Production of the carbonate radical anion during xanthine oxidase turnover in the presence of bicarbonate.

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Running title: CO$_3$\textsuperscript{-} production from xanthine oxidase turnover

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ABSTRACT

Xanthine oxidase is generally recognized as a key enzyme in purine catabolism but its structural complexity, low substrate specificity and specialized tissue distribution suggest other functions that remain to be fully identified. The potential of xanthine oxidase to generate superoxide radical anion, hydrogen peroxide and peroxynitrite has been extensively explored in pathophysiological contexts. Here we demonstrate that xanthine oxidase turnover at physiological pH produces a strong one-electron oxidant, the carbonate radical anion. The radical was shown to be produced from acetaldehyde oxidation by xanthine oxidase in the presence of catalase and bicarbonate on the basis of several lines of evidence such as oxidation of both dihydrorhodamine 1, 2, 3 (DHR) and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and chemiluminescence and isotope labeling/mass spectrometry studies. In the case of xanthine oxidase acting upon xanthine and hypoxanthine as substrates, carbonate radical anion production was also evidenced by the oxidation of DMPO and of DHR in the presence of uricase. The results indicated that Fenton chemistry occurring in the bulk solution is not necessary for carbonate radical anion production. Under the conditions employed, the radical was likely to be produced at the enzyme active site by reduction of a peroxymonocarbonate intermediate whose formation and reduction is facilitated by the many xanthine oxidase redox centers. In addition to indicating that the carbonate radical anion may be an important mediator of the pathophysiological effects of xanthine oxidase, the results emphasize the potential of the bicarbonate-carbon dioxide pair as a source of biological oxidants.

Key words: carbonate radical anion, xanthine oxidase, oxidative stress, bicarbonate-carbon dioxide pair, free radicals, peroxymonocarbonate.
INTRODUCTION

Xanthine oxidoreductase is a complex enzyme comprising two identical subunits of Mr 145,000 each containing one molybdenum, one FAD and two nonidentical iron sulfur centers (reviewed in 1-3). Largely because of its easy isolation from cow milk, xanthine oxidoreductase has been used as a model protein for structural and mechanistic studies of redox enzymes for over 60 years. Although xanthine oxidoreductase has broad specificity for both oxidizing and reducing substrates, its conventionally accepted role is in purine metabolism where it sequentially catalyzes the oxidation of hypoxanthine to xanthine and of xanthine to uric acid. In mammals, the enzyme exists in two interconvertible forms xanthine dehydrogenase (EC 1.1.1.204) which predominates \textit{in vivo}, and xanthine oxidase (XO; EC 1.1.3.22) which is the form usually isolated from biological sources. The enzyme is widely distributed throughout different mammalian tissues but particularly high activities have been found in the liver and intestine (reviewed in 1-3).

Both, xanthine dehydrogenase and XO can reduce molecular oxygen with production of superoxide radical anion and hydrogen peroxide at proportions that depend on the substrate and oxidation conditions (REACTIONS 1-3) (4-8).

\[
\begin{align*}
S + H_2O \xrightarrow{XO} S_{ox} + 2e^- + 2H^+ & \quad \text{(Reaction 1)} \\
2O_2 + 2e^- \xrightarrow{XO} 2O_2^- & \quad \text{(Reaction 2)} \\
O_2 + 2e^- + 2H^+ \xrightarrow{XO} 2H_2O_2 & \quad \text{(Reaction 3)}
\end{align*}
\]

Thus, the enzyme has been extensively used as a source of the so-called reactive oxygen species, particularly as a source of the superoxide radical anion. The potential of the enzyme to generate superoxide radical anion and hydrogen peroxide has led to widespread interest in its roles in numerous pathological states, including ischemia-reperfusion,
hypoxia-reoxygenation, multiple organ dysfunction, preservation-transplantation and vascular disease (3, 9, 10). Possible roles of XO in pathological processes have been reinforced by the recent demonstration that it may also generate reactive nitrogen species such as peroxynitrite that is a potent biological oxidant. Indeed, XO has been shown to be able to use both NADH and xanthine to reduce nitrite to nitric oxide (11-15), and the concomitant production of the superoxide radical anion, that reacts with nitric oxide in a diffusion-controlled process, results in peroxynitrite formation (16).

Relevantly, XO has been proposed by Hodgson and Fridovich (17) to be able to generate yet another strong one-electron oxidant, the carbonate radical anion. Formation of this radical through Fenton chemistry was proposed to explain the low-level chemiluminescence detected in incubations of acetaldehyde, XO and bicarbonate at pH 10.2 (Reactions 1-9) (17, 18).

\[
\begin{align*}
O_2^{-} + 2 H_2O_2 \xrightarrow{M^{+n} \text{catalysis}} & \cdot OH + \cdot OH + O_2 \quad \text{(Reaction 4)} \\
CO_2 + H_2O & \leftrightarrow H_2CO_3 \rightarrow HCO_3^- + H^+ \quad \text{pKa} = 6.37 \quad \text{(Reaction 5)} \\
HCO_3^- + H_2O & \leftrightarrow CO_3^{2-} + H^+ \quad \text{pKa} = 10.32 \quad \text{(Reaction 6)} \\
\cdot OH + HCO_3^- & \rightarrow H_2O + CO_3^{*-} \quad k = 8.5 \times 10^6 \text{M}^{-1}\text{s}^{-1} \quad \text{(Reaction 7)} \\
\cdot OH + CO_3^{2-} & \rightarrow OH^- + CO_3^{*-} \quad k = 3 \times 10^8 \text{M}^{-1}\text{s}^{-1} \quad \text{(Reaction 8)} \\
2 CO_3^{*-} & \rightarrow h\nu + \text{Products} \quad \text{(Reaction 9)}
\end{align*}
\]

There has been a renewed interest in the pathophysiological roles of the carbonate radical anion since its direct EPR detection from flow mixtures of peroxynitrite with the bicarbonate-carbon dioxide pair in aqueous solutions at physiological pH (19). In addition
of being considered an important mediator of peroxynitrite effects \textit{in vivo} (reviewed in \textit{20, 21}), the carbonate radical anion has been proposed as the diffusible oxidant produced during the peroxidase activity of the enzyme Cu,Zn-superoxide dismutase (22-27) whose relationship to the familial amyothophic lateral sclerosis disease remains under scrutiny.

Recently, we proposed that XO turnover at neutral pH could be another route for the biological formation of the carbonate radical anion (Fig. 1) (21). This proposition was based on the fact that the electrons coming from the oxidizing substrate flow through the many redox centers of XO, opening the possibility for production/escape of intermediates that are partially reduced/protonated such as the superoxide radical anion and the deprotonated peroxide (8). The latter could react with surrounding carbon dioxide to produce peroxymonocarbonate that either leaves the active site and acts as a two-electron oxidant (26, 28, 29), or is reduced at the active site to the carbonate radical anion (Fig. 1) (21). Here, we tested this proposal by using several methodologies to examine XO-mediated oxidation of acetaldehyde, hypoxanthine and xanthine in the absence and presence of bicarbonate and catalase at neutral pH.
MATERIALS AND METHODS

Chemicals - XO (from bovine milk, grade III) was purchased from Sigma Chemical Co. (St. Louis, MO) or Calbiochem (Darmstad, Germany). Both samples produced similar results and XO from Sigma was used in most of the reported experiments. DHR was purchased from Molecular Probes, Inc. (Eugene, OR). Isotope labeled water (H$_2^{18}$O; 96.9%) was purchased from Isotec (Miamisburg, OH). All other reagents were purchased from Sigma, Merck or Fluka and were analytical grade or better. Peroxynitrite was synthesized and quantified as previously described (19). All solutions and buffers were prepared with distilled water purified with a Millipore Milli-Q system. All buffers were treated with Chellex-100 to remove trace amounts of metal ion contaminants prior to use. Concentrations of carbon dioxide were calculated from the added bicarbonate concentrations by using pKa 6.4 (19).

XO activity - XO activity was monitored spectrophotometrically at 295 nm immediately before each set of experiments through its ability to oxidize xanthine to uric acid ($\varepsilon = 9,600$ M$^{-1}$s$^{-1}$) (30). In some experiments, XO activity was also monitored by superoxide radical anion production that was followed by cytochrome c Fe (III) (50 $\mu$M) reduction at 550 nm ($\Delta \varepsilon = 21,000$ M$^{-1}$cm$^{-1}$) (31).

DHR oxidation - DHR was dissolved in acetonitrile to a 10 mM final concentration and stored under argon protected from light. DHR (80-100 $\mu$M) was incubated with the various systems and its oxidation monitored either spectrophotometrically at 500 nm ($\varepsilon = 78,800$ M$^{-1}$s$^{-1}$) or fluorimetrically (32). In the latter case, a TECAN Spectrafluor Plus fluorimeter was employed and the wavelengths were selected through the use of optical filters as 492 and 535 nm for excitation and emission, respectively.
Chemiluminescence studies - Light emission was measured with a photocounting device consisting of a red-sensitive photomultiplier tube (9203BM Thor EMI Electron Tubes, UK) cooled to -20 °C with a thermoelectric cooler (FACT 50 MKIII, EMI Gencom, Plainview, NY) as described elsewhere (33, 34). The potential applied to the photomultiplier was -1,200 V. The phototube output was connected to an amplifier discriminator (model 1121, Princeton Instruments, NJ) which transmitted the signal to a computer. Light emissions at specified wavelength intervals were obtained with cut-off filters (Melles Griot Inc., Carlsbad, CA) placed between the cuvette and the photomultiplier. All sample components were mixed and poured into a mirrored walls glass cuvette (35 mm x 7 mm x 55 mm) maintained at 25 °C. Concentrated peroxynitrite or acetaldehyde stock solutions were then injected into the cuvette until they reached the concentrations specified in the figure legend. The infusion was made with a Harvar apparatus pump 22 and the mixture kept under continuous stirring. The chemiluminescence signal was monitored during the injection and after mixing of the reagents.

EPR spin trapping studies - EPR spectra were recorded at room temperature (25 ± 2 °C) on a Bruker EMX spectrometer equipped with an ER4122 SHQ 9807 high sensitivity cavity. The incubation mixtures were transferred to flat cells and the spectra typically recorded 1 min after substrate addition.

HPLC-mass spectrometry studies – The enzymatic incubations were maintained for 4 h at room temperature, transferred to a refrigerator and analyzed within 24 h. For analysis they were injected into a Shimadzu HPLC system (Shimadzu, Tokyo, Japan) connected to a Quatro II mass spectrometer (Micromass, Manchester, U. K.). The HPLC system was composed of two LC-10ADVp pumps connected to a SPD-10AVVP UV-visible detector
controlled by a communication bus module SLC-10ADV-CBM 10 A. HPLC separations were performed with a Phenomenex C-18 reverse phase column (50 x 1.0 mm, 3 μm particle size) (Phenomenex, Torrance, CA) equipped with a C-18 reverse phase guard column (20 x 2.1 mm, 5 μm, particle size) (Supelco, Bellefonte, PA). The columns were eluted with a gradient (0-20 min/ 0-40 % B) of solvent A (water containing 0.1 % acetic acid) and solvent B (acetonitrile/tetrahydrofuran (9:1 v/v containing 0.1 % acetic acid) at a flow rate of 0.25 ml/min. The eluent was monitored at 254 nm and analyzed by electrospray ionization mass spectrometry in the positive ion mode. The source temperature of the mass spectrometer was maintained at 110 °C and the cone voltage was set to 25 V. The capillary potential and the high electrode potential were set to 3.0 and 0.5 kV, respectively. Full scan data were acquired in the range of 70-270 m/z by means of the Mass Lynx NT software (Micromass, Manchester, U. K.).
RESULTS

*Production of radicals monitored by DHR oxidation*- DHR oxidation to rhodamine occurs by a radical mechanism and the process has been used to detect the production of radicals and ferryl intermediates (32). Here we show that DHR (80 µM) is oxidized in incubations containing acetaldehyde (5 mM) and XO (10 mU/ml) in phosphate buffer, pH 7.2 at 25 °C in the absence and presence of bicarbonate (100 mM) (Fig. 2). The small accelerating effect of bicarbonate on the initial rate of DHR oxidation (1.3 times) was reproducible but difficult to discriminate under these conditions where, most likely, Fenton chemistry in the bulk solution predominates (REACTIONS 1-8). Indeed, DHR is oxidized by the hydroxyl radical but not by superoxide radical anion or hydrogen peroxide (32) that are produced during XO turnover (REACTIONS 1-3) (4-8). However, the latter species can produce the hydroxyl radical (REACTION 4) because XO carries tightly-bound iron ions (35, 36).

Accordingly, addition of catalase (200 U/ml) greatly inhibited DHR oxidation and the accelerating effect of 100 mM bicarbonate became more pronounced (3.5 times) (Fig. 2). Further addition of Fe-superoxide dismutase (30 U/ml) and DTPA (0.1 mM) marginally affected DHR oxidation rate in the presence of bicarbonate (Fig. 2), suggesting that catalase addition was enough to minimize Fenton chemistry during acetaldehyde oxidation by XO. The Fe-superoxide dismutase enzyme was used in these experiments because it has not been reported to display the peroxidase activity that oxidizes the bicarbonate/carbon dioxide pair in contrast with the Cu,Zn-superoxide dismutase enzyme (22-27).

Thus, the possibility of XO turnover producing the carbonate radical anion by mechanisms other than Fenton chemistry (Fig. 1) (21) was further examined in the presence of catalase (200 U/ml). In most experiments, DTPA (0.1 mM) was also present. Under
these conditions, the presence of bicarbonate (50-200 mM) accelerated DHR oxidation to an extent that was roughly dependent on the anion concentration (Fig. 3). The possibility of a higher rate of DHR oxidation being the result of a modulating effect of bicarbonate on enzyme activity (37) was excluded by experiments showing that bicarbonate (100-200 mM) had marginal effects on cytochrome c-Fe (III) reduction by acetaldehyde/XO (data not shown). Relevantly, the rate of cytochrome c-Fe(III) reduction increased with the pH whereas the rate of DHR oxidation decreased (Fig. 4). This behavior further excludes that DHR oxidation is being mediated by carbonate radical anions produced through Fenton chemistry in the bulky solution. In this case, a higher oxidation rate would be expected at alkaline pH because the hydroxyl radical reacts much faster with the carbonate (REACTION 8) than with bicarbonate anion (REACTION 7) (17, 18).

Replacement of acetaldehyde (1-5 mM) with hypoxanthine or xanthine (80-200 µM) as the oxidizing XO substrate, abolished the accelerating effects of bicarbonate on DHR oxidation (see, for instance, Fig. 5 inset). This was not surprising because purines and their oxidation product uric acid, in particular, react very rapidly with the carbonate radical anion (21, 38). In agreement, the addition of uric acid (80 µM) to the incubations containing acetaldehyde/bicarbonate/catalase/XO strongly inhibited DHR oxidation (Fig. 3, inset). Although uric acid has been described as a XO inhibitor (39), its inhibitory efficiency is not enough to explain the lack of DHR oxidation under the experimental conditions of Fig. 3. In order to circumvent the fast reaction between the carbonate radical anion and uric acid, the experiments using xanthine and hypoxanthine as substrates were performed in the presence of uricase. In addition, DHR oxidation was monitored by fluorescence due to its greater sensitivity as compared to uv-visible spectroscopy. Under
these conditions, a clear effect of bicarbonate in accelerating DHR oxidation promoted by 
xanthine/XO/catalase and hypoxanthine/XO/catalase was observed (Fig 5). Bicarbonate 
effects were undetectable in the absence of uricase (Fig. 5, inset). These results further 
indicate that uric acid, the final oxidation product of XO-mediated oxidation of 
hypoxanthine and xanthine, precludes carbonate radical anion detection through DHR 
oxidation. Indeed, due to solubility constraints, DHR should be used at low concentration 
(≤ 100 µM) and such concentrations are probably not enough to compete with uric acid for 
the carbonate radical anion (see, also, below).

The above results suggest that oxidation of acetaldehyde, hypoxanthine or xanthine 
by XO in the presence of bicarbonate and catalase at neutral pH produces a strong one-
electron oxidant, most likely, the carbonate radical anion.

*Production of radicals monitored by spin-trapping* - The carbonate radical anion does not 
produce stable radical adducts with the currently available spin traps but it oxidizes DMPO, 
possibly to the corresponding cation radical that, upon water addition, produces the EPR 
detectable DMPO/•OH radical adduct (Fig. 6, inset) (22, 25). Thus, we examined the effect 
of bicarbonate addition (50-200 mM) on the yields of DMPO/•OH produced from 
acetaldehyde/XOD/catalase (Fig. 6). In the absence of bicarbonate, the resulting EPR 
spectrum was dominated by that of the DMPO/•COCH₃ radical adduct (labeled as o in Fig 
6) (40, 41) with some contribution of those of the DMPO/•OOH and DMPO/•OH radical 
adduct, particularly the first. Upon addition of increasing concentrations of bicarbonate, the 
resulting EPR spectra presented increasing levels of the DMPO/•OH radical adduct 
(labeled as x) (Fig. 6B-D).

The results shown in Fig. 6 are indicative of carbonate radical production but are
not unambiguous because the DMPO/•OH radical adduct is also produced from DMPO attack by the hydroxyl radical. To exclude a main role for the hydroxyl radical in the formation of the DMPO/•OH radical adduct detected under our experimental conditions (Fig 6), we repeated the experiments in the presence of DMSO (2 M) (Fig. 7A, B). The latter does not react with the carbonate radical anion but does react with the hydroxyl radical to produce the methyl radical which, in turn, reacts with DMPO to produce the DMPO/•CH₃ radical adduct (22, 25). This adduct, however, was not detectable upon DMSO addition to the acetaldehyde/XO/catalase/bicarbonate system nor the yield of the DMPO/•OH radical adduct was decreased (Fig 7A,B). In contrast, under our experimental conditions, tryptophan and a tyrosine analogue (HPA) that react fast with the carbonate radical anion (21) strongly inhibited the production of the DMPO/•OH radical adduct (Fig. 7C,D). Certainly, both targets were oxidized by the carbonate radical anion to the corresponding tryptophanyl and phenoxyl radical (21) but these species were not efficiently trapped by DMPO (Fig. 7C,D).

The above results strongly supported carbonate radical anion production during acetaldehyde oxidation by XO/bicarbonate and next, we used DMPO to examine XO-mediated oxidation of xanthine and hypoxanthine. In contrast to DHR oxidation in which, due to solubility constraints, the carbonate radical anion probe (DHR) was used at low concentration (≤ 100 µM) (Figs. 2-5), DMPO as a probe can be employed at high concentration (80 mM) (Figs. 6, 7). This high concentration was likely to outcompete the purines and their oxidation product, uric acid, for the carbonate radical anion eventually produced and, thus, uricase was not added in the incubations. In the absence of bicarbonate, incubations of xanthine/XO/catalase/DMPO produced an EPR spectrum that
matched that of the DMPO/•OOH radical adduct (Fig 8A) (42-44). Upon addition of increasing concentrations of bicarbonate the resulting EPR spectrum became dominated by that of the DMPO/•OH radical adduct as expected from DMPO oxidation by the carbonate radical anion (Fig 8B,C). Moreover, as was the case of acetaldehyde containing incubations (Fig. 7), the addition of DMSO did not alter the EPR spectrum obtained in the presence of bicarbonate (Fig. 8 D). The spectra shown in Fig 8 were obtained during the oxidation of hypoxanthine (80 µM) but practically the same spectra were obtained during XO-mediated oxidation of xanthine (160 µM) (data not shown). Taken together, these results strongly support carbonate radical anion formation during oxidation of both xanthine and hypoxanthine by XO/bicarbonate. In these systems, surprising at first, was the inhibitory effect of bicarbonate addition on the yields of the DMPO/•OOH radical adduct (Fig. 8). However, the superoxide radical anion reacts slowly with DMPO (k = 10 M⁻¹s⁻¹) (45) but reacts fast with both carbon dioxide (k~10⁵ M⁻¹s⁻¹ in aprotic media) (46) and carbonate radical anion (k~10⁸ M⁻¹s⁻¹) (47). Thus, in the presence of bicarbonate, the latter species probably inhibits the yields of the DMPO/•OOH radical adduct by competing with DMPO for the superoxide radical anion (Fig. 8).

Taken together, the above results demonstrate that XO turnover in the presence of bicarbonate produces a strong one-electron oxidant independently of the substrate being acetaldehyde, hypoxanthine or xanthine (Figs. 3-8). Thus, acetaldehyde was selected for further experiments aiming to a more unambiguous characterization of the produced radical.

Production of radicals monitored by chemiluminescence – As mentioned above, Hodgson and Fridovich attributed the light emission observed in incubations of acetaldehyde/XO at
pH 10.2 to chemiluminescent products arising from carbonate radical anion dismutation (REACTION 9) (17). By then, it was difficult to compare the observed light emission with a known source of the carbonate radical anion but presently, mixtures of peroxynitrite with the bicarbonate/carbon dioxide pair provide such a source (19). Thus, we compared the light emission profile of peroxynitrite/bicarbonate with that of acetaldehyde/XO/catalase/bicarbonate at pH 7.4 (Fig. 9). In both cases, most of the light was emitted in the region between 500-600 nm as reported before (Fig. 9B, D) (17, 48). Light emission from the peroxynitrite/bicarbonate system was detectable only under flow conditions as expected from the fast decay of the oxidant in the presence of bicarbonate at neutral pH (Fig. 9A) (19-21, 48). In contrast, light emission from the XO incubation increased stepwise after acetaldehyde addition and reached a plateau that was sustained for some minutes as expected from an enzymatic reaction (Fig. 9C). These results further support that the strong oxidant produced during XO turnover in the presence of catalase and bicarbonate at physiological pH is the carbonate radical anion.

Production of carbonate radical anion monitored by spin-trapping coupled to HPLC/MS-

To further prove that the carbonate radical anion was produced in incubations of acetaldehyde/XO/catalase/bicarbonate and was able to oxidize DMPO to the corresponding radical cation to produce the DMPO/$\bullet$OH radical adduct (Fig. 6, inset), we performed incubations in the presence of unlabeled and labeled water ($H_2^{18}O$) and analyzed the samples by HPLC-mass spectrometry. Both incubations presented peaks eluting at 8 and 15 min. The peak eluting at 8 min in both incubations showed mass spectrum with a major ion at m/z 114 corresponding to the molecular ion of DMPO ([M+H]$^+$ = 114) (data not shown). The peak eluting at 15 min showed a major ion at m/z 130 for the incubation in normal
water (H$_2^{16}$O) (Fig. 10A) and at m/z 130 and m/z 132 for the incubation in labeled water (H$_2^{18}$O) (Fig. 10B). The ion at m/z 130 corresponds to the protonated nitrone of the DMPO/$\cdot$OH radical adduct ([M+H]$^+$ = 130) whereas the ion at m/z 132 corresponds to the $^{18}$O-labeled DMPO/$^{18}$OH protonated nitrone ([M+H]$^+$ = 132). The incorporation of $^{18}$O atom into the DMPO/$^{18}$OH nitrone obtained in incubations in H$_2^{18}$O indicates the addition of water and thus, carbonate radical anion production (Fig. 6, inset). The fact that the nitrones and not the corresponding nitroxides or hydroxylamines were detected, emphasizes the oxidizing power of the XO incubations (49, 50).

The relative percentage of the DMPO/$\cdot$OH and DMPO/$^{18}$OH nitrone in the incubations in H$_2^{18}$O was estimated to be around 60 and 30%, respectively. This result is consistent with both experimental conditions and XO turnover. Thus, labeled water employed was 96.9 % H$_2^{18}$O and this was diluted to 80% H$_2^{18}$O in the incubations mixtures. Also, XO turnover in the presence of catalase and bicarbonate has at least two potential sources of the DMPO/$\cdot$OH radical adduct, the carbonate radical anion and the superoxide radical anion (Fig. 1). The carbonate radical anion oxidizes DMPO to the corresponding cation radical that produces a labeled DMPO/$^{18}$OH radical adduct in the presence of labeled water (Fig. 6, inset; Fig. 10). The superoxide radical anion is produced from molecular oxygen (unlabeled under our experimental conditions) (Fig. 1) and is trapped by DMPO to produce the DMPO/$\cdot$OOH radical adduct. In agreement, the DMPO/$^{18}$OOH radical adduct was detected by EPR in our incubation mixtures (Figs. 6A, 8A). This radical adduct, however, rapidly decays to the DMPO/$\cdot$OH radical adduct (45) and this fact explains why the unlabeled nitrone was also detected under our experimental conditions (Fig. 10).
DISCUSSION

Our results demonstrate that xanthine oxidase-mediated oxidation of acetaldehyde, hypoxanthine or xanthine in the presence of both catalase and bicarbonate produces the carbonate radical anion by a mechanism that is independent of Fenton chemistry in the bulky solution (Figs. 2-10). The carbonate radical anion was first characterized by indirect methodologies such as DHR (Figs. 3-5) and DMPO oxidation (Figs. 6-8). In the case of XO acting upon xanthine and hypoxanthine as substrates, bicarbonate-dependent DHR oxidation was detected only in the presence of uricase (Fig. 5). This fact reinforces carbonate radical anion identification because this radical reacts very rapidly with uric acid (21, 38) which, in turn, is produced from xanthine and hypoxanthine oxidation and is degraded by uricase. A more direct proof that the main strong one-electron oxidant formed during XO turnover in the presence of catalase and bicarbonate was the carbonate radical anion and not the hydroxyl radical was obtained from the similar EPR spectra produced by mixtures containing DMPO (80 mM) (Figs. 7A and 8C) and DMPO (80 mM) plus DMSO (2 M) (Figs. 7B and 8D). If the hydroxyl radical were the main radical produced, DMSO addition should render a spectrum dominated by that of the DMPO/•CH₃ radical adduct because the hydroxyl radical reacts at similar rates with DMSO (k=5.8 x 10⁹ M⁻¹s⁻¹) (51) and DMPO (k = 4.3 x 10⁹ M⁻¹s⁻¹) (52) but DMSO was present at a higher concentration. The spectrum obtained in the presence and absence of DMSO, however, was dominated by that of the DMPO/•OH radical adduct (Figs. 7A,B and 8C,D) a result that is consistent with carbonate radical anion production. Indeed, this radical reacts only with DMPO (22, 25), and not particularly fast (k = 2.5 x 10⁶ M⁻¹s⁻¹) (53), to produce the DMPO/•OH radical adduct (Fig. 6, inset). Accordingly, the yield of this adduct was strongly inhibited upon the
addition of targets that, in contrast with DMSO, react fast with the carbonate radical anion such as tryptophan (k = 1.4 x 10^8 M^{-1}s^{-1}) and a tyrosine analog (k = 4.5 x 10^7 M^{-1}s^{-1} for tyrosine) (54) (Fig. 7C,7D).

All tested XO substrates, acetaldehyde, xanthine and hypoxanthine provided qualitatively similar results in regard to the bicarbonate-dependent oxidation of DHR to rhodamine (Figs. 3-5) and of DMPO to the DMPO/•OH radical adduct (Figs. 6-8). More unambiguous characterization of the carbonate radical anion was pursued with acetaldehyde as the XO substrate because its two-electron oxidation product, acetate, is not expected to react fast with the carbonate radical anion (21). In acetaldehyde/XO/catalase/bicarbonate containing incubations, the carbonate radical anion was characterized by the best available methodologies (Figs. 9 and 10) except for direct EPR (19). The use of latter was precluded by the low flux of produced radicals (55). The similar emission spectra obtained with XO incubations and flow mixtures of peroxynitrite with the bicarbonate-carbon dioxide pair (Fig. 9), and the demonstration that a considerable part of the DMPO/•OH radical adduct produced in the enzymatic incubations resulted from water (H_2^{18}O) addition to DMPO (Fig. 10), however, provide strong support for carbonate radical anion characterization. The fact that acetaldehyde can also react with deprotonated peroxide to eventually produce radical intermediates (40, 41) does not challenge the conclusion that the carbonate radical anion is produced during XO turnover. The acetaldehyde-derived radicals produced by XO under different experimental conditions have been previously characterized as the acetyl and the methyl radical (40, 41). These species may oxidize DHR but they do not oxidize DMPO to the DMPO/•OH radical adduct. Rather, they add to DMPO to produce the DMPO/•COCH_3 and the DMPO/•CH_3
radical adduct. Under the experimental conditions employed here, only the DMPO//•COCH₃ radical adduct was detected (Fig. 6).

Our studies were performed in the presence of catalase to inhibit Fenton chemistry in the bulk solution that would make it difficult to prove the formation of the carbonate radical anion during XO turnover. The experimental conditions employed, however, are closer to physiological ones. In vivo, the presence of superoxide dismutases, peroxiredoxins, catalase and the low availability of redox active transition metal ions tend to minimize the occurrence of classical Fenton chemistry (REACTIONS 4-8) except in localized environments (56, 57).

The mechanism for carbonate radical anion formation that can be proposed from our studies relies on an intermediate that is likely to be produced during XO turnover and may react with the biologically ubiquitous carbon dioxide. Accordingly, the pH dependence for radical production under our conditions did not correlate with superoxide radical anion production (Fig. 4) or with the reactivity of the hydroxyl radical towards the bicarbonate/carbonate anion (REACTIONS 7 and 8) (17, 18) but rather, it correlated with the equilibrating concentration of carbon dioxide (REACTIONS 5 and 6). This result, a faster electron than proton transfer during XO turnover (8), and the recent NMR characterization of the peroxymonocarbonate anion in aqueous mixtures of bicarbonate/hydrogen peroxide (K= 0.33) (28, 29), argue for the mechanism shown in Fig 1. Thus, the carbonate radical anion is likely to be produced at the XO active site from carbon dioxide addition to deprotonated peroxide which, in turn, is reduced by electrons flowing from the substrate to molecular oxygen through the many redox centers of XO. The carbonate radical anion can leave the active site and react with external targets as
demonstrated here for DHR and DMPO (Figs. 3-5 and 6-8).

Production and escape of radical intermediates during biological oxidations catalyzed by oxidases and oxygenases occurs frequently, particularly when the electrons flow through many redox centers as is the case for the mitochondrial electron transport chain, cytochrome P<sub>450</sub> microsomal system, nitric oxide synthases, and xanthine oxidase (3, 15, 57-60). The latter is an important example because xanthine oxidase produces considerable yields of the superoxide radical anion (4-8), displays peroxidase/oxidase activity towards aldehydes (41) and is considered to play important roles in cytotoxic processes (3, 9-15). The latter activities and roles have been mostly attributed to hydroxyl radical production through Fenton chemistry (see, above). In this context, the results reported here are particularly relevant because they demonstrate that xanthine oxidase turnover at physiological pH can produce a strong one-electron oxidant from the biologically ubiquitous bicarbonate-carbon dioxide pair by a mechanism unrelated to classical Fenton chemistry. In addition to indicating that the carbonate radical anion may be an important mediator of the pathophysiological effects of xanthine oxidase, our results add to recent studies demonstrating the importance of the bicarbonate-carbon dioxide pair as a source of biological oxidants (21-29).

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**FOOTNOTES**

1 The abbreviations used are: bicarbonate, the sum of bicarbonate (HCO$_3^-$) and carbon dioxide (CO$_2$) unless specified; DHR, dihydrorhodamine 1, 2, 3; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DTPA, diethylenetriaminepentacetic acid; HPA, 4-hydroxyphenylacetic acid; peroxynitrite, the sum of peroxynitrite anion (ONOO$^-$, oxoperoxonitrate (-1)) and peroxynitrous acid (ONOOH, hydrogen oxoperoxonitrate) unless specified; XO, xanthine oxidase.
FIGURE LEGENDS

FIG 1- Schematic representation of a route proposed for carbonate radical anion production during XO turnover in biological environments. Modified from ref. 21 to account for the data presented in refs. 28, 29 and here. S and S_{ox} represents a general substrate and its two-electron oxidation product, respectively.

FIG 2- Initial rate of DHR oxidation in incubations of acetaldehyde/XO in the absence and in the presence of bicarbonate and radical scavengers. The reaction mixtures contained acetaldehyde (5 mM), XO (10 mU/mL) and DHR (80 µM) in 200 mM phosphate buffer, pH 7.2, and were incubated at room temperature (25 ± 2°C). When added, bicarbonate (100 mM), catalase (200 U/ml), Fe-superoxide dismutase (30 U/ml) and DTPA (0.1 mM) had the specified final concentrations. The values shown are the mean ± standard deviation of three independent determinations.

FIG 3- Oxidation of DHR in incubations of acetaldehyde/XO/catalase in the absence and in the presence of bicarbonate. The reaction mixtures contained acetaldehyde (5 mM), XO (20 mU/mL), catalase (200 U/ml), DHR (80 µM), DTPA (0.1 mM) and bicarbonate at the specified concentrations in 250 mM phosphate buffer, pH 7.2, and were incubated at room temperature (25 ± 2°C). The inset shows the inhibitory effect of uric acid (80 µM) upon DHR oxidation mediated by acetaldehyde/XO/catalase in the presence of 100 mM bicarbonate.

FIG 4- Initial rates of DHR oxidation and cytochrome c-Fe (III) reduction by acetaldehyde/XO/catalase/bicarbonate as a function of the pH. The reaction mixtures contained acetaldehyde (5 mM), XO (10 mU/mL), catalase (200 U/mL), bicarbonate (100 mM) and DHR (80 µM) or cytochrome c-Fe (III) (50 µM) in phosphate buffer (250 mM) of
the specified pHs, and were incubated at room temperature (25 ± 2°C). The values shown are the mean ± standard deviation of three independent determinations.

**FIG 5- Oxidation of DHR in incubations of hypoxanthine (A) and xanthine (B) with XO/catalase/uricase in the absence and in the presence of bicarbonate.** The reaction mixtures contained hypoxanthine (80 µM) or xanthine (160 µM), XO (20 mU/mL), catalase (200 U/ml), uricase (1 U/ml), DHR (80 µM), DTPA (0.1 mM) and bicarbonate at the specified concentrations in 200 mM phosphate buffer, pH 7.2, and were incubated at room temperature (25 ± 2°C). The inset in A shows total DHR oxidation at 8 min by hypoxanthine/XO/catalase in the absence of uricase in the absence and presence of 100 mM bicarbonate. DHR oxidation was monitored by fluorescence as described in the Experimental Procedures.

**FIG 6- EPR spectra of DMPO radical adducts obtained in incubations of acetaldehyde/XO/catalase/DMPO in the absence and presence of bicarbonate.** The reaction mixtures contained acetaldehyde (5 mM), XO (20 mU/mL), catalase (200 U/mL), DTPA (0.1 mM) and DMPO (80 mM) in phosphate buffer (200 mM), pH 7.2 and the spectra were run at room temperature 1 min after acetaldehyde addition. A, no bicarbonate added; B, 50 mM bicarbonate added; C, 100 mM bicarbonate added; and D, 200 mM bicarbonate added. The positions of the DMPO/•COCH₃ (o) and DMPO/•OH (x) peaks are labeled as specified; the peaks of DMPO/•OOH radical adduct are also evident but were not labeled for simplicity. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 1 G; time constant, 164 ms; scan rate 0.6G/s; and gain, 2x10⁵. The inset shows a schematic representation of the mechanism by which the carbonate radical anion produces the DMPO/•OH radical adduct.
FIG. 7- EPR spectra of DMPO radical adducts obtained in incubations of acetaldehyde/XO/catalase/bicarbonate/DMPO in the absence and presence of radical scavengers. The reaction mixtures contained acetaldehyde (5 mM), XO (20 mU/mL), catalase (200 U/mL), DTPA (0.1 mM), bicarbonate (200 mM) and DMPO (80 mM) in phosphate buffer (200 mM), pH 7.2, and the spectra were run at room temperature (25 ± 2°C) 1 min after acetaldehyde addition. A, no additions; B, 2 M DMSO added; C, 25 mM HPA added; 25 mM tryptophan added. Instrumental conditions; microwave power, 20 mW; modulation amplitude, 1 G; time constant, 164 ms; scan rate, 0.6 G/s; and gain 2 x 10^5.

FIG. 8- EPR spectra of DMPO radical adducts obtained in incubations of hypoxanthine/XO/catalase/DMPO in the absence and in the presence of bicarbonate. The reaction mixtures contained hypoxanthine (80 µM), XO (20 mU/mL), catalase (200 U/mL), DTPA (0.1 mM) and DMPO (80 mM) in phosphate buffer (200 mM), pH 7.2, and the spectra were run at room temperature (25 ± 2°C) 1 min after hypoxanthine addition. A, no bicarbonate added; B, 50 mM bicarbonate added; C, 100 mM bicarbonate added; and D, 100 mM bicarbonate plus 2 M DMSO added. The spectrum shown in A matches that of the DMPO/•OOH radical adduct. The spectra shown in B-D are a composite of those of the DMPO/•OOH and DMPO/•OH radical adduct but only the peaks of latter were labeled as (x) Instrumental conditions; microwave power, 20 mW; modulation amplitude, 1 G; time constant, 164 ms; scan rate, 0.6 G/s; and gain 2 x 10^5.

FIG 9- Comparison of light emission by mixtures of peroxynitrite/bicarbonate and of acetaldehyde/XO/catalase/bicarbonate. Peroxynitrite was infused at 0.4 mM/s in a solution of bicarbonate (100 mM) in phosphate buffer (250 mM), pH 7.4 at 25°C. Acetaldehyde was injected to a final concentration of 15 mM in the cuvette containing XO
(50 mU/ml), catalase (200 U/ml), bicarbonate (100 mM) in phosphate buffer (250 mM), pH 7.4 at 25 °C. A and C show total light emission as a function of time; B and D show light emission as a function of the wavelength for peroxynitrite and XO containing incubations, respectively. Light emission values represent the percentage of total light (without filters) that passed through each specified cut-off filter. The values shown are the mean ± standard deviation of three independent determinations.

FIG. 10- Mass spectra of the DMPO·OH radical adduct obtained in incubations of acetaldehyde/XO/catalase/bicarbonate/DMPO in unlabeled and labeled water. The incubation mixtures contained acetaldehyde (5 mM), XO (10 mU/mL), catalase (200 U/mL), bicarbonate (200 mM) and DMPO (80 mM) in phosphate buffer (200 mM), pH 7.4 and were incubated at room temperature (25 °C ± 2 °C) for 4 h. The samples were kept in the refrigerator and analyzed within 24 h by HPLC-MS as described in Experimental Procedures. The shown mass spectra were obtained by selecting the 15 min retention time peak for the incubations performed in H$_2^{16}$O (A) or H$_2^{18}$O (80% final $^{18}$O abundance).
S + H₂O $\xrightarrow{XO} S_{\text{ox}} + 2e^- + 2H^+$  \hspace{1cm} (Reaction 1)

2 O₂ + 2e $\xrightarrow{XO} 2 O_2^-$  \hspace{1cm} (Reaction 2)

O₂ + 2e⁻ + 2 H⁺ $\xrightarrow{XO} 2 H₂O₂$  \hspace{1cm} (Reaction 3)
\[ \text{O}_2^- + 2\text{H}_2\text{O}_2 \xrightarrow{\text{M}^{+n} \text{catalysis}} \text{HO}^+ + \text{HO}^- + \text{O}_2 \]  
(Reaction 4)

\[ \text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \rightarrow \text{HCO}_3^- + \text{H}^+ \quad \text{pKa} = 6.37 \]  
(Reaction 5)

\[ \text{HCO}_3^- + \text{H}_2\text{O} \leftrightarrow \text{CO}_3^{2-} + \text{H}^+ \quad \text{pKa} = 10.32 \]  
(Reaction 6)

\[ \text{HO}^- + \text{HCO}_3^- \rightarrow \text{H}_2\text{O} + \text{CO}_3^{2-} \quad k = 8.5 \times 10^6 \text{ M}^{-1} \text{s}^{-1} \]  
(Reaction 7)

\[ \text{HO}^- + \text{CO}_3^{2-} \rightarrow \text{OH}^- + \text{CO}_3^{2-} \quad k = 3 \times 10^8 \text{ M}^{-1} \text{s}^{-1} \]  
(Reaction 8)

\[ 2\text{CO}_3^{2-} \rightarrow \text{hv} + \text{Products} \]  
(Reaction 9)
Fig. 1
Fig. 2

DHR oxidation
(μM/min)

- HCO₃⁻
- HCO₃⁻ + catalase
+ HCO₃⁻ / + catalase / + SOD
+ HCO₃⁻ / + catalase / + SOD / + DTPA
- HCO₃⁻ / + catalase / + SOD / + DTPA

*
Fig. 3

Absorbance vs. Time (min)

- HCO$_3^-$
+50 mM
+100 mM
+200 mM

Urate (µM)
0
80
Fig. 4

DHR oxidation (µM/min)

pH

Citc Fe+3 reduction (µM/min)
Fluorescence (arbitrary units)

Time (min)

Fig. 5
Fig. 6

\[
\begin{align*}
\text{N} & \xrightarrow{\text{CO}_3^{2-}} \text{NO}_2^+ \xrightarrow{\text{H}_2\text{O}} \text{NO}_2^-
\end{align*}
\]

A

\[
\text{-HCO}_3^-
\]

B

+50 mM

C

+100 mM

D

+200 mM

Fig. 6
Fig. 7
Fig. 8
Fig. 9
Fig. 10
Production of the carbonate radical anion during xanthine oxidase turnover in the presence of bicarbonate
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