Superoxide Dismutase:
Improved Assays and an Assay Applicable to Acrylamide Gels

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Received April 21, 1971

Superoxide dismutase has been isolated from bovine erythrocytes (1),
bovine heart (2), and Escherichia coli (3). This enzyme has been de-
tected in a wide range of living things and has been implicated as being
an essential defense against the potential toxicity of oxygen (4). It has
also proved its utility as a probe for the involvement of O$_2^-$ in a variety
of reactions (5–10). The reaction it catalyzes may be represented as
follows:

$$\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$

Because of the instability of its substrate, all available assays of super-
oxide dismutase are indirect and depend upon its ability to scavenge O$_2^-$
from reaction mixtures and thus to inhibit reactions caused by O$_2^-$. In
the case of the assay used to monitor the purification of superoxide
dismutase (1), a flux of O$_2^-$ was generated by the aerobic action of
xanthine oxidase on xanthine. This O$_2^-$ was measured in terms of its
ability to reduce cytochrome c. Superoxide dismutase was quantitated
in terms of its ability to inhibit this reduction of cytochrome c. One
unit of superoxide dismutase was defined as the amount that caused
50% inhibition of the rate of reduction of cytochrome c under specified
conditions. This assay suffered from several shortcomings: (1) Agents
capable of the oxidation of ferrocytochrome c interfered and the assay
could not therefore easily be applied to crude extracts that might con-
tain cytochrome oxidase or cytochrome peroxidase activities. (2) The
assay was also dependent upon the availability of pure xanthine oxidase
and cytochrome c and was not readily applicable to acrylamide gels.

The observations that photochemically reduced flavins generate O$_2^-$

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1 The work reported here was supported in full by Grant GM-10287 from the
National Institutes of Health, Bethesda, Maryland.

2 Predoctoral trainee of the National Institutes of Health.
ASSAYS OF SUPEROXIDE DISMutASE

upon reoxidation in air (8) and that $O_2^-$ reduces nitro blue tetrazolium (11,12) suggested an assay for superoxide dismutase which could be applied both in free solution and in polyacrylamide gels and which could be made independent of other enzymes or proteins. The assays developed, utilized photochemical events to generate $O_2^-$ and used nitro blue tetrazolium to detect this radical. Superoxide dismutase inhibited the formation of the blue formazan and could be quantitated on this basis. When the reactions were performed on acrylamide gels, superoxide dismutase signaled its location by causing achromatic zones on otherwise uniformly blue gels. Nitro blue tetrazolium also proved to be a useful detector of $O_2^-$ generated by the xanthine oxidase system and provided the basis for an assay which was free of interference by other catalytic activities and which could be used to assay for superoxide dismutase in crude extracts.

MATERIALS AND METHODS

Xanthine oxidase, which had been isolated from raw cream by a procedure that avoided exposure to proteolytic agents (13), was kindly provided by K. V. Rajagopalan. Superoxide dismutase was prepared from bovine erythrocyte (1), bovine heart (2), and E. coli B (3). Bovine tissues were obtained at a local abattoir and were stored frozen until needed. Extracts were prepared by homogenization of each tissue in a Waring blender with 2 vol 0.05 M potassium phosphate at pH 7.8 followed by centrifugation at 20,000g for 10 min to remove particulate matter. Erythrocyte lysates were prepared by adding 1 vol water to packed bovine erythrocytes that had been washed with isotonic saline solution. E. coli B was obtained from Miles Laboratories. The cell paste was stored in the freezer. After thawing, 0.6 gm of this paste was suspended in 10 ml 0.05 M potassium phosphate at pH 7.8 and was disrupted by sonication at 0° for 1 min with a Branson Sonifier at a power setting of 125 W. This homogenate was then clarified by centrifugation. Nitro blue tetrazolium, grade III, was obtained from Sigma. Riboflavin was purchased from Eastman. All other dyes were products of Fisher Chemical Co.

The reduction of nitro blue tetrazolium (NBT) was followed at 560 nm in a Gilford model 2000 absorbance indicator equipped with a thermostated cell compartment. Reactions under controlled atmospheres were performed in cuvets that allowed the reaction mixture to be swept with the prepared gas (14). All photoinduced reactions were performed in an aluminum foil lined box fitted with a 15 W fluorescent lamp. Polyacrylamide disc gel electrophoresis was performed according to Davis (15) with the modification that both the resolving and the concentrating gels were photopolymerized with riboflavin. Samples were placed on the
gels in 20% glycerol and electrophoresis was performed at 80 to 120 V (2 mA/tube) until the bromphenol blue marker dye had swept through most of the gel. Protein was stained by immersion of the gels in 0.2% amido black in 7% acetic acid for 1 hr followed by destaining in 7% acetic acid.

Superoxide dismutase was localized by soaking the gels in $2.45 \times 10^{-3} M$ nitro blue tetrazolium for 20 min, followed by an immersion, for 15 min, in a solution containing $0.028 M$ tetramethylethylenediamine, $2.8 \times 10^{-4} M$ riboflavin, and $0.036 M$ potassium phosphate at pH 7.8. The gels were then placed in small dry test tubes and illuminated for 5 to 15 min. During illumination the gels became uniformly blue except at positions containing superoxide dismutase. Illumination was discontinued when maximum contrast between the achromatic zones and the general blue color had been achieved. The gels were then photographed.

RESULTS

Reduction of NBT by Xanthine Oxidase

Xanthine oxidase is able to transfer electrons to NBT either directly or via oxygen. The direct transfer occurs under anaerobic conditions and is not affected by the presence of superoxide dismutase, whereas the indirect route predominates in the presence of oxygen and may be inhibited by superoxide dismutase. Thus, when $2.2 \times 10^{-9} M$ xanthine oxidase acted upon an anaerobic solution containing $1 \times 10^{-4} M$ xanthine, $2.5 \times 10^{-5} M$ NBT, $10^{-4} M$ EDTA, and $0.05 M$ sodium carbonate at pH 10.2 and 25°, the absorbancy at 560 nm increased at a rate of 0.015/min due to accumulation of blue formazan, and 2.6 μg superoxide dismutase/ml was without effect. Under identical conditions, but in solutions equilibrated with air, the rate was 0.0102/min and superoxide dismutase at 2.6 μg/ml inhibited by 90%. As shown in Fig. 1 the initial rate of reduction of NBT in the aerobic xanthine oxidase system was a linear function of the concentration of xanthine oxidase. For reasons which have been discussed previously (1,16), the sensitivity of an $O_2^-$ detecting system toward superoxide dismutase will depend upon the rate of generation of $O_2^-$, the concentration of the compound used to detect the $O_2^-$, and pH. Conditions giving a rate of increase of absorbancy of 0.0165/min at 560 nm were arbitrarily chosen and the effect of superoxide dismutase was investigated. The results, illustrated in Fig. 2, demonstrate great sensitivity to superoxide dismutase. Thus, 50% inhibition was achieved at 0.0068 μg superoxide dismutase/ml, an amount defined as one unit of this enzyme under the specified conditions of assay. It is noteworthy that the maximum inhibition that could be achieved was
ASSAYS OF SUPEROXIDE DISMUTASE

Fig. 1. Rate of reduction of NBT as function of xanthine oxidase. Cuvets contained $2.5 \times 10^{-2} \, M$ NBT, $1 \times 10^{-4} \, M$ xanthine, $1 \times 10^{-4} \, M$ EDTA, $0.05 \, M$ sodium carbonate, and indicated concentrations of xanthine oxidase in final volume of 3.0 ml at 25°, pH 10.2, and equilibrated with air. Rate of reduction of NBT was followed at 660 nm.

90%—indicating that 10% of the reduction of NBT, in this aerobic assay system, was accomplished by a direct interaction of NBT and xanthine oxidase and did not involve $O_2^-$ as an electron-carrying intermediate.

Fig. 2. Inhibition by superoxide dismutase of NBT reduction in aerobic xanthine oxidase system. Cuvets contained $2.5 \times 10^{-2} \, M$ NBT, $1 \times 10^{-4} \, M$ xanthine, $3.3 \times 10^{-5} \, M$ xanthine oxidase, $1 \times 10^{-4} \, M$ EDTA, $0.05 \, M$ sodium carbonate, and indicated concentrations of bovine erythrocyte superoxide dismutase in total volume of 3.0 ml at 25°, pH 10.2, and equilibrated with air. Inhibition (%) of rate of NBT reduction as followed at 560 nm is presented as function of concentration of superoxide dismutase.
Photoreduction of NBT

Illumination of solutions containing riboflavin, methionine, and NBT resulted in a linear accumulation of the blue formazan, whether oxygen was present or not. In the absence of oxygen, superoxide dismutase was without influence whereas, under aerobic conditions, superoxide dismutase inhibited the reduction of NBT. We may conclude that photoreduced riboflavin can interact directly with NBT or alternately can reduce it by a pathway that utilizes $O_2^-$ as an electron-carrying intermediate. In the presence of oxygen the latter pathway was preferred. Thus, illumination of reaction mixtures which contained $2.4 \times 10^{-6} M$ riboflavin, $0.01 M$ methionine, $1.67 \times 10^{-4} M$ NBT, and $0.05 M$ potassium phosphate at pH 7.8 and 25° caused an increase in absorbance at 560 nm of 0.105/min in anaerobic and of 0.075/min in aerobic solutions. This anaerobic photoreduction of NBT was unaffected by $1.3 \mu g$ superoxide dismutase/ml, whereas the aerobic rate was inhibited 95% by this level of superoxide dismutase. The aerobic photoreduction of NBT obviously constitutes a superoxide dismutase sensitive reaction and as such can be exploited for its assay. It has the virtues of being readily standardized and independent of other enzymes and proteins, such as xanthine oxidase and cytochrome c.

Cuvets containing $1.17 \times 10^{-6} M$ riboflavin, $0.01 M$ methionine, $2 \times 10^{-5} M$ sodium cyanide, $5.6 \times 10^{-5} M$ NBT, and $0.05 M$ potassium phosphate at pH 7.8 and 30° were illuminated. The absorbance at 560 nm increased linearly with time of illumination. The light intensity was such as to cause an increase of absorbancy of 0.142/6 min. Figure 3 presents

![Fig. 3. Inhibition by superoxide dismutase of NBT reduction in photoreduction system. Cuvets contained $5.6 \times 10^{-5} M$ NBT, $1 \times 10^{-5} M$ methionine, $1.17 \times 10^{-6} M$ riboflavin, $2 \times 10^{-5} M$ sodium cyanide, $0.05 M$ potassium phosphate, and indicated amounts of bovine erythrocyte superoxide dismutase in total volume of 3.0 ml at 30°, pH 7.8, and equilibrated with air. Reduction of NBT, during 6 min of illumination, was measured in terms of increased absorbancy at 560 nm and inhibition (%) caused by superoxide dismutase was plotted as function of concentration of this enzyme.](image-url)
ASSAYS OF SUPEROXIDE DISMUTASE

the effects of superoxide dismutase on this system. One unit of superoxide dismutase, which caused 50% inhibition under these conditions, was equivalent to 0.045 μg of the pure bovine erythrocyte enzyme per milliliter. The maximal degree of inhibition that could be achieved was 94%. Cyanide was present as an inhibitor of peroxidases, which might interfere by causing a peroxidation of the blue formazan. This level of cyanide had no effect on the activity of bovine superoxide dismutase. This assay was found to be applicable to crude tissue extracts as well as to pure samples of superoxide dismutase. The maximum inhibition achievable by the addition of crude extracts was less than that which could be reached with pure superoxide dismutase, i.e., 70% as compared with 95%. The crude extracts thus appear to contain components which provide an oxygen-independent route of transfer of electrons from photoreduced riboflavin to NBT. These electron-transferring components were not dialyzable. The problem did not seriously mar the applicability of the assay to crude extracts, since a unit of superoxide dismutase could be defined as the amount needed to cause half-maximal inhibition. A unit of superoxide dismutase, when so defined, would not be affected by the existence of O₂⁻-independent routes for the reduction of NBT.

Localization of Superoxide Dismutase on Polyacrylamide Gels

The insolubility of the blue reduction product of NBT suggested that the assay described above might be used, with minor modifications, to detect superoxide dismutase on polyacrylamide gels. This proved to be the case. As a test of the sensitivity of the method a serial dilution of superoxide dismutase, purified by the method of McCord and Fridovich (1) but omitting the DE-32 chromatography, was electrophoresed on duplicate gels. One gel of each set was then stained for protein and the other for superoxide dismutase activity, as described under “Methods.” Visual examination of these gels demonstrated that 0.016 μg superoxide dismutase produced a detectable band when localized by the activity stain whereas 0.40 μg was required to produce a visible band when stained with amido black. Figure 4, which illustrates the results of this experiment, demonstrates that several electrophoretically separable forms of superoxide dismutase exist in these preparations.

This staining procedure has been applied to a number of sources of superoxide dismutase and the results are shown in Fig. 5. A sonicate of E. coli revealed at least two dozen protein bands (1c) and two well separated bands of superoxide dismutase activity (1b). The enzyme purified from this source (3) revealed only one band of activity. It is clear that E. coli B contains two species of superoxide dismutase, the basis of whose differences remain to be elucidated. A crude extract of
FIG. 4. Activity and protein stain applied to polyacrylamide gel electrophoretograms of serial dilution of bovine erythrocyte superoxide dismutase. The following amounts of superoxide dismutase were applied to duplicate gels: (a) 50 µg, (b) 10 µg, (c) 2 µg, (d) 0.4 µg, (e) 0.08 µg, (f) 0.016 µg, (g) 0.032 µg. After electrophoresis, the gels in set 1 were stained for protein with amido black in 7% acetic acid. The gels in set 2 were stained for superoxide dismutase activity as described under “Methods.”

bovine heart gave multiple bands of protein (2c) but only one band of superoxide dismutase activity (2b), which coincided with the band produced by the enzyme when purified (2a) from this source. Crude extracts of bovine brain (set 3), bovine lung (set 4), or bovine erythrocytes (set 5) similarly exhibited multiple protein bands but only one zone of superoxide dismutase activity. The diffuseness of the superoxide dismutase zone in gel (5b) was caused by the presence of massive amounts of hemoglobin which, although devoid of superoxide dismutase activity,
Fig. 5. Activity and protein stains applied to polyacrylamide electrophoretograms of several crude extracts and purified preparations of superoxide dismutase. In all cases samples were applied to the gels and electrophoresis was performed before the staining procedures were used: (1a) 1.4 μg *E. coli* superoxide dismutase, stained for activity; (1b) 7.5 μl crude extract *E. coli*, stained for activity; (1c) 15 μl crude extract *E. coli*, stained for protein; (2a) 0.07 μg purified bovine heart superoxide dismutase, stained for activity; (2b) 1.0 μl crude extract bovine myocardium, stained for activity; (2c) 5.0 μl crude extract bovine myocardium, stained for protein; (3a) 1.0 μl crude extract bovine lung, stained for activity; (3b) 12.5 μl crude extract bovine lung, stained for protein; (4a) 1.0 μl crude extract bovine brain, stained for activity; (4b) 15 μl crude extract of bovine brain, stained for protein; (5a) 0.049 μg purified bovine erythrocyte superoxide dismutase, stained for activity; (5b) 1.0 μl bovine hemolyzate, stained for activity; (5c) 3 μl bovine hemolyzate, stained for protein.
did modify the color of the blue formazan deposited in the gel, and this resulted in an apparent achromatic zone in black and white photographs. The superoxide dismutase bands obtained with extracts of bovine heart, lung, brain, and erythrocytes all migrated identically under these conditions. This result suggests their identity. Bovine heart and erythrocyte superoxide dismutase have already been found to be identical by several criteria (2) as have the corresponding proteins from human brain, liver, and erythrocytes (17).

**Survey of Dyes**

A variety of dyes have been tested for their abilities to generate $O_2^-$ when illuminated in the presence of tetramethylethylenediamine and oxygen. The criterion of $O_2^-$ production was the ability to cause a photoreduction of NBT, which was inhibitable by superoxide dismutase. Thus, reaction mixtures contained $6.7 \times 10^{-6} M$ or $8.34 \times 10^{-7} M$ dye, $1.7 \times 10^{-4} M$ NBT, $10^{-2} M$ tetramethylethylenediamine, and $0.05 M$ potassium phosphate at pH 7.8 and equilibrated with air at 25°C. These reaction mixtures were illuminated for 10 min in the presence and in the absence of 1.3 μg superoxide dismutase/ml. All the dyes tested, whose behavior is summarized in Table 1, were capable of the photoproduction of $O_2^-$.  

**DISCUSSION**

The assays described for superoxide dismutase utilize NBT as the detector of $O_2^-$ and define superoxide dismutase activity in terms of its ability to inhibit the reduction of NBT due to $O_2^-$. The existence of re-

**Table 1**

<table>
<thead>
<tr>
<th>Dye</th>
<th>Dye class</th>
<th>Concentration, $M$</th>
<th>$\Delta$OD/10 min in presence of superoxide dismutase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein</td>
<td>Xanthene</td>
<td>$6.07 \times 10^{-6}$</td>
<td>0.305</td>
</tr>
<tr>
<td>Eosin yellowish</td>
<td>Xanthene</td>
<td>$6.67 \times 10^{-6}$</td>
<td>0.203</td>
</tr>
<tr>
<td>Azure C</td>
<td>Thiazine</td>
<td>$8.34 \times 10^{-7}$</td>
<td>0.295</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>Thiazine</td>
<td>$8.34 \times 10^{-7}$</td>
<td>0.161</td>
</tr>
<tr>
<td>Proflavin</td>
<td>Acridine</td>
<td>$6.67 \times 10^{-6}$</td>
<td>0.331</td>
</tr>
<tr>
<td>Acridine yellow</td>
<td>Acridine</td>
<td>$6.67 \times 10^{-8}$</td>
<td>0.205</td>
</tr>
</tbody>
</table>
ASSAYS OF SUPEROXIDE DISMUTASE 285

actions which lead to the reduction of NBT without involving O$_2^-$ do not interfere with these assays provided that the sum total of such reaction pathways is not much greater than the O$_2^-$-dependent pathway. This is the case because a unit of superoxide dismutase is defined as the amount causing 50% of the maximum inhibition that can be achieved by superoxide dismutase. In fact, at least 90% of the NBT reduction seen in the standard assay conditions described was mediated by O$_2^-$. Crude tissue extracts did contain electron-transferring components which provided O$_2^-$-independent pathways of NBT reduction but the assays were readily applicable, even to these crude extracts. The sensitivity and specificity of these assays is quite remarkable. Thus, 0.016 μg bovine erythrocyte superoxide dismutase gave a visible band on acrylamide gels and crude extracts of a variety of bovine tissues gave only one zone staining for superoxide dismutase activity which migrated identically with the enzyme that had been purified from bovine erythrocytes.

Crude extracts of *E. coli* B have been found to contain two superoxide dismutases, whereas the enzyme purified from that source (3) contained only one. It follows that the purification procedure eliminated one of the enzymes. The properties of this superoxide dismutase isozyme would obviously be a matter of great interest.

The erythrocyte superoxide dismutase is obviously subject to minor modifications by the rigorous procedures (1) used in its purification. This enzyme, when analyzed by disc gel electrophoresis, gave several closely spaced bands of activity prior to chromatography on DE-32 (1) and only one band of activity thereafter. Stansell and Deutsch (18), working with erythrocuprein from human erythrocytes, observed that treatment with chloroform, ethanol, or lead acetate modified its electrophoretic and sedimentation properties. Bannister and Wood (19) found that a preparation of erythrocuprein which gave two protein bands on disc gel electrophoresis gave only one band on isoelectric focusing. They attributed this to a size alteration that had occurred during the isolation procedure. Erythrocuprein was one of the names applied to superoxide dismutase before its enzymic activity had been discovered (1).

It is interesting that assays, similar to the one described here for locating superoxide dismutase activity on polyacrylamide gels, have already been performed by other workers who were not aware of the significance of their observations. Thus, Brewer (20) stained starch gel electrophoretograms of hemolyzates by treatment with phenazinium methylsulfate, a tetrazolium, and light and noted the presence of reproducible achromatic zones. He thought that these achromatic zones were caused by a tetrazolium oxidase and went on to discover a genetic variant of this activity.
These "tetrazolium oxidases," which were found to be widely distributed in the animal kingdom (21), would now appear to have been due to superoxide dismutase.

The generation of $O_2^-$ by dyes illuminated in the presence of an electron donor and of oxygen has been proposed by several investigators (22–24). The data in Table 1 demonstrate that a variety of dyes share the ability to generate $O_2^-$ under such circumstances. The probable sequence of events involves excitation of the dye by absorption of a photon, reduction of the excited dye by reaction with the electron donor, and reaction of the reduced dye with oxygen to yield $O_2^-$. Massey et al. (8) have demonstrated the production of $O_2^-$ by illuminated tetracetylriboflavin through the use of cytochrome c and superoxide dismutase.

SUMMARY

Nitro blue tetrazolium has been used to intercept $O_2^-$ generated enzymically or photochemically. The reduction of NBT by $O_2^-$ has been utilized as the basis of assays for superoxide dismutase, which exposes its presence by inhibiting the reduction of NBT. Superoxide dismutase could thus be assayed either in crude extracts or in purified protein fractions. The assays described are sensitive to ng/ml levels of superoxide dismutase and were applicable in free solution or on polyacrylamide gels. The staining procedure for localizing superoxide dismutase on polyacrylamide electrophoretograms has been applied to extracts obtained from a variety of sources. E. coli has been found to contain two superoxide dismutases whereas bovine heart, brain, lung, and erythrocytes contain only one.

REFERENCES