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Manganese-dependent disproportionation of hydrogen peroxide in bicarbonate buffer

(superoxide anion/oxygen radicals/hydroxyl radicals/catalase mimic)

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ABSTRACT **At physiological concentrations of HCO_3^- and CO_2, Mn(II) catalyzes disproportionation of H_2O_2. This catalase-like activity is directly proportional to the concentrations of Mn(II) and H_2O_2, and it increases exponentially with increases in pH. The effect of increasing pH is almost completely attributable to the concomitant increase in HCO_3^- concentration. The rate is proportional to the third power of the HCO_3^- concentration, suggesting that 3 equivalents of HCO_3^- combine with 1 equivalent of Mn(II) to form the catalytic complex. It is presumed that the redox potential of the Mn(II) $\rightleftharpoons$ Mn(III) couple in such a complex permits H_2O_2 to carry out facile reactions with Mn(II) comparable to those that occur with Fe(III) and Cu(II) chelate complexes, in which OH· and $O_2^{\cdot-}$ are established intermediates. The Mn-catalyzed disproportionation of H_2O_2 does not occur at physiological pH in the absence of HCO_3^-. Hepes, inorganic phosphate, and inorganic pyrophosphate inhibit the reaction catalyzed by the Mn/HCO_3^- system. These results are similar to those of Sychev *et al.* [Sychev, A. Y., Pfannmeller, U. & Isak, V. G. (1983) *Russ. J. Phys. Chem.* 57, 1690–1693]. The catalase-like activity of Mn(II)–bicarbonate complexes reported here, together with the superoxide dismutase activity of Mn complexes demonstrated by Archibald and Fridovich [Archibald, F. S. & Fridovich, I. (1982) *Arch. Biochem. Biophys.* 214, 452–463], strengthen the proposition that Mn may play an important role in the protection of cells against oxygen radical-mediated damage.**

There is growing evidence that Mn(II) may play an important role in protecting cells from oxygen-radical damage. Low molecular weight complexes of Mn(II) have been shown to protect *Lactobacillus planetarium* and related bacteria against oxygen toxicity (1, 2); the oxidative damage to glutamine synthetase that occurs during growth of *Escherichia coli* in Mn(II)-deficient media is prevented when Mn(II) is added to the growth medium (3); the *in vitro* O_2-dependent inactivation of many enzymes by enzymic and nonenzymic metal ion-catalyzed reactions is inhibited by Mn(II) (3–6). These protective effects of Mn(II) have been attributed to the ability of Mn(II) complexes to catalyze the dismutation of $O_2^{\cdot-}$ (1, 7–9) and to inhibit the reduction of Fe(III) to Fe(II) by $O_2^{\cdot-}$-dependent and possibly $O_2^{\cdot-}$-independent pathways (4). When Mn(II) is complexed with inorganic pyrophosphate it is readily oxidized by $O_2^{\cdot-}$ to Mn(III), reaction **1** (1, 2). Mn(III) in strong perchloric acid solution (10), or at pH 6.5 when complexed with inorganic pyrophosphate (1), is reduced to Mn(II) by H_2O_2 or by $O_2^{\cdot-}$, reactions **2** and **3**. Coupling of reactions **1** and **2**, or of reaction **3** and half of reaction **1**, could therefore account for the ability of Mn(II) complexes to catalyze the dismutation of $O_2^{\cdot-}$, reaction **4** (1, 8).

$$2O_2^{\cdot-} + 4H^+ + 2Mn(II) \rightarrow 2H_2O_2 + 2Mn(III) \quad [1]$$

$$2Mn(III) + H_2O_2 \rightarrow O_2 + 2H^+ + 2Mn(II) \quad [2]$$

$$O_2^{\cdot-} + Mn(III) \rightarrow O_2 + Mn(II) \quad [3]$$

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2 \quad [4]$$

In the course of studies to determine the effect of Mn(II) on the oxidative deamination of amino acids by Fe(II) or Fe(III) plus H_2O_2 in bicarbonate buffer (pH 7.6), it was noted that Mn(II) catalyzes rapid dismutation of H_2O_2.

$$2H_2O_2 \rightarrow O_2 + 2H_2O \quad [5]$$

We report here the results of studies on the catalase-like activity of the Mn/HCO_3^- system.

MATERIALS AND METHODS

Manometric Measurement of O_2 Production. A Warburg apparatus was used to measure the changes in pressure associated with the production of O_2 during the decomposition of H_2O_2. Reaction mixtures (1.9 ml) containing variable amounts of Mn(II) and $NaHCO_3$ were placed in the main compartment of calibrated double-sidearm Warburg flasks, and 0.1 ml of 0.6 M H_2O_2 was placed in the *non*vented sidearm. The flasks were immersed in the Warburg bath at 37°C and, with shaking, were flushed with a mixture of 5% CO_2/95% N_2 for at least 15 min. The vented sidearm stopper of the flask was then closed and after 5 min, the reaction was initiated by mixing the H_2O_2 in the other sidearm with the mixture in the main compartment. The change in pressure was followed with time, and the amount of O_2 produced was calculated as described by Umbreit *et al.* (11). To determine the effect of pH and HCO_3^- concentration on the rate of H_2O_2 utilization, the reaction mixtures containing various concentrations of $NaHCO_3$ were equilibrated with N_2 gas mixtures containing 5, 10, 15, or 20% CO_2. The relationship between pH and HCO_3^- concentration and the partial pressure of CO_2 in the gas phase was determined as described (11). Solutions of H_2O_2 were prepared by dilution of 30% stock solutions from Fisher and were standardized by absorbance measurements at 240 nm (ε = 44 $M^{-1}\cdot cm^{-1}$). The content of H_2O_2 in reaction mixtures was measured by the method of Thurman *et al.* (12).

Materials. Reagents used were obtained from commercial sources as follows: $NaHCO_3$ from Mallinckrodt; H_2O_2 (30% solution), $Fe(NH_4)_2(SO_4)_2$, KSCN, EDTA, glycine, and in-

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organic pyrophosphate from Fisher; $FeSO_4 \cdot 7H_2O$ from Allied Chemical (Morristown, NJ); JMC Puratronic grade $MnCl_2$ from Alfa Products (Danvers, MA); Hepes from Fluka; desferrioxamine mesylate (Desferal) from Sigma; boric acid and HPLC-grade methanol from EM Science; Chelex 100 from Bio-Rad; 5% CO_2/95% N_2 from M. G. Scientific (Buffalo Grove, IL); 20% CO_2/80% N_2 from Matheson Gas; 10% CO_2/90% N_2 and 15% CO_2/85% N_2 from Puritan Bennett (Charlotte, NC). All buffers were treated with Chelex 100 prior to use.

RESULTS

Kinetics of the Mn(II)-Dependent Reaction. Semilogarithmic plots of H_2O_2 concentration versus time, at various concentrations of either HCO_3^- or Mn(II), at different partial pressures of CO_2, and at various pH values, are all linear for at least 20 min (Fig. 1*C*). However, the data are consistent also with a second-order process since plots of $[H_2O_2]^{-1} - [H_2O_2]_0^{-1}$ versus time are also nearly linear (Fig. 1*A*).

Deviation from apparent first- or second-order kinetics with time could be due to a time-dependent loss of catalytic efficiency of the Mn(II)–bicarbonate complexes formed. To examine this possibility, the reaction was followed until the H_2O_2 was nearly all consumed. Then, the concentration of H_2O_2 in the reaction mixture was restored to the original level and the rate of O_2 production was again monitored. The apparent first-order rate of O_2 production following readjustments of the H_2O_2 concentration to the original level was about half that observed initially (data not shown). Thus, the loss of catalytic activity with time is most likely due to a decrease in the catalytic efficiency or concentration of the Mn(II) complex. Indeed, the time-dependent change in O_2 production can be described by either first- or second-order rate law with respect to H_2O_2 concentration, together with a first-order decay of catalytic efficiency (Fig. 1 *B* and *D*). Whereas this analysis could not differentiate between first- and second-order reaction mechanisms, discrimination between these possibilities was obtained by measuring the initial rate of O_2 production at various initial concentrations of H_2O_2. As shown in Fig. 2, the initial rate of O_2 production is directly proportional to the initial H_2O_2 concentration, which is consistent with a first-order process.

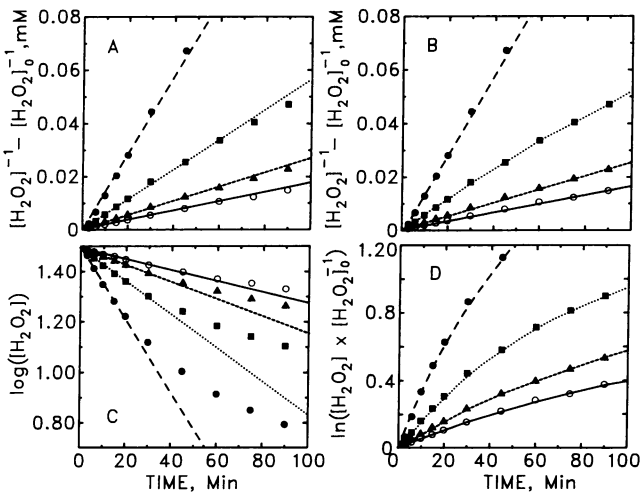

FIG. 1. Kinetic analysis for the time course of H_2O_2 consumption, measured by O_2 formation. Reactions were carried out in a Warburg apparatus as described in *Materials and Methods*. $[H_2O_2]_0$ = 31 mM; $[Mn(II)]_0$ = 0.05 mM; CO_2 was measured at 10% balanced by N_2. The reaction solution was buffered by HCO_3^-/CO_2 at pH 7.4, $[HCO_3^-]$ = 30 mM (○); pH 7.5, $[HCO_3^-]$ = 37 mM (▲); pH 7.55, $[HCO_3^-]$ = 42 mM (■); or pH 7.65, $[HCO_3^-]$ = 53 mM (●). Temperature was kept at 37°C. The data were analyzed as follows. (*A*) Second-order reaction with respect to $[H_2O_2]$. (*B*) Second-order reaction accompanied by a first-order inactivation of the catalyst, Mn–bicarbonate complex. (*C*) Semilogarithmic analysis for first-order reaction. (*D*) First-order reaction with a slow first-order inactivation of the Mn catalyst. The calculated lines in *A* and *C* were obtained using the rate constants computed by linear analysis of the first five data points on each line. The calculated lines in *B* and *D* were obtained with the rate constants determined by computer curve-fitting using an equation derived for a second-order reaction accompanied by a first-order inactivation of the catalyst, $[H_2O_2]^{-1} - [H_2O_2]_0^{-1} = k[Mn(II)]_0(1 - \exp(-k_i t))/k_i$, and for a first-order reaction accompanied by a first-order inactivation process, $\ln([H_2O_2]/[H_2O_2]_0) = k[Mn(II)]_0(1 - \exp(-k_i t/k_i))/k_i$, respectively. k and k_i are rate constants for the reaction (second-order rate constant for *B* and first-order rate constant for *D*) and for the inactivation process, respectively.

The apparent first-order rate constant k_{app} for H_2O_2 disproportionation is directly proportional to the Mn(II) concentration (Fig. 3) and is an exponential function of pH (Fig. 4*A*).

In bicarbonate/CO_2 buffers, the HCO_3^- concentration increases with increasing pH over the range 7.0–8.0, and at any given pH the HCO_3^- concentration is proportional to the partial pressure of CO_2 (11). Therefore, the effects of pH may be due to variations either in the H^+ concentration or in the HCO_3^- concentration, or both.

It is evident from the data in Table 1 and Fig. 4*B* that the effects of CO_2 and pH on the Mn-dependent decomposition of H_2O_2 are attributable to variations in HCO_3^- concentration alone. Thus, the concentration of HCO_3^- that is required to yield a given value of k is nearly the same (±10%), irrespective of variations in pH or in the partial pressure of CO_2 (Table 1). In fact, the value of k is a linear function of the HCO_3^- concentration raised to the third power (Fig. 4*B*), suggesting, among other possibilities, that 3 equivalents of HCO_3^- are coordinated with Mn to form the catalytic complex. Accordingly, a 2-fold increase in HCO_3^- concentration results in an 8-fold increase in the rate of H_2O_2 decomposition.

Effect of Desferrioxamine. The possibility that the above observations were due to the presence of trace amounts of Fe in the Mn(II) preparation used is contraindicated by the data in Fig. 5. Desferrioxamine inhibits completely the decomposition of H_2O_2 when Fe(II) is supplied as the divalent cation,

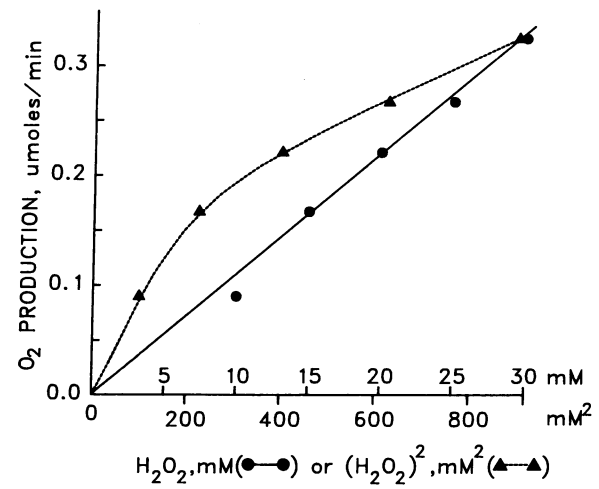

FIG. 2. Effect of H_2O_2 concentration on the initial rate of O_2 production. Mixtures containing 29.5 mM $NaHCO_3$ and 50 μM $MnCl_2$ in the main compartment of Warburg flasks were equilibrated with 5% CO_2/95% N_2 at 37°C. Reactions were started by mixing H_2O_2 in the sidearm with contents of the main compartment. O_2 produced was measured manometrically; the initial rate refers to the amount of O_2 produced during the first few minutes when the rate was apparently linear with time.

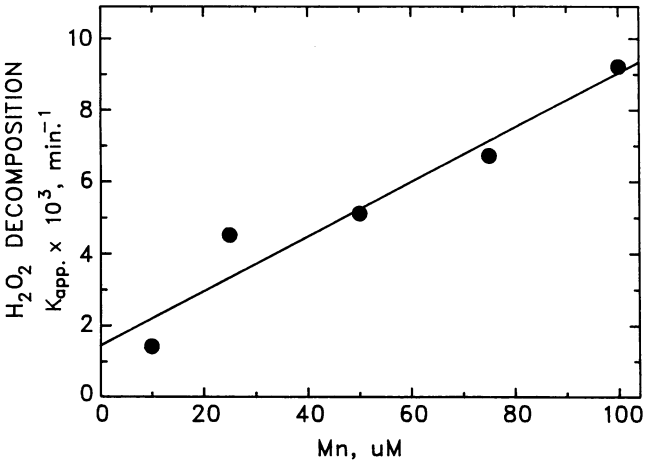

FIG. 3. Effect of Mn(II) concentration on the rate (k_{app}) of H_2O_2 decomposition. Reaction mixtures equilibrated with 5% CO_2/95% N_2 (pH 7.6) contained 23.5 mM $NaHCO_3$, 30 mM H_2O_2, and various amounts of Mn(II) as indicated. The half-times, $t_{0.5}$, for H_2O_2 decomposition used in calculations of the apparent first-order rate constant ($k_{app} = 0.693/t_{0.5}$) were estimated from the linear portion (≈20 min) of semilogarithmic plots of H_2O_2 concentration versus time.

but it stimulates the decomposition of H_2O_2 in the Mn(II)/HCO_3^- system.

DISCUSSION

The demonstration here that Mn(II) catalyzes the dismutation of H_2O_2 under physiological conditions (5% CO_2, 25 mM HCO_3^-) may account for its ability to replace catalase in preventing some superoxide dismutase-insensitive, metal ion-catalyzed oxidation reactions (3–5) and lends further support to the nation (1, 2, 8) that Mn(II) may aid in protecting cells against oxygen radical-mediated tissue damage: However, OH· and $O_2^{\cdot-}$ are generated from H_2O_2 in the presence of Mn/HCO_3^- mixtures, and amino acid-derived radicals are formed during the oxidation of amino acids by H_2O_2 in such mixtures (13). This serves notice that under some physiological conditions, Mn(II) might also contribute to oxygen-radical damage. In fact, the Mn(II)–bleomycin complex was shown to catalyze the cleavage of DNA in the presence of H_2O_2 (14). Mn–porphyrin complexes catalyze the cleavage of DNA in the presence of $O_2^{\cdot-}$-generating systems or in the

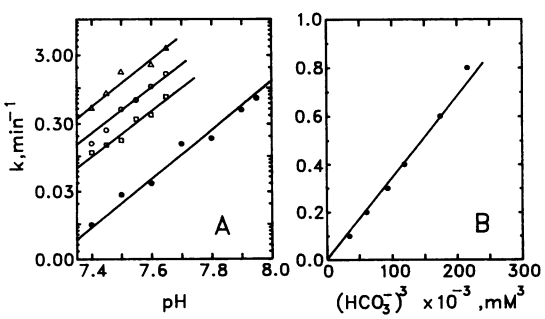

FIG. 4. Influence of pH and HCO_3^- concentration on the first-order rate constant for H_2O_2 decomposition. (*A*) Reaction mixtures contained initially 50 μM Mn(II), 30 mM H_2O_2, and various amounts of $NaHCO_3$ as needed to yield the pH values as indicated after equilibration with N_2 gas mixtures containing either 5% (●), 10% (□), 15% (○), or 20% (△) CO_2. The first-order rate constant, *k*, was calculated as described in Fig. 1. (*B*) The average concentrations of HCO_3^- (data from the last column in Table 1) raised to the third power were plotted against the corresponding *k* value (data from the first column in Table 1).

Table 1. Dependence of *k* on the HCO_3^- concentration, CO_2 partial pressure, and pH

	5% CO_2		10% CO_2		15% CO_2		20% CO_2		Average
k, min^{-1}	pH	HCO_3^-, mM	pH	HCO_3^-, mM	pH	HCO_3^-, mM	pH	HCO_3^-, mM	HCO_3^-, mM
0.1	7.71	29.7	7.43	31.2	7.35	39.5	7.10	29.5	32.5
0.2	7.78	35.2	7.51	37.5	7.43	47.0	7.21	37.8	39.4
0.3	7.84	40.6	7.55	41.9	7.47	51.9	7.30	47.1	45.4
0.4	7.88	44.0	7.56	42.4	7.50	55.9	7.37	55.6	49.2
0.6	7.93	49.3	7.63	50.3	7.55	62.3	7.42	61.2	55.8
0.8	7.96	52.9	7.66	54.2	7.58	66.0	7.46	66.8	60.0
1.0	7.99	56.9	7.69	57.4	7.60	70.0	7.48	70.8	63.8

For each partial pressure of CO_2, the pH required to obtain a given value of *k* shown in the first column was estimated (by interpolation) from the curves in Fig. 3*A*. The HCO_3^- concentration corresponding to each pH value was calculated by means of the Henderson–Hasselbalch equation, as described by Umbreit *et al.* (11).

presence of O_2 and ascorbate (15); and in nonaqueous media, Mn–porphyrin complexes catalyze the imidazole-dependent dismutation of H_2O_2 and the oxidation of alkanes and alkenes by H_2O_2 (16).

The dismutation of $O_2^{\cdot-}$ can be explained by the coupling of reactions **1** and **2** or reactions **1** and **3**. However, the decomposition of H_2O_2 by reaction **2** cannot proceed catalytically in the absence of a mechanism (not reaction **1**, which produces H_2O_2) for the regeneration of Mn(III). Perhaps the redox potential of a $Mn^{II}(HCO_3^-)_3$ complex is sufficient to permit reactions **6** and **7**; these, together with reaction **2**, would lead to redox cycling and the dismutation of H_2O_2.

$$2Mn(III) + H_2O_2 \rightarrow 2Mn(II) + O_2 + 2H^+ \quad [2]$$

$$H_2O_2 + Mn(II) \rightarrow OH\cdot + OH^- + Mn(III) \quad [6]$$

$$OH\cdot + Mn(II) \rightarrow Mn(III) + OH^- \quad [7]$$

$$\text{(sum)} \quad 2H_2O_2 \rightarrow O_2 + 2H_2O \quad [4]$$

Alternatively, H_2O_2 dismutation could occur by the coupling of reactions **6**, **8**, and **9**.

$$H_2O_2 + Mn(II) \rightarrow OH\cdot + OH^- + Mn(III) \quad [6]$$

$$OH\cdot + H_2O_2 \rightarrow O_2^{\cdot-} + H^+ + H_2O \quad [8]$$

$$O_2^{\cdot-} + Mn(III) \rightarrow Mn(II) + O_2 \quad [9]$$

$$\text{(sum)} \quad 2H_2O_2 \rightarrow O_2 + 2H_2O \quad [4]$$

The latter mechanism is consistent with the demonstration that $O_2^{\cdot-}$ and OH· are produced under our experimental conditions (13), but simultaneous operation of both mechanisms is possible.

From our data, the rate of H_2O_2 decomposition (R_{obs}) is described by the relationship $R_{obs} = k'[Mn][H_2O_2][HCO_3^-]^3$, where $k' = 3.5 \pm 1.5 \times 10^{-6}$ $mM^{-4}\cdot min^{-1}$. This suggests that 3 equivalents of HCO_3^- combine with Mn(II) to form the catalytic complex $Mn^{II}(HCO_3^-)_3$. Perhaps the redox potential of Mn in such a complex permits facile reaction with H_2O_2 to generate OH· and $O_2^{\cdot-}$ radicals that facilitate H_2O_2 dismutation. Complexation of Mn(II) with anions usually causes a decrease in the redox potential of the Mn(III) ⇌ Mn(II) couple (10). In any case, direct determination of the redox potential of Mn–bicarbonate complexes may help to explain the unique requirement for HCO_3^- in the decomposition of H_2O_2. Carbonate ion is required for the generation of luminescence associated with the oxidation of H_2O_2 by periodate (17) and for the luminescence produced during the aerobic oxidation of substances by xanthine oxidase at pH 10 (18).

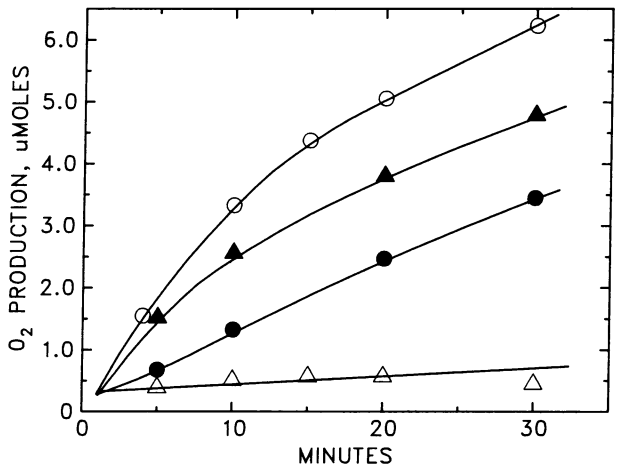

FIG. 5. Effect of desferrioxamine on the ability of Fe(II) and Mn(II) to catalyze the decomposition of H_2O_2 to O_2. Oxygen production was measured manometrically in a Warburg apparatus. Initially, the reaction mixtures contained 30 mM H_2O_2, 23.5 mM $NaHCO_3$, and either 20 μM Fe(II) (○); 20 μM Fe(II) plus 100 μM desferrioxamine (△); 50 μM Mn(II) (●); or 50 μM Mn(II) plus 100 μM desferrioxamine (▲). The gas atmosphere was 5% CO_2/95% N_2 (pH 7.6) at 37°C.

Because light emission varied as the square of the CO_3^{2-} concentration, it was suggested that OH· generated in these reactions reacts with CO_3^{2-} to form $CO_3^{\cdot-}$ radicals and that light emission is associated with dimerization of $CO_3^{\cdot-}$ radical (18). The ability of OH· to react with CO_3^{2-} and HCO_3^- is firmly established (19, 20).

$$OH\cdot + HCO_3^- \rightarrow H_2O + CO_3^{\cdot-} \quad [10]$$

Substitution of $CO_3^{\cdot-}$ for OH· in reaction **8** would lead to reaction **11**.

$$CO_3^{\cdot-} + H_2O_2 \rightarrow HCO_3^- + O_2^{\cdot-} + H^+ \quad [11]$$

The coupling of reactions **10, 6, 11,** and **9** could provide still another mechanism for the dismutation of H_2O_2. Whereas reactions **10** and **11** would be expected to occur under our experimental conditions (20, 21), it seems unlikely that they can account for the almost complete requirement for HCO_3^- in the Mn-catalyzed peroxidation reactions studied here. This follows from the consideration that $CO_3^{\cdot-}$ is most likely formed by the reaction of HCO_3^- with OH·. There is no reason to believe that the $CO_3^{\cdot-}$ radical generated by reactions with OH· would be more effective in promoting H_2O_2 decomposition than OH· alone (see also ref. 13). It is noteworthy that the $CO_3^{\cdot-}$ radical has been shown to react with $O_2^{\cdot-}$ to yield a more stable adduct, possibly CO_5^{2-}, which is presumed to undergo decomposition to form O_2 and CO_3^{2-} (20).

$$CO_5^{2-} \rightarrow O_2 + CO_3^{2-} \quad [12]$$

A role, if any, for such an intermediate in the decomposition of H_2O_2 is not evident.

Our results are generally similar to those of earlier studies by Sychev and coworkers (21–25). However, the Sychev group found the reaction to be second-order with respect to HCO_3^- concentration, whereas our results are more consistent with third-order kinetics. The studies of Sychev and coworkers were carried out in unbuffered solutions at 25°C, and during incubation, reaction mixtures were continuously titrated with perchloric acid to maintain the pH at a fixed level, and the concentration of HCO_3^- at a given pH was calculated using known values for the dissociation constant of $H_2CO_3 \rightleftharpoons HCO_3^- + H^+$ at equilibrium. Whether the rise in pH observed under their experimental conditions could be accounted for by the further decomposition of carbonic acid (i.e., $H_2CO_3 \rightleftharpoons H_2O + CO_2$) and concomitant loss of CO_2 to the atmosphere is not evident from the description of their experimental conditions. In our studies, changes in pH and the associated changes in HCO_3^- concentration were prevented by carrying out the reaction in bicarbonate/CO_2 buffers.

The potential biological importance of HCO_3^- in Fenton-type chemistry is underscored by the fact that this ion stimulates also the Fe-catalyzed disproportionation of H_2O_2 (25, 26) and the peroxidation of amino acids (26). In contrast to the Fe/HCO_3^- system, the reactions catalyzed by the Mn/HCO_3^- system are strongly inhibited by Hepes, inorganic phosphate, and inorganic pyrophosphate buffers (data not shown). It may be relevant that Tris and Hepes buffers inhibit the oxygen radical-mediated modification of tryptophan residues in proteins exposed to ^{60}Co irradiation, whereas bicarbonate buffers enhanced the rate of such protein damage (27).

The demonstration that several nonheme catalases possess more than one Mn atom per subunit (28) has led to the proposition that the disproportionation of H_2O_2 involves redox cycling between Mn species in binuclear or polynuclear complexes (28, 29). This has stimulated interest in the synthesis and properties of binuclear Mn complexes as models for the biologically active systems and the demonstration that Mn in such complexes may undergo redox cycling between Mn(II), Mn(III), and Mn(IV) (29–31). Results of these studies raise the possibility that Mn(IV) in addition to Mn(II) and Mn(III) may be implicated in the Mn-catalyzed H_2O_2 disproportionation and ligand peroxidation reactions. Since most studies implicating Mn(IV) were carried out under nonphysiological conditions, the results may not apply to the Mn/HCO_3^- system studied here. A role for Mn(II) in the HCO_3^--dependent dismutation of H_2O_2 has been postulated, but efforts to demonstrate such a role appear inconclusive (23).

Within the physiological pH range, the concentration of HCO_3^- in plasma can vary between 27.2 and 28 mM, and in cell water between 19.6 and 21.3 mM (ref. 32, p. 169). At concentrations of Mn found in plasma (2.6–3.6 μM; ref. 32, p. 21), the rate of H_2O_2 decomposition would be relatively small. Nevertheless, since the catalase-like activity of Mn is greatly augmented by the presence of amino acids and is extremely sensitive to small changes in HCO_3^- concentration, local variations in these parameters and in the intracellular distribution of Mn(II) may permit Mn to assume an important physiological role in oxygen-radical metabolism.

Fridovich and Archibald (1, 8) have noted that Mn complexes can substitute for superoxide dismutase in preventing oxygen toxicity in cells deficient in the enzyme. However, for complete protection, the H_2O_2 formed in the dismutation of $O_2^{\cdot-}$ must also be destroyed. The catalase-like activity of Mn-complexes may, together with the superoxide dismutase-like activity of such complexes, offer more complete protection against oxygen toxicity.

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