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TITLE: Genetic Variation in Resistance to Ionizing Radiation

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INCREMENTAL REQUEST FOR YEAR 3 (1992) AND PROGRESS REPORT FOR YEAR 2 (JANUARY - DECEMBER, 1991)

ABSTRACT

We proposed an investigation of genetically-determined individual differences in sensitivity to ionizing radiation. The model organism is *Drosophila melanogaster*. The gene coding for Cu,Zn superoxide dismutase (SOD) is the target locus, but the effects of variation in other components of the genome that modulate SOD levels are also taken into account. SOD scavenges oxygen radicals generated during exposure to ionizing radiation. It has been shown to protect against ionizing radiation damage to DNA, viruses, bacteria, mammalian cells, whole mice, and *Drosophila*. Two alleles, S and F, are commonly found 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 an ideal model system to investigate SOD-dependent radioresistance, because each allele yields different levels of SOD, so that S > F >> CA1.

The roles of SOD level in radioresistance are being investigated in a series of experiments that measure the somatic and germ-line effects of increasing doses of ionizing radiation. We have completed a number of experiments and are proceeding with many others. We have made progress along all the research lines anticipated for the first year and a half of this grant, as summarized in the following pages. In addition, we have pursued an unexpected genetic event—namely the nearly simultaneous transformation of several lines homozygous for the SOD "null" allele into predominantly *S* lines. Using specifically designed probes and DNA amplification by means of the *Taq* polymerase chain reaction (PCR) we have shown that (1) the null allele was still present in the transformed lines, but was being gradually replaced by the *S* allele as a consequence of natural selection; and (2) that the transformation was due to the spontaneous deletion of a 0.68 Kb truncated P-element, the insertion of which is characteristic of the CA1 null allele. The possibility of line contamination by *S* flies could be unambiguously excluded, since 9 bp (6 from the 5'

end and 3 from the 5' end) of the excised fragment remain in the transformed *S* genes as a tale-telling footprint of the truncated P element.

A. Introduction

The superoxide anion O_2^- is generated during cell respiration as well as during exposure to ionizing radiation. 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: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. SOD has been shown to protect against ionizing radiation damage to DNA, viruses, bacteria, mammalian cells, whole mice, and *Drosophila*. The role of SOD in the defense against the toxicity of oxygen has been demonstrated in other ways as well.

We proposed to elucidate further the role of genetic variation in radioresistance by investigating in *Drosophila melanogaster* the response of different SOD genotypes to ionizing radiation. Two alleles, S and F, exist at the SOD locus in natural populations. The allozymes encoded by them differ in specific activity. Moreover, we have a naturally occurring (not obtained by mutagenesis) "null" SOD allele, CA1, which is transcribed and translated, but exhibits only 3.5% of normal SOD levels. We have thus a convenient system for investigating the role of SOD in radioresistance, because we have three alleles in *D. melanogaster* that code for products with a considerable range in SOD activity: S > F >> CA1 ("null"). The F and S alleles are present in all natural populations of *D. melanogaster*, although their frequencies vary from one to another locality. Moreover, the CA1 allele was also obtained from a natural population.

We summarize the results obtained during the second year of funding of this project. We will briefly summarize our main results under subheadings 1-6, which correspond to those listed in the original proposal under D. Experimental Design and Methods (except that under 6 we also summarize the molecular work by which we discovered the unanticipated event of the transformation of the "null" into the *S* allele). It should be noted at this point that most of the experiments proposed and being carried out call for testing sets of numerous genetically independent lines, so that the SOD genotype is the same within each set, but the genetic background differs among the lines; and, moreover, most experiments call for considerable replication. The experimental blocks are so designed as to include in each block the variables under investigation (the SOD genotypes), whereas experiments with different lines and additional replications are gradually carried out over time. Experiments with additional lines and replications are in progress (and some will continue through the remaining 19 months of this research project, which terminates December 31,

1992), so that the numerical results hereafter summarized are not final. This is a "progress" report with respect to every one of the components of the project.

B. Results

1. Strains and Crosses. Using *Drosophila melanogaster* flies collected from a natural population, 54 experimental lines were generated, 18 homozygous for each of the three *Sod* genotypes, F/F, S/S, and N/N. (For simplicity, we use the symbol N for the naturally occurring null allele that we have characterized as CA1). In all experiments the irradiated individuals (larvae or adults) are progenies from two different experimental lines, so as to avoid the effects of inbreeding. One example of the crosses made in the experiments in which the larvae are irradiated is shown in Figure 1: 2F, 3F, 4F, and 5F represent four different lines homozygous for the *F* allele. One example for a case in which adult females are irradiated is shown in Figure 2.

The use of multiple lines of each genotype is necessitated by two related objectives that are essential to this proposal: (1) to insure that effects attributed to SOD are due to this locus rather than to the genetic background; and (2) to evaluate how genetic variation at other loci modulates the effects attributable to the SOD locus.

2. Lethal-Dose-50 for Different SOD Genotypes. Larvae of the same age are collected from each genotype, washed in a 20% sucrose solution, and placed in small glass Petri dishes for irradiation. Groups of 30 irradiated larvae are placed in separate vials and the adults emerging from the vials are counted.

We have continued testing the viability of irradiated third-instar larvae and obtained results consistent with those reported in our previous Program Report. As noted there the *F/F* and *S/S* maintained a high rate of survival through levels of irradiation up to 4 Kr (>85% relative to unirradiated controls). We have, therefore, increased the irradiation levels so as to ascertain whether differences in radioresistance occur between these two genotypes (and also to be able to obtain more accurate estimates of LD50). Our results to date indicate that at doses greater than 6 Kr the S/S genotype provides much greater protection than the F/F genotype, as expected if SOD protects against radiation damage. In one experiment, for example, at 6.5 Kr the rates of irradiated larvae surviving to adulthood were 28.8% and 0.5% for the *S/S* and *F/F* genotypes respectively.

3. Genotype-Dependent Longevity Effects of Irradiation. The continuing experiments yield results similar to those reported in last year's Progress Report (see Table 2, p. 4), although considerable variation occurs from line to line, apparently showing the importance of the genetic background with respect to longevity, both without and with irradiation.

Figure 1.

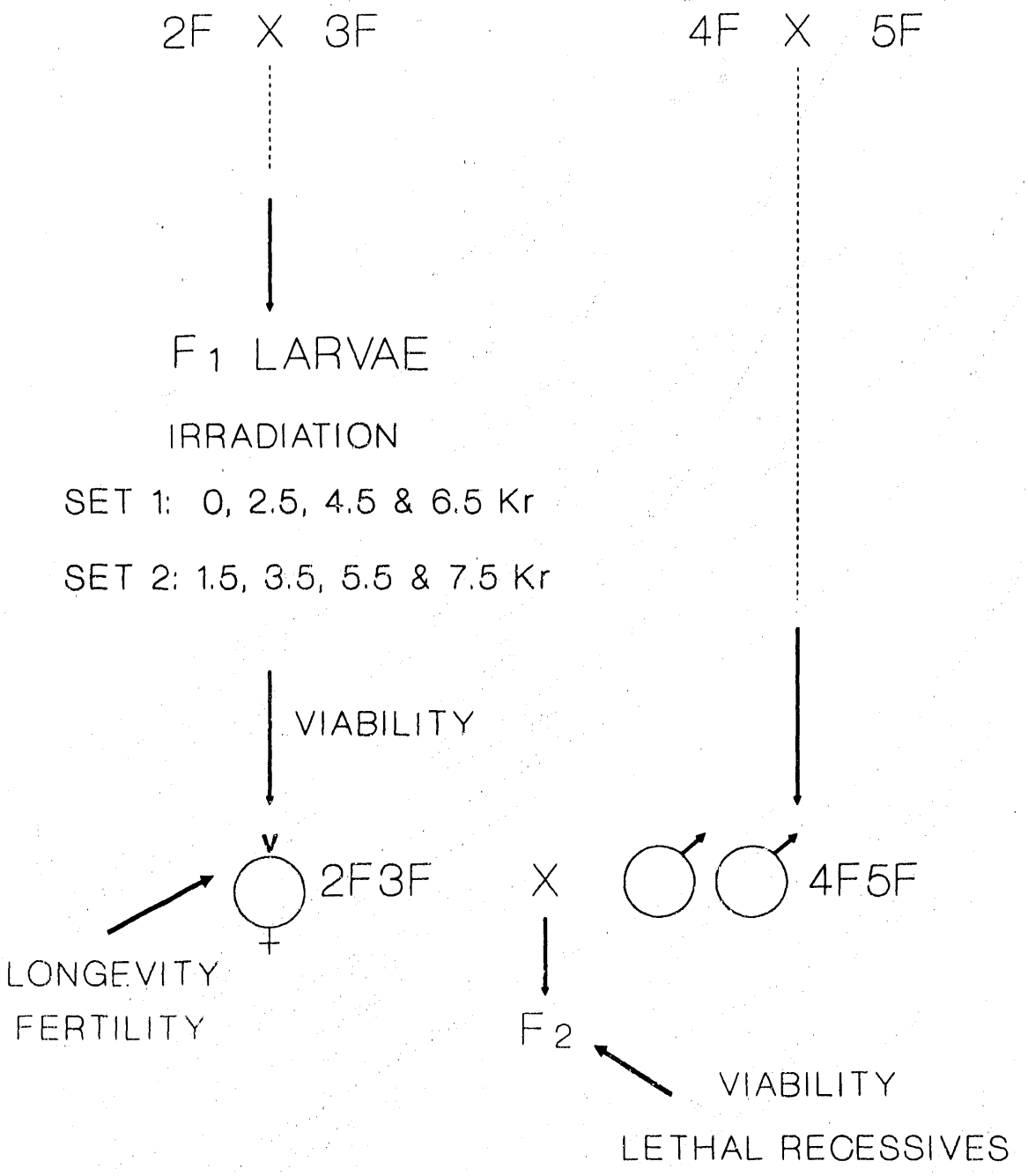
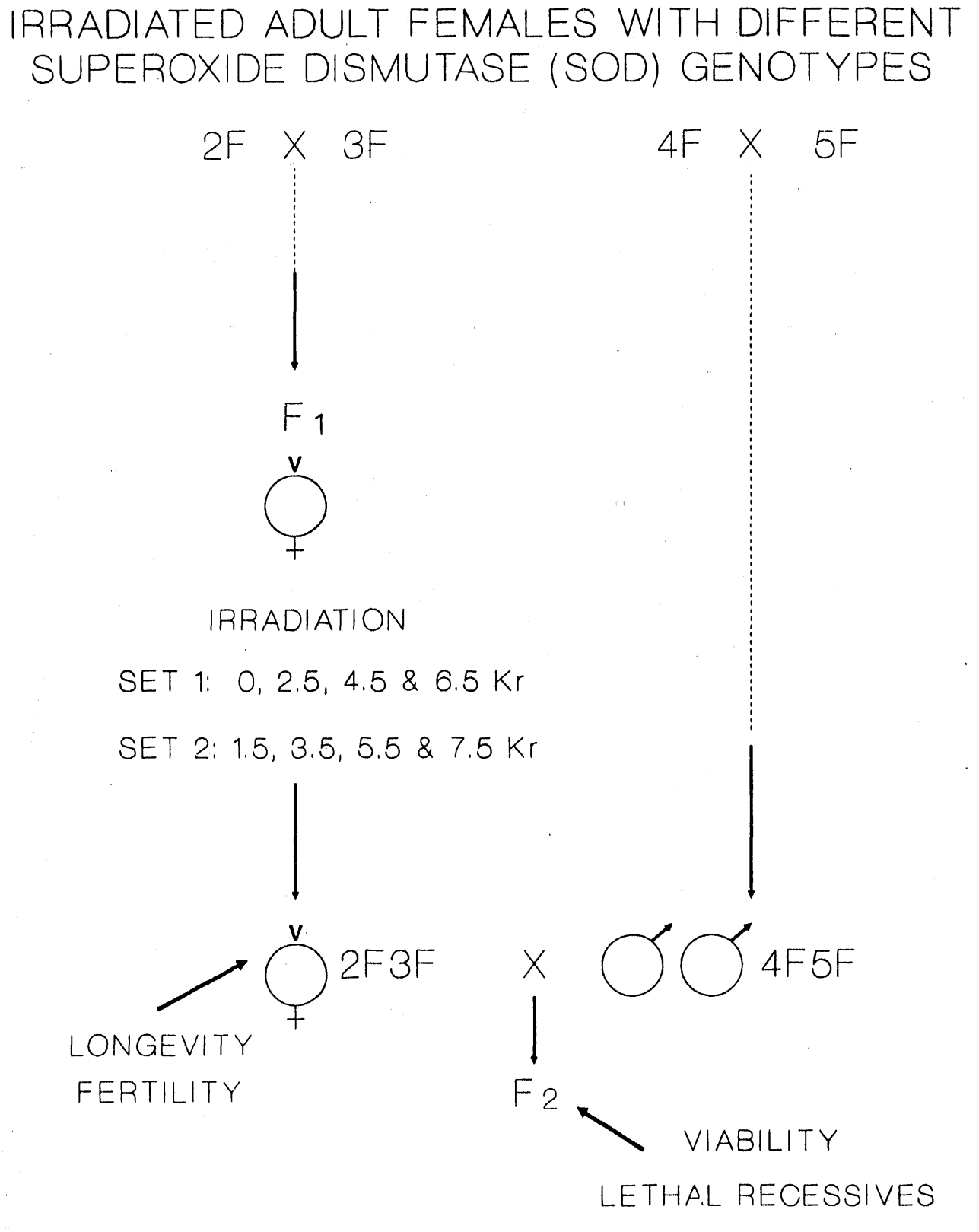

IRRADIATED THIRD INSTAR LARVAE WITH DIFFERENT SUPEROXIDE DISMUTASE (SOD) GENOTYPES.
2F X 3F
4F X 5F
F1 LARVAE
IRRADIATION
SET 1: 0, 2.5, 4.5 & 6.5 Kr
SET 2: 1.5, 3.5, 5.5 & 7.5 Kr
VIABILITY
2F3F
X
4F5F
LONGEVITY
FERTILITY
F2
VIABILITY
LETHAL RECESSIVES

Figure 2.


IRRADIATED ADULT FEMALES WITH DIFFERENT SUPEROXIDE DISMUTASE (SOD) GENOTYPES
2F X 3F
4F X 5F
F1
IRRADIATION
SET 1: 0, 2.5, 4.5 & 6.5 Kr
SET 2: 1.5, 3.5, 5.5 & 7.5 Kr
2F3F
X
4F5F
LONGEVITY
FERTILITY
F2
VIABILITY
LETHAL RECESSIVES

4. Sterility-Dose-50 for Different Genotypes. The results reported in the previous Progress Report showed that (1) considerable fertility variation occurs among females; (2) there is considerable variation in fertility as a function of age; and (3) when approximately large numbers of replications are carried out, the pattern of fertility differences among genotypes persists through age. This last result is of practical significance, because it implies that the fertility effects of SOD as a function of irradiation dose can be measured in females of any given age.

Our results to date show that the genetic background has a major effect on fertility (as a function of irradiation dose or in controls). The results of a particularly noteworthy experiment are shown in Figure 3, which is based on the observation of about 130 females for each point, with bars showing the standard error due to variation among sets of five females. One atypical result is the low fertility of the control *F/F* females (obtained by crossing ♀2F x 3F♂). Another is that the fertility of these particular *F/F* females decreases only very little or not at all as the irradiation dose increases.

We have, in addition, tested for germ-line radiation damage arising as a consequence of larval irradiation, and the SOD role in protecting against this damage. One kind of experiment tests for dominant lethal mutations (i.,e., examines the viability of eggs laid by females developed from irradiated larvae). A significant effect exists, accounting for a reduction of 3--5% in the viability of eggs per 1000 r. (The viability reduction appears to be lesser for *S/S* than for *F/F* individuals, as expected, if SOD provides protection; e.g., in one experiment egg viability from 0 to 4.5 Kr decreased by 12.2% in *S/S* individuals, but 18.2% in *F/F* individuals. But the relevant data are too limited as of this writing.) A second kind of experiment tests for recessive X-chromosome mutations, by examining the sex ratio (♀/♂) of the progenies (see Figure 4).

5. Genotype-Dependent Rate of Lethal Mutations. Immature oocytes (stage-7) are powerful indicators of modification in radiation response. 2-3 days old virgin females of the appropriate genotype are irradiated and then mated with excess males (see Figure 2). Eggs are collected from the second 24-hours brood, which represents irradiated immature oocytes at about stage-7. The incidence in one experiment of sex-linked recessive lethals, X-chromosome losses, and dominant lethality (as indicated by the rate of eggs that develop to adulthood) is shown in Figure 5. The procedures are as described in the grant application. Variation is large among the different lines, so that no conclusions can be drawn before these experiments are completed and the data analyzed. We have also tested for the rate of sex-linked recessive lethal mutations by examining the ♀/♂ sex-ratio among the surviving adults. The results of one experiment (the same as shown in Figure 5) are given in Table 1. At the highest dose (6.5 Kr), the sex ratio is much greater for *F/F* than for *S/S* females, consistent with

Figure 3.

FERTILITY OF FEMALES IRRADIATED AS THIRD INSTAR LARVAE. Number of eggs laid by 5 females in a period of 24 h. The vertical bars are the standard errors.

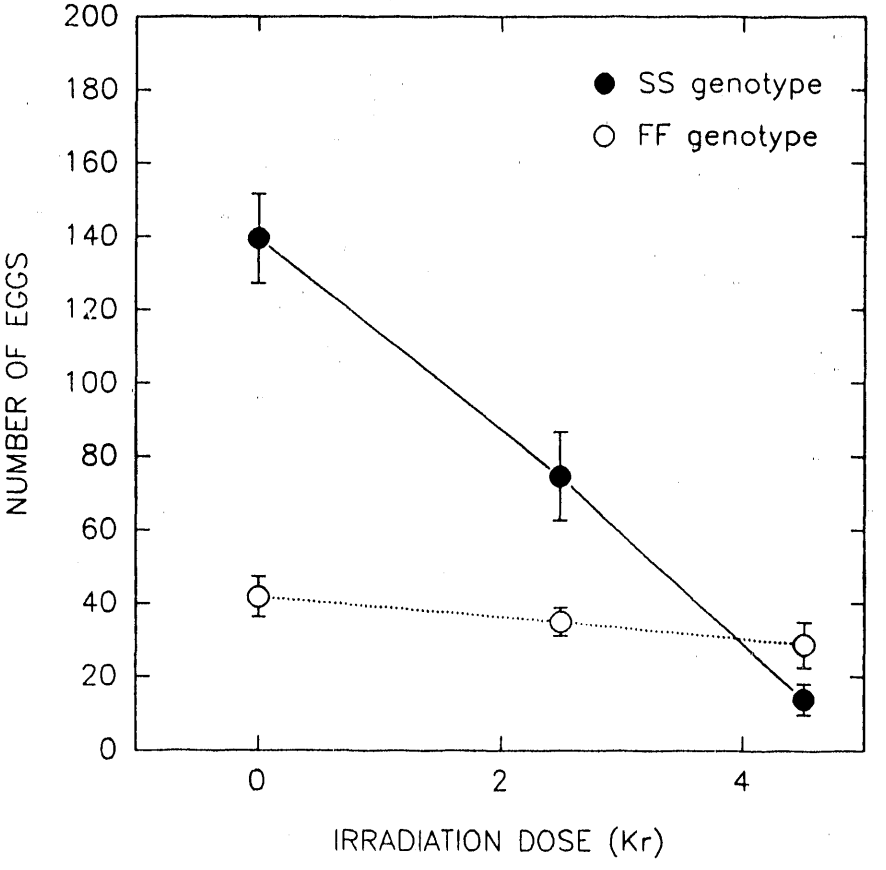


Figure 4.

X-chromosome lethal mutations in the progenies of females irradiated as third-instar larvae. The graph shows the ♀/♂ sex ratio.

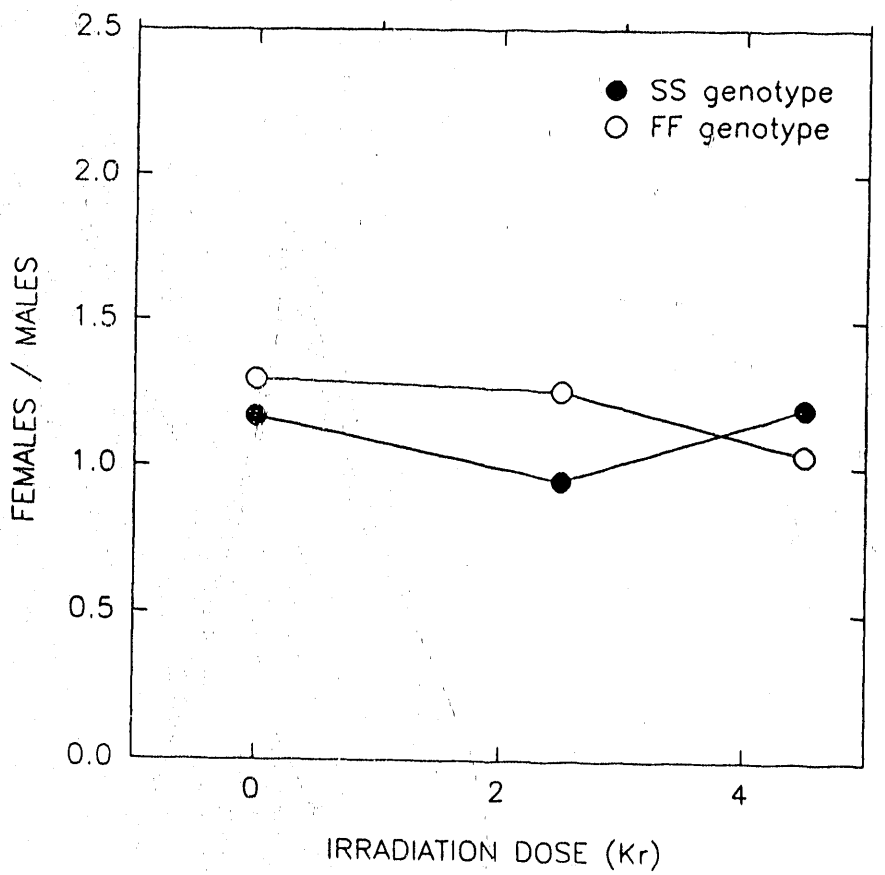
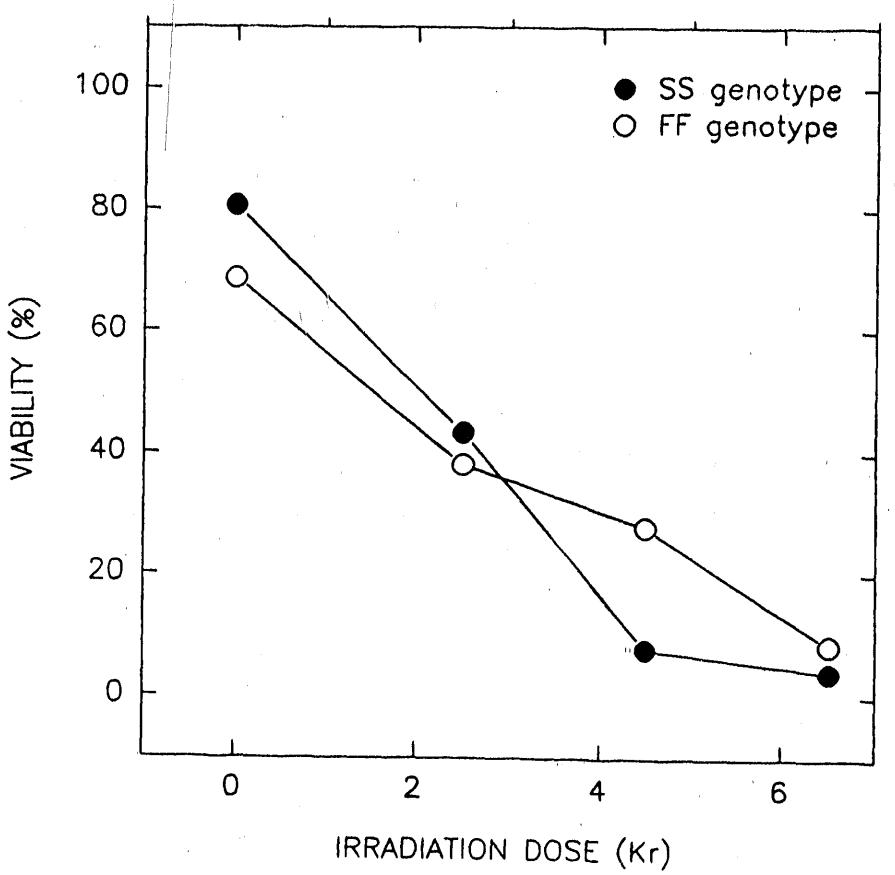


Figure 5.

VIABILITY OF EGGS LAID BY IRRADIATED FEMALES AT DIFFERENT DOSES OF IRRADIATION.



the hypothesis that the *S* allele provides greater protection against radiation damage.

Table 1. Sex ratio (females/males) among adults developed from irradiated immature oocytes at about stage-7.

DOSE (Kr)	GENOTYPE SS	FF
0	1.47	1.09
1.5	1.01	1.03
4.5	0.93	0.89
6.5	0.86	1.83

6. Molecular Characterization of the "Null" SOD^{CA1} Allele and of the Transformed *N → S* Lines. This "null" (N) allele was isolated from a natural population. Strains 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 occur with our N allele.

We have cloned and obtained the complete sequence of the N allele. As reported in last year's Progress Report, the N gene carries a 680-bp insertion that starts 47 bp downstream from the start of transcription. The insertion derives from a truncated P element and is identical to it, except for one nucleotide substitution at 424. The rest of the sequence is identical to the sequence of an F allele obtained from a Canton-S strain except for a point mutation in the second exon that results in a replacement of Asn by Lys at position 96 of the polypeptide, which confirms that the mature protein encoded by the N allele is the same as S. The deletion in the P element yields a stop codon. In addition the

inserted P-element fragment carries three poly-A signals, two of them adjacent to one another. The diminished expression of *N* is most likely due to a reduction in the rate of transcription, attributable to the insertion of the P element. Because the *CA1* "null" allele of SOD has less than 5% of wild-type SOD levels, it is very helpful in experiments that test for SOD function in vivo, such as the ones carried out in the present project.

We test routinely the S, F, and N lines by starch gel electrophoresis and SOD assay, so as to make sure that they are not contaminated. One such test showed that several N lines were apparently contaminated, since several (in some cases all) of the 10 or more 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 apparently independently in several *N* lines. By PCR amplification (and subsequent sequencing) using primers derived one from the SOD (wild-type) gene and the other 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 relevant 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.

C. Concluding Remarks

That SOD plays a role in protecting the cell against ionizing radiation has been conjectured by a number of investigators with various degrees of supporting evidence. The studies we have undertaken seek (1) to confirm the protecting role of SOD against ionizing radiation; (2) to ascertain the magnitude of the effects attributable to the SOD locus; (3) to investigate the germline and somatic cell effects of SOD and the magnitude of the effects for various life-cycle components; and (4) to ascertain the effects attributable to the genetic background. The population genetics approach followed in these studies should be of particular interest to the DOE, precisely because such approach is rarely followed in studies of this kind. Yet, the investigation of SOD effects on germline and somatic cells and various life history components provides invaluable guidance to further investigation of the vulnerable physiological processes protected by SOD. Moreover, the effects of the genetic background must be investigated because genetic variation is important in order to ascertain the effects of ionizing (and other) radiation in natural populations, and because

the effects of a particular enzyme or gene locus might be attributable to the genetic context rather than to the locus per se. *Drosophila* provides an ideal model system to investigate some of these issues, which are intractable or extremely costly with mammals and other larger organisms, whereas microorganisms and bacterial systems lack cellular and organ diversity. The genetic manipulations possible with *D. melanogaster* are also of great advantage for the present purposes. Now approaching the mid-point of the three-year research project, we report herein that the experiments proposed are proceeding according to plan and should, by the end of the three-year term, yield answers to the questions posed. We anticipate that these answers will be published in a number of papers (one already in press and another one in the writing stage).

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