Repair of Gamma Radiation-Induced X-Linked Lethal Mutations in the Nematode *Panagrellus redivivus*

by

Yvonne M. Jordan

A thesis
presented to the University of Manitoba
in partial fulfillment of the
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in
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ABSTRACT

The free-living nematode *Panagrellus redivivus* can repair gamma radiation-induced DNA damage. For single doses of radiation, the mutation rate decreases as dose increases. Studies using fractionated dose regimes suggest the presence of two types of recovery systems, one which operates after low doses of radiation and one which is induced after higher doses.

The effect of gamma radiation on non-disjunction was also examined. The results show that non-disjunction frequency increases as dose increases.

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INTRODUCTION

Historical

The first suggestion that ionizing radiation could affect the genetic material was made in 1927, when Muller observed that X-rays cause gene mutations in *Drosophila*. Many studies stemmed from this observation, and evidence began to accumulate suggesting that biological systems can recover from induced DNA damage. As early as 1944, it was reported that a dose of X-rays is less effective in the production of chromosome breaks and lethal effects if delivered in prolonged rather than acute doses (see Howard-Flanders 1978). In retrospect, the idea that repair is occurring at the lower dose rate seems obvious. However, a correlation between dose rate effects and repair was not suggested until over a decade later.

The delay was in part due to the fact that most subsequent studies of the genetic effects of ionizing radiation used *Drosophila* spermatozoa. These cells do not show a dose rate effect (Timofeeff-Ressovsky 1939, cited in Auerbach 1976), and so the idea of repair was not invoked. However, in 1958, Russell discovered that dose rate did affect mutation rates in mouse spermatogonia and oocytes.

He suggested that these cells have the ability to repair or prevent genetic damage after exposure to ionizing radiation (Russell *et al.* 1958, Russell 1963, Russell 1965).

The concept of repair was further explored by Elkind using mammalian cells in culture. Elkind and Sutton (1959) found that fractionation of a radiation dose into two exposures separated by a lag period led to an increase in survival over that observed when the same dose was given as a single exposure. Although the nature of this recovery was explored in a series of papers dealing with the effects of hypoxia (Elkind *et al.* 1964), temperature (Elkind *et al.* 1965), and various inhibitors (Elkind *et al.* 1967), no attempt was made to explore the molecular mechanisms involved.

Auerbach (1976) has stated that "X-radiation, which was the first discovered mutagen, is also the one whose primary mechanism is least understood. The difficulty lies not in the shortage of possible mechanisms, but rather in their multiplicity." Studies of the effects of ionizing radiation on DNA have shown that these include base changes, base loss, single or double strand breaks, DNA-DNA crosslinks (intra- or interstrand) and DNA-protein crosslinks (Auerbach 1976). One approach to elucidating the molecular basis of DNA repair might be to predict, on the basis of the types of lesions, the types of repair systems required to correct the

damage. Most work in this area has been done using DNA in solution. If the effect of ionizing radiation on bases is studied, different results are obtained when the bases are irradiated in different forms (for example,as free bases, as nucleosides, as nucleotides or as components of a helix). This is to be expected, as bases will be protected by their associations with other molecules. However, the complicated conditions in an intact cell or organism are not likely to be duplicated *in vitro*, and there is some doubt as to whether the *in vitro* results can be used to predict reliably the *in vivo* consequences of irradiation. As Lehmann (1978) has pointed out, two of the lesions observed *in vitro* (DNA-DNA crosslinks and DNA-protein crosslinks) have not been detected as a consequence of gamma or X-irradiation of whole cells.

The approach of dissecting repair pathways using mutants and biochemical assays has, in contrast, been very successful, and has provided a good understanding of the molecular mechanisms mediating DNA repair. Most of the work in this area has been done using ultraviolet rather than ionizing radiation. However, the existence of single mutants sensitive to both types of radiation suggests that some common steps are involved.

The first direct demonstration of repair at the molecular level came from the work of Setlow and Carrier (1964) which

showed that *Escherichia coli* is capable of excising pyrimidine dimers induced by ultraviolet radiation. The many studies of excision repair which began after this discovery had two consequences. The immediate benefit was that the individual steps involved in excision repair were well characterized. Secondly, it became apparent that excision repair is only one of several repair pathways available to cells after exposure to a damaging agent. Thus, it seems likely that the various DNA lesions caused by ionizing radiation can be acted upon by different repair pathways, and the pattern of recovery observed after irradiation may be the consequence of several different pathways acting separately or in concert.

Repair Pathways

Bacterial repair pathways have been the most widely studied, although recent work with yeast (Haynes *et al.* 1978, McKee and Lawrence 1979) and mammalian cells (Doniger 1978, Hanawalt *et al.* 1979) suggests that these systems also possess several recovery routes which appear (at this early stage of investigation) to be similar to those found in bacteria. The repair pathways may be divided into two broad categories: error-free and error-prone repair. Error-free repair may be further subdivided as follows:

1. Repair occurring prior to replication.

Excision repair (reviewed by Hanawalt *et al.* 1979) falls into this category, since it occurs prior to the first post-irradiation replication. Nucleotide excision repair is initiated when an endonuclease monitoring the DNA helix detects a lesion and introduces a nick in the damaged strand. The resulting site is then acted upon by DNA polymerase I (in bacteria) and its associated 5′-3′ exonuclease. The exonuclease activity removes the damaged nucleotide in a short fragment, while the polymerase resynthesizes the excised region. Finally, ligase seals the gap between the newly synthesized DNA and the original strand.

In addition to nucleotide excision repair, a cell may employ base excision repair. If a base alteration occurs, and is detected as such, repair may be effected by an N-glycosylase. This enzyme removes the damaged base, leaving a gap. The gap may be filled in one of two ways. An enzyme, base insertase, may fill the gap using the opposite strand as a template. Such an insertase activity has been found in human fibroblasts (Hanawalt *et al.* 1979). Alternatively, an endonuclease may nick the DNA strand at the site of the lesion, initiating excision and resynthesis (Linn 1978).

2. Repair occurring during replication

If a lesion is still present when the first post-irradiation replication begins, it may be bypassed by a process called strand displacement and branch migration (Hanawalt and Smith 1978). In this process, replication proceeds normally until a lesion is encountered. At this point, replication halts on the damaged strand, but proceeds a short distance (presumably to the end of the replicon) on the undamaged strand. The replication block causes the parental strands to reassociate. The daughter strands are displaced, and pairing between them can occur. In this conformation the intact daughter strand can provide an error-free template for the daughter strand which was blocked at the lesion. Replication can now proceed normally. Although this is not repair in its strictest sense since the original lesion remains in the DNA, it does allow the cell to replicate normally, and the resulting daughter strands are error-free.

3. Repair occurring after replication

It has been shown that after the first post-irradiation replication, gaps may persist in the daughter strands opposite lesions on the parental strand (Cleaver 1974). These gaps can be filled by a

postreplication repair system called daughter strand gap repair (Soyfer 1979). In this system, gaps on daughter strands are filled by exchanges with the undamaged parental strand having the same polarity. The resulting gap in the parental strand is then resynthesized. As with strand displacement and branch migration, the original lesion remains in the DNA, although the daughter strands are error-free.

Error-prone repair results when repair or synthesis occurs using a damaged template. There are two types of error-prone repair, constitutive and inducible.

1. Inducible error-prone repair

The presence of replication gaps (as described for daughter strand gap repair) may, in some instances, be the signal for induction of an error-prone repair system (reviewed in Witkin 1976). When DNA polymerase III (the major replicative polymerase in *E. coli*) attempts to fill in the gaps, it is blocked by the presence of the lesion. The result is that the polymerase "idles" at the damaged region, repeatedly inserting and removing bases opposite the lesion. The removal of bases opposite the non-coding region is effected by the 3′-5′ exonuclease activity associated with pol III. It is believed that the increase in concentration of deoxynucleotide

monophosphates produced by this reaction is the actual inducing signal. The sequence of events following this increase has been determined in bacteria.

Two gene loci are important in the inducible error-prone repair pathway. One (lexA) produces a product which acts as a repressor of the second important locus, recA. It is thought that low levels of the recA product (called protein X) are constitutively produced. The increase in deoxynucleotide monophosphates resulting from idling apparently activates protein X. The activated protein X cleaves the lexA repressor protein and other repressor proteins in the cell. This mass derepression has many associated effects, among which is one resulting in mutagenic repair. There is some controversy over the exact nature of this effect, but it appears to be one of two possibilities. A new polymerase may be induced which does not have the strict coding requirements of pol III and will simply add any base to get past the lesion (Witkin 1976). Alternatively, a product may be induced which modifies pol III, suppressing the 3′-5′ editing function, and allowing synthesis past a non-coding template (Bridges 1978).

2. Constitutive error-prone repair

Constitutive error-prone repair occurs prior to DNA replication, and appears to be related to excision (Bridges 1978). If two lesions occur close together but on opposite DNA strands, excision repair may result in mutagenesis if the excision gap resulting from attempted repair of one lesion overlaps the second lesion. If this occurs, the polymerase will not have an undamaged template for resynthesis, and mutagenesis may result. The nature of the enzymes involved in circumventing the lesion is not known, but there is some evidence implicating pol III in mutagenesis at excision gaps (Bridges 1978).

Radiation Biology of Nematodes

Nematodes have been found to be extremely resistant to ionizing radiation (Alicata and Burr 1949, Thomas and Quastler 1950, Gomberg and Gould 1953,Wood and Goodey 1957, Myers 1960, Siddiqui and Viglierchio 1970). For example, Panagrellus redivivus has an LD50 of 750,000 rads (Myers 1960). This resistance may be due to the eutelic nature of nematode tissue. However, an alternative explanation is that nematodes possess an efficient repair system.

Previous studies in this laboratory (Denich 1980) have suggested that the nematode *Panagrellus redivivus* possesses the ability to repair some damage after radiation exposure. The present study explores the repair capacity of *P. redivivus* using both single and fractionated doses of gamma radiation. In addition, the effect of radiation on non-disjunction is investigated.

MATERIALS AND METHODS

The Culture Media

1. M9 Buffer (Brenner 1974): 6 g. Na_2HPO_4, 3 g. KH_2PO_4, 5 g. NaCl, 0.25 g. $MgSO_4$. Make up to 1 litre with distilled water and autoclave.
2. Food Source (M9-Y)(Samoiloff *et al*. 1980): 1000 ml. M9 buffer, 0.05 mg/ml Baker's yeast, 1 ml. of cholesterol in ethanolic solution (5 mg. cholesterol/ml.). Autoclave.
3. Agar plates: Stocks are maintained on 1.7% agar plates.

The Organism

The free-living nematode *Panagrellus redivivus* is dioecious, and sexually dimorphic at the adult stage. Females are larger than males, and have a darker appearance due to the presence of eggs. Males can be easily identified by the presence of a spicule. Females have a chromosome complement of four pairs of autosomes and two X chromosomes, while males are 4AA plus a single X (Hechler 1970).

The postembryonic development of *P. redivivus* is characterized by a series of larval stages separated by molts. Hatching and the first larval stage are completed *in*

utero thus the first free-swimming stage is the second larval (L2) stage. The L2 progresses through the third (L3) and fourth (L4) larval stages prior to becoming an adult. This progression (L2 to adult) takes about 96 hours (Samoiloff and Pasternak 1968).

The strains utilized in these experiments are the wild-type (c15) strain, an uncoordinated mutant (b7), and a doubly marked line, jlc64. The b7 mutation was induced by EMS (ethyl methane sulphonate) (Burke and Samoiloff 1980). This mutant can be picked out both on agar plates and in liquid by its phenotype. Unlike wild-type worms (which are very active both on solid or in liquid media), b7 shows very limited movement and typically assumes a coiled posture in both instances. The b7 allele is a recessive X-linked mutation. Thus, females must be homozygous for the allele in order to express the phenotype. Males, being hemizygous for alleles on the X chromosome, need only a single copy of b7 to show the phenotype. The uncoordinated phenotype is expressed throughout the life cycle.

The semidominant jl mutation was induced by cold shock (Jordan, unpublished). Nematodes homozygous or hemizygous for this X-linked mutation are approximately 50% longer than wild-type *P. redivivus* and about 25% thinner. Heterozygous females are longer than normal. This mutation can be recognized in L4s and adults.

The c64 mutation was induced by EMS and is expressed as a slightly uncoordinated phenotype (Samoiloff, unpublished). When tapped on the head, worms expressing this X-linked recessive mutation do not respond (unlike wild-type worms which reverse when tapped). In addition, c64 mutants are less active than wild-type. The mutation is expressed throughout the life cycle.

The doubly marked jlc64 line was obtained by crossing worms carrying the jl mutation to ones carrying the c64 mutation and isolating crossover progeny.

Mapping studies (Samoiloff, unpublished) have been done with these three alleles, and the results indicate that b7 lies between jl and c64, being 8.93 map units from the former and 9.56 map units from the latter.

Protocol

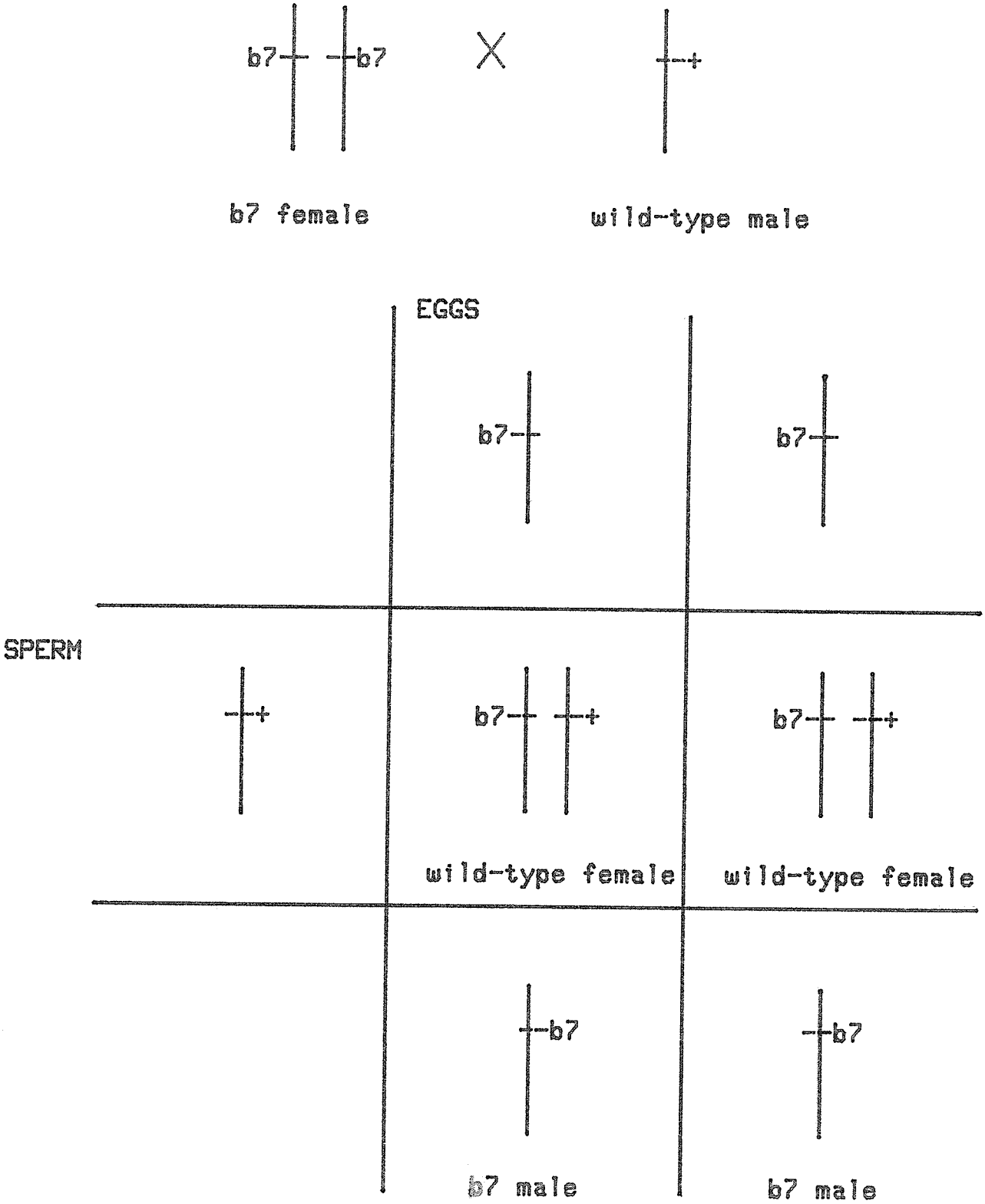
1). Single doses of gamma radiation

Virgin b7 females were collected by isolating individual worms at the L4 stage. These worms were held for 24 hours in 2.5 ml. Autoanalyzer cups (Fisher) containing 0.5 ml. of M9-Y. At the end of this time, males and females were categorized. Virginity is ensured by the fact that sexual activity does not begin until the adult stage is reached.

Virgin females isolated in this manner were exposed to gamma radiation in an AECL cobalt-60 gamma cell (dose rate:4308 rads/min.). The doses used were 1077, 2154, 3231, 4308, 5385, 6462, 7539, 8616, 9693, 10770, 11847 and 12924 rads. In total, 50 females were irradiated at each dose. Fifty unirradiated control females were run at the same time. Within one hour of irradiation, the females were mated to c15 males. The induction of X-linked recessive lethals can be detected in the progeny of this cross (see Fig. 1).

Both X chromosomes of the irradiated females are exposed to gamma radiation. When these females are mated to c15 males, we expect (if no lethal mutations have occurred) to have male and female progeny in a 1:1 ratio (Denich 1980). Moreover, these male and female progeny will be distinguishable by their phenotypes. Males will receive a single X chromosome from the female parent. Since this chromosome carries the b7 allele, the male progeny will be uncoordinated. Female progeny will receive an X chromosome carrying the b7 allele from their maternal parent, and an X chromosome carrying the wild-type allele at this locus from their paternal parent. Since b7 is a recessive mutation, the wild-type allele will mask its expression and the female progeny will show wild-type movement. Thus, if no lethal mutations are induced, we expect to see uncoordinated male progeny and moving female progeny in a 1:1 ratio. Any

Fig. 1 Cross of b7 females by c15 males. If a recessive lethal mutation is induced in an oocyte, it will be manifested by lethality in the hemizygous male.


b7
b7
X
+
b7 female
wild-type male
EGGS
b7
b7
SPERM
+
b7
+
b7
+
wild-type female
wild-type female
b7
b7
b7 male
b7 male

deviation from this ratio in favour of female progeny can be considered as being due to the induction of a recessive lethal which will be expressed in the hemizygous male but not in the heterozygous female.

The b7 by c15 matings were done on agar plates at room temperature (approximately 22°C). Progeny do not appear until the second day after mating. Each day after the initial appearance of progeny, the mated pairs were transferred to fresh plates and the young remaining on the old plate were counted and scored as male or female on the basis of phenotype. This can be done quite simply, as the worms tend to remain at the centre of the plate if a drop of food has previously been placed at that point. This central area can then be covered with several drops of M9. In the liquid, the b7 progeny can be easily distinguished from the wild-type progeny by their coiled posture. Using a micropipette, one can remove the larvae from the plate while counting and scoring as to phenotype.

2). Fractionated doses of gamma radiation

Virgin females were collected as outlined in the methods for single dose irradiation. A total dose of 12924 rads was administered, 50 females being irradiated at each of the following fractionation regimes: 1077+11847, 2154+10770, 3231+9693, 4308+8616, 5385+7539, 6462+6462, 7539+5385, 8616+4308, 9693+3231, 10770+2154, 11847+1077 and 12924 rads

total. In addition, 100 controls were run. The lag period between doses was two hours. Detection of X-linked recessive lethals was performed as outlined for single dose irradiation.

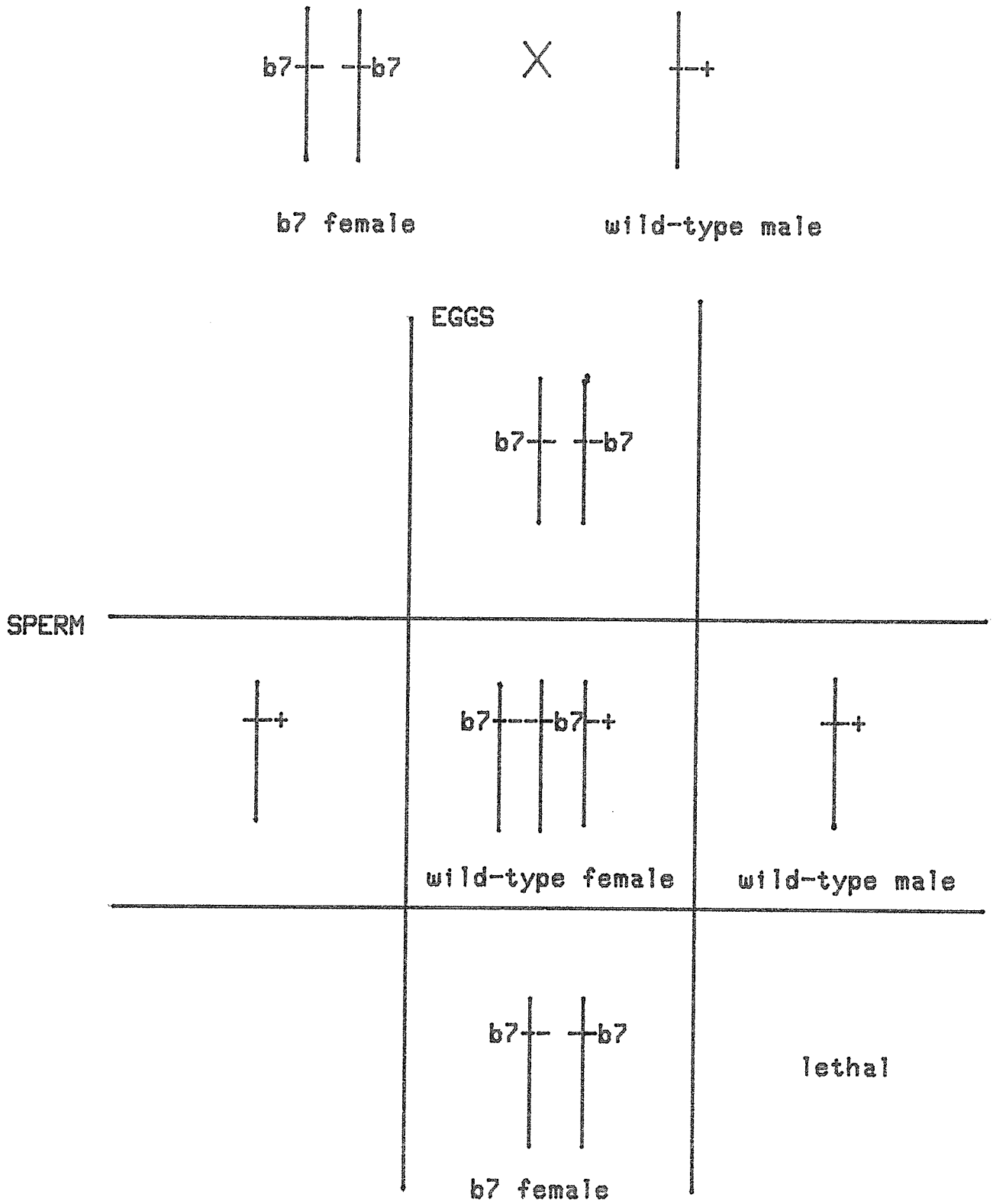
3). Determination of non-disjunction frequency

Non-disjunction is the failure of homologous chromosomes or sister chromatids to separate during mitosis or meiosis (Rothwell 1979). If non-disjunction frequencies change with radiation dose, the results of these experiments would be affected, since sex determination was done on the basis of phenotype only. Non-disjunction would result in wild-type males and b7 females (see Fig. 2). The effect of non-disjunction was tested in several experiments.

1. The effect of increasing radiation dose on non-disjunction frequencies

Virgin females were collected as previously outlined, and irradiated at doses of 2154, 4308, 6462, 8616, 10770 and 12924 rads (50 females per dose). Fifty control females were also tested. These females were mated to c15 males on agar plates and held at room temperature (approximately 22°C). When young appeared on the plates, the adult nematodes were transferred to fresh plates. These transfers were done daily. The young remaining on

Fig. 2 The effect of non-disjunction on a cross of b7 females by c15 males. Non-disjunction in the female produces eggs which contain two X chromosomes and eggs which contain no X chromosome.


b7
b7
X
+
b7 female
wild-type male
EGGS
b7
b7
SPERM
+
b7
b7
+
+
wild-type female
wild-type male
b7
b7
lethal
b7 female

the old plate were separated according to phenotype (uncoordinated versus movers) and placed on separate plates to mature. Once mature, the nematodes were checked to determine if phenotypic ratio was a reflection of sex ratio. That is, the plates of wild-type progeny (which should be female) were checked for the presence of males, and the plates of uncoordinated progeny (which should be males) were checked for the presence of females.

2. The effect of temperature on non-disjunction

Due to temperature fluctuations which were occurring in the lab, a study was undertaken to determine the effect of temperature on non-disjunction. Ninety virgin b7 females were collected and irradiated at 6462 rads of gamma radiation. These females were separated into three groups after irradiation and mated to c15 males. Each of the three groups was held at a different temperature (28° C, 22° C, or 13 °C) throughout the reproductive period. Non-disjunction frequencies at the different temperatures were determined as described above.

3. The effect of parental genotype on non-disjunction

To determine whether the phenotype expressed in the presumed non-disjunction products was indeed the result of non-disjunction, the experiment was performed using jlc64 males in place of c15 males. If the unexpected male offspring are receiving an X chromosome from the paternal parent, the use of jlc64 (which has a very distinctive phenotype) will allow the paternal X to be followed in the cross. Since both the jl and c64 mutations are recessive, they will be masked by the corresponding wild-type alleles on the maternal X chromosome carrying b7. Similarly, the wild-type allele at the b7 locus of the X chromosome carrying jl and c64 will mask the b7 effect. Thus, a worm having two b7 chromosomes and one jlc64 chromosome will have the wild-type phenotype. Non-disjunction frequencies in the b7 females (in the absence of any radiation) were determined as described in part (1) of this section.

RESULTS

Single Doses of Gamma Radiation

The effects of single doses of gamma radiation are presented in Table I. These figures are the additive results of several trials. To ensure that the data were sufficiently uniform to warrant adding the trials together, a homogeneity chi-square test was done (see Appendix I). The results of this test are given in Table II. A significant homogeneity chi-square value at a probability of 0.05 indicates that less than 5% of the time such a value will be obtained by chance alone. One point (12924 rads) has a homogeneity chi-square value which is significant at the 0.05 level of probability. The corresponding probability for this point is less than 0.001.

Mutations per locus and mutations per locus per rad are calculated using Target Theory (see Appendix II), and are shown in Fig. 3 and Fig. 4 (see Appendix IV for calculation of error bars). Mutations per locus remain essentially constant over the range studied. Mutations per locus per rad (a measure of mutagenicity per unit dose) decrease as dose increases.

TABLE I. RESULTS OF SINGLE DOSES OF GAMMA RADIATION

Dose (rads)	Females Tested	Male Progeny	Female Progeny	Sex Ratio (M/F)	Mutations/locus ($x10^{-4}$)	Mutations/locus/rad (10^{-8})
Control	50	2785	2897	0.961 ± 0.025	1.11	
1077	50	2515	2897	0.868 ± 0.037	3.93	36.50
2154	50	2887	3090	0.934 ± 0.024	1.89	8.76
3231	50	2767	2948	0.939 ± 0.061	1.76	5.45
4308	50	2903	3085	0.941 ± 0.024	1.69	3.92
5385	50	2671	2981	0.896 ± 0.024	3.05	5.66
6462	50	2499	2498	1.000 ± 0.062	0	0
7539	50	2317	2473	0.937 ± 0.027	1.81	2.40
8616	50	2637	2869	0.919 ± 0.025	2.34	2.72
9693	50	1861	1976	0.948 ± 0.030	1.67	1.72
10770	50	1922	2106	0.913 ± 0.029	2.54	2.36
11847	50	2085	2168	0.962 ± 0.036	1.08	0.92
12924	50	1952	2235	0.873 ± 0.117	3.76	2.91

TABLE II. HOMOGENEITY CHI-SQUARE FOR SINGLE DOSES OF GAMMA RADIATION

Dose (rads)	Additive chi-squares of separate trials	Chi-square of summed data	Homogeneity Chi-square	Degrees of Freedom	
Control	8.351	2.208	6.143	7	NS
1077	29.395	26.963	2.432	2	NS
2154	8.742	6.895	1.847	3	NS
3231	11.729	5.732	5.997	2	S
4308	8.098	5.532	2.566	3	NS
5385	17.638	17.003	0.635	2	NS
6462	4.910	0.0002	4.9098	3	NS
7539	5.560	5.081	0.479	3	NS
8616	11.278	9.775	1.503	3	NS
9693	5.573	3.447	2.126	3	NS
10770	10.745	8.405	2.340	3	NS
11847	3.062	1.620	1.442	1	NS
12924	37.814	19.128	18.686	2	S

NS - not significant

S - significant

Fig. 3 Mutation frequency (mutations per locus) following single doses of gamma radiation. The solid line (——) represents a plot of the actual data points, while the broken line (---) is a linear regression curve fitted to these points.

SINGLE DOSE

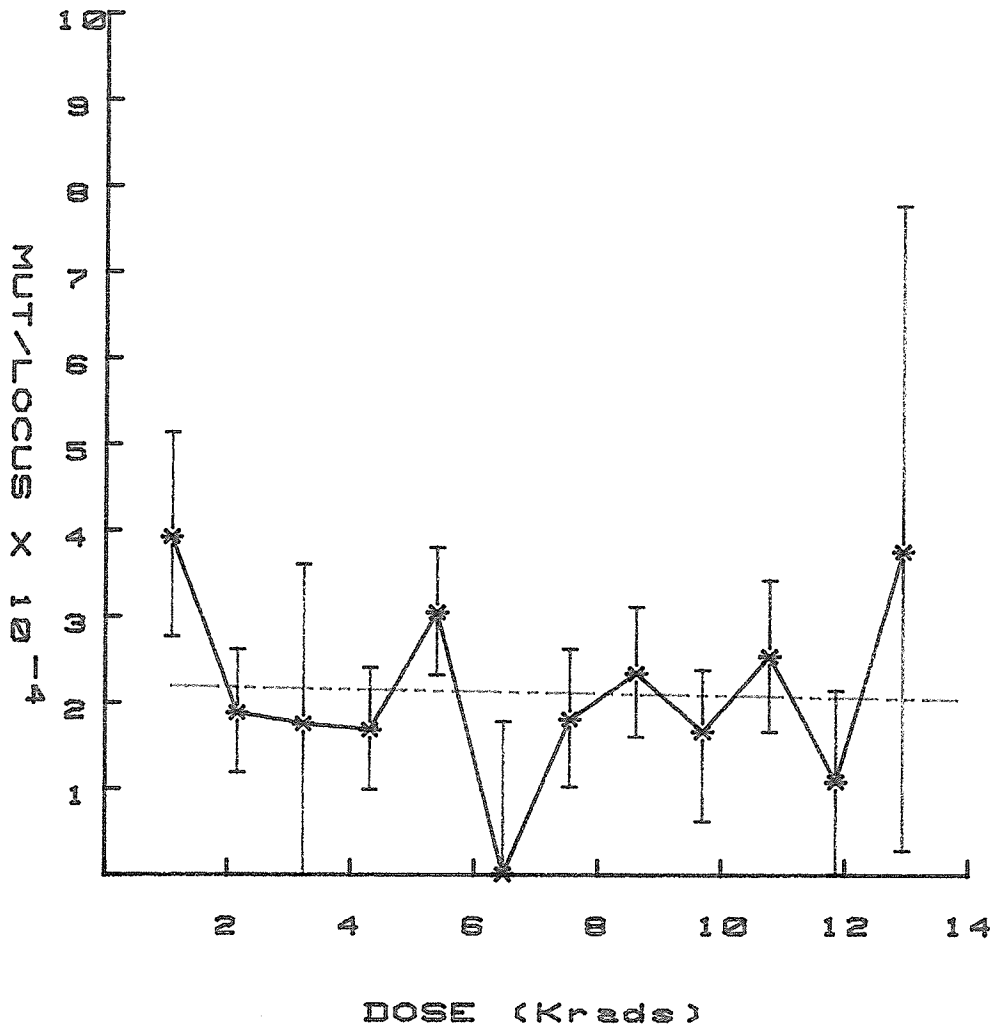
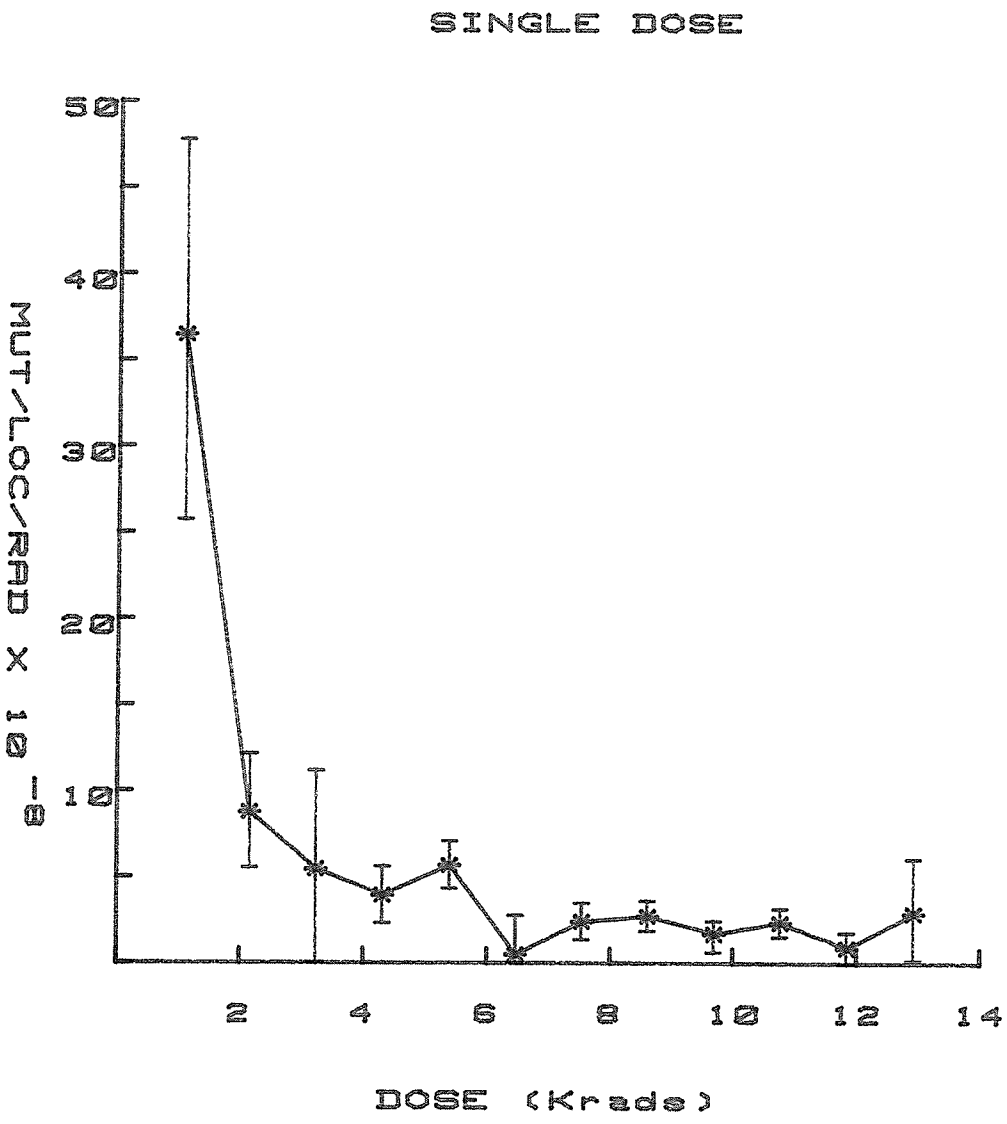


Fig. 4 Mutation rate (mutations per locus per rad) following single doses of gamma radiation.


SINGLE DOSE
MUT/LOC/RAD X 10 -8
50
40
30
20
10
2
4
6
8
10
12
14
DOSE (Krads)

Fractionated Doses of Gamma Radiation

The results of the fractionated dose experiments are presented in Table III, and the homogeneity chi-square tests for the data are shown in Table IV. The homogeneity chi-square values for control, 2154+10770 rads, and 12924 rads are significant at the 0.05 level of probability. The corresponding probabilities for these points are 0.0134, less than 0.001 and less than 0.001.

Mutations per locus is plotted against initial dose in Fig. 5. When a total dose of 12924 rads is fractionated and various combinations of first and second dose are studied, the pattern which emerges when a logarithmic regression line is fitted to the points is one of an increase in mutations per locus as the initial dose increases. If, however, the actual data points are examined, a different trend emerges. Mutations per locus increase with increasing dose up to 4308 rads. After this point, there is an abrupt decrease in mutation frequency which continues up to 6462 rads. After 6462 rads the number of mutations per locus rises once more, and plateaus at about 7539 rads.

Since the total dose is constant, the trends observed in Fig. 5 are repeated when mutations per locus per rad is plotted in Fig. 6.

TABLE III. RESULTS OF FRACTIONATED DOSES OF GAMMA RADIATION (TOTAL DOSE = 12924 RADS)

Dose (rads)	Females Tested	Male Progeny	Female Progeny	Sex Ratio (M/F)	Mutations/locus (10^{-4})	Mutations/locus/rad (10^{-8})
Control	100	4385	4375	1.002 ± 0.091		
1077+11847	50	1986	2006	0.990 ± 0.031	0.28	0.22
2154+10770	50	1993	2051	0.972 ± 0.126	0.80	0.62
3231+9693	50	1990	2121	0.938 ± 0.029	1.77	1.37
4308+8616	50	1872	2152	0.870 ± 0.062	3.87	3.00
5385+7539	50	1641	1749	0.938 ± 0.059	1.77	1.37
6462+6462	50	1961	2029	0.966 ± 0.066	0.95	7.33
7539+5385	50	1479	1662	0.890 ± 0.032	3.24	2.51
8616+4308	50	1180	1317	0.896 ± 0.036	3.05	2.36
9693+3231	50	1524	1715	0.889 ± 0.073	3.28	2.54
10770+2154	50	1625	1830	0.888 ± 0.030	3.30	2.55
11847+1077	50	1566	1790	0.875 ± 0.030	3.76	2.87
12924	50	1732	1983	0.873 ± 0.104	3.76	2.91

TABLE IV. HOMOGENEITY CHI-SQUARE FOR FRACTIONATED DOSES OF GAMMA RADIATION

Dose Regime (rads)	Additive chi-squares of separate trials	Chi-square of summed data	Homogeneity Chi-square	Degrees of Freedom	
Control	18.967	0.001	18.966	6	S
1077 + 11847	1.616	0.100	1.516	3	NS
2154 + 10770	16.508	0.004	16.504	2	S
3231 + 12924	4.302	4.174	0.128	2	NS
4308 + 8616	24.679	19.483	5.196	2	NS
5385 + 7539	6.823	3.441	3.382	2	NS
6462 + 6462	5.625	1.159	4.466	2	NS
7539 + 5385	11.698	10.662	1.036	2	NS
8616 + 4308	8.203	7.517	0.686	2	NS
9693 + 3231	16.883	11.263	5.620	2	NS
10770+ 2154	13.190	12.164	1.026	2	NS
11847+ 1077	16.712	14.951	1.761	2	NS
12924	29.452	16.959	12.493	1	S

NS - not significant

S - significant

Fig. 5 Mutation frequency (mutations per locus) as a function of the initial dose in fractionated dose regimes. Total dose is 12924 rads. The solid line (——) represents a plot of the actual data points, while the broken line (---) is a logarithmic regression curve fitted to these points.

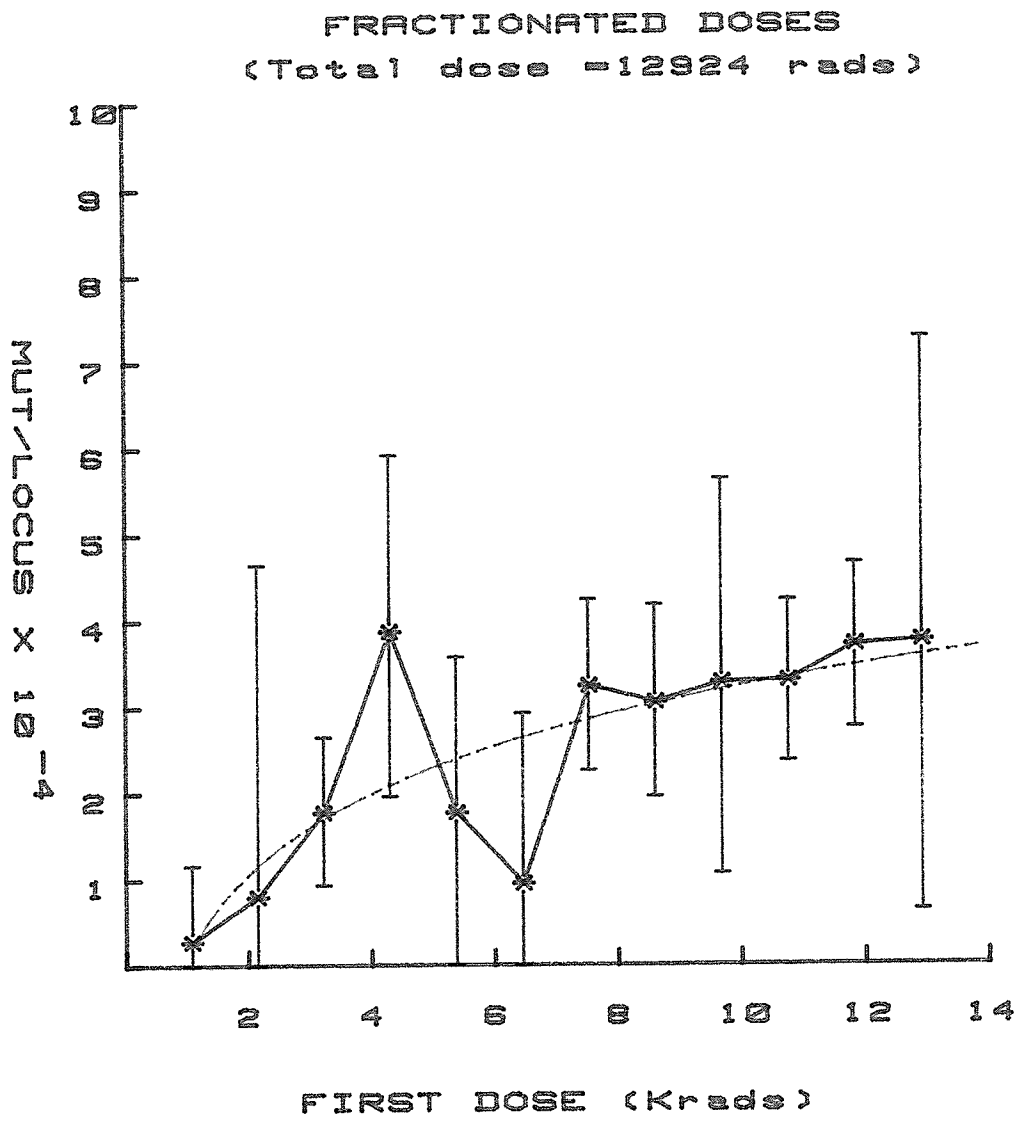
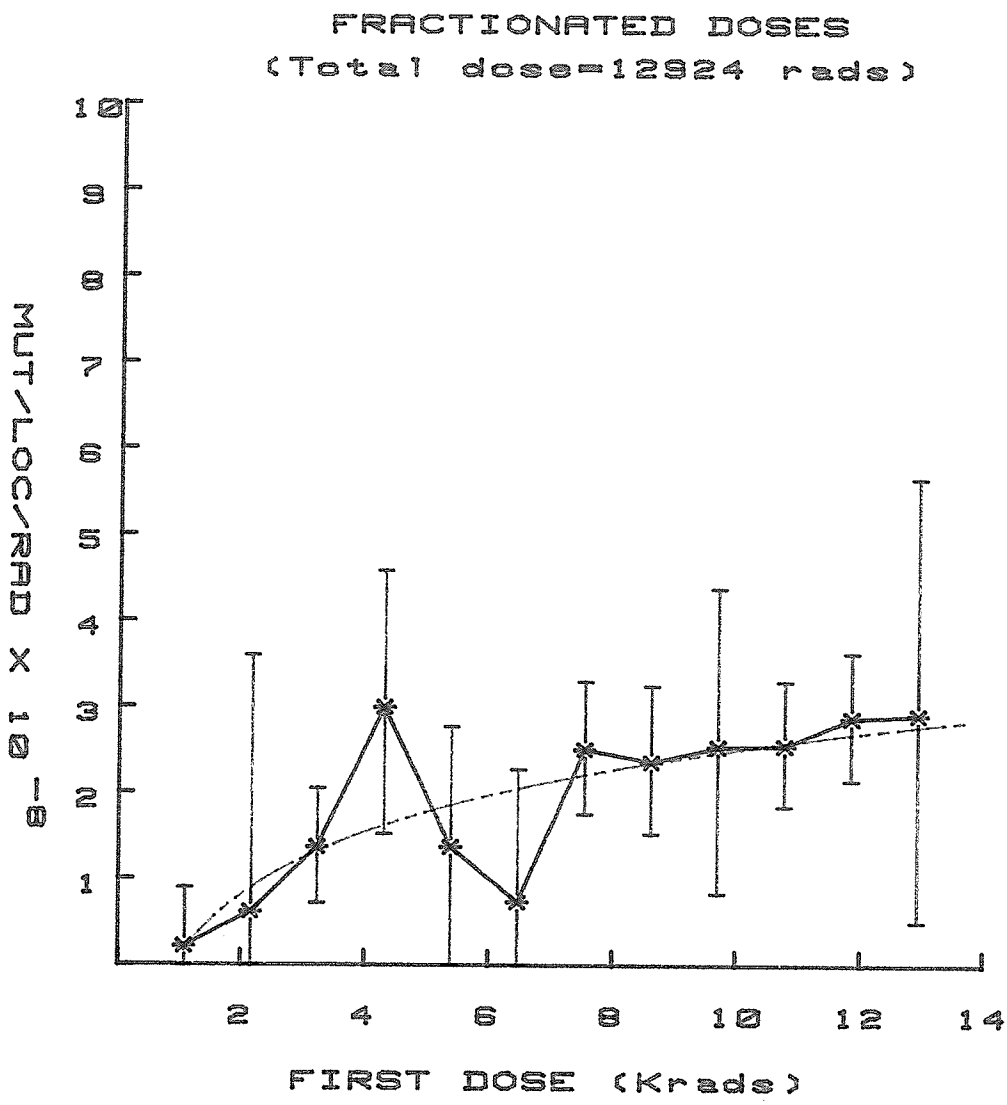

FRACTIONATED DOSES
(Total dose =12924 rads)
MUT/LOCUS X 10^-4
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10
12
14
FIRST DOSE (Krads)

Fig. 6 Mutation rate (mutations per locus per rad) as a function of the initial dose in fractionated dose regimes. Total dose is 12924 rads. The solid line (——) represents a plot of the actual data points, while the broken line (---) is a logarithmic regression curve fitted to these points.


FRACTIONATED DOSES
(Total dose=12924 rads)
MUT/LOC/RAD X 10 -8
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10
12
14
FIRST DOSE (Krads)

Non-disjunction

1) The effect of increasing radiation dose on non-disjunction frequency.

The effects of gamma radiation on non-disjunction frequency are presented in Table V. The results of homogeneity chi-square tests on three trials are contained in Table VI. The homogeneity chi-square values for 2154 rads and 10770 rads are significant at the 0.05 level of probability, and have probabilities of 0.039 and 0.009, respectively.

The frequency of non-disjunction is affected by radiation, and increases as dose increases over the dose range studied. However, this total frequency is not made up of equal numbers of male and female non-disjunction products. In all cases, the male non-disjunction products make up 87-100% of the total number of non-disjunction events (Table VII).

It should be noted that comparisons between mutation rates which might be generated from the non-disjunction data and those observed after single doses of radiation cannot be made for the following reasons:

1. Wide temperature fluctuations were occurring in the laboratory at the time of the non-disjunction study. This could have an effect on either non-disjunction frequency or the repair capacity.

TABLE V. NON-DISJUNCTION FREQUENCIES AS A FUNCTION OF RADIATION DOSE.

Dose (rads)	Moving Progeny	Uncoordinated Progeny	Wild Type Males	B7 Females	Frequency of Wild-type males	Frequency of B7 Females	Total non-Disjunction Frequency
Control	1749	1637	13	1	0.384%	0.030%	0.414%
2154	1595	1415	27	4	0.897%	0.133%	1.030%
4308	1532	1392	12	0	0.410%	-	0.410%
6462	1677	1369	33	2	1.083%	0.066%	1.149%
8616	1351	1320	26	4	0.973%	0.150%	1.123%
10770	1726	1503	62	4	1.920%	0.124%	2.044%
12924	1328	1182	48	5	1.912%	0.199%	2.111%

TABLE VI. HOMOGENEITY CHI-SQUARE FOR NON-DISJUNCTION DATA

Dose (rads)	Additive chi-squares of separate trials	Chi-squares of summed data	Homogeneity Chi-square	Degree of Freedom	
Control	6.956	3.705	3.251	2	NS
2154	17.663	10.764	6.899	2	S
4308	8.691	6.703	1.988	2	NS
6462	33.496	31.144	2.352	2	NS
8616	1.792	0.360	1.432	2	NS
10770	24.657	15.401	9.256	2	S
12924	8.687	8.492	0.195	2	NS

NS - not significant

S - significant

TABLE VII. DISTRIBUTION OF MALE AND FEMALE NON-DISJUNCTION PRODUCTS.

Dose (rads)	Frequency of Non-disjunction	Frequency of Wild type Males	Frequency of B7 Females	$\frac{\text{Freq. Wild Type males}}{\text{Total non-disjunction freq.}} \times 100$	$\frac{\text{Freq. B7 females}}{\text{Total non-disjunction freq.}} \times 100$
Control	0.414%	0.384%	0.030%	93%	7%
2154	1.030%	0.897%	0.133%	87%	13%
4308	0.410%	0.410%	-	100%	-
6462	1.149%	1.083%	0.066%	94%	6%
8616	1.123%	0.973%	0.150%	87%	13%
10770	2.044%	1.920%	0.124%	94%	6%
12924	2.111%	1.912%	0.199%	91%	9%

2. In the non-disjunction study, the offspring were not always separated according to phenotype at the L2 stage. As a result, instead of considering only lethals which act prior to the L2-L3 molt, there may be an added effect of lethals acting beyond this point.

Despite these problems, a general trend of increasing non-disjunction with increasing dose can be discerned.

2) Effect of temperature on non-disjunction

The effects of temperature on non-disjunction after 6462 rads of gamma radiation are presented in Table VIII. This study was undertaken in an attempt to determine the effects of the temperature fluctuations described above. Temperature does not seem to affect non-disjunction frequency, as the total frequency of non-disjunction events ranges from only 1.097% to 1.440%. It should be noted that the non-disjunction frequency of 1.097% observed at room temperature in this experiment agrees well with that observed after 6462 rads (1.149%) in the previous experiment.

3) Effect of parental genotype on non-disjunction

It was observed that the sex ratio of the progeny from a cross of b7 females by jlc64 males is skewed sharply in

TABLE VIII. THE EFFECT OF TEMPERATURE ON NON-DISJUNCTION AFTER 6462 RADS OF RADIATION.

Temperature (^{o}C)	Females Tested	Moving Progeny	Uncoordinated Progeny	Wild Type Males	Uncoordinated Females	Frequency of Wild Type Males	Frequency of B7 Females	Total Non-disjunction Frequency
28	30	679	610	17	1	1.319%	0.078%	1.397%
22	30	1194	1085	23	2	1.009%	0.088%	1.097%
13	30	630	690	17	1	1.288%	0.076%	1.364%

favour of female progeny (see Table IX). Therefore, quantitative comparisons between these data and those obtained using cl5 males are pointless. However, it was noted that the unexpected male progeny did appear to be the result of non-disjunction, since their phenotype corresponded to that of their male parent.

TABLE IX. THE EFFECT OF PATERNAL GENOTYPE ON NON-DISJUNCTION.

Females Tested	Moving Progeny	Non-Moving Progeny	j1c64 males	Wild Type Males	B7 Females	Frequency of non-disjunction
50	1673	1226	6	-	-	0.207%

DISCUSSION

It must be emphasized that the word "repair" as used in this discussion is an all-encompassing term. It includes both those processes which allow the cell to recover from radiation damage and those which allow the cell to prevent radiation damage. Radiation recovery may be due either to molecular repair processes similar to those outlined in the Introduction or to some other effect. Another effect which might result in recovery after irradiation is an increase in substances which act as radioprotectants. Examples of radioprotectants are compounds which act as radical scavengers (such as sulphur-containing compounds). Alternatively, the protective effect may be due to an increase in levels of an enzyme which is able to cope with radiation-induced radicals (such as superoxide dismutase). In any case, in this study it is not so much the source of the effect that is being studied, as the presence or absence of the effect itself.

This study uses the induction of recessive lethal X-linked mutations to probe the capacity of *P. redivivus* to recover from radiation-induced DNA damage. Recessive X-linked lethal mutations were chosen as an indicator for several reasons. Lethal mutations are more frequent than

viable visible mutations, they occur at large numbers of loci and can result from several different types of DNA lesions (Auerbach 1976). X-linked mutations were chosen over autosomal recessives because screening can be done in the first generation after irradiation (since males are hemizygous). These reasons, plus the fact that scoring of lethals is more objective than that of visibles, make X-linked lethal detection the best screen for this study.

In addition to recessive lethals, radiation can also produce dominant lethal effects. In Drosophila such effects have been attributed to the production of large deletions or the presence of unrestituted breaks (Auerbach 1976). Any dominant lethal mutation occurring in this study will affect both male and female progeny equally, since it is the chromosomal constitution of the sperm which determines the sex of the progeny. Thus, the measurement of recessive X-linked lethals will not be obscured by such an effect. That dominant lethality is occurring is shown by a drop in the number of female progeny per female from 57.9 in the control group to 44.7 after a single dose of 12924 rads (Table I).

As is the case with dominant lethals, differing sensitivities of cells during gametogenesis should not affect the measurement of recessive lethals, since this effect will be constant throughout the females tested. At the time of irradiation, the adult female contains germ

cells in varying stages of oogenesis up to and including metaphase I (Hechler 1970). Since each female will contain oogonia and oocytes in all of these stages, any difference in radiation sensitivity which may exist between stages will be constant from female to female.

Single doses of gamma radiation

The single dose data support the idea that P. redivivus can repair radiation-induced DNA damage. The radiation response pattern expected in the absence of repair is a linear increase in mutations with increasing dose. This linear pattern has been observed in many cell types or organisms, including repair-deficient Drosophila spermatozoa (Timofeeff-Ressovsky 1939, cited in Auerbach 1976). In Panagrellus the number of mutations per locus does not show a linear dose-effect relationship (see Fig. 3), suggesting that repair may be occurring.

When the data are examined in terms of mutagenicity per rad over a range of doses, the existence of a repair system becomes even more probable. Examination of Fig. 4 shows that the mutation rate decreases as dose increases. This may be due to a dose-dependent repair capacity. Alternatively, it may be indicative of an inducible repair system.

Fractionated doses of gamma radiation

Fractionation of a radiation dose into two exposures separated by a lag period can be used to study recovery from DNA damage. The rationale for such a procedure is as follows. If the damage generated by the first dose remains fixed in time, after the second exposure the net amount of damage produced will be equal to that observed when the same total dose is given in a single exposure. If, however, the damage resulting from the first dose is fully or partially repaired before the second dose is applied, the net damage will be less than that observed after a single exposure. In addition to repair of damage from the first dose, repair of the second dose may occur, decreasing the net damage even further.

Dose fractionation studies are commonly done with a constant first dose followed by a varying second dose (Elkind 1959). However, unequal split doses give an added amount of information (Malone _et al_. 1971, Purrott and Reeder 1978). If the recovery capacity is constant and unchanging, the number of mutations per locus should be the same for a low + high and a high + low dose regime (the result being a U-shaped curve when mutations are plotted against first dose). If the mutation yield from these two regimes is different, then there must be another aspect to the repair system. If Table III is examined, it can be seen

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that low + high and high + low fractionation schemes do not give the same results (although 4308+8616 rads and 8618+4308 rads are close). Correspondingly, the graph of mutations per locus (Fig. 5) does not give a U-shaped curve. Rather, the general trend is that as the initial dose increases both the mutation frequency and mutation rate increase (see Fig. 5 and Fig. 6).

There are several possible explanations for such a dose-response pattern after dose fractionation. The alternatives are as follows:

1). The amount of mutation is dependent on the first dose.

An increase in mutations per locus with increasing first dose would be observed if the repair system is itself damaged or inhibited as radiation dose increases. If the repair system can recover over a long time interval, this would not be detected after a single dose regime. At first glance, this may seem to contradict the dose-dependent repair suggested by the single dose data. In actual fact, it is conceivable that as dose increases both the repair capacity and the time required for that capacity to be fulfilled may increase. However, if the lag period used in this experiment (two hours) is not sufficient for recovery of the repair system, then a second dose could overload the repair system. Since both the extent of damage to and the recovery time for the repair system are dose-dependent, the

net effect would be an increased mutation frequency (decreased repair capacity) with increasing first dose.

This alternative involves a complex interplay of several phenomena. To explain the data in this manner, it is necessary to postulate the existence of three interacting factors: a dose-dependent repair capacity; a dose-dependent damage of the repair system; and a dose and time-dependent recovery of the repair system.

2). The amount of repair is dependent on the second dose.

Another possible explanation is that as the second dose increases, there is a dose-dependent increase in repair capacity. In this case, the damage of the first dose would be acted upon by a repair system. If the amount of repair associated with the initial dose given as a single dose is not completed by the time the second dose is administered, the remaining damage may be a signal for further (dose-dependent) stimulation of the repair system. The result would be a decrease in the observed mutation frequency as the second dose increases.

In actual fact, this explanation is only slightly different from the first. The major difference between the two is the question of which exposure determines the mutation frequency. Both alternatives require that a single repair system show more than one type of response.

3). There are two types of repair systems.

The major flaw in each of the above explanations is that they do not account for the fluctuation in the general response pattern that is observed between first doses of 4308 and 7539 rads (Fig. 5 and Fig. 6). Over this range, there is a sharp increase in mutation frequency (and rate) followed by a sharp decrease. To ignore these points would be a mistake, since the values are reproducible from trial to trial (see Table IV).

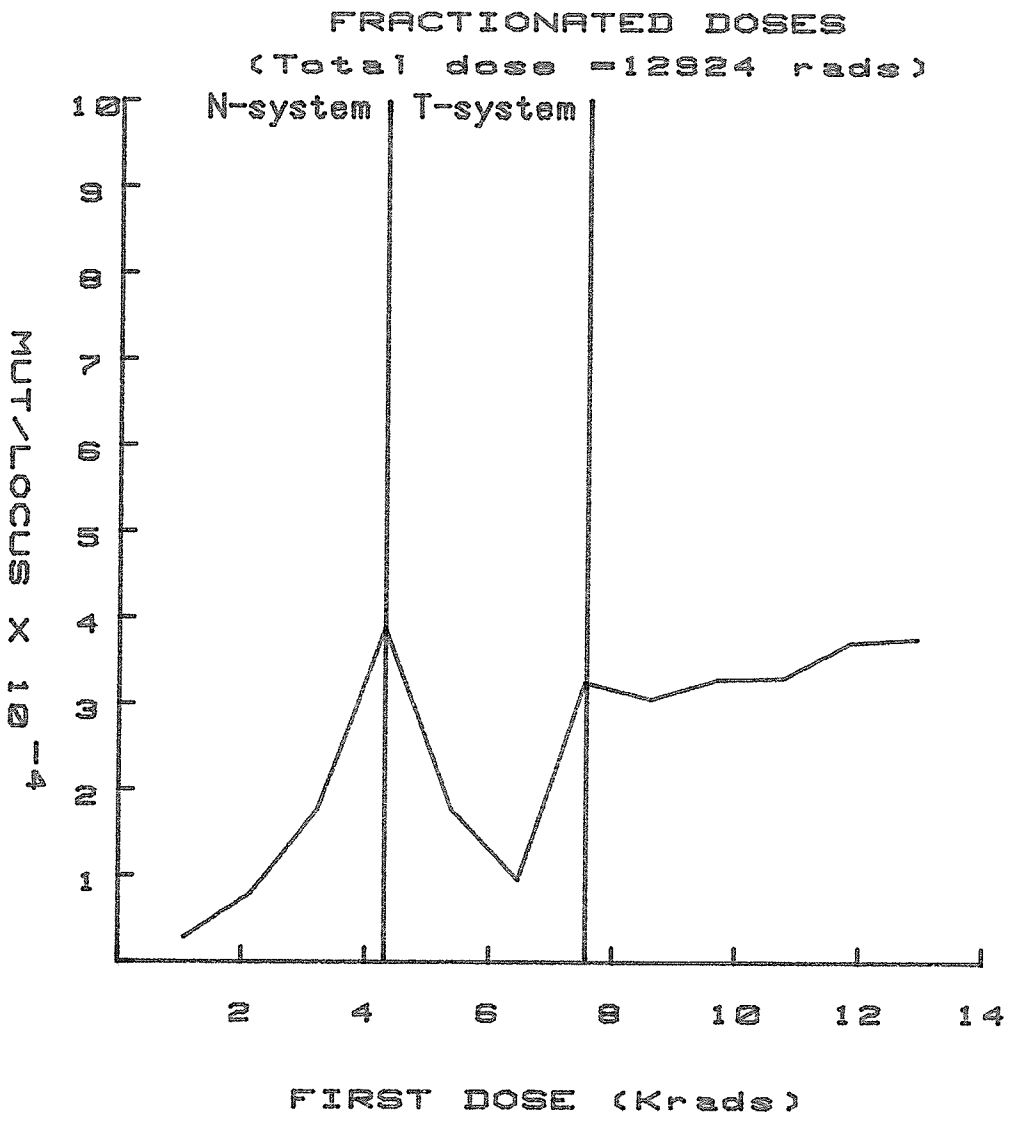
Such a fluctuation may be the result of the presence of more than one type of repair system. As outlined in the Introduction, it is known that there are several molecular repair processes. These repair processes interact to produce an overall radiation response pattern. The final effect observed in most organisms is either a single-hit or multi-hit survival curve, both of which are monotonic with respect to dose (Calkins 1967). However, non-monotonic response curves such as those obtained in this study have been observed in Tetrahymena pyriformis and Paramecium aurelia and are interpreted as being due to the interaction of two types of recovery systems (Calkins 1967). These two systems have been termed the 'N' and 'T' recovery systems and assigned the following properties (Calkins 1967). The N system repairs radiation damage after low doses. The T system does not operate after low doses and must be

activated by some stimulus. This stimulus is thought to be an accumulation of radiation damage. At very high doses, the radiation damage exceeds the capacity of the T system, and saturation is evident. The presence of these two systems is not apparent after single radiation doses, but fractionation appears to affect the threshold of the T system and the compound response curve manifests itself (Calkins 1977).

The response curves by Calkins are very similar to the curve shown in Fig. 5. If two repair systems exist in P. redivivus the pattern observed in the fractionation study can be explained if certain assumptions are made.

1. fractionation affects the T system threshold such that it is activated at a dose of approximately 4308 rads.
2. the T system is saturated in the region of 7539 rads.

Fig. 7 shows the proposed characteristics of the two systems with respect to the initial dose in a fractionated dose regime. The N system would deal with damage produced with doses of less than 4308 rads. The increase in mutations per locus with increasing dose up to 4308 rads represents the N system's decreasing ability to cope with the damage. Above 4308 rads the T system starts to function, causing an abrupt decline in mutations per locus.


FRACTIONATED DOSES
(Total dose =12924 rads)
N-system
T-system
MUT/LOCUS X 10^-4
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10
12
14
FIRST DOSE (Krads)

Above 6462 rads, the mutation frequency begins to increase until saturation of the T system occurs at about 7539 rads.

It is not possible at this point to suggest which of the known molecular repair mechanisms may constitute the T and N systems.

In this study, the scoring of recessive lethals was done on the basis of a deviation in sex ratio. The sex of the progeny was determined by phenotype. There are three things which could cause phenotype to be an unreliable indicator of sex. The first of these is reversion. If irradiation of an oocyte causes a reversion of the b7 mutation to wild-type, this will be manifested as a wild type phenotype in male progeny developing from that oocyte. However, this is highly unlikely, since b7 was induced by EMS and has proven to be fairly stable.

A second occurence which could affect the results is a deletion. If irradiation caused a deletion which eliminated the b7 phenotype and yet was not lethal, any male offspring receiving such a deleted chromosome would exhibit a wild-type phenotype. The b7 mutation is recessive, suggesting that it is either amorphic (complete absence of a product) or hypomorphic (reduced levels of a product). A deletion in such a mutation would presumably result in either extreme b7 or lethality. The only way a deletion in b7 could result in the wild-type phenotype being expressed

is if b7 is due to an addition (which would lead to a frameshift mutation). EMS does cause additions, but although b7 is fairly stable, the amount of spontaneous reversion observed is much higher than that associated with a frameshift.

The third event which could make phenotypic scoring of sex ratio unreliable is non-disjunction. As discussed in Materials and Methods, non-disjunction would result in wild-type male progeny and uncoordinated female progeny. However, if non-disjunction is occurring one would expect to get equal numbers of male and female non-disjunction products (if they have similar viabilities), and the net result would be no effect on the calculation of mutation frequencies and rates. However, studies with Drosophila have shown that non-disjunction frequencies are increased by exposure to radiation (Sobels 1980), and that more male than female non-disjunction products are produced (Mavor 1921,1922). To determine if this is the case with Panagrellus, studies were done on the effects of radiation dose on non-disjunction.

Non-disjunction frequencies do increase as radiation dose increases (see Table V). More importantly, the frequency of male non-disjunction products is greater than that of female non-disjunction products at any radiation dose (Table VII). However, the total frequency of non-disjunction is

relatively low and so should not drastically affect the results. This point is illustrated when the non-disjunction frequencies in Table V are applied to some of the single dose data. After correcting for non-disjunction (see Appendix III), the control sex ratio does not change, the sex ratio after 6462 rads changes from 0.99 to 0.98, and the sex ratio after 10770 rads changes from 0.91 to 0.90.

It is interesting to note that some of the exceptional male offspring were collected and mated to b7 females. Some of these males mated, producing both wild-type and uncoordinated progeny. However, the majority of these males did not produce offspring. This suggests that their phenotype may be the result of a gross chromosome abnormality which causes sterility. If this is the case, and another process in addition to non-disjunction is giving unexpected male offspring, this may be the source of the difference in the numbers of unexpected male and female progeny.

This possibility was examined to some extent when a study of the effect of paternal genotype on non-disjunction was performed. When jlc64 males were used as the parental line in a cross with b7 females, it was found that all the unexpected male progeny showed the phenotype of their paternal parent. This supports the idea that these males are the result of non-disjunction. However, the number of

females tested was not large and it is possible that wild-type male offspring may have been observed if the sample was larger.

The study of the effect of temperature on non-disjunction suggests that changes in temperature do not affect non-disjunction frequencies. However, the results are not conclusive.

This study has shown that *P. redivivus* has the capacity to repair gamma radiation-induced DNA damage, and that there are probably two repair systems working in this organism. Further studies will establish the nature and types of repair acting in *P. redivivus*.

APPENDIX I. HOMOGENEITY CHI-SQUARE

A homogeneity chi-square test is used to determine whether separate samples (or trials) are sufficiently uniform to warrant adding them together. The test is performed as follows:

1. A chi-square value is calculated for each individual trial, using the expected ratio of 50% male progeny:50% female progeny. The Yates correction factor is not used, since the individual chi-square values will eventually be summed.

2. The individual chi-squares are summed to give a total chi-square. Similarly, the degrees of freedom for each trial are summed to give a total value. The total chi-square value has two components: it is representative of both the departure of the data from the expected ratio, and the variation between trials.

3. A chi-square value is calculated for the pooled data. Again, the correction factor is not used, since this chi-square will later be subtracted.

4. The chi-square for the pooled data is subtracted from the total chi-square of the individual trials. At

the same time, the degrees of freedom are also subtracted. The resulting homogeneity chi-square value can be checked on a chi-square table to determine whether it represents a significant departure from chance.

APPENDIX II. TARGET THEORY

The effects of ionizing radiation on biological materials can be quantified by making use of target theory (reviewed by Maynard Smith 1968). Viewed simply, target theory proposes the existence of a number of "targets" in a cell or organism. A hit on any of these targets will cause a change - in this case, we are measuring lethality.

Suppose that each meiotic cell contains N targets, a hit on any one of which causes lethality (expressed at any point in development up to the L2 stage). If these cells are exposed to a dose of K particles, the probability that a particular target will be hit by a particular particle can be determined.

1. if the probability of a hit is p, then the probability that a particular target is not hit by a particular particle is 1-p.

2. the probability that a particular target is not hit by any of the K particles is $(1-p)^K$. It has been shown (Maynard Smith 1968) that if x is small and n is large, $(1-x)^n \simeq e^{-nx}$. Therefore,

$$(1-p)^K \simeq e^{-Kp}$$

3. if e^{-Kp} is the probability that a particular target is not hit, then the probability that none of the N targets is hit is

$$e^{-NKp}$$

Given the above, the proportion of survivors (S) will be equal to the probability that none of the targets is hit. That is,

$$S=e^{-NKp}$$

and

$$\ln S=-NKp$$

Once the data is gathered, this equation can be manipulated. The number of X-linked essential loci for development to the L2 stage (N) is 360 (Denich 1980). The experiment is designed to measure the survival of male progeny in comparison to that of female progeny. Assuming that an increase in X-linked lethal mutations is accompanied by a decrease in the number of males, S can be calculated as the ratio of male progeny to female progeny. The dose in rads (K) is known. A rad is a measure of absorbed radiation dose, and corresponds to an energy absorption of 100 ergs per gram (Hall 1978). The probability of a hit on a particular target can now be determined as

$$p=-\left(\frac{\ln S}{NK}\right)$$

APPENDIX III. APPLICATION OF NON-DISJUNCTION FREQUENCIES

The non-disjunction frequencies from Table V can be applied to the single dose data in order to determine the overall effect of non-disjunction. Using the data for a single dose of 10770 rads as an example, the correction is done in the following manner.

The frequencies of non-disjunction which were obtained after 10770 rads of gamma radiation are 1.920% wild-type males and 0.124% uncoordinated females. A single dose of 10770 rads resulted in a sex ratio of 0.91 (1922 male progeny: 2106 female progeny - see Table I). If the above non-disjunction frequencies are applied to the number of progeny, the number of male progeny becomes 1885 (1992-[.0192 x 1992]),and the number of female progeny becomes 2102 (2106-[.00124 x 2106]). The resulting sex ratio is 0.90.

APPENDIX IV. CALCULATION OF ERROR BARS

The error bars in Fig. 3,4,5 and 6 are calculated using the observed sex ratios at each dose and the corresponding homogeneity chi-square values. This is illustrated using two single dose data points:

1). If M=male progeny and F=female progeny, then R=M/F, and

$$\frac{\Delta R}{R} = \sqrt{\frac{\Delta M}{M} + \frac{\Delta F}{F}}$$
$$= \sqrt{\frac{1}{M} + \frac{1}{F}}$$

2). Examples:

1077 rads: $R=0.868$ $\Delta R/R=0.273$ $\Delta R=0.024$ $R \pm \Delta R=0.868 \pm 0.024$

The corresponding sex ratios are 0.844 and 0.892. These can be converted to mutations per locus and mutations per locus per rad as described in Appendix II.

3231 rads: $R=0.939$ $\Delta R/R=0.0265$ $\Delta R=0.025$ $R \pm \Delta R=0.939 \pm 0.025$

3). The homogeneity chi-square values for 1077 rads and 3231 rads are examined.

As a general rule, if the homogeneity chi-square value divided by the degrees of freedom is less than or equal to 1, the above correction factor is applied. If, however, the

chi-square value divided by the degrees of freedom is greater than 1, then a new correction factor is calculated as

$$(\Delta R)(\text{ chi-square value})$$

In the examples cited, the correction factor for 1077 rads remains the same (since 6.443/7 is less than 1). However, the correction for 3231 rads must be altered, since 5.997/2 is greater than 1. The new correction factor is calculated as (0.025)(5.997)=0.061.

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