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Evolution

Irradiation-resistance conferred by superoxide dismutase: Possible adaptive role of a natural polymorphism in *Drosophila melanogaster*

(evolution/natural selection/allozyme variation/ionizing radiation/null alleles)

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ABSTRACT **The toxic effects of ionizing radiation to DNA are thought to be due to the generation of the superoxide radical, $O_2^{-\cdot}$. Superoxide dismutase (SOD), which scavenges $O_2^{-\cdot}$, has been invoked as a protecting enzyme against ionizing radiation in viruses, bacteria, mammalian cells in culture, and live mice. We now demonstrate that SOD is involved in the resistance of *Drosophila melanogaster* against irradiation. The protection is greatest when flies carry the S form of the enzyme (which exhibits highest *in vitro* specific activity), intermediate when they carry the F form of the enzyme, and lowest when they are homozygous for *N*, an allele that reduces the amount of the enzyme to 3.5% of the normal level. Natural selection experiments show that the fitness of the high-activity *S* allele is increased in an irradiated population relative to the nonirradiated control. These results point towards a possible adaptive function of the *S/F* polymorphism found in natural populations of *D. melanogaster*.**

The superoxide dismutases (SOD; superoxide:superoxide oxidoreductase, EC 1.15.1.1) are a family of metalloenzymes that provide organisms with protection against oxygen toxicity by scavenging the superoxide radical anion, $O_2^{-\cdot}$ (1, 2). Cu,Zn SOD is found in the cytosol of animals, plants, and fungi.

In *Drosophila melanogaster* the gene (*Sod*) coding for this enzyme has been mapped in the third chromosome, locus 32.5 or 34.6 (3). The enzyme is a dimer consisting of two identical subunits, each with a molecular weight of 16,000 (4). Two alleles, *S* and *F*, are present with variable frequencies in natural populations, although the *F* allele is always the most abundant. (The *S*, for "slow," and *F*, for "fast," designations refer to the relative mobility of the encoded enzymes in assays by standard gel electrophoresis.) The F and S polypeptides differ by a single amino acid substitution and also in various biochemical properties (5). Relevant for the present purposes is that the S allozyme exhibits higher specific activity than the F form; the purified S enzyme is typically capable of scavenging $O_2^{-\cdot}$ at a rate about 2.7 times faster than the F enzyme (5).

SOD has been shown to protect against ionizing radiation damage to DNA, viruses, bacteria, mammalian cells in culture, and even whole mice (6–13). We have now tested this property in *D. melanogaster* in two ways: (*i*) by measuring the reduction in viability of larvae of different genetic constitutions exposed to increasing doses of ionizing radiation and (*ii*) by following the change in allelic frequencies over several generations in populations subject to x-irradiation every generation. Our results corroborate the role of this enzyme in providing biological protection against ionizing radiation. The results also show that the higher-activity S form of the enzyme provides greater protection than the F allozyme. This, in turn, identifies a possible adaptive role for the *S* and *F* polymorphism found in natural populations of *Drosophila*.

MATERIALS AND METHODS

Strains. More than one thousand *D. melanogaster* flies were collected in September 1981 from a natural population ("El Rio Vineyard," San Joaquin County, 40 miles southeast of Sacramento). The females were individually placed in vials. Sib-pair matings were made in each of four consecutive generations with the progenies of each individual female. The *Sod* genotype of the mating pairs was determined by starch gel electrophoresis (14). In the fourth generation, we isolated 20 strains homozygous for the *F* allele and 20 strains homozygous for the *S* allele.

Additional single-female lines of *D. melanogaster* were isolated from a collection made in 1981 near the Davis campus of the University of California. These lines were used for an experiment on divergent selection for high and low SOD activity. After three generations of selection, J.-D. Graf isolated in our laboratory one line carrying a mutation (herein called Sod^N, "null" allele) that in homozygous condition yields only 3.5% of the normal SOD activity, as measured by immunoassay [the cross-reacting material (CRM) levels, in ng/mg of tissue, are 2.35 and 67.43 for flies homozygous *N/N* and *F/F*, respectively]. This null mutation is *cis*-acting and maps at chromosome 3, locus 33.4, which suggests that it is either a mutation in a regulatory site closely linked to the *Sod* locus or a change in the coding sequence of the gene itself.

Determination of LD_{50}. The viability of third-instar larvae after irradiation was measured for the genotypes *F/F*, *S/S*, *F/S*, and *N/N* as follows. One hundred virgin females and 100 males homozygous for the appropriate allele were placed in a half-pint (235-ml) culture bottle with food. Four replicate cultures were set up for each genotype. After 24 hr, these flies were transferred without etherization to another bottle and left there to lay eggs for 24 hr, after which the parents were discarded; after 72 hr more, the larvae (of age 72–96 hr) were washed out with a 20% sucrose solution. Several hundred third-instar larvae of a given genotype (collected in approximately equal numbers from the four culture bottles) were placed in glass Petri dishes and subjected to the appropriate radiation dosage. The larvae were then separated in groups of 30, and each group placed in a separate vial. At least 10 replicate vials of each genotype were prepared for each radiation dose. Control vials were prepared in the same way, except that the Petri dishes were not exposed to radiation. The adults emerging from each vial were counted. All 20 strains of each genotype were equally represented in the experiments; the parental flies in each culture were the progenies of crossing two different strains of the appropriate genotype (i.e., the *S/S* homozygous parents were the F_1 of a cross between two different *S* strains, and similarly for the

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Abbreviation: SOD, superoxide dismutase.

other genotypes). All the experiments were conducted at 25°C. The irradiation dosages administered were 0, 1500, 2500, 3000, 3500, 4000, and 4500 roentgens (R, 1 R = 2.58×10^{-4} coulomb/kg). The statistical methods for determining the LD_{50} (the dose at which 50% of the individuals die) are given in *Results*.

Natural Selection Experiments. Three irradiated and three control populations were established. Within each set, one population was polymorphic for the *F* and *S* alleles, one polymorphic for *F* and *N*, and the third polymorphic for *S* and *N*. The founding flies were F_1 heterozygous progenies of parents homozygous for the appropriate alleles. The *F* and *S* parental homozygotes were themselves F_1 progenies of crosses between two different strains (i.e., the *F/F* homozygous parents were the F_1 of a cross between two different *F* strains, in such a way that all 20 *F* strains were equally represented in each population, and similarly for the *S/S* homozygotes). The *N/N* parental flies were obtained by intercrossing several *N* strains, but these had all been derived a few generations earlier from the single "null" strain isolated by J.-D. Graf.

Each population consisted of four culture bottles, each with approximately 100 pairs of parents. Radiation was administered to third-instar larvae, collected in the same way as for the LD_{50} experiment. Four sets of 10 replicate vials were prepared for each population, but the flies emerging from the vials were not counted. Instead, 25 males and 25 females were chosen for determining the *Sod* genotype by gel electrophoresis, and the rest were redistributed into four culture bottles in such a way that the progenies of the four cultures making up each population would be intermingled every generation. The radiation dose administered per generation was 4000 R, chosen because this was close to the LD_{50} as determined by the previous experiment. The populations were kept at 25°C.

In the *F* and *S* polymorphic population, all three genotypes are identifiable by electrophoresis and hence used for determining the allele frequencies. In the populations containing the *N* allele, only two phenotypic classes are identifiable by electrophoresis; the frequency of the *N* allele was determined as the square root of the frequency of the *N/N* homozygotes. This method of estimation is predicated on the assumption that the genotypic frequencies are in Hardy–Weinberg equilibrium, which cannot be tested in the populations with the *N* allele. We have made this test in the populations with the *S* and *F* alleles and found that only 2 out of the 26 samples do not fit the Hardy–Weinberg expectations at the 5% level, which is approximately what should be expected by chance.

Irradiation. This was done at the Laboratory for Energy Related Health Research in our university. X-rays were delivered with a GE Maximar therapy machine at a rate of 86 R/min (operating at 250 kV, 15 mA, with 0.25-mm Al filtration).

RESULTS

LD_{50} Experiments. The main results of the viability tests used for determining the LD_{50} are given in Table 1. In the absence of radiation, about 95% of the larvae reach the adult stage with no differences among the genotypes. As the radiation dose increases, the viability decreases, with the homozygotes for the null *Sod* allele showing the greatest sensitivity.

LD_{50} is calculated as follows. The viabilities given in Table 1 are first normalized by dividing them by the control value, and an arc sine transformation of the square root of the normalized viabilities is obtained. We assume that mortality is an exponential function of radiation dosage so that $v = ae^{-bD}$, where v is the transformed viability, D is the dose, and a and b are the parameters of the exponential regression, which are obtained by regression of v on D, given that $\ln v = (\ln a) - bD$. The LD_{50} is estimated by making $v = 45$ (notice that arc sine $\sqrt{0.50} = 45$) and then obtaining the value of $D = [-\ln(45/a)]/b$. The LD_{50} 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 LD_{50} 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).

Selection Experiments. The changes in allele frequencies in the six experimental populations are shown in Fig. 1. The experimental populations were treated with 4000 R administered every generation to third-instar larvae. The frequency of the *S* allele relative to the *F* allele is considerably higher in the irradiated than in the control population polymorphic for these two alleles. Like the LD_{50} results, this is consistent with the hypotheses that SOD protects against ionizing-radiation damage and that the degree of protection is a function of the form of the enzyme present. The higher *in vitro* activity of the S relative to the F allozyme is consistent with the higher frequency of S observed in the irradiated than in the control population polymorphic for these two allozymes.

The dynamics of the four populations involving the *N* alleles are puzzling. The frequency of the *N* allele is lower in the irradiated than in the control populations in most generations (the exceptions are two samples in the populations with the *S* allele and three samples in the populations with the *F* allele), but there is no evidence that the irradiation has decreased substantially the fitness of the *N* allele. One possible explanation of this unexpected result is that the *N/N* homozygotes have very low fitness even in the nonirradiated populations and that, therefore, the irradiation reduces their fitness little if at all.

We have evaluated the effects of natural selection in the populations by two different methods. First, we have used the method of Fisher (15, 16) to test whether the changes in gene frequencies can be explained as the result of random genetic drift alone. Fisher and Ford (15) have shown how the

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

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

The average number of replicates per observation is 16.

Table 2. Parameters of the exponential regression ($v = ae^{-bD}$) and LD_{50}

Sod genotype	a	b	r^2	LD_{50}, kR
S/S	102.1	0.154	0.82	5.31
F/F	93.7	0.159	0.97	4.61
S/F	97.5	0.185	0.95	4.19
N/N	106.9	0.274	0.94	3.16

The coefficient of determination, r^2, measures the fraction of the experimental variance explained by the regression.

effective size of the population in each generation and the sample taken from it can be used to generate an expected matrix of covariances between the gene frequencies observed in the various generations. This covariance matrix can then be used to test, by means of a χ^2, the hypothesis that random drift alone may account for the observed changes in gene frequency.

We need to know, however, the effective size of the population. Each experimental population consisted of about 800 individuals distributed into four cultures. The effective population size may reasonably be estimated as $n_e = 400$ individuals; that is, half the number of individuals in the population (17). We have used two additional estimates of effective population size, a conservative one ($n_e = 200$) and a generous one ($n_e = 600$). The results are given in Table 3. Significant deviations from random drift are observed in two of the six experimental populations. This method is, however, sensitive only to relatively large differences in selective value, of the magnitude $s \geq 0.05$ (16).

The second method is based on a model of linear frequency change due to selection (16), which may detect selection coefficients as small as 0.01. The magnitude of the selection differential per generation is measured by a linear parameter, γ, whereas statistical significance is evaluated by a χ^2 with one degree of freedom (16). The results of this test are given in Table 4. Significant selection effects are detected in three of the four populations polymorphic for the *N* allele, indicating that the *N* allele is being selected against. More important, however, are the results of the populations having both the *S* and *F* alleles, given that these are the only two alleles present in natural populations in nontrivial frequencies. In this case, the control population manifests a selective effect significantly different from zero, whereas the irradiated population does not. Interestingly, however, the coefficient of selection is negative (i.e., against the *S* allele) in the control population but positive (i.e., favoring the *S* allele) in the irradiated one, pointing to the disparate courses followed by these two populations.

Table 3. Tests of selective neutrality in experimental populations of *D. melanogaster* using the Fisher method (15), with three different values of the effective population size, n_e

Genetic composition	Treatment	Degrees of freedom	χ^2 $n_e = 200$	χ^2 $n_e = 400$	χ^2 $n_e = 600$
F and *S*	Irradiated	12	10.2	12.8	14.3
F and *S*	Control	12	21.2*	28.4†	32.7†
S and *N*	Irradiated	11	10.5	13.0	14.4
S and *N*	Control	11	12.0	16.4	19.2
F and *N*	Irradiated	11	15.6	20.7*	23.8*
F and *N*	Control	11	14.1	17.5	19.3

*$P < 0.05$.
†$P < 0.005$.

DISCUSSION

SODs protect against the toxicity of oxygen by catalyzing the dismutation of the superoxide radical anion, $O_2^{-\cdot}$, to molecular oxygen and hydrogen peroxide: $2O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$. That the toxicity of oxygen is largely due to the generation of superoxide radicals is supported by multiple sources of evidence (2), including paraquat studies showing that this herbicide is easily reduced to a relatively stable radical that in turn reacts with oxygen, generating $O_2^{-\cdot}$; the increased production of $O_2^{-\cdot}$ radicals is one cause of the lethality of paraquat (18, 19).

The toxic effects of ionizing-radiation damage to DNA are thought also to be due to the generation of superoxide radicals (13, 20, 21). Oxygen has long been known to enhance the lethality of ionizing radiation (2), and $O_2^{-\cdot}$ radicals are produced during the radiolysis of water (22). In any case, it has been shown previously that SOD protects against radiation damage to DNA (6), to viruses (7), bacteria (9), mammalian cells in culture (7), and whole mice (12). Protection was accomplished in these cases by adding SOD to the suspension medium or by injecting SOD into the mice after their irradiation.

We have now demonstrated in *D. melanogaster* a protective role against ionizing radiation for the SOD synthesized by the organism, and that the degree of protection is correlated with the specific activity of particular enzyme variants. The LD_{50} is considerably lower for individuals lacking SOD (null homozygotes) than for flies with active forms of the enzyme and is higher for flies having the allozyme with higher specific activity (S) than for those with the lower-activity allozyme (F). In addition, the natural selection experiments show that the fitness of the S enzyme relative to the F enzyme

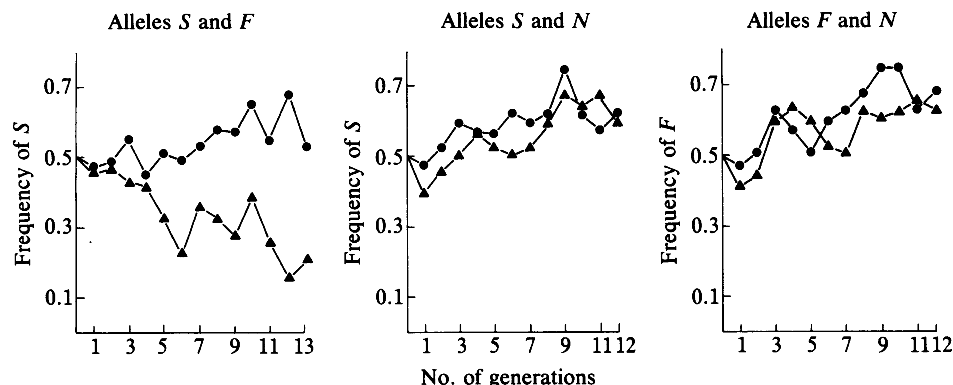

FIG. 1. Change in gene frequency in experimental irradiated (●, 4000 R per generation) and nonirradiated (▲) populations of *D. melanogaster*. Each population is polymorphic for two *Sod* alleles: *S* and *F* (*Left*), *S* and *N* (*Center*), *F* and *N* (*Right*).

Table 4. Tests of directional change in experimental populations of *D. melanogaster*

Genetic composition	Treatment	n_e = 200		n_e = 400		n_e = 600	
		γ	χ^2	γ	χ^2	γ	χ^2
S and *F*	Irradiated	0.021	1.6	0.022	2.8	0.022	3.8
S and *F*	Control	−0.047	8.0*	−0.047	12.8*	−0.046	16.2*
S and *N*	Irradiated	0.025	2.0	0.024	3.0	0.024	3.7
S and *N*	Control	0.043	5.9†	0.043	9.2*	0.042	11.6*
F and *N*	Irradiated	0.040	5.1†	0.040	8.3*	0.041	10.6*
F and *N*	Control	0.035	3.9	0.033	5.5†	0.032	6.6†

The coefficient of change, γ, measures the rate of change per generation of the first listed allele relative to the second one. The χ^2 values have one degree of freedom.
*$P < 0.005$.
†$P < 0.05$.

is considerably enhanced when the populations are exposed to ionizing radiation every generation.

The results reported here throw light on the general question of the maintenance of the pervasive enzyme polymorphisms present in natural populations. Our results show that the two SOD allozymes, F and S, found in natural populations of *D. melanogaster* may be functionally different *in vivo*, and not only *in vitro* as previously demonstrated (5). This is the case even though the two allozymes differ by a single amino acid substitution in a region of the protein that is not apparently involved in any essential function of the enzyme and that has evolved rapidly through evolutionary time (23).

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