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# The Antiozonant Ethylenediurea Does Not Act via Superoxide Dismutase Induction in Bean[1]

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## ABSTRACT

**It has been proposed that the mode of action of ethylenediurea, a very effective antiozonant, is via an increase in the antioxidant enzyme superoxide dismutase (EH Lee, JH Bennett [1982] Plant Physiol 69: 1444–1449). Data presented here refute that hypothesis. No ethylenediurea-associated increases in Cu/Zn-superoxide dismutase or Mn-superoxide dismutase activity, nor in steady-state Cu/Zn-superoxide dismutase protein levels, were found in soluble extracts of bean (*Phaseolus vulgaris* L. cv Bush Blue Lake 290) leaves. However, the cytosolic Cu/Zn-superoxide dismutase increased as a result of ozone fumigation and subsequent injury. Also noted was a developmentally related difference between chloroplastic and cytosolic Cu/Zn-superoxide dismutase, the latter declining during maturation of the leaf.**

In 1978, Carnahan *et al.* (7) reported the development of a powerful new chemical, EDU,[2] that protected foliage from ozone injury when applied as a soil drench or as a foliar spray. Their experimental data "implied the existence of an antagonistic interaction in definite proportions between EDU and ozone within the plants" (7). However, the nature of the interaction was not readily evident from the chemistry of EDU; it was not an exceptional reducing agent, nor did it appear to be an ozone scavenger (J.E. Carnahan, personal communication). In 1982, Lee and Bennett (17) published experimental results indicating that EDU-induced ozone tolerance in snap bean correlated with significant increases in SOD and catalase activities in leaf tissue, as measured by colorimetric solution assays and SDS-PAGE. Finding that young leaves, which are characteristically more ozone tolerant than older leaves, also had a higher SOD activity than older leaves, they speculated that increased SOD activity was the basis for ozone tolerance in EDU-treated plants. While their conclusion has been frequently cited, experimental data published by other researchers have not supported that conclusion. Although EDU protected other bean cultivars from ozone injury, increased SOD activity did not accompany that protection (8). Moreover, McKersie *et al.* (20) were unable to show a significant positive correlation between ozone tolerance and SOD activity in bean cultivars with differing ozone tolerances. Understanding the mode of action of EDU would not only elucidate an effective resistance mechanism but might also clarify the mechanism of ozone damage. It has been shown that EDU does not work via stomatal closure (4); nor does it scavenge superoxide ions (1), hydroxyl radicals (12), or hydrogen peroxide (L.H. Pitcher, unpublished data).

SOD in leaves is a primary scavenger for superoxide radicals generated both as a by-product of normal physiological activities and from exposure to pollutants such as ozone. Its importance as an antioxidant is demonstrated by the fact that it has been found in all aerobic organisms studied to date; also, mutants that are deficient in SOD are extremely sensitive to oxidative stress (11). Further, SOD activity increases in response to various oxidative stress conditions (2, 11, 23), including ozone (8, 20). Cu/Zn-SOD has been the focus of much of our research; therefore, based on Lee and Bennett's (17) report, we attempted to use EDU to study the regulation of SOD gene expression. However, we found no effect of EDU on SOD activity in our model system, pea, even though the plants were completely protected from ozone damage at doses comparable to those used in bean (Y. Shaaltiel, L.H. Pitcher, unpublished data). Thinking the effect might be genotype related, we studied the species and variety used by Lee and Bennett. Our goal was to test the hypothesis that the protective action of EDU was singularly dependent upon an increase in SOD. Our specific objectives were as follows: (a) to determine if EDU and/or ozone caused a significant change in SOD activity associated with cyt or chl Cu/Zn isoforms, or in Mn-SOD activity during a time period associated with EDU-induced ozone tolerance; (b) to determine if EDU and/or ozone caused a significant change in the steady-state levels of Cu/Zn-SOD immunologically detectable protein associated with the cyt or chl isoforms during a time period associated with EDU-induced ozone tolerance; and (c) to determine the long-term effects of EDU on overall SOD activity, as well as upon the relative activities of the various isozymes, by following SOD activity in leaves at different stages of development when EDU was administered to the plants.

Of major importance to the achievement of our objectives was the employment of two independent methods for the measurement of SOD, each one superior to colorimetric solution assays of leaf extracts or partially purified enzyme as

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[2] Abbreviations: EDU, ethylenediurea, *N*-[2-(2-oxo-1-imidazolidinyl)ethyl]-*N'*-phenylurea; SOD, superoxide dismutase; chl, chloroplastic; cyt, cytosolic.

performed by Lee and Bennett (17). Solution assays used without inhibitors, as employed by Lee and Bennett (17), do not distinguish among the various SOD isozymes but measure only total SOD activity; furthermore, they are well recognized to be fraught with technical difficulties (5, 10). We employed nondenaturing gel assays to measure activity of individual SOD isozymes and immunoblot assays to determine steady-state protein levels of the two Cu/Zn-SOD isozymes.

## MATERIALS AND METHODS

### Plant Growth, Experimental Design, Treatment, and Sampling

Snap beans (*Phaseolus vulgaris* L. cv Bush Blue Lake 290), obtained from E. Lee, were grown from seed in Jiffy Mix (Jiffy Products, West Chicago, IL) in 4-inch plastic or peat pots and fertilized weekly with Peter's 20–20–20 supplemented with Peter's micronutrients (W.R. Grace and Co., Fogelsville, PA). The plants were grown during the months of June, July, and August in a naturally lighted greenhouse supplied with charcoal-filtered air. Plants were used when the second set of trifoliate leaves was at approximately 80% expansion, because the leaf is maximally sensitive to ozone at this stage of development and because Lee and Bennett (17) showed a doubling of SOD in response to EDU at this stage.

The design for the main experiment consisted of four treatments with six or seven plants per variable (each variable within any one experiment containing the same number): control nontreated plants (C), EDU-treated plants (E), ozonated plants not pretreated with EDU (O), and ozonated plants pretreated with EDU (EO). The experiment was performed six times.

EDU (E.I. duPont de Nemours and Co.), prepared in aqueous solution at a concentration of 0.3 mg/mL, was applied to bean plants as a soil drench on the afternoon of the day before ozone exposure. Ozone fumigations (0.3 ppm for 6 h) were conducted in a dynamic flow system contained within a greenhouse (18); nonfumigated plants were held in a similar chamber without ozone during the fumigation. Ozone was produced by a generator (OREC model 0341–0) supplied with high-grade oxygen. Ozone concentration in the chamber was measured by the neutral potassium iodide method (13). One or two leaflets from the second trifoliate leaves were harvested and pooled 24 h after the end of ozone fumigation (which was 48 h after EDU treatment, as in Lee and Bennett [17]), frozen in liquid $N_2$, and stored at −70°C until extracts were prepared for analysis. Forty-eight hours after fumigation, the remaining second trifoliate leaflets were rated for visible injury on a scale of 0 to 10, where 10 equals 100% of the surface damaged.

### Preparation of Extracts

Leaves were ground to a powder with mortar and pestle under liquid $N_2$, then homogenized at a ratio of 1 g of leaf powder/5 mL of ice-cold buffer (100 mM sodium phosphate, pH 7.8, 0.1 mM EDTA, and 5 or 10 mM $\beta$-mercaptoethanol) with 6% insoluble PVP added. After filtration through Miracloth (Calbiochem), the filtrate was centrifuged at 39,000*g* for 15 min at 4°C. Aliquots of the supernatant were frozen in liquid $N_2$ and stored at −70°C. Protein concentration was determined by the Bradford method (6) with BSA as a standard.

### SOD Activity and Immunoblot Assays

SOD isozymes were separated by electrophoresis on 15% polyacrylamide gels using the Laemmli gel system (16) without SDS. To determine SOD enzyme activity, gels were stained as described (3) such that, where present, SOD inhibited the background staining reaction in proportion to its concentration. For the identification of Cu/Zn- *versus* Mn-SOD isozymes, gels were soaked in 2 mM KCN or 5 mM $H_2O_2$ prior to activity staining. KCN inactivates Cu/Zn-SOD; $H_2O_2$ inactivates Cu/Zn- and Fe-SOD (10). To quantify SOD protein levels, proteins within duplicate native gels were denatured by soaking the gels for 15 min at 70 to 90°C in transfer buffer without methanol (25 mM Tris-HCl, 192 mM glycine, and 0.1% [w/v] SDS). Following denaturation, proteins within the gels were electroblotted to 0.1 $\mu$m nitrocellulose paper in transfer buffer containing 20% (v/v) methanol. After the blot was blocked in Tris-buffered saline containing 3% BSA, it was incubated overnight with primary antibody raised against SDS-denatured pea cytosolic Cu/Zn-SOD (24), which recognizes both Cu/Zn-$SOD_{cyt}$ and Cu/Zn-$SOD_{chl}$ but does not cross-react with Mn-SOD. The blot was secondarily incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

Different isozymes on both activity gels and immunoblots were quantified by densitometric scanning with an Ultrascan XL Laser Densitometer (LKB, Sweden), using GELSCAN software (Pharmacia) to determine area under the peaks. One unit of SOD activity or protein was arbitrarily defined to correspond to 1 unit of peak area. Where appropriate, data were subjected to analysis of variance using the software package CSS Statistica (Statsoft Inc, Tulsa, OK).

## RESULTS AND DISCUSSION

Having utilized four different treatments (C, E, O, and EO, as described in "Materials and Methods"), we observed for all experiments, as shown in Table I, that: (a) ozone treatment was sufficient to cause visible necrosis to leaf surfaces and (b) EDU treatment completely protected ozonated plants from foliar injury. Thus, this work confirmed the antiozonant property of EDU. If EDU's protective action were indeed via an increase in SOD activity, that increase should have been apparent in treatments EO and E.

Figure 1 shows a representative SOD activity gel and immunoblot. In the activity gel (Fig. 1A), the top band, closest to the cathode, was identified as Mn-SOD on the basis of its insensitivity to $H_2O_2$ and KCN (data not shown). As previously reported (24), that band was not recognized by the Cu/Zn-SOD antibodies (data not shown). This was expected, because it is well documented that the Cu/Zn- and Mn-SODs are structurally and phylogenetically very distinct enzymes

**Table I.** *SOD Activity and Cu/Zn-SOD Protein Levels in Bean Leaf Soluble Extracts Separated by Electrophoresis on a Native 15% Polyacrylamide Gel and Then Either Stained for SOD Activity or Denatured in Situ, Transferred to Nitrocellulose Paper, and Immunoblotted with Antibody Raised Against Pea Cu/Zn-SOD$_{cyt}$*

Densitometric units represent the means ± SE from six experiments. Corresponding leaf injury data for ozone-treated plants are presented as percentage of leaf surface showing visible necrosis. Plants were treated with EDU the day before ozonation. Samples of second trifoliate leaves were taken 24 h after the end of ozone fumigation (0.3 ppm for 6 h). All samples from one treatment (six or seven plants per treatment) were pooled for extraction of soluble proteins. Because the activity and protein level of the Cu/Zn-SOD$_{chl}$ are higher than that of the Cu/Zn-SOD$_{cyt}$ (as is clear in Figs. 1 and 2), to achieve greater precision of the assay and to ensure that measurements were within the linear region of the assay, the protein samples were loaded at different concentrations. For activity determinations, concentrations of 40 to 45 μg/lane were used to quantify Cu/Zn-SOD$_{cyt}$ and Mn-SOD activity, whereas lower concentrations of 15 μg/lane were used for the Cu/Zn-SOD$_{chl}$ activity. For SOD protein level determinations by immunoblot analysis, concentrations of 400 to 800 μg/lane were used to quantify Cu/Zn-SOD$_{cyt}$, whereas a lower concentration of 200 μg/lane was used for Cu/Zn-SOD$_{chl}$. As determined by analysis of variance, there were no differences between treatment means compared within any one isozyme and assay.

| Treatment | SOD Activities | | | SOD Protein Levels | | % Leaf Injury |
|---|---|---|---|---|---|---|
| | Cu/Zn-SOD$_{chl}$ | Cu/Zn-SOD$_{cyt}$ | Mn-SOD | Cu/Zn-SOD$_{chl}$ | Cu/Zn-SOD$_{cyt}$ | |
| Control | 357 ± 37 | 394 ± 76 | 81 ± 3 | 1797 ± 43 | 770 ± 112 | 0 |
| +EDU | 315 ± 30 | 374 ± 65 | 84 ± 7 | 1759 ± 129 | 755 ± 120 | 0 |
| +$O_3$ | 323 ± 31 | 391 ± 52 | 86 ± 7 | 1692 ± 124 | 802 ± 94 | 35 ± 5 |
| +EDU + $O_3$ | 331 ± 35 | 374 ± 70 | 86 ± 8 | 1725 ± 97 | 708 ± 113 | 0 |

(2). On the basis of published information (23), Mn-SOD was assumed to be located primarily in mitochondria. It constituted about 10% of the SOD activity in our bean leaf samples. The two major bands, closest to the anode, were identified as Cu/Zn-SODs on the basis of their sensitivity to $H_2O_2$ and KCN (data not shown). Also, as seen in Figure 1, these bands were recognized by the Cu/Zn-SOD antibody. These identifications are in agreement with those of Kono *et al.* (14) and Kwiatowski and Kaniuga (15) for bean. In leaf tissue, Cu/Zn-SODs are located in the cytosol and in the chloroplast (21, 23, 24). The band closest to the anode, which was designated Cu/Zn-SOD$_{chl}$, was assumed to be a chl isozyme because it was the predominant band in mature leaf tissue. The second band, designated Cu/Zn-SOD$_{cyt}$, was assumed to be located in the cytosol inasmuch as it was the predominant band in root tissue (data not shown) and was approximately equal to the chloroplastic isozyme in sink leaves, thereafter declining with the maturation of the leaf (Fig. 2). No Fe-SOD activity was detected in these experiments.

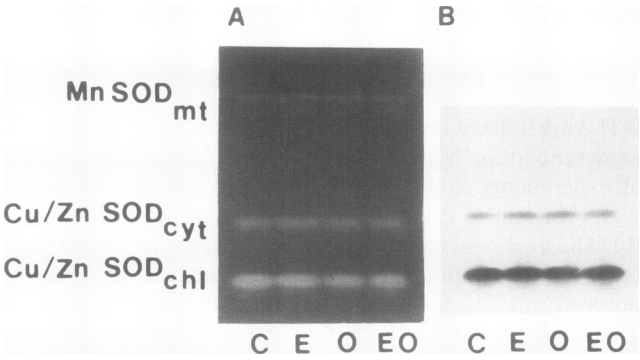


**Figure 1.** Effect of EDU (E), ozone (O), or a combination thereof (EO) on Cu/Zn-SOD activity (A) and protein levels (B) from soluble extracts of bean leaves sampled 24 h after the end of ozone fumigation (which was 48 h after EDU treatment) as compared with untreated controls (C). Treatments and conditions for the SOD activity gel (A) and immunoblot (B) were as described in "Materials and Methods." Sixty micrograms of protein/lane was loaded in A; 500 μg in B. This is a representative experiment (one of six) used to compile data presented in Table I.

As illustrated for a representative experiment in Figure 1, EDU treatment of snap beans did not result in any change in Cu/Zn-SOD$_{chl}$, Cu/Zn-SOD$_{cyt}$, or Mn-SOD activity, nor in Cu/Zn-SOD$_{chl}$ or Cu/Zn-SOD$_{cyt}$ immunologically detectable protein. Table I presents the summation of activity and immunoblot data obtained from six experiments. The bean plants had been treated with EDU at the time when the second trifoliate leaf was 80% expanded. In plants of this same age, Lee and Bennett (17) demonstrated a twofold increase in SOD activity in the second trifoliate. Our data are contradictory to their findings.

We performed two experiments to determine if leaves at different stages of development would respond to EDU treatment with increased SOD activity immediately after or 2 weeks after EDU treatment. EDU-treated plants were tested for SOD activity prior to, 48 h after, and 2 weeks after EDU application. In one experiment, plants were treated when the first or second set of trifoliate leaves was one-third expanded; in another experiment, EDU was applied when the third trifoliate set was one-third expanded (Fig. 2). In no case were there differences in the pattern of changes of SOD activity that could be attributed to EDU treatment. In all three sink leaves, the activities of Cu/Zn-SOD$_{chl}$ and Cu/Zn-SOD$_{cyt}$ were about equal; Cu/Zn-SOD$_{cyt}$ activity dropped by about 30 to 50% at 2 weeks in EDU- and non-EDU-treated plants, whereas Cu/Zn-SOD$_{chl}$ activity did not change, thus becoming the predominant isozyme in mature leaf tissue. When mature first and second trifoliates were assayed 2 weeks after EDU treatment (Fig. 2), once again there were no differences associated with the EDU treatment. These results contradict those of Lee and Bennett (17), who showed that EDU-induced SOD activity in mature leaves was retained by the trifoliate leaves for at least 2 weeks after application of EDU. Although we saw no increase in SOD due to EDU, the

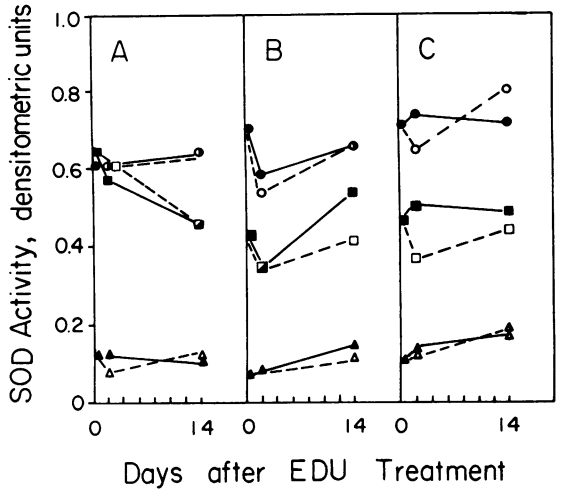


**Figure 2.** Long-term effect of EDU on Cu/Zn-$SOD_{chl}$ (●), Cu/Zn-$SOD_{cyt}$ (■), and Mn-SOD (▲) activity in soluble extracts of bean leaves as compared with Cu/Zn-$SOD_{chl}$ (○), Cu/Zn-$SOD_{cyt}$ (□), and Mn-SOD (△) activity from control plants that received no EDU. Lines (solid, control; broken, +EDU) are drawn between points only for ease of visualizing the general pattern; no time course of change in activity is implied. SOD activity was measured in native gels as described in the text; 80 μg of protein was loaded for each determination. EDU was applied to plants immediately after zero time sampling; leaves were also sampled at 2 and 14 d after EDU application. At the time of EDU application, third (A), second (B), and first (C) trifoliate leaves were 30, 81, and 100% expanded, respectively.

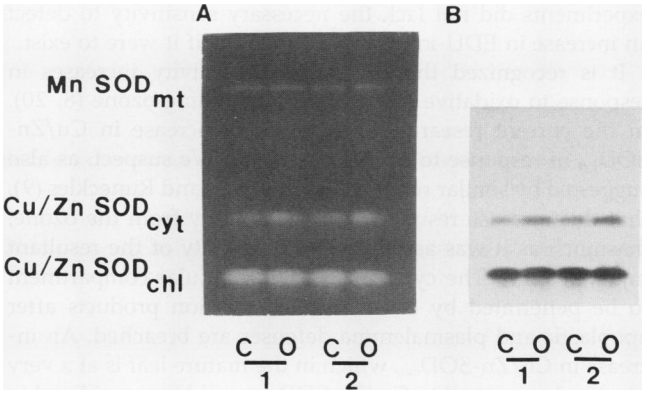


**Figure 3.** Effect of severe ozone injury (O) on Cu/Zn-SOD activity (A) and protein levels (B) in soluble extracts of bean leaves sampled 24 h (experiment 1) or 48 h (experiment 2) after end of ozonation (0.3 ppm for 6 h [experiment 1] or 3 h [experiment 2]) as compared with nonfumigated controls (C). In both experiments, leaves were severely injured over 80 to 100% of the adaxial surface. Conditions for the SOD activity gel (A) and immunoblot (B) were as described in "Materials and Methods." Sixty micrograms of protein/lane was loaded in A; 500 μg in B. There is a doubling of activity and protein level for the Cu/Zn-$SOD_{cyt}$ due to ozonation but no change in the Cu/Zn-$SOD_{chl}$ isozyme.

developmentally related change in SOD activity, noted when young leaves were followed to maturity, is to be expected as a result of chloroplast maturation over this time period. It has been shown by others (21) that cytosolic SOD is the major enzyme in nongreen tissue, whereas the chloroplastic isozyme predominates in green tissue. As well as illustrating once again that EDU did not enhance SOD activity, this experiment confirmed that the methodology we employed was sufficiently sensitive to demonstrate changes in SOD activity in the range reported by Lee and Bennett.

For the experiments reported in Table I, ozone injury to the second trifoliate leaf ranged from an average of 17% of the surface damaged per experiment to 53%. In additional experiments with ozonated, non-EDU-treated bean plants that were more heavily injured (over a range of 46–100% of the leaf surface), an approximate doubling of Cu/Zn-$SOD_{cyt}$ activity was noted in several cases. Figure 3 illustrates such increases in activity and protein levels in leaves from two representative ozonations where visible foliar injury was very severe. We combined the data from these additional experiments with ozonation data reported in Table I and expressed the increase in SOD activity of the ozonated samples over each control as a function of foliar injury (Fig. 4). An increase in Cu/Zn-$SOD_{cyt}$ was strongly and significantly associated with increasing foliar injury; there was a slight increase in Cu/Zn-$SOD_{chl}$ with increasing injury, which was only marginally significant. Mn-SOD activity did not change with increased foliar injury. In addition to standing on its own as

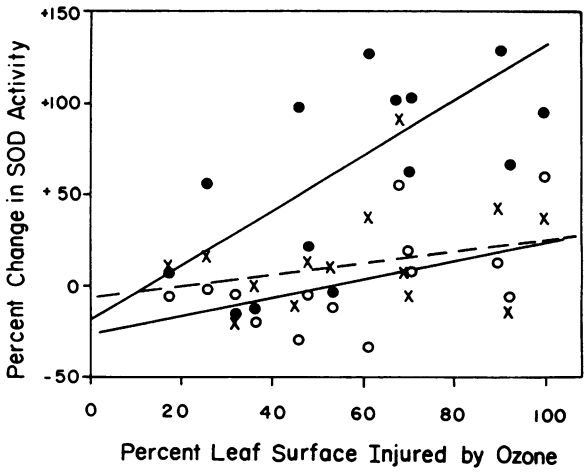


**Figure 4.** Percentage change in activity (*y* intercept) for bean leaf Cu/Zn-$SOD_{cyt}$ (●), Cu/Zn-$SOD_{chl}$ (○), and Mn-SOD (X) as a function of ozone-induced leaf surface necrosis (*x* axis). Data from 14 experiments are presented. Each data point representing foliar injury is an average rating from second trifoliate leaves of all ozonated plants within one experiment. SOD activity values from second trifoliate leaves of ozonated plants were compared with nonozonated controls to obtain the percentage change. Ozone fumigations were 0.3 ppm for 3 or 6 h. There was significant correlation ($Y = -16.429 + 13.011X$; $r = 0.6387$; $P = 0.05$) between increase in Cu/Zn-$SOD_{cyt}$ activity and foliar injury. There was only marginal biological significance ($Y = -31.474 + 5.8320X$; $r = 0.5310$; $0.05 < P < 0.10$) between increase in Cu/Zn-$SOD_{chl}$ activity and foliar injury. There was no correlation between Mn-SOD and foliar injury ($Y = -5.3553 + 3.6924X$; $r = 0.109$).

a finding, this indicated again that methods employed in our experiments did not lack the necessary sensitivity to detect an increase in EDU-induced SOD activity if it were to exist.

It is recognized that overall SOD activity increases in response to oxidative stress (2, 23), including ozone (8, 20). In the current research, a preferential increase in Cu/Zn-$SOD_{cyt}$ in response to ozone was noted. We suspect, as also suggested by similar research of Chanway and Runeckles (9), that this increase resulted only secondarily from the ozone, inasmuch as it was associated with severity of the resultant injury (Fig. 4). The cytosol is the first cellular compartment to be penetrated by ozone and its reaction products after apoplastic and plasmalemma defenses are breached. An increase in Cu/Zn-$SOD_{cyt}$, which in the mature leaf is at a very low level compared to Cu/Zn-$SOD_{chl}$, could be considered to be in the first line of defense. Although, to our knowledge, we are the first to report the preferential induction of Cu/Zn-$SOD_{cyt}$ by ozone injury (22), there have been at least three other reports of differential expression of SOD isozymes related to oxidative stress. Matters and Scandalios (19) reported that hyperbaric oxygen preferentially caused an increase in cyt SOD isoforms in maize. Perl-Treves and Galun (21) reported that drought caused an increase in cyt SOD activity and its transcript but an increase in chl SOD activity only. Tsang *et al.* (25) reported for *Nicotiana plumbaginofolia* that chilling in the dark and heat shock caused a preferential increase in the steady-state transcript for the cyt Cu/Zn-SOD as compared with that for the chl SOD isozyme.

In conclusion, we have been unable to detect any increase in SOD enzyme activity or immunologically detectable Cu/Zn-SOD protein levels due to EDU under conditions in which it acted as an effective antiozonant. Although we cannot explain the discrepancy between our results and those of Lee and Bennett (17), it can be said that whatever caused the increase in SOD in their experiments was not singularly associated with EDU. Our results convince us that the mode of action of this antiozonant does not involve stimulation of SOD activity or synthesis of new SOD protein. We appreciate the extensive antioxidative role of SODs; the current results do nothing to diminish their overall importance. However, because of the results reported herein, and those of others (8) also unable to ascertain a role for EDU in stimulating SOD activity, we conclude that the mode of action of EDU as an antiozonant is still unknown.

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