The overexpression of antioxidative enzymes such as CuZn-superoxide dismutase (SOD), Mn-SOD, and catalase has been previously reported to extend life span in transgenic flies (Drosophila melanogaster). The purpose of this study was to determine whether life-extending effects persist if the recipient control strains of flies are relatively short-lived. Accordingly, the life spans of large numbers of replicate control and overexpressor lines were determined in two long-lived genetic backgrounds involving a combined total of >90,000 flies. Significant increases in the activities of both CuZn-SOD and catalase had no beneficial effect on survivorship in relatively long-lived y w mutant flies and were associated with slightly decreased life spans in wild type flies of the Oregon-R strain. The introduction of additional transgenes encoding Mn-SOD or thioredoxin reductase in the same genetic background also failed to cause life span extension. In conjunction with data from earlier studies, the results show that increasing the activities of these major antioxidative enzymes above wild type levels does not decrease the rate of aging in long-lived strains of Drosophila, although there may be some effect in relatively short-lived strains.

The free radical hypothesis of aging postulates that senescence is due to an accumulation of molecular oxidative damage, caused largely by oxidants that are produced as by-products of normal metabolic processes (1). A logical prediction based on this hypothesis is that the elevation of antioxidative defenses should delay aging and extend life span (2).

CuZn-superoxide dismutase (SOD) and catalase act in tandem to eliminate superoxide anion radical and hydrogen peroxide, respectively, thereby constituting the primary line of intracellular, enzymatic, and antioxidative defense (2). Mn-SOD serves to eliminate superoxide radicals in the mitochondrial matrix, whereas thioredoxin reductase regenerates both reduced glutathione and thioredoxin in the fruit fly, Drosophila melanogaster (3). Several early studies revealed little or no increase in life span following overexpression of these enzymes in transgenic flies (4–7). Subsequently, three groups reported life span extensions, ranging from 33 to 48%, in flies overexpressing CuZn-SOD alone or in conjunction with catalase (2, 8, 9).

While the latter studies ostensibly confirmed the free radical hypothesis, the strength of their conclusions has been questioned on the basis that insufficient numbers of control strains were used (10) or that the controls had artificially short life spans, possibly because of inbreeding depression or other genetic background effects (11, 12). For instance, in one study (9), the life spans of control flies ranged from 25 to 65 days, whereas life span extension ranged from −3 to +48% in flies overexpressing CuZn-SOD. The greatest proportional increase in longevity occurred in the shortest-lived genetic background, extending the life span to only 37 days, whereas the 3% decrease in mean survival time was observed in a background with a control life span of 59 days. An additional complicating factor is the discordant findings that overexpression of the mitochondrial enzyme, Mn-SOD, either extends life span in a dose-dependent manner (13) or has no positive effect on life span (14).

The experiments reported here were undertaken to clarify the effects of simultaneous overexpression of different antioxidative gene combinations in relatively long-lived strains. The combinations employed were as follows: (i) CuZn-SOD and catalase; (ii) CuZn-SOD, catalase, and Mn-SOD; (iii) CuZn-SOD and thioredoxin reductase; (iv) catalase and thioredoxin reductase; and (v) CuZn-SOD, catalase, and thioredoxin reductase. A large number of replicate lines were used to control for insertional position effects of the transgenes. Increases in the gene dosages and activities of these enzymes were shown not to extend the life span of Drosophila in two outbred genetic backgrounds.

**EXPERIMENTAL PROCEDURES**

**Construction of Transgenic Fly Lines**—In this study, 15 Drosophila lines were constructed by recombination of transgenes containing the Drosophila genomic CuZn-SOD and catalase sequences, each inserted at one of five distinct loci, onto a single chromosome 2 homologue, which was subsequently maintained over the balancer chromosome CyO. Similarly, 17 control lines were constructed with transgenes containing empty vector sequences inserted at two of seven different loci and balanced over CyO. The construction of the individual transgenes and their effects on longevity have been described previously (6, 7).

Additional transgenic lines were generated by recombination of a thioredoxin reductase transgene at one of six different loci (15) with either CuZn-SOD (19 combinations), catalase (14 combinations), or both CuZn-SOD and catalase (28 combinations). Similarly, 28 combinations were made using CuZn-SOD, catalase, and Mn-SOD transgenes (the Mn-SOD transgenes were inserted at one of seven different loci). For the experiments involving three antioxidative transgenes, controls were generated by recombination of groups of three empty vector transgenes inserted at a total of seven loci (20 combinations).
The presence of all of the transgenes in each stock maintained over the CyO balancer chromosome was verified by Southern analysis. For antioxidant overexpressor stocks, separate probes were used for each antioxidant gene sequence. For controls, vector sequences were used in the probe.

**Life Spans and Enzyme Assays**—Male transgenic flies were backcrossed to parental y w females, and heterozygous male progeny were collected for measurement of antioxidant enzyme activities. CuZn-superoxide dismutase activity was measured by the method of Spitz and Oberley (16) using 2% sodium dodecyl sulfate pretreatment for 30 min to remove Mn-SOD activity (17) as described previously (18). Catalase activity was measured by monitoring rates of H$_2$O$_2$ consumption at 30°C as also described previously (15, 19). Thioredoxin reductase activity was measured using a surrogate assay with 5,5′-dithiobis(2-nitrobenzoic acid) as the substrate (15).

For life span experiments, male transgenic flies balanced over CyO were backcrossed to virgin y w females or outcrossed to Oregon R (wild type) females. Male CyO progeny, heterozygous for each transgene, were collected 1 ± 1 day post-eclosion and maintained at 25 ± 1°C. Fresh vials containing standard medium (yeast-cornmeal-sugar-agar) were provided, and survivorship was scored every second day initially and every single day beginning 20–30 days after collection.

**Statistical Analysis**—Within each life span experiment, the mean value for each transgenic line was calculated as recommended by Tatar (10) and compared using an unpaired Student’s t test. Enzyme activities (overexpressor versus control) were also compared using unpaired Student’s t tests. Correlation analysis involving enzyme activity and life span data for individual fly lines in different experiments was performed using Microsoft Excel software. Critical values of the correlation coefficient (r) were obtained from published tables (20).

**RESULTS**

**Overexpression of CuZn-SOD and Catalase**—Enzyme activities were determined for the 15 lines containing heterozygous CuZn-SOD and catalase transgenes in a y w background and 17 controls containing two empty-vector transgenes (Fig. 1). The CuZn-SOD activity of the SOD/catalase strains was increased by 50 ± 29% (mean ± S.D.; range: +19–128%) in comparison with the mean of the control values, whereas catalase activity was increased by 61 ± 41% (range: +18–144%). The differences in activity between overexpressor and control lines were highly significant for both enzymes (p < 0.0001).

**Effects of Different Transgene Combinations on Life Span: CuZn-SOD and Catalase**—The mean life spans of the SOD/catalase and control lines were 69.4 and 67.8 days, respectively, in the first of two independent experiments in the y w background (Fig. 2A). The respective life spans in the second experiment were 63.0 and 67.0 days. The net differences in survival times in the two experiments (SOD/catalase versus control) were +2.3% and −6.0% (Table I). All of the transgenic lines were also outcrossed to wild type (Oregon-R) females in two independent experiments, yielding life spans of 58.9 versus 61.3 days (−3.8%) and 60.0 versus 64.7 days (−7.2%). Among the four experiments, only the life span reduction of the latter cohort in the wild type background reached statistical significance (p < 0.01) (Fig. 2B). Comparisons among the individual lines overexpressing CuZn-SOD and catalase demonstrated that there was no significant correlation between the activities of the two enzymes, or between the activity of either enzyme and life span in any of the four experiments, or among the life spans of the individual lines in replicate experiments in either genetic background.

**CuZn-SOD, Catalase, and Mn-SOD**—In a separate study, a total of three life span experiments were conducted in two genetic backgrounds with flies containing CuZn-SOD, catalase,
and Mn-SOD transgenes (Fig. 3A). Survivorship data were obtained for a total of >25,000 flies. In each experiment, there was a non-significant decrease (<5%) in the mean life spans of flies containing antioxidative transgenes. None of these differences was statistically significant.

**CuZn-SOD, Catalase, and Thioredoxin Reductase—**Another experiment was conducted to examine the effects of CuZn-SOD and thioredoxin reductase transgenes in a y w background as well as two experiments with catalase and thioredoxin reductase transgenes. Overexpression of both enzymes was verified experimentally in the latter case (results not shown), whereas in the former case it was inferred from increased enzymatic activity in the ancestral transgenic lines prior to recombination. Each experiment involved in excess of 2800 experimental and 3000 control flies, but there were no significant changes in survival times (Table I).

Finally, in an independent study, the CuZn-SOD, catalase, and thioredoxin reductase transgenes were introduced into the same flies. An initial study of 5995 backcrossed experimental flies demonstrated a significant 7.9% increase in life span in comparison with 4300 control flies (p < 0.05) (Fig. 3D). However, a second experiment failed to replicate this result (Fig. 3C).

**DISCUSSION**

The main finding of this study is that the introduction of transgenes resulting in overexpression of major antioxidative enzymes had no significant effect on survival times if relatively long-lived fly lines were used as controls. A grand total of >90,000 flies were studied using two genetic backgrounds and large numbers of replicate lines with transgenes inserted at different loci to control for insertional position effects. These results demonstrate unequivocally that overexpression of the *Drosophila* CuZn-SOD, Mn-SOD, catalase, and thioredoxin reductase genes in the normal spatial and temporal patterns has no beneficial effect on longevity in long-lived outbred backgrounds. These findings differ from those of previous studies in which the pattern of gene expression was altered and/or the life spans of the control populations were relatively short.

The absence of life span extension in this study is seemingly at odds with the conclusions of several existing studies involving overexpression of SOD and/or catalase (2, 8, 9, 13). It has been noted previously (12) that reports of large relative increases in longevity in *Drosophila* have been based on control populations with life spans as short as 25–35 days. Such results should be interpreted with great caution, because the reference point is barely half of the normal value. The “extended” life spans of experimental populations in these studies do not exceed those of healthy control strains of the same species maintained under...

### TABLE I

**Summary of life span effects resulting from overexpression of enzymatic antioxidants**

| Transgenes (background)                        | Overexpressor life span<sup>a</sup> | Control life span<sup>b</sup> | Increase in life span<sup>c</sup> |
|------------------------------------------------|-------------------------------------|-------------------------------|-----------------------------------|
|                                                 | days                                | days                          | %                                |
| CuZn-SOD/catalase (y w)                        | 69.4 ± 7.9                          | 67.8 ± 6.8                    | +2.3                              |
| CuZn-SOD/catalase (y w) in a wild type background | 63.0 ± 10.4                         | 67.0 ± 7.4                    | −6.0                              |
| CuZn-SOD/catalase (black squares)              | 58.9 ± 4.6                          | 61.3 ± 8.6                    | −3.8                              |
| CuZn-SOD/catalase (wild type)                  | 60.0 ± 4.7                          | *64.7 ± 4.2*                  | −7.2<sup>c</sup>                  |
| CuZn-SOD/catalase/Mn-SOD (y w)                 | 63.6 ± 6.3                          | 65.8 ± 7.3                    | −2.2                              |
| CuZn-SOD/catalase/Mn-SOD (wild type)           | 60.3 ± 5.4                          | 61.7 ± 6.1                    | −3.8                              |
| CuZn-SOD/thioredoxin reductase (y w)           | 72.8 ± 5.0                          | 72.6 ± 6.6                    | +0.3                              |
| Catalase/thioredoxin reductase (y w)           | 63.6 ± 6.1                          | 63.4 ± 6.3                    | +0.3                              |
| Catalase/thioredoxin reductase (y w)           | 72.1 ± 6.7                          | 66.1 ± 10.7                   | +9.0                              |
| CuZn-SOD/catalase/thioredoxin reductase (y w)  | 67.4 ± 6.9                          | 62.5 ± 8.1                    | +7.9<sup>c</sup>                  |

<sup>a</sup> Results are expressed as mean ± S.D. of the mean life spans of individual lines of flies.

<sup>b</sup> Percent differences in mean life spans are of overexpressor versus control lines. Negative numbers indicate that the controls lived longer on average than the antioxidant overexpressors. Italics indicate experiments shown in Figs. 2 and 3.

<sup>c</sup> Boldface indicates statistically significant differences between overexpressor and control life spans (p < 0.05).
optimal conditions. This point is underscored by the finding of Sun and Tower that a single transgene insertion (SOD3B2) caused a significant 16–20% extension of life span in a short-lived TM3, Sb background, and a non-significant 1–3% decrease in survival times in a long-lived DrMIO background (9).

However, a second line (SOD3A1) had a significant 10–14% increase in life span following SOD overexpression in either background. This indicates that both the starting life span and epistatic interactions with other loci can affect the life span extension resulting from transgene overexpression. If the maximum life span extensions from all of the published studies of antioxidative enzyme overexpression are considered together, it appears that the beneficial effects are minimized or disappear completely in animals with relatively long reference life spans (Fig. 4A). If all of the data from each study are considered, i.e., the average of the life span extensions instead of the maximum beneficial effect, then a similar trend is observed, but the extension of life span associated with shorter-lived backgrounds becomes much smaller in magnitude (Fig. 4B).

The clearest exception to this generalization is the targeted expression of human CuZn-SOD in Drosophila motor neurons (8), which resulted in some extension of life span even when the SOD allele contained mutations associated with amyotrophic lateral sclerosis (23). However, the level of expression of CuZn-SOD in the central nervous system of adult Drosophila is normally very low (24). Thus, targeted expression may rescue...
an insufficiency in this tissue type, rather than supporting a general conclusion that increasing antioxidant levels slows the aging process. Furthermore, in the absence of replication in alternative genetic backgrounds, the possibility that this is a strain-dependent effect cannot be ruled out. It should also be noted that the complete abolition of Drosophila Cu/Zn-SOD and its replacement with human SOD at 5–10% of wild type activity levels were recently reported to have no detectable impact on survivorship or other biochemical and physiological parameters pertaining to oxidative stress and the rate of aging (25).

Given that mortality rates are either unaffected or even increased in both humans and mice with bolstered levels of superoxide dismutase or other antioxidants (26–29), it is striking to find that in lower organisms the antioxidant theory of aging is also less strongly supported than has been previously maintained. It is also crucial to recognize that these results do not contradict the broader oxidative stress hypothesis of aging, according to which the key parameter is oxidative damage arising from an imbalance among oxidant production, antioxidant defenses, and repair processes. However, the available evidence backed by the current findings suggests that antioxidant levels are not the limiting factor in this imbalance.

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Effects of Overexpression of Copper-Zinc and Manganese Superoxide Dismutases, Catalase, and Thioredoxin Reductase Genes on Longevity in *Drosophila melanogaster*

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