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Hydroxyl Radical Is Not a Product of the Reaction of Xanthine Oxidase and Xanthine

THE CONFOUNDING PROBLEM OF ADVENTITIOUS IRON BOUND TO XANTHINE OXIDASE*

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The reaction of xanthine and xanthine oxidase generates superoxide and hydrogen peroxide. In contrast to earlier works, recent spin trapping data (Kuppusamy, P., and Zweier, J. L. (1989) *J. Biol. Chem.* 264, 9880–9884) suggested that hydroxyl radical may also be a product of this reaction. Determining if hydroxyl radical results directly from the xanthine/xanthine oxidase reaction is important for 1) interpreting experimental data in which this reaction is used as a model of oxidant stress, and 2) understanding the pathogenesis of ischemia/reperfusion injury. Consequently, we evaluated the conditions required for hydroxyl radical generation during the oxidation of xanthine by xanthine oxidase. Following the addition of some, but not all, commercial preparations of xanthine oxidase to a mixture of xanthine, deferoxamine, and either 5,5-dimethyl-1-pyrroline-*N*-oxide or a combination of α-phenyl-*N*-tert-butyl-nitrone and dimethyl sulfoxide, hydroxyl radical-derived spin adducts were detected. With other preparations, no evidence of hydroxyl radical formation was noted. Xanthine oxidase preparations that generated hydroxyl radical had greater iron associated with them, suggesting that adventitious iron was a possible contributing factor. Consistent with this hypothesis, addition of H_2O_2, in the absence of xanthine, to "high iron" xanthine oxidase preparations generated hydroxyl radical. Substitution of a different iron chelator, diethylenetriaminepentaacetic acid for deferoxamine, or preincubation of high iron xanthine oxidase preparations with chelating resin, or overnight dialysis of the enzyme against deferoxamine decreased or eliminated hydroxyl radical generation without altering the rate of superoxide production. Therefore, hydroxyl radical does not appear to be a product of the oxidation of xanthine by xanthine oxidase. However, commercial xanthine oxidase preparations may contain adventitious iron bound to the enzyme, which can catalyze hydroxyl radical formation from hydrogen peroxide.

The discovery that the reaction of xanthine oxidase with xanthine leads to the formation of $^{\cdot}O_2^-$ [1] as well as H_2O_2 initiated interest in the role of these reactive oxygen intermediates in biology (1). In the intervening years the xanthine/xanthine oxidase reaction has provided a simple and reliable methodology for investigating the impact of controlled fluxes of $^{\cdot}O_2^-$ and H_2O_2 on biological systems (2–6). Furthermore, recent studies have suggested a role for this reaction in the pathogenesis of ischemia/reperfusion injury (7).

In addition to $^{\cdot}O_2^-$ and H_2O_2, Beauchamp and Fridovich (8) presented evidence that the reaction of xanthine and xanthine oxidase also yields hydroxyl radical as a secondary product. They postulated a direct reaction between the enzyme's two primary reaction products $^{\cdot}O_2^-$ and H_2O_2 according to the mechanism originally proposed by Haber and Weiss (9). However, subsequent work by Fridovich and colleagues, as well as other investigators (10–13), strongly suggested that a transition metal catalyst, such as iron, was required for the xanthine/xanthine oxidase reaction to generate hydroxyl radical. It seemed likely that hydroxyl radical generation reported in the earlier work (8) resulted from adventitious iron contaminants present in reaction buffers (10, 13).

More recently, however, the possibility that hydroxyl radical is a direct product of the xanthine/xanthine oxidase reaction was again proposed. Kuppusamy and Zweier (14) presented EPR spin trapping data, using DMPO, that implied that the oxidation of xanthine by xanthine oxidase could generate hydroxyl radical by the direct enzymatic one-electron reduction of H_2O_2. The EPR data presented in their report (14) differed from those obtained under similar experimental conditions by ourselves (13, 15, 16) and others (17, 18) which do not suggest that hydroxyl radical is generated by this reaction.

Given its critical importance to data interpretation from *in vitro* and *in vivo* experimental systems in which the xanthine/xanthine oxidase reaction is ongoing, we investigated the conditions required for this reaction to generate hydroxyl

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[1] The abbreviations used are: $^{\cdot}O_2^-$, superoxide actually the equilibrium mixture of $^{\cdot}O_2^-$ and HO_2; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide; DTPA, diethylenetriaminepentaacetic acid, often referred to as DETAPAC; ferrozine, 3-(2-pyridyl)-5-6-bis(4-phenyl-sulfonic acid)-1,2,4 triazine; Me_2SO, dimethyl sulfoxide; MNP, 2-methyl-2-nitrosopropane; SOD, Cu-Zn superoxide dismutase, PBN, α-phenyl-*N*-*tert*-butyl nitrone.

radical. In the present work we provide evidence that the xanthine/xanthine oxidase reaction does not lead to the generation of hydroxyl radical in the absence of exogenous iron. Nevertheless, we found that some commercial xanthine oxidase preparations contain high levels of adventitious iron tightly bound to the enzyme which catalyzes hydroxyl radical generation from H_2O_2.

MATERIALS AND METHODS

Reagents—Cu-Zn superoxide dismutase, catalase, deferoxamine mesylate, diethylenetriaminepentaacetic acid, xanthine, α-phenyl-*N*-tert-butyl nitrone, 3-(2-pyridyl)-5-6-bis(4-phenyl-sulfonic acid)-1,2,4 triazine (ferrozine), and H_2O_2 (30%) were obtained from Sigma. Ethanol and dimethyl sulfoxide were purchased from Aaper Alcohol and Chemical Company, Shelbyville, KY, and Fischer Scientific, respectively. Different lots and grades of buttermilk-derived xanthine oxidase were purchased from Sigma (grade 1 (lot 39F-3820, 128F-3870 and 119F37961), grade IV (lot 88F-3837), and grade IV (lot 59F-3897) and Boehringer-Mannheim (lot 11546524-90, 11640426-91, and 12251820-96). The activity of these xanthine oxidase preparations varied for each preparation (7.7–50 units/mg protein). However all experiments were performed by adding the necessary amount of each lot such that the reaction mixture contained 0.04 units/ml. DMPO was obtained from Sigma or synthesized according to the method of Bonnett *et al.* (19). *N*-*t*-butyl hydroxylamine was prepared by adding 10% NaOH to *N*-*t*-butyl hydroxylamine hydrochloride (Aldrich). The white precipitate of *N*-*t*-butyl hydroxylamine was extracted into ether. The solvent was dried with sodium sulfate and then evaporated to give *N*-*t*-butyl hydroxylamine.

In some experiments, xanthine oxidase was employed that had either been incubated overnight at 4 °C in the presence of Chelating Resin (Sigma) in phosphate-buffered saline, pH 7.40, or placed in 50,000-dalton membrane exclusion tubing and dialyzed overnight at 4 °C against 0.5 liter of 1 mM deferoxamine in phosphate-buffered saline, pH 7.40. Neither procedure altered the ability of xanthine oxidase to generate $^{\cdot}O_2^-$ as quantitated by the SOD-inhibitable reduction of ferricytochrome *c* (see below).

Spin Trapping—Desired reaction mixtures (0.5 ml) were prepared in glass tubes and transferred immediately to a quartz EPR flat cell, which was in turn inserted into the cavity of the EPR spectrometer, Varian E104A or E109 (Varian Associates, Palo Alto, CA). Unless otherwise noted, spectrometer settings were microwave power, 20 milliwatts; modulation amplitude, 1 G; time constant, 1 s; modulation frequency, 100 kHz; sweep rate, 12.5 G/min; and receiver gain, 3.2×10^4.

Superoxide Generation—The rate of superoxide generation was measured as the SOD-inhibitable reduction of ferricytochrome *c* (Sigma) as previously described (20).

Iron—Iron content of xanthine oxidase preparations was measured spectrophotometrically as formation of the Fe^{2+}-ferrozine complex (A_{560}) in the presence of 0.34 mM ascorbate according to the method of Carter (21). Results were compared with standard concentrations of ferrous ammonium sulfate incubated under the same conditions and expressed as nanomoles of iron per unit of xanthine oxidase.

Reactive Iron—Redox active iron in preparations of xanthine oxidase was determined by measuring the magnitude of the ascorbyl radical signal detected by EPR following the addition of iron-free EDTA (250 μM) and ascorbic acid (250 μM) to the xanthine oxidase solutions, 50 mM demetalled phosphate buffer, pH 7.40 ± 0.02 (22). The iron-free EDTA was allowed to incubate with the xanthine oxidase solutions for 2–3 h before the addition of ascorbic acid. The ascorbyl radical EPR signal intensity was determined immediately after the addition of ascorbic acid to the air-saturated solutions, using an EPR flat cell. Redox active iron concentrations were determined relative to standard solutions of Fe^{3+}-EDTA, 250 μM EDTA and varying amounts of iron.

RESULTS

Possible Formation of Hydroxyl Radical by Xanthine/Xanthine Oxidase—In preliminary work, using the experimental conditions of Kuppusamy and Zweier (14), we noted considerable variation among EPR spectra resulting from the reaction of xanthine oxidase (0.04 units/ml) and xanthine (0.4 mM) in the presence of deferoxamine (1.0 mM) and DMPO (50 mM). This variation was consistently related to the particular preparation of xanthine oxidase employed. Therefore, we examined eight different lots of xanthine oxidase obtained from two different commercial suppliers. Most xanthine oxidase preparations yielded EPR spectra comprised of both DMPO/$^{\cdot}$OOH (A_N = 14.3 G, A_H = 11.7 G; A_H^{γ} = 1.25 G) and DMPO/$^{\cdot}$OH ($A_N = A_H$ = 14.95 G). These spectra were dominated by the DMPO/$^{\cdot}$OOH spin adduct, whose peak amplitude initially increased and then plateaued or decreased over time (Fig. 1, *A* and *B*). However, with one xanthine oxidase preparation (39F-3820) the initial EPR spectra, which contained both DMPO/$^{\cdot}$OOH and DMPO/$^{\cdot}$OH signals, rapidly evolved to a spectrum characteristic of only DMPO/$^{\cdot}$OH (Fig. 2, *A* and *B*).

With most xanthine oxidase preparations, DMPO/$^{\cdot}$OOH and DMPO/$^{\cdot}$OH signals were inhibited 100 and 75–100%, respectively, by the inclusion of SOD (30 units/ml) in the system (Fig. 1*C*). Addition of catalase (500 units/ml), however, had no effect (Fig. 1*D*). In the cases in which SOD failed to totally eliminate DMPO/$^{\cdot}$OH, complete eradication could be obtained if both SOD and catalase were included in the reaction mixture (not shown).

The effects of SOD and catalase were considerably different when added to the xanthine/39F-3820 xanthine oxidase system. SOD eliminated detection of the DMPO/$^{\cdot}$OOH signal observed early, but had a negligible effect on the DMPO/$^{\cdot}$OH spectrum that evolved later (Fig. 2*C*). In contrast, catalase markedly decreased the intensity of this DMPO/$^{\cdot}$OH signal (Fig. 2*D*).

Additional Evidence for Generation of Hydroxyl Radical—These data were consistent with the possibility that the reaction of xanthine oxidase and xanthine did on occasion lead to generation and subsequent spin trapping of a "hydroxyl radical-like" oxidant. To further confirm that this species was indeed hydroxyl radical, additional experiments were performed. First, we examined the effect of the inclusion

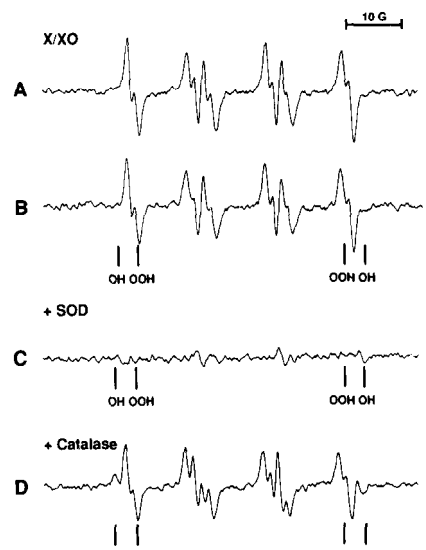

FIG. 1. *A* and *B* are sequential EPR spectra (8-min scans) obtained following the addition of low iron xanthine oxidase (*XO*) (0.04 units/ml) to xanthine (*X*) (0.4 mM), DMPO (50 mM), and deferoxamine (1 mM). EPR spectra shown in *C* and *D* were collected under the same conditions and are comparable in time sequence to *B* except that the reaction mixtures also contained SOD (30 units/ml) and catalase (500 units/ml), respectively. Results are representative of the 8–10 separate experiments using two different low iron commercial preparations of xanthine oxidase. Locations of high and low field peaks corresponding to DMPO/$^{\cdot}$OH and DMPO/$^{\cdot}$OOH are designated *OH* and *OOH*, respectively.

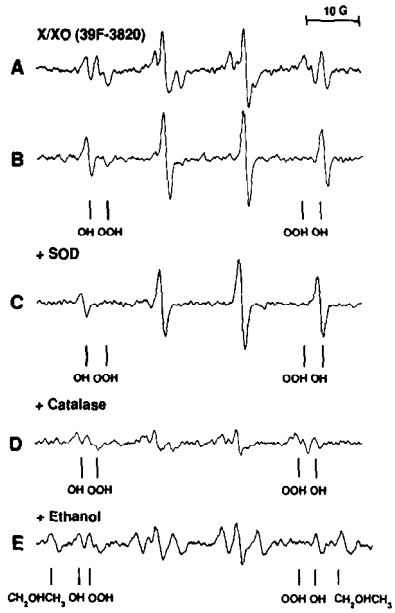

FIG. 2. *A* and *B* are sequential EPR spectra (8-min scans) obtained under the same conditions as in Fig. 1, *A* and *B*, except that a different xanthine oxidase (*XO*) preparation (39F-3820), high iron, was employed. EPR spectra shown in *C–E* were performed using the same reagents and are comparable in timing to *B* except that the reaction mixtures also contained SOD (30 units/ml), catalase (500 units/ml), or ethanol (0.14 M). Results are representative of four to six experiments. Locations of high and low field peaks corresponding to DMPO/$^{\cdot}$OH, DMPO/$^{\cdot}$OOH, and DMPO/$^{\cdot}CH_2OHCH_3$ are designated *OH*, *OOH*, and CH_2OHCH_3, respectively. *X*, xanthine.

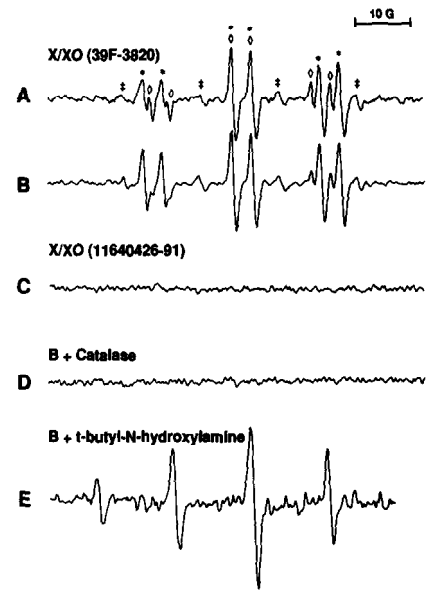

FIG. 3. *A* and *B*, sequential 8-min EPR spectrum obtained beginning 8 min after the addition of 0.04 units/ml xanthine oxidase (*XO*) (39F-3820), high iron, to xanthine (*X*) (0.4 mM), PBN (10 mM), Me_2SO (0.14 M), and deferoxamine (1 mM). *C*, EPR spectrum obtained under the identical conditions to that of *B* except the xanthine oxidase preparation used in Fig. 1 was employed. EPR spectra in *D* resulted from the conditions of *B* except catalase (500 units/ml) was included in the reaction mixtures. EPR spectrum shown in *E* was obtained 6 min after the addition of the xanthine oxidase used in *A* and *B* to xanthine, deferoxamine, and *t*-butyl-*N*-hydroxylamine (90 mM). Sweep rate was 12.5 G/min and receiver gain was 3.2×10^4 (*A–D*) or 1.25×10^5 (*E*). Locations of peaks corresponding to PBN/$^{\cdot}OCH_3$ (◇), PBN/$^{\cdot}CH_3$ (*), and MNP/$^{\cdot}$H (‡) are shown.

of ethanol or Me_2SO in the reaction mixture. Hydroxyl radical reacts with ethanol and Me_2SO to yield α-hydroxyethyl and methyl radical, respectively (23). The presence of these compounds in a DMPO spin trapping system should result in the generation of the corresponding DMPO spin adduct at the expense of DMPO/$^{\cdot}$OH. As shown in Fig. 2*E*, inclusion of ethanol or Me_2SO with a mixture of xanthine, DMPO, and deferoxamine to which was added the 39F-3820 xanthine oxidase preparation yielded DMPO/$^{\cdot}CH_2OHCH_3$ (A_N = 16.0 G, A_H = 22.8 G) or DMPO/$^{\cdot}CH_3$ (A_N = 16.5 G, A_H = 23.3 G), respectively.

As further confirmation that these reaction mixtures were generating hydroxyl radical, we used a recently developed spin trapping system for hydroxyl radical that is composed of Me_2SO and PBN (24). In this spin trapping system, when hydroxyl radical is generated under oxygen replete conditions, a stable spin adduct consistent with PBN/$^{\cdot}OCH_3$ is detected (24). When the oxygen availability becomes limited, PBN/$^{\cdot}CH_3$ is generated. In contrast to hydroxyl radical-derived DMPO spin adducts, PBN/$^{\cdot}OCH_3$ is resistant to $^{\cdot}O_2^-$-mediated destruction (24).

Therefore, the 39F-3820 xanthine oxidase preparation was added to a mixture of xanthine, deferoxamine, Me_2SO (140 mM) and PBN (10 mM). The resulting EPR spectrum (Fig. 3, *A* and *B*) was composed of three different nitroxides, none of which were detected if PBN or Me_2SO was omitted from the system. These species were decreased or non-detectable when other xanthine oxidase preparations were employed (Fig. 3*C*). Formation of all three species was inhibited by catalase (Fig. 3*D*). The two dominant nitroxide species observed had hyperfine splitting constants consistent with PBN/$^{\cdot}OCH_3$ (A_N = 15.08 G, A_H = 3.52 G), and PBN/$^{\cdot}CH_3$ (A_N = 16.5 G, A_H = 3.62 G). Initial spectra showed similar magnitudes of PBN/$^{\cdot}OCH_3$ and PBN/$^{\cdot}CH_3$. But, over time PBN/$^{\cdot}CH_3$ came to dominate, likely reflecting depletion of O_2 in the buffer by the ongoing xanthine/xanthine oxidase reaction, and $^{\cdot}$OH generation under oxygen-limited conditions (24).

The splitting constants of the third nitroxide species (A_N = A_H = 14.4 G) detected were similar to those previously reported (25) for the one-electron reduction of the spin trap 2-methyl-2-nitrosopropane, MNP/$^{\cdot}$H. This suggested the possibility that PBN was hydrolyzed in the above reaction to yield *t*-butyl-*N*-hydroxylamine, which was subsequently oxidized to give the corresponding nitroxide, MNP/$^{\cdot}$H. This possibility was examined. Experiments were repeated using the same conditions as in Fig. 3*A* except that 90 mM *t*-butyl-*N*-hydroxylamine was substituted for PBN and Me_2SO was omitted. The EPR spectrum obtained (a single species with A_N = A_H = 14.4 G) corresponded to the same nitroxide observed with PBN/Me_2SO (Fig. 3*E*) confirming the ability of the reaction mixture to oxidize *t*-butyl-*N*-hydroxylamine to MNP/$^{\cdot}$H. This spin adduct decayed rapidly in the presence of a flux of superoxide.

Iron Content of Commercial Xanthine Oxidase Preparations—Commerical xanthine oxidase preparations contain iron beyond that expected from the enzyme's iron-sulfur centers, and the enzyme has been reported to possess one high affinity binding site for exogenous iron (26). Therefore, it seemed possible that the variable hydroxyl radical generation, which we observed among xanthine oxidase preparations, could be related to differences in adventitious iron associated with the protein. Accordingly, we measured both total iron and redox active iron of some of the different xanthine oxidase preparations employed (Table I). Both techniques revealed a considerable variation in the amount of iron found in the different lots of xanthine oxidase. However, a direct correlation between the magnitude of hydroxyl radical generation detected by spin trapping, using each xanthine

TABLE I
Iron content of commercial xanthine oxidase preparations

Xanthine oxidase lot no.	[Iron] in nanomoles/unit xanthine oxidase	
	Total iron[a]	Redox iron[b]
39F-3820	54.8	37.0[c]
39F-3820 after chelation[d]	41.2	13.0
39F-3820 after dialysis[e]	5.5	0.4
59F-3897	123.7	—[f]
119F-37961	12.7	0.2
11640426-91	23.5	0.4

[a] Median value (n = 3–7) of total iron by ferrozine method.
[b] Median value (n = 4) of redox active iron from ascorbate-EPR method.
[c] 37 nmol/unit of xanthine oxidase would add adventitious redox active iron equivalent to 1.5 μM Fe-EDTA when using 0.04 units/ml as done in the spin trapping experiments reported here.
[d] Overnight treatment with chelating resin.
[e] Dialysis against 1 mM deferoxamine overnight.
[f] No sample available.

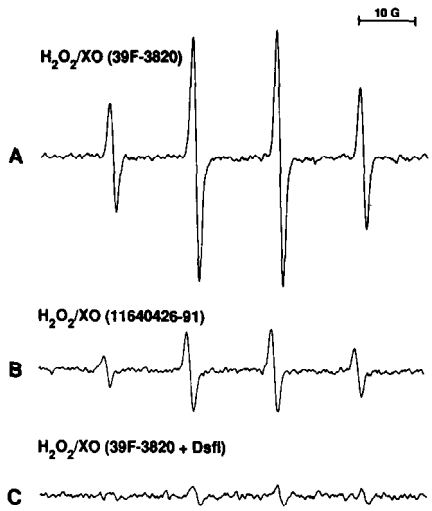

FIG. 4. **EPR spectra obtained following the addition of H_2O_2 (0.9 mM) to a mixture of xanthine oxidase (*XO*) (0.04 units/ml), deferoxamine (1 mM), and DMPO (50 mM).** In *spectrum A*, the high iron xanthine oxidase (39F-3820) preparation was present, whereas in *B* a low iron preparation (11546524-90) was substituted. *Spectrum C* was obtained under the same conditions as *A* except the high iron xanthine oxidase (39F-3820) had been incubated with deferoxamine (*Dsfl*) for 1 h prior to the addition of DMPO and H_2O_2. Results are representative of three to five experiments. The spectrum in each scan is that of DMPO/$^{\cdot}$OH.

oxidase preparation, and the amount of iron associated with the enzyme preparation was found. That is, those preparations with the greater amount of iron associated with them yielded the greater magnitude of hydroxyl radical-derived spin adducts. Preparations having high total iron had corresponding high redox active iron with the same pattern observed for low total and redox active iron preparations. Thus, the correlation between ability to generate hydroxyl radical and the enzyme's iron content was present regardless of whether total or redox active iron was the parameter used.

Formation of Hydroxyl Radical by the Reaction of Hydrogen Peroxide and Xanthine Oxidase—Since our data suggested the possibility that some xanthine oxidase preparations were capable of functioning as catalytic iron chelates, H_2O_2 was added to the high iron xanthine oxidase (39F-3820) in the presence of DMPO but in the absence of xanthine. The resulting EPR spectrum revealed large hydroxyl radical-derived spin adducts (Fig. 4*A*). Substitution of xanthine oxidase preparations with low levels of adventitious iron yielded little or no hydroxyl radical (Fig. 4*B*). Similar results were obtained with the PBN/Me_2SO spin trapping system (not shown).

Xanthine Oxidase Associated Iron Responsible for Hydroxyl Radical Generation Is Not an Integral Component of the Enzyme—The variable iron content of different xanthine oxidase preparations and the well recognized problem of adventitious iron that can contaminate chemical systems (27) strongly suggested that iron contamination of the xanthine oxidase preparations was responsible for our results. Subsequently, several lines of evidence in support of this hypothesis were obtained. Relative to experimental systems containing deferoxamine (Fig. 5, *A* and *B*) or no chelator (not shown) substitution of the iron chelator DTPA for deferoxamine, resulted in a considerable decrease in the magnitude of hydroxyl radical-derived spin adducts detected in xanthine/xanthine oxidase or H_2O_2/xanthine oxidase systems (Fig. 5, *C* and *D*). Preincubation of xanthine oxidase preparations for up to 1 h with either deferoxamine or DTPA further decreased, but did not eliminate, hydroxyl radical-derived spin adducts resulting from the reaction of xanthine oxidase with H_2O_2 (Fig. 4*C*). Similarly, prior overnight incubation of the xanthine oxidase suspension with chelating resin beads produced an approximately 30% decrease in the magnitude of hydroxyl radical-derived spin adducts detected following its reaction with xanthine or H_2O_2 (not shown). Most noteworthy, however, was that dialysis of 39F-3820 xanthine oxidase overnight against 1 mM deferoxamine eliminated the generation of hydroxyl radical whether the enzyme was added to xanthine or H_2O_2 (Figs. 6 and 7). None of the above procedures affected the enzyme's capacity to generate $^{\cdot}O_2^-$ as measured by SOD-inhibitable ferricytochrome *c* reduction (not shown).

DISCUSSION

The reaction of xanthine oxidase and xanthine has long been recognized to generate $^{\cdot}O_2^-$ and H_2O_2 (1), prompting routine use of this reaction as a means of studying the effects of these oxidant species on biologic systems (2–6). More

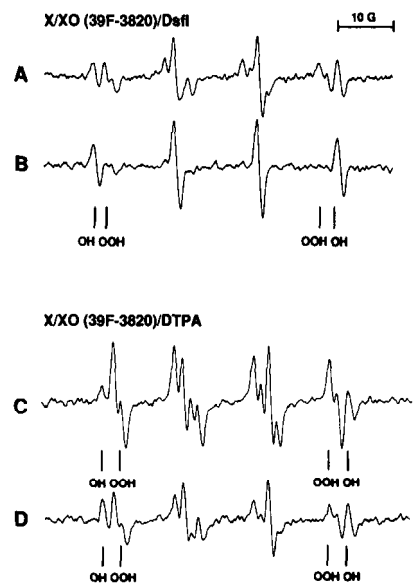

FIG. 5. EPR *spectra A* and *B* are sequential scans obtained following the addition of high iron xanthine oxidase (*XO*), 39F-3820, (0.04 units) to a solution containing xanthine (*X*) (0.4 mM), deferoxamine (*Dsfl*) (1 mM), and DMPO (50 mM), as in Fig. 2, *A* and *B*. Conditions under which EPR *spectra C* and *D* were obtained are identical to *A* and *B* except that DTPA (1 mM) was substituted for deferoxamine. Results are representative of three experiments. Location of high and low field peaks corresponding to DMPO/$^{\cdot}$OH and DMPO/$^{\cdot}$OOH are designated OH and OOH, respectively.

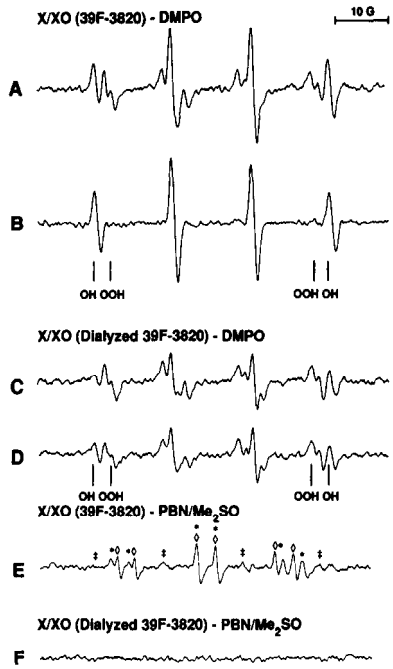

FIG. 6. EPR *spectra A* and *B* are sequential scans obtained following the addition of high iron (39F-3820) xanthine oxidase (*XO*) (0.04 ml) to xanthine (*X*) (0.4 mM), deferoxamine (1 mM), and DMPO (50 mM) as in Figs. 2, *A* and *B* and 5, *A* and *B*. *Spectra C* and *D* were obtained under the same conditions as *A* and *B* except the high iron (39F-3820) xanthine oxidase preparation had been dialyzed overnight against 0.5 liters of 1 mM deferoxamine. *Scans E* and *F* were obtained under the conditions of *B* and *D* except that PBN (10 mM) and Me_2SO (0.14 M) were present instead of DMPO. Results are representative of three experiments. For DMPO experiments (*A–D*) location of high and low field peaks corresponding to DMPO/$^{\cdot}$OH and DMPO/$^{\cdot}$OOH are designated *OH* and *OOH*, respectively. For PBN/Me_2SO experiments (*E* and *F*), the peaks corresponding to PBN/$^{\cdot}OCH_3$ (◇), PBN/$^{\cdot}CH_3$ (*), and MNP/$^{\cdot}$H (‡) are shown.

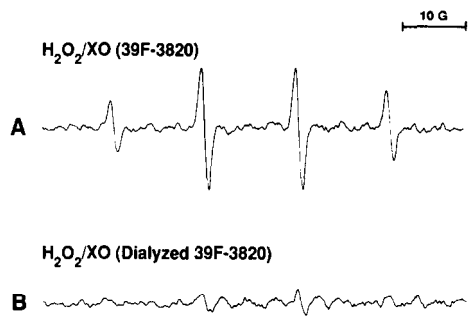

FIG. 7. **EPR spectra obtained following the addition of H_2O_2 (0.9 mM) to a mixture of deferoxamine 1 mM, DMPO (50 mM) and high iron (39F-3820) xanthine oxidase (*XO*) (0.04 units) (*A*), or high iron (39F-3820) xanthine oxidase (0.04 units) (*B*) which had been previously dialyzed overnight against pH 7.4 PBS containing 1 mM deferoxamine (0.5 liter).** Note there is no xanthine oxidase substrate in this system. Results were respresentative of three experiments and are the spectrum of DMPO/$^{\cdot}$OH.

recently, evidence has been presented that this reaction may play an important role in tissue injury resulting from ischemia/reperfusion (7). Some studies (28–30), however, have suggested that hydroxyl radical rather than $^{\cdot}O_2^-$ or H_2O_2 is the primary mediator of injury in such systems. Thus, an important issue in understanding the mechanism whereby xanthine oxidase induces cell injury is whether hydroxyl radical is a direct product of the oxidation of xanthine by this enzyme or a secondary product resulting from the iron-catalyzed Fenton reaction.

Consistent with the recent report of Kuppusamy and Zweier (14) we also found evidence, using two different spin trapping systems, for the formation of hydroxyl radical during the reaction of xanthine and *some* commercial preparations of xanthine oxidase, even in the presence of deferoxamine which was added to complex "free," *i.e.* adventitious, iron. In both of the spin trapping systems employed, the formation of hydroxyl radical-derived spin adducts were inhibited by catalase suggesting that hydroxyl radical arose as a consequence of the Fenton reaction.

Although our observations were consistent with those of Kuppusamy and Zweier (14), the magnitude of hydroxyl radical formation detected by spin trapping was highly variable, and, in fact, hydroxyl radical was not detectable at all with certain preparations of xanthine oxidase. All experiments were performed under conditions in which the rates of $^{\cdot}O_2^-$ generation were similar, suggesting that the reaction mixtures varied because of the presence of catalytic transition metals which are necessary to catalyze formation of hydroxyl radial from $^{\cdot}O_2^-$ and H_2O_2. Consistent with this hypothesis, we found that the amount of iron associated with commercial preparations of xanthine oxidase varied considerably. A direct correlation existed between both total iron and redox active iron associated with the xanthine oxidase preparation and the magnitude of hydroxyl radical-derived spin adducts detected with either spin trapping system employed. Furthermore, we were able to spin trap hydroxyl radical by the simple addition of H_2O_2 to "high iron" xanthine oxidase.

These results strongly suggested that hydroxyl radical detected as a result of the reaction of xanthine oxidase and xanthine was due to the presence of adventitious catalytic iron in some xanthine oxidase preparations. In support of this hypothesis, substitution of DTPA for deferoxamine, or preincubation of the xanthine oxidase with chelating resin, or overnight dialysis of the enzyme against deferoxamine all decreased or eliminated hydroxyl radical generation without affecting the ability of the enzyme to generate $^{\cdot}O_2^-$. Vile and Winterbourn (26) have preiously reported that xanthine oxidase contains one high affinity iron binding site/monomer having a K_a of 5×10^{12} M^{-1}. The failure of deferoxamine to prevent hydroxyl radical formation when added to the reaction mixture just prior to addition of xanthine oxidase, as observed by both Kuppusamy and Zweier (14) and ourselves, was likely due to insufficient time for the chelator to remove adventitious iron bound to the enzyme. Buettner (22) has observed that at neutral pH chelation of iron by deferoxamine is a relatively slow process.

In the PBN/Me_2SO spin trapping system those xanthine oxidase preparations that generated hydroxyl radical-derived spin adducts also produced an additional nitroxide species. This species (A_N = A_H = 14.4 G) has splitting constants identical to those reported for MNP/$^{\cdot}$H (25), suggesting that under the experimental conditions employed, PBN was hydrolyzed to yield *t*-butyl-*N*-hydroxylamine, which was then in turn oxidized to MNP/$^{\cdot}$H. Consistent with this hypothesis, substitution of *t*-butyl-*N*-hydroxylamine for PBN in these reaction mixtures yielded the same nitroxide (MNP/$^{\cdot}$H). The oxidation of an *N*-hydroxylamine to the corresponding nitroxide by superoxide or hydrogen peroxide in the presence of transition metals is known (31) and is the method of choice for preparing stable nitroxides (32).

Consistent with earlier studies (10–13, 15–18), our data suggest that hydroxyl radical is neither a direct product of the reaction of xanthine oxidase and xanthine nor is it due to the direct one-electron reduction of H_2O_2 by xanthine oxidase. However, contamination of commercial preparations of xan-

thine oxidase with high levels of adventitious iron will result in generation of variable amounts of hydroxyl radical. Although the detection of hydroxyl radical following the reaction of various commercial sources of xanthine oxidase with H_2O_2 suggests the iron is present in its reduced state (Fe^{2+}), there remains open the possibility that hydroxyl radical was generated via the reaction of H_2O_2 with a ferric iron complex such as those described by others (33–37). Investigators using the xanthine/xanthine oxidase reaction to study the impact of $^{\cdot}O_2^-$ and/or H_2O_2 on biologic systems need to be aware that hydroxyl radical generation may also be occurring simultaneously and that this hydroxyl radical generation is not necessarily prevented by the simple inclusion of iron chelators. Whether xanthine oxidase located at intracellular sites has catalytic iron bound to it is unknown but could have important ramifications for understanding the mechanism(s) of ischemia/reperfusion injury.

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