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ESTIMATE OF UNOBLIGATED BALANCES

No unobligated balances are anticipated at the end of the current budget period.

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TABLE OF CONTENTS

Title Page 1

Table of Contents 2

Introduction 3

Molecular Studies 4
1. Molecular Characterization of the "Null" *Sod*^{CA1} Allele 4
2. Molecular Characterization of Transformed N → S Sod .. 5
3. Error Rate of the Polymerase Chain Reaction 6
4. Exon/Intron Organization of the Cu,Zn Sod gene 7
5. G + C Content and Codon Use 9
6. Molecular Variation of *Sod* alleles 11

Radio-Resistance Studies 11
Strains and Crosses 11
Irradiation Treatment 12
7. Stage-3 Oocytes 12
7a. Germ Cell Dominant Lethals 12
7b. Larval Dominant Lethals 14
7c. Sex-Linked Recessive Lethals 14
8. Stage-7 Oocytes 15
8a. Germ Cell Dominant Lethals 15
8b. Larval Dominant Lethals 16
8c. Sex-Linked Recessive Lethals 17
9. Stage-14 Oocytes 18
9a. Germ Cell Dominant Lethals 18
9b. Larval Dominant Lethals 19
9c. Sex-Linked Recessive Lethals 20
10. First-Instar Larvae 20
10a. Viability Dominant Lethals 21
10b. Aging Dominant Mutants 21
11. Third-Instar Larvae 21
11a. Viability Dominant Lethals 21
11b. Fertility Dominant Mutants 22
12. Adult Females: Aging 23

Natural Selection 24
13. Overdominance 25
14. Overcompensation and Frequency-Dependent Selection ... 27

Publications Supported by DOE Grant ER60713 (1990-1992) ... 29

References Cited 30

INTRODUCTION

We proposed an investigation of the gene coding for Cu,Zn superoxide dismutase (*Sod*) in *Drosophila melanogaster* seeking to understand the enzyme's role in cell protection against ionizing radiation. Components of the investigation include molecular characterization of the gene; measuring the response of different genotypes to increasing levels of radiation; and investigation of the processes that maintain the *Sod* polymorphism in populations.

Two alleles, S and F, are commonly found at the Sod locus in natural populations of *D. melanogaster*; in addition we have isolated from a natural population a "null" (CA1) mutant that yields only 3.5% of normal SOD activity. The S, F, and CA1 alleles provide a model system to investigate SOD-dependent radioresistance, because each allele yields different levels of SOD, so that S > F >> CA1. The radioprotective effects of SOD can be established by showing protective effects for the various genotypes that correspond to those inequalities. Because the allele variants studied are derived from natural populations, the proposed investigation avoids problems that arise when mutants obtained my mutagenesis are used. Moreover, each allele is studied in multiple genetic backgrounds, so that we correct for effects attributable to other loci by randomizing these effects.

The superoxide anion O_2^- is generated during cell respiration as well as during exposure to ionizing radiation (Fridovich, 1975; Smith and Hays, 1968; Van Hemmen, 1971). Organisms have evolved different mechanisms to protect against the deleterious effects of reduced oxygen species. The copper-zinc superoxide dismutase (Cu,Zn SOD, referred to hereafter simply as SOD) is a eukaryotic cytoplasmic enzyme that protects the cell by scavenging superoxide radicals and dismutating them to hydrogen peroxide and molecular oxygen: $2O_2^- + 2H \rightarrow H_2O_2 + O_2$ (Fridovich, 1978). SOD had been shown to protect against ionizing radiation damage to DNA, viruses, bacteria, mammalian cells, whole mice, and *Drosophila* (Peng et al., 1986, and references herein).

We present a brief summary of our results. Some results have been published (or are in press) and additional details can be found in the publications. The results are reported under 14 headings that encompass research supported by this DOE grant.

MOLECULAR STUDIES

1. Molecular Characterization of the "Null" *Sod*^{CA1} Allele

In the process of selecting for low-activity *Sod* genotypes, we obtained a "null" (N) allele from a collection of *D. melanogaster* flies from Davis, California. Flies homozygous N/N have enzymatic activity about 3.5% of the F/F homozygotes. The N allele is, therefore, useful for assaying the radioprotective and other physiological roles of SOD, because together with the two commonly occurring alleles, *F* and *S*, provides a three level gradation of enzymatic activity: S/S > F/F >> N/N. In *Drosophila* studies, null alleles are typically obtained by mutagenesis, which induces other mutations besides the targeted ones and, hence, handicaps the attribution of particular effects to the mutated locus. Such problem does not arise with the N allele.

We constructed an EMBL3 phage genomic library of *D. melanogaster* N/N flies. We isolated 4 positive EMBL3 clones, which after digestion of their DNA with EcoRI proved to contain a 2.5 kb EcoRI fragment hybridizing to a *D. melanogaster* SOD cDNA probe. This fragment was subcloned into pGEM4Z plasmid and restriction mapped. The clone exhibits a 0.7 kb-long foreign DNA stretch, starting at position around 400, when compared to a wild type SOD region of *D. melanogaster*.

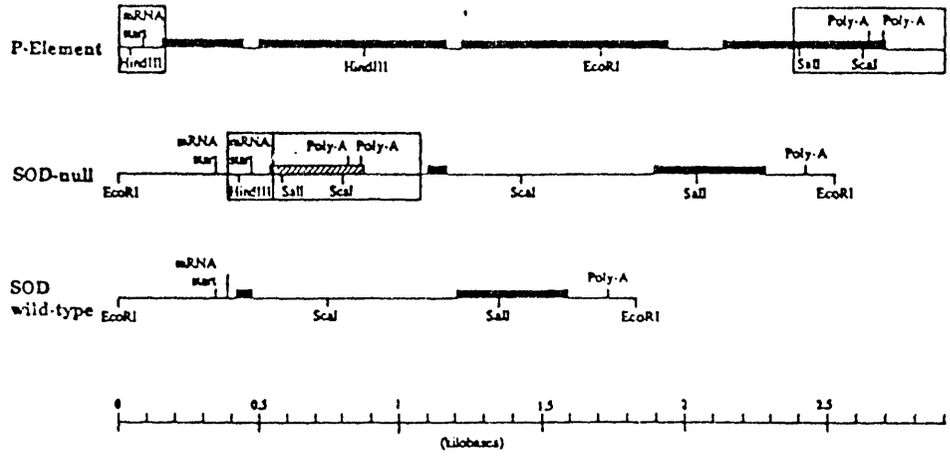
Outside this insertion, only one dfference occurs between the SOD "null" clone and wild type SOD from a Canton-S strain. This point mutation, a substitution of cytosine in the wild type gene by adenine in the "null," is in the translated region of the second exon and gives rise to a replacement of Asn by Lys at position 96 of the mature protein. Thus the protein encoded by the SOD^{CA1} is the same one encoded by the *S* allele, commonly found in natural populations.

The 0.68 kb insert in the SOD locus of the SOD^{CA1} strain proved to be the truncated P element derived from an autonomous 2.9 kb P element by internal deletion, as identified by computer-assisted sequence analysis. It contains 31 bp inverted repeats at both ends and creates an 8 bp duplication of a target sequence. The deletion starts and ends at positions 163 and 2389 of a complete 2.9 kb P element, bringing together the first and the fourth exons of transposase (Figure 1). However, a deletion event within the fourth (TGC) codon of the transposase changes it into a stop codon (TGA), leaving the last open reading frame without ATG as a starting codon and thus possibly preventing the translation of a truncated transposase.

The sequence data provided account well for the diminished level of Cu,Zn SOD expression in the SOD^{CA1} strain of *D. melanogaster*. The postulated mechanism seems to involve a decrease in transcription by providing additional polyadenylation signals

upstream from the translated region of the SOD gene due to the insertion of the truncated P element. We are investigating this mechanism by hybridization analysis of mRNA from the SOD "null" strain.

Figure 1



The organization of the Cu,Zn SOD locus in the SOD^{CA1} strain of *D. melanogaster*. Dark boxes represent coding regions. The cross-hatched bar is the portion of the 4-th P element ORF. Transcription start points and polyadenylation signals are indicated.

2. Molecular Characterization of Transformed N → S Sod

The "null" *Sod* allele was separately introduced into 18 different *D. melanogaster* lines, derived from wild-collected females. These lines were prepared for testing the radioprotective role of Sod in the experiments reported below. The lines used in these experiments are examined from time to time by starch gel electrophoresis in order to ascertain that they retain the proper SOD genotype (and are not contaminated). During a routine set of tests, we observed that several N lines were apparently contaminated, since some or all of the flies tested exhibited the S electrophoretic pattern. We were able to ascertain, however, that the lines were not contaminated by *S* individuals, but rather that the 0.68 kb truncated P-element that characterizes the *CA1* allele had excised spontaneously—and independently in these lines.

By PCR amplification (and subsequent sequencing) using primers derived (1) from the SOD (wild-type) gene and (2) from the P element we were able to show that the *CA1* allele was still present in the transformed lines. The frequency of the *CA1* allele in the lines was, however, low enough so that in some lines all individuals (typically, about 10) assayed by electrophoresis for each line were either *S/S* or *S/N* (both of which give the *S* electrophoretic pattern). Amplification using only SOD specific primers produced in each tested line a Southern band characteristic

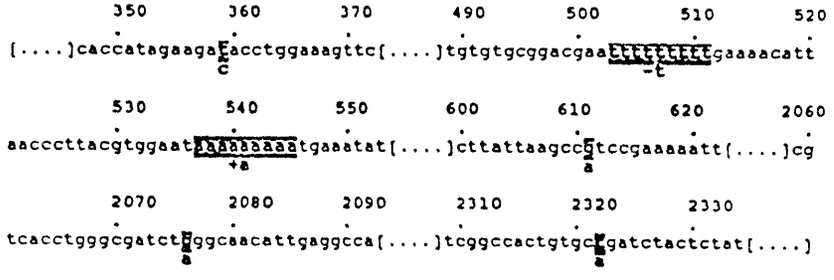
of the wild-type SOD. The revertant nature of this allele was shown by sequencing the insertion-bearing portion of the gene. The revertant gene retains, at the P-element insertion site, 9 bp of the inserted fragment, six (CATGAT) from the 5′ end of the insertion and three (GAT) from the 3′ end.

3. Error Rate of the Polymerase Chain Reaction

The *Taq* PCR reaction is a rapid means for DNA amplification, with many applications in genetics and evolution. The fidelity of the reaction is, however, a case of concern since the *Taq* polymerase has not detectable 3′ → 5′ proofreading exonuclease activity. The error rate of the reaction is, in particular, significant for investigations of genetic polymorphisms, in which DNA sequence variation is a relevant parameter.

We have used the 2.5 kb EcoRI fragment containing the *Sod* null allele for testing the PCR rate of error as follows. We amplified this fragment by six separate PCR reactions and cloned in a pUC19 plasmid and sequenced one fragment from each reaction. We have sequenced a total of 13,513 bp (1,658 bp from one clone, 2,293 from another, 2,388 from each of two more, and 2,393 from each of the last two). Compared with the sequence previously obtained from directly cloned genomic DNA, two of these sequences exhibit no differences, the third exhibits a 1-bp deletion (at about 505), the fourth a transition (G → A at 611), the fifth a transversion (T → A at 2322), and the sixth two transitions (T → C at 359 and G → A at 206) and a 1-bp insertion (at about 540) (Figure 2). The frameshifts are both associated with oligomers of the base inserted or deleted. The cumulative frequency of observed errors per nucleotide is 3.0×10^{-4} substitutions and 1.5×10^{-4} frameshifts.

Figure 2



Substitutions and additions/deletions induced in six amplifications of 2,533-bp fragment comprising Sod^{CA1} gene. Changes are shown below the nucleotide sequence of relevant segments. Two single-base frameshifts (at 503–512 and 537–545) and one transition (611) occur in the inserted P-element fragment, which extends from 393 to 1073; one transition (359) is 13 bp downstream from the start of transcription, and another (2322) is 33 bp downstream from the end of the coding sequence; the only transversion (2076) is within the second exon coding sequence (CTG→CTA, both coding for leucine).

The error rate of incorporation of the PCR can be estimated from the observed error frequency by taking into account the number of doubling cycles, according to the formula (2 x observed error frequency) / (number of cycles). We amplified the fragment for 40 cycles. The incorporation error rate per nucleotide may then be estimated as 1.5×10^{-5} for substitutions and 0.7×10^{-5} for frameshifts.

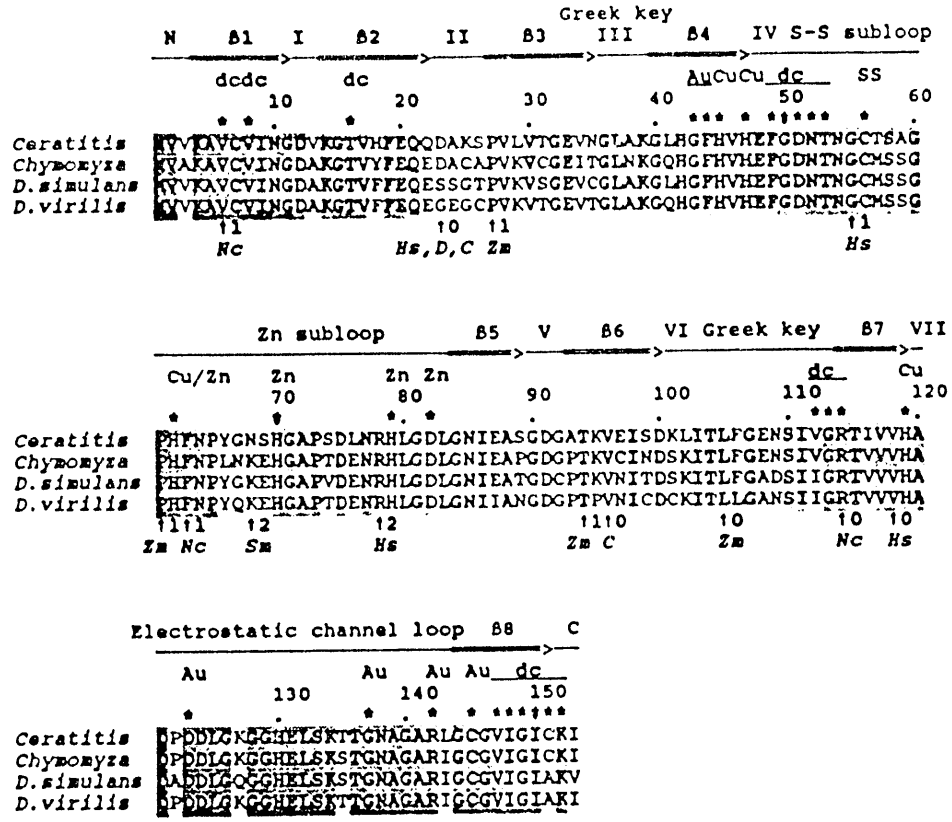
It may very well be that this rate of experimental error may be acceptable in many types of study. But we have proposed that whenever the nucleotide sequence of the amplified fragment must be unambiguously known, three separate amplifications would likely yield the correct sequence at all sites (Kwiatowski et al., 1991). Two of these products should be completely sequenced, but only the discordant sites need to be sequenced in the third. The probability that two amplifications will yield the same error at the same site is $< (4.5 \times 10^{-4})^2$, or $< 2 \times 10^{-7}$. If the primers are available, three separate amplifications of a genomic DNA fragment that are followed by getting one clone and one sequence from each of the three products will usually require less effort and cost than obtaining the DNA sequence by the alternative method of making a genomic library and then cloning and sequencing the genomic DNA fragment of interest.

4. Exon/Intron Organization of the Cu,Zn Sod gene

The *Sod* gene of *D. melanogaster* consists of two exons separated by one intron (see Figure 1 above). This same intron/exon structure occurs in other *Drosophila* species (we have in our laboratory sequenced the *Sod* gene in more than 20 species). The two genera, *Chymomyza* and *Scaptodrosophila*, closely related to *Drosophila*, as well as the farther related medfly, *Ceratitis capitata*, all exhibit a second intron, which separates the codons for amino acid residues 95 and 96 in the encoded polypeptide (Figure 3). The conclusion is warranted that this second intron became deleted shortly after the divergence of the genus *Drosophila* from other dipterans, and before the radiation of the Sophophora and Drosophila subgenera (Kwiatowski et al., 1992).

The origin and evolution of introns has been subject of much debate. Although there is evidence that group I as well as group II introns can integrate into genes, the prevailing view is that introns have existed from the start of protein evolution, and even that they may derive from the early "RNA world" that preceded the appearance of DNA organisms (Doolittle, 1987; Gilbert, 1987). The discovery that group I introns are shared by chloroplasts and cyanobacteria (Kuhsel et al., 1990; Xu et al., 1990) supports the existence of introns at least by the time of the eukaryote/prokaryote split. The early occurrence of introns is also supported by the intron distribution of some genes, such as triosephosphate isomerase (Gilbert et al., 1986).

Figure 3



Amino acid sequence alignment of the Cu,Zn SOD polypeptide in four dipteran species: *Ceratitis capitata, Chymomyza amoena, Drosophila simulans,* and *D. virilis*. Structural features of the protein are represented above the sequences: arrows represent the eight β strands, lines represent the random-coil loops (I-VII). Residues with significant functions are marked with asterisks and their functions indicated by two-letter symbols as follows: active site metal-ligand (Cu or/and Zn); active site auxiliary function (Au); disulfide bridge (SS); dimer contact (dc) (Getzoff et al., 1983; see also Getzoff et al., 1992). The symbols below the sequences refer to the introns: position (small arrows), site within the coding triplet (numbers), and species, humans (*Hs*), fluke (*Sm*), maize (*Zm*), *Neurospora (Nc)*, *Drosophila* species (*D*), other dipterans (*C*).

The evolution of *Cu,Zn Sod* manifests the occurrence of both insertions and deletions of introns at different positions in the coding sequence. Figure 3 indicates (arrows below the sequence) the position of introns in five distantly related organisms: *Homo sapiens* (*Hs*; data of Levanon et al., 1985), dipterans (*Drosophila* species, *D*; Kwiatowski et al, 1989a,b; Kwiatowski and Ayala, 1989;

Ceratitis capitata and *Chymomyza amoena*, *C*; this paper and our unpublished results), *Schistosoma mansoni* (*Sm*; Simurda et al., 1988); *Zea mays* (*Zm*; Cannon, personal communication), and *Neurospora crassa* (*Nc*; Chary et al., 1990).

Evidence for intron insertion derives from the observation that all 13 intron sites are unique to each type of organism, except for the site between amino acids 22 and 23, which is shared by humans and dipterans. In order to avoid postulating that intron insertions have occurred, one would have to postulate an ancestral gene with numerous introns, which seems extremely unlikely on the face of the evidence at hand (plus the likelihood that more intron sites will yet be discovered as the variety of organisms studied increases). One must, of course, consider the possibility that some intron sites are homologous but have changed their position in the amino acid sequence through evolution. Some intron sites are closely located: *Neurospora* and maize at 61 and 63; *Ceratitis* and maize at 94 and 96. But it seems unlikely that these are homologous, i.e., that they are derived from a preexisting intron located at a particular position accompanied by the insertion/deletion in one or both organisms of one (or two) amino acids on either side of the intron (Kwiatowski et al., 1992). In any case, intron insertions must have occurred at sites that are far removed from each other, unless we postulate the presence of very many introns in the ancestral gene. The dipteran data obtained in our laboratory support the evolutionary deletion of an intron site in the *Sod* gene.

5. G + C Content and Codon Use

Table 1 gives the G + C composition of the Cu,Zn *Sod* gene in *D. melanogaster* and two other dipterans. The G + C content is higher in *melanogaster* for all comparisons. The differences are greater for the third codon positions: G + C content is 40.0% on the average for *Chymomyza* and *Ceratitis*, but 74.7% in *D. melanogaster*, a proportional increment of 87%. The use of codons ending with G, for example, is 46% in *D. melanogaster*, but only 15% in *Chymomyza*.

Since the proportional increment in G + C use in *Drosophila* relative to the other two is three times larger for the third position than for all three, it is apparent that G + C use is similar, on the average, in both sets of species with respect to the first two codon positions. Table 1 (bottom row) shows that the strong bias favoring codons that end with G or C in the *Cu,Zn Sod* gene of *D. melanogaster* is also the case with respect to 412 other genes in this species.

Table 1. G+C Content (percent) in the *Cu,Zn Sod* gene of three dipteran species and in other genes of *D. melanogaster* and *C. capitata*.

Species	Coding Sequence		Introns	
	All sites	3rd sites	First	Second
Ceratitis capitata	47.4	41.6	27.8	22.7
Chymomyza amoena	45.7	38.3	32.2	17.2
Drosophila melanogaster	60.2	74.7	35.9	—
Other genes:				
C. capitata (four genes)[a]	—	46.60	—	—
D. melanogaster (412 genes)[b]	48.51	63.29	—	—

[a]Calculated from Rina and Savakis (1991) as the average for the four genes. [b]Calculated from GeneBank 63, produced by J. Michael Cherry.

The data just reviewed are consistent with the genome hypothesis of Grantham et al. (1980, 1981) that there is a species-specific bias in codon preferences (see also Sharp et al., 1988). Although the Cu,Zn SOD polypeptide is too short for drawing any definite conclusions, our data are consistent with the analysis of Starmer and Sullivan (1989) showing that codon use has a gene-specific component in addition to the species-specific component. The data in Table 1 taken at face value suggest that both components of codon preferences can evolve rapidly (see also Riley, 1989). The preference for synonymous codons ending with G or C is much higher in *D. melanogaster* than in *C. capitata* genes (63.3% vs. 46.6%, a relative increment of 36% in overall third-position use), two species that diverged no more than 100 million years ago. Notice that *Drosophila* and *Chymomyza* are more closely related to each other than either is to *Ceratitis*. The pattern observed in Table 1 suggests that *Ceratitis* and *Chymomyza* represent the ancestral preferences, which have, however, evolved in *Drosophila*. The evolution of the *Drosophila* preferences must have occurred rapidly after the divergence of the *Chymomyza* and *Drosophila* lineages, since they are shared by all other *Drosophila* studied in our lab.

Codon preferences are quite similar in all three dipterans (and other *Drosophila* species). However, in some cases, such as alanine and glutamine, there are differences between the *Drosophila* species as a set and the other two species; for example, all *Drosophila* species use GAG seven out of eight times for glutamine whereas *Ceratitis* uses this codon only one out of seven times and *Chymomyza* five out of nine times. In other cases, such as leucine,

there is considerable variation across all species. (For additional details, see Kwiatowski et al., 1992, in press.)

6. Molecular Variation of *Sod* alleles

As a follow-up of some of our studies of the *Sod* gene sponsored over a number of years by DOE, we presented a research proposal to NIH concerning allelic variation and rate of evolution of *Sod*. This proposal was funded starting July 1, 1991. One exciting result is that all S ("slow") alleles (sampled from three distant localities in two continents) are identical in sequence (whereas about 5% of nucleotide sites are polymorphic among F alleles). This and related results will not be reported here, but are noted because they motivate the focus of the research proposed for the next three years for the renewal of this grant. The molecular identity of the S alleles is, as far as we know, a unique finding in any organism to date. It indicates that the S allele has rapidly spread by natural selection and opens up for investigation a host of genetic and evolutionary questions.

RADIO-RESISTANCE STUDIES

The S and F *Sod* alleles are a naturally occurring polymorphism (which various sources of evidence indicates is maintained by natural selection). We proposed to investigate the response of different *Sod* genotypes to ionizing radiation.

Previous experience indicates that genetic differences in radioresistance exist among wild-type strains. The experiments were therefore designed to differentiate the effects due to (1) different *Sod* genotype; (2) genetic background. With respect to both, we proposed to investigate variation due to (1) life-cycle stage irradiated; (2) biological consequences for each irradiated stage; (3) different levels of irradiation.

The data accumulated are very large. Only a brief summary will be given below. No statistical analyses have been performed (except for some simple calculations, such as mean, variance, and the like), nor will we develop here any overarching interpretation of the results.

Strains and Crosses

We collected a large sample of *D. melanogaster* flies in Culver City, California, in which the frequency of the two common SOD alleles is 0.825 for F and 0.175 for S. The wild-collected females were individually placed in culture vials. Single-pair matings were made with their progenies and the SOD genotype of the mating pair ascertained after egg-laying. The process was repeated for seven generations for all lines, at which time 18 F/F and 18 S/S homozygous strains were chosen for the experiments, each one of the

36 lines derived from a different inseminated female collected in nature. (18 CA1/CA1 experimental strains were obtained by crossing the CA1/CA1 stock to each of 18 F/F strains for three generations. Notice that the CA1 allele codes for a "slow" allozyme that exhibits only about 3.5% as much activity as the wild-type slow allozyme.) All 54 experimental strains are homozygous for the standard 3-chromosome arrangement.

The eggs, larvae, and flies used in the experiments are prepared for each of the SOD genotypes in a similar way as will be herein described for the F/F homozygotes. The 18 F/F strains are symbolized as 1F, 2F, ... 18F. A set of crosses are first made as follows: ♀1F x 2F♂, ♀3F x 4F♂, etc. The F_1 progenies (symbolized 1F·2F, 3F·4F, etc.) are then intercrossed as follows: 1F·2F♀ x 3F·4F♂; 5F·6F♀ x 7F·8F♂; etc. Crosses are made so that in a particular experimental block any one of the original 18 strains is represented only once among the experimental organisms. These, of course, carry two genomes each derived from different original strains and, hence, are similar to wild organisms in that they are produced by the combination of two genomes randomly selected from the natural population.

Irradiation Treatment

All irradiation is administered with a "Model M Gammator," with Cesium 137 as the source. The irradiation is at a rate of 1 kR per 80 seconds following the specifications provided by the manufacturer's manual. The maximum dosage is 7.5 kR, requiring 10 min. exposure. 3-4 days old adult females are irradiated in vials, each with 20-50 individuals; larvae are placed in Petri dishes, each with 250-500 larvae. A piece of filter paper soaked in sucrose is enclosed in the container.

Immediately after irradiation the larvae are transferred to vials with food in groups of (typically) 40 per vial. The irradiated females are immediately crossed to non-irradiated males and placed in bottles in groups of (typically) 5 per vial. Eggs are collected from Petri dishes attached to the open-end of the bottle. Eggs are collected after 0-24 hours (which we will refer to as oocyte "stage 14"), 25-48 h ("stage 7"), and 49-72 h ("stage 3") (Bownes and Dale, 1982).

Experiments in any one day, any batch of food, etc., are always carried out with equal representation of the S/S and F/F genotypes, so that experimental uncontrolled variation will equally affect both genotypes (i.e., so as to randomize the experimental error between the two *Sod* genotypes).

7. Stage-3 Oocytes

7a. Germ Cell Dominant Lethals. The summary characteristics of the experiments are:

- Five genetic backgrounds (strain combinations) for each (S/S and F/F) *Sod* genotype.
- Eight irradiation levels: 0 kR (control), 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, and 7.5 kR.
- 10-16 replications for each irradiation level x genetic background x Sod genotype.
- 5 egg-laying females per replication.
- Approximately 150,000 eggs counted.

Figure 4

FERTILITY OF IRRADIATED FEMALES 48–72h AFTER IRRADIATION. Number of eggs laid by 5 females. The vertical bars are the standard errors.

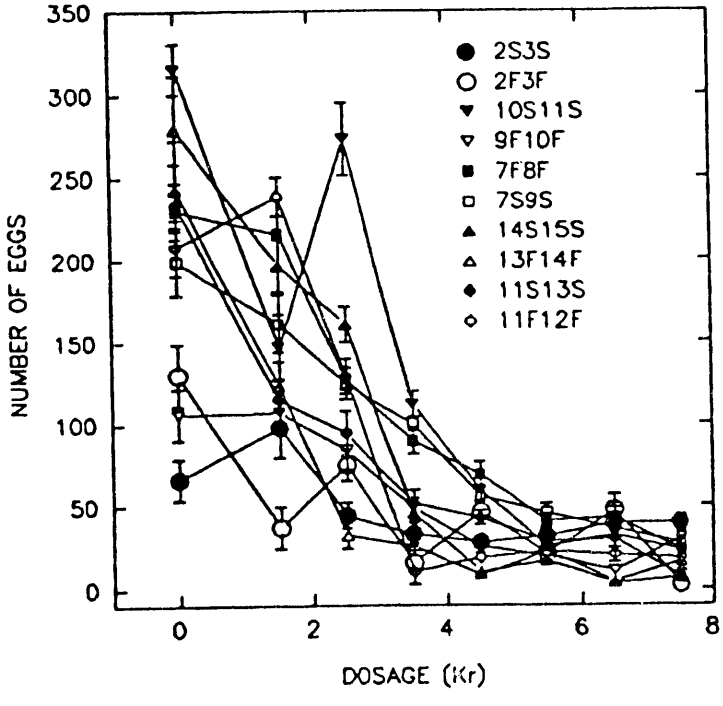
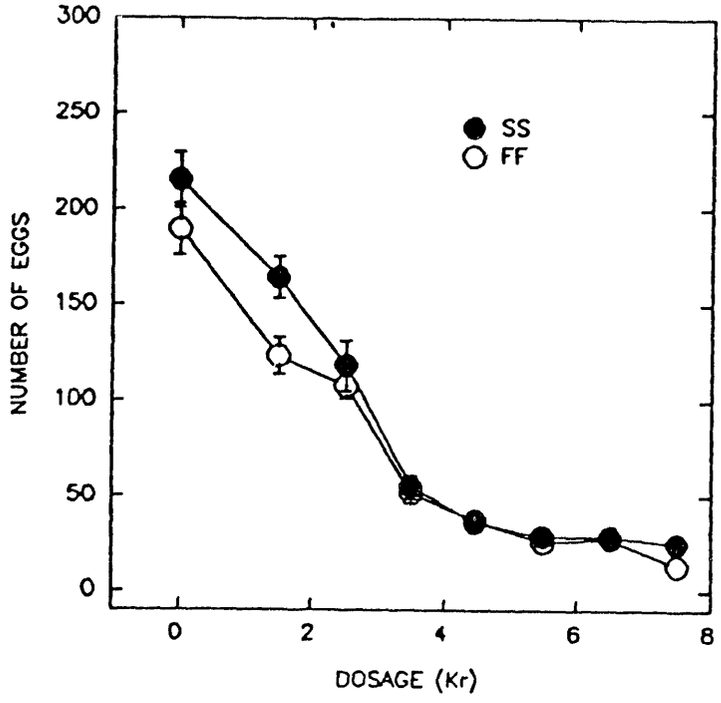


Figure 4 shows (for each of 10 genetically different lines and 8 irradiation levels) the average numbers of eggs laid by 5 females that were irradiated 48-72 hours earlier. Figure 5 averages the genetic backgrounds. The incidence of induced dominant lethals is, on the average, lower for S/S than for F/F (e.g., the incidence of lethals, for S/S and F/F respectively, is at 1.5 kR 23.6% and 35.0%; at 7.5 kR is 88.3% and 93.1%). This result is consistent with the hypothesis that SOD protects against ionizing radiation and with the observation that specific activity is S/S > F/F.

Figure 5

FERTILITY OF IRRADIATED FEMALES 48–72h AFTER IRRADIATION. Number of eggs laid by 5 females. The vertical bars are the standard errors.



7b. Larval Dominant Lethals. The summary characteristics of the experiments are:

- Four genetic backgrounds for each, S/S and F/F
- Eight irradiation levels 0 - 7.5 kR
- 20-50 replications
- 20-100 eggs per replication
- Approximately 150,000 eggs counted
- Approximately 50,000 emerging adults recorded.

Figure 6 shows the average percent of adults emerging for the two *Sod* genotypes. The percent larvae mortality is, for S/S and F/F respectively, 40.9 and 29.7 at 1.5 kR; 98.3 and 97.5 at 7.5 kR.

7c. Sex-Linked Recessive Lethals. The summary characteristics are the same as for 7b. The observations are the proportions of males and females among the emerging adults. The results for all replicates and all genetic backgrounds are summarized in Table 2.

Figure 6

EGG-TO-ADULT SURVIVAL OF EGGS LAID BY IRRADIATED FEMALES 48-72h AFTER IRRADIATION.
The vertical bars are the standard errors.

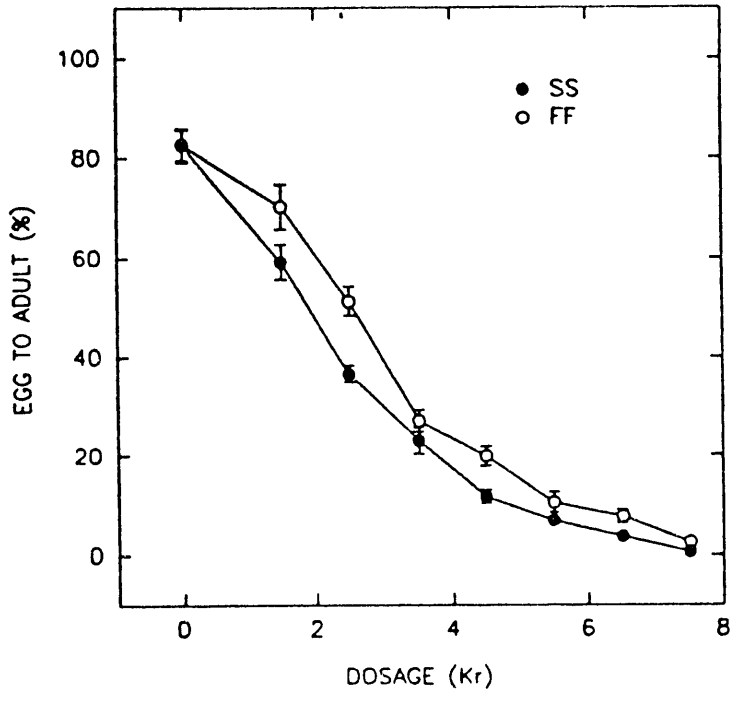


Table 2. Percent males emerging as adults from eggs irradiated as stage-3 oocytes.

	kR							
Sod genotype	0	1.5	2.5	3.5	4.5	5.5	6.5	7.5
F/F	46.2	41.8	36.7	41.0	54.3	51.2	43.4	7.7
S/S	49.0	51.1	45.6	52.5	53.5	50.0	47.5	37.5

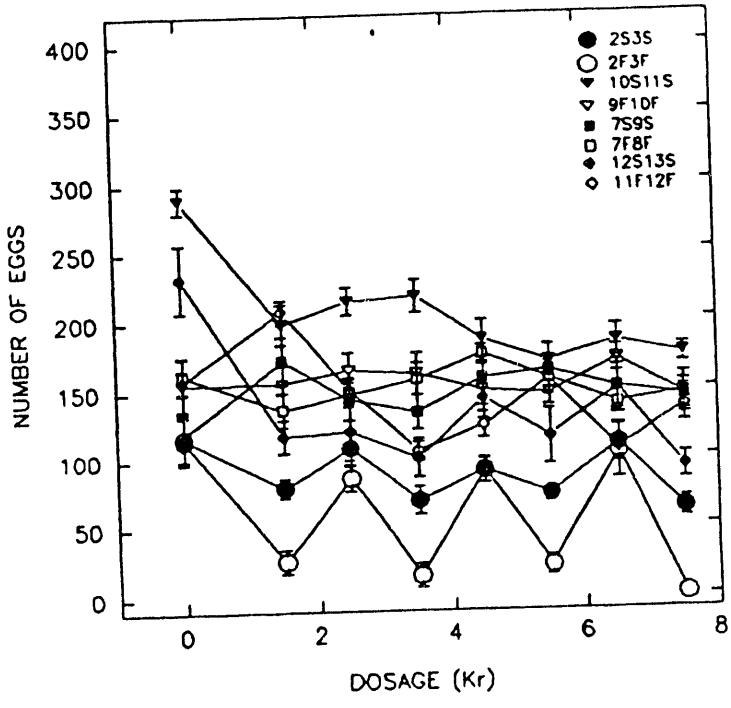
8. Stage-7 Oocytes

8a. Germ Cell Dominant Lethals. The summary characteristics of the experiments are:

- Four genetic backgrounds for each, S/S and F/F *Sod* genotypes
- Eight irradiation levels, 0-7.5 kR
- 10-16 replications for each background x genotype x irradiation level
- 5 egg-laying females per replication
- Approximately 130,000 eggs counted.

Figure 7

FERTILITY OF IRRADIATED FEMALES 24–48h AFTER IRRADIATION. Number of eggs laid by 5 females. The vertical bars are the standard errors.



The average numbers of eggs laid by all replicates for each given genetic background and radiation level are shown in Figure 7. The averages over genetic backgrounds for the S/S and F/F genotypes are shown in Figure 8.

Comparison between Figures 5 and 8 (or Figures 4 and 7) shows that incidence of germ-cell dominant lethals is much lower for stage-7 than for stage-3 irradiated oocytes. There is indication that the *S/S* genotype provides better radio-protection than the *F/F* genotype. The incidence of dominant germ-cell lethal mutations is, for S/S and F/F respectively, 3.1 and 19.5% for 1.5 kR, and 30.5 and 42.6% at 7.5 kR.

8b. Larval Dominant Lethals. The summary characteristics of the experiments are:

- Four genetic backgrounds for each, S/S and F/F
- Eight irradiation levels, 0 - 7.5 kR
- 7-10 replications
- An average of 30 eggs per replication
- Approximately 60,000 eggs counted
- Approximately 30,000 emerging adults recorded.

Figure 8

FERTILITY OF IRRADIATED FEMALES 24–48h AFTER IRRADIATION. Number of eggs laid by 5 females. The vertical bars are the standard errors.

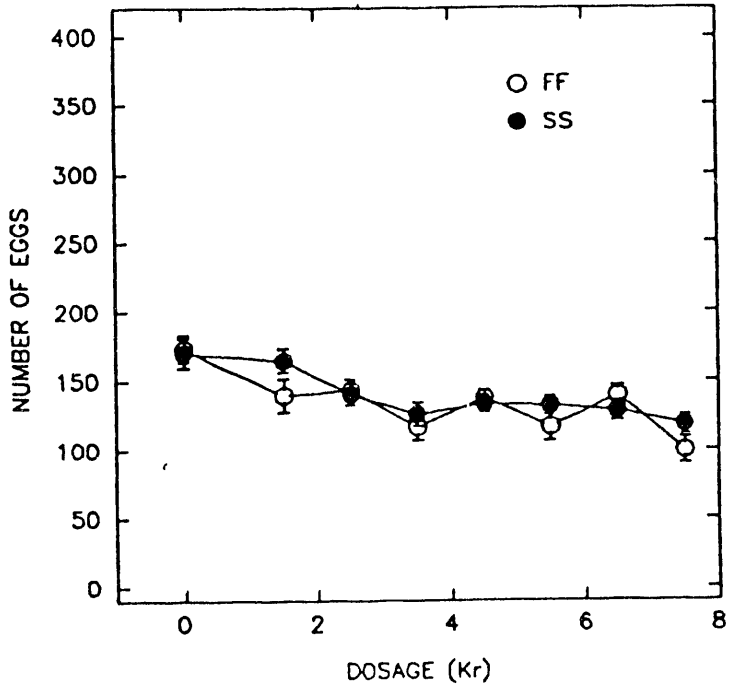


Figure 9 shows the percent of adults emerging, on the average for the two *Sod* genotypes. The percent larval mortality is, for S/S and F/F respectively, 14.9 and 22.5 for 1.5 kR, and 96.6 and 94.1 for 7.5 kR.

Notice that the incidence of larval lethals increases rapidly with irradiation dosage. This is also the case for stage-3 oocytes for both germ-cell and larval lethals. However, for irradiated stage-7 oocytes, the incidence of dominant germ-cell lethals increased at a much slower rate (Figs. 7 and 8).

8c. Sex-Linked Recessive Lethals. The summary characteristics are the same as for 8b. The observations are the proportions of males and females among the emerging adults. The overall results are summarized in Table 3.

Figure 9

EGG-TO-ADULT SURVIAL OF EGGS LAID BY IRRADIATED FEMALES 24-48h AFTER IRRADIATION.
The vertical bars are the standard errors.

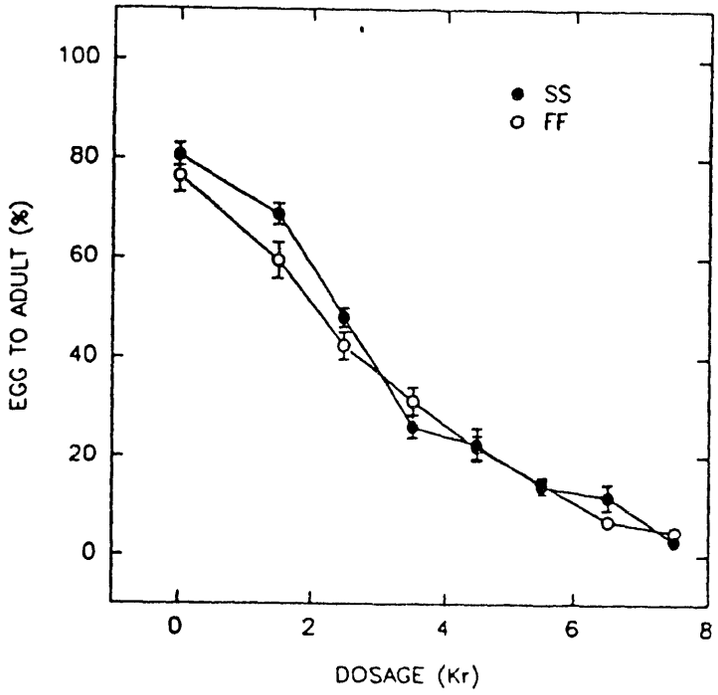


Table 3. Percent males emerging as adults from eggs irradiated as stage-7 oocytes.

	kR							
Sod genotype	0	1.5	2.5	3.5	4.5	5.5	6.5	7.5
F/F	50.9	49.3	53.6	52.9	47.6	47.0	52.6	41.1
S/S	48.3	54.3	53.0	52.7	45.7	54.7	50.8	50.0

9. Stage-14 Oocytes

9a. Germ Cell Dominant Lethals. The summary characteristics of the experiments are:

- Four genetic backgrounds of each of the two *Sod* genotypes
- Eight irradiation levels, 0-7.5 kR
- 10-16 replications for each background x genotype x irradiation level
- 5 egg-laying females per replication
- Approximately 50,000 eggs counted.

Figure 10

FERTILITY OF IRRADIATED FEMALES 0-24h AFTER IRRADIATION. Number of eggs laid by 5 females. The vertical bars are the standard errors.

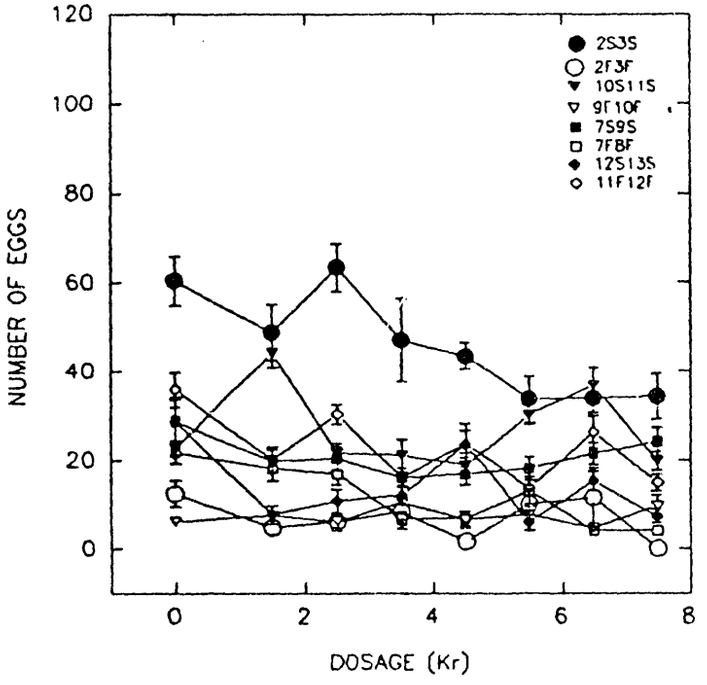


Figure 11

FERTILITY OF IRRADIATED FEMALES 0-24h AFTER IRRADIATION. Number of eggs laid by 5 females, the vertical bars are the standard errors (all lines)

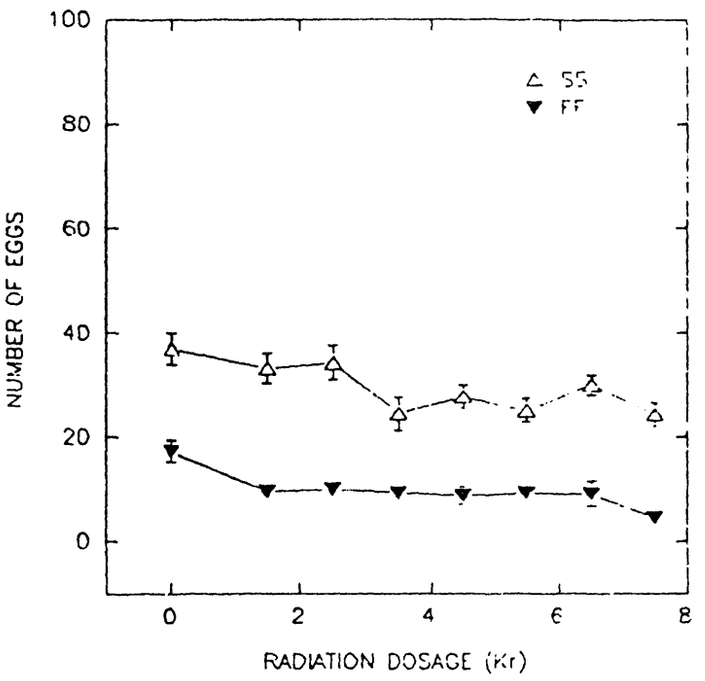


Figure 10 gives the replicate averages for each genetic background; Figure 11 gives the averages for each *Sod* genotype. The incidence of lethals increases only slowly with increasing irradiation dosage, as was the case for stage-7 oocytes, whereas for stage-3 oocytes the incidence of lethals increased more rapidly. The S/S genotype distinctly outperforms F/F for all dosages (and control). The incidence of lethal mutations is, for S/S and F/F respectively, 10.3 and 45.1% at 1.5 kR, and 34.5 and 74.2% at 7.5 kR.

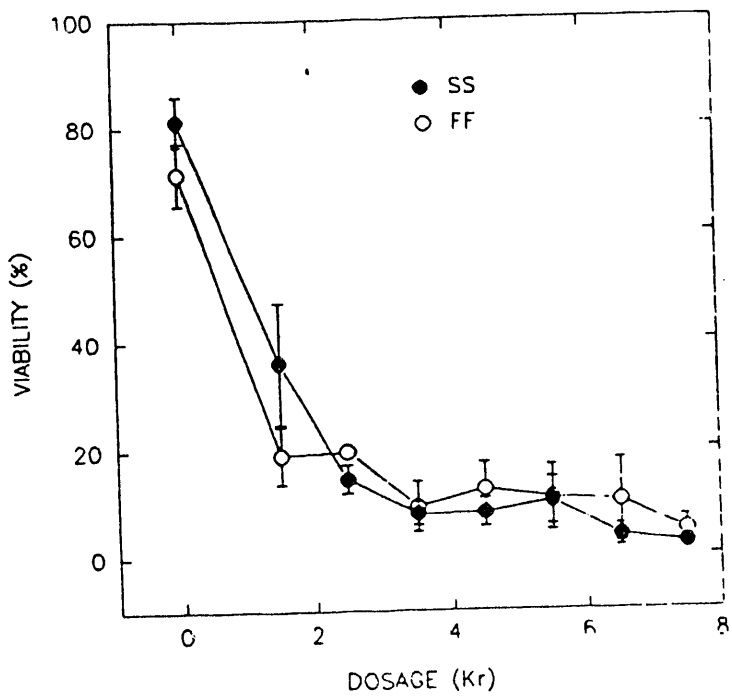
9b. Larval Dominant Lethals. The summary characteristics of the experiments are:

- Four genetic backgrounds for each S/S and F/F
- Eight irradiation levels, 0 - 7.5 kR
- 6-25 replications
- An average of 60 eggs per replication
- Approximately 100,000 eggs counted
- Approximately 40,000 emerging adults recorded.

Figure 12 summarizes the results. The incidence of larval mortality is, for S/S and F/F respectively, 55.5 and 73.2% for 1.5 kR, and 97.3 and 93.7% at 7.5 kR. The incidence of lethality increases very fast with radiation dosage.

Figure 12

EGG-TO-ADULT SURVIVAL OF EGGS LAID BY I IRRADIATED FEMALES 0-24h AFTER IRRADIATION.
The bars are the standard errors.



9c. Sex-Linked Recessive Lethals. The summary characteristics are the same as for 9b. The observations are the proportions of males and females among the emerging adults. The overall results are summarized in Table 4.

Table 4. Percent males emerging as adults from eggs irradiated as stage-14 oocytes.

	kR							
Sod genotype	0	1.5	2.5	3.5	4.5	5.5	6.5	7.5
F/F	48.7	48.3	53.4	46.7	51.0	50.5	63.1	37.5
S/S	50.9	46.6	49.9	45.6	49.6	50.4	50.2	59.3

10. First-Instar Larvae

These experiments with newly hatched larvae were reported in the first annual report (7/31/90) for this grant period. The "null" strains and crosses were confirmed to be still "null" (i.e., the "null" had not reverted to "slow" wild type by excision of the

inserted truncated P-element) at the end of the experiments herein reported.

10a. Viability Dominant Lethals. These experiments consist of 10 replicates, each with 30 larvae, at two levels of (0 and 1.5 kR) of irradiation. The results are summarized in Table 5.

Table 5. Percent adults emerging from irradiated first-instar larvae.

Genotype	Control	1.5 kR	% Reduction
F/F	97.3	84.7	14.0
S/S	95.0	77.7	18.2
N/N	93.7	58.0	38.1

10b. Aging Dominant Mutants. Experiments consist of 10 replicates, each with 30 adult males, at two levels (0 and 1.5 kR) of irradiation. Data are summarized in Table 6.

11. Third-Instar Larvae

These experiments are outlined in Figure 13. Newly-molted third-instar larvae (48-72 h after eggs laid, but checked for molting) are irradiated and placed in vials, 40 per vial. The emerging adults are sexed and scored ("viability"). 3-4 days old females are placed in groups of 5 per bottle and crossed to 10 males (unirradiated and with different genetic background). Starting 24 h later, eggs are collected in Petri dishes at 24 h intervals for three days ("fertility"). The 5 females are then placed (without males) in vials and their survival recorded at 4-day intervals until all 5 are dead ("longevity").

11a. Viability Dominant Lethals. The summary characteristics of the experiments are:

- Three genetic backgrounds for each (S/S and F/F) *Sod* genotype
- 8 irradiation levels (0 - 7.5 kR)
- About 70 replications for each *Sod* genotype, genetic background, and radiation level.
- 40 3rd-instar larvae per replication
- Approximately 100,000 3rd-instar larvae tested
- Approximately 50,000 emerging adults scored.

Table 6. Longevity of males irradiated (1.5Kr) as first instar larvae and of their controls.

DAY	CONTROL			IRRADIATED		
	F/F	S/S	N/N	F/F	S/S	N/N
0	30.0	30.0	30.0	30.0	30.0	30.0
5	30.0	29.7	29.3	29.5	29.5	28.8
10	29.5	29.6	27.9	26.3	28.8	24.5
15	29.0	29.4	27.1	24.8	28.3	21.2
20	28.5	29.1	25.6	21.7	26.0	18.2
25	27.9	28.8	23.9	17.9	23.4	15.1
30	26.6	28.2	22.2	16.8	23.0	14.4
35	26.1	27.9	20.6	15.4	22.3	13.9
40	25.8	27.5	19.3	14.2	21.2	12.8
45	24.7	26.0	18.1	12.6	20.9	11.2
50	24.0	24.9	17.5	12.0	18.8	10.5
55	22.4	23.6	17.3	11.4	17.2	9.2
60	21.3	20.4	17.1	10.5	15.3	7.1
65	19.4	17.7	15.6	9.2	13.0	5.1
70	18.0	16.9	15.2	8.6	11.4	3.1
75	16.2	16.0	14.9	7.3	8.7	1.8
80	14.8	13.0	14.2	4.8	6.7	1.1
85	11.3	8.8	10.7	3.2	5.5	.9
90	6.1	7.4	8.0	2.3	3.9	.3
95	4.6	6.0	5.8	1.5	2.8	.2
100	2.7	4.5	3.0	.6	1.4	0
105	2.2	2.6	1.0	.1	.4	•
110	1.4	1.6	0	0	.1	•
115	.6	.4	•	•	0	•
120	.3	0	•	•	•	•
125	.1	•	•	•	•	•
130	0	•	•	•	•	•
% reduction in longevity				25.7	17.3	34.7

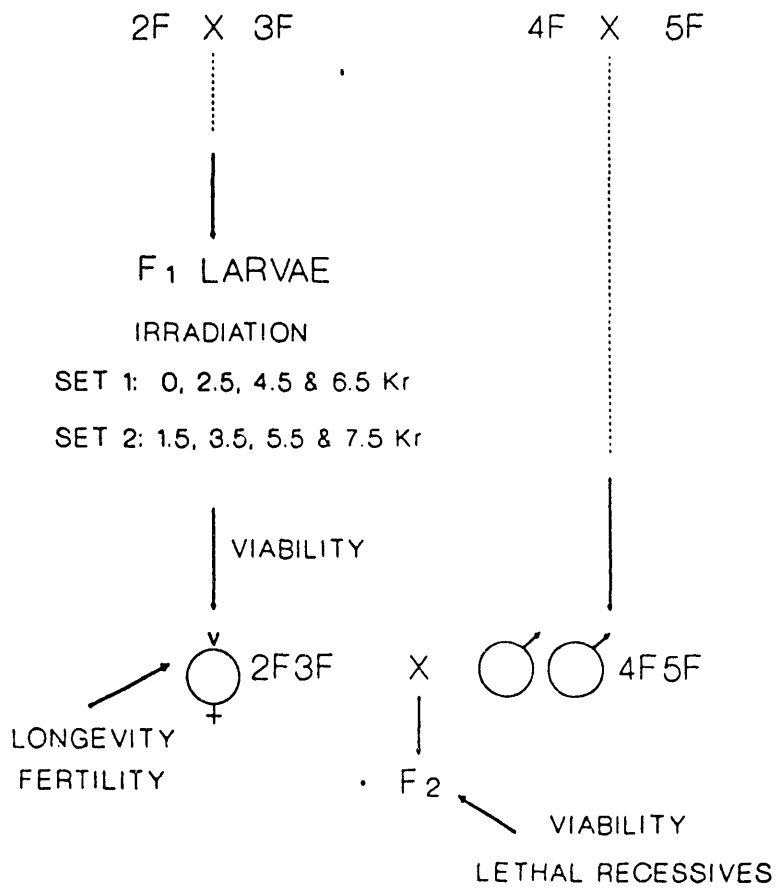
The results are summarized in Figure 14. Viability is higher for F/F at several dosages, most notably at 7.5 kR, a result that is consistent for all three genetic backgrounds.

11b. Fertility Dominant Mutants. The summary characteristics are:

- Three genetic backgrounds for each S/S and F/F
- 6 irradiation levels (0, 1.5, 2.5, 3.5, 5.5 and 7.5)
- About 20 replications per treatment, background, and genotype
- Approximately 150,000 eggs counted.

Figure 13

IRRADIATED THIRD INSTAR LARVAE WITH DIFFERENT SUPEROXIDE DISMUTASE (SOD) GENOTYPES.



The experiments are summarized in Figure 15. The S/S genotype exhibits greater fertility than F/F at all treatments.

12. Adult Females: Aging

The experiments designed with irradiated females are outlined in Figure 16. Virgin females are irradiated when they are 3-4 days old. They are then crossed to non-irradiated males with different genetic backgrounds. Longevity and fertility are measured as described in section 11 above. Only some longevity data are available now for reporting. Figures 17 and 18 give the average survivorship curves for the two *Sod* genotypes at each irradiation dosage. The data are for 8 irradiation treatments, three genetic backgrounds for each, F/F and S/S, and 10-50 replicates (of 5 females each) for each treatment, background, and *Sod* genotype (about 70,000 females were scored each about 10 times on the average).

Figure 14

THIRD INSTAR LARVAE TO ADULT SURVIVAL AT DIFFERENT DOSES OF IRRADIATION. Percent of adults emerged from irradiated third instar larvae.

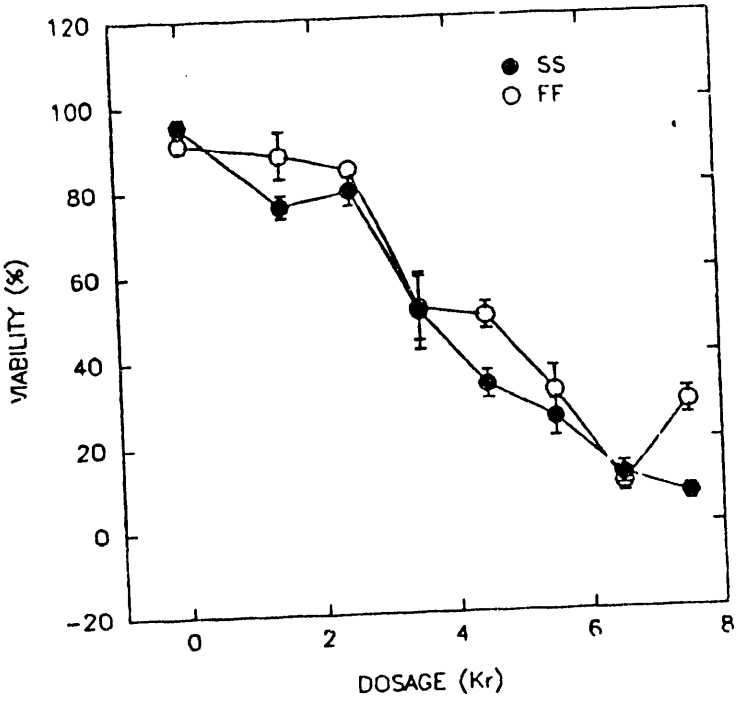
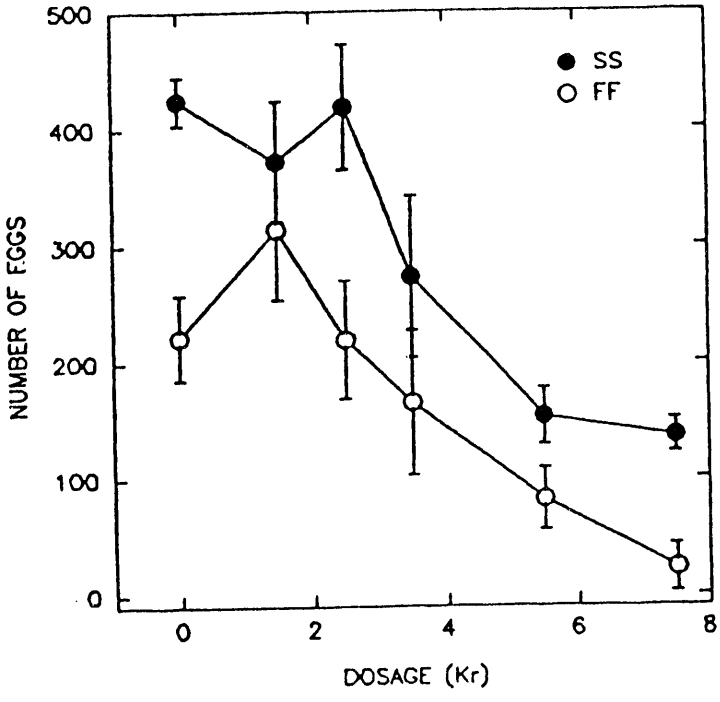


Figure 15

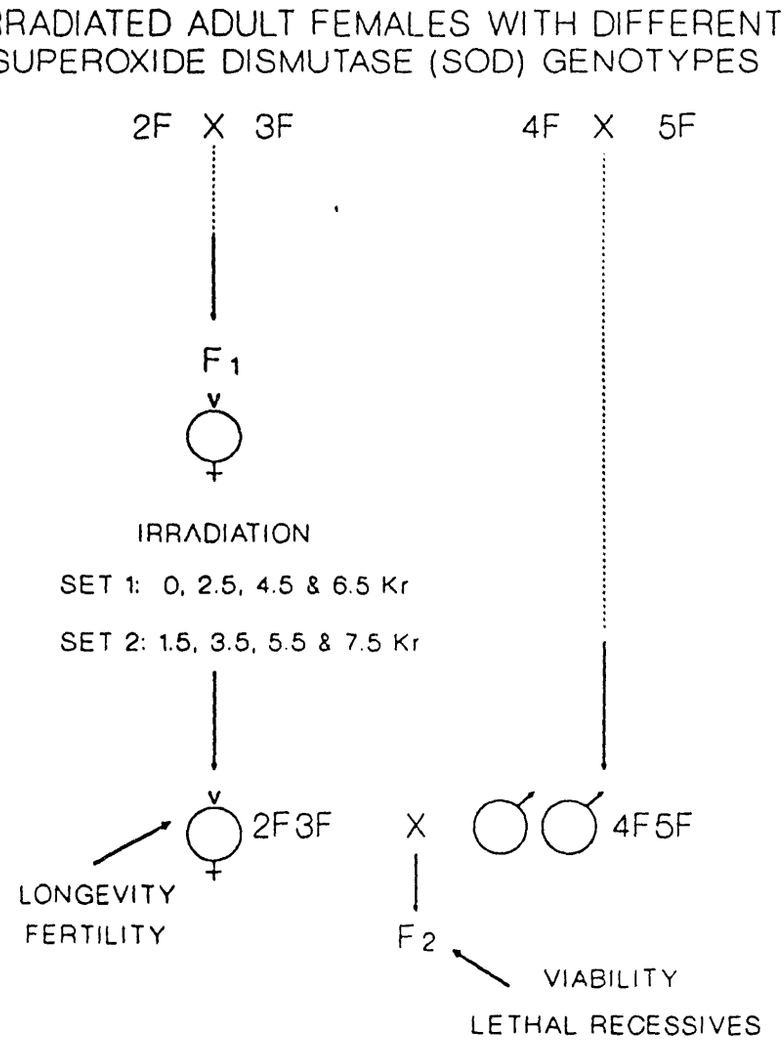
FERTILITY OF FEMALES IRRADIATED AS THIRD INSTAR LARVE. Number of eggs laid by 5 females in a 72 hours period.



NATURAL SELECTION

The F and S alleles of *D. melanogaster* Cu,Zn *Sod* represent a naturally occurring polymorphism. The question arises as to what processes account for this polymorphism. Of particular interest, of course, are processes related to the scavenging of superoxide radicals, the enzymatic function of *Sod*. Various sources of evidence, previously reviewed in our publications, grant applications, and reports to DOE, suggest that the *Sod* locus itself may be the target of selection in natural populations. This suggestion has received additional support by the discovery that the *S* alleles from distant localities exhibit identical nucleotide sequence (whereas about 20 substitutions differentiate any two F alleles on the average), which strongly suggests that the S allele is rapidly spreading throughout the world under substantial natural selection pressure. The experiments reported here seek to identify the population processes that would account for the *Sod* polymorphism. The experiments have been published (the three publications are referenced below) and hence the results will be only briefly alluded to. (Note that these experiments, although quite relevant to the objectives of this grant, have been carried out by visiting scientists who had their own support, and hence this grant's outlays for these experiments have been small, limited primarily to providing culture materials.)

Figure 16



13. Overdominance

Fitness superiority of the heterozygotes over the homozygotes is the most obvious process by which natural selection can account for genetic polymorphisms in populations.

We tested for *Sod* genotypic fitness effects (productivity per female) under controlled conditions in eight enviornmental set-ups (all combinations of two levels for each of three variables: genetic background, density, and temperature). The results have been published (Peng, Moya, and Ayala, 1991) and are partially summarized in Table 7. The heterozygous genotype (F/S) is superior to the homozygotes (S/S andF/F) in all cases and this overdominance is statistically significant in 13 of the 16 comparisons.

Figure 17

SURVIVORSHIP CURVE OF IRRADIATED FEMALES: ALL FF LINES

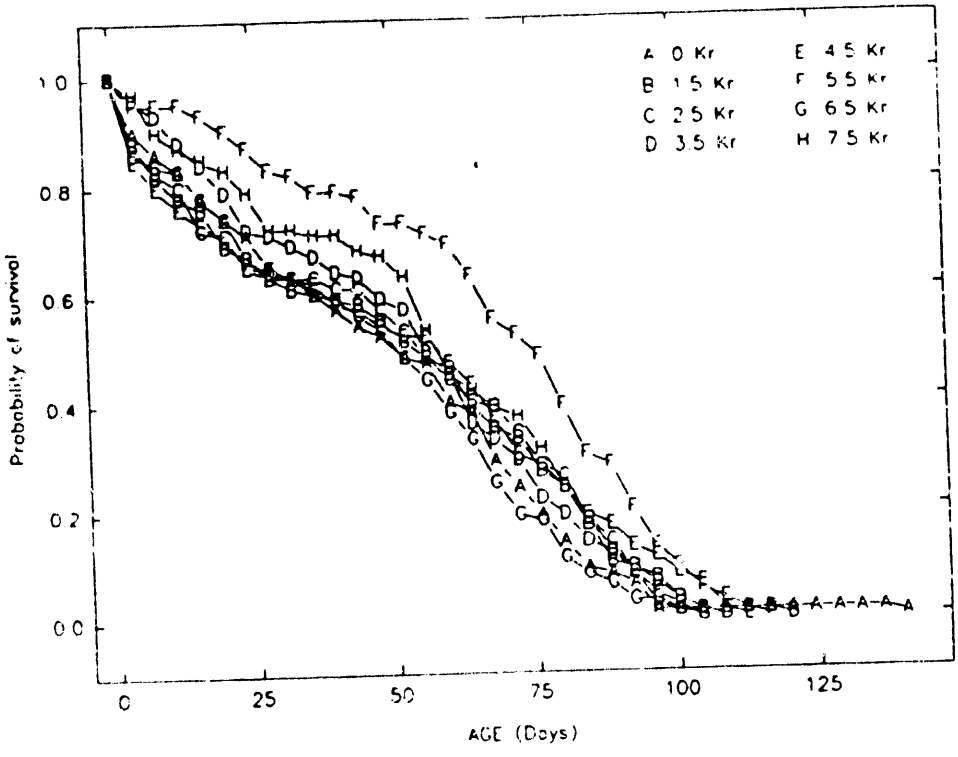
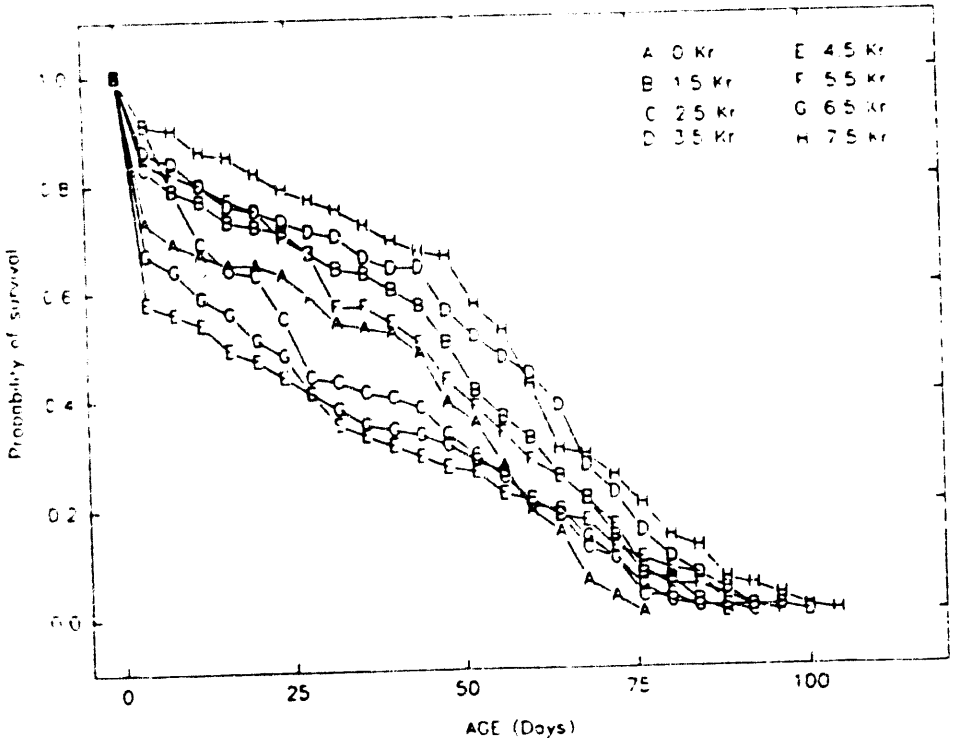


Figure 18

SURVIVORSHIP CURVE OF IRRADIATE FEMALES FOR ALL SS LINES



Fitness of the *Sod* genotypes was also measured by examining viability in four sets of environments (two levels of two variables). The heterozygotes were ostensively superior in all 8 comparisons with the two homozygotes, and this overdominance was statistically significant in two comparisons (see Table 3 in Milosević, Moya, and Ayala, 1990). Overdominance of the F/S genotypes occurs also with respect to female fertility but not male virility (Table 1 in Milosević, Moya, and Ayala, 1991).

Table 7

Values and statistical significance of *t* for comparisons between cultures with different *Sod* genotype (d.f. = 78 for each value; two-tailed tests)

Poly-morphism	*Sod* genotype	20°		28°	
		Scant	Crowded	Scant	Crowded
Low	*F/F-S/S*	1.02^{ns}	5.81***	2.47**	5.71***
	F/S-S/S	4.44***	6.88***	4.74***	9.62***
	F/S-F/F	3.07**	1.78^{ns}	2.25^{ns}	4.10***
High	*F/F-S/S*	1.51^{ns}	9.22***	2.61**	5.68***
	F/S-S/S	4.26***	10.14***	6.01***	11.30***
	F/S-F/F	2.73**	0.33^{ns}	3.45***	6.39***

$^{**}P < 0.01$; $^{***}P < 0.001$; ^{ns} not significant.

14. Overcompensation and Frequency-Dependent Selection

The significance of frequency-dependent selection as a process for maintaining poymorphism has become increasingly appreciated in recent times (see references in Milosević et al., 1990). In the case of competition for limiting resources, frequency dependence implies "overcompensation"—i.e., a mix of several genotypes exploits the limiting resources better than a single genotype. We have tested for overcompensation with respect to the *Sod* locus (and the small genetic region surrounding it) as well as with respect to the whole genome, using the *Sod* locus as a marker. The results (Peng et al., 1991; Milosević et al., 1991) show that overdominance is a rather general phenomenon and may indeed contribute to maintaining the *Sod* polymorphism. See, e.g., Table 8 showing that a mixture of all three genotypes (F/S, S/S, and F/F) exploits limited environmental resources better than any one genotype (including the fittest heterozygotes); this superiority is statistically significant in 10 out of 12 comparisons (13 out of 16 if the means are included).

Table 8

Values and statistical significance of *t* for comparisons between mixed and single genotype cultures (two-tailed tests)

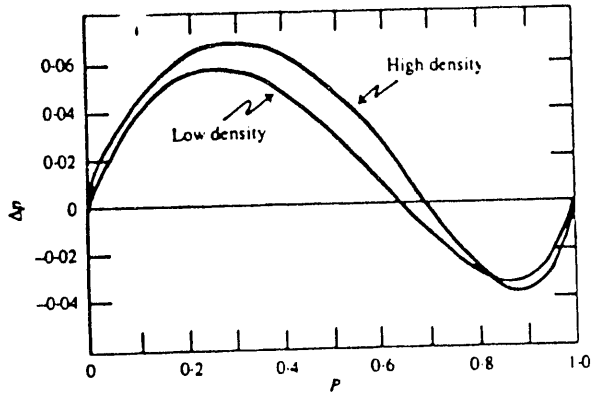
Sod genotype of the single cultures	d.f.	Scant		Crowded	
		20°	28°	20°	28°
S/S	78	9.26***	5.31***	19.09***	14.14***
F/F	78	8.04***	2.70**	5.80***	8.31***
F/S	78	5.82***	−0.80ns	5.40***	1.24ns
Mean[a]	158	7.02***	1.54ns	6.16***	5.61***

** $P < 0.01$; *** $P < 0.001$; ns not significant.

[a] For the comparisons with the mean of the single-genotype cultures, the three genotypes have been weighted according to their proportions in the mixed cultures: 3:4:3 for *F/F*, *F/S* and *S/S*, respectively.

Also interesting are the effects of mating type on maintaining the *Sod* polymorphism. We have shown that (contrary to a common assumption in population genetics theory) mating-type fitnesses are asymmetric and nonadditive. An exploration of this phenomenon (Milosević et al., 1991) and of the ensuing dynamics of *Sod* alleles indicates that a globally stable polymorphism will occur with both the S and F alleles at frequencies not very different from those observed in natural populations (Figure 19).

Figure 19



Dynamics of allele frequency change at the *Sod* locus in *Drosophila melanogaster* when fertility and viability are both taken into account. The change, Δp, per generation for a given frequency, p, of the *F* allele is shown. A globally stable polymorphic equilibrium occurs at $P = 0.641$ at low density, and at $p = 0.695$ at high density. The observed frequency of *F* in the natural population sample is $p = 0.873$.

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1990

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