (1963), using a dose of approximately 3000 R, found one translocation (which they attributed to a spermatocyte) in the brood tested 8 days after treatment, and none in later broods. They used a three day brood interval, and determined the age of their sampled gametes by the lethal brood pattern and induced crossing-over.

Slizynska (1963), whose results are discussed in more detail below, gave a dose of 2500 R and found in a cytological analysis that chromosome rearrangements were found in cells that were spermatogonial at the time of treatment.

Savhagen & Kristofferson (1963) irradiated 17 hour old male larvae with 900 R, and after eclosion three day broods were taken. On the 14th day a test for translocations gave a frequency of 0.12%.

Alexander & Berghendahl (1964) found no translocations in spermatogonia after a dose of 2000 R.

Savhagen (1964) irradiated 0-1 day old males with 4000 R, and found 0.57% translocations in the brood tested 14-18 days after treatment. The cells sampled were probably representative of spermatogonial stages.

**Mouse**

The results obtained with mice are of great practical importance, because of the interest in the effects of radiation on human populations. It is well established by the results of many investigators (Snell, 1933; Hertwig, 1938; Russell, 1954; Bateman, 1958; Craig et al., 1961), that within the range 300-1000 R, a period of fertility immediately following radiation is followed by an interval of sterility which varies according to the dose. Oakberg (1955) found that late spermatogonia and primary spermatocytes were by far the most sensitive to radiation, and were reduced
in number due to a killing of the cells. The period of sterility corresponds to the interval in which replacement of the later germ cell stages has not yet been completed owing to the depletion of spermatogonia and spermatocytes. Since matings made after the sterile period use sperm that were in the spermatogonial stage at the time of treatment, the sterile period is thus a useful marker for separating genetic changes induced in spermatogonia from those induced in later germ cell stages.

If a translocation is produced and transmitted to the offspring, the resulting individual will be heterozygous for it. This means that half his gametes will be unbalanced, and on fertilisation will produce inviable zygotes. This results in inherited semi-sterility.

Hertwig (1940) found no semi-sterile individuals among the offspring of post-sterile period matings of irradiated male mice exposed to doses of 1200-1600 R, and concluded that no translocations had been produced.

Griffen (1958) used a dose of 700 R to try to produce chromosomal aberrations in pre-meiotic germ cells of male mice. Following the period of sterility there was a period of 6-8 weeks in which spermatogonia appeared and normal spermatogenesis was resumed. The male offspring from the irradiated mice after this period were tested for semi-sterility. 706 progeny were tested, and 95 of them showed semi-sterility. Of these, 26 had been born more than 6 months after treatment, and there seems little doubt that the cells in which the aberrations had arisen were early or pre-spermatogonial. In addition, one translocation was detected cytologically. This was taken as proof that radiation could result in the storing of translocations in the early germ cells.
Looking at these results, two possibilities come to mind. Firstly, is the observed partial sterility due to translocations in every case, or to other factors affecting sterility? Secondly, what is the degree of effect on the fertility of animals that are heterozygous for a translocation? Translocations showing considerably more than 50% fertility in the heterozygote would not be detected by fertility tests. Koller (1944) showed that the degree of fertility depended on the structure and behaviour of the chromosomes involved in a translocation, while Auerbach & Slizynski (1956) considered that small translocations would not cause lethal aneuploidy of the zygote. They found that one male with an average litter size of nine, which was examined because it had produced two litters of seven, carried a translocation involving an exchange of minute segments. Thus similar translocations may have been present in fertile males which were not examined cytologically. Also Slizynski (1957) showed that both high and low fertility existed in translocation heterozygotes depending on the length of segments of chromosomes involved in the translocation, and further suggested that normal fertility could not be regarded as sufficient proof of the absence of a translocation. A cytological analysis on a much larger scale was obviously required in order to obtain more direct evidence of the production of translocations in spermatogonia, and only recently has such work been undertaken.

Griffen (1964) studied cytologically sterile and semi-sterile F1 mice from post-sterile period matings and translocations were confirmed; while Searle (1964) undertook a detailed investigation of the genetical effects of spermatogonial irradiation on the productivity of F1 female mice. The dose was 1200 R administered as two 600 R doses given 8 weeks
apart. He chose to collect data from females as they receive the
irradiated X-chromosomes from their fathers, so that sex-linked lethals
could be scored. In all, six factors which might be induced in sperma-
togonia were tested for - dominant lethals, dominant sterility, dominant
semi-sterility, reduced fertility, sex-chromosome aneuploidy, and
recessive sex-linked lethals. The results involving dominant semi-
sterility, the only ones considered here, are interesting. Searle found
that no fewer than 5.9% (6/104) of the daughters of the irradiated males
had inherited semi-sterility, and the presence of reciprocal trans-
locations in each semi-sterile stock was confirmed cytologically.
This finding was confirmed by Lyon et al. (1964) who found that 3.5%
of the sons of the same irradiated males as used by Searle carried such
translocations. Searle combined these two figures to give an overall
frequency of about 4%, i.e., 4% of the mature offspring of the irradiated
males carried reciprocal translocations which had been induced in sperm-
atogonia. Using this frequency, he calculated that spermatogonia were
1/6th as sensitive as spermatozoa to irradiation. However, Auerbach &
Slizynska (64) point out that a given number of breaks per genome has a
higher chance of yielding a translocation when the breaks occur in the
two genomes of a diploid spermatogonium than when they are distributed
over two genomes in separate spermatozoa. Thus if every break took part
in a translocation, a given frequency of breaks per genome would produce
roughly twice as many translocations in spermatoxon as in spermatozoa.
So if radiation response is measured in the two types of cell on the
basis of this, a ratio of 6:1 for observed translocations in spermatoxon
and spermatozoa means that the response of spermatozoa is 12 times that of
spermatoxon.
As mentioned above, in the experiments of Searle and Lyon et al., the irradiation consisted of two doses of 600 R given 8 weeks apart, i.e. high intensity radiation in two acute doses. Philips & Searle (1964) found that when 1200 R was given to male mice at a low dose rate (100 R per week), no significant induction of inherited semi-sterility was found. This they attribute to the fact that a long exposure time makes the required interaction of the pair of events necessary for the production of a translocation less likely; since the interval between the two events tends to increase and recovery processes leading to restitution intervene. The fact that the frequency of chromosome aberrations decreased following fractionated doses of X-rays was found in plants as early as 1939 by Sax in Tradescantia, and confirmed by Sax & Luippold (1952) and De Serres & Giles (1953).

Slijzynska (1963) showed that heterogeneity existed between Drosophila males with respect to genetic sensitivity to spermatogonial X-irradiation, as measured by the frequency of structural changes in salivary gland chromosomes of progeny. In some males, sensitivity was as high as when products of irradiated spermatids or spermatozoa were tested, but in most it was very low. One interesting fact was that the clustering of breaks in sensitive cells was highest in spermatogonia. Her data indicated that the spermatogonial cells are a heterogeneous population, consisting of cells that are either highly sensitive to X-rays or not sensitive at all. It was considered that the heterogeneity between males could be due to some of them having a much higher frequency of gonial mitoses at the time of irradiation, and that the genetic sensitivity of gonia undergoing mitotic division was much higher than of those in interphase. In mice, spermatogonial interphases seem very sensitive to the killing effects of radiation.
(Monesi, 1962) so that few germ cells at this stage would survive a large radiation dose. Carter et al. (1955) found great differences between surviving meiotic stages in radiosensitivity, so similar differences may well exist between mitotic stages. If only the most condensed stages have a sensitivity similar to that of spermatozoa then differential sensitivity might be the main reason for the much lower yield of translocations following spermatogonial irradiation than following spermatozoal. The heterogeneity found between males in Drosophila would not be expected in the mouse because of the presence of a "spermatogenic wave" travelling along the testis. There is no evidence for it either in Searle's and Lyon et al.'s experiments, since only two of the 22 F1 mice with inherited semi-sterility came from the same father, although up to 15 offspring were tested from each treated male. However, the heterogeneity between spermatogonial cells was suggested by Russell (1963) as an explanation of his dose fractionation experiments in mice.

One point which should be remembered when comparing mice with Drosophila is that since mice have 20 chromosome pairs while Drosophila has only 4, the chance of translocations being formed is proportionately higher.

Oftedal (1964a) found that at low dose rates, the frequency of sex-linked lethals induced in spermatogonia of Drosophila gave a non-linear dose-effect curve. He postulated that the effect was due to the induction of the mutations in a fraction of the spermatogonia, and that this same fraction was also more susceptible to the killing effect of radiation than the rest of the cell population. He tested this hypothesis
in the following way. On the assumption that genetic damage is not completely repaired, whereas "sub-killing" damage is repaired completely during each cell cycle as in mammalian cells grown in culture (Elkind & Sutton, 1960), treatment over several cell generations should lead to a higher yield of observable mutations. Since Drosophila gonia in late embryos or newly hatched larvae appear to have very short mitotic periods, protracted doses over a short time might show this effect, and Oftedal (1964b) showed that such protracted doses given to larvae do give a higher mutation yield, presumably as the lower doses kill fewer of the sensitive cells.

It can be seen that Oftedal and Slizynska (1963) both postulate that there is a percentage of sensitive cells in spermatogonia, and Russell (1963) comes to the same conclusion. If it is true that there is a sensitive stage, it might be suspected that chemicals, because of their less instantaneous action, might be able to act at the right stage for the production of translocations in spermatogonia. Sonbati & Auerbach (1960) considered this as one explanation of their finding that the ratio of mustard gas induced translocations to lethals in Drosophila was not depressed during the sensitive stage which occurs in late spermatogonia. They also considered that most or all of the mustard gas induced breaks might remain latent until after meiosis so that effectively translocations were produced in post-meiotic cells. However these conclusions were based on the results of one experiment which may not have given the true picture.

The fact that chemicals can produce translocations in spermatogonia was shown by Watson (1962), who found that 2-chloroethyl
methanesulphonate (CB 1506) produced 3% translocations in spermatogonial stages. However CB 1506 produces few rearrangements relative to lethals as 20% sex-linked lethals were found in the same experiment. There is some evidence that mutagenic delay is marked after treatment with CB 1506 (Mathew, 1961), and this could be the reason for the low frequency of translocations. Since CB 1506 has a selective action on spermatogonia and only acts weakly on spermatozoa (Fahmy & Fahmy, 1956), it seemed worthwhile to use chemical mutagens with less eccentric brood patterns for a comparison between the lethal/translocation ratio in spermatozoa and spermatogonia. If the ratio decreased by a smaller amount after chemical treatment than after irradiation, this would indicate that the shortage of translocations in spermatogonia was due to obstacles that could be overcome more readily by chemicals than by X-rays, although X-rays produce so few rearrangements in spermatogonial cells after irradiation that it is difficult to compare the change in breakage frequency from late to early germ cells.

Accordingly, the chemicals used in this investigation were chosen, with the exception of streptonigrin, because they were known to produce a reasonable effect on both pre- and post-meiotic germ cell stages of Drosophila, and were tested to see if they fitted with the above hypothesis.

As mentioned above, it is probably the difficulty of determining the age of the sampled gametes in Drosophila which gave conflicting results in previous studies. The determination of cell stages by means of the brood technique is necessarily indirect and much depends on the method used. When males are treated and mated to a succession of virgin
females, the lethals obtained can be plotted against each successive brood to give a brood pattern of sensitivity. To change this into a sensitivity pattern, the brood numbers must be substituted by developmental stages. Auerbach (1954) sets out a method of detecting various stages in spermatogenesis. When adult males are treated, the sperm used during the first few days is presumed to be mature at the time of treatment. The next stage is meiosis, which can be detected in three ways. Firstly, by the occurrence of induced cross-overs, secondly by the period of sterility caused by the lack of sperm from the spermatocytes and late spermatogonia which have been susceptible to the treatment, and thirdly by the induction of non-disjunction. The third stage is young gonias which can be detected in two ways; the appearance of clusters of identical mutations or crossovers, and germinal selection, which begins to act against sex-linked lethals at this time. Auerbach, in her own particular experiments, considered samples of spermatozoa collected 10-12 days after treatment as representative of spermatogonial stages, using 3 day broods of three females per male. Other authors, applying similar methods, reached the same conclusion (Sobels & Van Steenis, 1957; Chandley & Bateman, 1960). A more direct estimate of the timing of spermatogenesis was obtained by Chandley & Bateman (1962). They labelled the male germ cells of Drosophila with tritiated thymidine, and found that spermatozoa which had been at the spermatogonial stage at the time of treatment appeared in the ejaculate after ten days of sexual activity. More recently Puro (1964) and Olivieri & Olivieri (1964), using the appearance of clusters of X-ray induced cross-overs and lethals, showed that sperm
derived from spermatogonia was utilised 9-11 days after treatment.

It is obvious that there are many variable factors which could affect the correct determination of the age of gametes, including the strains of Drosophila used and the rate of sperm utilisation, but, taking the above experiments into consideration, it was decided to use three day broods, and to take brood (d) (10-12 days) as almost certainly representing spermatogonia, and brood (e) (13-15 days) as definitely representing them.

PART 2

Before dealing with the problem with which the second part of the investigation is concerned, it is necessary to look in some detail at the mutagenic alkylating agents, as the majority of the chemicals used in this investigation are of this type.

Chemicals which can act as alkylating agents under physiological conditions have attracted great interest in the past fifteen years, because of their pronounced biological effects which are similar in some ways to the effects produced by ionising radiation. It is because of this similarity that the name "radiomimetic" was given to some of these compounds.

The process of alkylation may be defined simply as the replacement of a hydrogen atom in a molecule by an alkyl group, and the term also applies to the addition of the radical to a molecule in a lower valency state. The group R is known as an alkyl radical, and a compound which can effect this replacement of a
hydrogen atom or addition is called an alkylating agent. The alkylations of biological interest apparently all involve attachment of the alkyl group to X through an oxygen, nitrogen or sulphur atom. In the process

\[ X^- + R : Y \rightarrow R : X + Y^- \]

RY is the alkylating agent and the extent of the displacement reaction will depend on the energy characteristics of the entities involved. In such a displacement reaction it can be considered that Y attached to the alkyl group R becomes more or less detached, together with the electrons which constituted the chemical bond, and the positively charged R group then seeks electrons. Thus alkylating agents can be considered as electrophilic centres which combine with electron rich or nucleophilic centres (i.e., electron seeking). The positively charged \( R^+ \) is known as a carbonium ion and its transfer in the process of alkylation can take place by at least two mechanisms. It can be mentioned here that according as to whether alkylating agents have one, two, or more alkylating groups, they are known as mono-, bi-, or poly-functional alkylating agents respectively.

The mutagenic properties of an alkylating agent were first shown for mustard gas by Auerbach & Robson (1946), and since then many other alkylating agents have been tested and found to be mutagenic. Amongst these are: nitrogen mustard derivatives on Drosophila (Bird, 1950, 1952; Fahmy & Fahmy, 1960), and Neurospora (Westergaard, 1957); epoxides on Drosophila (Rapoport, 1948; Bird & Fahmy, 1953), E. coli (Demerec et al., 1954), maize (Kreisenger, 1960), and Neurospora (Kölmark & Giles, 1955; Westergaard, 1957); ethyleneimine and
derivatives on Drosophila (Rapoport, 1947; Fahmy & Fahmy, 1954, 1955; Cardinali, 1954), Neurospora (Westergaard, 1957), and E. coli (Szybalski & Iyer, 1960); methanesulphonates on Drosophila (Fahmy & Fahmy, 1956, 1957a; Rohrbom, 1959), barley (Heslot et al., 1959; Ehrenberg, 1960), Neurospora (Kálmark & Giles, 1955; Westergaard, 1957), E. coli and S. typhimurium (Loveless & Howarth, 1959); and 3-propiolactone in Neurospora (Smith & Srb, 1951).

What of the mode of action of these chemicals? Studies of the cytotoxic activities of alkylating agents indicated that nucleic acids were probably an important site of alkylation. The greater cytotoxicity of the bifunctional agents was ascribed to their ability to cross-link fibrous macromolecules essential for cell division (Goldacre, Loveless & Ross, 1949). They suggested that two alkylating groups per molecule were required so that the molecule reacted at two separate points. Alexander, Cousens & Stacey (1957), using nitrogen mustard derivatives, demonstrated crosslinkage of DNA molecules both free and in association with protein, and found that bifunctional agents showed a much greater effectiveness.

Brookes & Lawley (1960) established the mode of action of mustard gas with nucleic acid at low doses in vitro and in vivo, and this was shown to apply to a variety of alkylating agents (Brookes & Lawley, 1961). It was found that the most reactive site for alkylation in DNA was the N-7 atom of guanine moieties (Lawley, 1957a; Lawley & Wallick, 1957), and it was confirmed that this was the site of alkylation under conditions when only the most reactive site was attacked, i.e. at low degrees of alkylation in vitro and in vivo (Brookes & Lawley, 1960).
Similar alkylation occurred in in vitro experiments with tobacco mosaic virus RNA (Fraenkel-Conrat, 1961), and Ehrlich ascites tumour cells (Rozman & Davison, 1961). It is significant to note that Nakajima & Pullman (1958) showed that the N-7 atom of guanine is the most nucleophilic centre not involved in hydrogen bonding in the Crick-Watson model of DNA.

Earlier, in an in vivo experiment, Lawley & Brookes (1959) had injected mustard gas into mice bearing 7 day old ascites tumours. After 15 minutes the tumour cells were removed and fractionated in order to obtain the RNA, DNA, and protein components. Alkylation of guanine moieties was the only reaction which could be established by paper chromatography and autoradiography of acid hydrolysates. A comparison was then made in vitro between Myleran, a weakly reactive bifunctional agent; half sulphur mustard, a highly reactive mono-functional agent; and mustard gas, a highly reactive bifunctional agent (Brookes & Lawley, 1961). With regard to reaction with nucleic acids, the only difference between the two mustards was found to be that the mustard gas yielded in addition to 7-alkylguanine, a cross-linked guanine product di-β-(guanin-7-yl) ethyl sulphide.

Following the initial alkylation of DNA, further slow chemical changes occur at neutral pH. The alkylated guanines split off (Lawley & Wallick, 1957; Lawley, 1957b; Brookes & Lawley, 1961) and fission of the sugar phosphate chain begins, the latter process continuing for many days after treatment.

If the difference in biological effect is to be ascribed to a difference in the way they react with nucleic acids, then the
formation of the di-(7 guaninyl) derivative must be of importance. Examination of the Crick-Watson model of DNA shows that two guanine moieties on opposite strands of the twin spiral of DNA could be linked by an alkyl chain of four or five atoms, provided that the sequence of bases along the DNA chain was guanine-cytosine in one direction only, i.e. so that one guanine molecule would be within the required distance of another on the other strand, viz.

\[
\begin{array}{c}
\text{G} \\
\text{C} \\
\text{C} \\
\text{T} \\
\text{A}
\end{array}
\]

Other evidence that the cross-linking of the twin strands of DNA by bifunctional alkylation does occur has been obtained, the most direct being that the process of "renaturation" of denatured DNA is more nearly reversible with DNA treated with a bifunctional alkylationg agent than with untreated DNA (Geiduschek, 1961; Lawley et al., as reported in Lawley & Brookes, 1963). The process of denaturation of DNA involves heating a solution of it so as to cause a separation of the strands, which on cooling do not normally return to the double stranded structure. But if this structure is anchored by interstrand cross-links, a return to the previous state is much more likely. Such an effect was shown by the above authors for DNA treated with the bifunctional agents di-2-chloroethyl methylamine and mustard gas, but not with the monofunctional agent 2-chloroethyl 2-hydroxyethyl sulphide. Also the yield of di-guaninyl derivatives decreases in passing from undenatured DNA to denatured DNA to RNA, i.e. as the extent of the twin spiral type structure decreases. Bifunctional alkylation is also more effective than monofunctional in
causing a decrease in the molecular weight of DNA (Lawley et al., 1960).

This latter effect can be explained by the fact that following alkylation of DNA by a monofunctional agent at a low extent of alkylation, breaks in either of the twin strands of the molecule at separated points would occur, which would not lead to a decrease in molecular weight, but when a bifunctional agent was used cross-linkage between the strands would eventually give rise to complete breaks and thus cause a decrease in molecular weight of the DNA.

Mutagenic effects of alkylation can be attributed to the splitting off of 7-alkylguanine products from alkylated DNA. Following deletion of alkylated guanine it is possible that the strand of DNA affected could still serve as a template for duplication, yielding a DNA identical with the original except for loss of a guanine-cytosine pair of bases. This would constitute mutation by loss. If a repair mechanism is envisaged, then the substitution of an incorrect base could be proposed as a mechanism for mutation. This subject is dealt with more fully in the discussion.

It has been claimed that bifunctional agents are more efficient chromosome breakers because of their ability to cross-link DNA (Fahmy & Fahmy, 1961). However it is known that some monofunctional agents are capable of breaking chromosomes, e.g. 3-propiolactone (Smith & Srb, 1951), and ethylene oxide (Nakao & Auerbach, 1961). Thus monofunctional alkylating agents are included in the chemicals used in this investigation so that the nature of the genetic changes induced by them can be compared with those of the bifunctional agents. A full list of all the chemicals used is given in the Materials & Methods Section.
The fact that some monofunctional alkylating agents are as efficient as bifunctional ones in chromosome breaking ability (Nakao & Auerbach, 1961; Alexander & Granges, 1964) means that the relative mutagenic efficiency of the two types of chemical is by no means clear. No qualitative difference has been found between them, although it has been claimed that quantitative differences exist (Fahmy & Fahmy, 1961).

In an attempt to clarify the situation, one phenomenon which might have an important connection with the action of alkylating agents is also investigated. Herskowitz (1955, 1956) first discovered that when mature *Drosophila* sperm were treated with triethylene melamine (a polyfunctional alkylating agent) and stored for periods of up to two weeks in females, the frequency of induced translocations rose steeply, while the recessive sex-linked lethal frequency remained essentially the same as did X-chromosome loss. Using nitrogen mustard, Schalet (1955) obtained similar results. However, as the sperm were treated in the females by means of post-copulatory vaginal douches, it is possible that indirect effects due to the cytoplasm or ovaries also being affected may have arisen. Evidence of this is shown by the fact that Schalet (1956) obtained a translocation between a paternal and a maternal chromosome in a storage experiment.

In 1963 Snyder carried out a similar experiment in order to see if point mutations and chromosome breaks responded differently to storage. He treated *Drosophila* males with triethylene melamine, and mated them to females in which the sperm were stored for six days. This eliminated the possibility of the maternal tissue being affected by the chemical. After the storage period, he found that the frequency
of translocations had risen sharply, while the sex-linked lethal frequency had risen to a lesser extent. This increase in lethals was correlated with a relative increase in translocation frequency in any one experiment, and on the assumption that sex-linked lethals related to chromosome breakage would be expected to increase in the same proportion as translocations, approximate agreement was obtained when the proportion of breakage-related lethals was estimated from the data in his experimental series. His data were consistent with the hypothesis that chromosome breakage, but not point mutations, increased during the storage period. This hypothesis is substantiated by the fact that Slizynska (unpublished) examined cytologically translocations induced before and after storage and found that after storage there were more breaks per translocation.

Since triethylene melamine is a polyfunctional alkylating agent, capable of cross-linking DNA as described above, it was decided to carry out a series of experiments similar to Snyder's, using ethylene imine, a monofunctional agent closely related to triethylene melamine, in order to see if the storage effect was related in any way to the structures of the two chemicals. Since the technique was slightly modified and different stocks of Drosophila used, the triethylene melamine experiment was repeated for comparison. (The results of this series of experiments have already been published (Watson, 1964).

A number of sex-linked lethals obtained in pre- and post-stored broods after treatment with triethylene melamine were examined cytologically for the presence of large rearrangements, the post-stored ones having first been located so that small deficiencies could also be
looked for. This cytological part of the investigation forms a corollary to the storage experiments, and was carried out in order to determine whether the number of lethals associated with chromosome breakage increased during the storage period.
MATERIALS AND METHODS

1. STOCKS

(a) The Oregon-K Stock

This is a wild-type stock of *Drosophila melanogaster* which has been kept in this laboratory for many years. Its spontaneous mutation rate is known to be about 0.3%.

(b) The 0-1;bw;st Stock

This stock was used to test simultaneously for sex-linked lethals and translocations involving the Y, II and III chromosomes. The genotype of the X-chromosome is \( Y^{s} so^{s1} \text{Im}^{49} so^{3} \). It includes the long inversion \( so^{3} \) within which is contained the inversion \( \text{Im}^{49} \). The entire X-chromosome is inverted and has the recessive marker yellow \( (y) \) at its base. The two inversions inhibit crossing-over along the whole length of the X-chromosome. In addition the second chromosome carries the recessive marker brown \( (bw) \), and the third carries the recessive marker scarlet \( (st) \).

(c) The 0-1 Stock

This stock was used for the detection of sex-linked lethals. It is similar in genetic constitution to the above stock except that it carries no recessive autosomal marker genes.

(d) The bw;st Stock

This stock was used for the detection of translocations. The autosomal recessive markers \( bw \) and \( st \) are carried on the second and third chromosomes respectively.
(e) The Muller-5 Stock (M-5)

This stock was used to carry lethals which were to be located. Its X-chromosome carries the dominant marker Bar (B) and the recessive marker apricot (w^a), and has two inversions, the sc^{S1} inversion and inversion-S, the latter being included in the former. These inversions completely inhibit crossing-over along the whole length of the X-chromosome. The formula of the stock can be written as sc^{S1} B In-S w^a sc^8.

(f) The Scar Stock

This stock was used for the detection of sex-linked lethals. In the males of the stock, the X-chromosome carries the recessive markers scute (sc), cut (ct), vermilion (v), forked (f), and carnation (car). The stock is maintained by crossing the males to attached-X females of the genetic constitution v v f^= f. The location of the five markers on the chromosome is shown on the following diagram.

<table>
<thead>
<tr>
<th>sc</th>
<th>ct</th>
<th>v</th>
<th>f</th>
<th>car</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>20</td>
<td>33</td>
<td>56.7</td>
<td>62.5</td>
</tr>
</tbody>
</table>

(g) The y w Stock

Males from this stock were crossed with females heterozygous for a sex-linked lethal so that the F_1 larvae required for cytology could be recognised and at the same time be transferred to a stock with a straight X-chromosome. The stock carries the recessive markers yellow (y) and white (w) on the X-chromosome.
II. TREATMENT

In all the experiments, only adult Oregon-K males were treated. They were collected when they were about 6 hours old and then kept at 25°C until they were two days old, when they were then treated. This ensured that the flies were of similar age and had had as uniform a development as possible. Kaplan (1954), using mustard gas, found a variation in dominant lethality between flies which were one day old and flies which were seven days old at the time of treatment, the older flies showing a higher frequency. The frequency of sex-linked lethals was not significantly different, but his results do show that some age effect does exist.

The chemicals used in this investigation were administered either by injection or by feeding. The techniques were as follows:

(a) Injection

The injections were carried out by means of a hand-drawn micropipette fitted with a rubber bulb and held firmly in a clamp. The flies were etherised, and using a pair of fine watch-maker's forceps one was picked up by the wings close to its body and brought against the tip of the pipette. A gentle pressure caused the tip to enter the soft lower abdomen, and then approximately 0.2 μl. of a solution of the chemical was administered. The fly was then withdrawn. If any liquid was seen to be escaping from the wound, the fly was discarded. If not, it was placed in a fresh vial to recover.

Carlson & Oster (1962) injected flies with methylene blue and found that most of them lost some of it as shown by the fact that spots of the dye were found on the side of the food vials. For this
reason it was not deemed necessary to measure with great accuracy the amount of chemical injected into each fly. Instead, one fly was injected with exactly 0.2µl by means of an Agla micrometer syringe before the experiments were begun, and the degree of swelling of the abdomen noted. This was used as a guide for all the other flies injected.

The injections were carried out under a binocular dissecting microscope of magnification 15X, and the chemicals were dissolved in 0.7% saline.

(b) Feeding

This particular method of adult feeding was first used by Pelecanos and Alderson (1963). Double layers of filter paper were placed at the bottom of half-pint milk bottles and a few drops of a solution of heat-killed yeast dropped on the centre of them. The paper was then saturated with a 5% solution of glucose containing the desired concentration of the chemical, and the Oregon-K males allowed to feed on this for the required length of time. During this time the filter paper was kept wet by the addition of a few drops of the glucose solution from time to time. The chemicals administered in this manner were diethyl sulphate, heliotrine, and dimethyl nitrosamine.

III. BREEDING TECHNIQUES

The treated males were mated individually to three virgin females of the O-1 bw; st stock, 24 hours after treatment in the case of the injected flies, and immediately in the case of the fed flies. After three days the males were mated again to fresh virgins and this was repeated every three days until four or five three day broods had been
obtained. The virgins used in the second and third broods served only as concubines, utilising the sperm of the males. This continual sexual activity of the males meant that progressively younger germ cells were sampled in each successive brood. The progeny of the first, fourth and fifth broods were tested according to the genetical tests outlined below, and in some cases only the first and fourth broods were tested. Only males which had given progeny in all broods were tested. The temperature throughout was 25°C.

The technique in the storage experiments was to prevent the P₁ females from laying eggs over a period of six days. This was done in the following manner. At the end of the third day the females (also of the O;1;byw;st stock) were placed in vials containing a food medium consisting of 4% agar and 2% sucrose. The vials were then kept at 25°C, underneath an electric fan which prevented the food from becoming sticky. After three days the females were transferred to fresh vials of the same food where they remained for the last three days of storage. Egg-laying was almost completely inhibited on this medium, and the survival rate was about 75%. After storage the females were placed on normal food medium and allowed to lay again for up to three three day periods in order to obtain as many progeny as possible.

The treated males used in these experiments were either discarded after the pre-stored period or later broods were obtained from them for testing as above. The pre-stored and post-stored broods were tested for sex-linked lethals and translocations.
IV. GENETICAL TESTS

(a) Test for recessive sex-linked lethals

The $F_1$ females were used for the sex-linked lethal test. They were mated to males of the O-1 stock, two males to each female. As the $F_1$ males also carried the O-1 chromosome, the females did not require to be virgin. The $F_2$ cultures were scored for lethals and the absence of wild-type males indicated the presence of one. The mating scheme is outlined below.

\[
\begin{array}{ccc}
O-1 & \times & O-1 \\
\hline
\end{array}
\]

$F_2$ males: $O-1$ : 

\[
\begin{array}{ll}
\text{yellow} & \text{wild-type} \\
\text{body} & \text{(dies if lethal)} \\
\hline
\end{array}
\]

Cultures containing ten or more yellow body and no wild-type males were scored as lethals. Doubtful cases were retested by mating the wild-type females to O-1 males and scoring the progeny as before.

(b) Test for translocations involving the Y, II and III chromosomes

The $F_1$ males were used for the translocation test. These males, which had an untreated X-chromosome, a treated Y-chromosome, and were heterozygous for treated II and III chromosomes, were mated individually to two virgin females of the bw;st stock. The $F_2$ cultures were examined without etherisation under the binocular microscope, and any doubtful culture was etherised and examined more closely.
If no translocation had occurred, the F₂ culture would consist of four phenotypic classes in equal proportions expected by Mendelian segregation; namely wild-type, brown, scarlet, and brown scarlet (white). If a translocation had occurred between the:

(i) II and III chromosomes, only wild-type and brown scarlet flies would be found.

(ii) Y and II chromosomes, all males would be wild-type and brown, while the females would be scarlet and brown scarlet.

(iii) Y and III chromosomes, all males would be wild-type and scarlet, while the females would be brown and brown scarlet.

(iv) Y, II and III chromosomes, all males would be wild-type and all females brown scarlet.

Markers in the chromosomes involved behave as though they were linked. The gametes which receive an unbalanced chromosome set from the males carrying a translocation would produce inviable zygotes, while balanced gametes would form a new linkage relationship for genes located within the translocated region. Schemes showing the method of detection of a translocation between II and III and between Y and II are outlined in figures 1a and 1b.

All presumed translocations were confirmed by mating the wild-type males to bw;st virgins and scoring as above.

(c) Location test for sex-linked lethals

Since the lethals to be located were kept balanced against the O-1 chromosome, females heterozygous for the lethal had first to be crossed to M-5 males, so that the lethal could be balanced against the M-5
If a translocation has been produced the resulting F$_6$ male will be heterozygous for it:

\[ \text{bw} \quad \text{st} \]
\[ \text{bw} \quad \text{st} \]

At meiosis balanced and unbalanced gametes are formed. w.r.t. II and III

Gametes:
(i) Balanced
(ii) Unbalanced due to duplication in one chromosome and a deficiency in the other.

On Fertilisation, only the balanced zygotes survive, therefore when the F$_6$ is crossed with a bw; at virgin $\exists$, only the following progeny are found:

10 wild type and white flies only are found.
Figure 1b  Scheme showing the detection of a translocation between chromosomes Y and II.

(b)  If a translocation has been produced the F1 will be:

\[
\begin{array}{c}
\ x \\
\ \downarrow \ \text{II} \\
\ \downarrow \ \text{III}_{a1} \ \\
\ \downarrow \ \text{at} \\
\end{array}
\]

At meiosis, balanced and unbalanced gametes are formed w.r.t. X, Y and II

(i) Balanced  
\[
\begin{array}{c}
\ \downarrow \ \text{bw} \\
\ \downarrow \ \text{at} \\
\end{array}
\]

(ii) Unbalanced  
\[
\begin{array}{c}
\ \downarrow \ \text{bw} \\
\ \downarrow \ \text{at} \\
\end{array}
\]

only the following progeny are found after crossing the F1 to a bw; at virgin R.

\[
\begin{array}{c}
\text{at} \\
\ \downarrow \ \text{bw} \\
\ \downarrow \ \text{at} \\
\text{dd} \\
\ \downarrow \ \text{bw} \\
\ \downarrow \ \text{at} \\
\end{array}
\]

wild-type and scarlet dd only.  whit and brown DD only.
chromosome instead. This was necessary as the M-5 chromosome carries
the dominant marker Bar which enables easy identification of a M-5/lethal
female. The transfer had to be carried out over two generations as it
was not possible to distinguish between M-5/lethal flies and M-5/0-1 flies
without crossing the F₁ females individually to M-5 males and examining
the progeny. If only M-5 males were produced then the F₁ female must
have been M-5/lethal. In that case, the heterozygous Bar daughters,
which would again be M-5/lethal, were used for the location test.
Virgins were crossed to scar males, and the F₁ non-Bar females (Scar/lethal)
were crossed to M-5 males. 400-500 F₂ males were scored for crossing-over
in respect to the five markers. From the types of fly obtained the lethal
could be placed in one of the four regions into which the X-chromosome is
divided by the five marker genes. The exact location was determined from
the number of flies showing one of the two markers limiting this region.
The following example shows how it is done.

<table>
<thead>
<tr>
<th>Classes</th>
<th>No. of flies scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>sc ct v f car</td>
<td>219</td>
</tr>
<tr>
<td>sc</td>
<td>0</td>
</tr>
<tr>
<td>ct v f car</td>
<td>57</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>0</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>71</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>23</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>87</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>25</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>0</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>1</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>1</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>16</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>2</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>2</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>8</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>1</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>1</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>1</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>1</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>1</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>1</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>1</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>1</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>1</td>
</tr>
<tr>
<td>sc ct v f car</td>
<td>1</td>
</tr>
</tbody>
</table>

Total 514
The above data shows that the lethal lies between vermilion and forked, as this region contains the complementary crossover classes. From the number of males it is possible to calculate the position of the lethal and its standard error. The more males in one class the nearer the lethal is to that marker.

<table>
<thead>
<tr>
<th>Single cross-overs</th>
<th>Double cross-overs</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>v + f</td>
<td>23</td>
<td>3</td>
</tr>
<tr>
<td>+ v f</td>
<td>87</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The region between vermilion and forked which is 23.7 crossover units long is divided in the ratio 105/26. The lethal is thus located as follows:

Distance between vermilion and lethal = \( \frac{105 \times 23.7}{131} \)

\[ = 18.9 \]

Thus distance from lethal to forked = 4.8

Standard Error = \( \sqrt{\frac{4.8 \times 18.9}{131}} \) = 0.83

Therefore lethal is located at 51.9 ± 0.83.

V. LIST OF CHEMICAL MUTAGENS USED

The chemical mutagens used in this investigation are listed below, along with their structure. Previous results obtained with them are discussed at more length in the Results and Discussion.

(a) Monofunctional alkylating agents

1. Diethyl sulphate (DES)

\[ \begin{align*}
\text{C}_2\text{H}_5 \quad \text{SO}_4^- \\
\text{C}_2\text{H}_5 
\end{align*} \]
This chemical was first tested for mutagenic effects in *Drosophila* by Rapoport (1947, 1961). Experiments by Pelecanos & Alderson (1963) and Alderson & Pelecanos (1964a, b) showed that it was a very effective mutagen in spermatozoa and reasonably effective in spermatogonia. Since it is not miscible with water, 33% alcohol was used in the feeding treatment instead of water in order to disperse the globules otherwise formed. It was obtained from British Drug Houses Ltd.

2. Dimethyl nitrosamine (DMNA)

\[
\begin{align*}
\text{CH}_3 & \quad \text{N-No} \\
\text{CH}_3 &
\end{align*}
\]

Clark (1960) and Pasternak (1962) found that DMNA was mutagenic in *Drosophila*. They administered it by injection, and found that it had a strong sterilising effect. Since Clark (1963) had found that the sterilising effect of heliotrine was lessened by feeding it to adults rather than injecting it, it was decided to use this method, and it was found that it was successful in producing mutations in pre- and post-meiotic stages, although a sterilising effect still was apparent. The DMNA used here was kindly given by Dr. P.N. Magee.

3. Ethylene Imine (EI)

\[
\begin{align*}
\text{H} & \\
\text{N} & \\
\text{H}_2\text{C} & \quad \text{CH}_2
\end{align*}
\]

This chemical was obtained from L. Light & Co. Rapoport (1947) first showed that EI was mutagenic in *Drosophila*, and since then many other investigators have shown it to be a potent mutagen.
4. Quinacrine Mustard (ICR 100)

2 methoxy-6-chloro-9-(3-(ethyl-2-chloroethyl)) aminopropylamino acridine dihydrochloride.

This chemical was kindly sent by Dr. E.A. Carlson. The work of Oster & Pooley (1960) and Carlson & Oster (1961, 1962) showed that this monofunctional agent showed a strong mutagenic effect on pre- and post-meiotic stages in *Drosophila*.

(b) Bifunctional alkylating agents

1. Diepoxybutane (DEB)

Butadiene diepoxide

\[
\text{CH}_2=\text{CH}-\text{HC}=\text{CH}_2
\]

This is a well known mutagen in *Drosophila* (Bird, 1950; Bird & Fahmy, 1952; and others). It was obtained from Columbia Organic Chemicals Co. Inc.

2. Triethylene melamine (TEM)

2:4:6-tri-(ethyleneimino)-1:3:5-triazine

[Diagram of Triethylene Melamine]
This chemical was obtained from Imperial Chemical Industries. Its mutagenic properties have been shown in numerous experiments (Fahmy & Bird, 1953; Fahmy & Fahmy, 1955a,b; Herskowitz, 1955; and others).

(c) Heliotrine.

Heliotrine is a pyrrolizidine alkaloid isolated from *Heliotropum europaeum*. The sample used here was kindly sent by Professor A.M. Clark, who in 1959 was the first to demonstrate its mutagenic activity in *Drosophila*.

It is known that heliotrine is probably responsible for liver damage in sheep (Bull, 1955), that it can produce liver tumours in rats (Schoental & Head, 1955; Schoental & Magee, 1955) and chromosome breakage in Plants (Avanzi, 1961) and rat intestinal epithelium (Bull, in Culvenor et al., 1962) as well as being mutagenic in *Drosophila*. It can thus be said that there is a resemblance between the alkaloid and the biological alkylating agents which also give rise to mutagenesis, chromosome breakage, and carcinogenesis, and it is thus possible that this similarity in biological properties is reflected in a similarity of mechanism of action. It was suggested by Culvenor et al. (1962) that as the pyrrolizidine alkaloids are alkylc esters, they might be able to function as alkylating agents by a mechanism involving alkyl-oxygen fission of the alkyl linkage, viz.
The basic formula of an alkaloid such as heliotrine is:

\[ \text{alkyl group} \]

Where \( R' = \text{H, OH, or O-acyl} \); \( R = \text{alkyl group} \).

The reaction would result in the displacement of the anion \( R'_2\text{CO}_2^- \) by a nucleophilic group \( X^- \). This would give either:

\[ \begin{align*}
R' & \quad \text{CH}_2X \\
N & \quad \text{N}
\end{align*} \]

or

A close parallel exists in several known alkylating agents which are esters and can react by the same mechanism, e.g., \( \beta \)-propiolactone (Ingold, 1953) and the alkylsulphonic esters such as Myleran (Davies & Kenyon, 1955). Culvenor et al. demonstrated in vitro that heliotrine reacted with a nucleophilic molecule as an alkylating agent, and preliminary experiments showed that it also reacted with cysteine. It is possible that the high degree of protection given by cysteine against chromosome breakage in Allium roots (Avanzi, 1961) might be due to preferential recombination with cysteine. It must be pointed out that the reactions of heliotrine as an alkylating agent are of a much lower order than those of the classical alkylating agents, and since in the present experiments heliotrine produced mutation frequencies comparable to those of the alkylating agents, it is possible that it acts by some other mechanism also.
(d) Streptonigrin (SN).

![Chemical Structure of Streptonigrin](image)

It was decided to carry out a small pilot experiment with this chemical in *Drosophila* after Cohen *et al.* (1963) found that it showed marked chromosome breaking abilities in cultured human leucocytes. It was isolated from *Streptomyces flocculus* by Rao & Cullen (1959). The sample used here was obtained from Charles Pfizer & Co.
VI. CYTOLOGICAL TECHNIQUES

Females carrying a sex-linked lethal balanced against the O-1 chromosome were crossed to \( yw \) males. Yellow can be distinguished in the larval stages as flies homozygous for the marker have brown mouthparts compared with the black ones of the wild-type. Since the progeny of the above cross consists of O-1 males which are yellow, O-1/\( yw \) females which are also yellow, and lethal/\( yw \) females which are wild-type in body colour, the larvae of the latter type, which are the ones required for cytology, can be easily recognised. Also, as the O-1 chromosome carries two inversions, the outcrossing to \( yw \) also puts the lethal over a chromosome free of any such rearrangement that would make cytological examination more difficult.

The salivary glands of the larvae were dissected out in a drop of solution made up of \( \bar{N} \) HCl in 0.7% NaCl. This solution greatly facilitates the dissection of the glands and the removal of the fat bodies from them. The glands were then transferred to a drop of stain which was aceto-carmine in the case of the pre-stored lethals, and aceto-orcein (containing 20% lactic acid) in the case of the post-stored lethals after it had been found to give a better result. The time of staining was five minutes in aceto-carmine and twenty to twenty five minutes in aceto-orcein. The glands were then squashed under an albuminised cover-slip on a slide which had been previously siliconised by immersing it in "Silicone Repelcote". The preparation was then examined under the microscope and if satisfactory, transferred to a slide jar containing 95% alcohol. The cover-slip floated free in a short time, and the preparation was made permanent by taking it through three changes of absolute alcohol, two
changes of xylol, and mounting it in Canada Balsam.

For cytological examination Bridges' reference system (1935) was used.
RESULTS

I. GENETICAL RESULTS

Before being considered as a whole, the results obtained with each chemical are dealt with in turn below.

(a) Triethylene melamine

Two experiments were carried out using TEM, and both gave similar results, which are summarised in Table 1. After it was found in the first experiment that 1% lethals and no translocations were found in brood (e), only broods (a) and (e) were tested in the second experiment. The overall mutation rate was lower than that obtained in previous experiments using the same concentration of the mutagen (Reddi & Auerbach, 1961; Snyder, 1963), but this could have been due to the fact that different strains of Drosophila were used or the age of the TEM when administered. It is however quite clear that a dose of TEM producing an average of 2.3% lethals in brood (d) gave rise to 0.15% translocations, i.e., in cells that were spermatogonial at the time of treatment.

(b) Ethylene imine

The results of the EI experiments are summarised in Table 2. In experiment 1 where the dose was $1.2 \times 10^{-2}$ M, the frequency of lethals in brood (a) was 3.4%, decreasing to 2.7% in brood (d). The lethal frequency in brood (a) is in good agreement with that obtained by Alexander and Granges (1964), who found 3.58% lethals using the same dose. However, these authors found 2.07% translocations compared with 0.65% in experiment 1. It was decided to repeat the experiment using double the dose to try to produce a greater effect even though the first dose produced 0.16% translocations in brood (d). This second concentration
was more effective in brood (a), producing 7.3% lethals and 1.5% translocations, but produced almost the same effect in brood (d) as the first experiment (2.6% lethals and 0.26% translocations), an interesting result, for one would expect higher frequencies after the increased dose.

(c) Heliotrine

As previously mentioned, Clark (1959) demonstrated the mutagenic activity of heliotrine in Drosophila. In these first experiments, he found that two sensitive periods appeared to exist after injection of adult males with 0.0% (0.001M), one in brood (a) and one in brood (d). He also found a sterilising effect, obtaining low numbers of progeny in the late broods. The chemical seemed to block the maturation of immature germ cells, so that as soon as the available spermatozoa and spermatids had been utilised, the males' breeding capacity was severely reduced, although they remained alive and active for up to three weeks after treatment. These findings of Clark were confirmed in the first heliotrine experiment, when approximately the same concentration was used. As can be seen in Table 3, the frequencies of sex-linked lethals in broods (a) and (d) were 5.5% and 6.6% respectively, while 0.6% translocations were produced in (a) and none in (d), the latter result being probably due to the fact that only a small number of chromosomes were tested owing to the sterility of the treated males. Brood (e) was completely sterile. After Clark (1963) had found that by reducing the dose considerably in one experiment, and feeding the heliotrine to the adult males in another, one peak of sensitivity was found in the fourth brood, it was decided to use the same methods, and the results of these two experiments are shown in Table 2. Since Clark used two day broods
instead of three day ones as in his first experiments, the peak of sensitivity probably occurred in brood (c), but nevertheless a reasonable mutagenic effect was produced in brood (d) and in brood (e) in the third experiment. Again no progeny was obtained in brood (e) in the second experiment, although in brood (d) a reasonable number of progeny were obtained, which gave rise to 6.5% lethals and 0.18% translocations. In the third experiment, the lethal and translocation frequencies are almost the same in broods (a) and (e). This suggests that spermatozoa and spermatogonia are equally sensitive to the mutagenic action of heliotrine when it is administered by feeding, but the data are too small to draw any definite conclusions.

(d) **Diepoxybutane**

One experiment was carried out using DEB, and the results are summarised in Table 4. They agree well with the results obtained by Bird & Fahmy (1953), who used the same concentration. The most interesting finding was that the lethal/translocation ratio showed the smallest increase from brood (a) to brood (d) of all the chemicals tested.

(e) **Quinacrine mustard**

It was decided to test this chemical after it had been found to exert a strong mutagenic effect on spermatogonia as well as spermatozoa (Carlson & Oster, 1962). The results, summarised in Table 5, show that ICR100 produced the highest lethal frequency in spermatogonia of all the mutagens tested (18.8%) as well as being very effective in spermatozoa, where the lethal frequency was 7.5%. Despite these high frequencies of lethals, ICR100 proved to be a very poor chromosome breaker, producing no rearrangements in broods (a) or (d). One translocation was found in brood (e), which
is surprising, since the other chemicals producing rearrangements at this stage also produced them in mature germ cell stages. Snyder & Oster (1964) also found that ICR100 was a poor chromosome breaker. They obtained 0.24\% translocations along with 12.26\% lethals using the same dose. This was in mature sperm, so perhaps the fact that no translocations were found in brood (a) in the present experiment is because the number of chromosome sets tested was insufficient.

(f) Dimethylnitrosamine

The results of two experiments using DMNA are summarised in Table 6. In the first experiment, using a $0.13 \times 10^{-4}$M concentration fed to adult males, the later broods were completely sterile, although the treated males remained alive and active. On reducing the dose to $0.6 \times 10^{-5}$M fertility was regained, but not until the fifth brood when a frequency of 10.7\% sex-linked lethals was produced. It is also seen from the results that no translocations whatsoever were produced.

Clark (1960) injected a 10\% solution of DMNA, and tested five three day broods for the production of sex-linked lethals and, obtained progeny from all the broods, although the numbers were very small indeed. The frequencies he obtained were 9\%, 11.7\%, 6.4\%, 2.7\% and 1.6\% respectively. The toxicity was also high in his experiment, only 20\% of the treated males surviving.

From the results obtained here, it appears as if DMNA exerts a killing effect upon spermatids, spermatocytes, and late spermatogonia, but that the early spermatogonia represented in brood (e) are partly resistant to this effect.
(g) Diethyl sulphate

When a 0.5% concentration of DES was fed to adult males, the same concentration as that used by Pelecanos & Alderson (1962), it was found to be completely toxic to the flies, and not until the dose was reduced to 0.2% did sufficient flies survive the treatment. The results are shown in Table 7. It is seen that the chemical induced a poor response in late broods, although 10% lethals were produced in brood (a). No translocations were found in any brood, and it was not considered worthwhile to carry out any more experiments using this chemical.

Recently Alderson & Pelecanos (1964) investigated the sensitivity of the Drosophila testis to DES and found that spermatogonia gave a sex-linked lethal rate of about 2-3% after treatment with 0.5%. They also found that no rearrangements were produced in any stage, agreeing with the results of this investigation, and those of Heiner et al. (1960) in barley.

(h) Streptonigrin

The results of the pilot experiments with SN are summarised in Table 8. The most striking feature of the results is the strong sterilising effect even at the low concentrations used. In the first experiment, using a concentration of $1 \times 10^{-6}M$, the males were completely sterile by the third brood, and fertility was not recovered. This sterility and the low numbers of progeny obtained in brood (a) indicate that SN has a strong killing effect on all stages of spermatogenesis. The fact that only 1.4% lethals and 0.5% translocations were produced in brood (a) is very puzzling, for with such a sterilising effect many more mutations would be expected. A dominant lethal test would help to elucidate this point.
When a reduced dose of $1 \times 10^{-9} \text{M}$ was injected, progeny was obtained from broods (a), (d) and (e), but the mutation rates were very low. However one translocation was produced in brood (e), although the lethal rate was below control values.

Cohen et al. (1963), studying the effect of SN on cultured human leucocytes, found that it inhibited mitoses and produced extensive chromosome breakage and rearrangements when administered in extreme dilutions ($10^{-7}$-$10^{-9} \text{M}$), while Kihlman (1964) found it to be a potent chromosome breaker in Vicia faba at a concentration of $10^{-5} \text{M}$. It thus seems likely that the strong sterilising effect of SN is due to the fact that the germ cells of Drosophila are extremely susceptible to the chemical. Another interesting feature is that in the experiments of Cohen et al., a significant increase in the frequency of chromosome aberrations was found when SN was added to the medium as late as two hours before the harvest of the culture. This suggests that SN is able to break the chromosomes in the $G_2$ period of interphase, i.e. after DNA synthesis.

General consideration of results

Only four of the chemicals used produced translocations in both spermatozoa and spermatogonia, namely TEM, EI, heliotrine, and DEB. In addition, ICR100 produced one translocation in spermatogonial cells. All the frequencies obtained in spermatogonia were low, ranging from 0.15% to 0.3%, and the results show that the chemicals producing the highest frequencies of translocations in spermatozoa did not necessarily do the same in spermatogonial stages. For example, the second EI experiment produced 2.5% translocations in brood (a) and 0.26% in brood (d), while the
third heliotrine experiment gave 0.18% and 0.25% respectively.

When the lethal/translocation ratios in spermatozoa and spermatogonia are compared, it increases in each case, but by varying degrees. It is doubled in the TEM and heliotrine experiments, trebled in the EI experiments, and increased by 50% in the DEB experiment. One fact which is quite clear from the results is that high sex-linked lethal frequencies were not reflected in a correspondingly high translocation frequency in any brood. Indeed, all the spermatogonial translocations were induced by chemicals which gave low lethal frequencies at this stage, with the exception of ICR100.

It is also seen from the results that when the monofunctional and bifunctional compounds are compared, no real difference can be made between them. The bifunctional agents (TEM and DEB) both produced lethals and translocations, while of the monofunctional agents, EI was more effective than the bifunctional ones in producing translocations, ICR100 produced the highest lethal frequencies, and DMNA and DES produced no breakage at all. As explained in the Materials section, heliotrine may act as an alkylating agent, though it is by no means certain that it does so. The fact that both types of alkylating agent can produce similar mutagenic effects gives a confusing picture when any attempts are made to differentiate between them. However, the results given in the next section show that a difference does exist.

The storage experiments

The results of the storage experiments are summarised in Tables 9-13 and in Figure 2. In the TEM and EI experiments, it is seen that in the pre-stored period, although much higher concentrations of EI
were used, the relative chromosome breaking abilities of the two compounds, expressed by the ratio of translocations to recessive sex-linked lethals in Table 10, were approximately the same. After storage, however, the translocation/lethal ratio in the TEM experiments rose steeply. This was due to the fact that the frequency of translocations increased 8-10 fold, while the frequency of lethals rose only by a small amount. The histogram in Figure 2 shows clearly the change after storage. This finding agrees well with the results of Snyder (1963). In contrast, there was no effect of storage on either the translocation or lethal frequencies in the EI experiments. This is a most important result, and its significance is discussed below.

There is some evidence that the environment of the sperm in the female might affect the breaks produced in the paternal chromosomes. It was shown by Bonnier (1954) and Bonnier & Löning (in Muller, 1954) that by ageing or irradiating eggs, the manner of reunion of broken chromosome pieces brought in by the sperm could be affected. Herskowitz (1958; 1963) reported that the physiological condition of the female could influence the gross chromosomal mutation frequency scored from X-rayed sperm. He found that when undernourished females were inseminated, then irradiated, and four four day broods taken from them, the frequency of translocations increased over the four broods. It must be pointed out, however, that the increase he obtained was of the order of 50% over the four broods compared with the 800% to 1000% increase found in the present experiments. Herskowitz put forward the hypothesis that the increase in breakage was due to some effect of the undernourishment of the females upon the rejoining of chromosome ends for, if the females were well nourished
throughout, no increase in translocation frequency was found. The P1 females used in the present experiments were kept well nourished before they were mated to the treated males, so presumably an effect similar to that of Herskowitz would not occur. Even if it did, it would not affect the results to any great extent.

Also it is quite clear that the storage effect is due solely to the chemical treatment. The fact that after treatment with EI, no storage effect was found, means that no other factors such as the condition of the females were responsible for any part of the storage effect observed in the TEM experiments. This is also confirmed by the results of the storage experiment using ICR100, the results of which are summarised in Table 13. This chemical, which is a monofunctional alkylation agent and has already been shown to be a very poor chromosome breaker (see Table 5), produced no translocations in either the pre- or post-stored periods, while the frequency of sex-linked lethals remained the same. If the period of storage caused any chromosome breakage other than might be due to chemical treatment, it would be expected that the post-stored brood might produce a few, but it obviously does not.

Table 11 gives the frequency of sex-linked lethals and translocations in three successive post-stored broods following treatment with TEM. It might be expected that the frequencies would increase with each successive brood, as the sperm has been in the females for three days longer in each, but there is no significant increase from brood to brood. This could conceivably be due to two reasons. Firstly, that the storage effect has reached its maximum after six days, or secondly, that the resumption of egg-laying by the females affects the sperm in some way,
preventing further effects. Snyder (1963) found a smaller storage effect at a lower temperature (12.5°C) and it seems most likely that the first hypothesis is the most plausible. The obvious experiment is to vary the time of storage and the temperature at which it is carried out. This would help to elucidate this problem. It could be mentioned here that Herskovitz (1955) found a continuing increase in translocation frequency over five three day broods.

Turning to the nature of the translocations produced in the TEM experiments, Table 12 shows that storage produced an equal increase in autosomal translocations and in those involving the Y chromosome. This indicates that there is no differential effect of storage on either the autosomes or the Y chromosome, a result also obtained by Snyder. The distribution of translocations in the HI experiments is also constant before and after storage which is expected since no storage effect exists.

II CYTOLOGICAL RESULTS AND DISCUSSION

The results of the cytological analysis of 19 pre-stored and 20 post-stored lethals are given in Tables 14 and 15. It is seen that the only type of rearrangement found in the pre-stored lethals were three inversions, giving a percentage of 15.3%; while in the post-stored lethals there were three translocations, four inversions, and two deficiencies (the latter type of change not being scored in the pre-stored lethals), giving a total percentage of 45%, or 35% when only the large rearrangements were taken into consideration. Even with the small numbers analysed, it is evident that there is an increase in rearrangements in the post-stored lethals, but it cannot be said with any
certainty that in the storage experiments the total increase in lethal frequencies after storage is due only to rearrangements.

The lethals analysed were from experiment TEM 2, in which the frequencies of lethals in the pre- and post-stored periods were 6.7% and 8.7% respectively. Taking the above figure of 15.8% as representing the breakage related component among the pre-stored lethals, the frequency of lethals not associated with breakage works out as 5.7%. Now if the increase in lethal frequency after storage is due to lethal rearrangements increasing in the same proportion as the translocations (which increased from 0.9% to 7.5% in expt. TEM 2), the formula set out by Snyder (1963) can be used to see if this assumption fits the data. The formula is as follows:

\[ L_S = L^P + (L^U - L^P)R \]

where \( L_S \) = % post-stored lethals; \( L^U \) = % pre-stored lethals; \( L^P \) = % lethals due to point mutations; \( R \) = % post-stored/pre-stored translocations.

If \( L_S \) is calculated taking \( L^P \) as 5.7, \( L^U \) as 6.7, and \( R \) as 7.5/0.9, the result is 14%, compared with the actual result of 8.7%. At first sight this seems to indicate that the frequency of lethals does not increase according to the above assumption. Several reasons can be put forward to try to explain this discrepancy. Firstly, the small numbers of lethals analysed means that the sampling errors are large. Secondly, the X-chromosome in which the lethals are located might respond in a different manner to storage than do the X-chromosome and the autosomes. Thirdly, it is also possible that the chromosome breaks occurring during storage do so in such a manner that the proportion of lethal rearrangements is lower than the proportion obtained in pre-stored spermatozoa.
The fact that the actual lethal frequency after storage is less than expected does mean the obtained increase can be explained by a rise in lethal rearrangements and not in point mutations, which might have been the case had more than 14% lethals been found in the post-stored period.
### Table 1: Frequencies of sex-linked lethals and translocations in broods (a), (d) and (e) following treatment with $1 \times 10^{-4}$ M. Triethylene Melamine.

<table>
<thead>
<tr>
<th>Brood</th>
<th>Expt. No.</th>
<th>Sex-linked lethal test</th>
<th>Translocation test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. chromosomes tested</td>
<td>No. lethals found</td>
<td>%</td>
</tr>
<tr>
<td>(a)</td>
<td>1</td>
<td>478</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>743</td>
<td>50</td>
</tr>
<tr>
<td>(d)</td>
<td>1</td>
<td>745</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>783</td>
<td>20</td>
</tr>
<tr>
<td>(e)</td>
<td>1</td>
<td>492</td>
<td>5</td>
</tr>
<tr>
<td>Brood</td>
<td>Conc.</td>
<td>Expt. No.</td>
<td>Sex-linked lethal test</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
<td>-----------</td>
<td>------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. chromosomes tested</td>
</tr>
<tr>
<td>(a)</td>
<td>$1.2 \times 10^{-2}$</td>
<td>1</td>
<td>64.1</td>
</tr>
<tr>
<td></td>
<td>$2.4 \times 10^{-2}$</td>
<td>2</td>
<td>715</td>
</tr>
<tr>
<td>(d)</td>
<td>$1.2 \times 10^{-2}$</td>
<td>1</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>$2.1 \times 10^{-2}$</td>
<td>2</td>
<td>695</td>
</tr>
<tr>
<td>(e)</td>
<td>$2.4 \times 10^{-2}$</td>
<td>2</td>
<td>512</td>
</tr>
</tbody>
</table>

Table 2. Frequencies of sex-linked lethals and translocations in broods (a), (d) and (e) following treatment with Ethylene Imine.
<table>
<thead>
<tr>
<th>Brood</th>
<th>Expt. No.</th>
<th>Sex-linked lethal test</th>
<th>Translocation test</th>
<th>Lethal/trans ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. chromosomes tested</td>
<td>No. lethals found</td>
<td>%</td>
</tr>
<tr>
<td>(a)</td>
<td>1</td>
<td>884</td>
<td>49</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>621</td>
<td>24</td>
<td>3.86</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>613</td>
<td>13</td>
<td>2.1</td>
</tr>
<tr>
<td>(a)</td>
<td>1</td>
<td>240</td>
<td>16</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>345</td>
<td>24</td>
<td>6.95</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>817</td>
<td>42</td>
<td>5.13</td>
</tr>
<tr>
<td>(e)</td>
<td>1</td>
<td>Sterile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Sterile</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>505</td>
<td>13</td>
<td>2.57</td>
<td></td>
</tr>
</tbody>
</table>

The heliotrine was injected in Expts. 1 and 2, and fed in Expt. 3. The time of feeding was 18 hrs. The concentrations used were: Expt. 1: 0.68 x 10^-2 M; Expt. 2: 0.34 x 10^-2 M; Expt. 3: 0.2 x 10^-2 M.

Table 3. Frequencies of sex-linked lethals and translocations in broods (a), (d) and (e) following treatment with Heliotrine.
<table>
<thead>
<tr>
<th>Brood</th>
<th>Sex-linked lethal test</th>
<th>Translocation test</th>
<th>Lethal/transloc ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. chromosomes tested</td>
<td>No. lethals found</td>
<td>%</td>
</tr>
<tr>
<td>(a)</td>
<td>359</td>
<td>32</td>
<td>8.9</td>
</tr>
<tr>
<td>(d)</td>
<td>654</td>
<td>19</td>
<td>2.9</td>
</tr>
<tr>
<td>(e)</td>
<td>423</td>
<td>7</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Table 4. Frequencies of sex-linked lethals and translocations in broods (a), (d) and (e) following treatment with approx. $1 \times 10^{-3}$ M Diepoxybutane ($\cdot 1\%$).
<table>
<thead>
<tr>
<th>Brood</th>
<th>Sex-linked lethal test</th>
<th>Translocation test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. chromosomes tested</td>
<td>No. lethals found</td>
</tr>
<tr>
<td>(a)</td>
<td>816</td>
<td>61</td>
</tr>
<tr>
<td>(d)</td>
<td>822</td>
<td>111</td>
</tr>
<tr>
<td>(e)</td>
<td>634</td>
<td>119</td>
</tr>
</tbody>
</table>

Table 5. Frequencies of sex-linked lethals and translocations in broods (a), (d) and (e) following treatment with $3 \times 10^{-4}$ M quinacrine mustard ($\ast 1\%$).
<table>
<thead>
<tr>
<th>Brood</th>
<th>Expt. No.</th>
<th>Sex-linked lethal test</th>
<th>Translocation test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. chromosomes tested</td>
<td>No. lethals found</td>
</tr>
<tr>
<td>(a)</td>
<td>1</td>
<td>632</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>329</td>
<td>18</td>
</tr>
<tr>
<td>(d)</td>
<td>1</td>
<td>Both sterile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(e)</td>
<td>1</td>
<td>Sterile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>419</td>
<td>45</td>
</tr>
</tbody>
</table>

Concentrations used: Expt.1: \(0.13 \times 10^{-4}\) M approx.
Expt.2: \(0.6 \times 10^{-5}\) M approx.

Table 6. Frequencies of sex-linked lethals and translocations in broods (a), (d) and (e) following treatment with Dimethylnitrosamine.
<table>
<thead>
<tr>
<th>Brood</th>
<th>Sex-linked lethal test</th>
<th>Translocation test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. chromosomes tested</td>
<td>No. lethals found</td>
</tr>
<tr>
<td>(a)</td>
<td>614</td>
<td>62</td>
</tr>
<tr>
<td>(d)</td>
<td>560</td>
<td>8</td>
</tr>
<tr>
<td>(e)</td>
<td>367</td>
<td>4</td>
</tr>
</tbody>
</table>

Time of feeding = 48hrs.

**Table 7** Frequencies of sex-linked lethals and translocations in broods (a), (d) and (e) following treatment with $1.3 \times 10^{-4}$ M Diethyl Sulphate.
<table>
<thead>
<tr>
<th>Brood</th>
<th>Expt. No.</th>
<th>Sex-linked lethal test</th>
<th>Translocation test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. chromosomes tested</td>
<td>No. lethals found</td>
</tr>
<tr>
<td>(a)</td>
<td>1</td>
<td>157</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>397</td>
<td>3</td>
</tr>
<tr>
<td>(a)</td>
<td>1</td>
<td>Sterile</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>232</td>
<td>2</td>
</tr>
<tr>
<td>(e)</td>
<td>1</td>
<td>Sterile</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>471</td>
<td>1</td>
</tr>
</tbody>
</table>

Concentrations used were: Expt. 1: $1 \times 10^{-6}$ M approx.  
Expt. 2: $1 \times 10^{-9}$ M approx.

**Table 8** Frequencies of sex-linked lethals and translocations in broods (a), (d) and (e) following treatment with the antibiotic streptonigrin.
<table>
<thead>
<tr>
<th>Agent</th>
<th>Molar conc.</th>
<th>Expt. No.</th>
<th>Sex-linked lethal test</th>
<th>Y-II-III translocation test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. chromosomes tested</td>
<td>No. lethals found</td>
</tr>
<tr>
<td>TEM</td>
<td>$2 \times 10^{-4}$</td>
<td>1</td>
<td>478</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>743</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>595</td>
<td>38</td>
</tr>
<tr>
<td>EI</td>
<td>$1 \times 10^{-2}$</td>
<td>1</td>
<td>641</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^{-2}$</td>
<td>2</td>
<td>715</td>
<td>52</td>
</tr>
<tr>
<td>TEM</td>
<td>$2 \times 10^{-4}$</td>
<td>1</td>
<td>204</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>847</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>708</td>
<td>86</td>
</tr>
<tr>
<td>EI</td>
<td>$1 \times 10^{-2}$</td>
<td>1</td>
<td>668</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>$2 \times 10^{-2}$</td>
<td>2</td>
<td>452</td>
<td>31</td>
</tr>
</tbody>
</table>

*Table 9* The frequencies of translocations and sex-linked lethals in progeny from TEM and EI treated $\Delta \delta$ before and after storage (6 days at $25^\circ$C).
Figure 2 Histogram showing the lethal and translocation frequencies before and after storage following treatment with TEM or EI.
<table>
<thead>
<tr>
<th>Agent</th>
<th>Expt.</th>
<th>Pre-stored</th>
<th>Post-stored</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM</td>
<td>1</td>
<td>0.12</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.13</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.28</td>
<td>1.42</td>
</tr>
<tr>
<td>EI</td>
<td>1</td>
<td>0.19</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.34</td>
<td>0.34</td>
</tr>
</tbody>
</table>

Table 10. Translocation/lethal ratios before and after storage.
<table>
<thead>
<tr>
<th>Brood</th>
<th>Sex-linked lethal test</th>
<th></th>
<th>Y-II-III translocation test</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. chromosomes tested</td>
<td>No. lethals found</td>
<td>%</td>
<td>No. chromosomes tested</td>
</tr>
<tr>
<td>1</td>
<td>256</td>
<td>30</td>
<td>11.8</td>
<td>379</td>
</tr>
<tr>
<td>2</td>
<td>254</td>
<td>34</td>
<td>13.4</td>
<td>337</td>
</tr>
<tr>
<td>3</td>
<td>198</td>
<td>22</td>
<td>11.1</td>
<td>243</td>
</tr>
<tr>
<td>Total</td>
<td>708</td>
<td>86</td>
<td>12.1</td>
<td>959</td>
</tr>
</tbody>
</table>

Table 11. Frequencies of sex-linked lethals and translocations in three successive post-stored broods of three-days each. (Storage expt. TEM 3).
Table 12. Distribution of translocations detected in the storage experiments.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Expt.</th>
<th>Translocations from unstored sperm</th>
<th>Translocations from stored sperm</th>
<th>Ratio of autosomal translocations to Y translocations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>II-III  y-II  y-III</td>
<td>II-III  y-II  y-III  y-II-III</td>
<td>Pre</td>
</tr>
<tr>
<td>TEM</td>
<td>1</td>
<td>6  -  -</td>
<td>17  1  2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>11  1  3</td>
<td>42  5  5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>11  1  1</td>
<td>137  14  12  2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Total)</td>
<td>28  2  4</td>
<td>196  20  19  2</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>5  1  -</td>
<td>6  1  -</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15  -  -</td>
<td>12  -  -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Total)</td>
<td>20  1  -</td>
<td>18  1  -</td>
<td></td>
</tr>
</tbody>
</table>
Table 13. Frequencies of sex-linked lethals and translocations before and after storage, following treatment with ICR 100 (6 days storage at 25°C).

<table>
<thead>
<tr>
<th></th>
<th>Sex-linked lethal test</th>
<th></th>
<th>Translocation test</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. chromosomes tested</td>
<td>No. lethals found</td>
<td>%</td>
<td>No. chromosomes tested</td>
</tr>
<tr>
<td>Pre-storage</td>
<td>355</td>
<td>28</td>
<td>7.9</td>
<td>574</td>
</tr>
<tr>
<td>Post-storage</td>
<td>737</td>
<td>61</td>
<td>8.3</td>
<td>695</td>
</tr>
</tbody>
</table>

Concentration used: $0.3 \times 10^{-4} \text{ M}$ approx. (0.1%).
<table>
<thead>
<tr>
<th>Lethal number</th>
<th>Location</th>
<th>Cytological Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No change</td>
</tr>
<tr>
<td>1</td>
<td>13.48 ± 0.8</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>22.92 ± 1.1</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>35.6 ± 0.78</td>
<td>X-3R</td>
</tr>
<tr>
<td>5</td>
<td>14.28 ± 1.3</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>55.7 ± 0.17</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>51.9 ± 0.83</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>2.17 ± 0.9</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>19.62 ± 0.36</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>0.25 ± 0.79</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>23.6 ± 0.9</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>41.92 ± 1.2</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>X-4</td>
</tr>
<tr>
<td>16</td>
<td>24.4 ± 0.95</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td>X-2R</td>
</tr>
<tr>
<td>22</td>
<td>0.68 ± 0.47</td>
<td>+</td>
</tr>
<tr>
<td>23</td>
<td>57.1 ± 0.38</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>12.2 ± 0.9</td>
<td>+</td>
</tr>
<tr>
<td>25</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>TOTAL</td>
<td>11</td>
<td>3</td>
</tr>
</tbody>
</table>

% of lethals with rearrangements = 45
% of lethals with large rearrangements = 35

Table 14. Cytological analysis of 20 post-stored lethals induced by T.E.M.

* = location test gave indeterminate result.
<table>
<thead>
<tr>
<th>No change</th>
<th>Translocation</th>
<th>Inversion</th>
<th>Deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
</tbody>
</table>

% of lethals with rearrangement = 15.8%

Table 15. Cytological analysis of 19 pre-stored lethals induced by T.E.M.
DISCUSSION

The object of this investigation was to find out whether chemicals were superior to X-rays in producing translocations in spermatogonia, and also to try and find a difference between the genetical effects of monofunctional and polyfunctional alkylating agents. A positive answer was found to both these problems, and in the following discussion, as in the Introduction, they are dealt with separately, taking the question of spermatogonial translocations first.

PART I

The hypothesis behind this part of the investigation was that chemicals might be able to overcome the obstacles which prevent the production of translocations in spermatogonia by X-rays, and that this hypothesis would be supported if the lethal/translocation ratio following chemical treatment showed a smaller change from spermatozoa to spermatogonia than it does after a dose of X-rays. Now there are two main difficulties concerning this hypothesis. Firstly, that chemicals are known to produce fewer numbers of translocations relative to lethals than X-rays, and also that spermatogonia have a low sensitivity to mutagenic action in the majority of cases. It was suggested by Auerbach (1951) that because certain chemicals produced potential breaks or delayed breakage, part of the shortage of translocations might be connected with the delayed effect of the mutagens, and Slizynaka (1957), following a study of formaldehyde induced rearrangements, proposed that potential breaks in the same chromosome tended to open simultaneously and hence favour the formation of intrachromosomal breaks above interchromosomal ones. In a
later paper, Slizynska (1963b) found that the ratio of inter- to intra-chromosomal breaks in *Drosophila* males after chemical treatment was approximately 1:4, compared with 1:2 for X-rays, and it was shown by Nasrat *et al.* (1954) using mustard gas, and Fahmy & Fahmy (1954) using TEM, that the low frequency of rearrangements following chemical treatment was not due to a shortage of breaks, but to a failure of the breaks to reunite, for the chemicals produced as many chromosome fragments as a comparable dose of X-rays.

As regards the low sensitivity of spermatogonia, two factors could affect the formation of translocations in these cells, according to the arguments set out in the Introduction. Firstly, the stage in the cell cycle, and secondly, the elimination of the translocations in meiosis. Taking the latter first, it was shown that although about half of any translocations formed are lost in meiosis, the chances for forming a translocation are twice as high in diploid spermatogonial cells as in haploid spermatocytes and spermatids, which should compensate to a large extent for the losses occurring during mitosis. It seems then as if the first factor is the most important one.

It will be recalled that Oftedal (1964a, b, c) proposed that there was a sensitive group of cells in spermatogonia which was very susceptible to the action of X-rays, so that there was a heterogeneity in the exposed population of cells as regards killing and mutation induction sensitivities. Slizynska (1963a) also found a heterogeneity in spermatogonial cells, agreeing with the results of Oftedal. Russell (1963) proposed that the increased mutation rate in spermatogonial cells of mice after two 500R doses given 24 hours apart was due to the fact that the
first dose synchronised the cell population so that more cells were in a sensitive stage at the time of the second. According to Muller (1954) the sensitive stage in the cell cycle is when they are undergoing mitosis, when the chromosomes are condensed and most susceptible to X-rays. It was then thought that chemicals, because of their prolonged action, might stand a better chance of being present at the right time for the production of translocations in spermatogonia.

Now there is some evidence from work with *Vicia faba* that chemicals differ from X-rays in the stage in the cell cycle in which they act. One of the well known differences between the action of X-rays and some radiomimetic chemicals in inducing chromosome aberrations in *Vicia* root-tips is that, with X-rays chromatid aberrations are seen at mitosis in the first few hours after irradiation of an asynchronously developing cell population, whereas with the chemicals no aberrations are seen until about 8-10 hours after treatment. In other words, there is a delay compared with the time of appearance of such aberrations after X-ray treatment. This delay has been reported for a large number of chemicals tested on *Vicia* roots; including nitrogen mustard (Ford, 1949; Revel, 1953), B-propiolactone (Smith & Srb, 1951; Swanson & Merz, 1959), maleic hydrazide (McLeish, 1953; Kihlman, 1956, Merz et al., 1961), triethylene melamine (Ockey, 1960), ethyl alcohol, ethyl methanesulphonate and Myleran (Reiger & Michaelis, 1960a, b) and N-nitroso-N-methylurethan (Kihlman, 1960).

In these experiments, however, no information on the mitotic delay induced by these chemicals was obtained, and in many instances no detailed information on mitotic frequency and aberration yields found soon after treatment was given. Ford (1949) and Revel (1953) had interpreted
the delayed effect after chemical treatment as due to the fact that aberrations were induced only in those cells which were in the early stages of interphase at the time of treatment. The autoradiographic studies of Howard & Pelc (1953) showed that the interphase period in *Vicia* root-tip cells could be divided into three phases; the pre-DNA synthesis phase (G\textsubscript{1}), the DNA synthesis phase (S), and the post-DNA synthesis phase (G\textsubscript{2}), and suggested that the results obtained by Revell (1953) indicated that only cells in the G\textsubscript{1} phase were sensitive to the chemical used. It was shown by Evans & Savage (1963) and Wolff & Luippold (1963) that irradiated G\textsubscript{1} cells yield chromosome-type changes and that chromatid-type changes are mainly induced in S and G\textsubscript{2}, so the finding that chemically induced aberrations in *Vicia* are of the chromatid-type suggests that although the cell may be sensitive to the initiation of aberrations only in early interphase, the actual production of the aberrations occurs at some later stage in development. Evans & Scott (1964) determined the average durations of G\textsubscript{1}, S and G\textsubscript{2} and also their sensitivity to the production of chromosome aberrations following X-ray or maleic hydrazide (MH) treatment. With MH, aberrations were induced only in cells undergoing replication, G\textsubscript{2} cells being undamaged when observed at their first post-treatment mitosis. This supports the idea that only cells in early interphase are sensitive to chemical treatment and that chromosome aberrations may be produced at the time of chromosome replication. Scott & Evans (in Evans & Scott, 1964) also found that nitrogen mustard produced chromatid aberrations only at the time of chromosome replication.

Since the majority of spermatogonial cells are in interphase, the above hypothesis might also explain why chemicals can produce trans-
locations at this stage, where DNA synthesis is continually occurring. However, conflicting results have also been obtained using *Vicia faba*. Kihlman (1955, 1961, 1962) has shown that with methylated oxypurines such as 8-ethoxycaffeine, chromatid aberrations are seen at mitosis shortly after treatment, suggesting that this group of chemicals may be similar to X-rays in terms of time of action, and more recently Scott & Evans (1964) also found that 8-ethoxycaffeine was most effective in producing aberrations in $G_2$. This has been found to be true for 5-fluorodeoxyuridine (Bell & Wolff, 1964) and deoxyadenosine (Kihlman et al., 1964). It should also be remembered that chemicals can produce rearrangements in mature *Drosophila* sperm where DNA synthesis does not take place, and also that storage of sperm treated with the same chemicals as used in the above experiments on *Vicia* e.g. nitrogen mustard and TEM, can lead to increased breakage (Herskowitz, 1955; Schalet, 1955; Snyder, 1963; and results obtained in the present investigation).

Regardless of whether the sensitive stage in spermatogonia to the action of chemicals is during chromosome replication or in the condensed stage of mitosis, one fact is quite clear. That is, that some of the chemicals used in this investigation did produce translocations in these cells, supporting the original hypothesis. It is true that the other chemicals used, i.e. DMNA, DES and ICR100, produced none, but they did not produce any in mature sperm either, a result which is discussed in Part II.

The lethal/translocation ratio produced by the first group of chemicals all show an increase from spermatozoa to spermatogonia. This increase is due mainly to a drop in the numbers of translocations
obtained in spermatogonia; but is also accompanied by a drop in the
number of lethals, indicating a drop in sensitivity, in the case of TEM,
EI and DEB. Heliotrine, however, which gave the highest increase in the
ratio, did so because there was an increase in the frequency of lethals.
This indicates an increase in sensitivity from spermatozoa to spermatogonia
and it might be said that this is borne out by the fact that in the third
heliotrine experiment the translocation frequency rose from 0.18% in brood
(a) to 0.25% in brood (d), although the numbers are far too small to make
this difference significant.

It is clear that the chemicals are less efficient than X-
rays in spermatozoa when the lethal/translocation ratio is taken as a
measure of effectiveness, but the position is reversed in spermatogonia as
the ratio for X-rays jumps to infinity in the majority of cases, no trans-
locations being produced.

One interesting feature of the results was that EI produced
very similar frequencies of sex-linked lethals and translocations in sper-
matogonia at both doses used, after producing markedly different ones in
spermatozoa. This means that in spermatogonia the frequency of mutations
was not proportional to the molar concentration. Nakao et al (1964) found
that doubling the dose of EI gave rise only to a 20% increase in lethals,
but a three-fold increase in translocations, and this was in mature sperm.
It is difficult to suggest an explanation for this phenomenon. It may be
that mutation damage induced by EI can reach a saturation point, which was
attained by the second dose used by Nakao et al, and already attained in
spermatogonia by the first dose in the present results. It may even be
that the surplus mutagen reacted with other cell constituents to form a
harmless by-product.
It is the purpose of the second part of this discussion to look at the nature of the mutations produced by the mono- and bifunctional alkylating agents in order to see if they differ in any respect. It has already been pointed out in the results that both types of chemical did produce some similar effects, but the fact that three of the monofunctional agents were capable of producing high sex-linked lethal frequencies but no chromosome breakage might be taken as an indication that the monofunctional agents tended to produce more intragenic or point mutations than intergenic ones involving some rearrangement of the chromosomes.

Now the problem of whether intergenic and intragenic changes are the results of two different processes is one which has been much discussed. A sharp distinction between the two types of change is not always possible for, in the case of radiation-induced lethals, these have been found to consist of apparent point mutations, small structural changes which behave as point mutations, and large structural rearrangements. Muller (1941, 1956) suggested that the chromosome is segmented in some manner at a level above the nucleotide units of DNA, and that there is a basic distinction between mutations referred to as point mutations and those associated with chromosome breakage, basing his arguments on various pieces of genetical evidence. Lea & Catcheside (1945) considered that radiation-induced lethals and chromosome rearrangements arose as a matter of chance from a single type of primary effect, and Herskowitz (1946) suggested that most of these lethals were produced at points of chromosome breakage. Both these assumptions were based on a consideration of linear dose-effects. It was also suggested by Muller & Altenburg (1930) and Muller (1954) that
lethals associated with gross structural changes were due to position effects.

The use of chemicals as mutagens opens up new possibilities for attempts to clarify the problem. If a chemical was found that produced point mutations but no chromosome breaks, i.e. intragenic changes only, this would give direct evidence of a difference between the two types of mutation. However, no clear example of such a chemical is known to exist, although they differ widely in their chromosome breaking abilities. In the results obtained in this investigation DES, DMNA and ICR100 produced no chromosome breakage but many lethals, but other investigators have shown that DES can produce low frequencies of chromosome breakage in barley (Heiner et al., 1962) and that ICR100 can do the same in Drosophila (Snyder & Oster, 1964).

Taking the opposite case, if a chemical existed which produced breaks or intergenic changes only, it would also produce small deficiencies which would act as recessive lethals, and position effect rearrangements which would appear as visible mutations. Some chemicals such as caffeine have been shown to break plant chromosomes but not to produce mutations in Drosophila, but unless it can be shown that they can also break Drosophila chromosomes it cannot be said that they produce only intergenic mutations. In any case, since plant chromosomes are so much more easily broken than those of Drosophila, a substance which breaks the former may not have the same effect on the latter. Also metabolic differences can exist as shown by the fact that such agencies as low doses of X-rays or excessive oxygen pressure (Conger, 1952) and anoxia (Mertz, 1959) can break plant chromosomes.

Newer ideas, based on the Watson-Crick model of DNA, can also be used to consider the problem of intra- and intergenic changes on a
more refined level, especially in view of recent developments concerning the nature of the genetic code.

As the result of much work pioneered by Crick et al. (1961) and Nirenberg & Matthaei (1961), it is now generally accepted that the amino acid sequence of protein molecules is determined by the sequence of bases along the DNA chain, that a codon of three consecutive nucleotides in the DNA specifies each amino acid residue in the polypeptide chain of the protein by means of messenger RNA, and that there might be "linkers" consisting of protein between the segments specifying different protein molecules, corresponding to the intergenic bonds proposed by Muller, providing a starting point for the reading of the code in each segment.

On the basis of this model molecular changes can be envisaged that could result in mutation. Firstly, the number of bases could remain constant, but their sequence could be changed by the substitution of one or more bases by different bases, i.e., a transition of the type envisaged by Freese (1958) who showed that 5-bromouracil could pair with adenine and thus replace thymine and that 2-aminopurine could pair with thymine and replace adenine. This could lead to an alteration in an amino acid sequence which appears as a mutation. An example of this is that a change in a single amino acid residue in haemoglobin is responsible for sickle-cell anaemia (Ingram, 1957). It could be mentioned here that Ludlam et al. (1964) studied the effect of alkylation of synthetic polynucleotides and found that treatment of polyadenylic acid with methyl methanesulphonate decreased its ability to code for polylysine synthesis.

Secondly, a loss of one or more base pairs could result in a deletion, and thirdly, chromosome breakage could also be caused by a loss of base pairs.
followed by failure of the ends to reunite. In the latter two cases, it
would be expected that whole polypeptide chains could not be made by the
mutant cells. An example of the third case might be in myelocytic leukaemia
in man, where the leukaemic cells carry the "Philadelphia" chromosome in
which a loss of part of the normal chromosome has occurred (Nowell &
Hungerford, 1960). As regards chromosome breakage it is not known whether
it could occur within the linkers or not.

Before considering further the question of whether mono-
functional alkylating agents tend to produce intragenic mutations, the
possible mechanism of mutation by alkylating agents can be discussed in
detail, assuming that this action is directly on the DNA of the chromosome.
As already mentioned in the Introduction, mechanisms for both types of
mutagenesis, i.e. intra- and intergenic changes, following treatment of
DNA with alkylating agents have been suggested in a series of papers
by Lawley & Brookes. They found that, following the alkylation of DNA,
the N-7 atom of the guanine moieties was the most susceptible, and that
the affected guanines were converted to 7-alkylguanine after monofunctional
alkylation, while after bifunctional alkylation the cross-linked product
di-(β-guanin-7-yl) ethylsulphide was also formed. Figure 3 shows how
the alkylated guanines could lead to the mutagenic changes outlined
above (Lawley & Brookes, 1963). In the diagram the base sequence along
the DNA strands is represented by the initial letters of the bases, while
the horizontal arrows show effects of hydrolysis of the alkylated DNA.
The inclined arrows show possible mechanisms for mutagenesis initiated
by faulty replication of the lower DNA strand.
Figure 3 Diagrammatic representation of alkylation of DNA by monofunctional and bifunctional agents and subsequent effects of alkylation.

Monofunctional alkylation

\[ \text{conversion of GC to AT} \]

\[ \text{deletion of GC} \]

\[ \text{molecular fission} \]

Bifunctional alkylation
A possible mechanism by which the initial alkylation of DNA at the N-7 atom of the guanine moieties could initiate mutation follows from its effect in causing an increased tendency for acidic ionisation at N-1 to occur, thus enhancing the probability of anomalous base pairing of ionised guanine with thymine, i.e. a transition (Lawley, 1961). However, since adenine and cytosine can also be alkylated to a small extent (Lawley & Brookes, 1963), this could also weaken the specificity of base pairing, and the conversion of G-C pairs to A-T pairs would probably not be the only mutagenic activity of this type. Alternatively, the alkylated guanines could leach out by hydrolysis, initiating the loss of a base pair causing a reading frame mutation similar to that envisaged by Crick et al. (1961) following treatment with acridine.

It could be proposed on the basis of the above mechanism that the monofunctional alkylating agents are more likely to give rise to point mutations and small deletions than chromosome breaks while bifunctional agents could produce breaks due to cross-linkages being formed. The results obtained with DES, DNA and ICR100 seem to support this hypothesis, together with the fact that the bifunctional agents TEM and DEB produced translocations.

If bifunctional alkylating agents produce breaks as a result of cross-linkage, then the question of how some monofunctional agents produce breaks is an important one. The capability of the latter chemicals in doing so is in no doubt (see Introduction), and in the present investigation, the monofunctional E1 produced more translocations in mature sperm than any of the bifunctional agents. Nakao et al. (1964) also found
that E1 was superior to TEM in the production of translocations, and Nakao & Auerbach (1961) showed that the monofunctional ethylene oxide was as effective as the bifunctional DEB in producing the same rearrangements.

It is true that in the above examples higher doses of the monofunctional agents were required relative to those of the bifunctional ones. This agrees with the hypothesis that two nearby single strand breaks could occur fortuitously in opposite strands of the DNA following monofunctional alkylation, giving rise to a complete break, and that the possibility of this occurring would be enhanced by a higher concentration. If this is so then it can be asked whether the molar concentration of the mutagen is a better measure of effectiveness in producing mutations.

However, it cannot be said that higher doses of monofunctional agents than of bifunctional ones are required to produce mutations in every case. For example, Bieseles et al. (1950) produced equal amounts of chromosome breakage in onion root-tip cells with equimolar concentrations of E1 and TEM, while the monofunctional ethylene oxide was found to be more effective on a molar basis than DEB in producing reverse mutations in Neurospora (Kölmark & Westergaard, 1953; Kölmark & Kondo, 1963). Also, in their investigation comparing mono- and bifunctional alkylating agents, Fahmy & Fahmy (1961) used $0.45 \times 10^{-2} \text{M}$ of the monofunctional methyl methanesulphonate and $2.42 \times 10^{-2} \text{M}$ of the monofunctional ethyl methanesulphonate to produce an average of 9% recessive sex-linked lethals in Drosophila, but required $8 \times 10^{-2} \text{M}$ of the bifunctional dimethanesulphonyl mannitol to produce an average of 5% lethals. Thus taking molar concentration as a measure of effectiveness, the monofunctional agents were 30 and 6 times more effective respectively than the bifunctional. In the present
investigation, it is seen that $0.3 \times 10^{-4}$ M ICR100 produced 7.5% sex-linked lethals in mature sperm; $0.13 \times 10^{-4}$ M DMNA produced 7.8%; and $1.3 \times 10^{-4}$ M DES produced 10%; while $2 \times 10^{-4}$ M TEM produced an average of 6.6% and $1 \times 10^{-3}$ M DEB produced 8.9%.

The above results show, if anything, that these monofunctional agents are more effective than bifunctional ones on a molar basis. This of course may be due to differences in penetration or in the reactions with cell constituents that the mutagen might undergo after it is administered. Because of this uncertainty, too much emphasis cannot be placed upon the molarity of the dose. Is there any other method, therefore, that can be used to try and distinguish between the two types of chemical?

In 1960 Fahmy & Fahmy put forward the hypothesis that the ratio of visible to lethal mutations induced in Drosophila could be used as an indicator of genetic differences between related chemical compounds, taking as their basis that visible mutations were the result of intramolecular changes within genes while lethal mutations involved material loss on the genic or subgenic level. Then in 1961 they carried out a series of experiments using monofunctional and bifunctional alkylating agents and claimed that the bifunctional agents gave a higher lethal to visible ratio, more complete than mosaic lethals, more chromosome breaks, and a higher frequency of small deficiency lethals, and that these observations constituted a significant difference between the two types of chemical.

However, there are certain factors to be taken into consideration on looking at these findings. Firstly, the classification of lethal mutations. The distinction between visible and complete mutations is by no
means a clear-cut one. In Drosophila a recessive lethal is defined as one that kills before emergence from the pupa, but if it was defined as one that kills before the egg hatches, then many recessive lethals would be defined as visibles if they killed in the larval or pupal stages. Also, one must consider the relative importance of the function affected by the mutation. An intramolecular reorganisation of the gene such as that discussed above could just as easily give rise to a lethal effect as could a visible one if, for example, it caused an enzyme to be affected which was indispensible to a vital function. Another problem also arises regarding the scoring of visible mutations. The personal factor involved in this is very important as it depends entirely on the scorer of the flies what mutations are classified. There will probably be a bias in the scoring also, for once one type of visible has been seen it is not so likely to be missed subsequently.

Secondly, it has been shown that a methodical error could be present in taking the complete/visible lethal ratio and the complete/mosaic lethal ratio as a basis for comparison. Browning & Altenburg (1961) found that as visible mutations arising as mosaics were readily observed while mosaic lethals were not detectable except by testing a further generation, this could lead to a higher number of visibles being detected. It has also been shown (Carlson & Oster, 1961; 1962; Oster & Carlson, 1963) that a large proportion of the visible and sex-linked lethal mutations induced by the monofunctional agent ICR100 were mosaically distributed in somatic and gonadal tissues. Thus the finding by the Fahmys that monofunctional agents produced more visible mutations relative to complete ones could be due to the fact that mosaic visibles would also be included.
The above results indicate that the classification of lethals and visibles cannot be used as a true indication of a genetical difference between mono- and bifunctional compounds. In the Fahmy's results the variations between experiments with the same compounds were ignored because they were not large enough to cause heterogeneity within compounds. However, it would have been better if an analysis of variance had been carried out, using the variation within compounds as a measure of control variation. Even taking their results as they stand, the average visible/lethal ratios for the two monofunctional compounds were 0.25 and 0.32 respectively, while that of the bifunctional compound was 0.21, which hardly reflects a great difference between the two types of chemical. More recently Snyder & Oster (1964) used the monofunctional ICR100 and the polyfunctional TEM to compare their capacity for producing mutations at the dummy locus. They found that although the monofunctional agent did produce significantly more mosaics at this locus than did the polyfunctional one, the difference was not nearly so great as that of Fahmy & Fahmy (1961), who stated that the complete/mosaic ratio was 1:2 for their monofunctional compounds and 2:1 for their bifunctional one. They based their conclusions on the incidence of mutations at an unselected sample of loci, whereas a study of mutation at a specific locus as carried out by Snyder & Oster was much more objective.

It can also be asked that if complete lethals are due to material loss or chromosome breakage, why some monofunctional agents such as those used in this investigation and others like ethyl methanesulphonate produce many lethals but little or no chromosome breakage. If it is assumed that lethals are produced by a mechanism essentially similar to that of
breaks, then translocations should also be produced in such cases. The fact that they are not indicates that lethals are produced in some other way.

Lastly the question of whether monofunctional agents are inferior to bifunctional ones in producing breaks can be considered. Once again there is no clear-cut difference, for in the results of this investigation, although three of the four monofunctional agents used were inferior to the bifunctional agents, the other monofunctional agent, EI, produced a higher translocation/lethal ratio than TEM, a result which was also obtained by Nakao et al. (1964) and Alexander & Granges (1964), and previously Nakao & Auerbach (1961) had shown that ethylene oxide produced the same translocation/lethal ratio as DEB. These results show that when the above ratio is taken as a measure of effectiveness, these particular chemicals produce an equal effect, so it cannot be said with any certainty that monofunctional alkylating agents are inferior in this respect in every case.

Apart from genetical tests, other evidence that there might be a difference between mono- and bifunctional alkylating agents has come from studies of their carcinostatic abilities. It has been observed (Haddow, Kon & Ross, 1948; Connors, Ross & Wilson, 1960) that almost all alkylating agents with effective carcinostatic ability possess two or more functional groups. However, exceptions are known. Quinacrine mustard (ICR100) has been shown by Creech et al. (1960) and Peck et al. (1961) to be a powerful inhibitor of mouse ascites tumours; ethylene imine was shown by Biese et al. (1950) to be effective in producing chromosome fragmentation in mouse sarcomas grown in tissue culture; and 2-chloroethyl methanesulphonate (CB1506) has been shown to possess tumour inhibitory properties (Haddow & Ross, 1956). The latter compound is interesting as it has been suggested
(Connors & Ross, 1958) that it might be converted into the bifunctional 2-chloroethyl cysteine. But 2-chloroethyl cysteine does not inhibit the growth of the Walker rat carcinoma under standard test conditions, and so its formation from CB1506 may not account for the latter's anti-tumour activity. It has also been suggested that CB1506 could form 2-chloroethylamines or sulphides within cell structures and that these could undergo reactions similar to other mustards, e.g. if a cysteine unit in a protein was chloroethylated this could react further to give cross-linked structures. Schmidt (1960) also investigated the carcinostatic effects of CB1506 and found that although it was inactive against a wide range of animal tumours, it was strikingly active against recently inoculated, and also against well established, Lymphoma 8. Returning to other compounds, Walpole (1958) found that at high doses the carcinogenic monoethyleneimides produced chromosome fragmentation and bridge formation in broad bean root-tips.

At the best, all the results discussed above show only quantitative differences between mono- and polyfunctional alkylating agents. The results of the storage experiments show, for the first time, a clear qualitative difference between the genetical effects of a monofunctional and a polyfunctional agent. While the frequency of translocations rose steeply after storage following treatment with TEM, no such effect was found when the monofunctional EI was used. This difference is presumably related to the manner in which the two chemicals produce chromosome breaks. On the assumption that they act directly on the DNA of the spermatozoa, relevant experiments can be discussed.

As mentioned above, according to Brookes & Lawley (1961) the most reactive site in the DNA molecule to alkylation is the N-7 atom
of the guanine moieties, and the most distinguishing feature between mono-
and bifunctional alkylation is the ability of bifunctional agents to form a
di-(7-guaninyl) derivative cross-linking two guanines, which could be on
opposite strands of the DNA molecule. Following the initial alkylation,
further slow chemical changes occur over a long period, leading to loss
of the alkylated guanines and scission of the sugar-phosphate chain.
This produces a break in the DNA, which will be complete if a cross-
linked derivative had been formed. Cross-linkage of DNA could also prevent
the separation of DNA strands prior to replication, thus inhibiting this
process. Monofunctional alkylation, on the other hand, leads only to
single guanine molecules being converted to 7-alkylguanine, which can
give rise to single strand breaks only. The delayed effect following
monofunctional alkylation was also much less pronounced (Lawley, 1961).

Loveless & Stock (1959) showed that the inactivation of
T2 bacteriophage by bifunctional alkylating agents proceeded in two steps,
corresponding to the reaction of first one, then the other, functional
arms of the molecule, and proposed that the effective inactivating reaction
was that of the second functional arm reacting with the DNA of the phage for,
if the treated phage were placed in 5% sodium thiosulphate solution, inacti-
vation was annulled, indicating that the thiosulphate had reacted with the
free second arms of the alkylating agent, thus preventing further alkylation.
Following monofunctional alkylation, inactivation was essentially a one step
reaction, although there was a slow second step due to the decay of the
alkylated products. Thiosulphate produced no significant effect, indicating
that the single arms of the monofunctional agent had already reacted with
the phage. A further observation relevant to this investigation was that
if phage were treated with the bifunctional nitrogen mustard and stored for at least two hours, maximal inactivation was achieved. Also Loveless (1961) showed that there was no difference between mono- and bifunctional agents when the phage φX174, which contains single stranded DNA, was used in inactivation experiments and in vitro.

The present results can be interpreted on the basis of the above findings. It may be assumed that in TEM-treated spermatozoa, breakage would continue to occur during storage due to cross-linkage, while no such effect would occur in EI-treated spermatozoa. This means, in effect, that potential breaks would become true breaks during storage after treatment with TEM, and this is supported by the unpublished results of Sliżynska, who found that after storage the number of mosaic translocations and repeats, both of which are an indication of potential breaks, fell sharply, while the number of translocations increased. More recent unpublished work by Sliżynska, where she compared the frequencies of mosaic and complete rearrangements in unstored sperm, sperm stored for six days at 12.5 °C, and sperm stored for the same length of time at 25 °C, showed that the rise in completes after storage at 12.5 °C was equal to the drop in mosaics, but that at 25 °C far more completes were found than could be accounted for by mosaics becoming completes. This suggests that at the higher temperature complete breaks are formed de novo as well as from mosaics, but it could also mean that the drop in mosaics was independent of the rise in completes, the results at 12.5 °C being a coincidence. A similar experiment on a larger scale might help towards determining the true picture.

As already mentioned, it has been found that the majority of alkylating agents with effective carcinostatic ability possess two or
more functional groups per molecule. The results of the storage experiments offer an explanation as to why this is so. Since the treatment of carcinomas involves a longish period of time during which the chemical can act, an increased effect due to cross-linkage would continue to occur after treatment with a polyfunctional alkylating agent, leading to chromosome breakage, mitotic inhibition and cell death, while a monofunctional agent would produce only its initial effect, which would not be increased by the length of the period of treatment. This hypothesis would explain why monofunctional agents can be efficient chromosome breakers, but poor carcinostatic agents. The question of the production of recessive lethals does not enter into the carcinostatic abilities of alkylating agents, as a cell can carry such mutations with no effect.

In conclusion some results recently obtained with bacteria are discussed, in which storage after alkylation appears to lead to a recovery. This is in contrast with the results obtained here, where storage led to increased damage of the genetic material.

It is known that the ability of bacteria to survive to produce colonies after treatment with ultraviolet light or ionising radiation is markedly dependent on post-irradiation cultural conditions, especially incubation under sub-optimal conditions. For example, minimal medium or low temperatures favour survival, although the response varies for different strains (Alper & Gillies, 1960). In the case of UV irradiation it is clear that survival is enhanced in conditions which permit metabolic repair of otherwise lethal primary lesions, i.e. excision and replacement of DNA nucleotides which have undergone dimerisation between adjacent
nucleotide moieties (Setlow & Carrier, 1964). The results of Strauss (1962, 1963) and Strauss & Wahl (1964), who investigated the consequences of the treatment of *B. subtilis* transforming principle with methyl methane-sulphonate, indicated that the same effect occurred. Harold & Ziporin (1958) had found that the survival of *E. coli* after treatment with bifunctional alkylating agents was higher on minimal medium than on nutrient agar, and Shield (in Loveless et al., 1965) found that the survival of nitrogen mustard treated *E. coli* K-12 was enhanced considerably by a variety of post-treatments including minimal media and non-optimal incubation temperatures. Also Patrick et al. (1964) reported that recovery occurred when nitrogen mustard treated yeast were stored in buffer. Loveless et al. (1965) found that there was a significant recovery in a certain strain of *E. coli* after storage in buffer following treatment with mustard gas. They suggested that recovery occurred by excision of the alkylated DNA regions followed by synthetic repair, similar to the mechanism envisaged by Setlow & Carrier (1964). The recovery, however, is in the absence of an energy source, and the lesions in the DNA would involve both strands of the DNA if cross-linkages had been formed. It is possible that the excision would occur in the buffer, but it is unlikely that synthetic replacement could proceed unless the cells had nutrients available. Loveless suggested that the excisions occurred in buffer, and on transfer to broth agar there was a lag in which replacement could occur prior to DNA replication. However, if the excision consisted of one base from one strand affected by cross-linkage, repair would not be necessary, as monofunctional alkylation need not impair the capacity for replication.

At first sight the above results appear to be in direct
contrast to the results of the storage experiments, but there are other factors to be considered. Firstly, Loveless et al. (1965) found no recovery after treatment of phage T2 with mustard gas, which indicates that the recovery may be restricted to bacteria, and as they also found a drop in survival after treatment of another strain of bacteria, it may even be restricted to certain strains of the latter. Secondly, the phenomenon of survival of bacteria is entirely different from the production of breaks in the chromosomes of a higher organism such as Drosophila. Thirdly, it is very unlikely that any recovery process could occur in sperm stored in the seminal receptacles of females as any breaks induced in the sperm remain open until after fertilisation.
SUMMARY

In order to determine whether chemicals are superior to X-rays in the production of translocations in spermatogonia of Drosophila melanogaster, and also to find out if there was a difference between the genetical effects of monofunctional and polyfunctional alkylating agents, a number of chemicals known to act on both pre- and post-meiotic germ cells were administered by injection or feeding to adult Drosophila males. The chemicals used were: heliotrine, a pyrrolizidine alkaloid; triethylene melamine (TEM) and diepoxybutane (DEB), both bifunctional alkylating agents; and ethylene imine (EI), quinacrine mustard (ICR100), dimethylnitrosamine (DMNA) and diethyl sulphate (DES), all monofunctional alkylating agents. In addition, the antibiotic streptonigrin was tested for possible mutagenic activity. The experiments carried out and the results obtained were as follows:

1. The treated males were mated every three days to fresh virgin females to obtain a maximum of five three day broods. The progeny of the first, fourth and fifth broods were tested for recessive sex-linked lethals and translocations, the latter two broods being taken as representative of spermatogonia at the time of treatment. It was found that four of the chemicals used, namely heliotrine, TEM, DEB, and EI were successful in producing translocations in spermatogonia, and that they were superior to X-rays in this respect. ICR100 also produced one translocation in the fifth brood. A pilot experiment using streptonigrin showed that it is weakly mutagenic, although it
produced a high degree of sterility, even at the low concentrations used. It is interesting to note that one translocation was produced in the fifth brood, i.e., in spermatogonia, providing additional evidence of the ability of chemicals to produce rearrangements at this stage.

2. On comparing the results obtained with the mono- and polyfunctional alkylating agents, it was found that both the bifunctional agents produced lethals and translocations in all broods tested, while ICR100, DMNA, and DES produced high lethal frequencies but no translocations apart from the one produced by ICR100. However, the remaining monofunctional agent, EI, produced the highest number of translocations relative to lethals of all the chemicals tested. This meant that no clear difference between the two types of chemical could be drawn from the above results.

3. Following treatment of the Drosophila males with EI and TEM, the treated sperm were stored for six days in the inseminated females. It was found that after storage the TEM translocation/lethal ratio rose steeply, due to a large increase in translocation frequency accompanied by a much smaller increase in sex-linked lethal frequency. The ratio for EI, however, remained constant before and after storage. This shows a clear qualitative difference between mono- and polyfunctional alkylating agents, which is presumably related to the mechanism by which they produce chromosome breaks. The hypothesis is put forward that the storage effect might be the main reason for the greater effectiveness of polyfunctional alkylating agents in carcinoostasis.

95.
A cytological analysis of a number of sex-linked recessive lethals produced before and after storage following treatment with TEM was carried out, the post-stored lethals having first been located. The results showed that the number of lethals associated with chromosome breakage increased after storage, supporting the hypothesis that chromosome breaks, but not point mutations, increased during the storage period.
ACKNOWLEDGEMENTS

It was my great privilege during the course of this investigation to be guided and encouraged by Dr. C. Auerbach, F.R.S., and I wish to extend my sincere thanks to her for all her help. I would also like to thank Professor C.H. Waddington, F.R.S., for providing laboratory facilities, Mr. E.D. Roberts for assistance with the figures, and the Medical Research Council for financial assistance.
BIBLIOGRAPHY


STRAUSS, B.S. & R. WAHL (1964). The presence of breaks in the deoxyribo-
nucleic acid of Bacillus subtilis treated in vivo with the alkylating
SWANSON, C.P. & T. MERZ (1959). Factors influencing the effect of β-propio-
SZYBELSKI, W. & V.N. IYER (1960) in Abstracts of the Biophysical Society
Meeting held in 1959 at Cambridge, Massachusetts.
TRAUT, H. (1960). Uber die Abkangigkeit der Rate strahleninduzierten
Translokationen und recessiv geslechtgebundener Letalfaktorenen vom
Stadium der Spermatogenese bei Drosophila melanogaster. Z. Indukt.
Abstamm.-u. Vererblehre. 91: 201.
Acad.Sci. 68: 750.
mutations in eight specific loci in the third chromosome of
WATSON, W.A.F. (1962). The production of translocations in spermatogonial
cells of Drosophila by chloroethyl methanesulphonate (CB 1506).
WATSON, W.A.F. (1964). Evidence of an essential difference between the
genetical effects of mono- and bi-functional alkylating agents.
WESTERGAARD, M. (1957). Chemical mutagenesis in relation to the concept of
Amer. Nat. 94: 85.


In order to determine whether chemicals are superior to X-rays in the production of translocations in spermatogonia of Drosophila melanogaster, and also to find out if there was a difference between the genetical effects of monofunctional and polyfunctional alkylating agents, a number of chemicals known to act on both pre- and post-meiotic germ cells were administered by injection or feeding to adult Drosophila males. The chemicals used were: heliotrine, a pyrrolizidine alkaloid; triethylene melamine (TEM) and diepoxynitrene (DEB), both bifunctional alkylating agents; and ethylene imine (EI), quinacrine mustard (ICRIO), dimethylamino (DMA) and diethyl sulphate (DES), all monofunctional alkylating agents. In addition the antibiotic streptonigrin was tested for possible mutagenic activity. The experiments carried out and the results obtained were as follows:

1. The treated males were mated every three days to fresh virgin females to obtain a maximum of five three day broods. The progeny of the first, fourth and fifth broods were tested for recessive sex-linked lethals and translocations, the latter two broods being taken as representative of spermatogonia at the time of treatment. It was found that four of the chemicals used, namely heliotrine, TEM, DEB, and EI were successful in producing translocations in spermatogonia, and that they were superior to X-rays in this respect. ICRIO also produced one translocation in the fifth brood. A pilot experiment using streptonigrin showed that it is weakly mutagenic, although it produced a high degree of sterility, even at the low concentrations used. It is interesting to note that one translocation was produced in the fifth brood, i.e. in spermatogonia, providing additional evidence of the ability of chemicals to produce rearrangements at this stage.

2. On comparing the results obtained with the mono- and polyfunctional alkylating agents, it was found that both the bifunctional agents produced lethals and translocations in all broods tested, while ICRIO, DMA, and DES produced high lethal frequencies but no translocations apart from the one produced by ICRIO. However, the remaining monofunctional agent, EI, produced the highest number of translocations relative to lethals of all the chemicals tested. This meant that no clear difference between the two types of chemical could be drawn from the above results.

3. Following treatment of the Drosophila males with EI and TEM, the treated sperm were stored for six days in the inseminated females. It was found that after storage the TEM translocation/lethal ratio rose steeply, due to a large increase in translocation frequency accompanied by a much smaller increase in sex-linked lethal frequency. The ratio for EI, however, remained constant before and after storage. This shows a clear qualitative difference between mono- and polyfunctional alkylating agents, which is presumably related to the mechanism by which they produce chromosome breaks. The hypothesis is put forward that the storage effect might be the main reason for the greater effectiveness of polyfunctional alkylating agents in carcinostasis.
4. A cytological analysis of a number of sex-linked recessive lethals produced before and after storage following treatment with TEM was carried out, the post-
stored lethals having first been located. The results showed that the number of
lethals associated with chromosome breakage increased after storage, supporting the
hypothesis that chromosome breaks, but not point mutations, increased during the
storage period.