Free Radicals and Inflammation: Protection of Synovial Fluid by Superoxide Dismutase

Abstract. *Enzymatically generated superoxide radical, by reacting with hydrogen peroxide to produce the hydroxyl radical, depolymerized purified hyaluronic acid and bovine synovial fluid. Since phagocytizing polymorphonuclear leukocytes produce superoxide radicals, this reaction is suggested and shown to be quantitatively feasible as the in vivo mechanism of synovial fluid degradation in an inflamed joint. Superoxide dismutase and catalase protect synovial fluid against such degradation in vitro.*

While free radicals have long been postulated in biological processes such as aging and in certain disease states, definitive data as to the specific chemical species involved and the types of molecular damage that might ensue have been difficult to obtain. Such studies have been facilitated by the realizations that certain oxidative enzymes produce the superoxide free radical ($O_2^-\cdot$) by a one-electron reduction of molecular oxygen under physiological conditions and that nature has provided a protective enzyme, superoxide dismutase (SOD), having the apparent sole function of scavenging the radical by the dismutation reaction (*1, 2*)

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

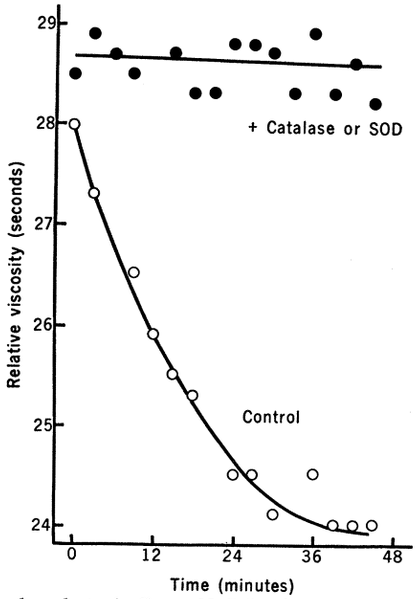
The data presented in this report show that enzymatically generated superoxide radical rapidly degraded bovine synovial fluid and solutions of purified hyaluronic acid prepared from human umbilical cords. The degradation was manifested by marked reductions in viscosity and by an inability of the degraded synovial fluid to form a "mucin clot" characteristic of healthy synovial fluid. The addition of either catalase or SOD to the synovial fluid or to the hyaluronate solutions provided protection against the degradation, indicating that both H_2O_2 and $O_2^-\cdot$ were necessary for the production of the degrading radical species, $OH\cdot$, produced as a secondary radical by the reaction

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

Hydrogen peroxide production always accompanies superoxide production since it is a product of the dismutation reaction, whether spontaneous or catalyzed (*1*). The synovial fluid contained a barely detectable amount of endogenous catalase (50 ng/ml or less) but did contain enough endogenous SOD (1 μg/ml) to provide partial protection from superoxide-induced degradation.

Hyaluronic acid was isolated from a homogenate of human umbilical cords by repeated fractional precipitation with cetylpyridinium chloride and with ethanol, as described by Danishefsky and Bella (*3*). The final product was isolated as the sodium salt and was lyophilized. On the basis of its glucuronic acid content and behavior on gel exclusion chromatography, the product was estimated to be hyaluronic acid of high purity and of molecular weight greater than 500,000. The material produced clear, viscous solutions when dissolved in phosphate buffer, *p*H 7.5. Superoxide dismutase was purified from human or bovine erythrocytes and assayed as previously described (*1*). Xanthine oxidase was chosen as the source of the superoxide radical since it has been well studied and documented and provides an easily controlled, reproducible flux of the radical (*1, 4*). The effects of exposing a solution of hyaluronate to superoxide are shown in Fig. 1, in terms of viscosity change with time. All operations were performed at room temperature. Relative viscosity was measured by recording the time (seconds) required for a given volume (0.6 ml) of the reaction mixture to drain by gravity from the barrel of a plastic 1-cm^3 syringe through a needle of appropriate size. The meniscus was timed as it passed between two calibration marks on the syringe barrel. The decrease in viscosity which resulted when hyaluronate was exposed to superoxide is apparent in Fig. 1. No change in viscosity occurred until xanthine oxidase was added and superoxide production began. Also apparent in Fig. 1 is the complete protection against decreases in viscosity provided by catalytic amounts of either SOD or catalase.

Because of the potential physiological significance of the free radical depolymerization of hyaluronic acid (discussed below), it was considered important to determine if exposure to superoxide led to similar decreased viscosity in whole synovial fluid. Earlier surveys of SOD activity in various mammalian tissues and extracellular fluids led to the conclusion that the enzyme was ubiquitously distributed among the tissues and organs, but that extracellular fluids contained negligible or no SOD. Careful assays of the bovine synovial fluid



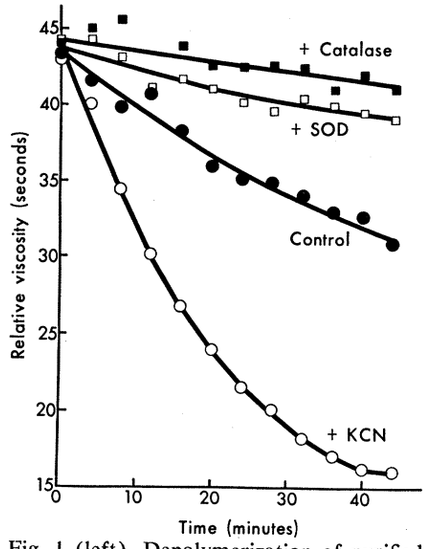


Fig. 1 (left). Depolymerization of purified hyaluronate solutions. Reaction mixtures contained 1.2 ml of 0.05*M* potassium phosphate buffer, *p*H 7.5, plus the following: sodium hyaluronate, 0.9 mg/ml; hypoxanthine, 0.67 m*M*; and xanthine oxidase (Sigma, grade I), 0.015 unit per milliliter. Data represented by solid circles were obtained in the presence of SOD (10 μg/ml) or catalase (Sigma, twice crystallized) (8.3 μg/ml). Superoxide was produced at less than 5 nmole min^{-1} ml^{-1}. Fig. 2 (right). Degradation of bovine synovial fluid. Each reaction mixture contained 1.3 ml of bovine synovial fluid, 0.1 ml of 10 m*M* hypoxanthine, 10 μl of 0.1*M* ethylenediaminetetraacetic acid (EDTA), and 0.018 unit of xanthine oxidase. Additional components as indicated were: catalase, 5 μg; SOD, 2 μg; and KCN, 1.0 μmole.

used in these studies (Pel-Freez, type 1, from steers) revealed a very low concentration of SOD (1 μg/ml) and a barely detectable concentration of catalase (0.05 μg/ml). These values are at least two or three orders of magnitude less than intracellular concentrations of the enzymes. The endogenous SOD in the synovial fluid was completely inhibited by 1 m*M* cyanide, indicating that the SOD is the copper-zinc enzyme found in cytoplasm rather than the manganoenzyme found in the mitochondrion (*5*). When the highly viscous synovial fluid was exposed to the flux of superoxide produced by xanthine oxidase, a marked decrease in viscosity resulted, as shown by the control line in Fig. 2. Two micrograms of SOD or 5 μg of catalase provided nearly complete inhibition of the degradative process. Conversely, when the endogenous SOD in the synovial fluid was inhibited by the addition of KCN, the synovial fluid was dramatically more susceptible to depolymerization by the radical, also shown in Fig. 2.

Deterioration of synovial fluid is a symptom which characterizes inflammatory types of arthritis. The condition of the synovial fluid is qualitatively assessed clinically by observing the fluid's ability to form a mucin clot when added to dilute acetic acid. Normal fluid forms a tight, ropy, white clot, whereas fluid from an inflamed joint may form a highly friable clot which breaks up on agitation. The effect of exposure to superoxide on the ability of synovial fluid to form a mucin clot is illustrated in Fig. 3. The fluids experienced identical exposures to superoxide. The one that formed the good clot was protected by endogenous SOD (1 μg/ml), while the other received no protection from SOD, as its endogenous SOD was inactivated by KCN. Cyanide alone had no effect on mucin clot formation. Since Fig. 2 indicates that the endogenous concentration of SOD is insufficient to provide complete protection of the synovial fluid, we suppose that the fluid in cuvette 1 of Fig. 3 was partly degraded, but not sufficiently depolymerized to cause the clot to disintegrate. Thus, poor mucin clot formation is indicative of extensively degraded synovial fluid.

The fact that the degradative process observed in these experiments was inhibited by either SOD or catalase indicates that neither the $O_2^-\cdot$ nor H_2O_2 alone could effect depolymerization of the polysaccharide, but must be present simultaneously for the process to occur.

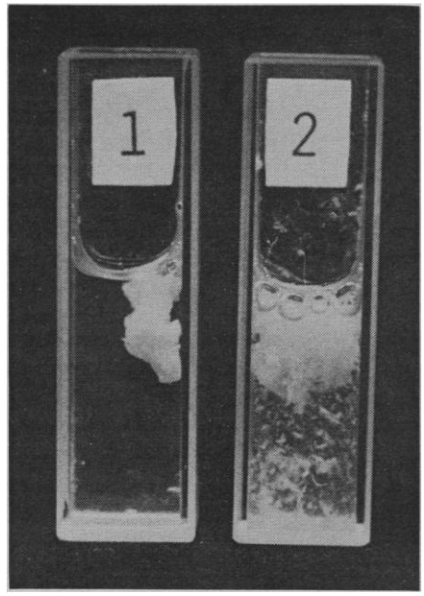


Fig. 3. Mucin clot formation. Reaction mixtures contained 1.5 ml of bovine synovial fluid, 0.1 ml of 10 m*M* hypoxanthine, 0.018 unit of xanthine oxidase, and 10 μl of 0.1*M* EDTA. Mixture 2 also contained 1 μmole of KCN. After incubation for 2 hours, each reaction mixture was added to a cuvette containing 1 ml of 7 percent acetic acid and shaken.

This implies that the actual depolymerizing species is generated secondarily by a reaction between $O_2^-\cdot$ and H_2O_2. The most likely candidate for this role is the reactive $OH\cdot$ generated by reaction 2. This reaction, proposed by Haber and Weiss (*6*), has been invoked by Beauchamp and Fridovich (*7*) to explain the production of ethylene from methional in the presence of superoxide. Additional support for $OH\cdot$ as the depolymerizing species in these experiments is given by the fact that 0.02*M* mannitol caused an almost complete preservation of the viscosity of a solution of hyaluronate under conditions as described in Fig. 1. Mannitol is considered to be an effective scavenger of $OH\cdot$ (*8*). The ability of $OH\cdot$ to depolymerize hyaluronic acid and other polysaccharides has been well studied by using pulse radiolysis as the source of the radical (*9*).

Intracellular concentrations of SOD and catalase are so high that the existence of physiological concentrations of $O_2^-\cdot$ and H_2O_2 sufficiently high to react and form $OH\cdot$ seems unlikely. In extracellular fluids, however, the feasibility of this reaction is apparent, provided there is a source of superoxide. Extracellular fluids other than synovial fluid were assayed for SOD and catalase with the following findings: human serum contained SOD (0.7 μg/ml) and catalase (0.8 μg/ml); human cerebrospinal fluid contained SOD (0.35 μg/ml) and no detectable catalase; bovine aqueous humor contained SOD (0.9 μg/ml) and no detectable catalase. The most likely physiological source of superoxide radical in the extracellular fluids now appears to be metabolically activated polymorphonuclear leukocytes (PMN). Babior *et al.* (*10*) found that phagocytizing PMN produce $O_2^-\cdot$ and release it into the surrounding medium. The observation has been confirmed in this laboratory. Evidence is mounting that the superoxide produced has a bactericidal role in the phagocytic vacuole (*11*), but its release into the surrounding medium is probably an unfortunate and potentially detrimental occurrence. A number of arthritic conditions are characterized by large influxes of PMN into the synovial fluid, and a strong correlation exists between decreased viscosity of the fluid or poor mucin clot formation and the number of PMN present in the inflamed joint (*12*). On the basis of our quantitative measurements of superoxide produced by activated PMN in vitro (approximately 1 nmole of $O_2^-\cdot$ per minute per 10^7 cells), the flux of superoxide produced in an inflamed arthritic joint could, at the upper limit, exceed by a factor of 3 the flux of $O_2^-\cdot$ produced under the experimental conditions described in Fig. 2. Thus, the quantity of superoxide which could be generated by phagocytes under physiological conditions is by no means trivial or insignificant. It seems entirely plausible that this mechanism could account for synovial fluid deterioration in inflammatory arthritic conditions. Release of lysosomal hyaluronidase by the invading PMN has not been a satisfactory explanation for the depolymerization of synovial fluid hyaluronate, since lysosomal hyaluronidase is totally inactive above *p*H 4.5 (*13*). Further, it seems unlikely that the damage caused by the free radicals would be limited to the depolymerization of hyaluronic acid, since $OH\cdot$ is one of the most potent oxidants known. It follows that injected SOD, to augment the low natural extracellular concentrations of the enzyme, might be expected to have an anti-inflammatory effect under certain conditions. This, in fact, appears to be the case (*14*).

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References and Notes

1. J. M. McCord and I. Fridovich, *J. Biol. Chem.* **244**, 6049 (1969).
2. J. M. McCord, B. B. Keele, Jr., I. Fridovich, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1024 (1971).
3. I. Danishefsky and A. Bella, Jr., *J. Biol. Chem.* **241**, 143 (1966).
4. I. Fridovich, *ibid.* **245**, 4053 (1970).
5. C. O. Beauchamp and I. Fridovich, *Biochim. Biophys. Acta* **317**, 50 (1973); R. A. Weisiger and I. Fridovich, *J. Biol. Chem.* **248**, 3582 (1973).
6. F. Haber and J. Weiss, *Proc. R. Soc. Lond. Ser. A Math. Phys. Sci.* **147**, 332 (1934).
7. C. O. Beauchamp and I. Fridovich, *J. Biol. Chem.* **245**, 4641 (1970).
8. J. M. McCord and I. Fridovich, *Photochem. Photobiol.* **17**, 115 (1973).
9. J. S. Moore, G. Phillips, J. Davies, K. Dodgson, *Carbohydr. Res.* **12**, 253 (1970).
10. B. Babior, R. Kipnes, J. Curnutte, *J. Clin. Invest.* **52**, 741 (1973).
11. I. Fridovich, *N. Engl. J. Med.* **290**, 624 (1974).
12. R. A. Jessar, in *Arthritis and Allied Conditions*, J. L. Hollander, Ed. (Lea & Febiger, Philadelphia, 1966), p. 76.
13. N. Aronson and E. A. Davidson, *J. Biol. Chem.* **242**, 441 (1967).
14. H. Marberger, W. Huber, G. Bartsch, T. Schulte, H. Swoboda, *Int. Urol. Nephrol.*, in press; S. Carson, E. Vogin, W. Huber, T. Schulte, *Toxicol. Appl. Pharmacol.* **26**, 184 (1973); D. S. Davies and D. L. Davies, *Fed. Proc.* **33**, 228 (1974); A. P. Autor, *Life Sci.* **14**, 1309 (1974).

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Chloral Hydrate: A Solvent for Biological Membranes

Abstract. *A buffer system containing chloral hydrate, taurine, and bromopyridinium lactate was used to dissolve several biological membranes and separate their protein components by polyacrylamide gel electrophoresis. This solvent system was capable of separating molecules of similar size on the basis of their charge and allows easy recovery of the proteins. Thus, aqueous chloral hydrate is an effective solvent for biological membranes.*

A major difficulty in studying biological membranes is the lack of solvent systems which permit complete disaggregation of membrane components and allow their recovery with biological activity. Present methods used to disaggregate the membranes involve sonication, detergents, and organic solvents (*1*), and they have not proved entirely satisfactory either because of incomplete dissolution or because when the components were totally dissolved it has been difficult to recover the dissolved components in a functional state. Buffer systems that contain sodium dodecyl sulfate (SDS) have been successfully used for completely dissolving membranes and subsequently separating the protein components by electrophoresis. However, in these systems complexing with SDS masks the net ionic charge of the native molecule, and separation on the basis of native ionic charge becomes difficult (*2*).

To study genetic variations in mammalian cell membranes we required a technique that can be used to dissolve membranes completely and also separate the components on the basis of their ionic charges because allelic variants of any kind would most often differ by amino acid substitutions and thus would not be separable on the basis of size. We here present a new method for the solubilization of membranes, which permits separation of the components on the basis of charge as well as size. In addition, in one complex system tested, biological activity of proteins was retained after the proteins were exposed to the solubilizing buffer.

Chloral hydrate (Mallinckrodt, U.S.P.) was recrystallized from chloroform, dried in a vacuum, and stored at −20°C. This step removes contaminating formic acid resulting from decomposition of chloral hydrate. Solubilizing buffer was prepared by dissolving 100 g of chloral hydrate in 33.3 ml of 0.6*M* taurine (2-aminoethanesulfonic acid), adding 0.58 ml of 3-bromopyridine (K & K Laboratories, Plainview, New York), and adjusting to *p*H 3.0 and a final volume of 100 ml by alternate addition of 85 percent lactic acid and water at room temperature. The membranes were dissolved in the solubilizing buffer as follows. A solution of 2-mercaptoethanol in water (40 percent by volume) was adjusted to *p*H 8.2 with 5*N* NaOH. Membranes were suspended at a concentration of 20 to 40 mg of protein per milliliter in dilute neutral buffer (or distilled water); seven parts of this suspension were mixed with one part of the mercaptoethanol solution (*p*H > 8), allowed to stand at room temperature for 5 to 10 minutes, and then dissolved in the solubilizing buffer to give a protein concentration of 1 mg/ml. The solid recrystallized chloral hydrate, 2.5 g per milliliter of the membrane-mercaptoethanol mixture, was added to give a final concentration of 1 g/ml. Neutral red (0.1 mg/ml) was added as a tracking dye, and 80 mg of dry Bio-Gel P6 (Bio-Rad, Richmond, California) was added per milliliter of solution to form a slurry.

Acrylamide gel buffer was prepared by the method described for the solubilizing buffer, except that the *p*H was adjusted to 2.8. Acrylamide (5 g) and 0.5 g of methylenebisacrylamide (Bio-Rad) were then dissolved in this buffer to a final volume of 100 ml. Gels were cast in nonsiliconized tubes (5 by 125 mm) with tight-fitting siliconized glass inserts to shape the gel (*3*). The tubes, sealed at the bottom with plastic holders (*3*), were flushed with nitrogen, the inserts were dropped to the bottom, and the tubes were again flushed with nitrogen and stoppered until ready for use.

The acrylamide was polymerized, with the use of a mixture of hydrogen peroxide and 1,1,3,3-tetramethyl-2-thiourea (Aldrich, Milwaukee, Wisconsin) (TMT) as catalyst. Because polymerization was very rapid under the conditions used, the gels were cast within 1 minute after the acrylamide solution was mixed with the catalysts. Oxygen was excluded from the system because it interferes with polymerization. For the preparation of five gels, 15 ml of the acrylamide solution in the gel buffer (*p*H 2.8) was gently degassed until bubbling began, and was then brought to atmospheric pressure with nitrogen. The process was repeated twice more, and then 6 ml was withdrawn into a 10-ml glass syringe that had been rinsed with the degassed acrylamide solution; 60 μl of 10 percent TMT in chloroform was added to the remaining 9 ml. A portion (6 ml) of the solution containing TMT was withdrawn into a second syringe which was rinsed as described above. Each solution was degassed by withdrawing the syringe plunger sharply while the syringe outlet was kept closed; the bubbles were then expelled. One-tenth milliliter of 6 percent hydrogen peroxide (freshly prepared) was added via a female-female Luer adapter to the first syringe; and the contents of the two syringes, now connected in the two syringes, were mixed by vigorous pumping back and forth. This mixture was quickly added to the gel tubes, which were then capped and kept at room temperature for 2½ hours to ensure complete polymerization of the gels. The inserts and any loose gel trapped by the inserts were then removed. At the time of electrophoresis, the gels were inverted and the sample was placed at the end originally con-