

# Oxygen Toxicity and the Superoxide Dismutase

EUGENE M. GREGORY AND IRWIN FRIDOVICH

Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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Oxygen caused an increase in the amount of superoxide dismutase in *Escherichia coli* B but not in *Bacillus subtilis*. *E. coli* B cells, induced by growth under 100% O<sub>2</sub>, were much more resistant to the lethal effects of 20 atm of O<sub>2</sub> than were cells which contained the low uninduced level of this enzyme. In contrast, *B. subtilis*, which could not respond to O<sub>2</sub> by increasing its content of superoxide dismutase, remained equally sensitive to hyperbaric O<sub>2</sub> whether grown under 100% O<sub>2</sub> or areobically. The catalase in these organisms exhibited a reciprocal response to oxygen. Thus, the catalase of *E. coli* B was not induced by O<sub>2</sub>, whereas that of *B. subtilis* was so induced. These results are consistent with the view that superoxide dismutase is an important component of the defenses of these organisms against the toxicity of oxygen, whereas their catalases are of secondary importance in this respect. The ability of streptonigrin to generate O<sub>2</sub><sup>-</sup>, by a cycle of reduction followed by spontaneous reoxidation, has been verified in vitro. It is further observed that *E. coli* B which contain the high induced level of superoxide dismutase were more resistant to the lethality of this antibiotic, in the presence of oxygen, than were *E. coli* B which contained the low uninduced level of this enzyme. This difference between induced and uninduced cells was eliminated by the removal of O<sub>2</sub>. These results are consistent with the proposal that the enhanced lethality of streptonigrin under aerobic conditions may relate to its in vivo generation of O<sub>2</sub><sup>-</sup> by a cycle of reduction and spontaneous reoxidation. In toto, these observations lend support to the hypothesis that O<sub>2</sub><sup>-</sup> is an important agent of oxygen toxicity and that superoxide dismutase functions to blunt the threat posed by this reactive radical.

The toxicity of oxygen has been well documented (4), but the bases of this toxicity and of the defenses which have evolved to deal with it have not yet been adequately explored (3, 5). The realization that O<sub>2</sub><sup>-</sup> is a common product of the biological reduction of oxygen (3, 7, 10) has led to the proposal that it is one of the reasons for oxygen toxicity and that the enzyme superoxide dismutase (8) functions as a defense against the reactivities of this radical (9). It has been reported (4a) that superoxide dismutase is induced by O<sub>2</sub> in both *Streptococcus faecalis* and in *Escherichia coli*, and that *S. faecalis*, which contained the high induced levels of this enzyme, were more tolerant of hyperbaric oxygen than were cells which contained a lower level of this enzyme. We now describe extensions of this work which utilize *E. coli* B and *Bacillus subtilis* and which support the proposal that O<sub>2</sub><sup>-</sup> is an important agent of the toxicity of oxygen and that superoxide dismutase provides an in vivo defense against this radical. It is also shown that the oxygen enhancement of the toxicity of streptonigrin, which has been sup-

posed to be due to the generation of O<sub>2</sub><sup>-</sup> (12, 13), is minimized by superoxide dismutase. This result is in full accord with the proposal that the streptonigrin quinone is reduced to a corresponding hydroquinone, which then reduces oxygen to O<sub>2</sub><sup>-</sup> (White et al., Abstr. Proc. Fed. Amer. Soc. Exp. Bio. 30:1145, 1971).

## MATERIALS AND METHODS

Trypticase soy broth was a product of the Bioquest Division of the Becton-Dickinson Co. Yeast extract and agar were obtained from the Difco Laboratories. *E. coli* B was obtained from D. Hall and *B. subtilis* from W. Guild, both of whom are members of this department. Streptonigrin was provided by Microbial Associates, Inc., Bethesda, Md., and streptonigrin monooxime by Charles Pfizer, Inc. Exposure to hyperbaric O<sub>2</sub> or N<sub>2</sub> was accomplished in shallow, magnetically stirred, liquid cultures in specially fabricated stainless steel pressure vessels. Soluble extracts were prepared by ultrasonic treatment of cells suspended in 0.05 M potassium phosphate, 10<sup>-4</sup> M ethylenediaminetetraacetic acid (EDTA), pH 7.8, and at 0 C, for 3 min with a Branson model W185, operated at a power output of 90 W, followed by centrifugation for 15 min at 27,000 × *g*. Superoxide

dismutase was assayed and units of activity were defined as previously described (8), while catalase was assayed by the method of Beers and Sizer (1). Protein was quantitated spectrophotometrically (11).

*E. coli* cells were grown in a medium whose composition in grams per liter was: glucose, 40.5;  $\text{NH}_4\text{Cl}$ , 5.0;  $\text{Na}_2\text{HPO}_4$ , 6.0;  $\text{KH}_2\text{PO}_4$ , 3.0; Bactotryptone, 0.5;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2;  $\text{NaCl}$ , 0.02;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 and ascorbic acid, 0.02. *B. subtilis* were grown in a medium composed of 30 g of Trypticase soy broth per liter and 5 g of yeast extract per liter. Cultures were grown at 37 C and were monitored turbidimetrically at 600 nm (1).

Both compressions and decompressions were carried out gradually over 3- to 5-min intervals. This was the case for compressions with  $\text{O}_2$  or with  $\text{N}_2$ .

## RESULTS

**Superoxide dismutase and oxygen toxicity in *E. coli*.** *E. coli*, grown anaerobically, gave cell-free extracts whose content of superoxide dismutase was 3.8 U/mg. In contrast, *E. coli* grown under oxygen yielded extracts containing 20 U of this enzyme per mg. Anaerobically grown and oxygen-grown *E. coli* were diluted into fresh medium which contained 0.5 mg of puromycin per ml to inhibit protein synthesis and thus to prevent enzyme induction during the test period. These cells were then incubated at 37 C under 20 atm of  $\text{O}_2$  and samples were removed at intervals, diluted into fresh medium, plated onto nutrient agar, and incubated aerobically at 37 C for the counting of surviving cells. Possible effects of puromycin or of pressure per se were assessed by controls which were treated in an identical fashion, except that the 20 atm of  $\text{O}_2$  was replaced by 20 atm of  $\text{N}_2$  during the test period. Figure 1 summarizes the results of these manipulations. Line 1 demonstrates that *E. coli* which had been grown under  $\text{N}_2$  and which therefore contained the low level of superoxide dismutase, were rapidly killed when exposed to 20 atm of  $\text{O}_2$ . Line 2 shows that *E. coli* which had been grown under  $\text{O}_2$  and which contained an elevated level of this enzyme were remarkably resistant to hyperbaric  $\text{O}_2$ . Lines 3 and 4, respectively, demonstrate that there were no very large differences between the survival of high or low superoxide dismutase-containing *E. coli* when incubated under 20 atm of  $\text{N}_2$ . These controls, when compared with line 2, also show that almost all of the modest mortality which was observed with these cells must have been due to the effects of puromycin and pressure changes.

**Superoxide dismutase and oxygen toxicity in *B. subtilis*.** *B. subtilis* 168 try<sup>-</sup> was grown under a range of oxygen concentrations, and cell-free extracts were then prepared and assayed

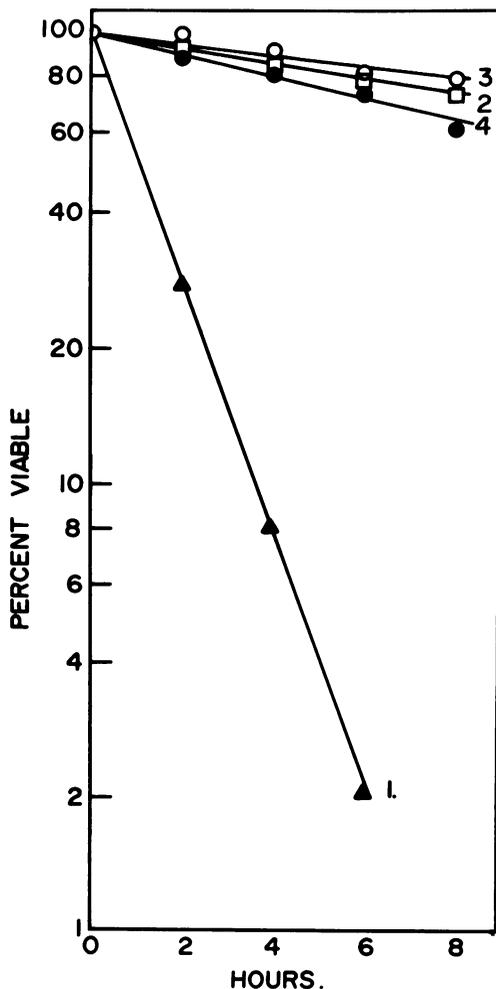


FIG. 1. Effects of superoxide dismutase on the lethality of hyperbaric  $\text{O}_2$  towards *E. coli*. *E. coli* were suspended to a density of  $10^7$  cells/ml in fresh growth medium containing 0.5 mg of puromycin per ml. They were then exposed to 20 atm of  $\text{O}_2$  or  $\text{N}_2$  at 37 C. At intervals, samples were removed from the hyperbaric chambers, suitably diluted, and plated onto nutrient agar for counting of surviving cells. Survivors are defined as those cells which gave rise to visible colonies after 12 h at 37 C. 1. (▲) Cells whose extracts contained 3.8 U of superoxide dismutase per mg, incubated under 20 atm of  $\text{O}_2$ . 2. (■) Cells whose extracts contained 21 U of superoxide dismutase per mg, incubated under 20 atm of  $\text{O}_2$ . 3. (○) Cells whose extracts contained 21 U of superoxide dismutase per mg, incubated under 20 atm of  $\text{N}_2$ . 4. (●) Cells whose extracts contained 3.8 U of superoxide dismutase per mg, incubated under 20 atm of  $\text{N}_2$ .

for superoxide dismutase and for catalase. The results, which are shown in Table 1, demonstrate that superoxide dismutase was not induced by oxygen in *B. subtilis* but that catalase

TABLE 1. *Effects of oxygen on superoxide dismutase and on catalase in B. subtilis*

Conditions of growth	Superoxide dismutase <sup>a</sup> (units/mg)	Catalase <sup>a</sup> (units/mg)
Still culture	11.3	0.8
Aerated	13.7	3.4
Gassed with 1.0 atm, O <sub>2</sub>	12.0	10.9
Gassed with 3.0 atm of O <sub>2</sub> <sup>b</sup> , 1 h	N.D. <sup>c</sup>	10.5
Gassed with 2.0 atm of O <sub>2</sub> <sup>b</sup> , 3 h	N.D.	20.0

<sup>a</sup> Assays performed on fresh cell-free extracts.

<sup>b</sup> Cells were grown to late log phase in still culture and then were gassed with 3 atm of O<sub>2</sub> for the indicated time.

<sup>c</sup> N.D., not done.

was so induced. This is thus the reciprocal of the case of *E. coli*, in which superoxide dismutase was oxygen induced while catalase was not (14). *B. subtilis* grown under air or under 100% into fresh media which contained 0.5 mg of puromycin per ml, and were exposed to 20 atm of O<sub>2</sub> at 25 C. Samples were removed at oxygen were diluted into fresh media which contained 0.5 mg of puromycin per ml and were exposed to 20 atm of O<sub>2</sub> at 25 C. Samples were removed at intervals for dilution, plating and growth of colonies at 37 C in air. Controls were treated identically except that 20 atm of N<sub>2</sub> replaced the exposure to 20 atm of O<sub>2</sub>. Figure 2 presents the results of these measurements. It is apparent that 20 atm of O<sub>2</sub> was equally lethal to *B. subtilis* whether grown under 20% O<sub>2</sub> or 100% O<sub>2</sub>. The control cultures demonstrate that *B. subtilis* was not adversely affected by puromycin or pressure per se. Since these cells differed in their content of catalase but not in their content of superoxide dismutase (Table 1), their failure to gain tolerance toward 20 atm of O<sub>2</sub>, when grown in an O<sub>2</sub>-enriched medium, suggests that catalase is not an important defense against hyperbaric O<sub>2</sub>.

**Superoxide dismutase and streptonigrin toxicity.** Oxygen enhances the toxicity of streptonigrin, and it has been suggested that this is due to the generation of O<sub>2</sub><sup>-</sup> by the streptonigrin (4a). The structure of streptonigrin is shown in Fig. 3. *E. coli*, which contain the high, induced level of superoxide dismutase, should be more resistant to streptonigrin than uninduced cells. *E. coli* B was grown to late log phase at 37 C either under vigorous gassing with 100% O<sub>2</sub> or under the essentially anaerobic conditions of deep, still, liquid culture in air. The former oxygen-grown cells gave cell-free extracts containing 20.0 U of superoxide dis-

mutase per mg while the latter gave extracts containing 3.8 U of this enzyme per mg. These cells were diluted to 10<sup>8</sup>/ml in Trypticase soy broth, which contained 1 μg of streptonigrin per ml, and were then incubated aerobically at 25 C. At intervals, samples were removed and were diluted and plated onto nutrient agar plates, which were incubated overnight at 37 C

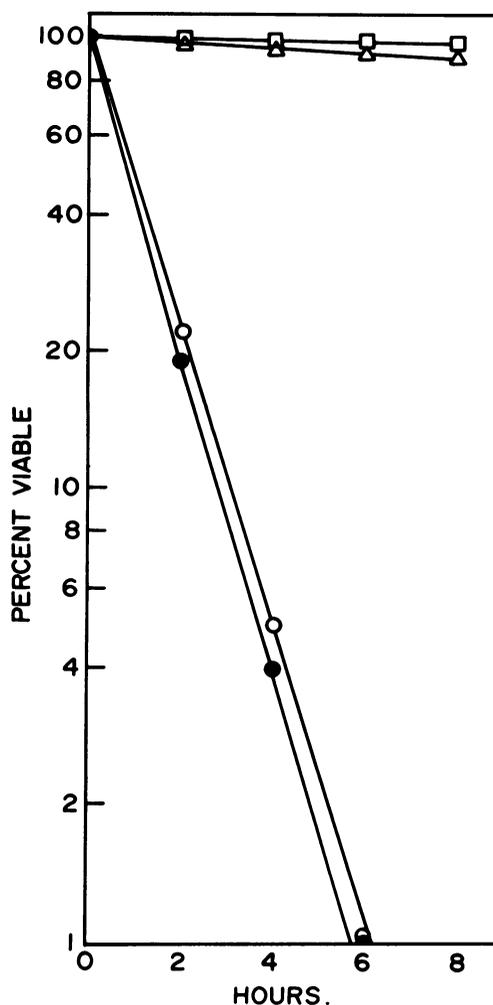


FIG. 2. *Effects of catalase on the lethality of hyperbaric O<sub>2</sub> towards B. subtilis 168 try<sup>-</sup>. B. subtilis were tested for their ability to survive exposure to 20 atm of O<sub>2</sub> or N<sub>2</sub> in a manner identical to that described for E. coli in the legend of Fig. 1. Symbols: ○, cells whose extracts contained 3.4 U of catalase per mg, incubated under 20 atm of O<sub>2</sub>; ●, cells whose extracts contained 10 U of catalase per mg, incubated under 20 atm of O<sub>2</sub>; △, cells whose extracts contained 3.4 U of catalase per mg, incubated under 20 atm of N<sub>2</sub>; □, cells whose extracts contained 10 U of catalase per mg, incubated under 20 atm of N<sub>2</sub>.*

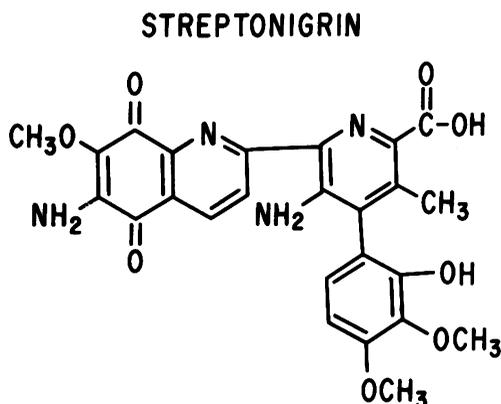


FIG. 3. Structural formula of streptonigrin.

for quantitation of surviving cells. Figure 4A, which presents the results of this study, demonstrates that the *E. coli* with the induced level of superoxide dismutase were much more resistant to streptonigrin than were *E. coli* which had been grown anaerobically and contained the lower level of this enzyme.

If the enhanced resistance towards streptonigrin, exhibited by the oxygen-grown cells, was indeed due to their elevated superoxide dismutase and thus to their greater ability to cope with  $O_2^-$ , then this advantage should disappear in the absence of oxygen. Oxygen-grown and aerobically grown *E. coli* were accordingly exposed to streptonigrin, as above, but under stringently anaerobic conditions, which were achieved by sweeping the cell suspensions with pure  $N_2$  for 1 h before the addition of streptonigrin. Controls were performed in which streptonigrin was not added. The results of these experiments are shown in Fig. 4A by the points  $\square$  and  $\Delta$ . It is clear that the elimination of oxygen removed those differences in sensitivity towards streptonigrin which had been apparent in the presence of oxygen. These results are in full accord with the view that the oxygen enhancement of streptonigrin toxicity is due to the generation of  $O_2^-$  and that superoxide dismutase dose protect against the lethal effects of this radical.

Streptonigrin monooxime should be unable to generate  $O_2^-$  because it cannot be readily reduced to a hydroquinone. Streptonigrin monooxime was tested at 1.0  $\mu\text{g}/\text{ml}$  and was found to be without detectable lethal effect on *E. coli* B, whether tested against oxygen-grown or anaerobically grown cells. These results are shown in Fig. 4B. The relative abilities of streptonigrin and of the corresponding monooxime to generate  $O_2^-$  were tested in the reaction system

described by White, Vaughn, and Yeh (Abst. Proc. Fed. Amer. Soc. Exp. Biol. 30:1145, 1971). Thus, when streptonigrin, at 5.0  $\mu\text{g}/\text{ml}$ , was added to a mixture containing  $3.3 \times 10^{-5}$  M nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH),  $10^{-5}$  M ferri-cytochrome *c*, 0.05 M potassium phosphate,  $10^{-4}$  M EDTA,  $2.4 \times 10^{-4}$  M  $O_2$ , and the spinach ferredoxin, NADP reductase at pH 7.8 and at 25 C, the reduction of cytochrome *c* was observed as an increase in absorbance at 550 nm of 0.016 per min. Superoxide dismutase ( $6 \times 10^{-7}$  M) inhibited this cytochrome *c* reduction, thus indicating that it was mediated by  $O_2^-$ . When the streptonigrin in these reaction mixtures was replaced by a corresponding amount of monooxime, there was no reduction of cytochrome *c*.

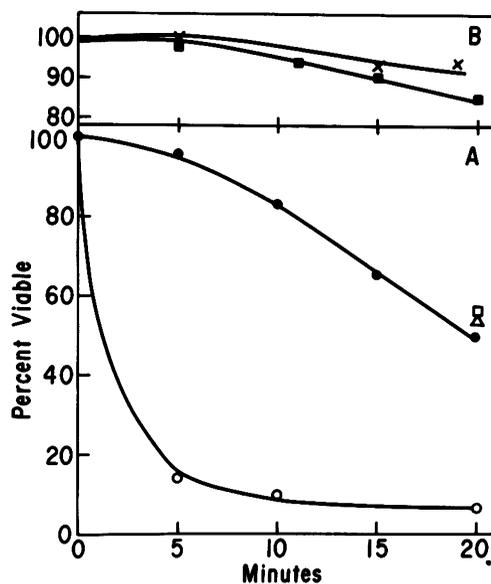


FIG. 4. Effects of superoxide dismutase on the lethality of streptonigrin towards *E. coli* B. *E. coli* were suspended in fresh growth media containing 1  $\mu\text{g}$  of streptonigrin (4A) or streptonigrin monooxime (4B) per ml. They were incubated at 25 C and samples were withdrawn at intervals for dilution and plating. Symbols:  $\circ$ , cells whose extracts contained 3.8 U of superoxide dismutase per mg, incubated aerobically;  $\bullet$ , cells whose extracts contained 21 U of superoxide dismutase per mg, incubated aerobically;  $\square$ , cells whose extracts contained 21 U of superoxide dismutase per mg, incubated anaerobically for 20 min;  $\Delta$ , cells whose extracts contained 3.8 U of superoxide dismutase per mg, incubated anaerobically for 20 min.

## DISCUSSION

The fact that oxygen induces superoxide dismutase but not catalase in the case of *E. coli* B (4a), while it induces catalase but not superoxide dismutase in the case of *B. subtilis* allowed independent manipulation of the levels of these enzymes and therefore facilitated the testing of their relative importance in protecting against the lethality of hyperbaric oxygen. The data shown in Fig. 1, 2, and 4 are typical of results obtained in repeated experiments. These results have demonstrated that increased levels of superoxide dismutase were associated with greater tolerance of hyperbaric oxygen, whereas increased levels of catalase were not. It should be emphasized that this relationship, which was a reliable indicator of oxygen tolerance within a given organism, need not predict relative oxygen tolerances when comparing different organisms. Thus, different organisms could differ in the extent of production of  $O_2^-$  at a given  $pO_2$  and could thus require different levels of superoxide dismutase to achieve the same degree of protection against this radical. The results obtained do indicate that increased concentrations of oxygen lead to increased rates of production of  $O_2^-$  and that superoxide dismutase is an important component of the defenses which deal with oxygen toxicity.

It has been shown by J. L. White, T. O. Vaughn, and W. S. Yeh (Abst. Proc. Fed. Amer. Soc. Exp. Biol. 30:1145, 1971) that streptonigrin is able to generate  $O_2^-$  *in vitro* by a cycle of reduction followed by reoxidation. This is similar to the behavior of menadione (10). We have confirmed this observation and have shown that the monooxime of streptonigrin is unable to function in this way. The enhancement of the lethality of streptonigrin by oxygen has been proposed to involve the intracellular generation of  $O_2^-$  by a similar cycle of reduction followed by reoxidation.

It has now been shown that *E. coli* B in which high levels of superoxide dismutase have been induced by growth under 100%  $O_2$  are resistant to the oxygen enhancement of streptonigrin lethality. Streptonigrin monooxime was without effect on *E. coli* B induced or uninduced,

whether tested aerobically or anaerobically. These results support the proposal that, in the presence of oxygen, a large component of streptonigrin toxicity is actually due to the generation of  $O_2^-$ .

## ACKNOWLEDGMENTS

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