The University of British Columbia

FACULTY OF GRADUATE STUDIES

PROGRAMME OF THE

FINAL ORAL EXAMINATION

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

of

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B.Sc., Presidency College, Calcutta, 1953
M.Sc., Calcutta University, Calcutta, 1955

THURSDAY, JUNE 17, 1965, at 10:30 A.M.
in Room 3332, Biological Sciences Building

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MODIFICATION OF RADIATION-INDUCED MUTATION FREQUENCIES BY ANTIBIOTICS IN DROSOPHILA MELANOGASTER

ABSTRACT

The experiments reported in the present dissertation were undertaken to obtain further evidence for the possible roles of protein, RNA and DNA macromolecules in radiation-mutagenesis in Drosophila melanogaster.

Several antibiotics were tested for their modifying effects on the frequency of radiation-induced sex-linked recessive lethals.

Pre-radiation treatment with actinomycin D significantly reduces the frequency of induced mutations in germ-cell stages assumed to include spermatids and spermatocytes. These results are consistent with the hypothesis of a role of proteins in the stabilization (repair) of radiation-induced premutational lesions.

Puromycin, a specific inhibitor of protein synthesis is ineffective in the modification of induced mutation frequencies in Drosophila melanogaster.

Mitomycin C is itself a potent mutagen in all germ-cell stages, peak mutagenicity occurring in spermatid stages. In combination with \( \gamma \)-rays, mitomycin C shows an overall additivity of effect. Mutation frequencies due to mitomycin C are not altered by pre- or concurrent treatment with actinomycin D. This may indicate different mechanisms for mutagenesis by mitomycin C and radiation.
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MODIFICATION OF RADIATION-INDUCED MUTATION FREQUENCIES
BY ANTIBIOTICS IN DROSOPHILA MELANOGASTER

by

RAMENDRA NATH MUKHERJEE
B.Sc.(Hons.) Presidency College, Calcutta, 1953
M.Sc. Calcutta University, 1955

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the Department of
ZOOLOGY

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA
June, 1965
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Department of Zoology

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Date The 17th June 1965
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ACKNOWLEDGEMENTS

I wish to express my sincere thanks and gratitude to DR. DAVID T. SUZUKI for his constant encouragement, enthusiastic guidance and critical supervision during the entire course of this research.

My grateful acknowledgements are given to DR. HAROLD BATHO and MR. KEN YUEN, both of the British Columbia Cancer Institute, for their generous assistance in radiating the flies.

My sincere thanks are due to DR. P. T. IVES for reading the manuscript and for his valuable suggestions and criticisms.

Thanks are due to DRS. C. V. FINNEGAN, G. M. TENER, J. R. MILLER and K. COLE for reading the manuscript and for their helpful suggestions.

I express my thanks to all my laboratory colleagues who have helped in the successful completion of this work.

I wish to thank MR. BASANTA SARKAR for his help in drawing the figures, MRS. MARGARET MORRIS for her meticulous typing of the manuscript and my wife UMA MUKHERJEE for her co-operation throughout the tenure of my research.

My study and stay in Canada was provided for by the award of a Commonwealth Scholarship by the Canadian Universities Foundation, Ottawa. Their assistance is gratefully acknowledged.
INTRODUCTION

Studies on the mechanism of mutation induction by radiation have been conducted extensively since MULLER (1927) reported the mutagenic effect of X-rays. However, the complexity of the process has prevented the formulation of a satisfactory model of general applicability.

Recent work indicates that one class of mutations induced by ionizing and non-ionizing radiation may result from a series of discrete steps beginning with the induction of an unstable premutational lesion in the genetic material and culminating in the fixation of the lesion as a stable self-replicating mutational event. Evidence for this path of mutation induction and the probable involvement of cellular macromolecular syntheses in these steps comes from the modification of the yield of mutations by post-radiation treatment with metabolic inhibitors (WITKIN 1956; DOUDNEY and HAAS 1959; KIMBALL, GAITHER and WILSON 1959; SOBELS and TATES 1961; SOBELS 1963a, b).

WITKIN (1956) studied the frequency of ultraviolet-induced reversions of tryptophan auxotrophs of Escherichia coli. She demonstrated that an inhibition of protein synthesis during the first hour after irradiation altered the yield of mutations by affecting the "fixation" of induced premutational lesions. KIMBALL, GAITHER and WILSON (1959) and KIMBALL (1961) showed that treatment of Paramecium aurelia with inhibitors of protein synthesis reduces the recovery of recessive deleterious mutations induced by ultraviolet light and X-rays. SOBELS and TATES (1961), SOBELS (1963a, b)
and CLARK (1963) obtained similar results in *Drosophila melanogaster*. WOLFF and LUIPPOLD (1955) and WOLFF (1959, 1960) have obtained evidence that oxidative phosphorylation and protein synthesis are involved in the repair of radiation-induced chromosome breakage in *Vicia faba*. The addition of ATP promotes repair, whereas chloramphenicol, a specific inhibitor of protein synthesis, prevents the rejoining of induced breaks. WOLFF (1960) has concluded that specific enzymes may be involved in the process of repair.

Recent findings in *E. coli* by SETLOW and CARRIER (1964) and BOYCE and HOWARD-FLANDERS (1964) indicate the existence of enzymes for the repair of U.V.-induced lesions (thymine-dimers) in DNA. Thus, the concept of the stabilization of radiation damage by an enzymatic process has been indicated in a number of organisms and provides a working hypothesis on which to base further critical experiments.

Studies of the role of macromolecules in radiation-mutagenesis have been facilitated by a better understanding of the molecular basis of gene structure and function and by the availability of a group of highly selective inhibitors of cellular metabolism. With the elucidation of their biochemical action, such inhibitors can be used for the investigation of the molecular mechanism of radiation-induced mutations. By testing the effects of metabolic inhibitors on the frequency of radiation-induced mutations in *Drosophila melanogaster*, the following question has been asked: Do protein synthesis, RNA synthesis and/or DNA replication play roles in the induction of mutations by radiation?
1. **Standard Mating Procedure**

Wild type males from an Oregon-R stock of *Drosophila melanogaster* were used in all of the experiments reported here. This stock had been maintained by brother-sister pair-matings for 369 generations to provide a completely isogenic genetic background.

The standard *Basc* chromosome (MULLER 1942) was used for detection of sex-linked recessive lethal mutations in all experiments reported here. The *Basc* chromosome (*sc^l B In(1)S w^a sc^8*) carries the dominant visible marker, Bar, a recessive visible, apricot and contains two inversions, In(1) scute^8^ and In(1)S (for a complete description see SPENCER and STERN 1948).

The following crosses were made:

\[
\begin{array}{cccc}
\text{P}_1 & \text{Oregon-R}^\text{369} & \text{Basc/Basc} & \text{X} \\
& X*Y & \text{♀} & (\text{Bar-apricot eyed}) \\
\text{F}_1 & \text{Basc/X*} & \text{♀} & \text{Basc/Y} & \text{♂} \\
& (\text{Red Bar eyed}) & \text{X} & (\text{Bar-apricot eyed}) \\
\text{F}_2 & \text{Basc/Basc} & \♀ & (\text{Basc}) \\
& \text{Basc/X*} & \♀ & (\text{Red Bar eyed}) \\
& \text{Basc/Y} & \♂ & (\text{Basc}) \\
& X*/Y & \♂ & (\text{non-Basc}) \\
& & & \text{absent if a lethal mutation is present} \\
\end{array}
\]

\[(X* \text{ represents the treated X chromosome)}\]
The absence of non-Basc males in an F₂ culture indicates the presence of a recessive lethal mutation on the X-chromosome carried by a sperm of the original treated male.

It has been established by various workers (MULLER 1930; LÜNING 1952; AUERBACH 1954; CHANDLEY and BATEMAN 1960; IVES 1963) using the criterion of mutation induction that different stages of germ-cell development of Drosophila melanogaster have different patterns of radiosensitivity.

The testis of an adult male at eclosion comprises an array of germ-cell stages which continually progress towards the formation of mature sperm. In order to detect differential stage sensitivity in a heterogeneous population of germ cells, two factors require control: (1) the age of the male at the time of treatment and (2) the sampling of these germ cells.

In order to control the age of the males to be tested, all adult flies were removed from the culture bottles on the day prior to treatment and only the males which had emerged within the following 18 hour period and in which the external chitin had hardened (indicated by a darkened body color) were used. These males were injected (as outlined in a later section) and 6 hours after injection were radiated. Thus they were about 24 hours old at the time of irradiation. Virgin females from the Basc stock were aged at least 3 days before they were used in matings.

A standard mating procedure was followed throughout the experiments. Each treated male was numbered and individually mated in a shell vial with a harem of six virgin Basc females. Clusters of lethals due to a single gonial event were detected by brood analyses of individual
males. The males were transferred without etherization to fresh vials with another six virgin females at 24 or 48 hour intervals for eight or more successive days after treatments (the exact times will be specified for each series). Offspring of each mating interval comprise a brood and represent a crude sample of sperm derived from cells of a particular stage at the time of treatment. After each mating period, each group of six mated females was transferred to vials of fresh medium at 48 hour intervals twice. This practice, suggested by IVES (1963), prevents overcrowding of progeny in any vial and assures maximal recovery of offspring derived from treated males. The time of male transfer was critically followed within 24 ± 1 or 48 ± 1 hour intervals throughout the experiments. The importance of maintaining uniform timing of transfers in order to permit valid correlations of the brood with the stage of germ cell development has recently been stressed by LEFEVRE and JONSSON (1964a, b).

The F₁ females which had emerged within 18 days of the introduction of parents into a vial were mated individually with their brothers and the resulting F₂ cultures scored for the presence of a sex-linked recessive lethal mutation on the treated X-chromosome.

All crosses were made on a standard-agar-cornmeal-dried yeast medium and carried out at room temperature which fluctuated around 23° ± 3° C. Each vial of F₂ cultures was examined under a binocular dissecting scope without etherization and a lethal event scored only in the complete absence of non-Basc males.
2. Injection of Chemicals into Males

In the experiments reported here, male flies were injected in the gonadal area in the manner outlined by SUZUKI (1963). Individual etherized males were placed in slots in a paraffin block. An Agla-microsyringe was attached to a glass microneedle by plastic catheter tubing (Figure 1). The tip of the needle (~2.0 μ in diameter) was inserted dorsally between the 4th and 5th tergite in the gonadal area and the test solutions injected until the abdomen was noticeably distended. Since CARLSON and OSTER (1962) have shown that the amount of liquid expelled after injection varies from fly to fly, no attempts were made to quantitate the amount injected into each fly.

3. Irradiation

In all experiments γ-rays delivered from a therapeutic 6000 curie Theratron Cobalt 60 "bomb" at the British Columbia Cancer Institute were used. Total doses of 600 rads were delivered at a rate of 52 rads/min at room temperature. Perforated size 00 gelatin capsules were loaded with etherized flies. In each experiment etherized flies were allowed to completely recover from anaesthesia before irradiation. IVES (personal communication) has suggested that mutation frequencies from radiated anesthetised flies may be affected by anoxic effects. The capsules containing the flies were placed between wax-slabs (½" thick)
Figure 1. A general view of the set up for injection of chemicals into the flies.
during exposure for accurate dosimetry. Immediately after radiation, the flies were transferred to culture vials and crossed with proper females.

4. Compounds Tested

(a) **Physiological saline**

Sodium chloride (NaCl) was dissolved in distilled water to make a 0.7N solution which is isotonic with the body fluid of the flies. In order to prevent cell damage by osmotic shock, all chemicals tested were first dissolved in physiological saline.

(b) **Actinomycin D**

Actinomycin D is an antibiotic first isolated by WAKSMAN and WOODRUFF (1940) from the actinomycetous fungus, *Streptomyces antibioticus*. Structurally, the antibiotic includes a chromophore group of compound ring systems with two attached identical polypeptide chains (Figure 2).

The antibiotic forms complexes with DNA and selectively inhibits the synthesis of DNA-mediated RNA (dm-RNA) by RNA polymerase (REICH et al. 1961a). At higher concentrations the antibiotic is also known to inhibit DNA replication (REICH 1964).

Solutions were prepared by dissolving the crystals in a few drops of 95% ethanol followed by further dilution with 0.7N saline to a final concentration of 1.0 µg/ml. Actinomycin D or 0.7N saline was injected into the males and half of each injected group was treated with 600 rads
Figure 2. Structure of actinomycin D; Sar, Sarcosine; L-N-meval, L-N-methylvaline; L-thr, L-threonine; D-val, D-valine; L-Pro, L-proline.
(ACTINOMYCIN D)
of γ-rays. Thus there were four sets of test conditions: actinomycin D + radiation, saline + radiation, actinomycin D and saline. In all three experiments (Series IA, IB and IC) males were transferred daily for eight successive single day broods.

(c) Puromycin dihydrochloride

Puromycin is an antibiotic first isolated from *Streptomyces alboniger* by PORTER et al. (1952). Structurally, it consists of an aminonucleoside linked to the amino acid p-methoxyphenylalanine (Figure 3). The antibiotic apparently blocks protein synthesis by acting at the level of the S-RNA-ribosomal complex (YARMOLINSKY and de la HABA 1959). It apparently substitutes for aminoacyl-S-RNA and becomes attached by its amino group to the incomplete polypeptide chain thus acting as a chain-interrupter (ALLEN and ZAMECNIK 1962; NATHANS 1964).

Solutions were prepared in the same manner as actinomycin D to a final concentration of 100 µg/ml. Puromycin or 0.7N NaCl was injected into the males and half of each injected group was treated with 600 rads of γ-rays, again resulting in four test conditions: puromycin + γ-rays, saline + γ-rays, puromycin and saline. Broods were transferred daily for eight successive days (Series 2A).
Figure 3. Structure of puromycin.
(PUROMYCIN)
(d) **Mitomycin C**

Mitomycin C is an antibiotic isolated from *Streptomyces cespitosus*. The chemical structure reveals a triazine ring, a methyl urethane side chain at C10, a tertiary methoxy group at C9 and 7-aminoquinone configuration representing active sites in the molecule (Figure 4). The antibiotic specifically inhibits DNA synthesis without concomitant effects on RNA and protein synthesis (SHIBA et al. 1959). SYBALSKI and IYER (1964) have reported that the antibiotic acts as a bifunctional alkylating agent forming cross-links with the complementary strands of DNA.

A solution was prepared at a concentration of 100 μg/ml and the four test conditions obtained: mitomycin C + γ-rays, saline + γ-rays, mitomycin C and saline. Two experiments were performed successively (Series 3A and 3B), both with daily brood transfer for eight successive days.

Since mitomycin C is itself mutagenic and an inducer of chromosome breaks (SHAW and COHEN 1965), a test for the modification of mitomycin C effects by actinomycin D was conducted. In this experiment, a mixture of mitomycin C and actinomycin D at concentrations of 100 and 1 μg/ml respectively, was injected into males. Two conditions were tested: mitomycin C + actinomycin D and mitomycin C alone. Males were transferred daily for 8 successive days (Series 4A). The combined actinomycin D and mitomycin C treatment was repeated with a modification in the schedule for injection of the antibiotics. 1 μg/ml actinomycin D or 0.7N NaCl was injected into two groups of males and 12 hours later both groups were injected with mitomycin C (100 μg/ml). Males were
Figure 4. Structure of mitomycin C.
(MITOMYCIN C)
transferred at 48 hour intervals for 10 days (Series 4B).

(e) 5-Fluorodeoxyuridine (FUDR)

FUDR is a halogenated derivative of deoxyuridine nucleoside synthesized by HEIDELBERGER et al. (1957) (Figure 5). The compound undergoes phosphorylation to form fluorodeoxyuridylate, an inhibitor of the enzyme thymidylate synthetase which catalyses the conversion of deoxyuridylate to thymidylate. The consequent deficiency of thymidylate results in an inhibition of DNA replication (COHEN et al. 1958). However, to some extent FUDR is also known to undergo conversion into fluorouridylate which can be incorporated into RNA (GORDON and STAHELIN 1958). To eliminate this possible side effect, an excess of uridine was supplied together with FUDR.

A mixture of FUDR (3 x 10^{-3} M) and uridine (2 x 10^{-3} M) or 0.7N NaCl was injected into the males. Half of the FUDR + uridine injected flies and all of the saline injected flies were irradiated with 600 rads of $\gamma$-rays. Three test conditions were obtained: FUDR + uridine + $\gamma$-rays, saline + $\gamma$-rays and FUDR + uridine. Males were transferred at 24 hour intervals for eight successive days (Series 5A).
Figure 5. Structure of 5-Fluorodeoxyuridine (FUDR).
(5-FUDR)
RESULTS

Test of preradiation treatments with actinomycin D were carried out in 3 experiments and the results of each test is shown in Tables 1A, 1B and 1C. A Chi-square analysis indicated that the data from the three experiments were homogeneous and they were lumped in Table 1D. It can be seen that injection of actinomycin D or saline has no mutagenic effect (columns 3 and 4, Table 1D). In the other two test conditions, mutation frequencies were increased in all broods (columns 1 and 2, Table 1D). The mutation percentage in each day brood of the two radiated series (A and S) in the pooled results and their 95% confidence limits are plotted in Figure 6. A maximum mutation yield of 6.0% is obtained in the 5th brood of the S series. Offspring recovered in this period of peak radio-sensitivity are presumed to represent a sample of cells that were in spermatocyte stages at the time of treatment. AUERBACH (1954) has shown that meiotic stages are most sensitive to radiation and brood 5 in these experiments showed the greatest induced sterility. These results are in accord with those of IVES (1963). IVES (1963) suggests that the use of large harems in daily matings of the treated males permits a exhaustive recovery of sperm batches and allows the early detection of spermatocytes in brood 5. Progeny recovered in the 7th and 8th broods which presumably represent gonial cells at the time of treatment show a sharp drop in mutations.

Data from the A series show a pattern similar to
Figure 6. Mutation percentages in successive one-day broods after radiation of saline and actinomycin D treated males.
ACTINOMYCIN D + Y-RAYS

SALINE + Y-RAYS

DAYS AFTER TREATMENT

PERCENT SEX-LINKED LETHAL MUTATIONS
that in the SY series in the frequency of induced mutations in successive broods, a peak of 3.3% being reached in the fifth brood (Figure 6). However, the mutation frequency is reduced with actinomycin D treatment in all the broods tested. The frequencies of mutation induction in the third to sixth broods (spermatid and spermatocyte stages) are significantly lower in the AY series than in the SY control. The low frequency of mutation induction in the gonial broods (7 and 8) prevents any comparison of actinomycin D effects on the induction of mutation in these stages.

The frequency distributions of treated males yielding mutations in different numbers of broods in the SY and AY are shown in Figure 7. Males in both series show a normal distribution. In the AY series a peak is reached at two broods (i.e. males in which mutations were detected in 2 out of 8 broods sampled) whereas the peak occurs at 3 in the SY series and the distribution tails out in the higher brood numbers.

In the absence of assays for the chemical effects of actinomycin D, sterility and induced mortality were used as biological indicators of the effectiveness of the antibiotic. Table 2 lists the percentage of males in each treatment surviving in successive broods. All deaths occurred within four days of treatment with actinomycin D increasing the mortality of males in both the radiated and non-radiated series (columns 1 and 3, Table 2). γ-radiation per se does not appear to affect viability (compare columns 2 and 4, Table 2).
Figure 7. Frequency distributions of males yielding mutations in different numbers of broods after each treatment.
Numbers of Broods

Percent males yielding mutations

Actinomycin + γ-Rays  M = 92
Saline + γ-Rays    M = 93

Numbers of Broods
Table 3 shows the average number of progeny obtained per treated male in each brood of each treatment. Actinomycin D does decrease the number of progeny per male both in radiated and non-radiated series, but the decrease is not statistically significant. Radiation alone does decrease the progeny size (compare columns 2 and 4, Table 3).

Using the biological criteria of changes in mortality and progeny size, actinomycin D does appear to be effective in *Drosophila*. SUZUKI (1965a) has come to a similar conclusion using these effects plus the genetic criterion of changes in crossover values.

The primary effect of actinomycin D on the inhibition of DNA-mediated RNA synthesis (REICH et al. 1961a) is expected to result in an inhibition in protein synthesis as a secondary effect. This gives rise to a possibility that the modification of mutation yields by actinomycin D may either be due to its direct effect on RNA synthesis or the secondary result of protein depletion. Recently, the antibiotic puromycin has been shown to block protein synthesis specifically, without concomitant effects on RNA or DNA synthesis (YARMOLINSKY and de la HABA 1959). In order to test this possibility, puromycin was used for pre-radiation treatment to study its effects on induced mutation frequency.

### Pre-radiation Treatment with Puromycin

Mutation frequencies under the test conditions puromycin + γ-rays (Pγ), saline + γ-rays (Sγ), puromycin (P)
and saline (S) are listed in Table 4. Treatment with puromycin alone, as with saline alone, had no effect on mutation induction (column 3 and 4, Table 4). The pattern of broodwise mutation frequency in the SY series is similar to previous patterns (Table 1D), a peak being reached in brood 5.

In the PY series, the pattern of mutation frequency in different broods is qualitatively and quantitatively similar to the SY series. The mutation percentages in every brood of the PY series are similar to the corresponding value in the SY control. Thus, unlike actinomycin D, puromycin fails to suppress the frequency of radiation-induced mutations in any of the broods tested.

Puromycin is known to inhibit protein synthesis in a wide range of biological systems such as bacteria (CREASSER 1955), mammals (MUELLER et al. 1961) and plants (CLICK and HACKETT 1963). No direct chemical assays for the effects of the antibiotic in Drosophila have yet been attempted. However, physiological effects of the antibiotic on sterility and induced mortality were used as biological indicators of the effectiveness of the antibiotic. Although the numbers of males in each treatment were small (PY = 30, SY = 28, P = 20 and S = 20), there was no indication of a significant effect of puromycin on mortality. Table 5 lists the number of progeny per male per brood after each treatment as a measure of fertility of the treated males. Again there is no indication of an effect of puromycin.

The lack of a marked physiological effect of puromycin
on *Drosophila*, together with the absence of any perceptible modifying effect on the frequency of radiation-induced mutations (Table 4) seem to indicate that the antibiotic is not effective in *Drosophila*.

However, high concentrations of actinomycin D may also inhibit DNA replication (Reich 1964). Thus there is the possibility that inhibition of DNA replication by actinomycin D is responsible for some of the reduction of radiation-induced mutations. In order to test this possibility, the effect of mitomycin C, a specific inhibitor of DNA synthesis (Shiba et al. 1959) was studied.

**Pre-radiation Treatment with Mitomycin C**

As in previous tests, four test series were carried out simultaneously: mitomycin C + γ-rays (Mγ), saline + γ-rays (Sγ), mitomycin C (MC) and saline (S), in two successive experiments the results of which are listed in Tables 6A and 6B. A Chi-square analysis indicated that the two experiments were homogeneous and the results were lumped in Table 6C. Saline injection alone (Table 6C) did not result in changes from the level of spontaneously occurring mutations as noted before. In the other test conditions, mutation frequencies were significantly increased.

The percentages of mutations induced in each day brood in the pooled data are shown in Figure 8. The pattern in the Sγ series is consistent with previous control results, peak radiosensitivity occurring in brood 5. Mitomycin C is
Figure 8. Mutation frequencies in daily broods following mitomycin C and γ-radiation treatments.
mutagenic in all cell stages of the testis including mature sperm which are recovered in the first and second broods. However, it can be seen that the pattern of mutagenesis by mitomycin C is different from that in the SY series, its peak being reached in the third brood instead of the fifth. The sum of the frequencies of lethals induced in the MC and SY series is greater than the frequencies from MY series in all but brood 8 (Table 7). However, only in brood 3 and 6 are these values significantly higher and the sum in brood 8 is significantly lower. Thus, additivity of effect by combined mitomycin C and radiation treatment is generally indicated.

The frequency distributions of treated males having mutations in different number of broods are shown in Figure 9. All the three test conditions MY, SY and MC have a unimodal distribution curve. However, the position and magnitude of the peak differs in different test conditions. The SY series has a peak at 3 with less than 20% of the males having mutations in more than 3 broods. The MY series shows a peak at 4 with over 30% of the males having mutations in more than 4 broods. In the MC series, the peak occurs at 5 with over 50% of the treated males having mutations in 5 or more broods. These results clearly indicate the potent mutagenic action of mitomycin C and that it is mutagenic in germ cells at all stages of development.

Table 8 shows the percent survival of males in each brood after different treatments. All of the mortality due to the treatments occur within the first three broods. The survival frequency of 75.6% in the S control series is signi-
Figure 9. Frequency distributions of males yielding mutations in different numbers of broods after each treatment.
PERCENT MALES YIELDING MUTATIONS

NUMBERS OF BROODS

- 33 -

MITOMYCIN C

M = 41

SALINE + RAY - λ

M = 46

MITOMYCIN C + RAY - λ

M = 51

PERCENT MALES YIELDING MUTATIONS

0 10 20 30 40 50 60
significantly higher than in the other three series, which in turn are not significantly different from each other. MC would appear to increase mortality (compare columns 3 and 4, Table 8) or have no effect (columns 1 and 2, Table 8). SUZUKI (1965b) has reported that MC injection has no effect on mortality of *Drosophila melanogaster* females.

Table 9 lists the average number of offspring per male per brood in each treatment. Here also the data indicate that in the S series the average number of offspring per male is significantly higher than in any other series which, in turn are not different from each other.

The genetic effects of mitomycin C reported here and by SUZUKI (1965b) indicate that it is physiologically effective in *Drosophila*, in spite of an absence of effect on fecundity and survival. Besides its primary effect of inhibition of DNA replication (SHIBA *et al.* 1959) mitomycin C has several other biological effects including mutagenicity (SZYBALSKI 1958; IIJIMA and HAGIWARA 1960; MUKHERJEE 1965b); fragmentation of chromosomes and other cell structures (SHATKIN *et al.* 1962; NOWELL 1964; SHAW and COHEN 1965) and stimulation of crossing over (IIJIMA and HAGIWARA 1960; HOLLIDAY 1964; SUZUKI 1965b). The reported biochemical effects of mitomycin C indicate that these effects are the secondary result of its initial inhibition of replication and enhanced depolymerization of DNA (SHIBA *et al.* 1959; KERSTEN and RAUEN 1961; REICH *et al.* 1961b). These findings pose an obvious question, can mitomycin C-induced mutations also be modified by actinomycin D? The preliminary report
by KIMBALL (1964) that lesions induced by triethyl melamine (TEM) and certain other alkylating agents could be altered by actinomycin D, makes this possibility likely. With this question in mind, male flies were injected with a mixture of actinomycin D and mitomycin C (MA) and were compared with an MC control. Table 10 lists the frequencies of mutations from these two test conditions. In the MC series a peak mutation frequency of 3.6% was obtained in the fourth brood, whereas the frequency of 1.60% in the corresponding brood of the MA series is significantly lower. However, in all other broods there was no indication of a suppression in mitomycin C-induced mutations by actinomycin D.

In another experiment, the flies were given an injection of actinomycin D 12 hours prior to the injection of mitomycin C. The results are shown in Table 11 using two-day brood analysis. In the MC series, a peak frequency of 3.8% was reached on the fourth day after treatment. In the corresponding brood, the MA series shows frequency of 2.8 which is not significantly lower. Unfortunately, the small sizes of the samples in these experiments do not allow a definite conclusion.

Although mitomycin C is an inhibitor of DNA replication (SHIBA et al. 1959) its mutagenicity complicates any interpretation of its effects in combination with radiation. This problem requires a study of the effect of DNA inhibition on the frequency of radiation induced mutations by a non-mutagenic agent. FUDR (5-fluorouracil deoxyriboside) was used for such a purpose.
Pre-radiation Treatment with FUDR

Table 12 lists the frequencies of radiation-induced mutations in three test conditions: FUDR + γ-rays (FY), SY and FUDR alone (F).

FUDR treatment alone is not mutagenic (column 3, Table 12) and the results of the SY series are as noted previously. In the FY series, the pattern of mutation induction is very similar to SY in all broods. Thus FUDR does not seem to modify radiation-induced mutation frequency.

By the criteria of increased mortality or reduced fecundity, FUDR does not exert an effect. These results cannot provide information on the role of DNA replication in the absence of evidence for the effects of FUDR on DNA replication.
The results indicate that the frequency of radiation-induced mutations in *Drosophila* can be reduced by pre-treatment with actinomycin D. This effect is specific for cells recovered in broods 3 to 6 which are presumed to have been spermatids and spermatocytes at the time of treatment. The primary effect of actinomycin D is the inhibition of all DNA-mediated RNA synthesis (REICH et al. 1961a). If it is assumed that this suppressible RNA bears the information necessary for the synthesis of cellular proteins, then treatment with actinomycin D would be expected to result secondarily in a reduction in protein synthesis. Indeed, in *Chironomus*, LAUFER et al. (1964) have found a marked depression in protein synthesis following treatment with actinomycin D.

The reduction in the frequency of radiation-induced mutations in *Drosophila melanogaster* by pre-radiation treatment with chloramphenicol (SOBELS and TATES 1961; SOBELS 1963a) and streptomycin (CLARK 1963) has been interpreted in terms of an inhibition of protein synthesis by the antibiotics. The similarity of the results reported here with those of SOBELS (1963a) and CLARK (1963) led MUKHERJEE (1965a) to suggest that the inhibition of protein synthesis could also be responsible for the modifying effects of actinomycin D. Numerous studies indicate that an enzymatic process ("repair") is involved in the stabilization of some types of damage induced in the genetic material by radiation (WITKIN 1956;
DOUDNEY and HAAS 1959; WOLFF 1959; SETLOW and CARRIER 1964; BOYCE and HOWARD-FLANDERS 1964). These reports show that those lesions fail to stabilize or repair in the absence of an enzyme moiety.

Mutagenesis by radiation may occur in a number of ways. It is generally agreed that the primary step in radiation mutagenesis is the establishment of a molecular alteration (premutational lesion) in the genetic material (which may correspond to the chromosome breaks observed by WOLFF 1959). Radiation-induced damage in the genetic material may immediately result in a self-replicating mutational event. Such events reflect instantaneous changes in the existing gene molecules (ALTENBERG and BROWNING 1961; MULLER, CARLSON and SCHALET 1961) and protein synthesis is not involved in their induction. On the other hand, another type of lesion may not be stabilized immediately and its conversion to a self-replicating mutation involves protein dependent metabolic steps. This latter class has been explained by the "Repair Hypothesis" of mutation induction (WOLFF 1960; SOBELS 1963a, b). This model supposes that an induced premutational lesion may encounter a number of possible fates depending on the metabolic state of the cell concerned: (1) the damage may not be fixed and may be detected genetically as a dominant lethal or cytologically as an unhealed chromosome break; (2) the lesion may be "repaired" to its original state before irradiation; or (3) the lesion may undergo "repair" with a genetic change which is detectable as a recessive lethal event incorporated
at the site of repair. If the second and the third processes require specific proteins, any suppression in the synthesis of proteins would be expected to decrease the recovery of recessive lethal mutations. The experiments reported here are consistent with this model, if one assumes that protein synthesis is inhibited by the absence of messenger-RNA after actinomycin D treatment.

The specific effects of actinomycin D on broods 3 to 6 are consistent with the known high metabolic activity of these cells. On the other hand, the absence of modification effects in broods 1 and 2 is expected on the basis of greatly reduced metabolic activities in mature sperm (KAUFMANN and GAY 1963). The apparent lack of effect of actinomycin D on the gonial cells recovered in the broods 7 and 8 may be due to the selective elimination of mutation-bearing cells during subsequent divisions, an absence of the repair mechanism in gonia or the induction of a different type of mutation in gonial cells. Although there are other possible explanations for actinomycin D effects, the repair hypothesis provides a satisfactory explanation for these experiments and permits further critical experimentation.

The effects of actinomycin D could also be due to its primary effect of inhibition of RNA synthesis rather than the secondary effect on proteins. The tests of puromycin, a specific inhibitor of protein synthesis, did not provide a solution to this problem. Although puromycin is known to inhibit protein synthesis in a wide range of biological
systems including bacterial and mammalian cells (MUELLER et al. 1961; CLICK and HACKETT 1963; BRACHET et al. 1964) the antibiotic seems to be ineffective in *Drosophila*. SUZUKI (personal communication) failed to find an effect of puromycin on viability, fertility and crossing over and BAKER (personal communication) has failed to detect any effect on viability and variegation. Thus, the failure to detect an effect of puromycin on the frequency of mutation induction is probably due to its ineffectiveness as a protein inhibitor in *Drosophila*.

At high concentrations, actinomycin D may also inhibit DNA replication (REICH 1964). This effect could be responsible for some of the modifications in the frequency of induced mutations in later broods by the antibiotic. Pre-radiation treatment with mitomycin C, an inhibitor of DNA replication (SHIBA et al. 1959) results in a frequency of mutations which is significantly lower in brood 6 than the sum of the frequencies in separate tests of mitomycin C and radiation (Figure 8, Table 7). The cells recovered in brood 6 presumably represent early spermatocyte stages in which DNA replication takes place. Thus, an inhibition of DNA replication may reduce the recovery of recessive lethals but this must be a small component of the actinomycin D effects. However, in view of the complicated biological effects of mitomycin C, including mutagenicity (IIJIMA and HAGIWARA 1960) and DNA depolymerization (SZYBALSKI and IYER 1964), any interpretation of the effects of the antibiotic in combination with radiation precludes definite conclusions.

The results presented in this report show that
mitomycin C is itself mutagenic in *Drosophila* in all cell stages of the testis. This is in contrast to its lack of mutagenicity in *Ustilago* and *Saccharomyces* (HOLLIDAY 1964), and is similar to the reported effects in *Escherichia coli* (IIJIMA and HAGIWARA 1960). LEWIS (personal communication) has also found the antibiotic to be mutagenic in *Drosophila*. The mechanism of mutation induction by mitomycin C is not clearly understood. IYER and SZYBALSKI (1963) and SZYBALSKI and IYER (1964) have reported that it acts as a bifunctional alkylating agent which forms cross-links with the complementary strands of DNA followed by a depolymerization of the DNA molecule. SHATKIN et al. (1962) found that mammalian cell cultures exposed to mitomycin C showed nuclear fragmentation, depolymerization of DNA and the appearance of DNA fragments in the cytoplasm. Recently NOWELL (1964) and SHAW and COHEN (1965) have found fragmentation of human leukocyte chromosomes by mitomycin C. This mechanism may also be responsible for the strong mutagenicity of the antibiotic in *Drosophila*. The period of peak mutagenicity in brood 3 indicates that the major mutagenic effect may be independent of DNA replication in chromosomes that are tightly compacted in the spermatids.

Since mitomycin C effects are radiomimetic (SHATKIN et al. 1962; SHAW and COHEN 1965), simultaneous treatment with mitomycin C and actinomycin D were carried out to determine whether the frequency of mitomycin C-induced mutations could also be modified by actinomycin D. The absence of a modifying effect by actinomycin D (Table 10) is not in accord with the effects of the antibiotic on
TEM-induced mutations in *Paramecium* (KIMBALL 1964) or on mitomycin C-induced DNA-depolymerization (CONSTANTOPOULOS and TCHEN 1964). These results may indicate that the lesions induced by mitomycin C are of a different nature from radiation-induced lesions and therefore are not subject to same repair mechanism.
SUMMARY

The modification of the frequencies of sex-linked recessive lethal mutations by some antibiotics was studied in *Drosophila melanogaster* to obtain evidence for the possible roles of protein, RNA and DNA synthesis in radiation-mutagenesis. The following results were obtained:

1. The antibiotic actinomycin D is not mutagenic by itself. However, pre-radiation treatment with actinomycin D significantly reduces the frequency of induced mutations in germ-cell stages assumed to include spermatids and spermatocytes. If it is assumed that protein synthesis is inhibited after actinomycin D treatment, these results are consistent with the model of a protein-dependent process of fixation of radiation-induced genetic damage.

2. Puromycin, a specific inhibitor of protein synthesis is ineffective in the modification of induced mutation frequencies in *Drosophila melanogaster*.

3. Mitomycin C is itself a potent mutagen in all germ cell stages of *Drosophila*, peak mutagenicity occurring in spermatid stages. In combination with γ-rays, mitomycin C shows an overall additivity of effect. Mutation frequencies due to mitomycin C are not altered by pre- or concurrent treatment with actinomycin D. This may indicate different mechanisms for mutagenesis by mitomycin C and radiation.
LITERATURE CITED


ALTENBERG, E. and L. BROWNING, 1961 The relatively high frequency of whole-body mutations compared with fractionals induced by X-rays in Drosophila sperm. Genetics 46: 203-212.


APPENDIX

Studies on the effects of actinomycin D on X-ray induced mutations in different germ cell stages of *Drosophila melanogaster* were also carried out at the University of Alberta, in Edmonton in 1963.

Males, about 24 hours old, from wild type Oregon-R stock were injected with a solution of actinomycin D dissolved in 0.7N saline at a concentration of 1.0 \( \mu \text{g/ml} \). Radiation was delivered from a constant-potential X-ray machine operated at 300 Kv and 10 mA and provided with a 1 mm Al filter. A total exposure of 600 roentgens* was delivered at a dose rate of approximately 109 r/min at room temperature.

The standard *C1B* test for sex-linked recessive lethal mutations (MULLER 1928) was used in all the experiments. The *C1B* chromosome carries an inversion (C) which prevents crossing over, a dominant visible marker Bar (B) and a recessive lethal (1). (See MULLER 1928 for a complete description of the chromosome.) The mating scheme using the *C1B* chromosome is similar to that with the *Base* stock (see page 3 in Materials and Methods section of the main text), except that *C1B/+* heterozygous females are used in the *F_1* cross and in the *F_1* only Bar eyed females are mated individually to their brothers. The absence of males in the *F_2* culture indicates the presence of a recessive lethal mutation on the X-chromosome carried by a sperm of the original treated male.

* roentgen = the physical unit of radiation equivalent to an energy absorption of 95 ergs/gm Water (at 4°C).
In the first experiment the treated males were individually mated with a harem of six virgin $C_{1B}/+$ females in every 24 hour interval for 10 successive days. Table 13 lists the results obtained in the four conditions tested: actinomycin D + X-rays (AX), saline + X-rays (SX), actinomycin D (A) and saline (S). It can be seen that injection of actinomycin D and saline alone has no apparent effect on the induction of mutations (column 3 and 4, Table 13). In the other two test conditions the frequencies of mutations are increased in most of the broods (column 1 and 2, Table 13). The mutation percentages and their 95% confidence limits in each day brood of the two radiated series are plotted in Figure 10. A maximum mutation yield of 1.7% is obtained in the 5th brood of the SX series, which presumably represents a sample of cells that were in the spermatocyte stages at the time of treatment. Progeny recovered in the 7th and subsequent broods show a sharp drop in mutations and presumably represent gonial cells at the time of treatment.

Combined treatment with actinomycin D and X-rays show a different brood pattern in the frequency of radiation-induced mutations (Figure 10). In this series, a much higher mutational peak is reached in the earlier broods. The mutation percentages of 3.6 and 4.2 reached in broods 3rd and 4th respectively, presumably represent spermatid stages at the time of treatment. These frequencies are significantly higher than the corresponding values in the SX series. On the other hand, in the AX series the mutation rate in the fifth brood is reduced significantly. Mature sperms sampled in the
Figure 10. Mutation percentages in successive one-day broods after radiation of saline and actinomycin D treated males.
first and second broods show similar frequencies of radiation-induced mutations with and without actinomycin D pretreatment.

The above experiment was repeated twice using the same technique but two-day brood samples for 12 successive days and the data are listed in Tables 14A and 14B. A Chi-square analysis indicated that the data from these two experiments were homogeneous and the data were therefore pooled in Table 14C. The mutation percentages in each brood of the pooled data are plotted with 95% confidence limits in Figure 11. Again it can be seen that saline or actinomycin D injection alone does not result in changes from the level of spontaneously occurring mutation rate.

In the SX series the induced mutation rate shows a progressive rise to a peak of 2.1% in the 4th brood (7-8 days) which presumably corresponds to spermatocyte stages. This late recovery of spermatocytes probably reflects a less exhaustive sampling of sperm. Results qualitatively similar to those in the first experiment were obtained in the AX series, peak mutation yields being obtained in broods 2 and 3. In the fourth brood, (7-8 days after treatment) presumably representing spermatocytes, the frequency of mutations in the AX series is lower than in the corresponding brood of the SX series, although the difference is not statistically significant (Figure 11). Thus the patterns of mutational frequency presented in Figures 10 and 11 are similar. It is possible that actinomycin D affects the cell cycle and thus shifts the speed of spermatogenesis so that
Figure 11. Mutation percentages in successive two-day broods after radiation of saline and actinomycin D treated males.
the cells having peak radiosensitivity normally recovered in brood 4, are recovered much earlier in the AX series. An attempt was made to test this possibility by standard translocation test. The frequency of radiation-induced translocations in the male germ-cell stages follows a definite pattern: it is highest in the spermatids and sharply decreases in the spermatocytes and spermatogonia. Thus it permits a precise determination of the time of transition from spermatids to spermatocytes.

The data in these experiments with actinomycin D and X-rays differ both qualitatively and quantitatively from those in the main text.

An overall lower mutational yield in these experiments for an equivalent X-ray dose (600 r) could partly be due to the tester stock (C1B) used. The inversion C in the C1B chromosome does not suppress crossing over completely and viable F2 males resulting from double crossovers within the inversion were occasionally recovered. This, probably leads to an under-estimate of the mutation frequency.

The earlier peak in the induced mutation frequency in AX series is not clearly understood. This apparent discrepancy in results obtained following an use of X-rays and Y-rays gives a note of caution in extrapolating the biological effects of one kind of radiation into another (IVES personal communication).
TABLE 1A (Series 1A)

Frequencies of radiation-induced recessive sex-linked lethal mutations in successive one-day broods after each treatment.

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>1 Actinomycin D + γ-rays</th>
<th>2 Saline + γ-rays</th>
<th>3 Actinomycin D</th>
<th>4 Saline</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Number tested</td>
<td>Mutations No. %</td>
<td>Number tested</td>
<td>Mutations No. %</td>
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<tr>
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<td>632</td>
<td>8 1.2</td>
<td>759</td>
<td>14 1.8</td>
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<tr>
<td>2</td>
<td>495</td>
<td>4 0.8</td>
<td>798</td>
<td>16 2.0</td>
</tr>
<tr>
<td>3</td>
<td>507</td>
<td>6 1.2</td>
<td>721</td>
<td>21 2.9</td>
</tr>
<tr>
<td>4</td>
<td>975</td>
<td>20 2.1</td>
<td>1005</td>
<td>46 4.6</td>
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<tr>
<td>5</td>
<td>481</td>
<td>15 3.2</td>
<td>504</td>
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<tr>
<td>6</td>
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<td>10 2.1</td>
<td>527</td>
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<tr>
<td>7</td>
<td>535</td>
<td>2 0.4</td>
<td>498</td>
<td>3 0.6</td>
</tr>
<tr>
<td>8</td>
<td>637</td>
<td>3 0.5</td>
<td>363</td>
<td>0 0</td>
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<td>68 1.4</td>
<td>5175</td>
<td>152 2.9</td>
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**TABLE IB (Series 1B)**

Frequencies of radiation-induced recessive sex-linked lethal mutations in successive one-day broods after each treatment.

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<th>4</th>
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<td></td>
<td>Actinomycin D + γ-rays</td>
<td>Saline + γ-rays</td>
<td>Actinomycin D</td>
<td>Saline</td>
</tr>
<tr>
<td></td>
<td>Number tested</td>
<td>Mutations No.</td>
<td>Mutations %</td>
<td>Number tested</td>
</tr>
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<td>-----------------</td>
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<td>1.6</td>
<td>737</td>
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<td>2</td>
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<td>8</td>
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<td>517</td>
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<td>501</td>
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<td>Totals</td>
<td>4456</td>
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### TABLE IC (Series IC)

Frequencies of radiation-induced recessive sex-linked lethal mutations in successive one-day broods after each treatment.

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<th>Actinomycin D + γ-rays</th>
<th>Saline + γ-rays</th>
<th>Acintomycin D</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number tested</td>
<td>Mutations No.</td>
<td>%</td>
<td>Number tested</td>
</tr>
<tr>
<td>1</td>
<td>368</td>
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<td>1.6</td>
<td>744</td>
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<tr>
<td>2</td>
<td>975</td>
<td>11</td>
<td>1.2</td>
<td>714</td>
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<td>3</td>
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<td>345</td>
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<tr>
<td><strong>Totals</strong></td>
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<td>112</td>
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<td>6219</td>
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<td>Days after treatment</td>
<td>1: Actinomycin D + γ-rays</td>
<td>2: Saline + γ-rays</td>
<td>3: Actinomycin D</td>
<td>4: Saline</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------------</td>
<td>-------------------</td>
<td>------------------</td>
<td>---------</td>
</tr>
<tr>
<td></td>
<td>Number tested</td>
<td>Mutations No.</td>
<td>Number tested</td>
<td>Mutations No.</td>
</tr>
<tr>
<td>1</td>
<td>1768</td>
<td>26</td>
<td>2240</td>
<td>38</td>
</tr>
<tr>
<td>2</td>
<td>2065</td>
<td>22</td>
<td>2288</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>2233</td>
<td>40</td>
<td>2322</td>
<td>65</td>
</tr>
<tr>
<td>4</td>
<td>2542</td>
<td>76</td>
<td>2877</td>
<td>148</td>
</tr>
<tr>
<td>5</td>
<td>1289</td>
<td>43</td>
<td>1777</td>
<td>107</td>
</tr>
<tr>
<td>6</td>
<td>1319</td>
<td>34</td>
<td>1590</td>
<td>63</td>
</tr>
<tr>
<td>7</td>
<td>1515</td>
<td>4</td>
<td>1561</td>
<td>11</td>
</tr>
<tr>
<td>8</td>
<td>1611</td>
<td>3</td>
<td>1523</td>
<td>0</td>
</tr>
<tr>
<td>Totals</td>
<td>14342</td>
<td>248</td>
<td>16178</td>
<td>468</td>
</tr>
</tbody>
</table>

TABLE 1D

Frequencies of radiation-induced recessive sex-linked lethal mutations in successive one-day broods after each treatment.
TABLE 2

Percentage of males surviving after each treatment in successive one-day broods.

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>Treatment and number of flies treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Actinomycin D + $\gamma$-rays (180 flies)</td>
</tr>
<tr>
<td>1</td>
<td>82.2</td>
</tr>
<tr>
<td>2</td>
<td>63.3</td>
</tr>
<tr>
<td>3</td>
<td>53.8</td>
</tr>
<tr>
<td>4</td>
<td>52.0</td>
</tr>
</tbody>
</table>
TABLE 3

Progeny per treated male per day brood after each treatment.

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>1 (Actinomycin D + γ-rays) (89 flies)</th>
<th>2 (Saline + γ-rays) (92 flies)</th>
<th>3 (Actinomycin D) (24 flies)</th>
<th>4 (Saline) (31 flies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>89.5</td>
<td>102.8</td>
<td>107.5</td>
<td>113.5</td>
</tr>
<tr>
<td>2</td>
<td>78.3</td>
<td>90.5</td>
<td>99.5</td>
<td>109.4</td>
</tr>
<tr>
<td>3</td>
<td>88.7</td>
<td>88.3</td>
<td>95.9</td>
<td>98.6</td>
</tr>
<tr>
<td>4</td>
<td>58.5</td>
<td>79.5</td>
<td>76.0</td>
<td>105.7</td>
</tr>
<tr>
<td>5</td>
<td>62.3</td>
<td>75.5</td>
<td>81.3</td>
<td>99.0</td>
</tr>
<tr>
<td>6</td>
<td>65.9</td>
<td>77.2</td>
<td>73.4</td>
<td>103.2</td>
</tr>
<tr>
<td>7</td>
<td>75.5</td>
<td>82.0</td>
<td>106.5</td>
<td>97.5</td>
</tr>
<tr>
<td>8</td>
<td>99.0</td>
<td>93.6</td>
<td>101.2</td>
<td>110.2</td>
</tr>
<tr>
<td>Totals</td>
<td>617.7</td>
<td>689.4</td>
<td>741.3</td>
<td>837.1</td>
</tr>
<tr>
<td>Mean #</td>
<td>(77.2)</td>
<td>(86.2)</td>
<td>(92.6)</td>
<td>(104.5)</td>
</tr>
</tbody>
</table>
TABLE 4 (Series 2A)

Frequencies of radiation-induced recessive sex-linked lethal mutations in successive one-day broods after each treatment.

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>Puromycin + $\gamma$-rays</th>
<th>Saline + $\gamma$-rays</th>
<th>Puromycin</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number tested</td>
<td>Mutations No.</td>
<td>%</td>
<td>Number tested</td>
</tr>
<tr>
<td>1</td>
<td>423</td>
<td>7</td>
<td>1.6</td>
<td>457</td>
</tr>
<tr>
<td>2</td>
<td>396</td>
<td>7</td>
<td>1.7</td>
<td>442</td>
</tr>
<tr>
<td>3</td>
<td>518</td>
<td>16</td>
<td>3.1</td>
<td>427</td>
</tr>
<tr>
<td>4</td>
<td>406</td>
<td>15</td>
<td>3.7</td>
<td>430</td>
</tr>
<tr>
<td>5</td>
<td>495</td>
<td>25</td>
<td>5.1</td>
<td>405</td>
</tr>
<tr>
<td>6</td>
<td>543</td>
<td>24</td>
<td>4.4</td>
<td>439</td>
</tr>
<tr>
<td>7</td>
<td>592</td>
<td>7</td>
<td>1.2</td>
<td>504</td>
</tr>
<tr>
<td>8</td>
<td>549</td>
<td>5</td>
<td>0.9</td>
<td>445</td>
</tr>
<tr>
<td>Totals</td>
<td>3922</td>
<td>106</td>
<td>2.7</td>
<td>3549</td>
</tr>
</tbody>
</table>
TABLE 5
Progeny per treated male per day after each treatment.

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>Puromycin + γ-rays (21 flies)</th>
<th>Saline + γ-rays (17 flies)</th>
<th>Puromycin (15 flies)</th>
<th>Saline (18 flies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>92.8</td>
<td>112.2</td>
<td>105.0</td>
<td>99.3</td>
</tr>
<tr>
<td>2</td>
<td>102.0</td>
<td>96.0</td>
<td>103.8</td>
<td>124.0</td>
</tr>
<tr>
<td>3</td>
<td>100.6</td>
<td>92.5</td>
<td>111.2</td>
<td>98.4</td>
</tr>
<tr>
<td>4</td>
<td>76.8</td>
<td>79.5</td>
<td>89.7</td>
<td>95.0</td>
</tr>
<tr>
<td>5</td>
<td>68.4</td>
<td>72.0</td>
<td>96.5</td>
<td>84.3</td>
</tr>
<tr>
<td>6</td>
<td>70.6</td>
<td>75.3</td>
<td>92.5</td>
<td>105.4</td>
</tr>
<tr>
<td>7</td>
<td>84.2</td>
<td>76.9</td>
<td>88.6</td>
<td>93.5</td>
</tr>
<tr>
<td>8</td>
<td>81.4</td>
<td>91.0</td>
<td>98.7</td>
<td>113.5</td>
</tr>
<tr>
<td>Totals</td>
<td>676.8</td>
<td>695.4</td>
<td>786.0</td>
<td>813.4</td>
</tr>
<tr>
<td>Mean #</td>
<td>(84.6)</td>
<td>(86.9)</td>
<td>(98.2)</td>
<td>(101.7)</td>
</tr>
<tr>
<td>Days after treatment</td>
<td>1 Mitomycin C + γ-rays</td>
<td>2 Saline + γ-rays</td>
<td>3 Mitomycin C</td>
<td>4 Saline</td>
</tr>
<tr>
<td>---------------------</td>
<td>------------------------</td>
<td>------------------</td>
<td>--------------</td>
<td>---------</td>
</tr>
<tr>
<td></td>
<td>Number tested</td>
<td>Mutations No. %</td>
<td>Number tested</td>
<td>Mutations No. %</td>
</tr>
<tr>
<td>1</td>
<td>519</td>
<td>13 2.5</td>
<td>522</td>
<td>8 1.5</td>
</tr>
<tr>
<td>2</td>
<td>1005</td>
<td>35 3.5</td>
<td>548</td>
<td>7 1.3</td>
</tr>
<tr>
<td>3</td>
<td>802</td>
<td>49 6.1</td>
<td>562</td>
<td>14 2.5</td>
</tr>
<tr>
<td>4</td>
<td>603</td>
<td>44 7.3</td>
<td>595</td>
<td>18 3.0</td>
</tr>
<tr>
<td>5</td>
<td>595</td>
<td>47 7.9</td>
<td>600</td>
<td>37 6.2</td>
</tr>
<tr>
<td>6</td>
<td>712</td>
<td>36 5.1</td>
<td>498</td>
<td>20 4.0</td>
</tr>
<tr>
<td>7</td>
<td>613</td>
<td>23 3.8</td>
<td>404</td>
<td>5 1.2</td>
</tr>
<tr>
<td>8</td>
<td>480</td>
<td>11 2.3</td>
<td>598</td>
<td>5 0.8</td>
</tr>
<tr>
<td>Totals</td>
<td>5329</td>
<td>258 4.8</td>
<td>4327</td>
<td>114 2.6</td>
</tr>
</tbody>
</table>
TABLE 6B (Series 3B)

Frequencies of induced recessive sex-linked lethal mutations in successive one-day broods after each treatment.

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>1 Mitomycin C + Y-rays</th>
<th>2 Saline + Y-rays</th>
<th>3 Mitomycin C</th>
<th>4 Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number tested</td>
<td>Mutations No.</td>
<td>%</td>
<td>Number tested</td>
</tr>
<tr>
<td>1</td>
<td>580</td>
<td>15</td>
<td>2.6</td>
<td>595</td>
</tr>
<tr>
<td>2</td>
<td>396</td>
<td>13</td>
<td>3.3</td>
<td>755</td>
</tr>
<tr>
<td>3</td>
<td>619</td>
<td>42</td>
<td>6.8</td>
<td>628</td>
</tr>
<tr>
<td>4</td>
<td>578</td>
<td>41</td>
<td>7.1</td>
<td>764</td>
</tr>
<tr>
<td>5</td>
<td>482</td>
<td>42</td>
<td>8.7</td>
<td>886</td>
</tr>
<tr>
<td>6</td>
<td>491</td>
<td>28</td>
<td>5.7</td>
<td>778</td>
</tr>
<tr>
<td>7</td>
<td>515</td>
<td>18</td>
<td>3.5</td>
<td>645</td>
</tr>
<tr>
<td>8</td>
<td>442</td>
<td>12</td>
<td>2.7</td>
<td>325</td>
</tr>
<tr>
<td>Totals</td>
<td>4103</td>
<td>211</td>
<td>5.1</td>
<td>5376</td>
</tr>
</tbody>
</table>
### TABLE 6C

Frequencies of induced recessive sex-linked lethal mutations in successive one-day broods after each treatment.

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>1 Mitomycin C + γ-rays</th>
<th>2 Saline + γ-rays</th>
<th>3 Mitomycin C</th>
<th>4 Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number tested</td>
<td>Mutations</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>1099</td>
<td>28</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1401</td>
<td>48</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1421</td>
<td>91</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1181</td>
<td>85</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1077</td>
<td>89</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1203</td>
<td>64</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1128</td>
<td>41</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>922</td>
<td>23</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td><strong>Totals</strong></td>
<td><strong>9432</strong></td>
<td><strong>469</strong></td>
<td><strong>4.9</strong></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 7

Mutation percentages in the Mitomycin C + $\gamma$-rays Series and the sum of the percentages in the Mitomycin C and $\gamma$-rays Series in daily broods.

<table>
<thead>
<tr>
<th>Brood Number</th>
<th>Treatment</th>
<th>1</th>
<th>2</th>
<th>3*</th>
<th>4</th>
<th>5</th>
<th>6*</th>
<th>7</th>
<th>8*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitomycin C + $\gamma$-rays</td>
<td>2.5</td>
<td>3.4</td>
<td>6.4</td>
<td>7.2</td>
<td>8.2</td>
<td>5.3</td>
<td>3.6</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>[\sum] Mitomycin C and $\gamma$-rays</td>
<td>2.8</td>
<td>3.4</td>
<td>7.7</td>
<td>7.7</td>
<td>8.7</td>
<td>7.6</td>
<td>3.9</td>
<td>2.1</td>
<td></td>
</tr>
</tbody>
</table>

* the values in these columns are significantly different
TABLE 8

Percentage of males surviving after each treatment in successive one-day broods.

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>Treatments and number of flies treated</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mitomycin C + ( \gamma )-rays</td>
<td>62.6</td>
<td>69.8</td>
<td>62.7</td>
<td>78.0</td>
</tr>
<tr>
<td></td>
<td>Saline + ( \gamma )-rays</td>
<td>(99 flies)</td>
<td>(86 flies)</td>
<td>(75 flies)</td>
<td>(41 flies)</td>
</tr>
<tr>
<td>1</td>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Mitomycin C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(75 flies)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(41 flies)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 9

Progeny per treated male after each treatment in successive one-day broods.

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>Treatments and number of males tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mitomycin C + ( \gamma )-rays (51 males tested)</td>
</tr>
<tr>
<td>1</td>
<td>76.2</td>
</tr>
<tr>
<td>2</td>
<td>70.0</td>
</tr>
<tr>
<td>3</td>
<td>71.0</td>
</tr>
<tr>
<td>4</td>
<td>58.4</td>
</tr>
<tr>
<td>5</td>
<td>60.7</td>
</tr>
<tr>
<td>6</td>
<td>65.0</td>
</tr>
<tr>
<td>7</td>
<td>59.0</td>
</tr>
<tr>
<td>8</td>
<td>57.5</td>
</tr>
<tr>
<td>Totals</td>
<td>517.8</td>
</tr>
<tr>
<td>Mean number</td>
<td>(64.7)</td>
</tr>
</tbody>
</table>
TABLE 10 (Series 4A)

Frequency of recessive sex-linked lethal mutations in successive day-broods after each treatment.

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>1 Mitomycin C</th>
<th>2 Mitomycin C + Actinomycin D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number tested</td>
<td>Mutations No.</td>
</tr>
<tr>
<td>1</td>
<td>384</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>363</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>518</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>448</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>387</td>
<td>7</td>
</tr>
<tr>
<td>6</td>
<td>564</td>
<td>9</td>
</tr>
<tr>
<td>7</td>
<td>538</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>431</td>
<td>4</td>
</tr>
<tr>
<td>Totals</td>
<td>3633</td>
<td>47</td>
</tr>
</tbody>
</table>
### TABLE 11 (Series 4B)

Frequencies of recessive sex-linked lethal mutations in successive two-day broods after each treatment.

<table>
<thead>
<tr>
<th>Brood Number</th>
<th>1 Mitomycin C</th>
<th></th>
<th></th>
<th>2 Mitomycin C + Actinomycin D</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Mutations</td>
<td>%</td>
<td>Number</td>
<td>Mutations</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>tested</td>
<td>No.</td>
<td>%</td>
<td>tested</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>207</td>
<td>3</td>
<td>1.4</td>
<td>204</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>261</td>
<td>10</td>
<td>3.8</td>
<td>283</td>
<td>8</td>
<td>2.8</td>
</tr>
<tr>
<td>3</td>
<td>279</td>
<td>6</td>
<td>2.1</td>
<td>310</td>
<td>9</td>
<td>2.9</td>
</tr>
<tr>
<td>4</td>
<td>264</td>
<td>6</td>
<td>2.3</td>
<td>287</td>
<td>6</td>
<td>2.1</td>
</tr>
<tr>
<td>5</td>
<td>266</td>
<td>5</td>
<td>1.8</td>
<td>233</td>
<td>4</td>
<td>1.7</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1277</strong></td>
<td><strong>30</strong></td>
<td><strong>2.3</strong></td>
<td><strong>1317</strong></td>
<td><strong>30</strong></td>
<td><strong>2.3</strong></td>
</tr>
</tbody>
</table>
TABLE 12 (Series 5A)

Frequencies of sex-linked recessive lethal mutations in successive day broods after each treatment.

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>1 FUDR + γ-rays</th>
<th>2 Saline + γ-rays</th>
<th>3 FUDR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number tested</td>
<td>Mutations No. %</td>
<td>Number tested</td>
</tr>
<tr>
<td>1</td>
<td>345</td>
<td>4 1.1</td>
<td>371</td>
</tr>
<tr>
<td>2</td>
<td>406</td>
<td>6 1.5</td>
<td>361</td>
</tr>
<tr>
<td>3</td>
<td>348</td>
<td>8 2.3</td>
<td>473</td>
</tr>
<tr>
<td>4</td>
<td>380</td>
<td>12 3.1</td>
<td>388</td>
</tr>
<tr>
<td>5</td>
<td>418</td>
<td>17 4.1</td>
<td>430</td>
</tr>
<tr>
<td>6</td>
<td>345</td>
<td>7 2.0</td>
<td>345</td>
</tr>
<tr>
<td>7</td>
<td>350</td>
<td>6 1.7</td>
<td>321</td>
</tr>
<tr>
<td>8</td>
<td>350</td>
<td>0 0</td>
<td>409</td>
</tr>
<tr>
<td>Totals</td>
<td>2942</td>
<td>60 2.04</td>
<td>3098</td>
</tr>
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</table>
TABLE 13

Frequencies of radiation-induced recessive sex-linked lethal mutations in successive daybroods after each treatment.

<table>
<thead>
<tr>
<th>Days after treatment</th>
<th>1 Actinomycin D + X-rays</th>
<th></th>
<th>2 Actinomycin D + X-rays</th>
<th></th>
<th>3 Actinomycin D</th>
<th></th>
<th>4 Saline</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number tested</td>
<td>Mutations No.</td>
<td>%</td>
<td>Number tested</td>
<td>Mutations No.</td>
<td>%</td>
<td>Number tested</td>
<td>Mutations No.</td>
</tr>
<tr>
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<td>580</td>
<td>7</td>
<td>1.2</td>
<td>485</td>
<td>3</td>
<td>0.6</td>
<td>375</td>
<td>0</td>
</tr>
<tr>
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<td>761</td>
<td>4</td>
<td>0.5</td>
<td>418</td>
<td>2</td>
<td>0.5</td>
<td>315</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>612</td>
<td>22</td>
<td>3.6</td>
<td>522</td>
<td>0</td>
<td>0</td>
<td>296</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>616</td>
<td>26</td>
<td>4.2</td>
<td>317</td>
<td>1</td>
<td>0.3</td>
<td>312</td>
<td>1</td>
</tr>
<tr>
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<td>600</td>
<td>2</td>
<td>0.3</td>
<td>402</td>
<td>7</td>
<td>1.7</td>
<td>221</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>764</td>
<td>2</td>
<td>0.3</td>
<td>382</td>
<td>5</td>
<td>1.3</td>
<td>229</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>430</td>
<td>1</td>
<td>0.2</td>
<td>764</td>
<td>2</td>
<td>0.3</td>
<td>327</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
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<td>0</td>
<td>458</td>
<td>0</td>
<td>0</td>
<td>275</td>
<td>1</td>
</tr>
<tr>
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<td>482</td>
<td>0</td>
<td>0</td>
<td>413</td>
<td>1</td>
<td>0.2</td>
<td>319</td>
<td>0</td>
</tr>
<tr>
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<td>443</td>
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<td>427</td>
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<td>0</td>
<td>285</td>
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</tbody>
</table>

Totals 5588 65 1.2 4888 21 0.4 2954 2 0.07 2977 3 0.1
TABLE 14A

Frequencies of X-radiation-induced recessive sex-linked lethal mutations in successive two-day broods after each treatment.

<table>
<thead>
<tr>
<th>Days after treatments</th>
<th>1 Actinomycin D + X-rays</th>
<th>2 Saline + X-rays</th>
<th>3 Actinomycin D</th>
<th>4 Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number tested</td>
<td>Mutations No.</td>
<td>%</td>
<td>Number tested</td>
</tr>
<tr>
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<td>714</td>
<td>13</td>
<td>1.8</td>
<td>547</td>
</tr>
<tr>
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<td>621</td>
<td>14</td>
<td>2.2</td>
<td>513</td>
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<tr>
<td>6</td>
<td>569</td>
<td>23</td>
<td>4.0</td>
<td>589</td>
</tr>
<tr>
<td>8</td>
<td>482</td>
<td>6</td>
<td>1.3</td>
<td>447</td>
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<tr>
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<td>489</td>
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<td>0.6</td>
<td>404</td>
</tr>
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<td>577</td>
<td>3</td>
<td>0.5</td>
<td>517</td>
</tr>
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<td>62</td>
<td>1.8</td>
<td>3017</td>
</tr>
<tr>
<td>Days after treatments</td>
<td>Actinomycin D + X-rays</td>
<td>Saline + X-rays</td>
<td>Actinomycin D</td>
<td>Saline</td>
</tr>
<tr>
<td>-----------------------</td>
<td>------------------------</td>
<td>----------------</td>
<td>--------------</td>
<td>--------</td>
</tr>
<tr>
<td></td>
<td>Number tested</td>
<td>Mutations No.</td>
<td>%</td>
<td>Number tested</td>
</tr>
<tr>
<td>2</td>
<td>503</td>
<td>5</td>
<td>1.0</td>
<td>329</td>
</tr>
<tr>
<td>4</td>
<td>545</td>
<td>8</td>
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<td>527</td>
</tr>
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<td>612</td>
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<td>414</td>
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<td>473</td>
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<td>398</td>
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<td>337</td>
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<tr>
<td>12</td>
<td>549</td>
<td>2</td>
<td>0.3</td>
<td>436</td>
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<tr>
<td>Totals</td>
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<td>35</td>
<td>1.1</td>
<td>2441</td>
</tr>
</tbody>
</table>
**TABLE 14C**

Frequencies of X-radiation-induced recessive sex-linked lethal mutations in successive two-day broods after each treatment.

<table>
<thead>
<tr>
<th>Days after treatments</th>
<th>Actinomycin D + X-rays</th>
<th>Saline + X-rays</th>
<th>Actinomycin D</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number tested</td>
<td>Mutations No.</td>
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</tr>
<tr>
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</tr>
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<td>22</td>
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<td>1003</td>
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<td>955</td>
<td>12</td>
<td>1.2</td>
<td>845</td>
</tr>
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<td>10</td>
<td>886</td>
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<td>1126</td>
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<td>0.4</td>
<td>953</td>
</tr>
<tr>
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<td><strong>6531</strong></td>
<td><strong>97</strong></td>
<td><strong>1.5</strong></td>
<td><strong>5458</strong></td>
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