Carbon dioxide mediates Mn(II)-catalyzed decomposition of hydrogen peroxide and peroxidation reactions

Stefan I. Liochev and Irwin Fridovich*

Department of Biochemistry, Duke University Medical Center, Durham, NC 27710

Mn(II) can catalyze the decomposition of H_2O_2 and, in the presence of H_2O_2, can catalyze the oxidation of NADH. Strikingly, these processes depend on the simultaneous presence of both CO_2 and HCO_3^-. This explains the exponential dependence of the rates on $[HCO_3^-]$, previously noted by other workers. These processes are inhibited by Mn-superoxide dismutase, establishing the generation of O_2^- and its role as an essential reactant. A scheme of reactions, consistent with the known properties of this system, is proposed. The large rate enhancements provided by $HCO_3^- + CO_2$, and the abundance of both of these species *in vivo*, suggest that similar reactions have relevance to the oxidative stress imposed by O_2^- and H_2O_2.

The Cu,Zn superoxide dismutase (SOD1) catalyzes the oxidation of a variety of substrates by H_2O_2 (1–8). This peroxidative activity was thought to depend on HCO_3^- (2–7), but was shown to actually require CO_2 (8). Carbonate radical (CO_3^-) is proposed to be the strong oxidant that is responsible for the peroxidations observed (3–8). HCO_3^--dependent peroxidations and H_2O_2 decomposition, catalyzed by Mn(II) and Fe(II), have been reported (9–17). The possibility that, in these reactions, CO_2 rather than HCO_3^- might be required, needs to be considered. One puzzling aspect of the Mn(II) + HCO_3^--catalyzed peroxidations and H_2O_2 decompositions was the exponential dependence of rates on $[HCO_3^-]$ within a given range of $[HCO_3^-]$. Thus, Sychev *et al.* (9, 10) reported dependence of the rate on more than the square of $[HCO_3^-]$, whereas the Stadtman group (13) reported a third-order dependence on $[HCO_3^-]$.

In what follows, we demonstrate a dependence on CO_2 and an apparent synergism between CO_2 and HCO_3^-, which largely explains the reported exponential responses to $[HCO_3^-]$. The abundance of CO_2 and HCO_3^- in biological systems and their role in the peroxidations catalyzed by SOD1 and metal cations, such as Mn(II), could have relevance to the oxidative stress experienced by aerobic organisms.

Materials and Methods

$NaHCO_3$, $MnCl_2$, sodium pyrophosphate, and H_2O_2 were from Mallinckrodt; Tris was from Merck; catalase was from Roche Molecular Biochemicals; human MnSOD was from Biotechnology General (Rehovot, Israel); and NADH was from Sigma. Reaction mixtures usually contained 10 mM H_2O_2, 0.1 mM $MnCl_2$, and 20 mM $NaHCO_3$ in 100 mM Tris buffer at pH 7.4 and room temperature (23°C). Reactions were followed spectrophotometrically, and in cases where the addition of reactants entailed sudden changes in absorbance, due either to dilution or the absorbance of the reagent added, such changes were corrected in the data shown. When absorbance changes with time were too rapid to record, they are shown as dashed lines as in Fig. 3. CO_2 was added as ice-cold water saturated with CO_2 gas.

Results

Absorption Spectra.
Addition of the third component (H_2O_2 or $MnCl_2$, or $NaHCO_3$), to Tris-buffered solutions of the other two, initiated the changes in absorption spectrum shown in Fig. 1. It should be noted that several buffering species were explored, including phosphate, cacodylate, and Hepes, but only Tris supported the changes observed. Fig. 1A shows that the most rapid (0–3 min) was an increase in absorbance in the range of 250–350 nm and a decrease at wavelengths <250 nm. At longer reaction times (0–20 min), absorbance appeared at ≈270 nm, whereas the absorbance <250 nm first decreased and then increased, as shown in Fig. 1B. At still longer times (Fig. 1C), the band at ≈270 nm became more pronounced, a shoulder developed at ≈300 nm, and absorbance <250 nm decreased.

The species responsible for the absorbance centered at 270 nm is some stable product of Tris oxidation, as similar absorbance is seen in Tris buffer that has aged for many months. The kinetics of the absorbance change at 270 nm is shown in Fig. 2. Starting the reaction by adding $NaHCO_3$ caused a small but rapid increase at 270 nm, which was followed by a slower increase that was not interrupted by late addition of 48 µg/ml MnSOD, 0.5 mM pyrophosphate, or 120 units/ml catalase, any of which inhibited if present at the outset. The initial rapid increase at 270 nm was due to the rapidly formed species absorbing in the 250- to 350-nm region. Evidently, the stable A_{270} species is produced from a preformed precursor and does not depend on the continued presence of H_2O_2, O_2^-, or Mn(III). The latter deduction is based on the abilities of catalase to remove H_2O_2, MnSOD to remove O_2^-, and pyrophosphate to complex and thus trap Mn(III).

The time course of the broad band followed at ≈300 nm is illustrated in Fig. 3. Addition of $NaHCO_3$ to the buffered solution of Mn(II) + H_2O_2 caused a rapid increase in absorbance that approached a plateau. This plateau evidently represented a dynamic steady state, as adding MnSOD (Fig. 3, line 1) or pyrophosphate (Fig. 3, line 2) caused a swift decline. The species followed at 300 nm could not have been Mn(III), because H_2O_2 was seen to instantly bleach the absorbance of Mn(III) acetate. These results indicate that both O_2^- and higher valent states of Mn(II) are needed for the formation of the A_{300} species. The rate of appearance of the A_{300} species was slower when $NaHCO_3$ was the last component added than it was when H_2O_2 was added last (Fig. 3). This could be explained by the relative slowness of the formation of CO_2 after the addition of the alkaline $NaHCO_3$ to the reaction mixture buffered at pH 7.4. This view is supported by the fact that carbonic anhydrase at 50 µg/ml, when present in the reaction mixture, sharply increased the rate seen when $NaHCO_3$ was added last and made it indistinguishable from line 2 in Fig. 3.

CO_2 Is a Reactant.
The addition of $NaHCO_3$ initiated a rapid increase in A_{300} that approached a plateau within 3 min, as seen

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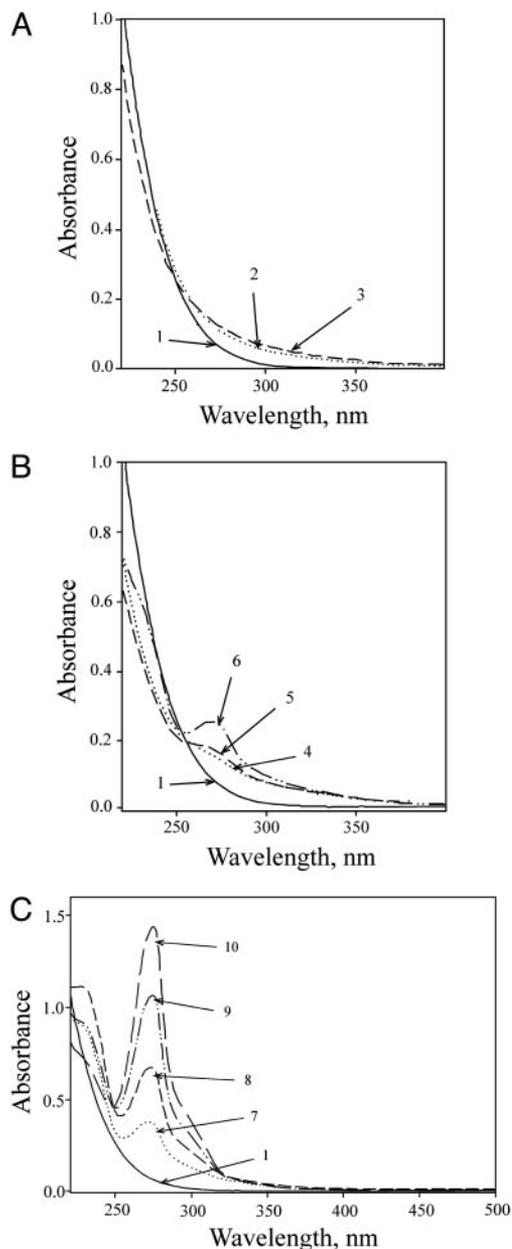
Fig. 1. Absorption spectra during the Mn + H_2O_2 + HCO_3^- reaction. Reactions were started by adding $NaHCO_3$ to 20 mM to 10 mM H_2O_2, 0.1 mM $MnCl_2$, and 100 mM Tris chloride at pH 7.4. (*A*) Spectra taken at 0, 1, and 3 min (lines 1, 2, and 3). (*B*) Spectra taken at 0, 6, 10, and 20 min (lines 1, 4, 5, and 6). (*C*) Spectra taken at 0, 30, 50, 80, and 120 min (lines 1, 7, 8, 9, and 10). The zero time spectrum was taken before the addition of HCO_3^-.

Fig. 2. Time dependence of absorbance at 270 nm. Reaction mixtures were as in Fig. 1, with the reaction started by the addition of HCO_3^-. At the arrows, MnSOD to 48 μg/ml, pyrophosphate (PP) to 0.5 mM, or catalase (Cat) to 120 units/ml was added.

than after, HCO_3^-, a very small and transient increase in A_{300} was seen, and the subsequent addition of HCO_3^- gave the modest and limiting increase in A_{300} that would have been observed in the absence of the CO_2 addition (Fig. 4*B*, line 3). Evidently, CO_2 greatly increases A_{300} only when HCO_3^- is also present. Clearly, CO_2 and HCO_3^- both are needed to support the reaction and behave synergistically.

The apparent difference between the effects of MnSOD and pyrophosphate, when added after the reaction was underway, as compared to their effects when added at the outset, is easily explained. Thus, there is overlap between the spectra of the A_{270} and A_{300} species. Thus, by the time the MnSOD or pyrophosphate had been added (Fig. 3), some of the stable A_{270} product had accumulated, and its absorbance at 300 nm prevented the complete ablation of the A_{300}. In contrast, when MnSOD or pyrophosphate was present at the outset, they prevented production of both the A_{300} and A_{270} species.

It thus appears that the rapidly forming A_{300} species is needed for production of the A_{270} species.

Consumption of H_2O_2. Absorbance at 240 nm reflects both H_2O_2 and the rapidly forming A_{300} species. Hence, the reaction between Mn(II), H_2O_2, HCO_3^-, and CO_2 produces a transient increase at 240 nm followed by a linear decrease (Fig. 5). Starting the reaction by adding $NaHCO_3$ last gave a slower increase in A_{240} than did adding $MnCl_2$ last (Fig. 5, compare lines 1 and 2).
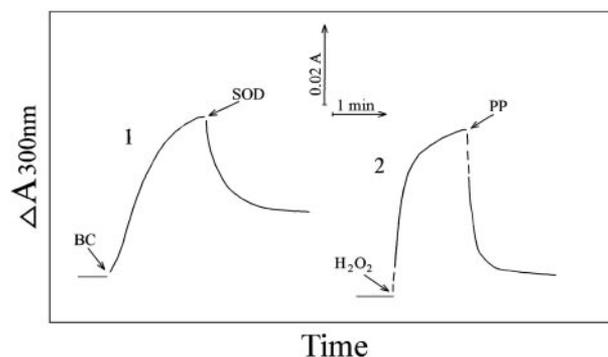
Fig. 3. Time dependence of absorbance at 300 nm. Reaction conditions were as in Fig. 1. Line 1, HCO_3^- (BC) added at the first arrow and MnSOD to 24 μg/ml at the second arrow. Line 2, H_2O_2 added at the first arrow and pyrophosphate (PP) to 0.5 mM at the second arrow.

in Fig. 3. When CO_2 was added, it caused a marked increase followed by a decline to the plateau that would have been reached in the absence of the addition of CO_2, as seen in Fig. 4*A*. MnSOD (24 μg/ml) or pyrophosphate (0.5 mM) present at the outset prevented the increase in A_{300} (data not shown). The effect of added CO_2 was even more striking when the amount of HCO_3^- used to start the reaction was halved. Thus, as shown in Fig. 4*B*, the increase in A_{300} was decreased by $\approx$5-fold when [HCO_3^-] was halved, in agreement with an exponential dependence of rate on [HCO_3^-]. Subsequent addition of CO_2 again caused an abrupt but temporary increase in A_{300} (Fig. 4*B*, line 1). The presence of carbonic anhydrase eliminated the effect of added CO_2 (Fig. 4*B*, line 2). When CO_2 was added before, rather

Fig. 4. Effect of CO_2 on absorbance at 300 nm. (*A*) Conditions were as in Fig. 1. Reaction was started by addition of HCO_3^- at the first arrow and 0.15 ml of ice water saturated with CO_2 added to the total volume of 3.0 ml at the second arrow. (*B*) Conditions were as in Fig. 1 except that HCO_3^- was added to 10 mM. Line 1, HCO_3^- added at the first arrow. Line 2, carbonic anhydrase (CA) was added to 50 μg/ml at the second arrow and CO_2 added at the third arrow. Line 3, CO_2 added at the first arrow and HCO_3^- added at the second arrow.

This is explained by the slowness of the uncatalyzed dehydration of H_2CO_3 to CO_2, as shown by the effect of carbonic anhydrase (Fig. 5, line 3). The linear decrease in A_{240} that followed the transient increase reflects consumption of H_2O_2, which was $\approx$0.5 mM/min, of H_2O_2, and it was not influenced by carbonic anhydrase (Fig. 5, compare lines 1 and 3). Ethanol at 1% did not inhibit, but MnSOD or pyrophosphate did when added after the linear rate had been achieved, after a lag of 5–10 s (data not shown).

Addition of CO_2 increased the consumption of H_2O_2 as illustrated by line 1 in Fig. 6*A*. Prior addition of carbonic anhydrase eliminated this effect of CO_2, as shown by Fig. 6*A*, line 2. The effect of CO_2 was even more striking when the concentration of HCO_3^- was decreased from 20 to 10 mM. Thus, as shown in Fig. 6*B*, the rate of H_2O_2 consumption was then much slower, $\approx$0.1 mM/min, and the effect of added CO_2 was more obvious. The presence of carbonic anhydrase eliminated the effect of added CO_2, as shown by Fig. 6*A*, line 2. CO_2 was able to enhance the rate of H_2O_2 decomposition only if HCO_3^- was present. Thus, as shown by line 1 in Fig. 6*C*, addition of CO_2 to the Tris-buffered mixture of Mn(II) plus H_2O_2 was without
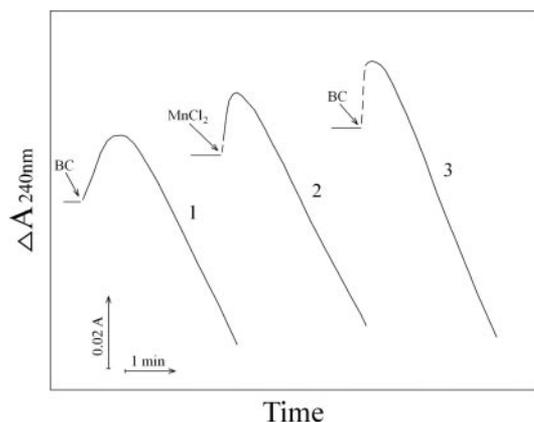
effect, and subsequent additions of 10 mM HCO_3^- gave the rates previously seen at these levels of HCO_3^- in the absence of CO_2 addition. Doubling the amount of CO_2 added, in the absence of HCO_3^- (Fig. 6*C*, line 2), had only a small effect, undoubtedly due to some conversion to HCO_3^-. These results again bespeak a synergism between CO_2 and HCO_3^-, due to the requirement for both to support the decomposition of H_2O_2.

Oxidation of NADH. Previous workers (11, 12, 14, 17) demonstrated that Mn(II) + H_2O_2 + HCO_3^- could cause the oxidation of diverse substrates, such as amino acids. We chose NADH because it absorbs at 340 nm, where the rapidly formed A_{300} species does not absorb significantly. Moreover, $CO_3^{\bullet-}$, considered a likely oxidant in this reaction system, is known to oxidize NADH with a rate constant of $\approx$10^9 M$^{-1} \cdot$s^{-1} at 25°C (18). It should also be noted that HO$^{\bullet}$ attacks NADH at sites that do not yield NAD$^+$, whereas $CO_3^{\bullet-}$ does so (18). Line 1 in Fig. 7*A* shows that addition of HCO_3^- to 20 mM to the Tris-buffered mixture of H_2O_2 + Mn(II) initiated oxidation of NADH at a rate of $\approx$0.02 mM/min after a brief lag. Halving the amount of HCO_3^- added decreased the rate by 5.3-fold, and subsequent addition of CO_2 greatly increased the rate (Fig. 7*A*, line 2). MnSOD inhibited the oxidation of NADH, and this inhibition was not overcome by subsequent addition of CO_2 (Fig. 7*A*, line 3). The lags seen when HCO_3^- was used to start the reaction were not as evident when Mn(II) or H_2O_2 was the last component added, or when carbonic anhydrase was present at the time HCO_3^- was added (Fig. 7*B*). It follows that the lags seen in Fig. 7*A* were due to the time required for conversion of some of the HCO_3^- to CO_2. Ethanol added to 1% did not inhibit NADH oxidation (Fig. 7*B*, line 1), whereas MnSOD did so (Fig. 7*B*, line 2). Thus, HO$^{\bullet}$ is not an intermediate in this process, but O_2^- is. In full accord with the deductions so far, adding CO_2 after HCO_3^- caused a marked increase in the rate of NADH oxidation (Fig. 7*C*, line 1), and this effect of CO_2 was eliminated if carbonic anhydrase was present (Fig. 7*C*, line 2). Addition of CO_2 in the absence of HCO_3^- transiently increased the rate due undoubtedly to the generation of some HCO_3^-, the second component needed.

Discussion

One aspect of the toxicity of O_2^- is thought to involve the univalent oxidation of the [4Fe–4S] clusters of dehydrases,

Fig. 5. Kinetics of absorbance changes at 240 nm. Conditions were as in Fig. 1. Line 1, reaction was started by the addition of HCO_3^-. Line 2, reaction was started by the addition of Mn(II). Line 3, carbonic anhydrase was present at 50 μg/ml, and reaction was started by the addition of HCO_3^-.
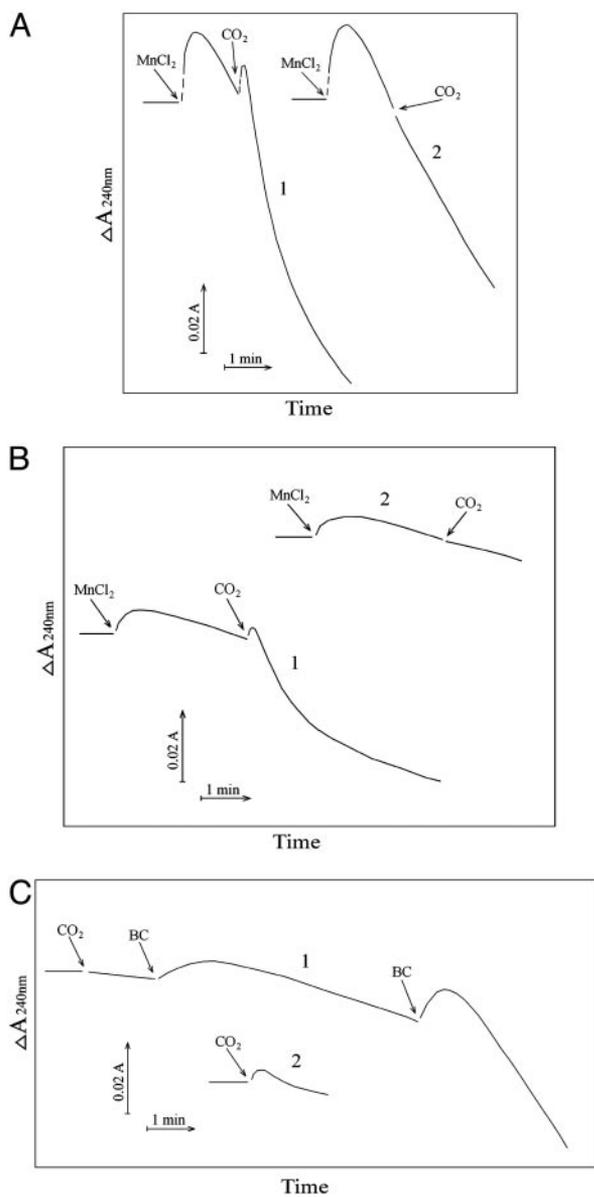
Fig. 6. Effects of CO_2 on absorbance changes at 240 nm. (*A*) Conditions were as in Fig. 1. Line 1, reaction was started by addition of Mn(II) at the first arrow, and then CO_2 was added at the second arrow. Line 2 was as in line 1, but carbonic anhydrase was present at 50 $\mu g/ml$. (*B*) Conditions were as in *A* except that HCO_3^- was present at 10 mM. (*C*) Line 1, CO_2 was added to Tris-buffered solution containing 10 mM H_2O_2 and 0.1 mM Mn(II) at the first arrow, and then HCO_3^- to 10 mM was added at the second arrow, and at the third arrow HCO_3^- was raised to 20 mM. Line 2, 0.3 ml of CO_2 solution was added to the H_2O_2 + Mn(II) reaction mixture at the arrow.

Fig. 7. Oxidation of NADH. NADH was present at 0.1 mM, and oxidation was followed at 340 nm. (*A*) Line 1, conditions were as in Fig. 1 except as noted below, and HCO_3^- was added to 20 mM at the arrow. Line 2, HCO_3^- was added to 10 mM at the first arrow, and 0.15 ml of CO_2 solution was added at the second arrow. Line 3, HCO_3^- was added to 10 mM at the first arrow, MnSOD was added to 30 $\mu g/ml$ at the second arrow, and 0.15 ml of CO_2 solution was added at the third arrow. (*B*) Mn(II) was added at 0.1 mM, H_2O_2 was added at 10 mM, and HCO_3^- was added at 10 mM. Line 1, Mn(II) was added at the first arrow, and ethanol to 1% was added at the second arrow. Line 2, reaction was started with H_2O_2 at the first arrow, and then MnSOD was added to 6 $\mu g/ml$ at the second arrow and to 18 $\mu g/ml$ at the third arrow. Line 3, as in lines 2 and 3 in *A* but carbonic anhydrase was present at 50 $\mu g/ml$. (*C*) Mn(II) was added at 0.1 mM, H_2O_2 at 10 mM, and HCO_3^-, when present, at 5 mM. Line 1, HCO_3^- was added at the first arrow, and 0.15 ml of CO_2 solution was added at the second arrow. Line 2, HCO_3^- was added at the first arrow, carbonic anhydrase to 50 $\mu g/ml$ was added at the second arrow, and 0.15 ml of CO_2 solution was added at the third arrow. Line 3, 0.15 ml of CO_2 solution was added at the arrow, but HCO_3^- was not present.

such as aconitase. This destabilizes the clusters causing release of Fe(II), and that released iron can then generate potent oxidants by reacting with hydroperoxides, in what is called Fenton chemistry (19). Iron is not the only metal cation that can participate in Fenton chemistry. Thus, Mn(II), Co(II), Cr(II), and Cu(II) can do likewise, and HCO_3^- or CO_2 has been shown to enhance the oxidations caused by several of these metals in the presence of H_2O_2 (11, 12, 14, 16, 20). In one of these cases, Xiao *et al.* (20) showed that CO_2 enhanced the luminescence of luminol caused by Co(II) + H_2O_2. H_2O_2 decomposition and oxidations, catalyzed by Mn(II) + H_2O_2, in the presence of HCO_3^-, have been studied by a number of

workers, such as Sychev *et al.* (9–12) and the Stadtman group (13–15); the exponential dependence of rate on [HCO_3^-] has been noted, and mechanisms have been proposed.

Our present demonstration that both HCO_3^- and CO_2 are required for this process explains the exponential dependence on $[HCO_3^-]$, as elevating $[HCO_3^-]$ also raises the $[CO_2]$ in equilibrium with it. Any valid mechanism must explain the dual requirement for HCO_3^- and CO_2 and also the involvement of O_2^-. Moreover, analogy with the Cu,ZnSOD + H_2O_2 system indicates that $CO_3^{\cdot-}$ is a likely participant, whereas $HO^\cdot$ is not (1, 3–8). The role of O_2^- could not be the reduction of Mn(III) to Mn(II), as suggested by the Stadtman group (13), because H_2O_2 itself rapidly accomplishes this reduction. On the other hand, the oxidation of Mn(II) to Mn(III) is also not likely to be the role of O_2^-, as H_2O_2 would then be produced from the O_2^-, yet rapid H_2O_2 consumption is a hallmark of this process.

The following scheme of reactions is in accord with the known properties of the system. It is proposed in the realization that it is an oversimplification that others may want to elaborate.

$$Mn(II) + HCO_3^- + H_2O_2 \rightleftharpoons$$

$$HCO_3^- - Mn(II) - {}^-OOH + H^+ \quad [1]$$

$$HCO_3^- - Mn(II) - {}^-OOH + H^+ \rightleftharpoons$$

$$HCO_3^- - Mn(III) - OH + HO^- \quad [2]$$

$$HCO_3^- - Mn(III) - OH + H_2O_2 \rightleftharpoons$$

$$HCO_3^- - Mn(III) + O_2^- + H_2O + H^+ \quad [3]$$

$$2HCO_3^- - Mn(III) + H_2O_2 \rightleftharpoons$$

$$2\,HCO_3^- - Mn(II) + O_2 + 2H^+. \quad [4]$$
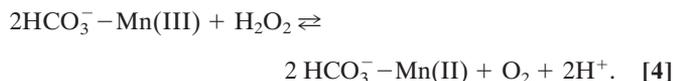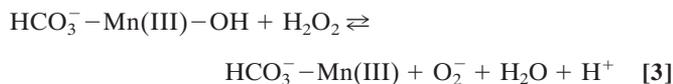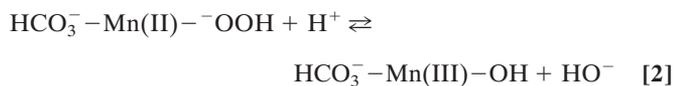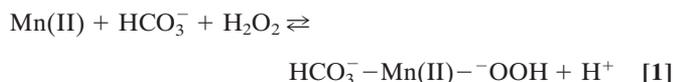
In reactions 1–4, H_2O_2 has been decomposed to $H_2O + O_2$, and O_2^- has been generated, in what may be viewed as the initiation phase, setting the stage for the chain reaction depicted by the following reactions. In what follows, the manganese may or may not be participating in the form of an HCO_3^- complex.

$$Mn(II) + CO_2 + H_2O_2 \rightleftharpoons HOO^- - Mn(II) - CO_2 + H^+$$

$$[5]$$

$$HOO^- - Mn(II) - CO_2 + O_2^- + H^+ \rightleftharpoons$$

$$HO - Mn(II) - CO_2 + O_2 + HO^- \quad [6]$$

$$HO - Mn(II) - CO_2 + H_2O_2 \rightleftharpoons$$

$$Mn(II) + CO_2 + O_2^- + H_2O + H^+ \quad [7]$$

$$HOO - Mn(II) - CO_2 + O_2^- \rightleftharpoons$$

$$Mn(II) + CO_3^{\cdot-} + O_2 + OH^- \quad [8]$$

$$CO_3^{\cdot-} + H_2O_2 \rightleftharpoons HCO_3^- + H^+ + O_2^- \quad [9]$$

$$CO_3^{\cdot-} + NADH \rightleftharpoons HCO_3^- + NAD^\cdot \quad [10]$$

$$NAD^\cdot + O_2 \rightleftharpoons NAD^+ + O_2^- \quad [11]$$

$$O_2^- + O_2^- + 2H^+ \rightleftharpoons H_2O_2 + O_2. \quad [12]$$
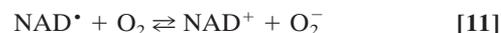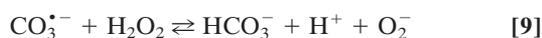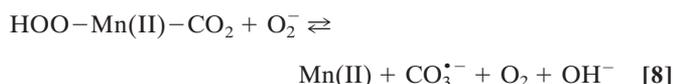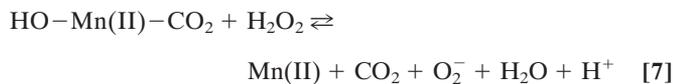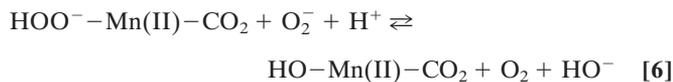
The O_2^- generated in reaction 3 is essential for reactions 6 and 8, and it may be viewed as the initiating radical. This is in accord with the observation by Sychev *et al.* (10) that tetranitromethane, which reacts with O_2^- at $\approx 10^9$ $M^{-1} \cdot s^{-1}$, strongly inhibited the decomposition of H_2O_2 and that the simultaneous addition of tetranitromethane and *N,N'*-dimethyl-*p*-nitrosoaniline prevented the oxidation of the *N,N'*-dimethyl-*p*-nitrosoaniline until all of the tetranitromethane was consumed. In our case, we used MnSOD, which scavenges O_2^- as efficiently as does tetranitromethane, but which acts catalytically rather than stoichiometrically. The product of reaction 6 may itself act as the oxidizing species, or carbonate radical, made as in reaction 8, may be the oxidant that can oxidize H_2O_2 (reaction 9) or NADH (reaction 10). Reactions 6 and 7 or 8 and 9 may be alternative or simultaneous pathways. Note the similarity of pathways 6 and 7 as well as 8 and 9 to the classical Haber–Weiss chemistry (21). Because O_2^- is merely acting as a reductant, other reductants in the cell could possibly trigger these potentially damaging processes. Reaction 12 is a termination reaction, and it explains the inhibition by MnSOD. The product of reaction 2 contains trivalent manganese, and it could be the site of inhibition by pyrophosphate. Oxidation of Tris by one or more of the oxidants generated in this scheme would account for the slowly forming and stable species followed at 270 nm. Sychev *et al.* (9, 10) worked without buffer and maintained pH by titration. Hence, it cannot be the case that Tris is needed for the process under study. We must rather conclude that Tris did not significantly interfere whereas phosphate, Hepes, and cacodylate did so.

The biological significance of Fenton chemistry has been questioned in part because the rate constants for the reactions of reduced metals, and their complexes, with H_2O_2 are not rapid, and their *in vivo* metal ligands are unknown (22). The present observations of the synergistic rate accelerations by $HCO_3^- + CO_2$ plus the work of Sychev *et al.* (9–12) and the Stadtman group (13–16) overcome this bottleneck and may well be of importance in the imposition of oxidative stress in biological systems. In this regard, the observation of Stadtman and Berlett (16) that HCO_3^- greatly enhances the ability of Fe(II) plus H_2O_2 to cause the oxidation of amino acids is also very interesting. The exponential dependence of rates on $[HCO_3^-]$ can have two components. Thus, the second-order dependence reported by Sychev *et al.* (10) is explained by the dual need for HCO_3^- and CO_2. To this can be added the effect of HCO_3^- in decreasing the amount of free hexaquo Mn(II) available for consumption of O_2^- by the reaction $Mn(II) + O_2^- + 2H^+ \rightleftharpoons Mn(III) + H_2O_2$. This, together with the need for $CO_2 + HCO_3^-$, could explain the third-order dependence noted by the Stadtman group (13).

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