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Superoxide Dismutase

AN ENZYMIC FUNCTION FOR ERYTHROCUPREIN (HEMOCUPREIN)*

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SUMMARY

An enzyme which catalyzes the dismutation of superoxide radicals ($O_2 \cdot^- + O_2 \cdot^- + 2H^+ \rightarrow O_2 + H_2O_2$) has been purified by a simple procedure from bovine erythrocytes. This enzyme, called superoxide dismutase, contains 2 eq of copper per mole of enzyme. The copper may be reversibly removed, and it is required for activity. Superoxide dismutase has been shown to be identical with the previously described copper-containing erythrocuprein (human) and hemocuprein (bovine).

Stable solutions of the superoxide radical were generated by the electrolytic reduction of O_2 in an aprotic solvent, dimethylformamide. Slow infusion of such solutions into buffered aqueous media permitted the demonstration that $O_2 \cdot^-$ can reduce ferricytochrome *c* and tetranitromethane, and that superoxide dismutase, by competing for the superoxide radicals, can markedly inhibit these reactions.

Superoxide dismutase was used to show that the oxidation of epinephrine to adrenochrome by milk xanthine oxidase is mediated by the superoxide radical.

An assay of several tissues indicates that superoxide dismutase is widely distributed within mammalian organisms.

A blue copper protein was isolated from bovine erythrocytes by Mann and Keilin (2) in 1939. This crystalline protein, called "hemocuprein," contained 0.34% copper and had a molecular weight of about 34,000. No enzymic activity was found for this protein. More recently a copper protein from human erythrocytes has been characterized (3, 4). This protein was called "erythrocuprein" and was similar in copper content and size to hemocuprein. Erythrocuprein, like hemocuprein, lacked an apparent enzymic function. Furthermore, copper proteins of similar size and copper content have been isolated from tissues other than blood. A copper protein called "cerebrocuprein" was isolated from human brain (5). "Hepatocuprein" has been isolated from bovine (2) and equine (6) liver. These proteins, like the erythrocyte proteins, have not been associated with any apparent enzymic function.

Work in this laboratory (7) led to the proposal of a previously unsuspected enzymic activity which catalyzes the dismutation or disproportionation of superoxide free radical anions.

$$O_2 \cdot^- + O_2 \cdot^- + 2H^+ \rightarrow O_2 + H_2O_2 \quad (1)$$

It is the purpose of the present report to provide additional evidence for this dismutase activity and to show that this activity is associated with the copper-containing proteins described above (2–6).

EXPERIMENTAL PROCEDURE

Spectrophotometric assays were performed with a Gilford model 2000 recording spectrophotometer equipped with a thermostated cell compartment. Other spectral data were obtained with a Cary 15 spectrophotometer. Ultracentrifugal studies employed Beckman analytical ultracentrifuges and were performed in 0.1 M NaCl at pH 6.0 or 7.0, buffered with approximately 0.02 M phosphate. Total copper was assayed by use of 2,2′-biquinoline in acetic acid (8). Hydrogen peroxide was assayed by a colorimetric method (9). Adrenochrome formation was followed as an increase in absorbance at 480 mμ (10).

Reduction of tetranitromethane was monitored by following the increase in absorbance at 350 mμ due to the accumulation of the nitroform anion. An extinction coefficient of 14,800 was used (11). The tetranitromethane was washed with water to remove colored impurities, and stock solutions were prepared in ethanol. Milk xanthine oxidase was purified as described previously (12) or was prepared by a method not involving proteolysis.[1] Horse heart cytochrome *c*, type III, was a product of Sigma. The molar extinction coefficients given by Massey (14) were used, and its reduction was followed at 550 mμ. The concentration of purified superoxide dismutase solutions was determined by drying deionized aliquots of the solutions over P_2O_5 under reduced pressure at 70° to constant weight.

Electrolytic Production of Superoxide in Dimethylformamide—Solutions of tetrabutylammonium superoxide were produced by the electrolytic univalent reduction of molecular oxygen dissolved in dry *N*,*N*-dimethylformamide. These solutions of the superoxide radical were stable for hours at room temperature or for weeks at liquid nitrogen temperature. The method is a modification of the procedure used by Maricle and Hodgson (15). We have avoided the need for an aqueous-organic liquid-liquid

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[1] F. O. Brady, unpublished data.

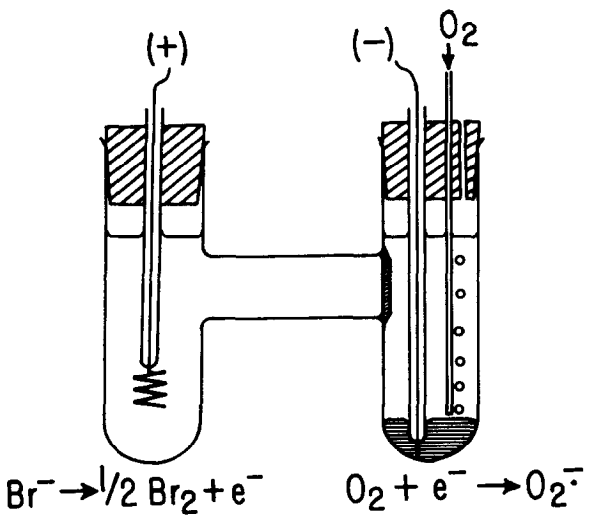

FIG. 1. Cell for the electrolytic reduction of O_2 to $O_2^{\cdot-}$ in *N,N*-dimethylformamide. Details described under "Experimental Procedure".

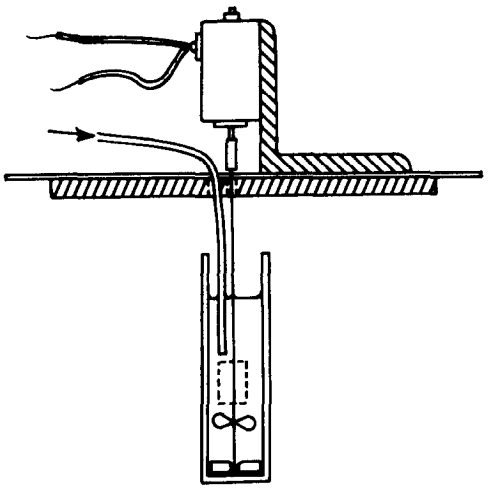

FIG. 2. Cuvette stirrer for infusion experiments. The *dashed line* approximates the light path through the 1-cm cuvette. A lucite bearing in the bottom of the cuvette prevents shaft wobble. See description under "Experimental Procedure".

interface by replacing the calomel anode by the oxidation of bromide to free bromine at a platinum electrode, allowing both chambers of the cell to be filled with the same solution, 0.1 M tetrabutylammonium bromide or tetrapropylammonium bromide in dimethylformamide. The necessity of maintaining absolutely anhydrous conditions cannot be overemphasized, as the superoxide radicals can spontaneously dismute only if protons are available (Equation 1). These solutions were stored over anhydrous calcium sulfate before being placed in the electrolytic cell, which is diagrammed in Fig. 1. This glass cell holds approximately 60 ml. The two chambers are separated by a sintered glass disc, 1 cm in diameter. The cathode is a pool of mercury, and the anode is a coil of platinum wire. Oxygen, which had been dried by bubbling through concentrated sulfuric acid, was continuously bubbled through the cathode chamber. A direct current was applied as indicated, and a current of 1 ma was maintained for several hours. At the end of this period the anode solution was bright yellow because of the accumulation of free bromine, and the cathode solution was pale brownish yellow and contained tetrabutylammonium superoxide. For the infusion experiments, a Manostat digital microburette was filled with the cathode solution (tetrabutylammonium superoxide in dimethylformamide) and was driven by a synchronous motor at such a rate that 2.6 μl per min were delivered through a polyethylene capillary tube attached to the tip of the microburette.

Fig. 2 shows the cuvette stirrer which allowed the solutions of superoxide prepared as described above to be slowly added, with rapid mixing, to the contents of a cuvette in the recording spectrophotometer. The superoxide solution was infused through the tube indicated by the *arrow*. The rate of stirring was controlled by varying the direct current voltage applied to the small motor. The apparatus fit snugly over the sample compartment of the Gilford 2000, replacing the usual sample compartment cover, thereby allowing the absorbance of the cuvette contents to be monitored continuously as the infusion of superoxide proceeded. The propeller shaft intercepted a small but constant fraction of the light path and thus offered no hindrance to the recording of absorbance changes.

Definition of Unit of Superoxide Dismutase Activity—The standard assay is performed in 3 ml of 0.05 M potassium phosphate buffer at pH 7.8 containing 10^{-4} M EDTA in a 1.0-cm cuvette thermostated at 25°. The reaction mixture contained 1×10^{-5} M ferricytochrome *c*, 5×10^{-5} M xanthine, and sufficient xanthine oxidase to produce a rate of reduction of ferricytochrome *c* at 550 mμ of 0.025 absorbance unit per min. The concentration of xanthine oxidase in the cuvette was usually 6×10^{-9} M but may vary with different preparations of the enzyme.[2] Under these defined conditions, the amount of superoxide dismutase required to inhibit the rate of reduction of cytochrome *c* by 50% (*i.e.* to a rate of 0.0125 absorbance unit per min) is defined as 1 unit of activity.

Enzyme Purification—Fresh bovine blood, preserved with acid-citrate-dextrose solution, was generously provided by

[2] With a fresh preparation of native xanthine oxidase the reduction of cytochrome *c* is *totally* dependent on the presence of oxygen (in the absence of other electron carriers). We have noticed that with aged preparations of xanthine oxidase an ability to reduce cytochrome *c* anaerobically does appear to a small extent. As expected, the susceptibility of aerobic reduction rates to inhibition by superoxide dismutase correlates well with these observations. That is, with a fresh native preparation of xanthine oxidase, the aerobic reduction of cytochrome *c* may be inhibited essentially 100% by dismutase. With an aged preparation, a residual rate of cytochrome *c* reduction remains which is not susceptible to inhibition by dismutase. These data indicate that the reduction of cytochrome *c* by *native* xanthine oxidase is entirely mediated by the superoxide radical, $O_2^{\cdot-}$. In aged preparations of xanthine oxidase a second pathway of cytochrome *c* reduction is opened: a pathway which is direct and does not require the presence of oxygen as an electron carrier. Komai, Massey, and Palmer (13) have recently shown that deflavo xanthine oxidase possesses a cytochrome *c* reductase activity which is completely independent of the presence of oxygen, concluding (*a*) that flavin is somehow involved in the transfer of electrons to molecular oxygen, and (*b*) that the removal of flavin from the enzyme uncovers a site from which cytochrome *c* may accept electrons directly from reduced xanthine oxidase. Massey's holoenzyme, prepared by a method employing pasteurized cream and proteolysis by pancreatin, also showed a weak but definite ability to reduce cytochrome *c* anaerobically. We have shown that when native enzyme, prepared without proteolysis (see Footnote 1), is treated with pancreatin (0.1 mg per ml, overnight at 37°), it, too, develops a weak anaerobic rate (4% of the aerobic rate), not inhibitable by dismutase, representing a departure from true native characteristics.

TABLE I

Purification of bovine superoxide dismutase

Preparation stage	Volume	Total protein	Total units	Specific activity	Yield	Purification factor
	ml	*g*		*units/mg*	%	
Hemolysate	6000	~900[a]	≥1,040,000† (300 mg)	≥1.2[b]		
Supernatant from Tsuchihashi fractionation	4700	9.35	1,040,000	101	100	84
Ethanolic phase	1260	2.52	1,000,000	400	96	330
Acetone precipitate dissolved in water	90		900,000		87	
After chromatography on DE-32		0.190	630,000	3300	60	2750

[a] Estimated.

[b] The hemolysate could not be accurately assayed owing to optical interference with the standard assay. The figures quoted assume no loss in the subsequent step. This may be a valid assumption, since 300 mg is the total amount of erythrocuprein to be expected from an equivalent amount of packed *human* cells (17). All yield data are relative to the Tsuchihashi supernatant.

Swift. Cells were collected from approximately 7 liters of blood by centrifugation, then resuspended and washed in 0.9% NaCl by centrifugation. The 3 liters of packed cells were lysed by the addition of an equal volume of deionized water. Hemoglobin was then precipitated from the 6 liters of hemolysate by an adaptation of the Tsuchihashi chloroform-ethanol treatment (16). With stirring, 0.25 volume (1500 ml) of ethanol and 0.15 volume (900 ml) of chloroform were added in the cold. This mixture was allowed to stir for 15 min, during which time the mixture becomes very thick. It was diluted with 0.10 volume of water, and the precipitate was removed by centrifugation. The resulting supernatant (4.7 liters) was pale yellow and contained 1.04×10^6 units of superoxide dismutase activity. This solution was allowed to warm to room temperature, and 1400 g of solid K_2HPO_4 (300 g per liter) were added, resulting in the separation of two liquid phases. The denser phase was essentially aqueous and contained most of the salt. The lighter phase was water-ethanol and contained little salt. A brownish precipitate was present in the upper phase. The upper phase was collected (1260 ml) and centrifuged. The pale yellow supernatant contained essentially all of the dismutase activity (1.0×10^6 units). This solution was cooled to 4°, and 0.75 volume of cold acetone was added to precipitate the dismutase. The light blue precipitate was dissolved in water and dialyzed against 0.0025 M potassium phosphate at pH 7.4, then applied to a DE 32 column (2 × 17 cm) equilibrated with the same buffer. The column was eluted with a gradient of potassium phosphate ranging from 0.0025 to 0.2 M, pH 7.4. A blue-green protein was eluted from the column as a single peak, clearly separated from minor contaminating proteins. The blue-green band represented 190 mg of superoxide dismutase displaying 630,000 units of activity with a specific activity of 3300 units per mg. The results of this purification are summarized in Table I.

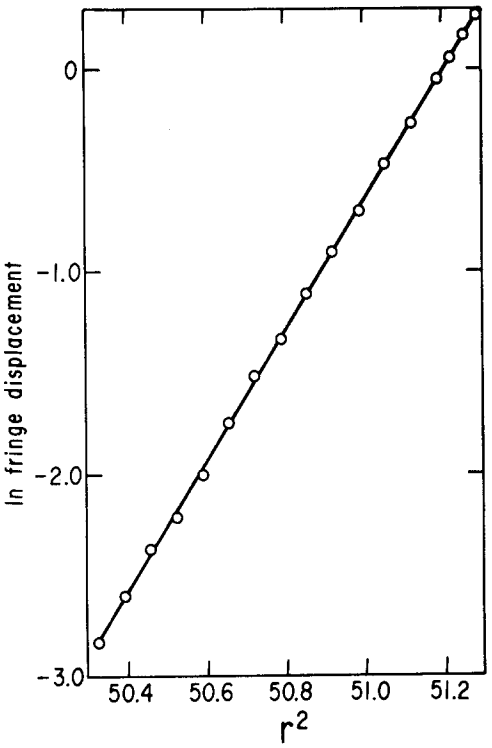

FIG. 3. Equilibrium sedimentation of bovine superoxide dismutase. Protein concentration was 0.24 mg per ml in 0.1 M NaCl buffered at pH 6.0 with 5 mM potassium phosphate. Rotor speed was 40,000 rpm.

RESULTS

Identity of Superoxide Dismutase as Erythrocuprein (Hemocuprein)—The superoxide dismutase isolated by the above procedure exhibited a pale greenish blue color and contained 0.38% copper. Gel filtration indicated a molecular weight slightly larger than bovine carbonic anhydrase (molecular weight 31,000). Subsequent determination of molecular weight by sedimentation equilibrium gave a value of 32,600 (Fig. 3) when the partial specific volume was assumed to be 0.720. The ultraviolet and visible absorption spectra of the enzyme are shown in Fig. 4. The enzyme exhibits a unique ultraviolet spectrum with a maximum at 258 mμ and a broad, weak visible absorption band with a peak around 680 mμ. A high degree of homogeneity was indicated by equilibrium sedimentation and sedimentation velocity experiments. Fig. 5 shows the single peak observed with schlieren optics during a sedimentation velocity run. An $s_{20,w}$ of 3.35 S was obtained at a protein concentration of 7.25 mg per ml. Polyacrylamide disc gel electrophoresis at pH 8.9 in Tris-HCl buffer revealed a single band.

All of the above data are in close agreement with the results of similar experiments performed on bovine hemocuprein (2) or human erythrocuprein (3, 4, 18). As further proof of the identity of superoxide dismutase with erythrocuprein, a sample of human erythrocuprein which had been prepared by a totally different purification procedure was kindly provided by Drs. H. F. Deutsch and Robert Carrico. This human erythrocuprein was assayed

by us for superoxide dismutase activity and displayed a specific activity of 3000 units per mg. Our preparations of bovine superoxide dismutase possess a specific activity of 3300 units per mg.

Studies with Electrolytically Produced $O_2 \cdot^-$—The pale brownish cathode solution resulting from the electrolytic reduction of O_2 in dimethylformamide was tested for the presence of the superoxide radical by adding a drop or two of aqueous KCl solution to a small aliquot of the cathode solution (15). This resulted in an immediate effervescence, because of the release of molecular oxygen, as would be predicted from Equation 1. If a few crystals of potassium perchlorate were added to an aliquot of the cathode solution, a flocculent yellow precipitate formed (potassium superoxide) which evolved oxygen vigorously on contact with water. These reactions indicated the presence of the superoxide anion in the cathode solution.

Reduction of Cytochrome c—When the cathode solution was placed in the motor-driven microburette and infused into a stirred cuvette containing a buffered solution of cytochrome *c*, reduction took place as indicated by Fig. 6, with the cytochrome *c* being essentially completely reduced. This reduction was quite sensitive to the presence of superoxide dismutase; a concentration of 2×10^{-8} M caused a 70% inhibition, even in the presence of 40 times as much cytochrome *c*.

Reduction of Tetranitromethane by $O_2 \cdot^-$—Fig. 7 points out the ability of superoxide to reduce tetranitromethane, producing the nitroform anion, $C(NO_2)_3^-$. Again, superoxide dismutase potently inhibited this reduction. Fig. 7 also indicates that superoxide dismutase activity was destroyed by boiling the enzyme, and that several equivalents of free cupric ion had no effect on the rate of reduction.

Preparation of Apoenzyme—Overnight dialysis of the holoenzyme against 0.05 M sodium acetate buffer at pH 3.8 containing 10^{-3} M EDTA resulted in the loss of about 90% of the copper, with a corresponding loss of about 85% of the activity. A brief incubation of the apoenzyme with 10^{-4} M $CuSO_4$ resulted in the reappearance of about 80% of the original activity.

Metal Replacement Tests—Apoenzyme (4×10^{-6} M) was incubated for a short time with the following metal ions at a concentration of 10^{-3} M: Ni^{++}, Co^{++}, Hg^{++}, Mg^{++}, Fe^{+++}, and

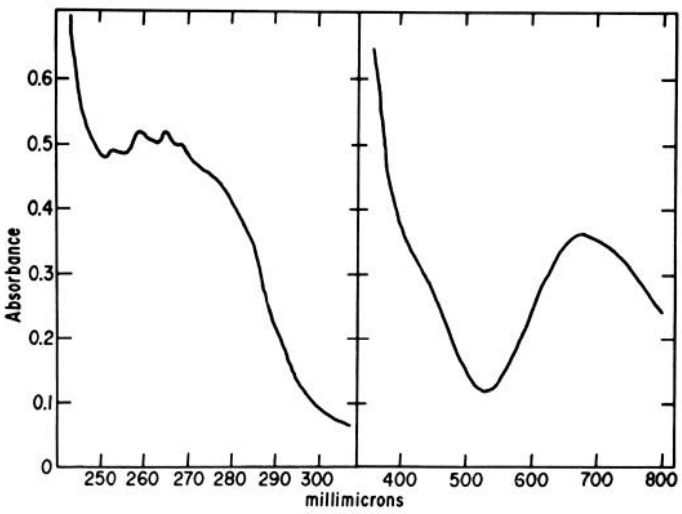

FIG. 4. Absorption spectrum of bovine superoxide dismutase. The concentration of the enzyme was 1.5 mg per ml for the ultraviolet spectrum, 47 mg per ml for the visible spectrum. A 1-cm path length was used.

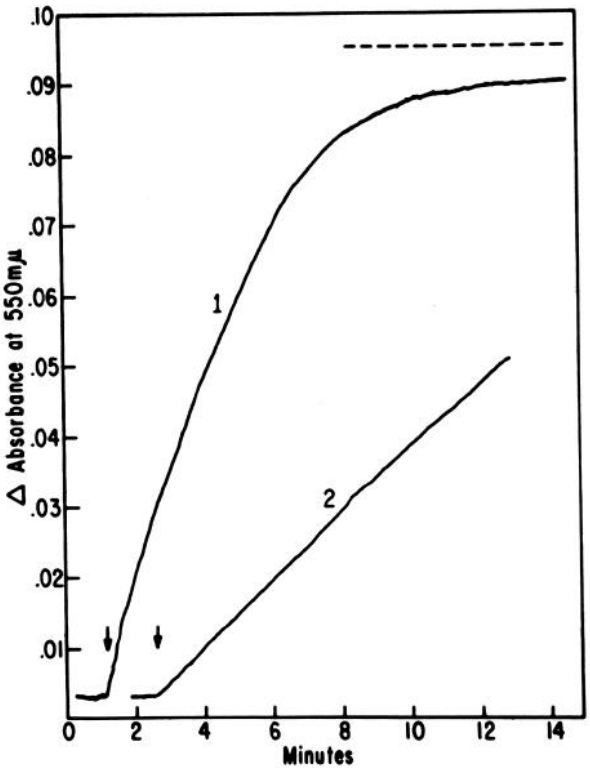

FIG. 6. Reduction of cytochrome *c* by infused $O_2 \cdot^-$ and inhibition by superoxide dismutase. Reactions were performed at 25° in a total volume of 3.0 ml, buffered at pH 7.8 by 0.05 M potassium phosphate containing 0.1 mM EDTA, in 1.0-cm cuvettes continuously stirred by the device shown in Fig. 2. Infusion of an electrolytically prepared solution of $O_2 \cdot^-$ was initiated at times indicated by the *arrows*. Additional components were *Curve 1*, 5×10^{-6} M cytochrome *c*. (The *dashed line* indicates total reduction.) *Curve 2*, 4×10^{-5} M cytochrome *c*, 3×10^{-8} M bovine superoxide dismutase. Both cuvettes contained approximately 20 μg of catalase.

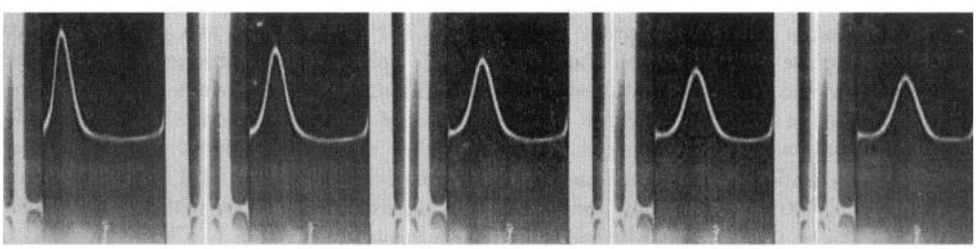

FIG. 5. Sedimentation velocity schlieren patterns of bovine superoxide dismutase. Protein concentration was 7.25 mg per ml in 0.1 M NaCl buffered at pH 7.0 with potassium phosphate. Pictures were taken at 16-min intervals after reaching 56,100 rpm. Direction of sedimentation is from *left* to *right*.

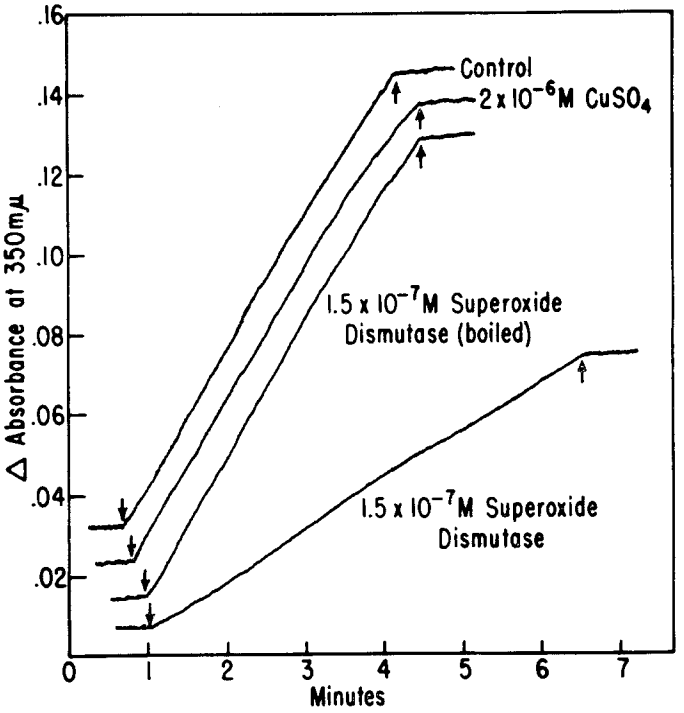

FIG. 7. Reduction of tetranitromethane by infused $O_2 \cdot^-$ and inhibition by superoxide dismutase. Reactions were performed at 25° in a total volume of 2.5 ml, buffered at pH 7.8 with 0.05 M potassium phosphate containing 1×10^{-4} M EDTA and 4×10^{-5} M tetranitromethane. At the *arrows* the infusion of an electrolytically prepared solution of $O_2 \cdot^-$ was started (↓) or stopped (↑). Additional components are indicated on the graph.

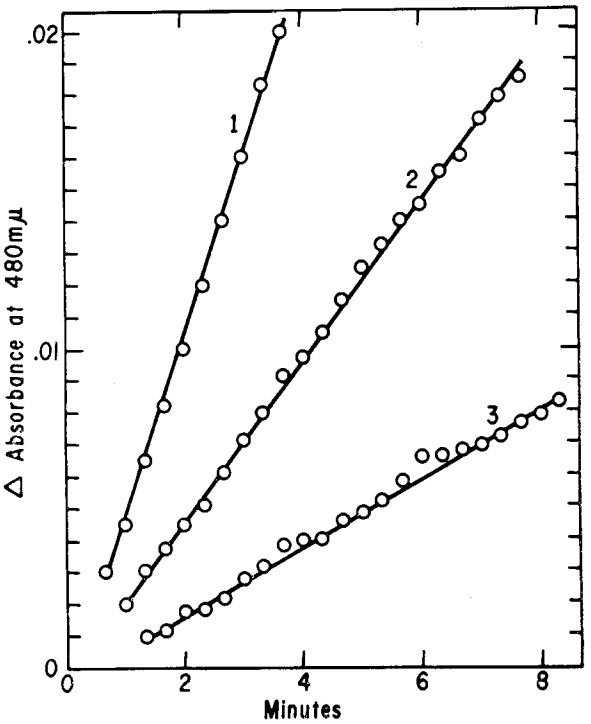

FIG. 8. Co-oxidation of epinephrine to adrenochrome by xanthine oxidase plus xanthine, and inhibition by superoxide dismutase. Reactions were performed at 25° in a total volume of 3.0 ml buffered with 0.01 M sodium borate, pH 10.1. Each cuvette contained 2×10^{-4} M epinephrine, 1×10^{-4} M xanthine, and 1×10^{-8} M milk xanthine oxidase. Superoxide dismutase concentrations were *Curve 1*, none; *Curve 2*, 11 ng per ml; *Curve 3*, 45 ng per ml.

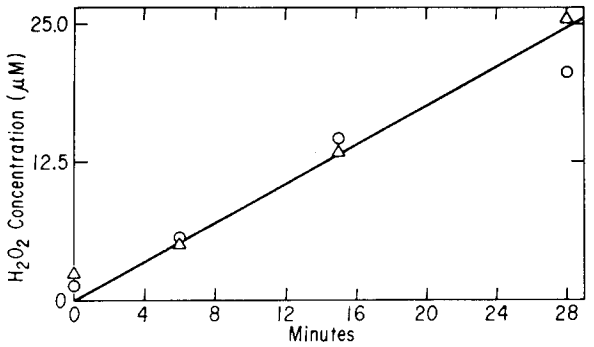

FIG. 9. Comparison of rates of production of hydrogen peroxide for the catalyzed and spontaneous dismutations of the superoxide radical. Each of two reaction mixtures contained a total volume of 8.0 ml buffered at pH 7.0 with 5 mM Tris-chloride, 10^{-4} M in xanthine, and 8.5×10^{-9} M in milk xanthine oxidase. One reaction mixture (○) contained no superoxide dismutase, the other (△) contained 1.6×10^{-8} M superoxide dismutase. Aliquots were taken at the times indicated and assayed (9) for accumulated hydrogen peroxide.

Zn^{++}. None of these was able to replace Cu^{++} in restoring dismutase activity to the apoenzyme.

Oxidation of Epinephrine to Adrenochrome by Xanthine Oxidase Plus Xanthine—It has been reported (19) that xanthine oxidase in the presence of an oxidizable substrate will catalyze the cooxidation of epinephrine to form adrenochrome. The data shown in Fig. 8 represent the appearance of adrenochrome in such a reaction mixture and demonstrate the great sensitivity of this reaction to the presence of minuscule amounts of superoxide dismutase. Under the conditions used, a 50% inhibition of the rate of oxidation of epinephrine was observed in the presence of 3×10^{-10} M superoxide dismutase, a concentration equivalent to 10 ng per ml.

Hydrogen Peroxide as Product of Dismutase-catalyzed Reaction—Reaction mixtures containing xanthine oxidase plus xanthine in the presence and absence of superoxide dismutase were assayed for hydrogen peroxide accumulation. The presence of superoxide dismutase had no effect on the rate of hydrogen peroxide production, as shown in Fig. 9. This indicates that the products of the spontaneous dismutation reaction are identical to the products of the enzyme-catalyzed dismutation.

Tissue Distribution of Superoxide Dismutase—Although superoxide dismutase has not yet been characterized from tissues other than blood, preliminary experiments have demonstrated that the activity exists in many tissues such as bovine heart (320 units per g, wet weight), brain (200 units per g, wet weight), and liver; horse heart; and porpoise skeletal muscle.

DISCUSSION

The superoxide dismutase isolated in these studies endures a rigorous and simple purification procedure with unusual stamina. After the Tsuchihashi (chloroform-ethanol) fractionation, the supernatant was warmed to room temperature and solid dibasic potassium phosphate was added, salting out an ethanolic phase which contained a nearly quantitative yield of the activity. The enzyme can remain in the solvent for hours at room temperature, without significant loss of activity. Early procedures for the purification of hemocuprein (2) or erythrocuprein (3) employed the Tsuchihashi fractionation. Stansell and Deutsch more recently published a lengthy purification procedure for *human* erythrocuprein (17) which avoids the use of such drastic methods.

They observed that exposure of their purified material to the chloroform-ethanol treatment led to an increased sedimentation constant, a decreased intrinsic viscosity, and the loss of about 8% of the copper. Such treatment also led to an apparent increase in the degree of heterogeneity (18). Our data suggest that the bovine enzyme is more resistant to modification by this drastic treatment. Our purified enzyme exhibits a full complement of copper (0.38%) and shows a very high degree of homogeneity as witnessed by the linearity of the equilibrium sedimentation data (Fig. 3), the single component revealed by the sedimentation velocity data (Fig. 5), and the appearance of a single band by disc gel electrophoresis. The specific activity of our *bovine* superoxide dismutase was slightly higher than the specific activity of the sample of *human* erythrocuprein prepared by Dr. Deutsch.

The ultraviolet absorption spectrum of bovine superoxide dismutase (Fig. 4) is quite unique and deserves some comment. The absence of a peak around 280 mμ indicates a very low content of tryptophan and tyrosine residues, perhaps zero. There is an absorbance maximum at 258 mμ with a molar extinction coefficient of 10,300. Human erythrocuprein displays an absorbance maximum at 265 mμ with a molar extinction coefficient of 18,400 (3) or 18,700 (18). The fine structure which is observed from 250 to 270 mμ with bovine superoxide dismutase has not been observed with the human enzyme. This fine structure is undoubtedly contributed by phenylalanine residues. There is an excellent correspondence between the ultraviolet spectrum of the enzyme and of phenylalanine. The absorption maximum in the visible region is quite weak and broad, peaking around 680 mμ with a molar extinction coefficient of about 300. The corresponding visible maximum for the human enzyme has been reported to be at 655 mμ (ϵ = 350) (18) or 670 mμ (20).

The recognition of superoxide dismutase as an enzymic activity and its final identification as erythrocuprein (hemocuprein) have provided explanations for many observations that have been made in this laboratory. The fact that a protein existed which was a competitive inhibitor of the reduction of cytochrome *c* by xanthine oxidase was first observed in 1962 (21), and again in 1967 (22). The data gave every indication that the proteins myoglobin and carbonic anhydrase were competitive inhibitors with respect to cytochrome *c* in the classical sense, that is, that they shared a common binding site on the surface of xanthine oxidase. When attempts to demonstrate this binding by physical methods failed, the mechanism of the reduction of cytochrome *c* by xanthine oxidase was probed more deeply, yielding data which finally proved that *neither* cytochrome *c* *nor* the protein inhibitors were bound to xanthine oxidase. Instead, the reduction of cytochrome *c* was shown to be mediated by univalently reduced oxygen (the superoxide radical), which is produced by xanthine oxidase and liberated into free solution (7). This insight suggested that the protein inhibitors were acting in free solution to destroy the superoxide radical catalytically. The superoxide dismutase activity was found not to belong to the proteins originally suspected of binding to xanthine oxidase, but to a trace impurity of amazing potency. This "impurity" has now been purified and identified as erythrocuprein.

Since superoxide dismutase was discovered as a competitive inhibitor in the xanthine oxidase-cytochrome *c* system, it was highly desirable to demonstrate the activity in systems involving neither xanthine oxidase nor cytochrome *c*. For this reason we chose the most direct approach practicable: the electrolytic production of stable solutions of $O_2 \cdot^-$ in an aprotic solvent. If such a solution were slowly infused into a stirred cuvette, the situation should be completely analogous to a cuvette containing xanthine oxidase plus xanthine. That is, superoxide radicals would be introduced into solution at a constant rate. As shown in Fig. 6, the reaction was completely analogous to the xanthine oxidase system with respect to cytochrome *c* reduction, thus demonstrating that the superoxide radical can indeed reduce cytochrome *c*, and that superoxide dismutase potently inhibits this reduction, exactly as predicted. Thus, xanthine oxidase has been eliminated from the system and replaced by a known source of $O_2 \cdot^-$.

To take the system one step further, cytochrome *c* was replaced by tetranitromethane. Tetranitromethane has been used by a number of investigators as a quantitative scavenger for the superoxide anion (11, 23, 24) producing the stable nitroform anion, $C(NO_2)_3^-$. The rate constant for the reaction

$$O_2 \cdot^- + C(NO_2)_4 \rightarrow O_2 + C(NO_2)_3^- + NO_2 \qquad (2)$$

has been found to be 1.9×10^9 M^{-1} sec^{-1} (23). Fig. 7 shows that tetranitromethane is effectively reduced by the infusion of $O_2 \cdot^-$ and that superoxide dismutase does indeed inhibit the process. At a concentration 270-fold less than the tetranitromethane, the enzyme gives more than 50% inhibition, indicating that the rate constant for the reaction of dismutase with the radical must be approximately 5×10^{11} M^{-1} sec^{-1}. The magnitude of this figure underscores the almost incredible activity of this enzyme. By judicious choice of assay conditions, the enzyme may be readily assayed at concentrations of less than 1 ng per ml.

The nature of the superoxide radical is such that it may act either as a reductant or as an oxidant. (In the dismutation reaction, half is oxidized to O_2 by the other half, which is in turn reduced to H_2O_2.) We have demonstrated that superoxide dismutase is quite capable of protecting cytochrome *c* or tetranitromethane from reduction by $O_2 \cdot^-$. Fig. 8 indicates that this enzyme is also capable of preventing the oxidation of a substance by $O_2 \cdot^-$. The recent observation (19) that epinephrine is oxidized to adrenochrome by xanthine oxidase in the presence of xanthine suggested that this oxidation might be mediated by the superoxide radical. The marked inhibition of this process by superoxide dismutase confirms this suspicion. A concentration of superoxide dismutase of 3×10^{-10} M competed equally with 2×10^{-4} M epinephrine for the $O_2 \cdot^-$.

Several early lines of indirect chemical evidence (25–27) had implicated the involvement of the superoxide radical in certain reactions catalyzed by xanthine oxidase, finally leading to the proposal in 1962 (28) that enzyme-bound superoxide radicals were involved. Research in this area was seriously limited by a lack of methodology for dealing with the proposed superoxide radical. The present descriptions of a method for producing stable solutions of the radical and of an enzyme which catalyzes its dismutation have shown themselves to be powerful tools for studying reactions involving $O_2 \cdot^-$.

The major part of the literature dealing with the superoxide radical has studied the radical's fleeting existence in aqueous media. The radical has been generated in oxygenated water by pulsed radiolysis (23, 29, 30), by flash photolysis (31), by a flow system involving irradiation by an electron beam (11), and by timed exposure to a γ-ray source (24). These studies have contributed much significant information concerning the nature of the superoxide radical. The value of the rate constant for the second order spontaneous dismutation (Equation 1) near neutral

pH is generally agreed upon as being about 2×10^7 M^{-1} sec^{-1} (11, 23, 29). The pK for the equilibrium

$$HO_2\cdot \rightleftharpoons H^+ + O_2\cdot^-$$

has been found to be around 4.5 (11, 23, 29).

Proof of the involvement of the superoxide radical in biological systems other than xanthine oxidase has been, up to now, virtually nonexistent, though its involvement has been from time to time postulated, particularly in mechanisms describing the spontaneous autoxidation of hemoglobin or myoglobin to the met- states (32, 33). X-ray irradiation of aqueous solutions of cytochrome *c* led to the postulation that the superoxide radical is capable of reducing cytochrome *c* (34), but no biological implications were made.

Yamazaki and Piette (35), in a study of the peroxidase-oxidase reaction, proposed the superoxide radical as the active intermediate involved in this reaction but lacked conclusive evidence for this proposal. They noted that, because of the rapid spontaneous second order dismutation reaction, $O_2\cdot^-$ could never accumulate to a concentration sufficiently large to be detected by electron spin resonance. More recently, however, a rapid-freezing technique has allowed electron spin resonance to confirm the production of the superoxide radical by xanthine oxidase (36).

The abundance of superoxide dismutase activity in the variety of animal tissues assayed suggests that the enzyme might play a significant, even vital, role in protecting the organism against the damaging effects of the superoxide radical. Investigations are currently under way in this laboratory to study these effects.

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