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The Interaction of Bovine Erythrocyte Superoxide Dismutase with Hydrogen Peroxide: Chemiluminescence and Peroxidation†

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ABSTRACT: Reaction of bovine erythrocyte superoxide dismutase with H_2O_2 was accompanied by a luminescence whose intensity was a function of the concentration of H_2O_2 and whose duration was coincident with the inactivation of the enzyme by this reagent. Oxygen, which protected against inactivation, also diminished the luminescence. Several other compounds which prevented the inactivation by H_2O_2 also modified the luminescence. Thus urate, formate, and triethylamine inhibited luminescence whereas imidazole and xanthine augmented it. These seemingly contrary effects can be explained by assuming that the compounds which protected the enzyme were peroxidized in competition with the sensitive group on the enzyme. The luminescence arises because that group on the enzyme was oxidized to a product in an electronically excited state, which could return to the ground state by emitting light. Imidazole and xanthine gave electronically excited products whose quantum efficiency was greater than that of the group on the enzyme, whereas urate, formate, and triethylamine gave products with much lower luminescent efficiencies. This superoxide dismutase could catalyze the peroxidation of a wide range of compounds, including ferrocytochrome *c*, luminol, diphenylisobenzofuran, dianisidine, and linoleic acid. In control experiments, boiled enzyme was inactive. This peroxidative activity can lead to unexpected effects when superoxide dismutase is added to H_2O_2-producing systems, as a probe for the involvement of O_2^-. Several examples from the literature are cited to illustrate the misinterpretations which this previously unrecognized peroxidative activity can generate.

The preceding paper (Hodgson and Fridovich, 1975) describes the inactivation of the copper- and zinc-containing superoxide dismutase by H_2O_2. A mechanism was proposed in which H_2O_2 first reduces the Cu^{2+} and then reacts with the Cu^+, so generated, to give a potent oxidant, which remains bound to the metal. This bound oxidant, in turn, attacks an adjacent histidine residue and so destroys the integrity of the catalytic site. In the course of these studies we observed a chemiluminescence during the reaction of H_2O_2 with the enzyme and noted additionally that superoxide dismutase can act as a peroxidase. Since superoxide dismutase is often used as a test for O_2^- in oxidative and in chemiluminescent reactions and since H_2O_2 is often a product of such reactions, this peroxidative action of superoxide dismutase can, if not appreciated, lead to misinterpretation of the observations. The chemiluminescence and the peroxidations which accompany the interaction of superoxide dismutase with H_2O_2 were therefore studied both to gain understanding of their mechanisms and to expose the dangers of uncritically applying superoxide dismutase as a test for O_2^- in peroxide-generating systems.

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Materials and Methods

The manganese-containing superoxide dismutase was prepared from *Escherichia coli* as previously described (Keele et al., 1970). Luminescent intensity was measured with the photometer described by Mitchell and Hastings (1971) which was calibrated with the stable standard light source described by Hastings and Weber (1963). Some of the measurements were made with a Nuclear Chicago Mark I liquid scintillation counter with the coincidence circuit inactivated. Rates of change of absorbance were recorded with a Gilford Model 2000. The absorption spectrum of cytochrome *c* was recorded with an Aminco DW-2 whereas the spectrum of linoleic acids was taken with a Cary Model 15. The bleaching of diphenylisobenzofuran was followed at 410 nm (Merkel et al., 1972). The peroxidation of dianisidine was followed at 460 nm (Fridovich, 1963). All other materials and procedures were exactly as described in the preceding paper (Hodgson and Fridovich, 1975).

Results

Chemiluminescence. Admixture of superoxide dismutase

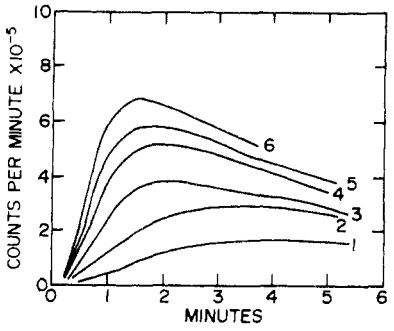

FIGURE 1: Luminescence accompanying the reaction of superoxide dismutase with H_2O_2. Reaction mixtures contained 0.11 mg/ml of enzyme in 50 m*M* sodium carbonate–0.1 m*M* EDTA at pH 10.2 and at 16°. The concentrations of H_2O_2 were as follows: line 1 = 0.23 m*M*; line 2 = 0.35 m*M*; line 3 = 0.40 m*M*; line 4 = 0.46 m*M*; line 5 = 0.52 m*M*; and line 6 = 0.58 m*M*.

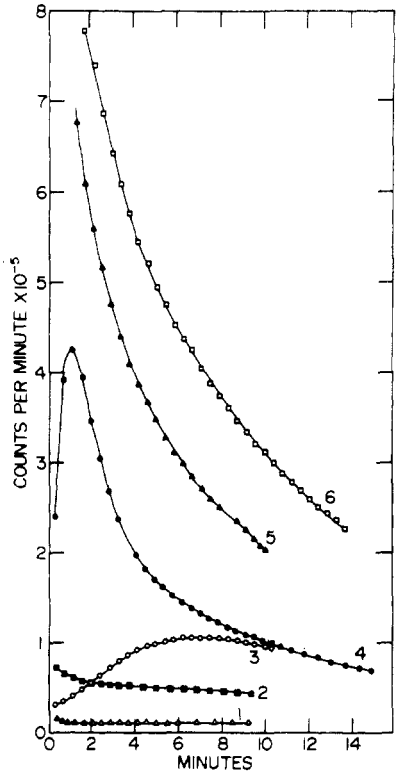

FIGURE 2: Modification of the luminescence by various compounds. Reaction mixtures contained 0.030 mg/ml of enzyme, 0.2 m*M* H_2O_2, 50 m*M* sodium carbonate, and 0.1 m*M* EDTA at pH 10.1 and 16°. The following test compounds were also present: curve 1 = 0.1 m*M* urate; curve 2 = 0.1% triethylamine; curve 3 = 40 m*M* formate; curve 5 = 40 m*M* imidazole; and curve 6 = 0.1 m*M* xanthine. Curve 4 presents the luminescence in the absence of test compounds while curve 1 also represents the luminescence seen in the absence of superoxide dismutase. The light intensity in the presence of 0.1 m*M* xanthine was too intense to be represented on this ordinate scale so curve 6 represents data divided by a factor of five.

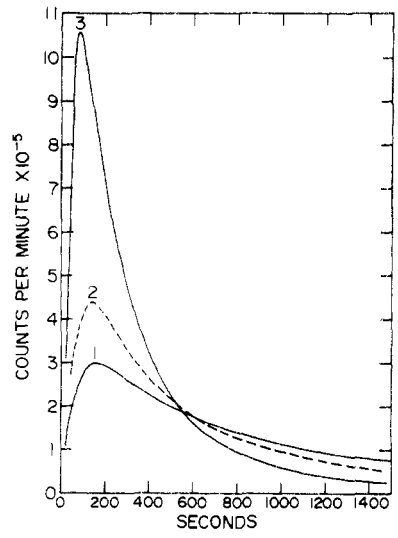

FIGURE 3: The effects of oxygen on luminescence. Reaction mixtures contained 0.085 mg/ml of enzyme, 0.07 m*M* H_2O_2, 0.1 m*M* EDTA, and 50 m*M* sodium carbonate at pH 10.2 and at 16°. Curve 1 was obtained with a reaction mixture which had been bubbled with oxygen for 20 min whereas in curve 2 equilibration was with air and in curve 3 with nitrogen.

with H_2O_2, under conditions which lead to irreversible inactivation of the enzyme, resulted in a luminescence whose time course is shown in Figure 1. The total light emitted during the reaction was directly proportional to the concentration of H_2O_2 and to the concentration of enzyme. When the rate of inactivation of superoxide dismutase by H_2O_2 was compared with the rate of decay of light emission under identical conditions, the pseudo-first-order rate constants were found to be approximately the same. Thus, when H_2O_2 was added at a final concentration of 5.8×10^{-4} *M* to a solution of 7.8×10^{-7} *M* superoxide dismutase in carbonate buffer at pH 10.1 and 5°C, the apparent first-order rate constant for the decay of light emission (obtained from a plot of ln cpm vs. time after H_2O_2 addition) was 0.048 min^{-1}. In a parallel experiment and under identical conditions, the inactivation of superoxide dismutase was followed and was found to have an apparent rate constant of 0.058 min^{-1}. By using a series of Kodak Wratten gelatin filters the wavelength of the light emitted was estimated to be between 420 and 480 nm.

Compounds which had been found (Hodgson and Fridovich, 1975) to protect the enzyme against H_2O_2 were tested for their effects on the luminescence. Figure 2 shows that urate, formate, and triethylamine inhibited luminescence whereas imidazole and xanthine augmented it. It should be noted that luminescence in the presence of xanthine was actually fivefold greater than shown in Figure 2. Indeed its intensity had to be divided by five for presentation on the ordinate scale of this figure.

Oxygen was seen (Hodgson and Fridovich, 1975) to protect the enzyme against inactivation by H_2O_2. If luminescence and inactivation were consequences of the same process, then oxygen should also inhibit luminescence. Figure 3 demonstrates that this was true. Thus, luminescence was most intense under nitrogen, less under air, and least under pure oxygen.

We previously observed that superoxide dismutase inhibited the luminescence of luminol, induced by the xanthine oxidase reaction (Hodgson and Fridovich, 1973). In contrast, when superoxide dismutase is added some time after the start of the xanthine oxidase reaction it actually augments luminescence. It seems likely that this is due to the ability of superoxide dismutase to catalyze a peroxidation of luminol, with attendant luminescence. Figure 4 illustrates the luminescence caused by the action of 2.8 μg/ml of superoxide dismutase on 1×10^{-5} *M* luminol and 5×10^{-5} *M* H_2O_2. The effects of several compounds on this luminol luminescence are also shown. Thus the manganic-superoxide dismutase from *E. coli*, triethylamine, xanthine, manni-

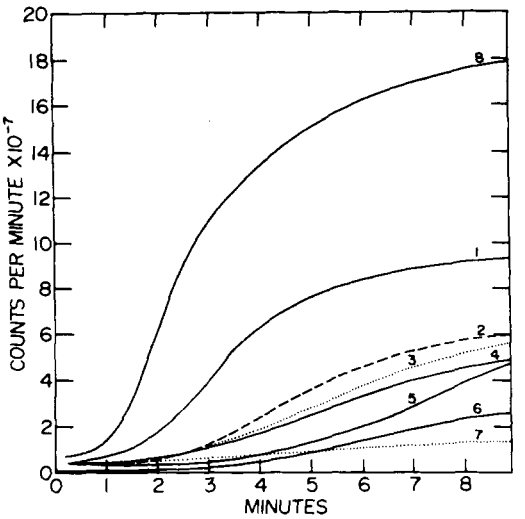

FIGURE 4: Luminescence during the peroxidation of luminol. Reaction mixtures contained 2.8 μg/ml of enzyme, 0.05 mM H_2O_2, 0.01 mM luminol, 0.1 mM EDTA, and 50 mM sodium carbonate at pH 10.2 and 16°. Additional components were: line 1 = none; line 2 = 6 mM formate; line 3 = 0.5% ethanol; line 4 = 50 mM mannitol; line 5 = 0.01 mM xanthine; line 6 = 0.016% triethylamine; line 7 = 15 μg/ml of manganic-superoxide dismutase, and line 8 = 0.1 mM azide.

tol, ethanol, and formate all inhibited, whereas azide augmented this luminescence.

Peroxidations. Several compounds protected the copper–zinc superoxide dismutase against inactivation by H_2O_2 (Hodgson and Fridovich, 1975). Such protective compounds might act by being oxidized in lieu of the active site histidine. In this case superoxide dismutase might be expected to catalyze the peroxidation of such compounds. The results with luminol strengthened this expectation. As shown in Figure 5, superoxide dismutase catalyzed the peroxidation of ferrocytochrome *c*. Thus the difference spectrum in Figure 5 was recorded 1 min after adding H_2O_2 to a mixture of superoxide dismutase plus ferrocytochrome *c*. This spectrum shows that the process was a simple oxidation to ferricytochrome *c*. The magnitude of these spectral changes increased with time of reaction. There was no reaction in the absence of superoxide dismutase. The effects of various compounds on the rate of change of absorbance at 550 nm are shown in Figure 6. Xanthine, azide, or urate accelerated, mannitol or manganic-superoxide dismutase was without effect and formate or triethylamine inhibited this peroxidation of ferrocytochrome *c*. Controls demonstrated that none of these compounds acted on cytochrome *c* itself.

The peroxidative activity of superoxide dismutase is relatively nonspecific. Figure 7 shows that diphenylisobenzofuran was also attacked and that xanthine or azide augmented this process whereas urate, formate, or triethylamine inhibited. Mannitol or the *E. coli* manganic-superoxide dismutase had no effect. Bilirubin and dianisidine were peroxidized by superoxide dismutase.

There is, at present, great interest in the possibility that O_2^-, or radicals derived therefrom, may be instrumental in initiating the peroxidation of unsaturated lipids. Consequently superoxide dismutase is being tested for its effects on lipid peroxidation (Fong et al., 1973; Goda et al., 1973; Pederson and Aust, 1972, 1973; Zimmerman et al., 1973). It would certainly be important to know whether superoxide dismutase could cause the peroxidation of unsaturated lipids. Figure 8 demonstrates that it could. Thus superoxide dismutase plus H_2O_2 acted upon linoleic acid to cause the

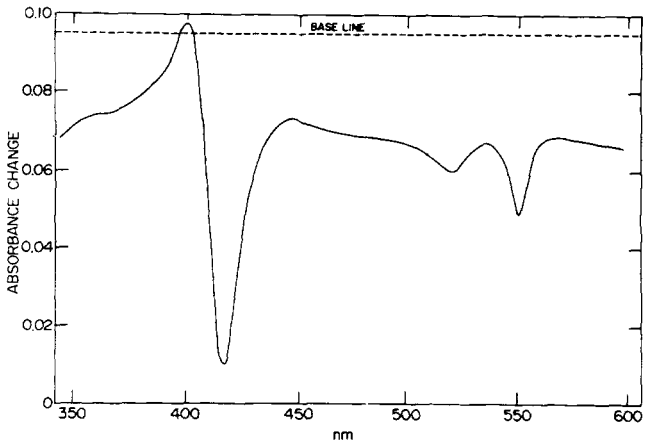

FIGURE 5: Peroxidation of ferrocytochrome *c*. Reaction mixtures contained 55 μg/ml of enzyme, 0.08 mM H_2O_2, 7.6 μM ferrocytochrome *c*, 0.1 mM EDTA, and 50 mM sodium carbonate at pH 10.2 and 25°. Blank and sample cuvettes were identical except that H_2O_2 was added to the sample only and this difference spectrum was recorded after 2 min. The baseline position was raised arbitrarily.

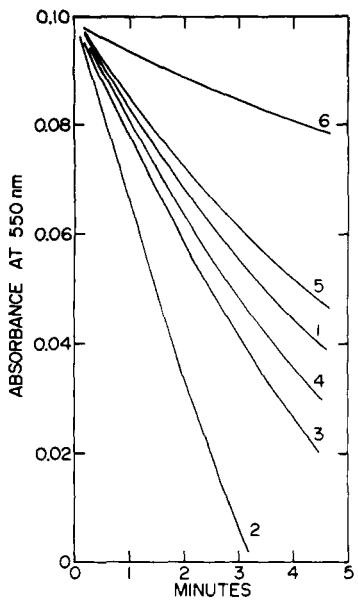

FIGURE 6: Effects of various compounds on the peroxidation of ferrocytochrome *c*. Reaction mixtures contained 2.66 μM of enzyme, 0.15 mM H_2O_2, 8 μM ferrocytochrome *c*, 0.1 mM EDTA, and 50 mM sodium carbonate at pH 10.0 and 25°. Additional components were: line 1 = none or 50 mM mannitol or 3.0 μM of manganic-superoxide dismutase; line 2 = 1.0 mM xanthine; line 3 = 1.0 mM azide; line 4 = 0.01 mM urate; line 5 = 0.1% triethylamine, and line 6 = 50 mM formate.

progressive appearance of an absorption band at 232 nm which is characteristic of conjugated dienes (Privett et al., 1955) and in this case indicates the formation of the conjugated diene hydroperoxide of linoleic acid. There was no spectral change if either H_2O_2 or superoxide dismutase was omitted. Furthermore, thin-layer chromatography of chloroform–methanol extracts of the reaction mixtures showed that a spot corresponding to the linoleic hydroperoxide was formed only in the combined presence of H_2O_2 and superoxide dismutase.

Discussion

The luminescence which accompanies the inactivation of the bovine erythrocyte superoxide dismutase by H_2O_2 and

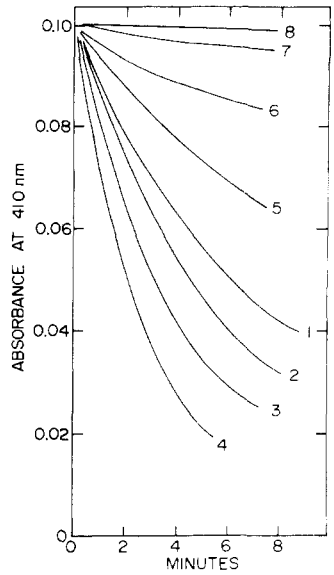

FIGURE 7: Peroxidation of diphenylisobenzofuran. Reaction mixtures contained 30% ethanol, 0.1 mM H_2O_2, 2.66 μM of enzyme, 3.3 M diphenylisobenzofuran, 0.1 mM EDTA, and 50 mM sodium carbonate at pH 9.8 and 25°. Additional components were: line 1 = none or 0.1 M mannitol or 120 μg/ml of manganic-superoxide dismutase; line 2 = 0.1 mM xanthine; line 3 = 0.1 mM azide; line 4 = 1.0 mM azide; line 5 = 0.1% triethylamine; line 6 = 30 mM formate; line 7 = 1.0 mM urate. Line 8 was a control in which enzyme was omitted.

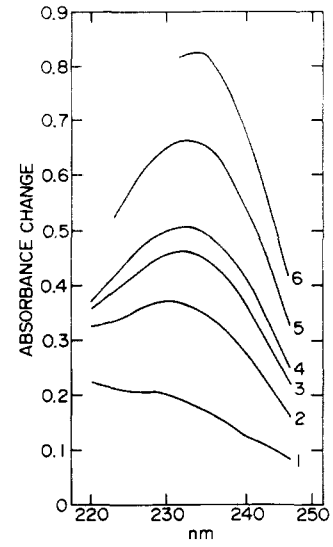

FIGURE 8: Difference spectra of the peroxidation of linoleic acid. Reaction mixtures contained 1.88 μM of enzyme, 3.3% dimethoxyethane, 0.1 mM H_2O_2, 1.0 mM linoleic acid, 0.1 mM EDTA, and 50 mM sodium carbonate at pH 9.8 and 22°. Blank and sample cuvettes were identical except that H_2O_2 was added only to the sample and difference spectra were then recorded at 2.5-min intervals.

the peroxidations catalyzed by this enzyme can be explained. Thus H_2O_2 first reduces the Cu^{2+}, at the active site, to Cu^+ which reacts with additional H_2O_2 to generate Cu^{2+}-OH· or $Cu^{2+}O\cdot^-$ (Hodgson and Fridovich, 1975). This powerful oxidant could then attack an adjacent histidine residue, yielding an electronically excited oxidation product, which emits light during its return to the ground state. The inactivation is thus accompanied by luminescence. When exogenous electron donors, such as dianisidine or linoleic acid, compete with the active site histidine, for reaction with the bond oxidant, they prevent inactivation of the enzyme and are themselves peroxidized. If the exogenous electron donor oxidizes to an electronically excited product, which emits light with greater efficiency than does the product obtained from the endogenous donor, then it will augment the luminescence. Conversely, if the product of oxidation of the exogenous electron donor emits less efficiently than that of the endogenous donor, then it will inhibit luminescence. Xanthine and imidazole belong in the former category; while formate, urate, and triethylamine belong in the latter category (Figure 2).

If the bound oxidant could be generated only as a consequence of the interaction of Cu^+ with H_2O_2, then oxygen could, by reoxidizing the Cu^+ to Cu^{2+}, diminish the concentration of bound oxidant. This would then decrease the rate of attack on the endogenous electron donor and thus decrease the luminescence and the rate of inactivation of the enzyme. Figure 3 demonstrated that this was the case.

Luminol greatly augmented the luminescence of the enzyme plus H_2O_2. This is not surprising in view of the abilities of diverse oxidants to elicit light from this compound (Hodgson and Fridovich, 1973). As previously discussed (Hodgson and Fridovich, 1973), oxidants remove one electron from luminol giving a luminol radical, which reacts with oxygen giving O_2^-, which can, in turn, react with a second luminol radical to yield an adduct, which then decomposes to yield electronically excited aminophthallate, which emits light. Because of this mechanism, luminol luminescence, in buffered aqueous systems, is inhibited by superoxide dismutase, whatever the nature of the oxidant used to start the chain of events. In fact the manganic-superoxide dismutase, which does not itself react with H_2O_2, did inhibit the luminescence of luminol caused by the copper–zinc superoxide dismutase plus H_2O_2. This was shown in Figure 4 and it demands additional comment. Thus why was the copper–zinc superoxide dismutase unable to scavenge the O_2^- essential to the luminol luminescence, while the manganic enzyme could do so? This could be answered by a competition between H_2O_2 and O_2^- for the active site on the copper–zinc enzyme. Thus H_2O_2 rapidly reduces the Cu^{2+} in this enzyme, so it certainly competes with O_2^- for the cupric form of the enzyme. Furthermore H_2O_2 appears to react with the cuprous enzyme to generate a bound oxidant, as described previously (Hodgson and Fridovich, 1975), so it also competes with O_2^- for the cuprous form of the enzyme. H_2O_2 must therefore act as a competitive inhibitor of the copper–zinc superoxide dismutase, in addition to causing its slow irreversible inactivation. H_2O_2 does not have this effect on the manganic enzyme which was therefore an effective scavenger of O_2^-, even in the presence of H_2O_2. Compounds such as xanthine, formate, or triethylamine inhibited luminol luminescence by competing with luminol for the bound oxidant. This is in keeping with their ability to prevent the inactivation of the enzyme by H_2O_2. Ethanol and mannitol, which did not protect the enzyme against H_2O_2, cannot have prevented luminol luminescence by this mechanism. We suppose they acted by reacting with the luminol radical which is also an intermediate in this luminescence (Hodgson and Fridovich, 1973).

The peroxidase action of superoxide dismutase is nonspecific. Thus ferrocytochrome c, dianisidine, diphenylisobenzofuran, bilirubin, and linoleic acid were all attacked. Other compounds, which are themselves peroxidized, can either act to inhibit or to activate the peroxidation of these substances. Which effect is seen will depend upon the reactivity of the radical products formed during the peroxidation of these compounds. Thus formate inhibited the peroxidation

of ferrocytochrome *c* by H_2O_2 plus superoxide dismutase, whereas xanthine accelerated this reaction (Figure 6). The formate effect could be explained as follows:

$$Enz\text{-}Cu^{2+}\text{-}OH\cdot + HCOO^- \rightarrow Enz\text{-}Cu^{2+} + OH^- + HCOO\cdot \quad (a)$$

$$HCOO\cdot + O_2 \rightarrow CO_2 + H^+ + O_2^- \quad (b)$$

In this case formate reacts with the bound oxidant as in reaction a and in so doing spares ferrocytochrome *c*, which would otherwise be oxidized in its stead. Furthermore the formate radical can react with oxygen, as in reaction b, to yield O_2^- which in turn could reduce ferricytochrome *c* and could compete with H_2O_2 for reaction with the cuprous form of the enzyme. All of these actions would have the apparent effect of reducing the rate of oxidation of ferrocytochrome *c*. In contrast, we can suppose that the univalent oxidation of xanthine, by the enzyme-bound oxidant, is an efficient process which yields a xanthine radical which itself oxidizes ferrocytochrome *c*. In this case xanthine would augment the rate of oxidation of ferrocytochrome *c* if it reacted more rapidly with the bound oxidant than did ferrocytochrome *c*. Azide also augmented several of these peroxidations (Figures 4, 6, and 7). In this case one must propose an azide radical, i.e., N_3^0 which would be produced by the oxidation of azide and which would survive long enough in solution to react with such compounds as ferrocytochrome *c* or bilirubin. The azide radical has been proposed as an intermediate in various free radical reactions (Minisci, 1975).

The reaction of the copper–zinc superoxide dismutase with H_2O_2 and its ability to luminesce and to catalyze peroxidations must be considered each time this enzyme is used as a probe for O_2^- in H_2O_2-producing systems. Failure to consider these aspects of its chemistry can lead to confusion or to faulty conclusions. Studies of the effects of superoxide dismutase on galactose oxidase (Kwiatkowsky and Kosman, 1973) provide a case in point. Thus superoxide dismutase inhibited galactose oxidase activity when the latter activity was estimated manometrically, but not when it was measured colorimetrically with a coupled assay, dependent upon the peroxidation of dianisidine. Presumably superoxide dismutase really does inhibit galactose oxidase as was shown in the uncomplicated manometric assay. In the coupled assay this inhibition could have been masked by the ability of superoxide dismutase to catalyze the peroxidation of dianisidine. It was also reported (Kwiatkowsky and Kosman, 1973) that superoxide dismutase inhibited the manometric assay, when it was present at the start of the reaction, but not when it was added after galactose oxidase had been acting for 15 min. This could also be explained by the interaction of H_2O_2 with the copper–zinc superoxide dismutase. Thus during its action, galactose oxidase produces H_2O_2 and after 15 min sufficient H_2O_2 could have accumulated to inactivate the superoxide dismutase.

Another case in point arises from studies of the peroxidation of lipids initiated by the microsomal oxidation of NADPH or by the aerobic action of xanthine oxidase. In one such study it was reported (Fong et al., 1973) that superoxide dismutase augmented lipid peroxidation. From our present vantage point this effect is readily explained in terms of the peroxidation of the lipids catalyzed by the superoxide dismutase added. The H_2O_2 needed for this peroxidation was produced by the microsomal NADPH oxidase or by xanthine oxidase. The literature contains additional examples. It should be noted that the manganic-superoxide dismutase from *E. coli* (Keele et al., 1970) is not readily inactivated by H_2O_2 and does not catalyze peroxidations. It can therefore be used in place of the copper–zinc enzyme whenever there is possibility of equivocal results due to interaction of H_2O_2 with the superoxide dismutase.

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