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Enclosure 1

WRITTEN INFORMATION TO BE PROVIDED

BY THE PROJECT PRINCIPAL INVESTIGATOR

1. Project Title "GENETIC VARIATION IN RESISTANCE TO IONIZING RADIATION"

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Otherwise, indicate: "Not Applicable."

MASTER

fp

3. Principal Project Personnel

Francisco J. Ayala, Donald Bren Professor of Biological Sciences

- A. Principal Investigator
- B. Population genetics and evolution
- C. 10%
- D. University of Madrid, Spain, B.Sc., 1955, Physics
Columbia University, New York, M.A., 1963, Genetics
Columbia University, New York, Ph.D., 1964, Genetics
Rockefeller University, New York, Postdoc, 1964-65, Genetics
- E. Assistant Professor (1965-67), Providence College, Providence, RI; Assistant Professor (1967-71), Rockefeller University, New York, NY; Associate Professor (1971-74) and Professor of Genetics (1974-87), University of California, Davis; Director of Institute of Ecology (1977-81), University of California, Davis; Chairman of the Division of Environmental Studies (1977-81), University of California, Davis; Distinguished Professor of Biological Sciences (1987-89), University of California, Irvine; Donald Bren Professor of Biological Sciences (1989-present), University of California, Irvine.
- F. Member, National Academy of Sciences, American Academy of Arts and Sciences, American Philosophical Society; Fellow, California Academy of Natural Sciences, American Association for the Advancement of Science; Member, U.S. Association of the Club of Rome, Sigma Xi; Career Development Award from the National Institutes of Health (awarded 1968; declined 1971); Guggenheim Fellow (1977-78); Fulbright Fellow (1979 and 1981); Johns Hopkins University Centennial Scholar (1977); Outstanding Article Award for Volume IV of *San Jose Studies* (1978); Medal, College of France (1979); Honorary Member, Federation of Yugoslavian Genetics Societies (1981); Doctor Honoris Causa, Universidad de Leon (1982); Honorary Member, Sociedad Espanola de Genetica (1985); W.E. Key Award of the American Genetics Association (1985); Doctor Honoris Causa, Universidad de Madrid (1986); Doctor Honoris Causa Universidad de Barcelona (1986); Honorary Member, Golden Key National Honor Society (1986); Scientific Freedom and Responsibility Award of the American Association for the Advancement of Science (1987).

G. Relevant Publications (1986-89). Partial sample list.

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4. Additional Project Personnel

Douglas Skarecki, Technician, M.S., Molecular Biology.
 Robert Tyler, Graduate Student, B.A., University California,
 Irvine, 1986, Population Genetics.

5. Project Overview

A. Specific Project Objectives

1) Past Objectives.

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.

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 an ideal model system to investigate SOD-dependent radioresistance, because each allele yields different levels of SOD, so that $S > F \gg CA1$. The radioprotective effects of SOD can be definitely 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 by mutagenesis are used. Moreover, each allele is studied in multiple genetic backgrounds, so as to avoid effects attributable to other loci, by randomizing them.

The very reactive superoxide anion O_2^- is generated during cell respiration as well as during exposure to ionizing radiation (Fridovich, 1975; Smith and Heys, 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).

Evidence that genetic differences may affect sensitivity to ionizing radiation has been shown in *Drosophila* in two ways: first, differences have been shown to exist between strains (review in Sankaranayanan and Sobels, 1976); second, resistance to radiation can evolve under natural selection (Marques, 1973; Nöthel, 1987).

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2) Current Objectives.

We proposed a variety of experiments that were grouped under three categories of objectives. The first set of experiments as well as all but the last one of the second set are the objectives actively pursued at present. They are:

Objective 1. Response of different *SOD* genotypes to ionizing radiation. We proposed:

- To determine the Lethal-Dose-50 (LD_{50}) by measuring the rate of survival to adult state in larvae with different *SOD* genotypes subject to increasing levels of ionizing radiation.
- To determine the effect of *SOD* genotype on longevity after irradiation.
- To determine the fertility of irradiated females with different *SOD* genotypes.
- To measure the rate of dominant lethal mutations in irradiation late spermatids with different *SOD* genotypes, as well as the rescue effects of the maternal genotype.
- To measure the rate of dominant lethal mutations in irradiated stage-7 oocytes with different *SOD* genotype and the rescue effects of the sperm genotype.

We would like to emphasize, first, that as pointed out above we possess an ideal assay system to evaluate the radioprotective effects of *SOD* in that we have two naturally occurring alleles, S and F, that differ in specific enzymatic activity, plus one more, CA1 (or N for "null") that was also obtained from a natural population but exhibits only traces of enzymatic activity. Thus, radioprotection differences between genotypically different individuals that are causally attributable to *SOD* should occur according to the specific relationship $S > F \gg N$. This gradation makes possible a more critical analysis of the contribution of *SOD* to radioresistance than it is possible in other experiments of this kind where only two genetic alternatives exist—presence of absence of a functional gene.

Secondly we would like to emphasize that the experimental measurements for each *SOD* genotype are made in a set of (at least) four different genetic backgrounds. This is important for two reasons: (1) in order to make sure that the effects associated to *SOD* are attributable to that locus, rather than being due to a particular genotype at a different locus that might be accidentally associated with the *SOD* genotypes. This could indeed be the case if the experiments were made with only one particular strain of each *SOD* genotype (as it is often the case in studies of this kind). (2) In order to find out what the effects attributable to *SOD* are in different genetic backgrounds.

Objective 2. Molecular characterization of the null allele SOD^{CA1}. This gene contains a 0.68 Kb insert within the transcribed region. We proposed:

- To complete the sequence of the 2.5 Kb clone of the CA1 gene.
- To characterize the relevant elements of the CA1 clone.
- To characterize the level, size, and rate of synthesis of the transcript(s) from the CA1 gene.

The characterization of the CA1 gene and of its transcription (and translation) characteristics is an important step towards determining the genetic/physiological role of SOD in resistance to ionizing radiation.

3) Planned future work.

During years 2 and 3 of this grant, we will continue the investigations currently underway and carry out the other experiments proposed in our project:

- To sample natural populations of *D. melanogaster* seeking to isolate other naturally occurring SOD "null" alleles, and to characterize them and determine their frequency.

Objective 3. To investigate the correlation between radiation resistance and levels of SOD activity attributable to loci other than the SOD locus. We propose:

- To measure SOD activity levels in 30 strains homozygous by descent for different F alleles.
- To measure radiation resistance in the three strains with highest, and three with the lowest, SOD activity; and test for correlation between SOD activity levels and radioresistance.
- To determine whether inter-strain differences in SOD levels are due to differences in transcription or translation rates, or to some other process (e.g., rate of denaturation).

Objective 3 will make it possible to differentiate the contributions made to radioresistance by the amino acid difference between the S and F allozymes and the SOD levels per se. Genetic variation affecting SOD levels exists within a given allozyme class (such as homozygotes for the F allele; see Graf and Ayala, 1986). Objective 3 can be further pursued in a variety of ways that can be chosen after preliminary experiments are concluded.

The directions in which this project might be continued beyond year 3 will depend on the results of the current investigations. Along the lines of Objective 1 we would want to investigate, for example, why particular stages of the life cycle are more responsive to SOD levels than other stages. Along the lines of objective 2 we might want to investigate other "null" alleles found in natural populations (if they are found) and why they are maintained in populations. If no such alleles are found (as the CA1 null was) we might want to induce null mutations by mutagenesis and characterize these alleles. Along the lines of objective 3

(and 1 and 2 as well) we might want to use P element-mediated transformation to investigate how the presence of more than two SOD genes in an individual affects SOD protein levels and, in turn, how this would affect sensitivity to irradiation. We would like also to investigate the precise gene regulation mechanisms of transcription and translation for SOD.

Additional possible lines of future research are: (1) A genetic investigation of the role of catalase in radiosensitivity; catalase is functionally associated with SOD because it catalyzes the breakdown of H_2O_2 (SOD catalyzes the dismutation of the superoxide anion to H_2O_2 and oxygen) to water and molecular oxygen. (2) Investigation of the roles of SOD and catalase in radiosensitivity under conditions of oxygen-enhancement of radiation effects or of inhibition of catalase activity (e.g., by the herbicide 3-amino-1,2,4-triazole).

B. How this project relates to the DOE mission.

This research is directly related to the DOE interest in understanding the health effects of ionizing radiation, and particularly in the genetic and molecular basis of radiosensitivity differences between individuals.

C. How this project relates to other projects being funded by DOE.

The P.I. does not have the relevant information.

D. Project History.

The grant "Genetic Variation in Resistance to Ionizing Radiation" is a new research project started on January 1, 1990, which has been funded at a level of \$65,650 direct costs, plus \$31,180 indirect costs for Year 1. No other extramural support is available for this project.

The P.I. had an extramural research contract with DOE (and precursor agencies) extending from 1971 to 1986 to study the "Genetic Structure of Natural Populations" which was supported at an approximate increasing level of \$50,000 to 100,000 per year, direct costs, plus 40-45% indirect costs. From July 1987 to December 1989, the P.I. received a grant from DOE at a yearly level of approximately \$65,000 direct costs and \$30,000 indirect costs.

6. Scientific and Technical Content

A. Schedule of major research activities.

The experiments listed above in the section on current objectives are in progress and will continue through years 2 and 3. The experiments listed in the section "Planned Future Work" will start during the second half of year 2 (1991) and continue through

Year 3 (1992).

B. Scientific issues currently being addressed and their significance.

The general issue is the genetic determination of individual difference in resistance to ionizing radiation. More concretely, the issue is the role of the gene coding for Cu,Zn superoxide dismutase (SOD), an enzyme that catalyzes the dismutation of the superoxide anion to hydrogen peroxide and oxygen. Production of the very reactive superoxide anion is enhanced during exposure to ionizing radiation, which accounts for much of the cellular damage resulting from such exposure.

The studies proposed are specifically significant in that they represent a unique effort to investigate the effects of specific genetic differences in sensitivity to ionizing radiation, and that somatic as well as germline radiation effects are quantified using a variety of radiation doses. No such comprehensive study of individual genetic differences has been previously done to our knowledge.

The significance of the studies proposed is enhanced by the fact that they are unique by the combined presence of three additional traits: (1) the genetic variants (S, F, and CA1) analyzed are naturally occurring rather than obtained by mutagenesis; (2) the genetic background is randomized so that systematic radioresistance effects can be attributed to the SOD locus rather than to other uncontrolled genetic differences; (3) the three isolated alleles provide a gradation of SOD activity levels (S>F>>CA1) rather than only a presence-absence situation, as is usually the case in radioresistance (and, more generally, molecular) investigations.

C. Experimental and theoretical approach taken, techniques used, and resources applied.

1. Rationale. A distinctive component of our experiments is that three different SOD genotypes are employed: S/S, F/S, and CA1/CA1. The levels of enzymatic activity corresponding to these genotypes are S/S > F/S >> CA1/CA1. Hence, any putative radioprotective effects attributable to the SOD locus must vary among the genotypes accordingly to the inequalities just shown.

Accidental associations between radioprotective effects and the genetic background (i.e., the genotype at loci others than SOD) are eliminated in the experiments by the use of multiple independent strains for each SOD genotype; these genetically heterogeneous strains are all derived from a natural population.

The conflation between effects due to the target locus and those due to other loci is a common problem for experiments seeking

to attribute particular phenotypic effects to particular genotypes. Indeed, this conflation may be particularly worrisome in experiments with mutants induced by mutagenesis, because other mutations are likely to have been also induced elsewhere in the genome; and in experiments that use only one mutant and one wild-type strain, because (1) these strains are likely to differ at other loci as well, and (2) the effects may be due to specific associations between particular genes at other loci and the target locus, even when the two experimental stocks differ only at the target locus.

We investigate the effects that SOD may have in genetically different individuals by studying the effects of each SOD genotype in at least four different genetic backgrounds.

2. Stocks. 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 four 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. The 18 CA1/CA1 experimental strains were obtained by crossing the CA1/CA1 stock to each of 18 F/F strains and backcrossing the progenies to the same 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 experimental eggs, larvae, or flies used in the experiments are prepared for each of the three 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: 91F x 2F♂, 93F x 4F♂, etc. The F₁ progenies (symbolized 1F·2F, 3F·4F, etc.) are then intercrossed as follows: 1F·2F♀ x 3F·4F♂ (the progenies of which, symbolized 1F·2F·3F·4F are the experimental organisms). Four genetic backgrounds are created for each SOD genotype by intercrossing the stock strains. In addition to 1F·2F·3F·4F, the other three genetic backgrounds for the F/F homozygotes are 5F·6F·7F·8F, 9F·10F·11F·12F, and 13F·14F·15F·16F. Crosses are made so that in a particular experimental block any one of the original 18 stock strains is represented only once among the experimental organisms. These, of course, carry two genomes each derived from a different original strain and, hence, are similar to wild organisms in that they are produced by the combination of two genomes randomly selected from the natural population. Inbreeding effects are thus avoided while testing a random sample of wild genomes.

3. Lethal-Dose-50 for Different SOD Genotypes. First-instar 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 counted. At least 40 replicate vials are prepared for each genotype (F/F, S/S, and CA1/CA1) and radiation intensity. The irradiation dosages are 0, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 kR, but additional levels may be used, if preliminary experiments so indicate. The LD₅₀ will be determined by exponential regression (see Peng et al., 1986).

Similar experiments are performed with 82-96 h-old larvae in order to determine the LD₅₀ for third-instar larvae.

4. Genotype-Dependent Longevity Effects of Irradiation. First-instar larvae are collected and irradiated or kept as controls. Emerging adult flies are collected daily and placed in groups of 30 virgin males of the same age in a vial. The survivors are counted every 5 days and then transferred, without anaesthesia, to fresh vials.

These experiments are performed with irradiated first- and third-instar larvae for each genotype (F/F, S/S, and CA1/CA1). The levels of irradiation are as in the previous section.

5. Sterility-Dose-50 for Different Genotypes. Female fecundity is measured by placing fertilized females in a large vial with food which is replaced at 24 h intervals. The eggs laid are recorded as function of female age, stage irradiated, dose, and genotype. Adults developed from the LD₅₀ experiments above are used to measure the sterility effects of larval irradiation. 2-days old females irradiated as larvae are mated with excess 2-days old non-irradiated males of a standard genotype (the same for the various female genotypes), and transferred after 48 hours, without anaesthesia, to the vials with sampling spoons. Eggs are counted for five consecutive days (female age, 5-9 days). Experiments are replicated 20 times for each irradiation stage (first or third larval instar), irradiation dose (0, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 kR), and genotype (F/F, S/S, CA1/CA1). Additional doses will be used if preliminary results so indicate. Regression methods are used to estimate the dose at which fecundity is reduced by 50% (LD₅₀).

The LD₅₀ is also be estimated for females irradiated as pupae and as 1-day old adults.

6. Genotype-Dependent Rate of Lethal Mutations. Immature oocytes (stage-7) are powerful indicators of modification in radiation response (Sankaranayanan and Sobels, 1976; Nöthel, 1987). 2-3 days old virgin females of the appropriate genotype are starved for 6 hours and then fed for 18 hours with 5% glucose in water. Immediately after the feeding period, the females are irradiated

and then mated with excess males of either S/S or CA1/CA1 genotype. Eggs are collected from the second 24-hour brood, which represents irradiated immature oocytes at about stage-7. Sex-linked recessive lethals, X-chromosome losses, and dominant lethality (as indicated by failure of eggs to develop to adulthood) are scored. Control and irradiation doses are listed for previous experiments. Eggs are collected by placing 20 inseminated females in a plexiglass cylinder with an attached bottom section with medium (and a cotton plug on top). At least 1000 eggs are collected for each genotype and dose.

7. Molecular Characterization of the "Null" CA1 Allele. We have constructed a genomic library of the CA1 strain and isolated a clone with the SOD gene region. Standard DNA amplification (by means of the Ta9 polymerase chain reaction) and sequencing techniques are used to obtain the complete sequence of this "null" mutant.

We have observed revertants of the SOD^{CA1} mutant to wild-type SOD^S. These appear as occasional revertants among the numerous cultures and experimental populations of the CA1 allele kept in our laboratory. In order to characterize these putative revertants, we use primer-directed PCR amplification of the SOD locus with primers that flank the insertion point of the P-element or that span the whole EcoRI fragment that encompasses the SOD gene.

D. Importance of solving the problem being addressed by the research.

This project deals with general questions of major importance: the genetic determination of resistance to ionizing radiation and the degree of individual genetic variation concerning resistance. The particular role that SOD may play, and how it may do so through the different stages of the life cycle, is a more particular issue of importance. More generally, the experiments deal also with questions of general significance in the fields of populations genetics and radiation biology.

7. Project Output

A. Major recent accomplishments with supporting data and their significance.

As of this writing, this research project is in the eighth month of funding. A number of experiments are in progress and some have been completed (see 4-8 below); some relevant previous results are also summarized (1-3 below).

1. Developmental Profile of SOD. We have shown that the two common genetically-determined variants of SOD, S and F, differ in specific activity, as well as in thermostability and other properties, and that differences exist between different S or

different F lines (Lee et al. 1981, Graf and Ayala 1986). One important question is whether differences in SOD levels observed at one stage of the life cycle (or responses attributable to SOD levels) persist throughout development. We have examined this question by measuring SOD concentration from early larval stages through adult life. The results, illustrated in Figure 1 for two F strains, indicate that SOD levels are strictly and consistently regulated throughout development.

Fig. 1. Developmental profile of SOD content in two strains of *Drosophila melanogaster*, F3 and F8. Each point is the mean of three independent measures. Adult levels are the means of male and female levels. Vertical lines indicate standard errors. The two strains are homozygous for the "fast" electromorph of SOD.

2. Lethal Dose-50 for Irradiated 3-Instar Larvae. Preliminary results showing that SOD affects sensitivity to radiation were obtained by irradiating 3rd-instar larvae homozygous for the S, F, or N alleles or heterozygous for S and F. The mean numbers of adults developing from 30 irradiated larvae (16 replicates per observation) are shown in the table below (Peng et al. 1986).

Table 1. Number of adults developed from 30 irradiated larvae of *D. melanogaster* exposed to x-irradiation

Sod genotype	No. of adults (mean $\pm$ SEM)						
	0 R	1500 R	2500 R	3000 R	3500 R	4000 R	4500 R
S/S	28.3 $\pm$ 0.4	28.1 $\pm$ 0.4	26.5 $\pm$ 0.5	23.8 $\pm$ 0.6	24.1 $\pm$ 0.7	23.2 $\pm$ 0.7	10.3 $\pm$ 1.0
F/F	28.5 $\pm$ 0.3	26.8 $\pm$ 0.5	22.2 $\pm$ 1.0	21.5 $\pm$ 0.9	21.2 $\pm$ 0.7	15.3 $\pm$ 0.7	13.3 $\pm$ 1.0
S/F	28.4 $\pm$ 0.4	26.8 $\pm$ 0.9	24.5 $\pm$ 0.9	20.9 $\pm$ 0.9	18.8 $\pm$ 1.2	11.4 $\pm$ 0.8	12.9 $\pm$ 1.0
N/N	28.2 $\pm$ 0.4	27.7 $\pm$ 0.4	18.5 $\pm$ 1.0	18.4 $\pm$ 1.0	14.6 $\pm$ 1.3	9.4 $\pm$ 0.8	5.0 $\pm$ 0.7

The LD-50's for the four genotypes are given in Table 2. The LD-50's are calculated by first normalizing the viabilities by dividing by the control value and obtaining an arc sine transformation of the square root of the normalized viabilities. It is assumed that mortality is an experimental function of radiation dosage so that $v = a \cdot e^{-bD}$, where v is the transformed viability, D is the dose and a and b are the parameters of the experimental regression, which are obtained by regression of v on D , given that $\ln v = (\ln a) - bD$. The LD₅₀ is estimated by making $v = 45$ (notice that arc sine $0.50 = 45$) and then obtaining the value of $D = [-\ln(45/a)]/b$. The LD₅₀ for the various genotypes is given in Table 2 together with the regression parameters and a measure (r^2) of the experimental variance explained by the regression function. The values of r^2 are very high, which indicates that our model explains most of the experimental variability.

The LD₅₀ is lowest for the N/N homozygotes and highest for the S/S genotypes. This is consistent with the hypothesis that SOD plays a significant role in protecting against irradiation damage. Indeed, the null homozygotes exhibit no SOD activity detectable by electrophoresis (and only 3.5% of the "normal" activity as measured by immunoassay), whereas the S enzyme has higher *in vitro* activity than the F enzyme (5).

3. Response of SOD to Natural Selection after Irradiation. We prepared two continuous populations starting with the S and F alleles of SOD at equal frequencies (all founders were SOD heterozygotes). In one population, 4000 R were administered each generation to the third-instar larvae from which the next generation adults develop. The other population was equally treated except for the irradiation. The results are graphically shown in Figure 2. In the non-irradiated control population, the frequency of the S allele decreases to a frequency of 10-20%, which is approximately the frequency it has in the natural population whence the founder flies come. In the irradiated population, however, the frequency of S after selection is 50-60% considerably higher than in the control. This is precisely the result expected if SOD is involved in radioresistance, given that the S allozyme exhibits higher enzymatic activity than the F allozyme.

Fig. 2. Change in gene frequency in experimental irradiated (•, 4000 R per generation) and nonirradiated (▲) populations of *D. melanogaster*.

4. 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. Two experiments are in progress. One tests larvae irradiated during their third instar, i.e., 82-96 hours after hatching: ten replicates have been completed for each of five radiation treatments in addition to the controls. The second experiment tests larvae irradiated during first instar: ten replicates have been completed for just one treatment and their control. The results to date are shown in Table 3 for both experiments.

Table 3. Viability of irradiated larvae with different *Sod* genotype.

Geno- type	Control	Third Instar					First Instar	
		1K	2K	3K	4K	5K	1.5K reduction	%
F/F	29.2	28.8	26.0	25.8	25.1	3.9	25.4	14.0
S/S	28.5	26.5	25.3	22.2	24.3	3.6	23.3	18.2
N/N	28.1	27.7	26.9	23.9	15.5	1.7	17.4	38.1

The dose is given in kilorads. The numbers in the body of the table are the adults emerging out of 30 irradiated larvae, with 10 replicates for each genotype and treatment.

Two results are noteworthy. (1) Differences in radioresistance attributable to SOD are apparent in both experiments. (2) First-instar larvae are much more susceptible than third-instar larvae to irradiation. When third-instar larvae are irradiated, a major decrease in survival rate appears only at doses of 4Kr for the N/N genotype, whereas for first-instar larvae the viability of the N/N genotype is reduced by 38% already at 1.5Kr.

5. Genotype-Dependent Longevity Effects of Irradiation. First-instar larvae are collected and irradiated or kept as controls. Emerging adult flies are collected daily and placed in groups of 30 virgin males of the same age in a vial. The survivors are counted every 5 days and then transferred, without anaesthesia, to fresh vials. Two sets of controls and two sets of 1.5Kr experiments have been completed. Each set consists of 10 replicate vials each started with 30 flies. The results are shown in Figures 3 and 4, where each point in the graph is the average of the number of flies surviving in the 10 replicate cultures. Although these are only preliminary and partial data, some results are noticeable. One is that longevity of the SOD genotypes is different in different genetic backgrounds. Second that, as expected, longevity is considerably reduced (between 20 and 40 percent) by exposure to irradiation (1.5 Kr) in early life (first-instar larvae): oxygen radicals have been postulated as major contributors to the aging process. Third that the reduced longevity induced by irradiation is genotype-dependent.

Fig. 3

MEAN LONGEVITY AT 1.5Kr

Fig. 4

6. Sterility-Dose-50 for Different Genotypes. We first measured female fertility by placing a virgin female with two males in a vial and replacing each day a cardboard spoon with standard *Drosophila* food. Results for two levels of irradiation and controls are shown in Table 4. Each entry is based on 60 replications and represents the number of eggs collected in three days. Variation between replicates was, however, enormous as can be inferred from the standard deviations shown in the table. There was also considerable variation from day to day in the same culture.

Table 4. Fertility of females irradiated as third-instar larvae.
Percent Reduction = P.R.

Genotype	Dose (Kr)				
	0	1	P.R.	2	P.R.
FF	202 ± 43	111 ± 11	45.4	122 ± 20	39.6
SS	172 ± 24	157 ± 29	8.7	127 ± 20	26.2
NN	183 ± 26	78 ± 18	57.4	85 ± 18	53.6

The data are averages for 60 females of the number of eggs laid by a single female in days 4, 7, and 10 after emergence.

We have, therefore, performed a series of experiments, first, with different media and culture conditions in order to find a system which yields less variation and, second, to ascertain age-dependent fertility. We found that single females with two males each in a vial with charcoal medium at the bottom, and changing the female and males daily to a new vial yielded the most consistent results. Figure 5 shows the mean fertilities and standard error ($N \approx 80$) for females of different genotypes (FS represents heterozygous females with a father F/F and a mother S/S; and the reciprocal for SF). The pattern of age-dependent fertility variation is shown in Figure 6. We are using the new culture conditions for the experimental treatments.

7. 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. Eggs are collected from the second 24-hours brood, which represents irradiated immature oocytes at about stage-7. The incidence of

sex-linked recessive lethals, X-chromosome losses, and dominant lethality (as indicated by failure of eggs to hatch or to develop to adulthood: "embryonic" and "total") are shown in Table 5. The procedures are as described in the grant application. The results to date do not show significant differences between the S/S and F/F genotypes but a greater radiosensitivity of the N/N at 1.5Kr and 2.5Kr doses.

Table 5. Lethals (%) induced by irradiation of stage 7 immature oocytes with different *Sod* genotypes.

Genotype	Embryonic lethals		Total lethals
	1.5K	2.5K	1.5K
F/F	52.5	70.6	56.6
S/S	53.1	71.2	62.5
N/N	69.4	77.5	78.2

8. Molecular Characterization of the "Null" SOD^{CA1} Allele.

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 shown in Figures 7 and 8, it 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 (see Figure 8). In addition the inserted P-element fragment carries three poly-A signals, two of them adjacent to one

another (857 - 868, see Figure 8, which correspond to the second one shown on the top of Figure 7). 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.

B. Bibliography of publications emanating from this project.

This new project (less than eight funded months) has not resulted yet in any publication, but one paper in press. Page proofs of this and two earlier publications are enclosed (10 copies).

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Figure 5. Number of eggs laid in 24 hours for 19-day old females with standard error bars.

Figure 6.

Mean number of eggs laid in 24 hours for females of different ages and genotypes.

- FF
- FS
- ▲ SF
- ◇ SS
- + NN

Figure 7. The organization of the Cu, Zn SOD locus in an *SOD^{CA1}* strain of *D. melanogaster*. Dark boxes represent coding regions. The cross-hatched box is a portion of the 4th P-element ORF. Transcription start points and polyadenylation signals are indicated.

Figure 8.

The sequence of the *SOD^{CA1}* locus adjacent to the insertion of a P-element. The figure shows the alignment of *SOD^{CA1}*, regions of the inserted P-element and Cu, Zn SOD of *Drosophila melanogaster*. The 8-bp target site duplications as well as the site of the internal deletion within the P-element are indicated.

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