The work embodied within this thesis is the result of a research programme which I have carried out, and no Degree has been conferred upon me for any part of this work unless indicated by the phrase,-Brink ; unpublished.

Signed: N.J.Brink

SOME ASPECTS OF THE MUTAGENIC ACTIVITY OF THE PYRROLIZIDINE ALKALOID HELIOTRINE IN DROSOPHILA MELANOGASTER

Ph. D.

N. G. Brink
Department of Zoology
University of Tasmania
Hobart
Tasmania.
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INTRODUCTION

The pyrrolizidine alkaloids are a group of chemical compounds which occur naturally in certain genera of the Compositae, Luguminosae and Boraginaceae. The alkaloid molecule consists of a basic methylpyrrolizidine ring with one or more hydroxyl groups esterified by mono- or di- basic carboxylic acids (Warren, 1955). Heliotrine is an open monoester, with one of the hydroxyl groups of the basic moiety heliotridine esterified with heliotric acid. Its structural formulae is shown in Figure 1.

The alkaloids possess considerable biological activity. The prevalence of hepatic cirrhosis in livestock which have grazed on pastures infected with Senecio, Crotalaria, Heliotropium and other plants containing these alkaloids has been reported on numerous occasions since its first recorded occurrence in New Zealand sixty years ago. The purified alkaloids extracted from various plant species produce liver cirrhosis and primary liver cancer in experimental animals (see for example, Harris, Rose and Chen, 1957; Bull, Dick, and McKenzie, 1958; Bull and Dick, 1959; Schoental and Magee, 1959; Cook, Duffy and Schoental, 1950). In addition, the alkaloids inhibit smooth muscle tonus and antagonize acetyl choline (McKenzie, 1958), block neuromuscular transmission in rat phrenic nerve diaphragm preparations (Gallagher and Koch, 1959), disrupt the TCA cycle and cause complete loss of activity of the DPN-dependent enzymes in the rat liver (Christie, 1958; Christie, LePage and Baille, 1961).

Histologically, one of the most characteristic changes produced in the hepatic cells of animals which have developed liver cirrhosis following treatment with pyrrolizidine alkaloids, is a marked increase in their size

(Bull, 1955; Schoental and Magee, 1959). Accompanying this increase in cell size, referred to as megalocytosis, is a corresponding increase in the size of the nucleus. Bull and Dick (1960) have suggested that the enlarged nuclei may be due to polyploidy and have shown that the amount of DNA in these nuclei is significantly greater than that normally present in the average hepatic cell. However, since the incidence of polyploidy in liver cells is known to increase with advancing age, it remains uncertain whether the increased DNA content observed by Bull is due to this or to polyploidy or polyteny induced by the alkaloid.

However, the pyrrolizidine alkaloids apparently affect the nuclear material, since they are mutagenic in Drosophila (Clark, 1959; 1960a) and produce chromosome and chromatid aberrations in Allium cepa (Avanzi, 1961). Recently it has been found that heliotrine produces chromosome breaks in the peripheral leucocytes of the rat kangaroo, Potorous tridactylis, in tissue culture (Clark and Bick, unpublished).

The mechanism whereby the alkaloids react with the nuclear material to produce genetic damage is not clear, although in the case of heliotrine both the acid and basic moieties appear to be necessary for mutagenic activity, since neither heliotridine nor heliotric acid were found to be mutagenic in Drosophila when injected alone (Clark, 1959). In addition, alkaloids lacking the 1 : 2 double bond in the methylpyrrolizidine ring (e.g. platyphylline) are not mutagenic (Clark, 1960a). Recently, Culvenor, Dann and Dick (1962) suggested that pyrrolizidine alkaloids act on cell nuclei by a process of alkylation. The fact that both the acid and basic moieties of the molecule, as well as the 1 : 2 double bond in the methylpyrrolizidine ring, are necessary

for

for producing genetic damage has led Culvenor et al. to propose that they may react with the genetic material by a process of alkyl-oxygen fission. Avanzi has suggested that oxygen tension may be an important factor in the mutagenic activity of pyrrolizidine alkaloids in Allium, since she found that cysteine reduces the frequency of chromosome and chromatid aberrations they produced. However, Culvenor et al. have shown that the pyrrolizidine alkaloids react with cysteine and conclude that the protective effect of this compound in Allium may result from its combination with the alkaloids rather than by reducing the oxygen tension within the treated cells.

The pyrrolizidine alkaloids are potent mutagens in Drosophila, irrespective of whether they are injected intra-abdominally or added to the food medium. For heliotrine, which is the alkaloid most thoroughly investigated so far for mutagenic activity, Clark (1963a) has suggested that the spermatocytes and possibly the early spermatid and late spermatogenial stages are the most sensitive germ cells in the male, both for the production of sex-linked lethals and chromosome breakage. Prolongued feeding on the alkaloid renders males reproductively sterile once their available supply of mature sperm have been utilized, whilst comparatively short feeding periods markedly affect the fertility of females (Brink, unpublished).

In the present investigation an experimental study has been made of:-

(i) the ability of heliotrine to produce sex-linked lethals, sex-linked lethal mosaics, dominant lethals, translocations, deletions, chromosome loss and delayed chromosome loss in Drosophila.

(ii) the distribution along the X-chromosome of 117 sex-linked lethals produced by heliotrine. This distribution is compared with those obtained by other workers for X-ray and chemically induced lethals.

(iii) the mutation frequencies produced by combined heliotrine and X-ray treatments in Drosophila.

(iv) the effect of the metabolic inhibitors sodium azide, potassium cyanide, sodium hydrosulphite and gaseous nitrogen as well as the antibiotic chloramphenicol on the mutagenic activity of heliotrine in Drosophila.

The sensitivity of the various germ cell stages in the Drosophila testis to the mutagenic action of heliotrine has been examined by the brood pattern technique. In most experiments the males were first mated twenty-four hours after treatment and subsequently transferred to three fresh females every three days. This method was initially used by Auerbach (1954) in her study of the sensitivity of the Drosophila testis to the mutagenic action of X-rays, although more frequent brood changes are now used by many investigators in order to minimize the mixing of different germ cells in a particular brood. However, the three day interval is generally considered adequate for an initial determination of the brood pattern of sensitivity produced by a particular mutagenic agent. Refinements to this pattern can be obtained subsequently by making use of a shorter brood interval.

Auerbach (1957) has drawn attention to errors which can be made in the interpretation of data obtained by brood pattern analysis. This applies particularly to comparisons of the brood patterns of sensitivity produced by different chemical mutagens or different concentrations of the same mutagen. Thus two chemical mutagens may produce identical brood patterns of sensitivity (i.e. maximum sensitivity occurs in the same brood), and from this it could erroneously be concluded that the germ cell

sensitivity pattern for the two mutagens was also identical. However, the chemical treatments may alter the rate of development and utilization of the germ cells so that a particular germ cell type is not used in the same brood in each case. Consequently, in order to equate the brood pattern of sensitivity with a particular germ cell type it is necessary to use some genetic marker (e.g. induced crossing-over or non-disjunction) to delimit certain stages of spermatogenesis.

MATERIALS AND METHODS

Throughout this investigation only male Drosophila have been treated. Heliotrine was used at a concentration of 0.001 M. It was made up in 0.7% saline and injected intra-abdominally. The micropipettes used for injection and the method of injection have been described by Clark (1963a; see also Appendix 1). In some earlier experiments the volume injected was between 0.11μl. and 0.12μl., but owing to the sterilizing effect of the alkaloid in the later broods it was reduced to 0.09μl. In subsequent experiments. Virgin males, aged between 16 and 48 hours, were treated and mated to three virgin females (strain depending on the type of mutation to be scored) with fresh females being provided every three days until four or five broods had been obtained. In some experiments a two day brood interval was used.

The flies were cultured in 4" x 1" glass vials on a semolina-molasses-agar medium (see Appendix II) which was supplemented with a drop of live yeast suspension the day before the flies were placed in the vials. The cultures were incubated in a constant temperature room maintained at 25^{o} $\pm$ 1^{o}C.

The actual crosses made for the detection of the various types of mutations are described in the appropriate sections.

PART I

RESULTS

I. DISTORTION OF THE F1 MALE/FEMALE RATIO AND THE FREQUENCIES OF SEX-LINKED RECESSIVE LETHALS AND DOMINANT LETHALS PRODUCED BY HELIOTRINE IN DROSOPHILA

a) Experimental Methods.

(i) Sex-linked lethals and distortion of the F1 sex ratio.

Sex-linked lethals produced by heliotrine in Canton-S, X^{C2} v f (ring-X) and y v / $sc^{8}Y^{B-S}$ males were detected by a modification of the Muller-5 method described by Spencer and Stern (1948). The treated males were mated to asc (sc^{S1} In - S w^{a} sc^{8}) females, since in the stocks available in this laboratory, females homozygous for this chromosome showed higher fecundity than the Basc females.

After scoring the F1 sex ratio, the flies were placed in half pint milk bottles on a honey-agar medium and allowed to mass mate for several days before the females were placed individually in small creamers for the detection of sex-linked lethals in the F2 generation. In one of the experiments in which y v / sc^{8} Y^{B-S} males were used, the F1 females were backcrossed to asc males to facilitate the scoring of lethals by eliminating the marked Y chromosome.

All F1 females were tested for sex-linked lethals to avoid any bias resulting from differential rates of development of normal females and those heterozygous for a sex-linked lethal (Yanders, 1958).

(ii) Dominant lethals.

For the detection of dominant lethals, treated Canton-S or X^{C2} v f males were mated to asc females. When the male was transferred to fresh females at each brood change, the females with which it had mated during the

previous three days were placed individually in 2" x ½" clearsite vials, the lid of which contained the usual Drosophila food medium mixed with a small amount of lampblack to give greater optical contrast between the eggs and the food medium. The lids were changed after twenty-four hours and the females discarded after forty-eight hours. The lids were then placed in fly-proof containers and the numbers of hatched and unhatched eggs counted twenty-four hours later. In a few of the "egg dishes" none of the eggs hatched. These were discarded from the count since it is likely that the females were not inseminated or had exhausted their supply of sperm.

b) Experimental Results.

(i) Sex-linked lethals

The spontaneous incidence of sex-linked lethals in Canton-S males was found to be 0.15% (3872 tested chromosomes), whilst in $X^{C2}v\ f$ males it was 0.61% (1797).

Three replicate experiments were carried out to determine the frequency of sex-linked lethals produced by heliotrine in Canton-S males.

In one of these experiments records were kept showing the lethal frequency induced by the alkaloid in individual males. The results, shown in Table 1, clearly indicate significant heterogeneity. Although the same quantity of alkaloid was injected into each fly, the amount subsequently reaching the germ cells within the testes of individual males probably varies considerably. This would certainly affect the yield of mutations produced. In addition, the rate of maturation and subsequent utilization of the germ cells may be affected thus leading to a shift in the brood pattern of sensitivity. Previous work (Brink, unpublished), in which the

sterilizing effect of heliotrine in Drosophila was investigated by administering the alkaloid along with the food supply, showed considerable variation in the degree of atrophy of the testes amongst males treated for the same length of time. This variation was probably due to different quantities of alkaloid being absorbed by the gonads of the individual males.

Despite this marked heterogeneity in the mutagenic activity of heliotrine in the individual males of a treated series, a comparison of several such series shows no significant heterogeneity (Table 2). Consequently the data for each of these three experiments has been combined and the overall mutation frequency, with 95% confidence limits, for each of the five broods is shown in Figure 2 (continuous line).

Two replicate experiments were carried out to determine the sex-linked lethal frequency produced by heliotrine in $\underline{X^{C2}v\ f}$ males. The data for the two experiments are shown in Table 3, and since there is no marked heterogeneity between the results, the figures have been combined and the resultant mutation frequencies, with 95% confidence limits, for each of the seven broods (two day brood interval) are also shown in Figure 2 (broken line).

Table 4 shows the results of two replicate experiments using $\underline{y\ v\ /\ sc^{8}\ Y^{B-S}}$ males. In the first experiment the treated males were mated to asc; bw; st females, whilst in the second, asc females were used. Since there is no significant heterogeneity between the two experiments the data have been combined and the resultant sex-linked lethal frequency, with 95% confidence limits, is shown in Figure 3 (broken line). The results obtained for Canton-S males are also shown in this figure (continuous line) for purposes of comparison. The marked Y chromosome carried

by the y v / sc^8 Y^{B-S} males also enabled chromosome loss and non-disjunction to be scored. These results will be considered below (page 20).

Figure 2 indicates that the brood pattern of sensitivity for Canton-S and X^{C2} v f males is similar except for those germ cells used on the fourth day after treatment, where a significantly lower frequency of lethals was found in the ring-X males (7.13 $\pm$ 1.22% lethals in rod-X's; 2.96 $\pm$ 1.25 % lethals in ring-X's). In both strains the maximum lethal frequency occurs in sperm used on the seventh to eighth days following treatment (9.33 $\pm$ 1.51% for Canton-S males and 8.60 $\pm$ 2.49% for X^{C2} v f males).

The brood pattern of sensitivity obtained for y v / sc^8 Y^{B-S} males is dissimilar to that obtained for Canton-S males (Figure 3). As was the case with the ring-X flies, the frequency of lethals induced in sperm used on the third to fourth days after treatment is significantly lower than that obtained for fourth day sperm from Canton-S males (2.13 $\pm$ 0.82% lethals for third day sperm in y v / sc^8 Y^{B-S} males, 7.13 $\pm$ 1.22% lethals for fourth day sperm in Canton-S males). In addition, the maximum lethal frequency obtained in the mutant stock is significantly lower than that observed in the wild type males (5.69 $\pm$ 1.52% lethals in the former and 9.33 $\pm$ 1.51% lethals in the latter stock). The maximum yield of lethals is obtained on the seventh day after treatment in the wild type stock and on the ninth day in the y v / sc^8 Y^{B-S} males.

(ii) Dominant lethals and distortion of the F1 sex ratio

The frequencies of inviable eggs laid by asc females following fertilization by untreated Canton-S or X^{C2} v f males are shown in Table 5. A significantly higher proportion of eggs fail to hatch when the females are mated to the ring-X males ($\chi^2 = 17.6$, $p < 0.0001$).

Two replicate experiments were carried out to test the effect of heliotrine on the control frequency of egg hatchability in Canton-S males, and two replicates were similarly undertaken using $\underline{X^{C2}v\ f}$ males. The results of these four experiments are shown in Table 6. Since there is no marked heterogeneity between the two replicates in each of the experimental series, the data have been combined and the resultant frequencies of unhatched eggs, with 95% confidence limits, for both Canton-S (continuous line) and $\underline{X^{C2}v\ f}$ (broken line) males are shown in Figure 4.

In both types of male, the germ cells used on the seventh and tenth days after treatment appear to be the most sensitive with regard to the production of dominant lethals by heliotrine. This is clearly indicated in Table 7, which shows the relative dominant lethal frequency produced by the alkaloid in both rod- and ring- X's. The relative dominant lethal frequency (RDF) is derived from the following expression:-

$$RDF = 1 - \frac{\text{frequency of hatched eggs in the experimental series}}{\text{frequency of hatched eggs in the control series}}$$

The RDF obtained in the second brood (fourth day sperm) is intermediate between that of the first and third broods in both the "rod" and "ring" series. This may indicate that the germ cells utilised in this brood are of intermediate sensitivity, or alternatively a mixture of sensitive and insensitive stages in this brood could account for the intermediate value observed. The RDF values indicate that the alkaloid is from 1.5 - 2 times more efficient in producing dominant lethals in ring-X males than in rod-X males in all broods.

The increase in the F1 male/female ratio resulting from the mating

of treated males to untreated females can be used to give a measure of the frequency of dominant lethals produced in the X-chromosome, since those female embryos inheriting a paternal-X which has rejoined to form an inviable chromosome rearrangement (e.g. sister chromatid union or an asymmetrical chromosome exchange) will die.

For untreated Canton-S males mated to asc females a sex ratio of 0.51 (2500 males and 4949 females) was obtained, whilst for untreated X^{C2}v f males crossed to asc females the ratio was 0.86 (3360/3892). Since the frequency of inviable eggs laid by asc females mated to untreated X^{C2}v f males was only slightly higher than that obtained when Canton-S males were used, the marked difference in the male/female ratio between the two stocks may have been due to the greater selective advantage of the Canton-S/asc heterozygous females over the asc males than was the case with X^{C2}v f/asc females and asc males.

The effect of heliotrine on the control sex ratio was scored in the sex-linked lethal experiments described previously, the sex of the F1 flies being scored preparatory to their being set up for the detection of lethals in the F2. The data for two replicate experiments in the Canton-S series and two replicates in the X^{C2}v f series have been pooled and the results are shown in Figures 5 and 6 respectively.

In both series, the brood pattern of sensitivity for distortion of the sex ratio is similar to that obtained for dominant lethals. In Table 8 are shown the relative increases in the male/female ratio produced by heliotrine in each brood with respect to the control values in both the "rod" and "ring" series. As was the case with dominant lethals, the increase in the sex ratio produced by the alkaloid in ring-X males is greater than

the corresponding increase in rod-X males in all broods.

Zygotic lethality is only one of several causes which may result in the non-hatching of eggs. Thus induced sterility in the male or physiological inactivation of the sperm could result in many unfertilized eggs being laid. A close correlation between the brood patterns of sensitivity for dominant lethals and distortion of the sex ratio produced by heliotrine is expected on the hypothesis that zygotic lethality accounts for the non-hatching of eggs. Calculation of the correlation coefficients showed a high degree of positive correlation between the relative dominant lethal frequency and the F1 male/female ratio in both rod- and ring- X males ($r = 0.84$ for Canton-S males, $r = 0.90$ for $X^{C2}v\ f$ males).

In the $X^{C2}v\ f$ stock the positive correlation between dominant lethals and sex ratio is less marked at the higher lethal frequencies ($>70\%$) where there appears to be a significant increase in the sex ratio with little change in the yield of dominant lethals. There appear to be two explanations for this discrepancy. Firstly, as the relative dominant lethal frequency approaches 100%, there will be an increase in the number of eggs carrying more than one lethal. Since these eggs will be recorded as possessing only one lethal, the observed frequency will be lower than the actual yield. Secondly, in the more sensitive germ cells there may be a relative increase in the frequency with which damaged X^{C2} chromosomes are lost. Thus potential female embryos become XO males, and this serves to decrease the number of female offspring whilst at the same time it increases the number of males. This is in contrast to the reduction in the number of female offspring without any corresponding increase in the number of males when the damaged X^{C2} chromosome behaves as a dominant

lethal. Where the relative number of females is low as a consequence of dominant lethals being produced in the treated paternal ring-X, only a small increase in the number of damaged "rings" being eliminated from otherwise viable gametes (as might occur during the reduction divisions) is needed to markedly alter the sex ratio.

c) Discussion of Results

(i) Sex-linked lethals

The sex-linked lethals produced by ionizing radiations in Drosophila have been divided into three classes (Lea, 1955). Type A lethals are those due to point mutations. These are undetectable cytologically and may or may not be associated with chromosome breakage followed by restitution. Type B lethals are those associated with deletions and these are usually detectable cytologically by the absence of one or more bands in the salivary gland chromosomes. Type C lethals include those associated with major structural rearrangements (e.g. inversions and symmetrical interchanges).

When ring-X males are treated, Type C lethals associated with interchanges are inviable as they result in the formation of dicentrics (Catcheside and Lea, 1945). However, Type A lethals not associated with breakage as well as a proportion of Type B and Type C (associated with inversions) lethals are viable in ring-X's. According to the estimates of Catcheside and Lea about half the deletions and inversions are viable. Many Type A lethals associated with chromosome breakage followed by restitution will be inviable in ring-X's, since twisted reunion or intrachromosomal crossing-over will lead to the formation of large dicentric rings or interlocking rings in a proportion of lethal bearing gametes.

Following heliotrine treatment, the yields of lethals obtained in rod- and ring- X's were similar except in those germ cells used on the fourth day, where the frequency induced in ring-X's was significantly lower than that obtained in "rods". This is similar to the results obtained by Clark (1963a). The lower yield of lethals in fourth day sperm from $\underline{X^{C2}v\ f}$ males may be due to the elimination of Type C lethals as well as those A and B lethals associated with chromosome breakage and intrachromosomal crossing-over during restitution. However, since the dominant lethal frequency and distortion of the sex ratio are similarly depressed in this brood in ring-X males, this explanation for the low yield of sex-linked lethals does not appear likely. On the other hand, the observed discrepancy between the mutation yields obtained in the germ cells used on the fourth day in the two stocks may be due to strain differences (see below, p 16).

In all other broods the sex-linked lethal frequency produced by the alkaloid in the two stocks is similar indicating that the majority of lethals are probably due to point mutations which are not associated with chromosome breakage. The Fahmy's (Bird and Fahmy, 1953; Fahmy and Fahmy, 1955a) have made the claim that some deficiencies produced by alkylating compounds are not the result of chromosome breakage but are due to "various degrees of failure of gene reproduction". If this claim proves to be correct and applicable to other chemical mutagens it is possible that many Type B lethals (i.e. small deletions) produced by these compounds will also be viable in ring-X's.

Further evidence that most of the lethals produced by heliotrine are of the A type comes from the cytological observations of Gunson (unpublished). She found that only about 5 - 10% of lethals produced by 0.001 M heliotrine were associated with cytologically detectable changes in the salivary gland chromosomes. Most of the changes were either deficiencies or translocations.

(ii) The mutagenic activity of heliotrine in different strains of Drosophila

Strømnaes (1958 ; 1959) has reported dissimilarities in the mutation frequency produced by ionizing radiations in the Oslo and Iso-Amherst strains of Drosophila melanogaster , whilst Auerbach and Moser (1953 a) and Auerbach (1956) have reported differences in the brood sequence of mutation frequencies for formaldehyde treated larvae of different strains. On the other hand, Oster (1958 a) failed to detect a significant difference in the mutation frequency produced by X-rays in several different strains of Drosophila. Strømnaes has suggested that the strain differences may be correlated with dissimilarities in the rate of maturation and utilization of germ cells in different stocks. Alternatively, they may be due to differences in the sensitivity of a particular stage of spermatogenesis. Lindsley, Edington and von Halle (1958) have reported differences in the radiation sensitivity of mature sperm when the sex chromosome constitution is altered.

In the present investigation three strains of flies were treated. The difference between the Canton-S and X^{C2} v f males, with regard to the frequency of sex linked lethals produced in those germ cells used on the fourth day after treatment, may be due to strain differences similar to those discussed above. This hypothesis is further supported by the sex ratio data. Thus the relative increase in the sex ratio in this germ cell stage in ring-X males is only 56% of the increase found in eighth day sperm, whereas in the Canton-S males the relative increases for broods two and three (fourth and seventh day sperm respectively) are of comparable magnitude. Furthermore, the brood pattern of sensitivity for $\frac{y\ v}{sc^8}\ Y^{B-S}$ males is dissimilar to that obtained for Canton-S males. Thus peak

sensitivity occurs on the ninth day following treatment (cf. the seventh day for Canton-S males), and in all broods, with the exception of the fifth, the mutation frequency is significantly greater in the wild type stock.

The data obtained in these experiments appear to indicate that the three stocks differ in their sensitivity to the mutagenic activity of heliotrine. Whether this dissimilarity is due to differences in rates of germ cell maturation or whether it is actually due to sensitivity differences has not been determined. In an attempt to solve this problem, it would be of interest to test other chemical mutagens, particularly those with a similar pattern of germ cell sensitivity to that of heliotrine, using these same stocks. If differences in the rate of germ cell maturation are responsible, then the strain differences should be detectable for each mutagen tested. On the other hand, if sensitivity differences are involved, the various strains may show a similar response to one and dissimilar response to another mutagen.

11. THE DELETION, CHROMOSOME LOSS, TRANSLOCATION AND SEX-LINKED LETHAL MOSAIC FREQUENCIES PRODUCED BY HELIOTRINE IN DROSOPHILA

a) Experimental Methods

(i) Hyperploid Females (Deletions)

The frequency of hyperploid females obtained from the mating of treated males with attached-X females can be used to give a measure of the deletion frequency (Bateman, 1957). In this investigation treated Canton-S males were mated to attached-X females homozygous for yellow body (yy). The brood interval was two days. Only two types of F1 offspring are expected - yellow females and wild type males - most of triplo-X and all the YY flies failing to survive. Non-yellow hyperploid females occasionally occur in the progeny. They carry a centric fragment of the paternal-X containing the allele y^+ in addition to the attached-X chromosome. If the centric fragment is large (i.e. there is only a small deletion), the hyperploid females are small with roughened eyes, scalloped wing margins and occasional malformation of the appendages as the triplo-X condition is more nearly approached.

(ii) XO males, translocations, sex-linked lethal mosaics and other delayed genetic effects

A group of three experiments were carried out, in each of which, one or more of these genetic effects were scored. In each case $\frac{y\ v}{sc^8\ Y^{B-S}}$ males were treated. These males possess an X chromosome which carries the marker genes y and v, whilst attached to the short arm of the Y chromosome is an X fragment bearing sc^8, y^+ and bb^+. In addition the Bar-Stone marker is attached to the long arm of the Y. Phenotypically, these males are non-yellow Bar vermilion-eyed (Brosseau, Nicoletti, Grell and Lindsley, 1961).

In the first experiment these males were mated to $y\ w^a$, bw, st females. A three day brood interval was used and translocations (2 - 3,

2 - Y, 3 - Y or 2-3-Y), chromosome loss (X, Y, Y^S or Y^L) and non-disjunction of the paternal X and Y were scored according to the methods outlined in Figure 7.

In the second experiment, the treated males were crossed to asc ; bw ; st females. A three day brood interval was used and XO males (loss of X, Y or Y^L and/or non-disjunction), sex-linked lethals, sex-linked lethal mosaics and translocations involving the second, third and/or Y chromosome were scored according to the methods outlined in Figure 8. The F1 females were collected as virgins and mated to asc males for the detection of sex-linked lethals (absence of yellow vermilion males in the F2 cultures indicating the presence of a lethal). A 30-50% sample of the non-lethal F2 cultures was selected at random and retested for the presence of mosaic lethals. Eight asc/y v heterozygous females from each of these non-lethal cultures were placed individually in culture vials and an F3 progeny obtained. If one or more of the eight females failed to produce yellow sons amongst her offspring, their mother (an F1 female) was assumed to be a gonadel mosaic for a sex-linked lethal. The F1 males were tested for translocations by crossing to bw ; st females. The male offspring of this cross were also scored for eye shape, and if two or more non-Bar male progeny were found (at least twelve males scored in each culture), then the F1 male was assumed to be a gonadel mosaic for loss of Y or Y^L or delayed non-disjunction,

although in this latter case the reciprocal class of flies (i.e. Bar eyed females) should also have been present.

In the third experiment, the treated males were mated to asc females, and the frequency of XO males and sex-linked lethals scored in the F1 and F2 generations respectively. XO males were scored as in the previous experiment, whilst the absence of vermilion eyed males in the F2 indicated the presence of a lethal. The scoring of lethals was carried out using the low power of a binocular microscope because of the presence of the Bar^{Stone} marker on the Y chromosome in the males. Delayed loss of the X or Y (appearance of non-Bar males in the F2) and delayed non-disjunction (appearance of non-Bar males and Bar eyed females in the F2) were also scored in this experiment.

b) Experimental Results.

(i) Hyperploid Females

Table 9 shows the frequency, with 95% confidence limits, of hyperploid females induced by heliotrine in two separate experiments. The deletions produced by the alkaloid in the treated paternal-X were apparently quite small, since most of the hyperploid flies exhibited scalloping of the wings and occasional malformation of the appendages. The brood pattern of sensitivity is similar in the two experiments, with a peak occurring in broods four, five and six, although the total yield of hyperploids in the second experiment was greater than that obtained in the first in all broods.

(ii) Translocations, XO males, sex-linked lethal mosaics and other delayed genetic effects

The results of the three experiments carried out to test for these various genetic effects are summarised in Table 10.

A very low frequency of translocations is produced by the alkaloid, only three being recovered out of 3502 gametes in all broods of both experiments.

All occurred in the later broods, corresponding to sperm used on or after the ninth day. One involved the two large autosomes, whilst the other two occurred between the Y chromosome and the second autosome and the Y and the third autosome respectively.

Chromosome loss and/or non-disjunction were scored in all three experiments. In the first, loss of Y^S and Y^L were scored in addition to XO males which arose as a result of complete loss of the Y or X or non-disjunction of these two chromosomes. It was found that complete loss (or non-disjunction) occurred comparatively rarely, only two cases being found out of 3210 tested gametes in all broods. On the other hand, three males were found in which the markers located on Y^S had been lost, and eight in which the marker on Y^L was missing. In addition, two mosaics were found in the fifth brood, one showing complete loss on one side and loss of the y^+ marker on the opposite side, whilst the other had one normal eye and one Bar eye. All thirteen males displaying partial loss of the Y chromosome were tested for fertility and as a result it was found that six ($3B^+ y^+$, 2 B y and the B y^+ / B^+ y^+ mosaic) produced offspring when mated to bw ; st females. This indicates that either the marker genes on the long and short arms of the Y had mutated to y and B^+ respectively, or alternatively, they may have been deleted without the accompanying loss of the fertility genes on these arms. In this experiment, brood five showed the maximum frequency (1.04%) for all types of chromosome loss and/or non-disjunction.

In the second experiment only three XO males were found amongst 1668 males scored giving a frequency of 0.18%. Two of the abnormal flies were found in brood four (0.60%) and the other in brood one (0.24%). The normal F1 males were mated to bw , st females for the detection of translocations and delayed Y chromosome loss. The progeny from this cross were narcotised with

CO_2 and examined under the low power of a binocular microscope in order to reduce the possibility of translocations and non-Bar males being overlooked. Non-Bar males may have occurred as a result of spontaneous loss of the Y, or the Bar^{Stone} marker, but where two or more were found in the same culture delayed loss of Y or Y^L as a result of heliotrine treatment probably accounted for the observed result. In this case loss may have occurred during the reduction divisions in some germ cells of the F1 male. Delayed loss was found to occur in all broods, with the maximum yield in brood five (2.97%).

In the third experiment only two non-Bar abnormal XO males were found amongst 1996 scored, one in each of the fourth and fifth broods. The F1 flies were inbred and the F2 scored for sex-linked lethals, delayed loss of X, Y or Y^L and secondary non-disjunction. The genotypes and phenotypes resulting from delayed loss or non-disjunction, as well as those normally expected are summarised in Table 11.

Delayed chromosome loss may occur during the cleavage divisions in which case gynandromorphs should occur amongst the normal F1 flies. Alternatively, delayed chromosome loss and/or non-disjunction may occur during meiosis in the spermatocytes or oocytes of the F1 flies and this will lead to unusal genotypes occurring amongst the normal F2 progeny (Table 11). No gynandromorphs were found amongst the F1 flies. It therefore appears unlikely that delayed loss occurs during mitosis. On the other hand, sixteen F2 cultures were found in which non-Bar apricot eyed males and Bar eyed females occurred amongst the expected Bar males and non-Bar females. These abnormal offspring apparently occur as a result of non-disjunction of the y v and asc chromosomes during meiosis in some of the oocytes of the F1 female. All sixteen cultures were found in the

fourth brood giving a frequency of 3.22% delayed non-disjunction in this brood. In addition to these cultures, there was one in brood five which contained non-Bar males indicating that one of the F1 males was a gonadel mosaic for Y chromosome loss (i.e. loss occurred during the cleavage divisions). Alternatively, loss may have taken place during some meiotic divisions in the F1 male.

The results obtained in the three experiments indicate that the maximum frequency of XO males (including those flies in which only the long arm of the Y chromosome had been lost) occurs in the later broods corresponding to germ cells used on the ninth and subsequent days following treatment. In the two experiments where delayed loss (including secondary non-disjunction) was scored, the sensitivity pattern appeared to be similar to that obtained for XO males in the F1 generation.

The sex-linked lethal results obtained in Experiments 2 and 3 have been described previously (see page 9). The frequency of mosaic lethals produced by the alkaloid in Experiment 2 is lower than that obtained for complete lethals, although the difference is not statistically significant. However, included amongst the twenty lethals scored as mosaics are thirteen in which only one of the eight females tested had no yellow sons amongst her offspring. There are two alternative explanations which can account for these cases. Firstly, several replications of the chromosome may be required before the lethal can become expressed in a particular cell, or secondly, these single cultures possessing no yellow males may represent spontaneously arisen lethals. Many, if not all of these thirteen cases recorded as mosaics, probably represent spontaneous lethals. Thus the actual frequency of mosaics probably lies between the limites of 0.87% and the 2.49% shown in the Table. As was the case for complete lethals, the most sensitive germ cell stage is that utilised in

brood four. If only those seven cases, in which two or more of the eight females failed to produce yellow male offspring, are considered, four of them occurred in the fourth brood giving a mosaic frequency of 2.27% compared with 4.55% when all twenty cases were considered as mosaics. The frequency of complete lethals in this brood is 6.23%.

c) Discussion of Results

Other chemical mutagens appear to be comparatively inefficient in producing interchromosomal rearrangements (Auerbach and Moser, 1953 b; Slizynska, 1957 ; 1963 b ; Sonbati and Auerbach, 1960 ; Nakao and Auerbach, 1961; Watson, 1962). Thus the ratio of sex-linked lethals to 2-3 translocations in the most sensitive germ cell stage following X-irradiation has been found to be between 1 and 1.5 (Oster, 1955 ; 1958 a , Traut, 1960; Chandley and Bateman, 1960), whilst the most sensitive stage for the production of translocations and sex-linked lethals following treatment with mustard gas gave a ratio of lethals to 2-3 translocations of almost 5 (Sonbati and Auerbach, 1960). Nakao and Auerbach obtained a lethal/translocation ratio ranging from 5 to 20 following treatment with ethylene oxide or diepoxybutane. Watson obtained a ratio of almost 100 in the sensitive spermatogonial stage following treatment with chloroethyl methanesulphonate (CB 1506), although selection may have operated in these germ cells to reduce the number of translocations.

Heliotrine, like the other chemical mutagens, produces few interchromosomal rearrangements. Thus in the most sensitive germ cells (those used on the ninth day after treatment) in $\frac{y\ v}{sc^{8}\ Y^{B-S}}$ males the lethal/translocation ratio was found to be twenty, a value which is comparable with those obtained for other chemical mutagens.

Auerbach (1951) has suggested that the low frequency of

interchromosomal rearrangements produced following treatment with chemical mutagens may be due to the primary lesion being a "potential break" which only opens and becomes available for participation in chromosomal rearrangements later in the cell cycle or after several replications have taken place. Thus more intra- than inter-chromosomal rearrangements tend to be formed due to the fact that "potential breaks" located on different chromosomes often become available for participation in interchanges at widely separated times. Slizynska (1957; 1963a ; 1963 b) has clearly demonstrated this in the case of formaldehyde. She found that the ratio of interchanges (translocations) to intrachanges (inversions, deficiencies and repeats) was about five or six times greater in formaldehyde treated flies compared with the ratio obtained following treatment with X-rays.

Evidence that "potential breaks" are produced following heliotrine treatment has been obtained in the present investigation. Thus the ratio of hyperploid females (intrachromosomal deletions) to translocations (symmetrical chromosome exchanges) obtained for ninth day sperm, which showed maximum sensitivity for both types of genetic damage, was between 12 and 20, whereas the X-ray data of Chandley and Bateman (1960) gave a ratio of < 1 for spermatids and about 3.5 for spermatocytes. The latter germ cells show maximum sensitivity for deleted-X's, whilst most translocations occur in the spermatid stage following X-irradiation.

Further evidence that "potential" genetic damage is produced by chemical mutagens comes from experiments in which mosaicism, delayed chromosome loss and secondary non-disjunction have been found to occur. Thus Auerbach (1946) and Auerbach and Moser (1953 b) have found that mosaicism

occurs following treatment with mustard gas and formaldehyde respectively, whilst Altenberg and Browning (1961) have reported a high percentage of fractionals compared with whole body mutations following treatment with phenylalanine mustard, dimethyl myleran and 2, 5 bis-ethylene-iminohydroquinine. In addition, Carlson and his coworkers (Carlson and Oster, 1962; Carlson and Southin, 1963) have reported a high incidence of mosaicism following treatment with the monofunctional quinacrine mustard ICR 100. They found that the post-meiotic germ cells produced a higher yield of mosaics than the pre-meiotic stages, apparently as a result of the sorting out of damaged and undamaged strands during mitosis and meiosis. Sex-linked lethal mosaics have been found to occur following heliotrine treatment, although their frequency does not appear to be as high as that obtained for other mutagens. Thus Carlson and Oster found almost four times as many mosaics as complete lethals following treatment with ICR 100, whereas the yield of mosaics produced by heliotrine may only be about 25% of the frequency of complete lethals. The maximum yields of complete and mosaic lethals produced by heliotrine occur in the same brood (brood four). In brood five no mosaics were found. If this brood corresponds to treated spermatogonia (see page38), then a sorting out of strands during mitosis and the subsequent reduction divisions, similar to that reported by Carlson and Southin, may account for the non appearance of mosaics in this brood. However, since only seventy non-lethal F2 cultures were retested in this brood, the sample is probably too small to draw any definite conclusions as regards this hypothesis.

Auerbach (1947) reported evidence of delayed non-disjunction and chromosome loss following treatment of Drosophila with mustard gas. She suggested that a change in the centromere region resulted in the

treated chromosome following its homologue at meiosis and either becoming lost or incorporated within the same cell. The change did not appear to affect the functioning of the centromere during mitosis since no gynandromorphs were found amongst the F1 offspring. On Auerbach's hypotheses, delayed loss and non-disjunction may not necessarily be correlated with "potential" genetic damage, it may merely represent the delayed appearance of a particular genetic effect which can only become expressed after meiosis has taken place. In this regard, it would be of interest to determine whether X-irradiation, which is apparently not as efficient in producing "potential" genetic damage as are chemical mutagens, can induce delayed non-disjunction similar to that reported for mustard gas.

The delayed non-disjunction and delayed chromosome loss results produced by heliotrine may be due to a similar type of mechanism to that proposed by Auerbach for nitrogen mustard. Lindsley and Novitski (1958) have suggested that the centromere is merely the inactive anchor point of the chromosome attaching it to the spindle fibre. The kinetic activity for chromosome separation is related to the constitution of the heterochromatic regions which lie immediately adjacent to the centromere. It has been reported by several workers, mostly using plant cells, that heterochromatic regions are more susceptible to breakage by chemical mutagens than are euchromatic regions (McLeish, 1953; Revell, 1953 ; Moutschen-Dahmen and Moutschen-Dahmen, 1958; Fahmy and Bird, 1953). If it is found that certain groups of chemical mutagens react with heterochromatic regions, then non-disjunction and chromosome loss during the reduction divisions in the germ cells of the F1 offspring may be a prevalent type of genetic damage produced by these mutagens.

Chromosome loss and/or non-disjunction occurred most frequently on the ninth and twelfth days following heliotrine treatment.

Both Strangio (1961; 1962) and Såvhagen (1961 a) observed a sharp peak in the incidence of XO males on the 7th and 8th days following X-irradiation, this peak coinciding with the appearance of the first non-disjunctional females indicating that cells treated in pre-anaphase 1 were being utilised on these days. Although a proportion of the XO males represented the complimentary class of non-disjunctional flies, the frequency of X or Y chromosome loss still reached a peak on these days (Såvhagen, 1961 a). Using the same type of male and thereby eliminating the sensitivity differences which may result when different strains of flies are used, Strangio (1962) found that the maximum frequency of XO males occurred two days later than the maximum sex-linked lethal frequency. Similarly, Såvhagen (1960) observed that the maximum yield of translocations following X-irradiation occurred prior to the peak frequency of paternal sex chromosome loss. These results suggest that following X-ray treatment chromosome loss has a different sensitivity pattern to that obtained for lethals and translocations in that it occurs most frequently during the first meiotic division (Såvhagen, 1961 b).

An explanation of this difference may be found in a consideration of the various mechanisms which could lead to chromosome loss in the various stages of spermatogenesis. Sister-union, following the formation of chromatids in broken chromosomes which have failed to restitute, could lead to chromosome loss (Pontecorvo, 1941; 1942), provided the break point was close to the centromere. If the dicentric forms a large bridge at anaphase (break point far from the centromere) cell division may not be completed and sister-union in this case leads to dominant lethality (Pontecorvo, 1942). Loss due to this mechanism may occur in any germ cell stage where breakage or "potential breakage" is produced by the mutagenic treatment.

However, during the first meiotic division chromosome loss may also result from the failure of chromosomes to pair correctly or their inability to separate completely at the onset of anaphase 1 so that a chromosome may follow its homologue and be incorporated in the same cell (non-disjunction) or it may be "left out" of both cells. Gershenson (1933) suggested that chromosome loss resulting from pairing failure may account for the observed discrepancy between the number of exceptional males and females recovered as a result of primary non-disjunction. Using structurally altered X chromosomes he found that pairing was affected by loss or redistribution of heterochromatin within the chromosome. His results have been confirmed by Sandler and Braver (1954) using a number of structurally altered X and Y chromosomes. They found that loss of the Y occurred more frequently than loss of the X except in XYY males. In addition, the proposal of Lindsley and Novitski (1958) that heterchromotic regions adjacent to the centromere are important in the separation of chromosomes at anaphase would indicate that damage to these regions may affect the disjunction of chromosomes and lead to an increase in the incidence of both chromosome loss and non-disjunction.

On the basis of these finding, it is to be expected that the highest frequency of exceptional (XO) males should occur prior to anaphase 1. Strangio abd Savhagen have confirmed this for X-ray treatments. It is reasonable to suppose that XO males will also be most prevalent in those germ cells treated prior to anaphase 1 when chemical mutagens are used provided that they are not refractory to the mutagenic activity of the particular compound. Consequently the peak incidence of chromosome loss and/or non-disjunction in the F1 following heliotrine treatment may be indicative that preanaphase 1 germ cells are being used on the ninth day after treatment.

111. THE GENETIC LOCALISATION OF SEX-LINKED LETHALS PRODUCED BY HELIOTRINE IN DROSOPHILA.

a) Experimental Methods

A total of 117 sex-linked lethals produced by heliotrine in the first, second, third and fourth broods of two experiments have been genetically localised using the Xple technique. Virgin females, heterozygous for a lethal, were crossed to Xple males which carry the marker genes yellow (y), apricot (w^a), crossveinless (cv), cut (ct), vermilion (v) and forked (f). The F1 lethal/Xple females were crossed to asc males and the F2 males scored for the various marker genes. In most cases between one and two hundred males were scored, the position of the lethal being determined by the amount of crossing-over between it and the marker genes located immediately to the left and right. Ten of the lethals were found to be associated with suppression of crossing-over between two or more of the markers on the chromosome, although cytological examination of the salivary gland chromosomes failed to show the presence of structural rearrangements in most cases. These ten lethals have been excluded from the calculations on the distribution, since a reduction in the amount of crossing-over between some markers could occur if two or more lethals were located on the one chromosome, or alternatively reduced crossing-over may be due to mutation in genes controlling the amount of crossing-over. Cytological observations on all 117 lethals have been kindly carried by Miss M. M. Gunson of the University of Melbourne.

b) Experimental Results

In Figure 11 the frequencies of heliotrine induced sex-linked lethals in thirteen chromosome regions, each five map units in length, are shown. There appear to be two sensitive regions, one at the extreme distal

end of the chromosome and the other at the proximal end between the forked locus and the centromere.

In Table 12 the number of heliotrine induced lethals occurring in each of the following four regions are shown.

1. between the distal end of the chromosome and cut.
2. between cut and vermilion.
3. between vermilion and forked.
4. between forked and the spindle attachment.

In addition, this Table shows the distribution of 85 visible loci (most useful loci listed in Bridges and Brehme, 1944), 324 X-ray (300r) (Ives, 1959), 52 Myleran (Rohrborn, 1959), 83 mustard gas (Slizynska and Slizynski, 1947), 567 alkylating compound (Fahmy and Fahmy, 1956; 1957), 151 TEM (Belitz, 1954) and 188 Chinon 1 (Belitz, 1957) induced lethals and 102 spontaneously arisen lethals (Belitz, 1957). Stern (1957) has suggested that the distribution along the X-chromosome of lethals produced by various mutagenic agents may vary according to the marker stocks used for the localisation. In addition, Stern has shown that heterogeneity in the recombination values between various markers, caused by both environmental and genetic changes, may be expected to produce variations in the location of lethals. In all the above investigations the markers yellow or scute (0.0), cut (20.0), raspberry (32.8) or vermilion (33.0) and forked (56.7) have been used. Thus a comparison of the lethal frequencies produced by the various mutagenic agents mentioned above in each of the chromosomal regions delimited by these markers can be carried out with a greater degree of confidence than if the lethals had been located in a smaller chromosome segment (e.g. 3 - 10 map units) on the basis of the recombination data.

The data set out in Table 12 show several interesting points. Firstly, the frequency distribution of lethals in each of the four regions after treatment with chemical mutagens appears to be similar for each mutagen except in the case of Myleran where there is a deficiency of lethals in the f - spa region (only 5.8% of lethals), compared with 18-24% for the other chemicals. On the other hand, the frequency of spontaneous visibles and lethals occuring in the sc - ct region appears to be in excess of the frequency of induced lethals in this region (48.1% spontaneous lethals and 42.4% spontaneous visibles compared with 30 - 37% induded lethals), whilst in the f - spa region the position is reversed, there being fewer spontaneous visibles and lethals than induced lethals (9.8% spontaneous lethals, and 11.8% spontaneous visibles compared with 18 - 24% chemically induced lethals). With regard to the frequency distribution of X-ray induced lethals, the pattern appears to be similar to that for chemically induced lethals, except for the f - spa region where there appear to be relatively fewer radiation produced lethals (14.3% X-ray induced lethals compared with 18 - 24% chemically induced lethals).

Many of the sex-linked lethals produced by heliotrine show an interesting characteristic. After about 7 - 10 generations in stock, wild type flies appear in the cultures. The frequency with which these wild type flies appear is too high to be accounted for by stock contamination or back mutation and probably represents a characteristic feature of some lethals produced by the alkaloid. It may result from a progressive sorting out of damaged and undamaged strands in a multistrand chromosome structure similar to that proposed by Steffenson (1959).

c) Discussion of Results

As Figure 9 shows, the extreme distal portion and the proximal sixth of the X-chromosome are the most sensitive regions with respect to the production of sex-linked lethals by heliotrine, whilst the intermediate regions are comparatively insensitive. Similar frequency patterns of distribution have been obtained following treatment with mustard gas (Slizynska and Slizynski, 1947), alkylating compounds (Fahmy and Fahmy, 1957), TEM (Belitz, 1954) and high doses (12.5 kr) of X-rays (Ives, 1959), although the incidence of lethals in the proximal region following heliotrine treatment appears to be much greater than for other mutagenic agents excepting TEM.

The Sliznyski's have attempted to relate the distribution of mustard gas induced lethals to the occurrence of heterochromatin along the chromosome and conclude that these supposedly less active regions contain fewer lethals, the maximum frequency occurring in regions adjacent to heterochromatin. Investigations with plant cells and Drosophila indicate that the maximum frequency of chromosome breakage following treatment with chemical mutagens may occur in heterochromatic regions (McLeish, 1953; Revell, 1953; Moutschen-Dahmen and Moutschen-Dahmen, 1958; Fahmy and Bird, 1953), although Slizynska (1957) has found fewer breaks in these regions following treatment with formaldehyde. However, she suggests that this apparent deficiency may be due to the different times at which breaks produced in eu- and hetero- chromatin become available for participation in structural rearrangements. Thus the liklihood of interactions between breaks produced in these two chromosomal regions is reduced, and the re-joining of two heterochromatic regions is not detectable cytologically (unless an exchange is produced). If it can be confirmed that following treatment with chemical mutagens, lethals are more prevalent in euchromatic

regions, whilst the breakage frequency is highest in heterochromatin it would be reasonable to conclude that sex-linked lethals produced by chemical mutagens (excluding those associated with structural rearrangements) are not associated with chromosome breakage. This should result in similar sex-linked lethal frequencies being produced in rod- and ring- X chromosomes. This has been found following heliotrine treatment (see page 10), however it would be premature to conclude from this that lethals occur in euchromatin and breaks in heterochromatin following treatment with the alkaloid until more extensive data on the distribution of breaks and lethals is obtained. Also a clearer picture in both chemical and genetical terms of the differences between eu- and hetero- chromatin is needed.

The large number of lethals produced by heliotrine in the extreme distal region of the chromosome is not unexpected, since a high frequency of spontaneous visibles (Bridges and Brehme, 1944) and lethals (Belitz, 1957) are also found in this region. Thus in the chromosome region between the yellow and apricot loci (2.3% of the genetic map) there are found 15.3% of the spontaneous visibles and 19.6% of the alkaloid induced lethals. It appears likely that the lethals and visibles in this region arise as mutations in the same population of loci which are either extremely numerous in this section or alternatively readily undergo spontaneous and induced change.

On the other hand, the second peak of lethals in the proximal region of the chromosome between the forked locus and the spindle attachment (14.1% of the chromosome length) may indicate a susceptability of this region to the mutagenic action of heliotrine since 23.4% of the lethals occurred in this region compared with 11.8% of visibles

and 9.8% of spontaneous lethals. However, the excess of heliotrine induced lethals in this region does not appear to be peculiar to the alkaloid, since TEM (Belitz, 1954 , Fahmy and Fahmy, 1956) and to a lesser extent some of the other chemical mutagens also produce a high yield of lethals in this region.

Auerbach and Woolf (1960), in their criticism of the Fahmy's claim (Fahmy and Fahmy, 1959) claim that there are two populations of gene loci, α and β , the latter of which differs in its response to the mutagenic action of X-rays and alkylating agents, suggest that the high frequency of chemically induced lethals in the proximal region offers a promising line of research to test the concept of mutagen specificity in particular chromosome regions. The large number of stocks now available in which parts of the proximal block of heterochromatin have been either lost or re-distributed suggests a possible basis for attacking this problem. If the large number of lethals induced in the f - spa region are due to position effects as a result of part of the basal heterochromatin being deleted, a change which may be undetectable cytologically, then a stock in which part of this heterochromatin has been transposed to another part of the chromosome could provide useful information.

GENERAL DISCUSSION

a) The genetic nature of dominant lethals produced by heliotrine.

Pontecorvo (1942) and others have discussed the nature of dominant lethals produced by X-irradiation and suggest that there are three main mechanisms which may result in dominant lethals being formed. Firstly, asymmetrical chromosome exchanges or chromatid interchanges, formed by the interaction of breaks produced by irradiation before or after chromosome splitting respectively, lead to the formation of dicentrics which are inviable. Secondly, deletions above a critical size induced in autosomes often cause chromosome inbalance which results in the death of the embryo. Thirdly, chromosome breaks which fail to restitute do not appear to "heal" (Herskowitz and Muller, 1953 ; Hwrskowitz, 1954), but undergo sister-chromatid union following the splitting of the chromosome. Where sister-union occurs in the centric fragment a dicentric chromosome is produced which behaves as a dominant lethal either through loss or bridge formation. The first type of dominant lethal requires two independently produced breaks for its production and the last type a single break. The second type may result from two breaks or a single break depending on whether the deletion is interstitial or terminal, although the absence of healing suggests that most of them are probably two hit interstitial deletions.

Fahmy and Fahmy (1954) have investigated the nature of dominant lethals produced by 2:4:6-tri (ethyleneimino)1:3:5-triazine (TEM) and on the basis of their findings suggest that they arise as result of mechanisms similar to those described above for X-irradiation (i.e. asymmetrical exchanges, interstitial deletions and sister-chromatid union),

although the relative frequencies may be different in each case.

In addition to the primary or actual breaks produced by chemical mutagens there are partial of "potential" breaks which will tend to modify the dose/frequency response curve (Fahmy and Fahmy, 1955 b) as well as the relative yields of the different types of dominant lethal. Slizynska (1963 a; b) reports a higher intra-/inter- chromosomal rearrangement ratio following formaldehyde treatment compared with that obtained after X-irradiation. Thus it is possible that more dominant lethals may result from deletions and isochromatid aberrations than from asymmetrical exchanges.

Heliotrine induced dominant lethals probably result from the same type of chromosome aberrations as do those produced by X-irradiation, although, like TEM, it probably produces the three types in different relative proportions. The low translocation frequency indicates that few result from asymmetrical exchanges unless it is assumed that these are produced at a much higher frequency than symmetrical exchanges. On the other hand the high frequency of hyperploid females produced by the alkaloid suggests that a number of dominant lethals may result from interstitial deletions. In addition, the high deletion/translocation ratio indicates that potential breaks are produced by the alkaloid and this could result in a high yield of isochromatid aberrations. As discussed previously (see page 28), chromosome loss may occur as a consequence of sister chromatid union when the break point is close to the centromere. The resultant dicentric is small and is therefore more likely to be eliminated from both daughter nuclei due to "lagging" at anaphase than is a large dicentric, (formed when the break point is far removed from the centromere) which will form a bridge at anaphase and consequently behave as a cell lethal. Thus

the number of sister-unions should be proportional to the yield of chromosome losses. Since chromosome loss following heliotrine treatment occurs at a low frequency (see page 21) it is to be expected that the frequency of isochromatid aberrations also occurs relatively infrequently. On the other hand breaks may not be produced randomly along the length of the chromosome. Thus a deficiency of breaks in the proximal region could mean that the majority of sister-unions lead to cell lethality rather than to chromosome loss.

Further data on the nature and distribution of breaks produced by heliotrine as well as information on the dose/frequency response curve is needed before the relative proportions of the different types of chromosome aberrations contributing to dominant lethals can be positively determined.

b) The germ cell sensitivity pattern following heliotrine treatment in Drosophila.

To determine which germ cell stage is the most sensitive to the mutagenic activity of heliotrine, or indeed any mutagenic agent, it is desirable to have some genetic marker which can be used to characterize a particular type of germ cell or group of germ cells, since different chemical mutagens or different concentrations of the same mutagen may affect the rate of development and utilization of sperm (Auerbach) 1957). Induced crossing-over or non-disjunction are useful in this regard as they clearly delimit the post-meiotic from the pre-meiotic germ cells, whilst the occurrence of bunches of identical or complimentary crossovers delimit the early spermatogonial stage.

Induced crossing-over in a dual purpose stock which enabled sex-linked lethals as well as crossing-over to be detected has been successfully used by Auerbach and Sonbati (1960) and Reddi and Auerbach (1961)

to delimit the sensitive germ cell stages following treatment with mustard gas and TEM respectively whilst induced non-disjunction has been used by Såvhagen (1961 a) and Strangio (1962) in their studies on the germ cell sensitivity pattern after X-irradiation. Preliminary investigations (Clark, 1963 a) indicate that induced crossing-over occurs comparatively rarely following heliotrine treatment.

Both Såvhagen and Strangio have shown that the maximum chromosome loss frequency occurs in pre-anaphase 1 germ cells following X-irradiation. Possible reasons for this have been discussed previously (see page 28). In the present investigation, the maximum yield of XO males (whether they resulted from chromosome loss or non-disjunction) occurred in broods four and five corresponding to treated germ cells used on the ninth to twelfth days after treatment. Whether the XO males found in these broods result from chromosome loss, non-disjunction or a combination of both factors is uncertain. However, if the peak frequencies for the two types of damage coincide, as was found to be the case following X-irradiation, then it is likely that the germ cells used in brood four correspond to treated spermatocytes. Since the sex-linked lethal results using $\frac{y\ v}{sc^{8}\ Y}$ B-S males also showed a maximum frequency in brood four it is possible that primary spermatocytes represent the most sensitive germ cell stage for the production of this type of mutation following heliotrine treatment. Clark (1963 a), on the basis of his results and the spermatogenesis timing experiments of Chandley and Bateman (1962), also suggests that the spermatocyte stage may be the most sensitive for the induction of sex-linked lethals and chromosome breakage by heliotrine. The fact that the maximum yield of lethals was obtained on the seventh day following the treatment of Canton-S males may be due to the stock

differences discussed previously (see page 16), with treated spermatocytes becoming available for fertilization on the seventh day in this stock. In their experiments Chandley and Bateman showed that spermatocytes first became available at this time.

The dominant lethal and sex ratio data indicate that several stages of spermatogenesis may be equally sensitive as the peak frequency extends over four to seven days. This is similar to the pattern observed by Luning (1952) and Bateman (1956) for the production of dominant lethals by X-rays.

Germ cell sensitivity patterns for a number of alkylating agents have now been obtained. For some (e.g. mustard gas and TEM), peak sensitivity occurs in the early spermatocyte and late spermatogonial stages (Auerbach and Sonbati, 1960; Reddi and Auerbach, 1961), whilst for others (e.g. CB 1506) peak sensitivity appears to be confined to the spermatogonial stage (Fahmy and Fahmy, 1956 b). The most sensitive germ cells following formaldehyde treatment appear to be the auxocytes (Auerbach and Moser, 1953 a). Auerbach and Sonbati have pointed out that different sensitivity patterns obtained for different mutagens does not necessarily involve a difference in action on the genetic material as the different chemicals may affect other cullular processes which could influence the ultimate fate of the mutation. Thus cellular metabolism may be affected and this could enhance the mutagenic activity of the chemical compound or affect the repair of premutational damage.

In addition, two other factors appear to be important in determining the germ cell sensitivity pattern produced by chemical mutagens. The first of these involves the penetrance of the chemical into the nucleus of the treated cell. A number have a high molecular weight and con-

sequently entrance to the nucleus may be restricted to periods of cell division when the nuclear membrane breaks down. This may partly account for the fact that the spermatocyte and spermatogonial stages appear to be the most sensitive after treatment with some chemical mutagens, although other factors are likely to be involved. The second factor involves the stability of the mutagen following its injection into the organism. If the chemical remains active for several hours it has a greater chance of reacting with the nuclear material during the most sensitive stage of the cell cycle than if it is rapidly metabolised. Recent studies by Slizynska (1963 c) indicate that spermatogonia are a heterogenous population of cells and thus show considerable variation in their response to the mutagenic action of X-rays. Thus a mutagenic chemical which remains active in these cells for some hours should produce more damage than rapidly metabolised mutagens since all spermatogonial cells would pass through the sensitive stage during this time.

Oxygen tension during treatment with chemical mutagens may be another important factor in determining germ cell sensitivity. Kihlman (1961) has summarised the available data on the effect of oxygen on the frequency of chromosome aberrations produced by various mutagens in plant cells. However, the chromosome breaking ability of alkylating agents appears to be independent of oxygen concentration. Thus the germ cell sensitivity pattern produced by these compounds in Drosophila may not be affected by this gas, although that produced by other chemicals may well be affected.

CONCLUSIONS

In the light of the results obtained in the present investigation the following conclusions can be made with regard to the mutagenic activity of heliotrine in *Drosophila melanogaster*:-

1. Heliotrine is a potent mutagen, producing many different types of genetic damage. High sex-linked and dominant lethal frequencies are produced in the sensitive germ cell stages. Deletions are also produced with a high frequency, although translocations, chromosome loss and/or non-disjunction occur comparatively rarely.

2. Although the sensitive germ cell stages could not be positively identified from the experimental results, there is some evidence which suggests that the primary spermatocytes show the greatest susceptability to the mutagenic activity of the alkaloid, although peak sensitivity may extend into the spermatid and late spermatogonial stages. The brood pattern of sensitivity for sex-linked lethals, deletions, chromosome loss and/or non-disjunction showed a clearly defined peak on or about the ninth day indicating a particularly sensitive stage of spermatogenesis. On the other hand, the pattern of sensitivity for dominant lethals (and sex ratio distortion) failed to exhibit this peak, but showed a "plateau" of sensitivity extending over several days suggesting that several germ cell stages may be equally affected.

3. The genetic localisation of sex-linked lethals produced by heliotrine indicates that very few lethals are associated with major structural rearrangements. The fact that the sex-linked lethal frequency produced by the alkaloid in rod- and ring- X's is similar also indicates that the majority of lethals are point mutations not associated with

chromosome breakage.

4. The frequency distribution of the alkaloid induced lethals along the X chromosome shows a cluster at the extreme distal end with a second cluster in the basal (proximal) region. The distal clustering is probably associated with a large number of loci (or mutable loci) within this region as other workers have reported its occurrence in the case of spontaneous lethals and visibles and lethals induced by X-rays and various chemical mutagens. On the other hand, the basal clustering appears to be more specific , being confined to heliotrine, mustard gas, TEM and certain of the alkylating agents investigated by the Fahmy's. The clustering in this region may in some way be associated with the basal heterochromatin although further work is needed, with the possible use of rearranged X's, to confirm this hypothesis.

5. A low translocation frequency is produced by the alkaloid, but dominant lethals, hyperploid females (deletions) and sex-linked lethals occur with a high frequency. Thus the sex-linked lethal/translocation ratio is considerably greater than that obtained following X-ray treatments and is comparable with the ratio produced by other chemical mutagens. There are more intra- than inter- chromosomal rearrangements produced by the alkaloid, and as has been found with other chemical mutagens this probably indicates that "potential" chromosome breaks are produced.

6. The occurrence of sex-linked lethal mosaics and delayed chromosome loss following heliotrine treatment indicates that a part of the genetic damage produced only becomes expressed after several replications of the genetic material, further supporting the hypotheses that "potential" genetic damage is produced by the alkaloid.

7. Different strains of Drosophila melanogaster appear to vary

in their sensitivity to the mutagenic action of heliotrine. Whether this difference is related to dissimilarities in the sensitivity of particular germ cells in the various stocks or whether it is related to differences in rates of germ cell maturation and utilization has not been positively determined.

PART II

MODIFICATION OF THE MUTAGENIC ACTIVITY OF HELIOTRINE IN DROSOPHILA MELANOGASTER

a) Introduction.

It is now a well established fact that the chromosome aberration frequency produced by ionizing radiations and various chemical mutagens shows considerable variation depending on the stage of the cell cycle as well as the type of cell treated. In addition, temperature, oxygen tension and other factors which affect cellular metabolism have been found to modify the yield of chromosome breaks and structural rearrangements produced by various mutagenic agents. Evans (1962) and Kihlman (1961) have written comprehensive reviews on the factors which modify the yield of chromosome aberrations produced by ionizing radiations and chemical mutagens respectively.

Investigations utilizing Escherichia coli, Salmonella, Paramecium, Drosophila and mice indicate that the point mutation as well as the chromosome aberration frequency produced by ionizing radiations can be modified by altering the metabolism of the irradiated cells with various treatments given prior to, during or after irradiation, or by changing the dose rate. These treatments (if effective) may either increase or decrease the amount of primary damage produced by sensitizing or protecting the genetic material respectively, or alternatively they may enhance or inhibit natural repair processes.

Russell, Russell and Kelly (1958; 1960) have found that the irradiation of mouse spermatogonia and oocytes at low intensities produces less damage than if high dose rates are used. However attempts to show an intensity effect in Drosophila have not proved successful (see Clark, 1960 b, for review), although Oster, Zimmering and Muller (1959) did

find an intensity effect for treated oogonia. However, in a more recent publication (Muller, Oster and Zimmering, 1963) they suggest that this conclusion may still be open to question. Purdom (1963) failed to detect an intensity effect for treated spermatogonia, although for the lowest dose rate tested (0.01 r/min.) there was an indication of an intensity effect. On the other hand, Tazima and his coworkers (Tazima, Kondo and Sado, 1961; Tazima and Kondo, 1963) have shown an intensity effect in the oogonia and the spermatogonia of the silkworm, the effect being more pronounced in the former. In the light of their results with mice, Russell (1963) has proposed that the dose rate effect is due to the repair of premutational damage being "saturated" or blocked at high irradiation intensities.

Recent experiments indicate that there is repair of premutational genetic damage following ultra-violet irradiation in Escherichia and Salmonella and after X-irradiation in Paramecium. Although there is some disagreement amongst the workers in this field as to the nature of the primary lesion and the exact mechanism of its repair, they generally agree that there is an unstable state (the "potential mutation") which may be either "lost" or "repaired" as a result of various post-irradiation treatments. The final step resulting in "mutation incorporation" is DNA synthesis.

There is some evidence that the primary lesion occurs in the DNA molecule itself (see Witkin, 1961), although the original proposal of Haas and Doudney (1957) that the initial damage is to the nucleic acid precursors cannot be ruled out. Loss or repair of premutational damage, a process referred to as "mutation frequency decline", is enhanced by amino acid deprivation or inhibition of protein synthesis by chloramphenicol (Witkin, 1956; Doudney and Haas, 1960 a ; b), photo-reactivating light

(Haas and Doudney, 1960) and the basic dye pyronin (Witkin, 1961) in Escherichia. In Paramecium, post-irradiation treatment with streptomycin, chloramphenicol or caffeine, reduced the incidence of recessive lethals induced in this organism (Kimball, Gaither and Perdue, 1961). On the basis of their results, Kimball and his coworkers suggest that repair of premutational damage in Paramecium is a metabolically controlled process. The rate of repair is lower in the presence of metabolic inhibitors, but there is more time available for repair before the terminal event (probably DNA synthesis) when the remaining premutational damage is converted into mutation. On the other hand, Doudney and Haas and Witkin propose that "mutation stabilisation", the antagonistic process of "mutation frequency decline", is prevented by the post-irradiation inhibition of protein synthesis, i.e. there is an interruption in the chain of events leading to the stabilisation of premutational damage. However, "mutation frequency decline" is not a passive process, but one which requires respiratory energy (Doudney and Haas, 1960 b). In Escherichia, as in Paramecium, the final step in mutation incorporation is apparently the synthesis of DNA (Haas and Doudney, 1960).

Sobels has found that post-irradiation treatment with cyanide (1960 ; 1962 a) or chloramphenicol (Sobels and Tates, 1961; Sobels, 1963) either increases or decreases the mutation frequency produced by X-rays in Drosophila. In addition, post-irradiation treatment with purified nitrogen (Sobels and Tates, 1961; Sobels, 1963) or 2, 4 dinitrophenol (Sobels, 1963) has been found to increase the frequency of sex-linked lethals produced by X-irradiation. Sobels (1963) interprets these results along the same lines as Kimball et al. (1961) explained the modifying effects of chloramphenicol and streptomycin in Paramecium. However, the nature of the terminal event in Drosophila remains unknown although it presumably is not associated with

DNA synthesis.

Sobels (1963) has found that following irradiation in nitrogen, treatment with oxygen significantly reduces the sex-linked lethal frequency in spermatids whereas leaving the flies in nitrogen appears to have no effect on the mutation frequency. On the other hand, post-irradiation treatment with either oxygen or nitrogen appears to have no effect if the X-irradiation is carried out in oxygen. He suggests that more damage is caused to a repair mechanism when irradiation is carried out in oxygen. This is similar to the model proposed to account for part of the increase in the chromosome aberration yield following irradiation in an oxygen atmosphere (Wolff and Atwood, 1954; Wolff and Lindsley, 1960). Apparently irradiation in air does not damage the repair process, since post-irradiation treatment with nitrogen increases the mutation frequency (Sobels and Tates, 1961). In view of the earlier observations of Sobels (1960 ; 1961) that post-irradiation treatment with cyanide increases the sex-linked lethal frequency irrespective of whether the irradiation was given in oxygen or nitrogen, it is likely that damage to the repair mechanism resulting from irradiation in oxygen is not irreversible. Thus the post-treatments with nitrogen may have been too short to produce an effect whereas the narcotizing effect of cyanide, which lasts for some time following removal of the flies from the gas, could be effective in increasing the amount of genetic damage.

Recovery from genetic damage in treated sperm which has been stored in males Drosophila is still a controverisal point among geneticists. Independent investigations carried out by Nordback and Auerbach (1957), Abrahamson and Telfer (1956) and Luning (1958) showed that fewer translocations are found in mature sperm used on the second day after irradiation than on the first day irrespective of whether the males were mated on the

first day. The frequency of sex-linked lethals and Y chromosome loss also appears to be lowered by storage of irradiated sperm in treated males. However, it has been noted that X-irradiation in nitrogen eliminates the difference between first and second day sperm (Baker and von Halle, 1953; Luning, 1961 and Alexander, 1962), whilst Oster (1961) has observed that neutron irradiation has the same effect. On the basis of these findings Oster has suggested that the difference in mutation frequency between the two batches of sperm may be due to differences in sensitivity, the sperm used in the first mating being irradiated at a higher oxygen concentration than that used in the subsequent mating because of its closer proximity to the genital orifice.

Recently Mossige (1963) has extensively analysed this problem by observing the mutation frequencies obtained in the first and second matings following irradiation of males at various intervals after eclosion. Irradiations were carried out in nitrogen, air and oxygen. For young males (0 - 4 hours) a lower sex-linked lethal frequency was obtained on the second day after treatment irrespective of whether the males were first mated immediately after irradiation or twenty-four hours later. In addition, this result was obtained irrespective of whether the X-rays were given in nitrogen, air or oxygen and from this it was concluded that mature sperm in very young males is relatively anoxic. This result may be related to Khishin's (1955) observation that the mature sperm present in the seminal vesicles of newly emerged males does not become functional until about seven hours after eclosion. For seven day old males, the sex-linked lethal frequency in sperm used in the second mating was found to be lower than that obtained in the first mating irrespective of whether the first copulation takes place immediately after treatment or ten

hours later. However, the yield of lethals in the first used sperm is not reduced as a result of increasing the time interval between treatment and mating.

On the basis of these findings, Moesige has suggested that there are two mechanisms which account for the different mutation frequencies observed in first and second day sperm. In young males, where the mature sperm are apparently relatively anoxic, recovery accounts for the different lethal frequencies in the two batches of sperm irrespective of whether the males are mated on the first day. In older males (i.e. about seven days) differential sensitivity appears to account for the different mutation yields in the two matings. Apparently no recovery occurs in these older males.

In addition to induced mutation yields being altered by treatments which either enhance or inhibit natural recovery processes, they may also be altered by other treatments which may increase or decrease the amount of primary damage produced. Thus oxygen, anoxia, cysteamine and other chemicals containing sulphydryl groups, cyanide, dinitrophenol and other respiratory inhibitors have all be found to modify the amount of primary genetic damage produced by ionizing radiations in various organisms.

Oxygen has been found to enhance the X-ray induced reversion frequency of certain biochemical mutants in Escherichia coli (Anderson, 1951; Hollaender, 1957). It also enhances the sex-linked lethal frequency produced by X-irradiation in both rod- and ring- X chromosomes in Drosophila (Baker and Sgourakis, 1950, Baker and Edington, 1952; Baker and von Halle, 1955) and the X-ray induced mutation frequency in Paramecium (Kimball and Gaither, 1951; Kimball, 1954) and Aspergillus (Stapleton and Hollaender, 1952). Experiments carried out by Howard-Flanders and Moore

(1958) on the killing effect of X-rays in bacteria, indicate that oxygen must be present during irradiation to exert an enhancing effect, since the gas was found to have no effect when applied within twenty milliseconds after the conclusion of the irradiation.

The mechanism whereby oxygen enhances the mutagenic activity of X-rays is not clearly understood although it has been suggested (see Bacq and Alexander, 1961) that the gas may react with a damaged target thus reducing the liklihood of it being restored. This hypothesis appears to be supported by the observation that nitric oxide enhances the X-ray induced chromosome aberration frequency in mouse ascites tumour cells (Gray, Green and Hawes, 1958) and root tips of Vicia faba (Kihlman, 1958; 1959) as well as the sex-linked and dominant lethal frequencies in Drosophila (Capps, 1961). However, Capps has found that nitric oxide produces an effect over and above that produced by oxygen, suggesting that the mechanism of action in each case is at least partly dissimilar.

There are a number of chemical compounds which reduce the mutation and chromosome aberration frequencies as well as the killing effect of X-rays when they are present during irradiation. Hollaender and Kimball (1956) have broadly classified these compounds according to their effect.

Treatment with sodium hydrosulphite has been found to increase the survival in irradiated bacteria (Burnett, Morse, Burke and Hollaender, 1952) and reduce the X-ray induced chromosome aberration frequency in Vicia and Tradescantia (Wolff, 1954; Mikaelson, 1954). The protective effect of this compound apparently results from its reducing properties. Another compound, 2, 3 dimercaptopropanol (BAL), also appears to reduce the chromosome aberration frequency produced by X-irradiation as a result

of its lowering the oxygen tension (Wolff and Atwood, 1954). Kimball and Gaither (1951) failed to obtain a reduction in the X-ray induced mutation frequency in Paramecium following treatment with sodium hydrosulphite although they did find that the compound did reduce the lethal action of X-rays in this organism.

The sulphydryl compounds cysteine and cysteamine have a marked protective effect against X-ray induced killing (see Bacq and Alexander, 1961, for review), as well as the production of chromosome aberrations by ionizing radiations (see Evans, 1962, for review). Cysteamine has been found to reduce the yield of biochemical reverse mutations produced by X-rays in bacteria (Hollaender, 1957). Hollaender also found that the protective effect of cysteamine + sodium hydrosulphite was more than additive suggesting that the protective effect of the two compounds may involve different mechanisms. This has been confirmed by Vergroesen, Vos and Budke (1962) in their work on the protective effects of cysteamine and nitrogen in tissue culture cells. Apparently the sulphydryl compounds exert their protective effect by reacting with the free radicals produced by ionizing radiations (Bacq and Alexander, 1961).

AET (2-aminoethylisothiourea) protects against X-ray induced killing in mammals (see Doherty, 1960), although it has been found to increase the dominant and sex-linked recessive lethal frequency in Drosophila when given as a pre-irradiation treatment (Edington, 1958). Recently, Mittler (1963) showed that neither AET nor MEA (2-mercaptoethylamine) affected the sex-linked lethal, hyperploid or translocation frequencies produced by X-irradiation in Drosophila.

A number of investigations have been carried out in recent years

using antibiotics in an attempt to modify the yields of mutations produced by ionizing radiations. The work with chloramphenicol in Paramecium has already been alluded to whilst the reduced yield of X-ray induced sex-linked lethals in spermatids observed by Sobels and Tates (1961) following chloramphenicol pretreatment in Drosophila has been confirmed by Clark (1963 b). Wolff (1960) found that chloramphenicol inhibited the restitution of chromosome breaks after X-irradiation in Vicia. However, Browning and Altenburg (1963) failed to detect an antimutagenic effect for chloramphenicol when the polar cap cells of Drosphila were irradiated with UV. Streptomycin has been found to reduce the frequency of radiation induced recessive lethals in Paramecium (Kimball, Gaither and Perdue, 1961) and sex-linked lethals in Drosphila (Clark, 1963 b), whilst penecillin G (Burdette, 1961 a ; Clark, 1963 b) and actinomycin D (Burdette, 1961 b) have been found to have a similar effect in Drosophila.

The way in which the various antibiotics modify the induced mutation frequencies is not clearly understood at the present time. Many have a similar effect in that they reduce the yield of induced mutations, however, this does not necessarily indicate a similar mode of action in each case but may merely represent identical end points of a number of different modifying mechanisms. In the case of chloramphenicol, the inhibition of protein synthesis has been implicated as the basis of its modifying effect, at least in micro-organisms, although there is little evidence to justify this conclusion as far as more complex organisms are concerned.

From this brief resumé it is evident that the mutation frequency produced by ionizing radiations is subject to modification by treatments which either affect the amount of primary damage produced or modify

natural recovery processes. In view of these results it is of interest to test whether the mutation frequency produced by chemical mutagens can also be modified. These investigations are made difficult by the fact that many chemical compounds may remain mutagenically active for many hours. Thus gaseous and chemical treatments used in an attempt to modify their mutagenic activity may be ineffective if not given for a sufficient length of time. In addition, it is difficult to determine whether a particular modifying treatment is affecting the amount of primary damage produced by the mutagen or whether it is affecting the amount of repair since the two processes are probably proceeding concurrently.

Despite these difficulties, our present knowledge concerning the nature of the primary genetic damage produced by various chemical mutagens would indicate that it could be profitable to investigate factors which may modify their mutagenic activity. The occurrence of "potential" chromosome breakage" and possibly other types of premutational damage following treatment with various mutagenic chemicals suggests a possible basis for investigating this problem as the ultimate fate of this primary lesion - i.e. whether it is repaired or fixed as a mutation - depends upon the integrated processes of cellular metabolism and these can be modified or inhibitied by various treatments.

Kihlman and others (see Kihlman, 1961, for review) have investigated the effect of different oxygen tensions and various respiratory inhibitors on the chromosome aberration frequency produced by several chemical mutagens. On the basis of the results obtained, Kihlman has proposed a classification of chemical mutagens depending on whether their ability to produce chromosome aberrations is affected by changes in

oxygen tension, and if so, whether it is suppressed by respiratory inhibitors and uncoupling agents. The production of chromosome aberrations by alkylating agents appears to be independent of oxygen concentration, a fact which has been confirmed for the induction of sex-linked lethals by mustard gas in Drosophila (Auerbach and Moser, 1951). On the other hand, the frequency of aberrations produced by N-methylated oxypurines (e.g. 8- ethoxycaffeine) and the phenylnitrosamines (e.g. N-Methylphenylnitrosamine) is dependent on the presence of oxygen, whilst the action of the former group of compounds is suppressed by respiratory inhibitors and uncoupling agents. Apparently the mutagens discussed by Kihlman affect the amount of primary damage produced, although further work could show that recovery processes are also affected.

Sobels (1956 a) and Sobels and Simons (1956) have shown that the mutagenic activity of injected formaldehyde solutions in Drosophila is enhanced by pretreatment with cyanide gas. In both males and females the sex-linked lethal frequency was enhanced by the cyanide treatment, whilst the incidence of induced crossing-over was almost as high as that produced by X-rays. Sobels suggested that the increased mutation rate was probably due to the formation of dihydroxydimethyl peroxide produced as a result of the reaction of hydrogen peroxide (formed as a result of the inhibition of catalase by cyanide) and formaldehyde. This result is in agreement with the earlier observations of Dickey, Cleland and Lotz (1949) and Jensen, Kirk, Kolmark and Weatergaard (1951) in Neurospora where the frequency of reversions following combined formaldehyde and hydrogen peroxide

treatments was greater than that expected on an additive basis.

Herskowitz (1951) and Auerbach (1956) have found that respiratory inhibitors were ineffective in modifying the mutation frequencies produced by formaldehyde, whilst Clark (1958) has found that they have no effect on the mutagenic activity of pyronin. However, Strauss and Okuba (1959) have found that they can reduce the number of revertants to prototrophy in Escherichia coli by altering cellular metabolism following treatment with alkylating agents.

Finally, mention must be made of several investigations which report an increase in the mutation frequency when spermatozoa treated with mustard gas or TEM (tri-ethylene melamin) are stored in the spermathecae of inseminated females for varying periods of time (Herskowitz, 1955; 1956; Schalet, 1955, Auerbach and Sonbati, 1960; and Snyder, 1963). Herskowitz abd Schalet reported that the frequency of translocations following treatment with TEM or mustard gas is increased with storage, whilst the frequencies of XO males abd sex-linked lethals appeared to be unaffected by storage. Schalet's results for mustard gas appear to have been confirmed by Auerbach and Sonbati (1960) in that they observed an increase in the frequency of Minutes with storage whilst the lethal frequency was not significantly altered. However, Snyder found that TEM increased the frequency of lethals, translocations and Y chromosome loss with storage.

A possible interpretation of these results may be that the increase in the translocation frequency is due to the opening of "potential" breaks during storage, particularly if breaks do not rejoin until after fertilization as shown by Muller (1940) to be the case following X-irradiation. However, the unaltered frequency of XO males observed by Herskowitz and Schalet do not appear to fit this hypothesis. Both these workers treated spermatozoa within the spermathecae of inseminated females and this may well have resulted in female germ cells being affected by the mutagen. Auerbach and Sonbati have discussed the possible significance of this. In addition, the increase in the sex-linked lethal frequency observed by Snyder appears to be too large to be accounted for solely on the basis of an increase in the number of lethals associated with chromosome breakage and rearrangements resulting from the delayed opening of breaks. On the other hand, the increase may result from the realization of pre-mutational damage (i.e. "potential" point mutations) produced by THM. Auerbach (1951) has formulated three alternative hypotheses whereby delayed mutation may occur, two dependent on division and one, the stabilization of a metastable molecular equilibrium, which is independent of division. On the basis of this latter hypothesis, sperm stored at comparatively high temperatures should show a greater increase in mutation frequency than sperm stored at a lower temperature compared with the yield of mutations obtained in unstored sperm. This appears to fit with Snyder's observations.

In the present investigation preliminary experiments were carried to test the effect of temperature on the mutagenic activity of heliotrine. Although the experiments were carried out on a small scale there was some indication that the distortion of the sex ratio produced by the alkaloid in mature sperm was lower at 20°C than at 25°C.

These investigations indicate that the chromosome aberration and mutation frequencies produced by at least some chemical mutagens can be modified by various treatments. It was therefore considered worthwhile to test the effect of several metabolic inhibitors on the mutagenic activity of heliotrine. Potassium cyanide, sodium azide, sodium hydrosulphite, nitrogen and chloramphenicol have been used in this attempt. In several of the experiments males carrying a ring-X chromosome as well as those with rod-X's were treated in an attempt to determine whether the amount of primary genetic damage or repair processes were being affected.

b) Experimental Methods

Where the flies received treatment with potassium cyanide, sodium azide, sodium hydrosulphite or chloramphenicol in addition to heliotrine, the two chemicals were injected simultaneously. Cyanide was used at a concentration of 0.007 M, azide 0.003 M, sodium hydrosulphite 0.01 M and chloramphenicol at a concentration of 0.23% unless otherwise indicated.

In those experiments where gaseous nitrogen was used, the males were placed in a 4" x 1" glass vial through which was passed a stream of commercial nitrogen saturated with water vapour. All traces of oxygen were removed by passing through a solution of pyrogallic acid and potassium hydroxide. After one hour in nitrogen the flies were removed, etherized, and placed on a fine wire mesh fitted to the stage of a binocular microscope which enabled nitrogen gas to be circulated around them whilst they were being injected with the alkaloid. Although the conditions were by no means completely anoxic during injection, the anaesthetic and the circulating nitrogen probably prevented a complete

recovery of metabolic activity following the pre-injection treatment in anoxia. Immediately after the administration of the alkaloid the flies were returned to the glass vials and supplied with stream of pure nitrogen for a further period of one hour. The sealed glass vial containing the flies was immersed in a water bath maintained at 25$^{+}_{-}$ $\frac{1}{2}$°C. during the gas treatment. Control flies were treated similarly except that an air stream, saturated with water vapour was used in place of the nitrogen stream.

The treated males were mated to asc, or in some experiments to Basc females. A three day brood interval was used in all experiments except those in which gas treatments were used where the brood interval was two days.

Sex-linked lethals, distortion of the sex ratio and dominant lethals were scored in the same way as described previously (see page 7). The genetic localisation of sex-linked lethals produced by combined heliotrine and potassium cyanide treatments was carried using the Xple technique described on page 30.

c) Experimental Results.

1. The absence of an effect of KCN and NaN_3 on the spontaneous mutation frequency.

Neither cyanide (0.005 M) nor azide (0.002 M) significantly increased the spontaneous sex-linked lethal frequency. A yield of 0.25% (1588 tested gametes) and 0.18% (1697) lethals were obtained following cyanide and azide treatments respectively compared with the spontaneous frequency of 0.15% (3672). Likewise cyanide failed to affect the spontaneous breakage of ring-X chromosomes. The F1 male/female ratio following cyanide treatment was 0.82 (1136 males and 1391 females)

compared with 0.86 (3360/3892) in the control series.

2. The effect of KCN and NaN_3 on the heliotrine induced mutation frequency.

The effect of cyanide and azide on the sex-linked lethal frequency produced by heliotrine in both rod- and ring-X males is shown in Table 13, whilst the effects of the inhibitors on the F1 sex ratio distortion and dominant lethal frequency are shown in Tables 14 and 15 respectively.

It is evident, from the combined brood totals in each of the experimental series, that both cyanide and azide have produced a statistically significant increase in all three types of genetic damage produced by the alkaloid (last two columns of Tables 13, 14 and 15), except for the sex-linked lethals in the ring-X series where cyanide failed to produce a statistically significant increase. The failure of cyanide to produce a statistically significant increase in this series is possibly due to the sterilizing effect of the alkaloid in the younger germ cells (Clark, 1959; Brink, unpublished) and this has resulted in too few chromosomes being tested.

The enhancing effect of cyanide and azide on the mutagenic activity of heliotrine is most apparent in the second, third and fourth broods although there is some indication that it may also extend forward into the first brood. However, the long brood interval employed in these experiments, together with the possibility that the inhibitors may have retarded the development rate of maturing germ cells, makes it difficult to delimit a particularly sensitive stage of spermatogenesis. The metabolic inhibitors may affect the maturation rate of sperm thus causing shifts in the brood pattern of sensitivity. Although little is known about the

effect of inhibitors on the process of spermatogenesis in Drosophila, there is evidence from work with the Cecropia silkworm (Schneiderman, Ketchel and Williams, 1953) that in vitro spermatogenesis is delayed by various metabolic inhibitors. It was found that a concentration of 8×10^{-4} M sodium azide produced a 50% inhibition of spermatogenesis. However, in Vicia, Read (1959) found that cyanide only causes an initial reduction in growth rate, the normal rate as well as the lost growth subsequently being regained.

In Figure 10 is shown the distribution of 110 sex-linked lethals produced by heliotrine + cyanide treatment and it is compared with the distribution obtained following heliotrine treatment alone (see pages 30-31). It is evident that cyanide has not altered the distribution of heliotrine induced lethals despite the increase in frequency which it has produced.

3. The effect of $Na_2S_2O_4$ on the heliotrine induced mutation frequency.

The effect of sodium hydrosulphite on the sex-linked lethal frequency produced by heliotrine in rod-X males is shown in Figure 11, whilst its effect on the F1 male/female ratio distortion resulting from alkaloid treatment in rod- and ring- X males is shown in Figures 12 and 13 respectively.

In each case there is a statistically significant reduction in the amount of genetic damage produced by the alkaloid in broods three and four, corresponding to sperm used on the seventh to twelfth days after treatment. The mutation frequencies in other broods do not appear to be significantly affected by sodium hydrosulphite treatment.

In Table 16 the sex-linked lethal results obtained after treatment of ring-X males are shown. It is evident that in these males sodium hydrosulphite has had no effect on the yield of lethals produced by the alkaloid.

4. The effect of gaseous nitrogen on the heliotrine induced mutation frequency.

The effect of gaseous nitrogen on the sex-linked lethal frequency produced by heliotrine in ring-X males in two separate experiments is shown in Table 17, whilst the effect of the gas on the sex ratio distortion produced by the alkaloid in the same two experiments is shown in Table 18.

Pre- and post- injection treatment with pure nitrogen does not appear to significantly affect the yield of lethals produced by the alkaloid, although in one experiment it did produce a slight reduction, but the decrease was not statistically significant.

On the other hand, nitrogen treatment does reduce the sex ratio distortion produced by the alkaloid, particularly in broods four, five and six. This decrease is particularly marked in the second experiment.

5. The absence of an effect of chloramphenicol on the heliotrine induced mutation frequency

Chloramphenicol does not appear to modify the sex-linked lethal frequency (Table 19), F1 sex ratio distortion (Table 20) or the yield of dominant lethals (Table 21) produced by heliotrine.

Two experiments were carried out to test the effect of the antibiotic on the alkaloid induced sex-linked lethal frequency. The first experiment showed a slightly increased lethal frequency in broods three and four of the heliotrine + chloramphenicol series. However, this increase was not confirmed in the second experiment, where the yield of

lethals was actually slightly lower than that obtained in the heliotrine series.

Neither the dominant lethal frequency nor the sex ratio distortion obtained in the heliotrine + chloramphenicol series showed any consistent increase or decrease relative to the results obtained in the corresponding broods of the heliotrine series.

d) Discussion of Results.

Neither cyanide nor azide were found to be mutagenic when injected alone. This is in agreement with the findings of Sobels (1954 a) for Drosophila, although cyanide has been found to produce chromosome aberrations in Vicia (Lilly and Thoday, 1956; Kihlman, 1957) and mutations in micro-organisms (Wyss, Clark, Haas and Stone, 1948; Wagner, Haddox, Fuerst and Stone, 1950). The failure of cyanide to ellicit a mutagenic response in Drosophila cannot be attributed to the non-penetrance of the chemical into the germ cells since Sobels has shown that HCN gas can be used effectively to modify the mutation frequency produced by X-irradiation (Sobels, 1962 a) and formaldehyde (Sobels, 1956 a, Sobels and Simons, 1956) in this organism. Cyanide probably produces mutations in micro-organisms as a consequence of hydrogen peroxide accumulation due to the inhibition of catalase. Indeed Wagner et al. have found that hydrogen peroxide is mutagenic in Neurospora. Hydrogen peroxide, or organic peroxides formed from this compound, may also account for the chromosome aberrations produced by cyanide in plant cells. The failure of the inhibitor to produce mutations in Drosophila may be a consequence of hydrogen peroxide being rapidly decomposed in this organism. There is some evidence form this in Sobels and Simons observation that the injection of formaldehyde-hydrogen peroxide mixtures failed to enhance the mutagenic activity of formaldehyde.

When either cyanide or azide were injected along with heliotrine a significantly higher mutation frequency was obtained than that produced by the alkaloid alone. The enhancing effect was obtained irrespective of whether rod- or ring- X males were treated. When the mutation frequencies obtained in the first four broods are combined it is found that azide increases the alkaloid induced sex-linked lethal frequency in the "ring" series by 40.6% and in the "rod" series by 33.0%. The corresponding increases in the male/female ratio are 13.3% and 22.7% respectively, whilst the increases in the dominant lethal frequency are 27.5% for "rings" and 33.6% for "rods". Cyanide has increased the heliotrine induced sex-linked lethal frequency by 42.3% in the "ring" series and by 45.2% in the "rod" series, whilst the corresponding increases in the dominant lethal frequency are 15.5% and 18.7% respectively.

The combined data for the first four broods in all experiments indicate that the sex-linked lethal frequency produced by all types of treatment in ring-X males is lower than the corresponding frequency induced in rod-X males. (for heliotrine, χ^2 = 8.42; p = 0.003 and χ^2 = 1.96; p = 0.15; for heliotrine + azide, χ^2 = 8.96; p = 0.003; for heliotrine + cyanide, χ^2 = 3.27; P = 0.07). This may be correlated with the elimination of lethals associated with structural rearrangements in ring-X's, although the fact that both inhibitors increase the lethal frequency in "rings" and "rods" to a similar extent suggests that it is the frequency of point mutations that is being enhanced and not the number of lethals associated with structural rearrangements. Consequently the dissimilar mutation yields in the two stocks may be correlated with strain differences (see page 16).

When sodium hydrosulphite was injected along with heliotrine or treatment with gaseous nitrogen preceded and followed the injection of the alkaloid a significantly smaller distortion in the sex ratio was produced in sperm used on the sixth to twelfth days after treatment irrespective of whether rod- or ring- X males were treated. Thus sodium hydrosulphite has decreased the alkaloid induced distortion of the sex ratio in these germ cells by 26.1% in Canton-S males and by 19.6% in $\underline{X^{C2}\ v\ f}$ males, whilst nitrogen produced decreases of 19.3% and 17.2% respectively in the two experiments undertaken using $\underline{X^{C2}v\ f}$ males. Canton-S males were not treated in the nitrogen experiments. Sodium hydrosulphite produced a statistically significant reduction ($\chi^2 = 16.35$; $p < 0.0001$) in the sex-linked lethal frequency in these same germ cell stages in Canton-S males, although neither it nor nitrogen appeared to have any marked effect on the yield of lethals produced by heliotrine in ring - X males.

With regard to chromosome breakage, cyanide and azide may block restitution as Wolff and Luippold (1955) have found following X-irradiation in Vicia faba. If the initial amount of breakage produced by heliotrine in rod and ring chromosomes is the same, and the alkaloid does not prevent restitution, then the frequency of viable restitutions in "rods" will be greater than in "rings" since twisted restitution or intra-chromosomal crossing-over in the latter case will result in inviable dicentrics being formed (Catcheside and Lea, 1945). Consequently, if repair is blocked by cyanide and azide, the relative increase in the male/female ratio produced by the inhibitors in ring chromosomes will be less than in "rods". Similarly the increase in the alkaloid induced dominant lethal frequency produced by cyanide and azide

should be lower in ring-X males than in rod-X males. The results obtained in this investigation agree with this "repair hypothesis" in that the increases in the sex ratio distortion produced by azide and the increased yield of dominant lethals produced by both inhibitors is smaller in $\underline{X^{C2}v\ f}$ than in Canton-S males, although the increase in ring-X's may be too large relative to that found in "rods" to be explained solely on the basis of this hypothesis. In addition, if cyanide and azide inhibit restitution then there should be an increase in the frequency of structural rearrangements which may be expected to increase the sex-linked lethal frequency in rod-X's but not in ring-X's. However, the experimental results indicate that the increases in the sex-linked lethal frequency produced by the two inhibitors in "rods" and "rings" is of the same magnitude. Also, the genetic localisation of lethals produced by heliotrine and heliotrine + cyanide treatments does not show an increase in the frequency of lethals associated with structural rearrangements in the case of the latter treatment.

As discussed previously (see pages 25-26), there appears to be some evidence which suggests that "potential" chromosome breaks are produced following heliotrine treatment. If the enhancing effect of cyanide and azide results from the inhibition of repair of "potential" breaks and other types of premutational damage, then the increases which they produce in the mutation frequency should be similar irrespective of whether rod- or ring-X's are treated, as these lesions should be equally reparable in both types of chromosome. Sobels (1962 b, 1963) has suggested that the modifying effects of cyanide, nitrogen and chloramphenicol on the X-ray induced mutation frequency in Drosophila results from the inhibition of the repair of premutational genetic damage,

although at the same time they increase the amount of time in which repair may occur. A similar mechanism may account for the enhancing effect of cyanide and azide on the heliotrine induced mutation frequency.

Sodium hydrosulphite has decreased the distortion in the sex ratio produced by heliotrine to the same extent in the sensitive germ cell stages of rod- and ring- X males. Nitrogen treatment has produced a similar decrease in ring-X males, suggesting that the mechanism of action of the two treatments may be the same. If the protective effect of nitrogen and sodium hydrosulphite is correlated with a reduction in the oxygen tension within the treated cells, then it is possible that azide and cyanide act in a converse manner by blocking the cytochrome respiratory pathway and thereby increasing the oxygen tension. This may in turn lead to an increase in the genetic damage produced by exposure to heliotrine.

Avanzi (1961) suggests that the protective effect of cysteine following heliotrine treatment in Allium results from the lowering of oxygen tension within the treated cells. However, Culvenor, Dann and Dick (1962) have shown that pyrrolizidine alkaloids react with cysteine and suggest that the protective effect of this compound in Allium may result from its combination with the alkaloids. In addition, the alkylating properties attributed to this group of chemical mutagens by Culvenor et al. suggests that oxygen may not be involved in their mutagenic activity, since the mutation frequencies produced by the alkylating agents nitrogen mustard, β - propiolactone or N-Nitroso-N-methyl urethan are not affected by changes in oxygen tension (Kihlman, 1955; Auerbach and Moser, 1951; Swanson and Merz, 1959; Kihlman, 1960).

The results obtained in the present investigation clearly indicate that cyanide, azide, sodium hydrosulphite and nitrogen have all affected the mutagenic activity of heliotrine, but the mechanism whereby they

modify its effect is not clear at the present time. If sodium hydrosulphite and nitrogen act by reducing the oxygen tension, then they will only be effective in those germ cells which normally have a supply of oxygen greater than that which can be utilised in respiration and other metabolic activities. This may account for the fact that these metabolic inhibitors only produced an effect in some broods, however it does not explain the unchanged point mutation frequency (sex-linked lethals in "ring" chromosomes) which accompanies the reduced amount of chromosome breakage (indicated by sex ratio distortion). Cyanide and azide interfere with a number of metabolic processes, thus their enhancing effect on the mutagenic activity of heliotrine may result from causes other than the inhibition of repair or increase in oxygen tension. These include the sensitization of the genetic material to the mutagenic action of the alkaloid. Since cyanide has been found to react with the genome in certain organisms to produce mutations it may also sensitize the genetic material to the mutagenic action of other mutagens. The inhibitors may also decrease the rate at which the heliotrine molecule is rendered inactive thus increasing the amount of time in which the alkaloid can be mutagenically active. Another complicating factor is that heliotrine itself may affect metabolic processes within the treated cell thus influencing the amount of primary damage that ultimately becomes converted into mutation.

The failure of chloramphenicol to modify either the sex-linked lethal or chromosome breakage frequencies produced by heliotrine may be a consequence of different rates of penetration into the germ cells of the two chemicals. Clark (1963 b) found that injection of the antibiotic 30 minutes prior to irradiation resulted in a significantly lower sex-linked

lethal frequency being obtained in the spermatid stage than when the irradiation was given alone, indicating that the antibiotic was present in these cells at the time of irradiation. Thus different rates of penetration probably do not account for the failure of chloramphenicol to modify the heliotrine induced mutation frequency since the alkaloid is apparently mutagenically active within at least fifteen minutes of its being injected (Clark, unpublished; see also page 78).

In the many investigations which have been carried out in recent years in an attempt to modify the mutation frequency produced by ultra-violet light and ionizing radiations in a variety of organisms by pre-irradiation treatment with chloramphenicol, the inhibition of protein synthesis has been suggested as the underlying cause of the modifying effect of the antibiotic (Witkin, 1956; Doudney and Haas, 1961 a; b; Kimball, Gaither and Perdue, 1961; Lieb, 1960; Ryan, Rudner, Nagata and Kitani, 1959; Wolff, 1960; Sobels and Tates, 1961; Sobels, 1963). However, Kimball, Gaither and Perdue suggest that DNA and RNA synthesis may also be affected by chloramphenicol in Paramecium and consequently may play a part in the reduced mutation frequency produced by X-irradiation in the presence of chloramphenicol.

Clark (1963 b) has confirmed the experimental findings of Sobels and Tates that chloramphenicol reduces the yield of sex-linked lethals obtained in the spermatid stage following X-irradiation in Drosophila, although he disagrees with the interpretation of these authors that the modifying effect of the antibiotic in this organism resulted from the inhibition of protein synthesis. This criticism stems from the fact that a number of other antibiotics, which apparently have little or no effect on protein synthesis, have been found to

modify the mutation frequency in Drosophila, Paramecium and plant cells. Thus Burdette (1961 a) has found that actinomycin D reduces the yield of sex-linked lethals in Drosophila, as does penecillin G (Burdette, 1961 b; Clark, 1963 b), whilst streptomycin lowers the frequency of sex-linked lethals in Drosophila (Clark, 1963 b) and recessive lethals in Paramecium (Kimball, Gaither and Wilson, 1957; Kimball, Gaither and Perdue, 1961). Mitomycin C, on the other hand, increases the frequency of simple breaks and exchanges in Vicia cells, apparently as a result of the inhibition of DNA synthesis (Matsuura, Tinifuji, Saho and Iwabuchi, 1963). Wolff (1960) had previously suggested that the increase in the chromosome breakage frequency produced by chloramphenicol in Vicia resulted from the inhibition of protein synthesis and was not in any way dependent on DNA synthesis.

The work of Witkin, Doudney and Haas, Lieb and others clearly indicates that chloramphenicol enhances "mutation frequency decline" by way of the inhibition of protein synthesis. However, it may be premature to extrapolate these findings to the mature sperm of Drosophila, although the results of Alfert (1959) which showed a change in the composition of protein during the transition from the spermatid stage to the mature spermatozoon indicates that protein synthesis may occur during the late spermatid stage. If the inhibition of protein synthesis is responsible for the modifying effect of chloramphenicol in the spermatid stage of Drosophila following X-irradiation, it must be concluded in the light of the results obtained with heliotrine that protein synthesis has no affect on the yield of mutations produced by the alkaloid.

e) Conclusions.

In the light of the results obtained, the following conclusions can be drawn concerning the effects of potassium cyanide, sodium azide, sodium hydrosulphite, nitrogen and chloramphenicol on the mutagenic activity of heliotrine in Drosophila melanogaster:-

1. Potassium cyanide and sodium azide have a similar effect in that they both increase the sex-linked lethal and chromosome breakage frequencies produced by heliotrine, particularly in those germ cell stages utilised on the fourth to twelfth days following treatment.

2. Potassium cyanide has no affect on the distribution along the chromosome of sex-linked lethals produced by heliotrine.

3. Neither potassium cyanide nor sodium azide were found to be mutagenic when injected alone.

4. Both sodium hydrosulphite and gaseous nitrogen produced a similar effect in that they reduced the chromosome breakage frequency produced by heliotrine in germ cells used on the seventh to twelfth days after treatment, although they appeared to have no effect on the sex-linked lethal frequency except when rod-X males were treated.

5. The similarity of the effects produced by sodium hydrosulphite and nitrogen suggests that the removal of surplus oxygen (i.e. oxygen not utilised in respiration) affords some protection against the mutagenic activity of heliotrine, although other causes cannot be eliminated, particularly as there is some evidence obtained by other workers which suggests that oxygen may not affect the mutagenic action of pyrrolizidine alkaloids.

6. The effect of potassium cyanide and sodium azide may partly be due to the inhibition of repair of premutational damage caused by the alkaloid. However, they may also act through increasing oxygen tension, through sensitizing the genetic material or by some other

mechanism.

7. The antibiotic chloramphenicol has no effect on either the sex-linked lethal or chromosome breakage frequencies produced by heliotrine.

PART III

THE MUTATION FREQUENCY RESULTING FROM COMBINED TREATMENTS OF HELIOTRINE AND X-IRRADIATION IN DROSOPHILA MELANOGASTER

a) Introduction.

When cells are treated with two or more mutagenic agents the resultant chromosome aberration or mutation frequency may represent the sum of the frequencies obtained when each treatment is given separately (i.e. their effects are additive), or alternatively the mutation yield may be greater than that expected on the basis of an additive effect (i.e. they produce a synergistic effect). Synergism may result from four alternative mechanisms :-

i) Chromosome breaks produced by one mutagenic agent may interact with those produced by another so that the nett yield of chromosome and/or chromatid exchanges will be greater than the added frequencies when the two treatments are given separately. This type of synergism is restricted to "two hit" chromosome aberrations (e.g. translocations), those mutations or chromosome rearrangements resulting from a single "hit" always show an additive increase.

ii) If a chemical mutagen disrupts certain metabolic processes in a treated cell as well as producing mutations it may enhance the mutagenic activity of another physical or chemical mutagenic agent, either by blocking a repair mechanism or increasing the amount of primary damage induced. Thus the resultant mutation frequency will be greater than the additive yields of the two treatments when these are given separately. Both "one hit" and "two hit" mutations and chromosome rearrangements can be affected by this mechanism.

iii) Synergism may result if one treatment sensitizes the genetic material to the mutagenic action of a second treatment, since this results in a higher yield of mutations being produced by this latter mutagenic agent.

Both "one hit" and "two hit" genetic changes will probably be affected.

iv) Two mutagenic agents (both usually chemical mutagens) which are weakly mutagenic when used alone, may interact following a combined treatment to form a highly potent mutagen. Consequently, the resultant genetic damage produced by this reaction product will be considerably greater than the added yields of the two weak mutagens when these are given in separate treatments.

Synergism of the first type has been found to occur following treatment with X-rays and β - propiolactone (Merz, Swanson and Cohn, 1961), X-rays and 8-ethoxycaffeine (ibid.), X-rays and maleic hydrazide (ibid.) and X-rays with either urethane or mustard gas (Oster, 1958 b). With regard to chemical mutagens, simultaneous treatments with diepoxybutane and 2, 2' bipyridine (Cohn, 1961 a) and KCN and diepoxybutane or 2, 2' bipyridine (Cohn, 1961 a; b) produce a synergistic effect. Also ethyl alcohol used in conjunction with either Myleran or TEM and simultaneous treatment with Myleran and ethyl-methane sulphonate (see Michaelis and Rieger, 1963) all produce synergistic effects. However, a number of other chemical mutagens tested for synergism did not produce a chromosome aberration frequency greater than that expected when the yields of the two treatments are additive.

An example of the second type of synergistic effect is shown in experiments with Vicia, where cyanide was found to increase the mutagenic effectiveness of X-irradiation as a consequence of an increased intracellular oxygen concentration brought about by the inhibition of respiration (Kihlman, Merz and Swanson, 1957; Kihlman, 1958). Cyanide itself produces chromosome aberrations (Lilly and Thoday, 1956; Kihlman, 1957). It is possible that part of the synergistic effect resulting from com-

bined KCN and X-ray treatments (Merz, Swanson and Cohn, 1961) is due to the interaction of chromosome breaks, whilst the balance of the effect results from increased oxygen tension enhancing the damage produced by X-rays.

Synergism resulting from the sensitization of the genetic material is probably exemplified by the combined treatments of nucleotide analogues and X-irradiation. The analogue 5-bromodeoxyuridine, which produces a low frequency of chromosome aberrations, has been found to markedly enhance the frequency of chromosome aberrations produced by ionizing radiations (Somers and Humphrey, 1963; Koo, 1963). Koo found that the yield of both bridges and acentric fragments produced by X-rays is increased by BUDR indicating that the synergistic effect of the two treatments does not result from the interaction of chromosome breaks. Somers and Humphrey showed that the genetic damage produced by the combined treatments is more marked in certain chromosome regions, which the authors suggest may be related to a high adenine-thymine content and hence a high frequency of BUDR incorporation during DNA synthesis. However, it is not clear whether the synergism between ionizing radiations and BUDR results from the sensitizing effect of the analogue or whether it is due to a delay in the rejoining of chromosomes due to the inhibition of DNA synthesis caused by the analogue. The synergism between ultraviolet light and tertiary-butyl hydroperoxide observed by Altenberg (1955) in Drosophila may also be due to a sensitizing effect of the chemical (or the radiation), since the frequency of second autosome lethals produced by the combined treatment was about six times greater than the additive frequencies of the two treatments when these were given separately.

The fourth type of synergism, resulting from the chemical interaction of two weak mutagens to produce a new compound which is a much more effective mutagen, is illustrated by the interaction of formaldehyde and

and hydrogen peroxide to form dihyroxydimethyl peroxide (Dickey, Cleland and Lotz, 1949; Sobels, 1956 a; b; Sobels and Simons, 1956), which appears to be quite an effective mutagen in bacteria and Drosophila.

b) Experimental Methods.

Two experiments were carried out in order to test for a possible synergistic effect between heliotrine and X-irradiation. In both, $y\ v\ /sc^{8}\ Y^{B-S}$ males were treated.

In the first experiment the following treatments were given:-

Group 1. Injected with 0.09 μl. of 0.001 M heliotrine in 0.7% saline.

Group 2. Received 1500 r of X-rays at a dose rate of 150 r/min. (235 kvp, 15 m.a., 1 mm. Al. added filter giving a half value layer of 0.5 mm. Cu.).

Group 3. Injected with 0.09 μl. of 0.001 M heliotrine in 0.7% saline and two hours later X-irradiated with 1500 r delivered at 150 r/min..

Group 4. Injected with 0.09 μl. of 0.001 M heliotrine in 0.7% saline and twenty minutes later X-irradiated with 1500 r delivered at 150 r/min..

Following treatment, the males were mated individually, each to three $y\ w^{a}$; bw ; st females, fresh females being provided every seventy two hours until five broods had been obtained.

Loss of Y^{S} or Y^{L}, together with the frequency of XO males (loss of X or Y and/or non-disjunction) were scored in the F1 generation according to the methods described previously (see pages 18-21). Translocations involving the second, third and/or Y chromosome were scored in the progeny of the F1 males when these were backcrossed to bw ; st females.

In the second experiment the following treatments were carried out:-

Group 1. Injected with 0.09 μl. of 0.001 M heliotrine in 0.7% saline.

Group 2. Received 900 r of X-rays at a dose rate of 150 r/min. (235 kvp ; 15 m.a. ; 1 mm. Al. added filter giving a half value layer of 0.5 mm. Cu.).

Group 3. Injected with 0.09 μl. of 0.001 M heliotrine in 0.7% saline and fifteen minutes later X-irradiated with 900 r delivered at 150 r/min..

Group 4. X-irradiated with 900 r delivered at 150 r/min. and fifteen minutes later injected with 0.09 μl. of 0.001 M heliotrine in 0.7% saline.

The treated males were mated to asc ; bw ; st females. The brood interval was three days.

The frequency of XO males (loss of X or Y and/or primary non-disjunction) was scored in the F1. The F1 females were collected as virgins and mated to asc males for the detection of sex-linked lethals, the absence of yellow vermilion male offspring indicating the presence of a lethal.

These flies which received X-ray treatment were irradiated in $\frac{3}{4}$" x $\frac{1}{4}$" thin walled plastic containers to minimise radiation scatter. Irradiations were not carried out until the flies had recovered from the anaesthetization under which they were injected with the alkaloid. This precaution was taken in order to reduce the possibility of different mutation yields being produced in anaesthetized and non-anaesthetized flies due to differences in the metabolic activity of the two groups.

All three groups which received X-ray treatment were irradiated together except in the first experiment where the heliotrine treated flies were irradiated separately from those which received irradiation only. In this case it was estimated that the dose error between the two treatments would not have amounted to more than four per cent. All irradiations were kindly carried out by Mr. D. Madden of the Peter McCallum Clinic, Royal Hobart Hospital.

c) Experimental Results.

The results of the first experiment are shown in Tables 22 (XO males and loss of Y^S, Y^L) and 23 (translocations).

Chromosome loss and/or non-disjunction are "one hit" genetic changes. When the injection of the alkaloid and irradiation were separated by twenty minutes the resultant frequency of XO males was found to be greater than that expected if the genetic damage produced by the two treatments was additive. The synergistic effect produced was found in the first, second and fourth broods. However, when the injection of the alkaloid preceded irradiation by two hours this synergistic effect was no longer apparent.

Translocations are "two hit" chromosomal rearrangements. With a twenty minute interval between alkaloid injection and irradiation, the two treatments produced a synergistic effect with regard to this type of genetic damage. Based on the combined brood totals, the translocation frequency produced by X-rays alone is significantly lower than that produced by the combined treatments when the interval separating them is twenty minutes ($\chi^2 = 4.75$; $p = 0.03$). However, the synergistic effect is no longer apparent when the interval between treatments is extended to two hours.

The results of the second experiment are shown in Tables 24 (XO males) and 25 (sex-linked lethals).

As in the previous experiment, pre-irradiation treatment with heliotrine produces a synergistic effect in the frequency of XO males. This effect is evident in all broods. On the other hand, post-irradiation treatment with the alkaloid appears to produce no synergistic effect with regard to this type of genetic damage. If the brood totals are combined, it is found that the frequencies of XO males produced by pre- and post- irradiation treatments are significantly different ($\chi^2 = 9.99$; $p = 0.0015$).

Neither pre- nor post-irradiation treatment with heliotrine produces a synergistic effect in the frequency of sex-linked lethals, the mutation yields produced by the two treatments being additive. There is some indication that pre-irradiation injection of the alkaloid has produced a synergistic effect in broods one and three, but in all other broods the lethal frequencies are additive.

d) Discussion of Results.

The results obtained in the two experiments indicate that pre-irradiation treatment with heliotrine is effective in increasing the yield of chromosome aberrations above that expected on the basis of an additive effect (i.e. the two treatments exhibit synergism). This synergistic effect is no longer apparent if a period of two or more hours elapses between the injection of the alkaloid and X-irradiation. Post-irradiation treatment with heliotrine also appears to be ineffective in producing a synergistic effect.

Since both "one hit" (i.e. chromosome loss and/or non-disjunction) and "two hit" (i.e. translocations) chromosome aberrations are affected

by pre-irradiation treatment, it is evident that synergism cannot result only from the interaction of chromosome breaks produced by the alkaloid and X-rays, since in this case only the frequency of "two hit" rearrangements should be affected. If heliotrine produces a high frequency of "potential breaks" (see pages 25-26), then the interaction of breaks produced by the alkaloid and X-irradiation should rarely occur since most of the breaks produced by the latter treatment would have either restituted or rejoined with other radiation induced breaks to form new rearrangements before the "potential breaks" produced by the alkaloid became available for participation in exchanges.

Since post-irradiation treatment with the alkaloid does not produce a synergistic effect, it is possible that injection of heliotrine prior to X-irradiation affects the metabolic activity of the germ cells so that they are rendered more sensitive to the mutagenic action of ionizing radiations (either by increasing the amount of primary damage or blocking repair), or alternatively, the alkaloid may sensitize the genetic material to the mutagenic action of X-rays.

Although nothing is known concerning the possible effects of heliotrine on the metabolic activity of the various germ cell stages of Drosophila, the work of Christie (1958)and Christie, LePage and Baille (1961) has shown that the alkaloid disrupts the TCA cycle and causes loss of activity of DPN-dependent enzymes in the mitochondria of the rat liver. If the alkaloid produces a similar inhibitory effect on the respiratory cycle in the germ cells of Drosophila, then a resultant increase in the intracellular oxygen tension or an inhibition of energy requiring repair processes could lead to an increase in the frequency of chromosome aberrations produced by X-rays in the presence of heliotrine. This would be similar to the enhancing effect of KCN on the X-ray induced chromosome

aberration frequency reported by Kihlman (1958).

e) Conclusions.

The following conclusions may be drawn with regard to the mutagenic effectiveness of combined heliotrine and X-ray treatments:-

1. Treatment with heliotrine fifteen minutes prior to X-irradiation resulted in a significantly higher yield of XO males and translocations than the added frequencies obtained when the two treatments were given separately.

2. A two hour interval between injection of the alkaloid and irradiation eliminated the synergistic effect observed when only fifteen minutes separated the treatments.

3. Post-irradiation injection of the alkaloid failed to produce a synergistic effect.

4. The synergism observed following pre-irradiation treatment is probably due to a disruption in the metabolic activity of the treated germ cells caused by heliotrine. This disruption has enhanced the mutagenic activity of X-irradiation.

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APPENDIX 1

The micropipettes used for injecting the flies are made by drawing out fine glass capillaries in a microflame. Each capillary is drawn out to a fine point and then sealed into the tapered end of a hollow glass shank with a small drop of paraffin wax, leaving about $\frac{1}{8}$" of the capillary protruding from the end of the glass tubing which serves as a holder. The volume of the pipette is determined by titrating the volume of hydrochloric acid it delivers against NaOH delivered from an Agla micrometer syringe using a standard volume of bromothymol blue as indicator. The titration is carried out on a waxed slide. Adequate mixing of the acid, alkali and indicator during the titration is effected by a gentle air stream directed to one side of the droplet which causes it to spin in the one place. Since the pipettes are only about $\frac{1}{2}$" long, they are self filling when the pointed end is dipped into a drop of liquid. The liquid is expelled from the pipette by gently blowing through a length of rubber tubing attached to the end of the glass shank.

A small drop of the solution to be injected is placed on a waxed slide near the etherized flies, and the pipette filled by placing the tip in this solution and thence into the dorsal surface of the abdomen. The liquid is injected by gently blowing through the rubber tubing, until all the fluid is expelled. The pipette is then quickly withdrawn to prevent the body fluids of the fly from being drawn into it. Care must be taken during injection to avoid blowing air into the abdomen of the fly.

APPENDIX 11

The food medium used in the present investigation consisted of 21% treacle (molasses) 1.2% agar, 0.3% propionic acid (to inhibit mould and bacterial growth) and 10% semolina; all measurements are by weight. The treacle, agar, propionic acid and two thirds of the water were brought to the boil when the semolina and the balance of the water were added and the mixture brought to the boil again prior to dispensing. The medium was supplemented with one drop of live yeast suspension twenty-four hours prior to use.

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Table 1: The sex-linked lethal frequency produced by heliotrine in the individual males of an experimental series.

Heliotrine = 0.001 M. Brood interval three days.

Male no.	Brood 1		2		3		4		Total	
	% leth.	n.	% leth.	n.	% leth.	n.	% leth.	n.	% leth.	n.
H1	-	7	-	10	3.8	26	-	0	2.3	43
H2	-	55	18.3	36	9.6	52	-	17	5.0	160
H3	-	46	6.5	31	8.8	57	-	14	4.7	148
H4	-	47	15.0	40	10.4	48	6.3	16	7.9	151
H5	-	33	5.9	17	-	17	-	17	1.2	84
H6	2.6	38	14.7	34	8.2	49	-	0	8.3	121
H7	-	45	8.3	24	11.8	34	5.9	17	5.8	120
H8	-	62	7.5	40	7.7	26	2.9	35	3.7	163
H9	3.6	56	4.7	43	20.0	10	-	50	3.8	159
H10	2.0	49	7.1	42	7.7	13	-	0	4.8	104
H11	-	31	2.4	41	9.4	64	2.2	46	4.4	182
H12	2.4	41	5.0	20	-	36	4.5	22	2.5	119
H13	-	57	16.0	25	12.8	47	6.7	15	7.6	144
H14	-	25	-	26	9.1	22	3.7	27	3.0	100
H16	2.5	40	-	26	5.3	19	-	35	1.7	120
H17	2.2	45	25.0	16	6.5	46	-	0	7.5	107
H18	4.1	49	9.1	11	4.4	46	-	23	4.0	148

Table 3: The results of two replicate experiments in which the sex-linked lethal frequency produced by heliotrine in $\frac{C2}{X^{C2}vf}$ males has been scored.

Heliotrine = 0.001 M; brood interval two days.

	Experiment 1.		Experiment 2.	
	% leth.	n. chrom.	% leth.	n. chrom.
Brood 1.	1.62	309	1.62	433
Brood 2.	1.17	426	0.85	353
Brood 3.	3.01	366	2.91	344
Brood 4.	8.33	156	6.10	295
Brood 5.	10.66	122	7.49	227
Brood 6.	7.70	39	5.47	201
Brood 7.	3.70	27	3.44	262
Total	3.53	1445	3.55	2115

$\chi^2 = 0$; $p = 1$

Table 4: The results of two replicate experiments in which the sex-linked lethal frequency produced by heliotrine in $yv/sc^{8}Y^{B-S}$ males has been scored.

Heliotrine = 0.001 M ; brood interval three days.

	Experiment 1.		Experiment 2.	
	% leth.	n. chrom.	% leth.	n. chrom.
Brood 1	1.93	571	0.72	415
Brood 2	2.05	730	2.24	491
Brood 3	5.75	400	4.41	522
Brood 4	6.23	401	5.24	496
Brood 5	5.29	170	2.32	436
Total	3.65	2272	3.09	2360

$\chi^2 = 1.04$

$p = 0.3$

Table 5 : The frequency of inviable eggs laid by asc females following fertilization by untreated Canton-S or $X^{C2}vf$ males.
Brood interval = three days.

	Canton-S		$X^{C2}vf$	
	% u.h.	no.	% u.h.	no.
Brood 1	15.7	1481	28.6	1492
Brood 2	23.1	1244	16.9	1093
Brood 3	10.0	759	12.3	713
Brood 4	7.5	610	10.3	399
Total	15.7	4094	20.0	3697

$\chi^2 = 17.6$

$p < 0.0001$

u.h. = eggs unhatched

Table 6 : The percentage of unhatched eggs laid by asc females following their fertilization by heliotrine treated Canton-S or $X^{C2}vf$ males.

Heliotrine = 0.001 M: brood interval three days.

	Canton-S males				$X^{C2}vf$ males			
	Experiment 1		Experiment 2		Experiment 1		Experiment 2	
	% u.h.	n.eggs	% u.h.	n.eggs	% u.h.	n.eggs	% u.h.	n.eggs
Brood 1	23.0	3278	17.8	1974	36.5	1319	35.4	724
Brood 2	52.6	1634	36.2	2318	59.2	781	54.2	1024
Brood 3	51.2	1491	43.9	919	75.8	846	74.9	837
Brood 4	48.3	658	50.2	994	84.7	72	66.1	174
Brood 5	-	-	31.3	1302	-	-	-	-
Total	38.2	7061	33.3	7507	45.5	3018	43.7	2759

u.h. = unhatched eggs

Table 7 : The relative dominant lethal frequency produced by heliotrine in Canton-S and $X^{C2}vf$ males.

Type of male	Brood 1	2	3	4
Canton-S	0.66	0.26	0.43	0.45
$X^{C2}vf$	0.11	0.47	0.72	0.68

Table 8 : The relative increase in the F1 male/female ratio produced by heliotrine in Canton-S and $X^{C2}vf$ males.

Canton-S males		$X^{C2}vf$ males	
Brood 1	0.84	Brood 1	1.43
Brood 2	1.33	Brood 2	1.19
Brood 3	1.39	Brood 3	1.65
Brood 4	1.29	Brood 4	2.06
Brood 5	0.98	Brood 5	1.95
		Brood 6	2.76
		Brood 7	1.29

Table 9 : The frequency of hyperploid females, with 95% confidence limits, produced by heliotrine in two replicate experiments. Heliotrine = 0.001 M; brood interval two days.

	Experiment 1		Experiment 2	
	% hyper.	n. gametes	% hyper.	n. gametes
Brood 1	-	256	0.86 ± 1.19	233
Brood 2	1.49 ± 1.30	335	2.87 ± 1.96	279
Brood 3	0.61 ± 0.69	493	1.86 ± 1.48	322
Brood 4	1.63 ± 1.59	245	5.42 ± 2.67	277
Brood 5	3.16 ± 2.73	158	4.90 ± 2.96	204
Brood 6	2.23 ± 2.16	179	4.22 ± 3.06	166
Brood 7	-	-	-	133

Table 10 : Chromosome loss, non-disjunction, translocation, sec-linked lethal and sex-linked lethal mosaic frequencies produced by heliotrine in yv/ $sc^{8}Y^{B-S}$ males.

Heliotrine = 0.001 M; brood interval three days.

Effect scored	Experiment 1		Experiment 2		Experiment 3	
s.l.l.			% leth.	no.	% leth.	no.
Brood 1			1.93	571	0.72	415
Brood 2			2.05	730	2.24	491
Brood 3			5.75	400	4.41	522
Brood 4			6.23	401	5.24	496
Brood 5			5.29	170	2.32	436
Total			3.65	2272	3.09	2360
s.l.l. mos.						
Brood 1			1.73	173		
Brood 2			2.79	215		
Brood 3			1.79	168		
Brood 4			4.55	176		
Brood 5			-	70		
Total			2.49	802		
transloc.	%transloc.	no.	%transloc.	no.		
Brood 1	-	502	-	342		
Brood 2	-	383	-	410		
Brood 3	-	423	-	267		
Brood 4	0.24	422	0.35	289		
Brood 5	0.28	363	-	101		
Total	0.09	2093	0.07	1409		

Table 10 : (cont.)

Effect scored	Experiment 1		Experiment 2		Experiment 3	
* XO males	%XO	n.males	%XO	n.males	%XO	n.males
Brood 1	0.47	837	0.24	418	-	356
Brood 2	0.33	609	-	473	-	392
Brood 3	0.18	567	-	313	-	452
Brood 4	0.16	622	0.60	336	0.23	442
Brood 5	1/22	575	-	128	0.28	354
Total	0.47	3210	0.18	1668	0.10	1996
† Delayed XO males						
Brood 1			0.58	342	-	415
Brood 2			0.24	410	-	491
Brood 3			0.75	267	-	522
Brood 4			0.69	289	3.22	496
Brood 5			2.97	101	0.23	436
Total			0.71	1409	0.70	2360

* Also includes those males which had lost only the long or short arm of the Y.

† Includes secondary non-disjunction in third experiment.

Table 11. The normal and abnormal progeny resulting from the mating of asc/ $sc^{8}Y^{B-S}$ males with y v/ asc females. Abnormal offspring may arise as a result of loss of X or Y or non-disjunction. See text.

♀ \ ♂	asc	$sc^{8}Y^{B}$	O	asc/$sc^{8}Y^{B}$
yv	yv/asc	yv/$sc^{8}Y^{B}$	yv/-	yv/asc/$sc^{8}Y^{B}$
asc	asc/asc	asc/$sc^{8}Y^{B}$	asc/-	asc/asc/$sc^{8}Y^{B}$
O	asc/-	$sc^{8}Y^{B}$/-	-	asc/asc/$sc^{8}Y^{B}$
		Dies.		
yv/asc	yv/asc/asc	yv/asc/$sc^{8}Y^{B}$	yv/asc	yv/asc/asc/$sc^{8}Y^{B}$
	Dies			Dies

Loss in F1 ♀ - non-Bar apricot eyed males

Loss in F1 ♂ - non-Bar yellow vermilion males

non-Bar apricot eyed males.

Non-disjunction in F1 ♀ -

non-Bar apricot eyed males

Bar-eyed females.

Non-disjunction in F1 ♂ -

non-Bar males, Bar females and Bar apricot females.

Table 12 : The distribution of sex-linked lethals produced by ionizing radiation and various chemical mutagens in four X-chromosomal regions delimited by the markers sc, ct, v, and f. The frequency of sponteous visibles and lethals is shown for comparison.

Mutagens	Chromosome Region								Total
	sc - ct		ct - v		v - f		f - spa		
	no.	%	no.	%	no.	%	no.	%	
Heliotrine (a)	40	37.4	12	11.2	30	28.0	25	23.4	107
X-rays (b) (Ives, 1959)	101	31.2	49	15.1	117	36.1	57	14.5	324
Myleran (c) (Rohrborn, 1959)	19	36.5	7	13.5	23	44.2	3	5.8	52
Mustard Gas (d) (Slizynska and Slizynski, 1947)	29	34.9	15	18.1	24	28.9	15	18.1	83
Alkylating Comp. (Fahmy and Fahmy, 1956, 1957)	172	30.4	92	16.2	182	32.1	121	21.3	567
TEM (f) (Belitz, 1954)	52	34.4	18	11.9	46	30.5	35	23.2	151
Chinon 1 (g) (Belitz, 1957)	58	30.9	32	17.0	62	33.0	36	19.1	188
Spontaneous visibles (Bridges and Brehme, 1944)	36	42.4	16	18.8	23	27.1	10	11.8	85
Spontaneous lethals (Belitz, 1957)	49	48.1	11	10.8	32	31.4	10	9.8	102

Table 13. The effect of potassium cyanide and sodium azide on the sex linked lethal frequency induced by heliotrine. Brood interval 3 days.

Expt. No.	Male Type	Treatment	Brood 1 % leth.	Brood 1 no. chrom.	Brood 2 % leth.	Brood 2 no. chrom.	Brood 3 % leth.	Brood 3 no. chrom.	Brood 4 % leth.	Brood 4 no. chrom	Brood 5 % leth.	Brood 5 no. chrom.	Total % leth.	Total no. chrom.	
3	Rod-X	Heliotrine	1.24	726	7.88	482	8.01	612	2.10	334			4.78	2154	X^2 = 7.84
		Heliotrine + Cyanide	1.38	725	10.17	403	10.79	667	7.69	208			6.94	2003	P = 0.006
4	Rod-X	Heliotrine	0.97	516	6.53	505	11.62	482	5.05	463	0.22	460	5.07	2426	X^2 = 6.27
		Heliotrine + Azide	3.10	580	9.05	453	14.65	314	9.18	411	1.34	522	6.71	2280	P = 0.013
5	Ring-X	Heliotrine	1.74	461	5.53	253	8.05	87	-	17			3.55	818	X^2 = 2.14
		Heliotrine + Cyanide	0.65	460	6.23	273	17.21	122	11.11	36			5.05	891	P = 0.15
6	Ring-X	Heliotrine	0.72	555	3.47	461	9.32	236	6.42	265			3.89	1517	X^2 = 4.35
		Heliotrine + Azide	0.69	582	7.00	357	10.22	362	7.38	271			5.47	1572	P = 0.04

Heliotrine = 0.001M, KCN = 0.007M, NaN_3 = 0.003M. Volume injected = 0.09/Ml.

Table 14 : The F1 male/female ratios obtained from Canton-S of $\frac{X^{C2}vf}{}$ males mated to abc females. Males treated with heliotrine, or with heliotrine + either potassium cyanide or sodium azide. Brood interval three days.

Expt. No.	Male Type	Treatment	Brood 1	Brood 2	Brood 3	Brood 4	Brood 5	Total	
4	Rod-X	Heliotrine	0.60 (364/608)	0.68 (384/567)	0.71 (420/590)	0.66 (381/573)	0.51 (298/580)	0.63 (1847/2918)	X^2 = 20.87
		Heliotrine + Azide	0.62 (403/655)	0.89 (451/509)	0.99 (354/358)	0.85 (397/466)	0.57 (342/602)	0.75 (1947/2590)	P < 0.0001
5	Ring-X	Heliotrine	1.10 (601/548)	1.40 (431/307)	3.37 (330/98)	3.65 (73/20)	*	1.47 (1435/973)	X^2 = 7.96
		Heliotrine + Cyanide	1.14 (597/524)	1.71 (536/314)	3.43 (470/137)	3.85 (158/41)	*	1.63 (1661/1016)	P = 0.005
6	Ring-X	Heliotrine	1.11 (781/704)	1.60 (906/568)	2.40 (1006/420)	1.94 (648/334)	0.69 (405/590)	1.43 (3746/2616)	X^2 = 14.72
		Heliotrine + Azide	1.26 (1001/795)	1.89 (1051/557)	2.77 (1073/387)	2.31 (678/294)	0.60 (265/441)	1.64 (4068/2474)	P < 0.0001

Heliotrine = 0.001M, KCN = 0.007M, NaN_3 = 0.003M. Volume injected = 0.09µl * Not scored

Table 15 : The effect of potassium cyanide and sodium azide on the dominant lethal frequency induced by heliotrine. Brood interval = 3 days.

		Brood 1		2		3		4		5		Total		
Male	Treatment	% leth.	no.	% leth.	no.	% leth.	no.	% leth.	no.	% leth.	no.	% leth.	no.	
Canton-S	Heliotrine	23.0	3278	52.6	1634	51.2	1491	48.3	658	*		38.2	7061)	χ^2 = 18.9
	Heliotrine+KCN	20.9	2665	59.3	1649	62.5	1620	65.3	721	*		45.4	6655)	P < 0.0001
$X^{C2}vf$	Heliotrine	36.5	1319	59.2	781	75.8	846	84.7	72	*		54.5	3018)	χ^2 = 13.26
	Heliotrine+KCN	40.1	1057	60.9	1183	78.7	1479	75.0	280	*		63.0	3999)	P = 0.003
Canton-S	Heliotrine	34.5	591	63.6	385	45.0	773	37.1	140	18.4	376	40.5	2265)	χ^2 = 30.0
	Heliotrine+NaN_3	42.9	748	67.4	454	73.8	808	53.9	284	1518	385	53.6	2679)	P < 0.0001
$X^{C2}vf$	Heliotrine	35.4	724	54.2	1024	74.9	837	66.1	174	*		56.3	2759)	χ^2 = 24.9
	Heliotrine+NaN_3	61.1	766	72.8	441	86.1	346	83.7	233	*		71.8	1786)	P < 0.0001

Heliotrine = 0.001 M, KCN = 0.007 M, NaN_3 = 0.003 M. Volume injected = 0.09 μl. * Not Scored

Table 16 : The effect of sodium hydrosulphite on the sex-linked lethal frequency produced by heliotrine in $X^{C2}vf$ males.

Heliotrine = 0.001 M, $Na_2S_2O_4$ = 0.01 M. Brood interval 3 days.

	Heliotrine		Heliotrine + $Na_2S_2O_4$	
	% leth.	no.	% leth.	no.
Brood 1	0.72	555	1.53	589
Brood 2	3.47	461	3.24	370
Brood 3	9.32	236	8.31	325
Brood 4	6.42	265	6.51	261
Total	3.89	1517	4.21	1545

Table 17 : The effect of gaseous nitrogen on the heliotrine induced sex-linked lethal frequency.

Heliotrine = 0.001 M; brood interval two days.

	Experiment 1.				Experiment 2.			
	Nitrogen		Air		Nitrogen		Air	
	%leth.	no.	%leth.	no.	%leth.	No.	%leth.	No.
Brood 1	3.98	176	1.62	309	0.82	364	0.74	537
Brood 2	1.46	274	1.17	426	0.67	598	0.73	684
Brood 3	3.42	234	3.01	366	1.46	618	2.25	577
Brood 4	9.23	130	8.33	156	2.69	446	4.72	381
Brood 5	4.29	140	10.66	122	6.23	305	4.23	355
Brood 6	3.64	55	7.69	39	2.29	218	1.30	230
Brood 7	-	40	3.70	27	1.43	279	1.44	209
Total	3.72	1049	3.53	1445	1.98	2828	2.05	2973

Table 18: The effect of gaseous nitrogen on the F1 sex ratio distortion produced by heliotrine.

Heliotrine = 0.001 M; brood interval two days.

	Experiment 1						Experiment 2					
	Nitrogen			Air			Nitrogen			Air		
	♂	♀	♂/♀	♂	♀	♂/♀	♂	♀	♂/♀	♂	♀	♂/♀
Brood 1	250	183	1.37	372	319	1.17	551	406	1.36	749	587	1.28
Brood 2	304	278	1.09	409	443	0.92	677	639	1.06	791	758	1.04
Brood 3	488	269	1.81	617	389	1.59	607	563	1.08	880	618	1.42 *
Brood 4	254	135	1.88	359	168	2.14	461	377	1.22	676	405	1.67 *
Brood 5	326	151	2.16	431	141	3.06 *	640	311	2.06	846	386	2.19
Brood 6	154	58	2.66	167	53	3.15	337	219	1.54	488	254	1.92
Brood 7	56	45	1.24	92	70	1.31	297	277	1.07	238	219	1.09
Total	1832	1119	1.64	2447	1583	1.55	3570	2792	1.28	4668	3227	1.45 *

* Significant at the 5% level.

Table 19 : The effect of the antibiotic chloramphenicol on the sex-linked lethal frequency produced by heliotrine.

Heliotrine = 0.001 M, Chloramphenicol = 0.23 % ; brood interval three days.

	Experiment 1				Experiment 2			
	Heliotrine		Heliotrine+Chl		Heliotrine		Heliotrine+Chl	
	%leth.	no.	%leth.	no.	%leth.	no.	%leth.	no.
Brood 1	1.29	464	0.63	473	1.05	949	1.36	1322
Brood 2	4.53	287	5.77	312	7.06	737	6.09	986
Brood 3	7.57	370	9.06	287	8.43	332	8.10	395
Brood 4	2.93	205	5.19	212	7.04	355	6.60	530
Brood 5	2.14	281	0.39	259	2.02	397	0.93	429
Total	3.67	1607	3.82	1543	4.44	2770	4.07	3662

Chl = Chbramphenicol

Table 20 : The effect of the antibiotic chloramphenicol on the dominant lethal frequency produced by heliotrine.

Heliotrine = 0.001 M, Chloramphenicol = 0.23%; brood interval three days.

	Heliotrine		Heliotinre + Chl	
	% leth.	no. eggs	% leth.	no. eggs
Brood 1	17.6	2115	13.6	2822
Brood 2	36.6	2330	35.2	3480
Brood 3	43.9	919	45.7	1178
Brood 4	50.2	994	49.8	1572
Brood 5	31.3	1302	29.8	1219
Total	66.9	7660	68.0	10271

Chl = Chloramphenicol

Table 21 : The effect of the antibiotic chloramphenicol on the F1 sex ratio distortion produced by heliotrine.

Heliotrine = 0.001 M, Chloramphenicol = 0.23%; brood interval three days.

	Heliotrine			Heliotrine + Chl		
	Males	Females	M/F	Males	Females	M/F
Brood 1	366	1097	0.33	622	1698	0.37
Brood 2	553	820	0.67	680	1126	0.60
Brood 3	246	360	0.68	340	481	0.71
Brood 4	246	372	0.66	403	638	0.63
Brood 5	238	483	0.49	264	448	0.59
Total	1649	3132	0.53	2309	4391	0.53

Chl = chloramphenicol

Table 22 : The frequency of XO males (including loss of Y^S and Y^L) produced by heliotrine, X-irradiation and combined heliotrine and X-ray treatments.

Heliotrine = 0.001 M, X-irradiation = 1500r at 150r/min., Brood interval three days.

	Heliotrine		X-irradiation		Heliotrine-20mins-X-rays		Heliotrine-2hrs.-X-rays	
	%XO	n.males	%XO	n.males	%XO	n.males	%XO	n.males
Brood 1	0.48	837	0.56	1066	2.01	498	0.71	702
Brood 2	0.33	609	1.16	773	3.09	518	1.25	560
Brood 3	0.18	569	4.04	668	3.67	245	3.83	313
Brood 4	-	621	2.59	424	4.88	123	4.11	292
Brood 5	1.05	574	0.76	791	0.71	280	1.46	343
Total	0.4	3210	1.6	3722	2.6	1664	1.8	2210

Table 23 : The 2-3 translocation frequency produced by heliotrine, X-irradiation and combined heliotrine and X-ray treatments.

Heliotrine = 0.001 M, X-irradiation = 1500r at 150r/min. Brood interval three days.

	Heliotrine		X-irradiation		Heliotrine - 20mins- X-rays		Heliotrine - 2hrs. - X-rays	
	%t.	n. gametes	%t.	n. gametes	%t.	n. gametes	%t.	n. gametes
Brood 1	-	502	1.17	511	1.93	311	1.72	522
Brood 2	-	383	2.25	489	5.99	384	3.45	406
Brood 3	-	423	5.90	390	9.37	160	8.00	200
Brood 4	-	422	5.86	273	7.32	82	2.53	198
Brood 5	0.28	363	0.79	378	0.46	219	0.41	241
Total	0.05	2093	2.89	2041	4.41	1156	2.87	1567

Table 24 : The frequency of XO males (including loss of Y^S and Y^L) produced by heliotrine, X-irradiation and combined heliotrine and X-ray treatments.

Heliotrine = 0.001 M, X-irradiation = 900r at 150r/min.; brood interval three days.

	Heliotrine		X-irradiation		Heliotrine–15 mins–X-rays		X-rays – 15 mins – Heliotrine	
	%XO	n.males	%XO	n.males	%XO	n.males	%XO	n.males
Brood 1	0.24	418	-	335	0.77	259	-	340
Brood 2	-	473	-	479	0.42	240	0.25	402
Brood 3	-	313	-	270	1.52	132	-	241
Brood 4	0.60	336	0.82	364	5.20	173	1.57	254
Brood 5	-	128	0.96	209	1.67	180	0.52	191
Total	0.18	1668	0.30	1657	1.73	984	0.42	1428

Table 25 : The sex-linked lethal frequencies produced by heliotrine, X-irradiation and combined heliotrine and X-ray treatments.

Heliotrine = 0.001 M, X-irradiation = 900r at 150r/min., Brood interval three days.

	Heliotrine		X-irradiation		Heliotrine - 15 mins-X-rays		X-rays - 15 mins Heliotrine	
	%leth.	n.chr.	%leth.	n.chr.	%leth.	n.chr.	%leth.	n.chr.
Brood 1	1.93	571	1.33	451	6.76	296	2.72	441
Brood 2	2.05	730	2.65	718	3.82	340	5.44	698
Brood 3	5.75	400	5.69	457	17.68	164	11.43	315
Brood 4	6.23	401	5.76	451	9.35	213	13.62	279
Brood 5	5.29	170	1.01	298	2.15	186	5.36	224
Total	3.65	2272	3.37	2375	7.17	1199	6.95	1957

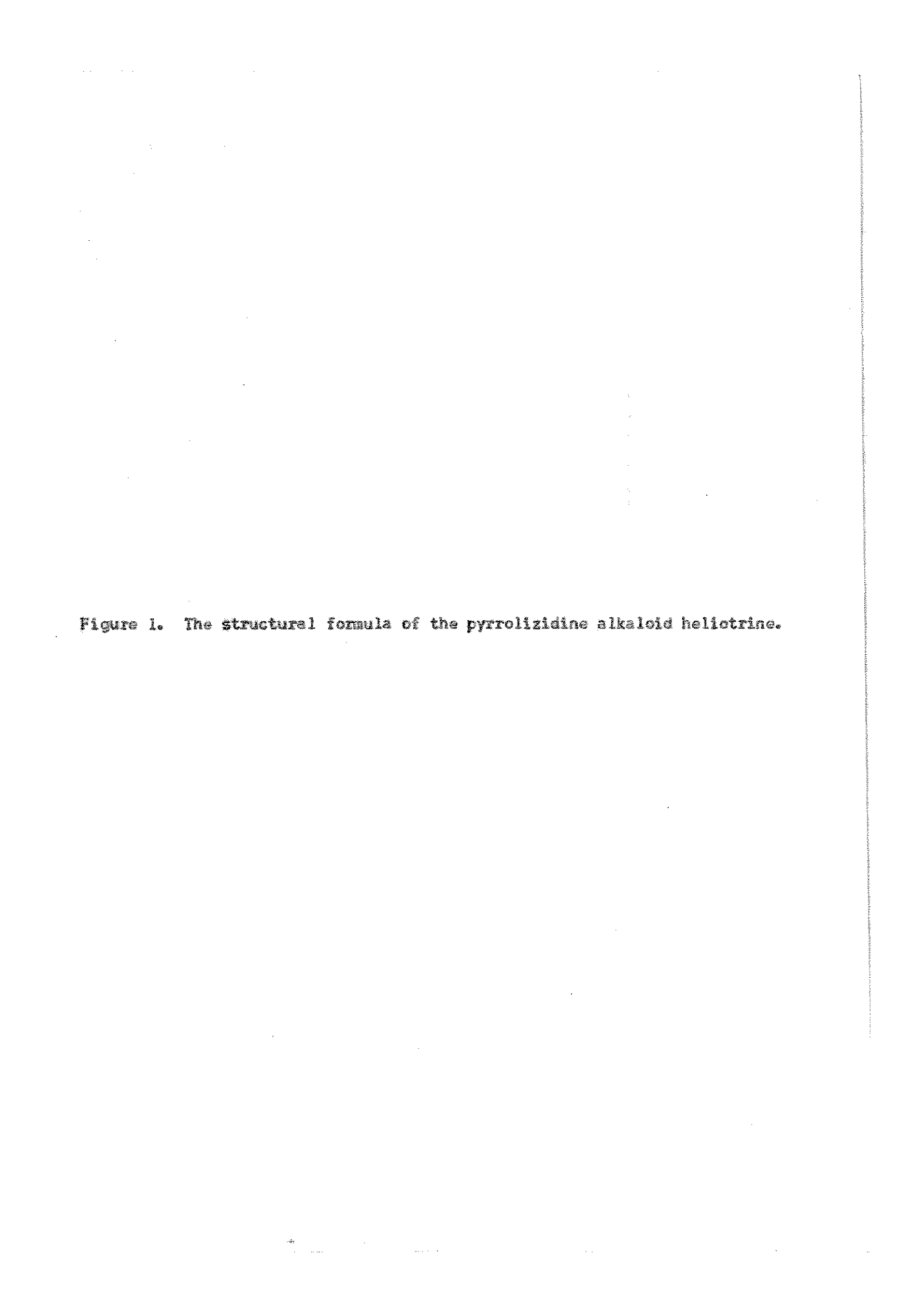


Figure 1. The structural formula of the pyrrolizidine alkaloid heliotrine.

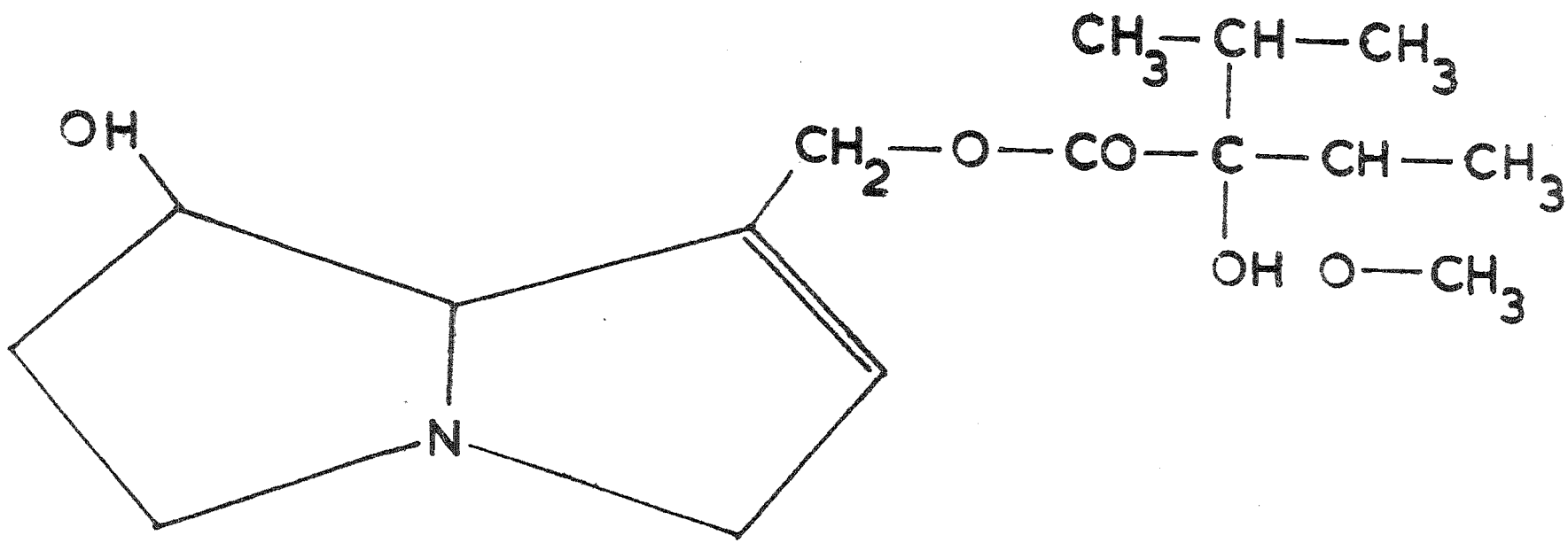
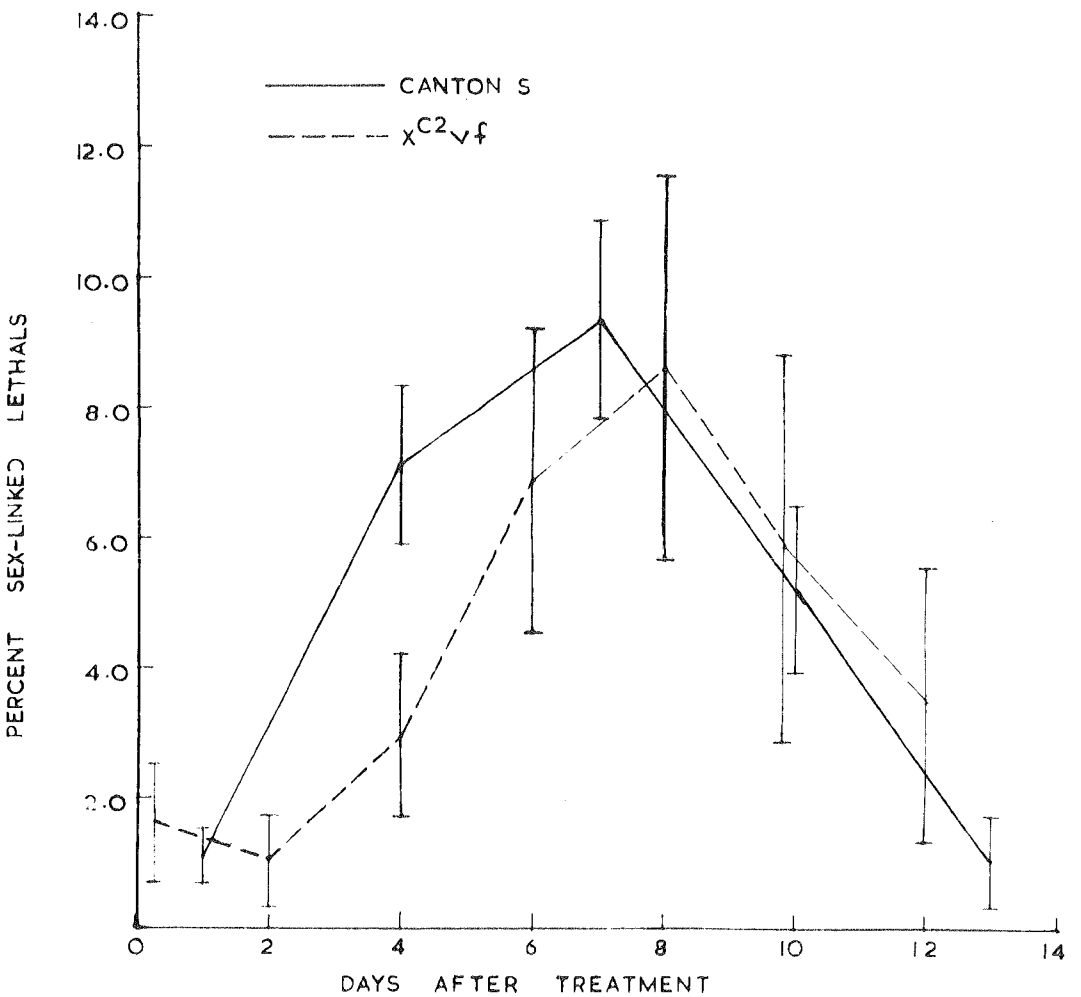

OH
N
CH2—O—CO—C—CH—CH3
CH3—CH—CH3
OH O—CH3

Figure 2. The sex-linked lethal frequency, with 95% confidence limits, produced by heliotrine in Canton-S (unbroken line) and $\underline{X^{C2}vf}$ (broken line) males. Heliotrine = 0.00M, brood interval = two days for $\underline{X^{C2}vf}$ males and three days for Canton-S males.

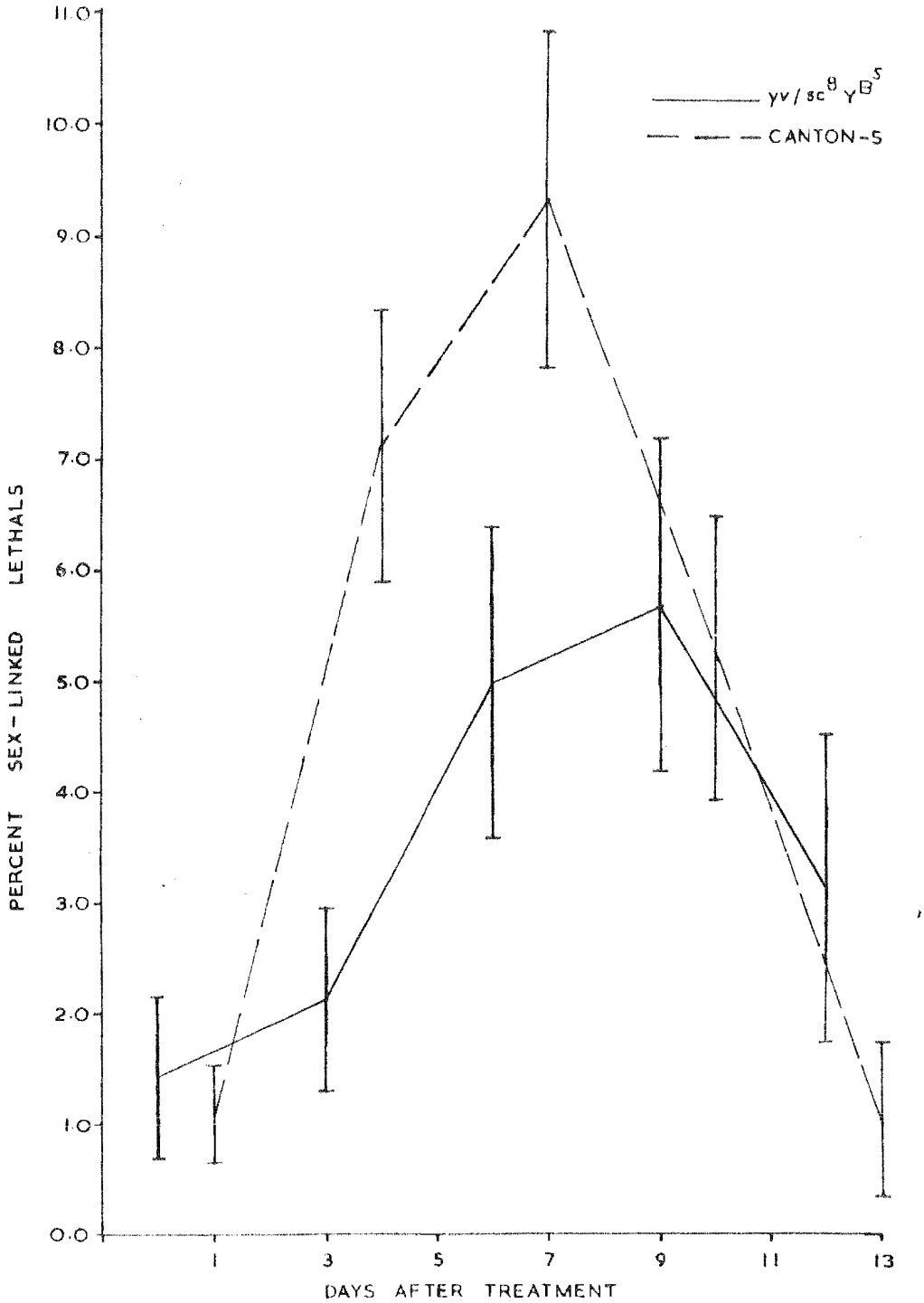


NO. LETH.	24		123		133	60	9
NO. GAMETES	2191		1724		1426	1152	857
NO. LETH.	12	8	21	31	30	14	10
NO. GAMETES	742	779	710	451	349	240	289

ROD

Figure 3. The sex-linked lethal frequency, with 95% confidence limits, produced by heliotrine in Canton-S (continuous line) and yv/ $sc^{8}Y^{B-S}$ (broken libe) males.

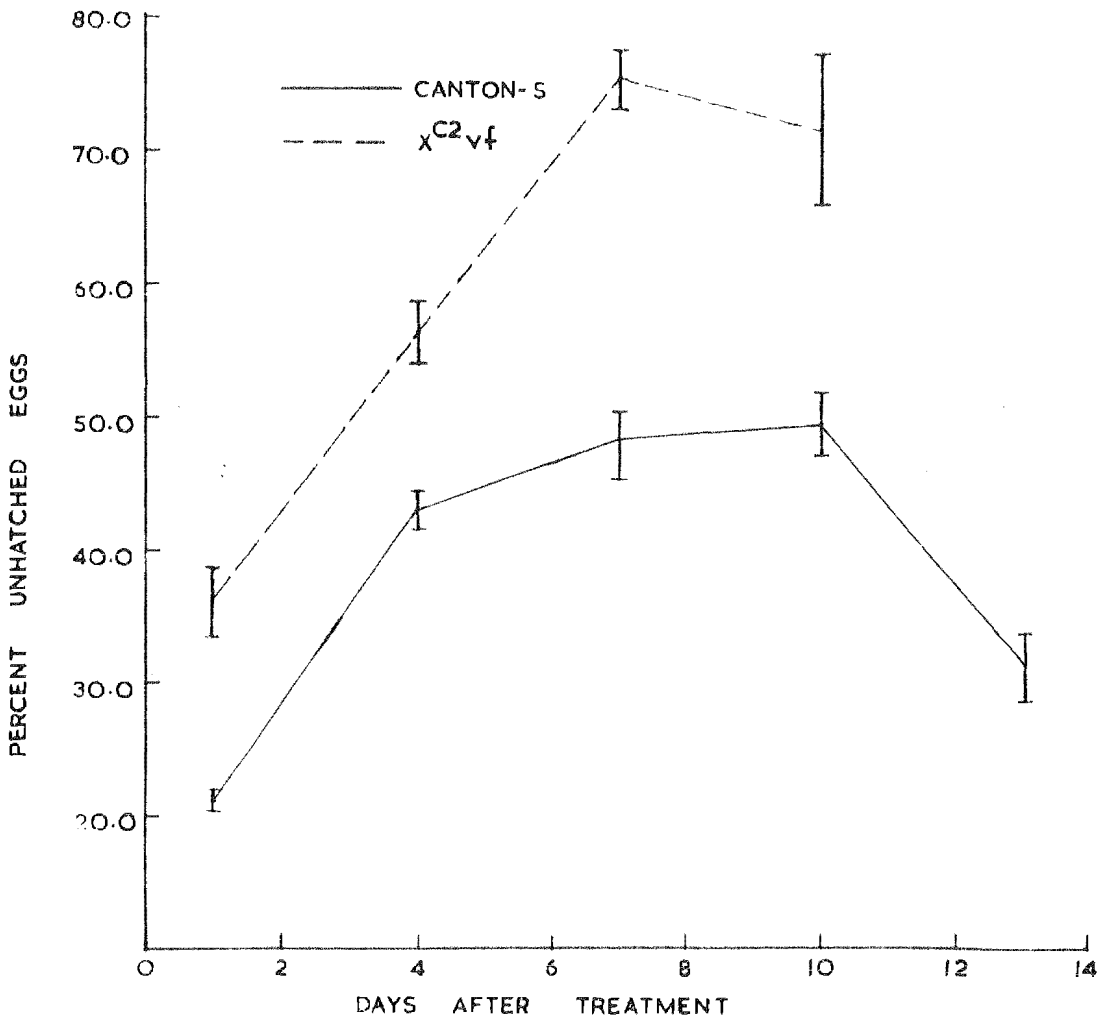
Heliotrine = 0.001 M, brood interval = three days.



NO LETH	14	26	46	51	19	yv/sc^B Y^B^S
NO GAMETES	986	1221	922	897	606	
NO LETH.	24	123	133	60	9	CANTON-S
NO GAMETES	2191	1724	1426	1152	857	

Figure 4. The frequency of unhatched eggs, with 95% confidence limits, produced by asc females when mated to heliotrine treated Canton-S of $\frac{C2}{X}$vf males.

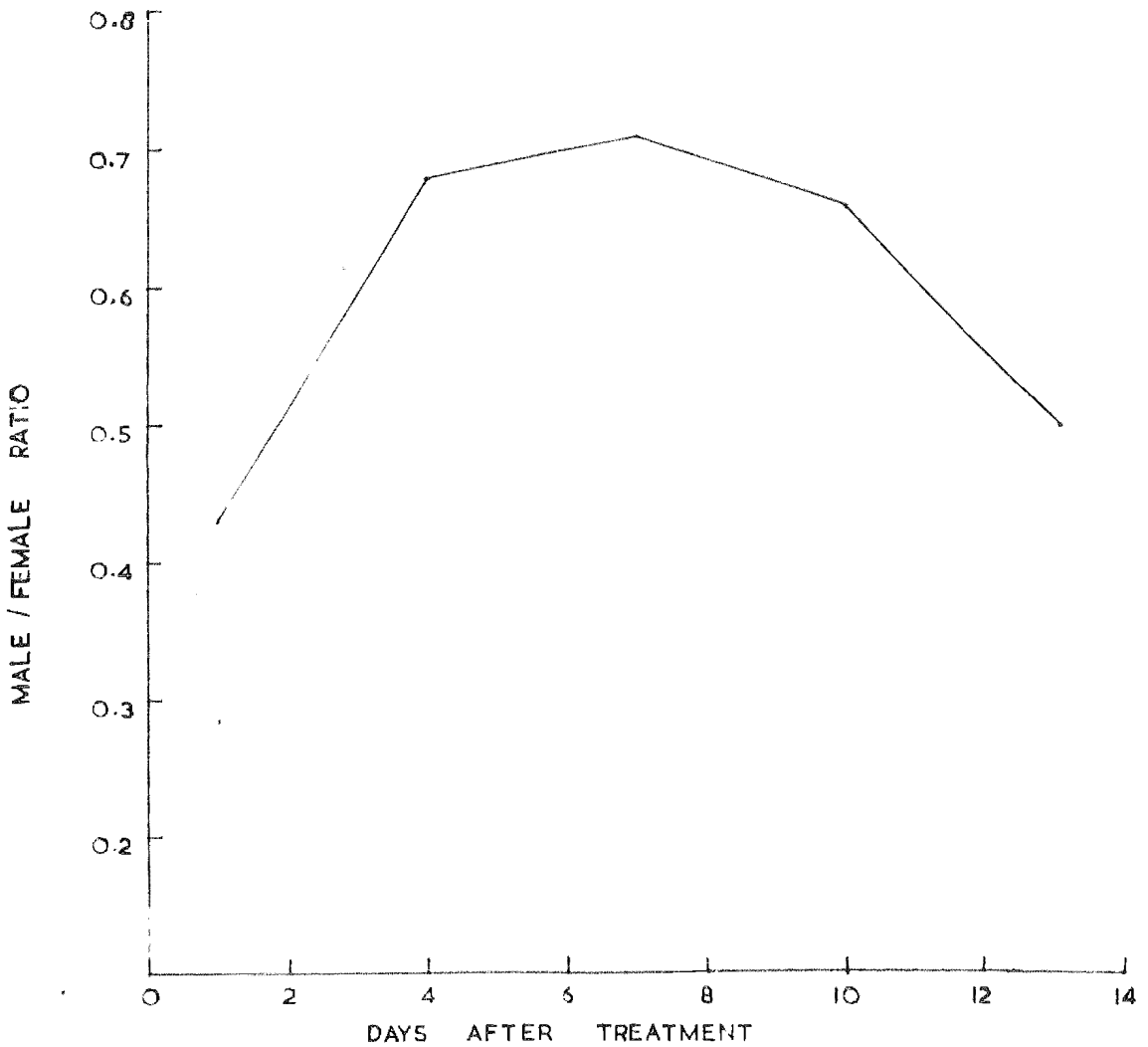
Heliotrine = 0.001 M, brood interval three days.



EGG POD	NO. EGGS UNHATCH.	1105	1700	1167	817	407
	NO. EGGS LAID	5252	3952	2410	1652	1302
EGG	NO. EGGS UNHATCH.	1306	1017	1268	176	
	NO. EGGS LAID	2043	1805	1683	246	

Figure 5. The F1 male/female ratio produced when heliotrine treated Canton-S males are mated to asc females.

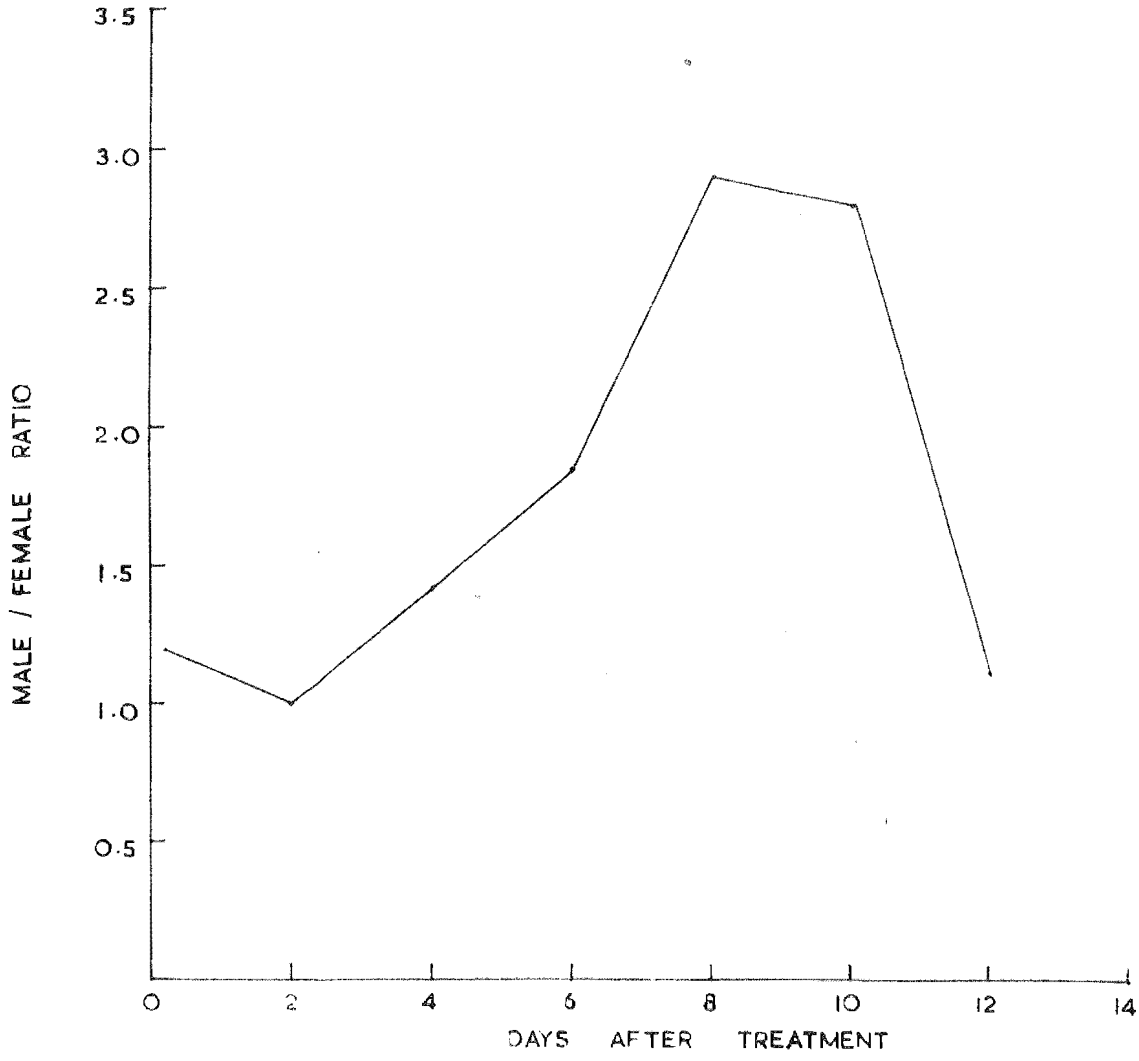
Heliotrine = 0.001 M; brood interval three days.



NO. MALES	730	937	558	627	535
NO. FEMALES	1705	1387	946	945	1064

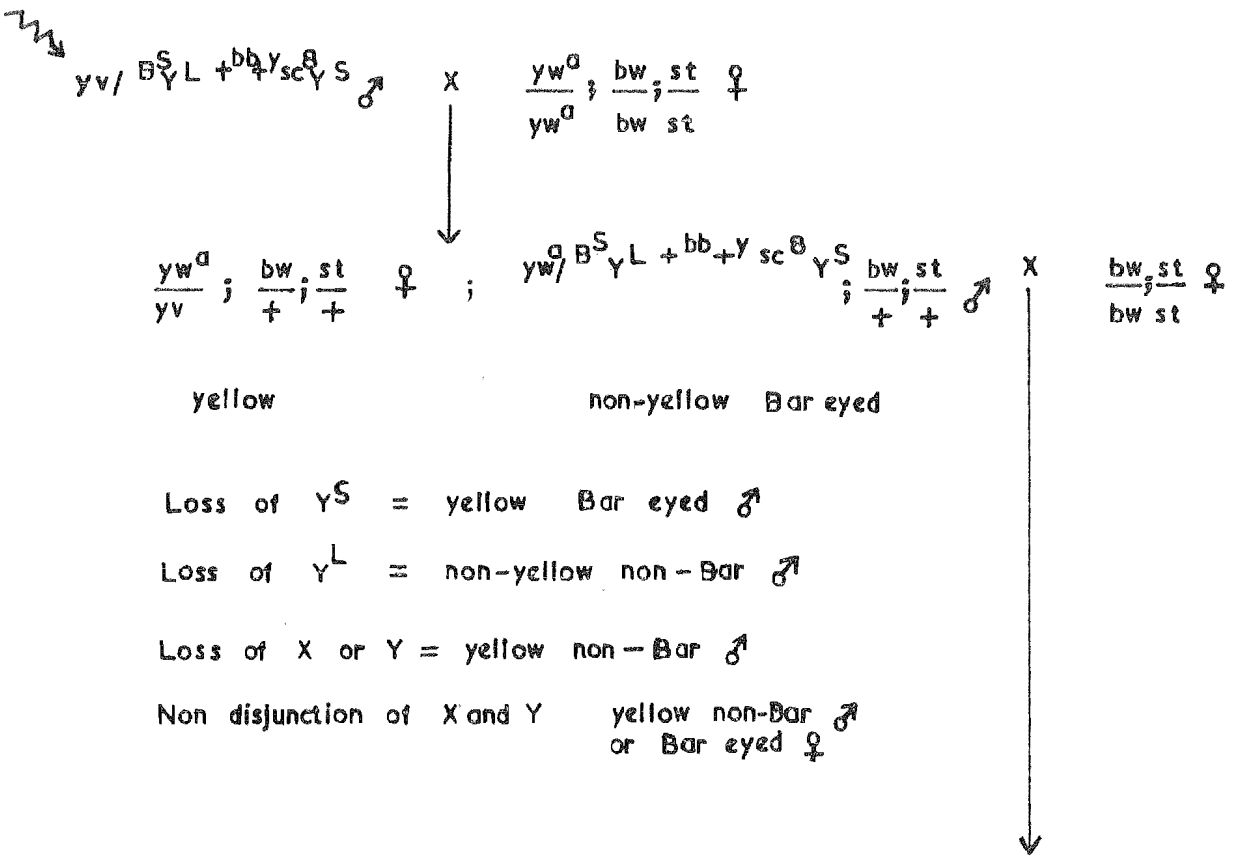
Figure 6. The F1 male/female ratio produced when heliotrine treated $\underline{X^{C2}vf}$ males are mated to $\underline{asc}$ females.

Heliotrine = 0.001 M; brood interval two days.



NO. MALES 950	837	1107	898	1134	761	388
NO. FEMALES 797	845	781	486	392	273	346

Figure 7. Crosses made for the detection of XO males, loss of Y^S or Y^L, and translocations involving the second, third and/or Y chromosomes.



2/3 Translocations = absence of bw and st flies

2/Y Translocations = absence of bw and w males

3/Y Translocations = absence of st and w males

2/3/Y Translocations = only wild type males and w females present

Figure 8. Crosses made for the detection of XO males, sex-linked lethals, sex-linked lethal mosaics, translocations and delayed chromosome loss or secondary non-disjunction.

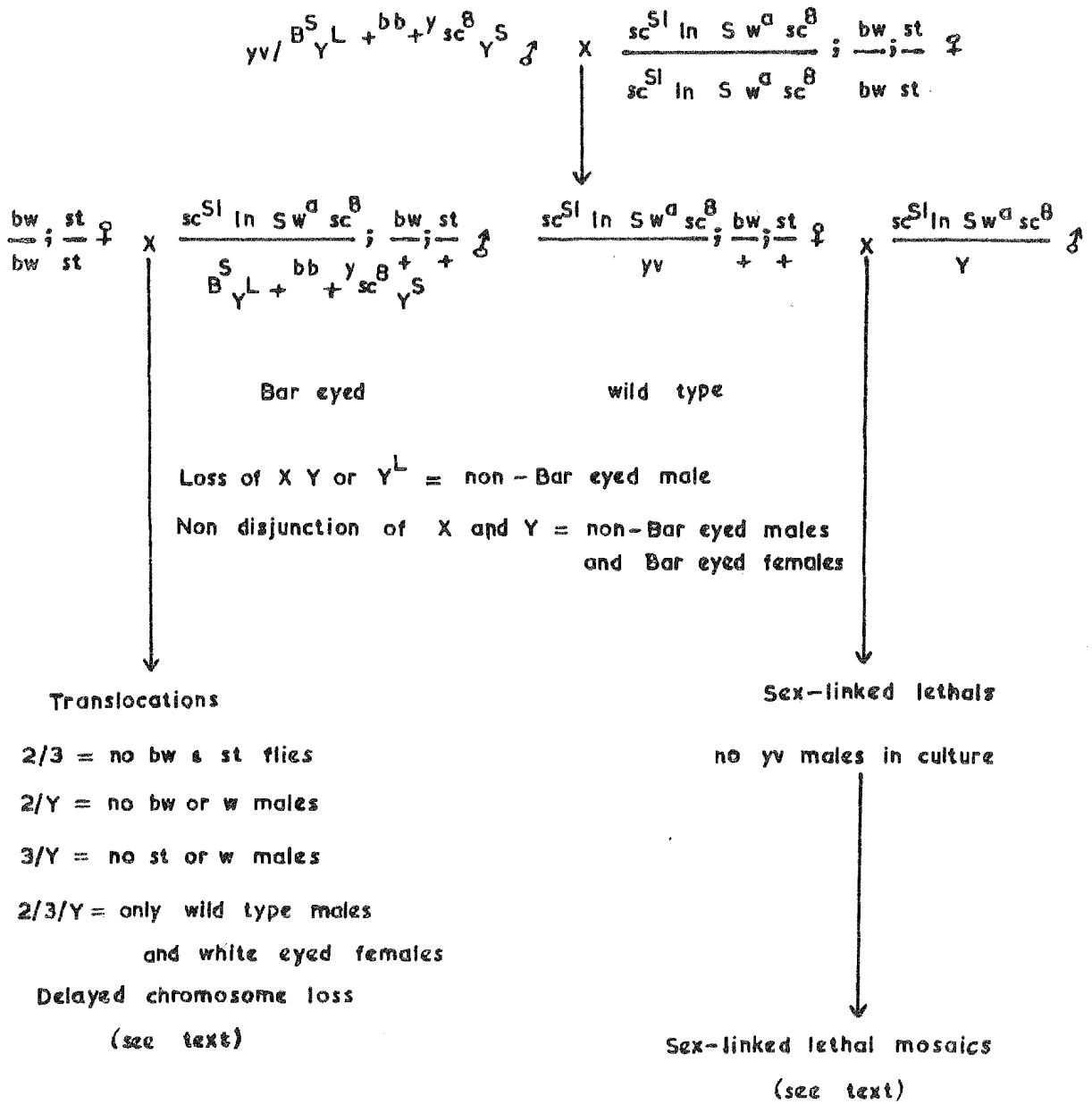

yv/ B^S Y^L + bb + y sc^8 Y^S ♂
X
sc^SI In S w^a sc^8 ; bw ; st / sc^SI In S w^a sc^8 ; bw ; st ♀
bw ; st / bw st ♀
X
sc^SI In S w^a sc^8 ; bw ; st / B^S Y^L + bb + y sc^8 + / Y^S + ♂
sc^SI In S w^a sc^8 ; bw ; st / yv + + ♀
X
sc^SI In S w^a sc^8 / Y ♂
Bar eyed
wild type
Loss of X Y or Y^L = non - Bar eyed male
Non disjunction of X and Y = non-Bar eyed males and Bar eyed females
Translocations
2/3 = no bw & st flies
2/Y = no bw or w males
3/Y = no st or w males
2/3/Y = only wild type males and white eyed females
Delayed chromosome loss
(see text)
Sex-linked lethals
no yv males in culture
Sex-linked lethal mosaics
(see text)

Figure 9. The frequency of heliotrine induced sex-linked lethals in thirteen chromosome regions each five map units in length.

Heliotrine = 0.001 M.

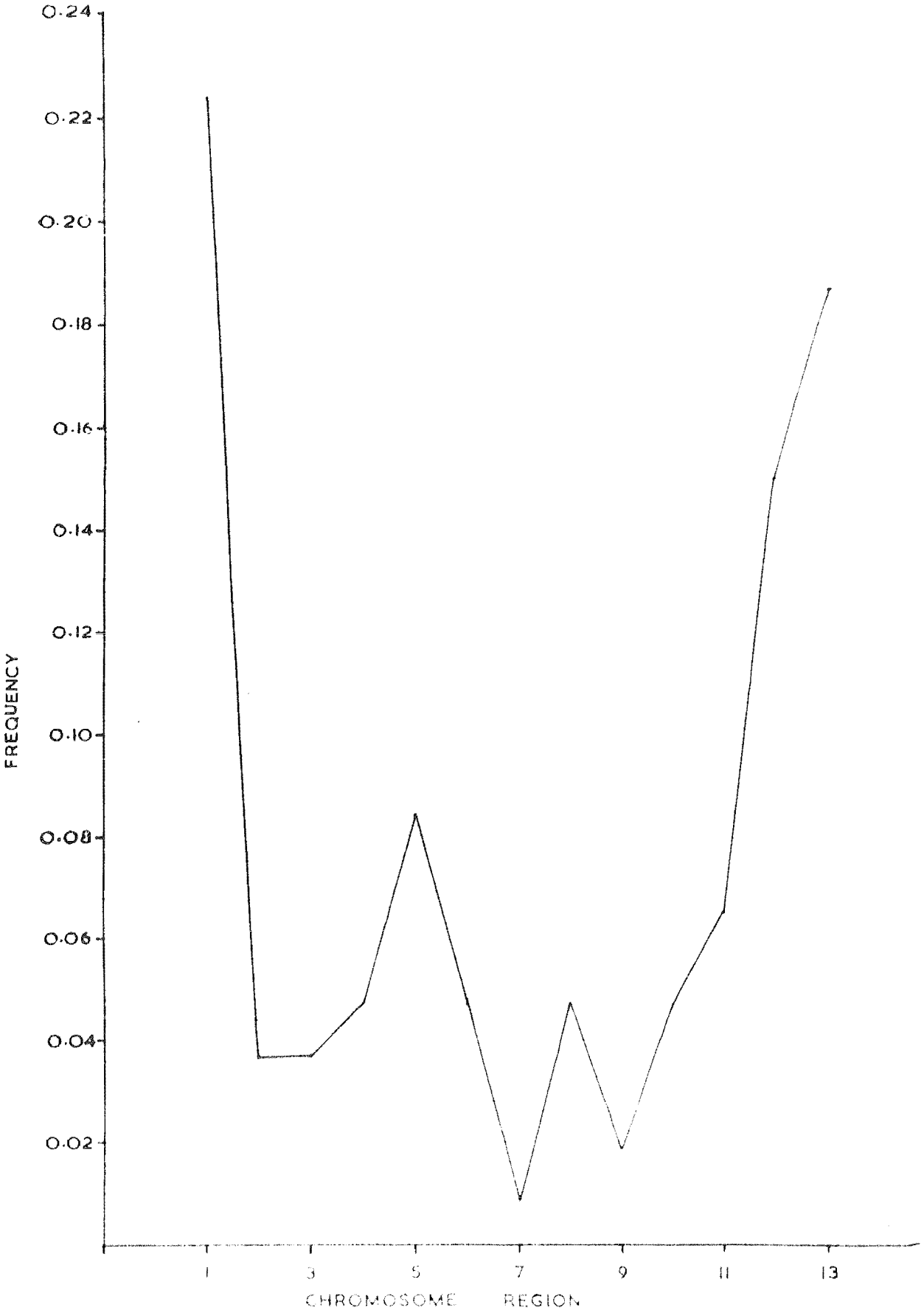

0·24
0·22
0·20
0·18
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
FREQUENCY
1
3
5
7
9
11
13
CHROMOSOME REGION

Figure 10. The frequency distribution of 107 sex-linked lethals produced by heliotrine and 110 sex-linked lethals produced by combined heliotrine + KCN treatment within thirteen chromosome segments each five map units in length.

Heliotrine = 0.001 M, KCN = 0.007 M.

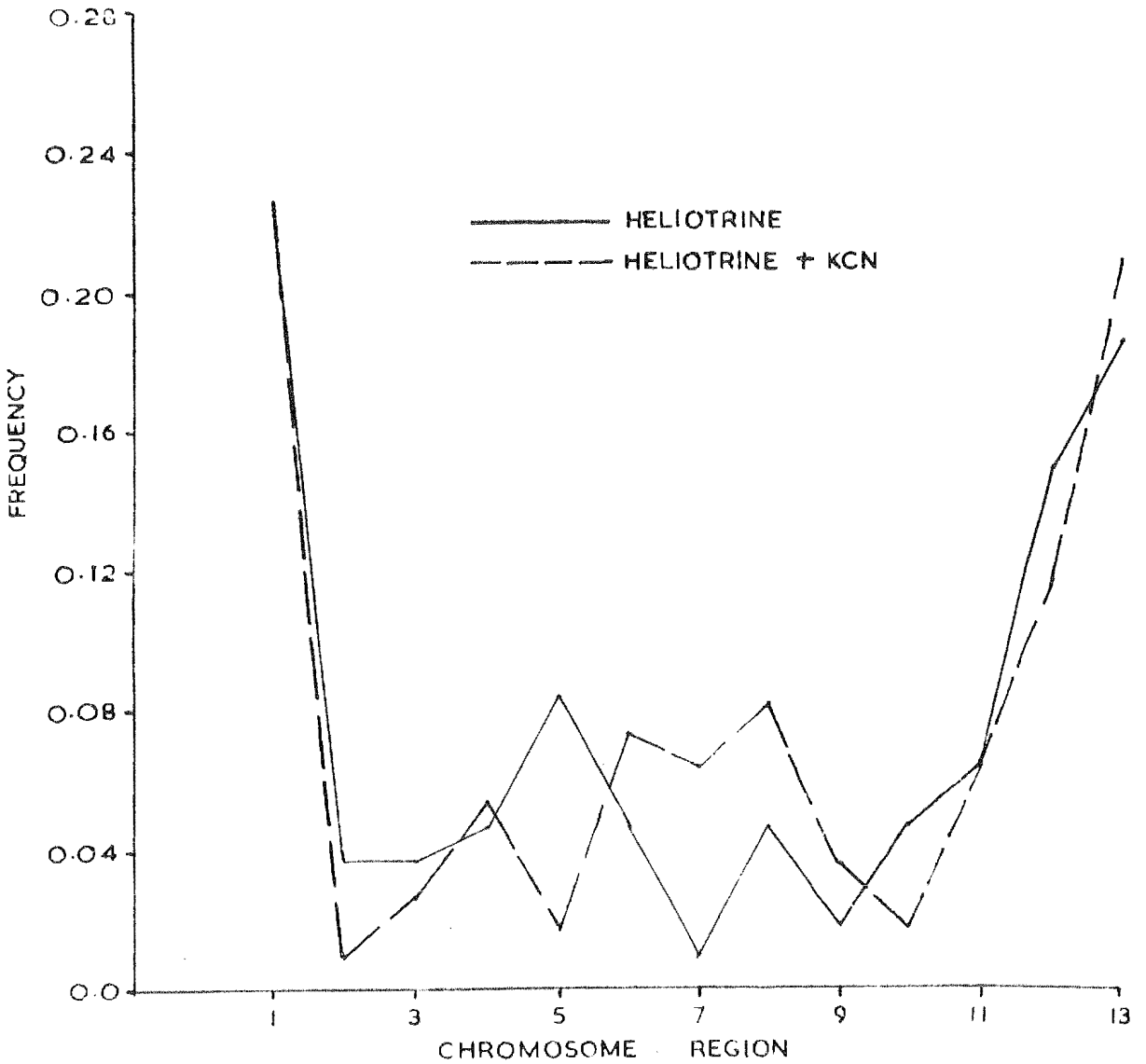
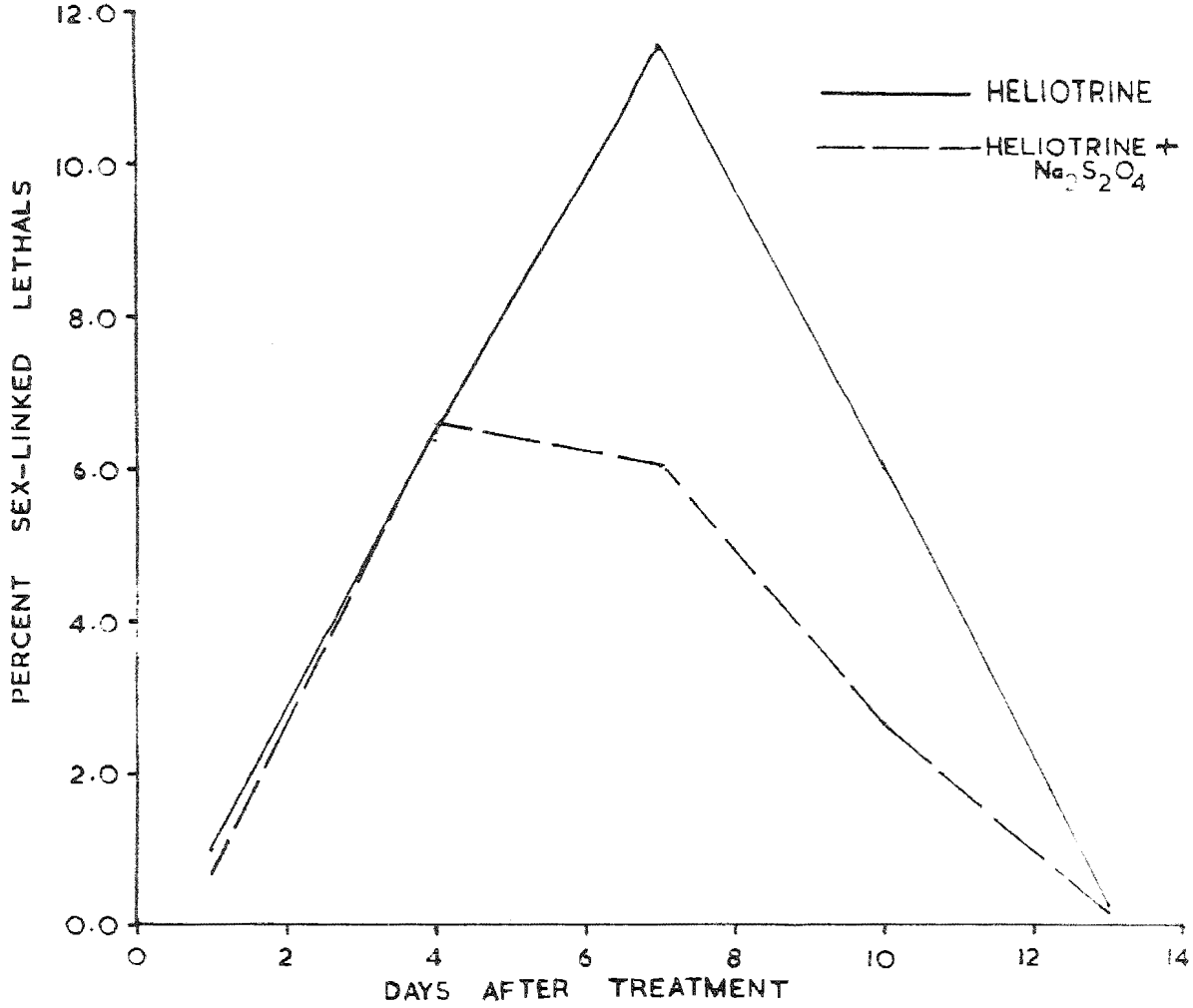
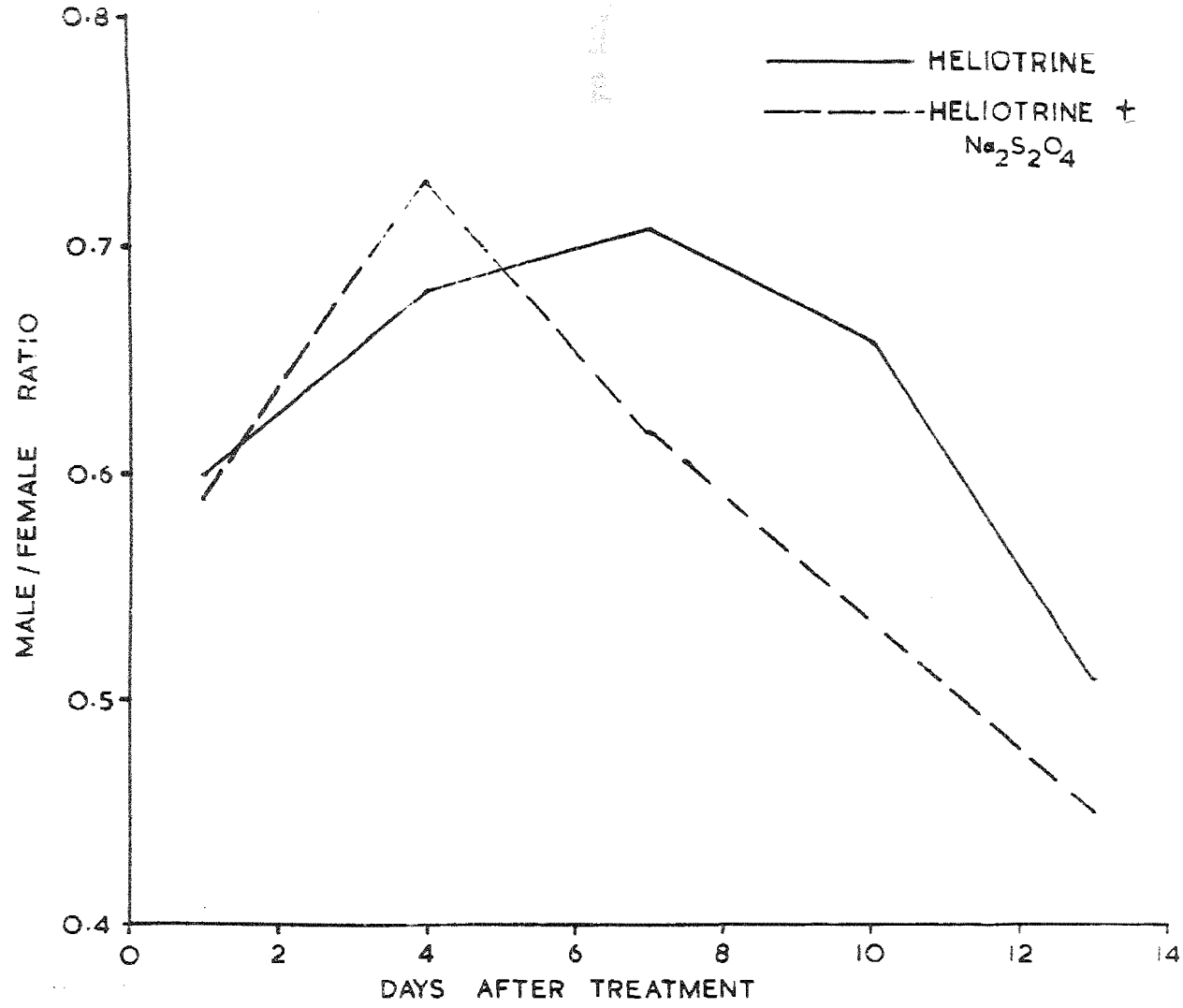

0·28
0·24
0·20
0·16
0·12
0·08
0·04
0·0
FREQUENCY
HELIOTRINE
HELIOTRINE + KCN
1
3
5
7
9
11
13
CHROMOSOME REGION

Figure 11. The effect of sodium hydrosulphite on the sex-linked lethal frequency produced by heliotrine in Canton-S males.
Heliotrine = 0.001 M, Sodium hydrosulphite = 0.01 M.
Brood interval three days.



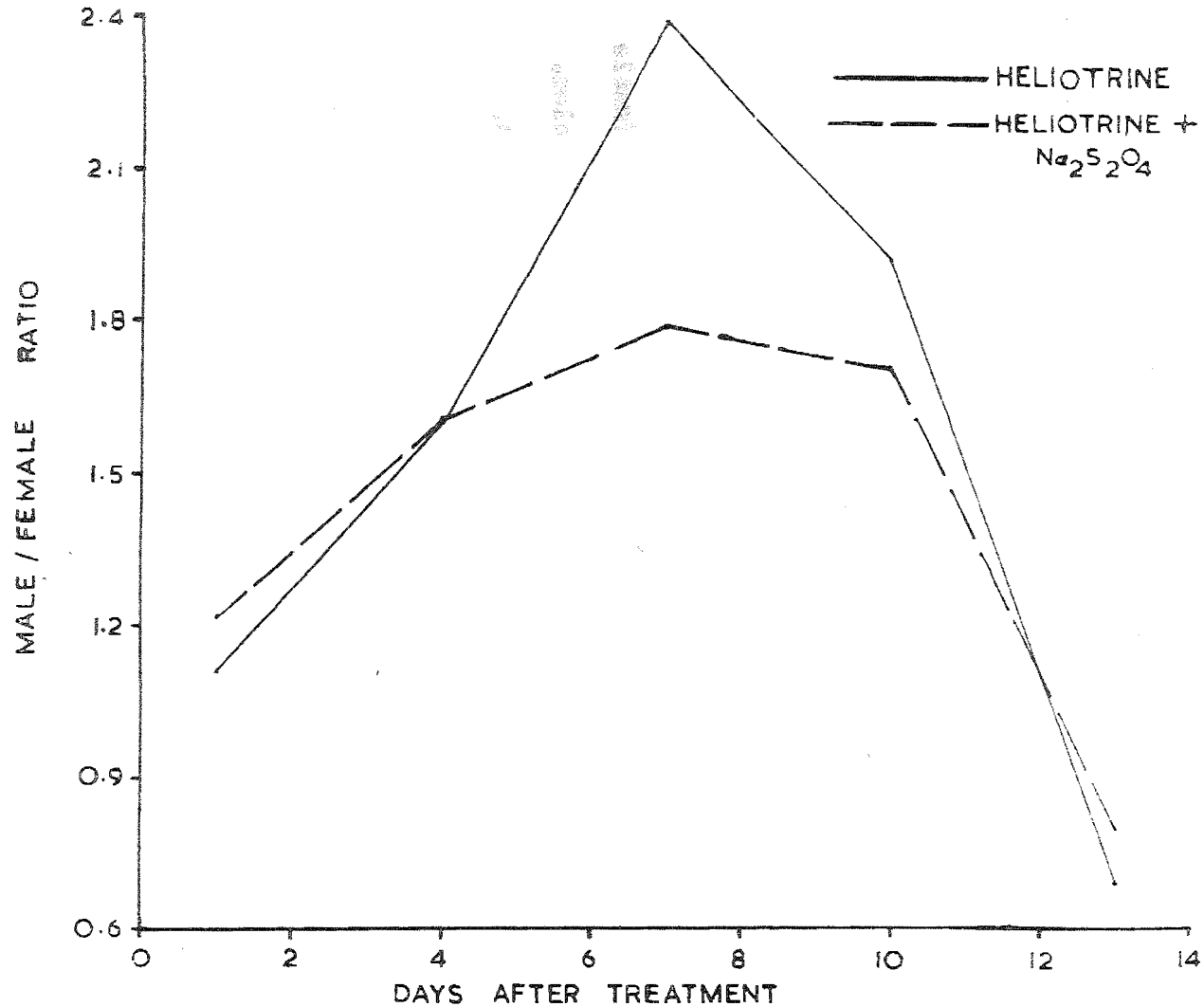
HELIOTRINE	NO. LETHALS	5	33	56	28	1
	NO. GAMETES	516	505	482	463	460
HELIOTRINE + $Na_2S_2O_4$	NO. LETHALS	5	38	41	18	1
	NO. GAMETES	713	595	677	674	590

Figure 12. The effect of sodium hydrosulphite on the F1 male/female ratio distortion produced by heliotrine in Canton-S males.
Heliotrine = 0.001 M, Sodium hydrosulphite = 0.01 M.
Brood interval three days.



HELIOTRINE	M/F	364/608	384/567	420/590	381/573	298/580
HELIOTRINE +$Na_2S_2O_4$	M/F	554/942	525/722	534/859	389/960	377/836

Figure 13. The effect of sodium hydrosulphite on the F1 male/female ratio distortion produced by heliotrine in $\underline{X^{C2}vf}$ males. Heliotrine = 0.001 M, Sodium hydrosulphite = 0.01 M. Brood interval three days.



HELIOTRINE	M/F	781/704	906/568	1006/420	648/334	405/590
HELIOTRINE + $Na_2S_2O_4$	M/F	826/678	747/464	670/373	566/329	536/669