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# Bicarbonate-Mediated Peroxidase Activity of the Manganese(II)-Gluconate Complex

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A influência de íons bicarbonato na atividade peroxidásica do sistema manganês(II)-gluconato foi investigada, a 303,0 K e pH = 10, visando detectar sua possível mediação na geração de espécies reativas de oxigênio. Experimentos cinéticos, com monitoramento espectrofotométrico do peróxido de hidrogênio remanescente em solução, indicaram um mecanismo radicalar da reação, ao contrário do verificado na ausência desses íons. Um aumento significativo na velocidade de oxidação do ligante e de substratos adicionados, como etilenoglicol, foi observado: $k_{obs} = (0,99 \pm 0,05)\ 10^{-3}$ e $(2,42 \pm 0,11)\ 10^{-3}\ s^{-1}$, na ausência e na presença de bicarbonato, respectivamente. Uma dependência de pseudo-primeira ordem, seguida de um efeito de saturação para $[HCO_3^-] > 0,02$ mol $dm^{-3}$, também foram verificados. Através de ressonância paramagnética eletrônica (RPE), foi possível detectar a presença de radicais hidroxila, com o uso de captadores de spin.

The effect of bicarbonate ions on the catalytic activity of the dimeric manganese(II)-gluconate complex was observed. Kinetic experiments, by monitoring the remaining peroxide in alkaline solution, at 303.0 K and pH = 10, showed an increase in the rate constant: $k_{obs} = (0.99 \pm 0.05)\ 10^{-3}$ and $(2.42 \pm 0.11)\ 10^{-3}\ s^{-1}$, respectively, in the absence and in the presence of bicarbonate. A pseudo-first order dependence on $[HCO_3^-]$ was observed, followed by a saturation effect at concentrations higher than 0.02 mol $dm^{-3}$. Evidence for the presence of hydroxyl radicals was obtained by EPR measurements, using the spin-trapping method, which indicated a radicalar mechanism, in contrast to previous results in the absence of bicarbonate ions.

**Keywords:** *manganese(II)-gluconate complex, oxygen reactive species, peroxidase activity*

## Introduction

Transition metal-promoted radical reactions constitute an emergent field in modern synthetic organic chemistry, where titanium-, vanadium-, manganese-, iron-, cobalt-, and copper-mediated reactions are used in carbon-carbon bond formation[1]. However, oxidative cleavage of these bonds frequently occurs when transition metal ions catalyze reactions with hydrogen peroxide, or molecular oxygen[2], especially when radical intermediate species are involved.

Hydroxylated ligands, such as sorbitol, gluconate, or mannitol, can form stable complexes with manganese ions, in different oxidation states[3], which further participate in the catalysis of the oxidation processes. Gluconate complexes have been described as attractive model compounds for biological systems[4], since they can apparently resist irreversible oxidation[5,6]. A monomeric Mn(III)-gluconate complex was reported, exhibiting a catalase activity, by a nonradical mechanism, through the formation of a peroxo-Mn(III)-complex[7].

On the other hand, a manganese-sorbitol complex was used as the redox catalyst to promote the high-pH autoxidation of hydrazine, in a chain reaction propagated by hydrogen peroxide, and oxy radicals[8]. In addition, the oxidation of amino acids by $H_2O_2$, catalyzed by Mn(II)-bicarbonate complexes[9], was observed with the formation of a transient "caged" hydroxyl radical in the inner coordination sphere of Mn(II). Both superoxide and hydroxyl radicals were detected by EPR measurements, using the spin-trapping method. Evidence for the presence of hydroxyl radicals was also obtained in the oxidation of ethylene glycol by $H_2O_2$, using the same Mn(II)-bicarbonate catalytic system[10].

We recently investigated the catalase and the peroxidase activity of the dimeric Mn(II)-gluconate complex, $[Mn_2(GH_3)_4(\mu\text{-}OH_2)_2]^{4-}$ ($GH_3$ = bidentate gluconate ligand), in strong alkaline solution, in the presence of a large excess of ethylene glycol[11]. The oxidative cleavage of both the ligand and the substrate was verified, with the formation of formaldehyde, although neither hydroxyl nor superoxide radicals were detected, when the reaction was carried out under strictly anaerobic conditions. However, when the pH was adjusted with a carbonate-bicarbonate buffer an appreciable increase in the product formation was observed.

In order to verify the influence of the bicarbonate ions on the catalytic activity of the manganese(II)-gluconate complex, we carried out further kinetic experiments at pH 10, controlled by a carbonate-bicarbonate buffer. Under these experimental conditions, the generation of oxygen-free radicals was detected, indicating a different mechanism for the reaction with a higher rate constant.

## Materials and Methods

Hydrogen peroxide, 35% in weight and free from stabilizers, was kindly supplied by Peróxidos do Brasil Ltda. Solutions of this reagent were prepared by dilution, and analyzed spectrophotometrically as described below. The sodium salt of D-gluconic acid was purchased from Aldrich, and $MnCl_2.4H_2O$ from Merck. Manganese ions were analyzed in stock solutions by titration with edta[12]. Ethylene glycol, from Oxiteno or Labsynth, was purified by distillation under reduced pressure as recommended[13]. Other chemicals from different sources and of analytical grade were used as supplied. Nanopure deionized water was used in the preparation of all solutions.

In the EPR measurements, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, 0.1 mol $dm^{-3}$) was used as the spin-trap, previously purified by filtration through charcoal[14]. EPR spectra were obtained in a Bruker ER 200D-SRC spectrometer, with a 20 MW microwave power, and a scan rate of 0.1 G $s^{-1}$, using a standard flat-faced quartz cell.

Hydrogen peroxide was monitored in the reaction mixture through the metavanadate method[15,16] based on the formation of a vanadium(V)-peroxo complex, with a maximum absorbance at 454 nm ($\varepsilon = 360$ $mol^{-1}$ $dm^3$ $cm^{-1}$). All measurements were carried out at (303.0 ± 0.5) K, and pH = 10, adjusted with a carbonate-bicarbonate buffer. The ethylene glycol substrate was added, under nitrogen atmosphere, to a solution of the dimeric manganese(II)-gluconate complex, $[Mn_2(GH_3)_4(\mu\text{-}H_2O)_2]^{4-}$ ($GH_3$ = bidentate gluconate ligand), prepared by dilution of aliquots of the manganese(II) stock solution with 0.100 mol $dm^{-3}$ of sodium gluconate[11]. The exclusion of oxygen was necessary since the manganese(II) catalyst reacts much more quickly with oxygen than with the peroxide in alkaline solution, forming the apparently inactive Mn(IV) complex[3]. The reaction was initiated by the addition of hydrogen peroxide.

The kinetic analysis was based on the experimental rate law as a function of the total ligand and total metal concentrations, assuming that all manganese is complexed by gluconate. The rate constants were obtained from at least four determinations within ±5% of each other.

In the product analysis, solutions of 2,4-dinitrophenylhydrazine were prepared by dissolving 2.0 g of the reagent (2,4-DNPH, from Aldrich) in 100 mL of methanol, adding 4 mL of concentrated sulfuric acid, and heating the solution.

The formation of carbonyl compounds in the reaction was verified by the precipitation of the corresponding hydrazones with 2,4-DNPH. After 2 hours of reaction, 15 mL of the reaction mixture ($10^{-3}$ mol $dm^{-3}$ $[Mn(II)]_T$, $10^{-2}$ mol $dm^{-3}$ $[H_2O_2]$, 0.100 mol $dm^{-3}$ $[Gluc]_T$, and 1.0 mol $dm^{-3}$ [ethylene glycol]) were mixed with 20 mL of the 2,4-DNPH stock solution. The obtained precipitate was then filtered, dried, redissolved in acetone, and analyzed by gas chromatography, coupled to mass spectrometry. In these experiments a Varian GC-3400 instrument was used, coupled to a INCOS 50 mass spectrometer.

Formaldehyde was also detected by the specific Hantsch reaction[17]. 10.0 mL of the reaction solution were added to 10.0 mL of the Nash reagent (acetylacetonate 0.02 mol $dm^{-3}$, and acetic acid 0.05 mol $dm^{-3}$, in 2.0 mol $dm^{-3}$ ammonium acetate). The formation of 3,5-diacetyl-1,4-dihydrolutidine was then monitored at 412 nm, after heating the mixture to 60 °C for 5 min.

## Results

### *Kinetic measurements*

In all the kinetic measurements, a total concentration of gluconate, $[Gluc]_T = 0.100$ mol $dm^{-3}$, and ethylene glycol, [RH] = 1.00 mol $dm^{-3}$ were used. Plots of ln $[H_2O_2]$ *vs.* time indicate a first-order dependence on the peroxide concentration, as shown in Fig. 1. The calculated rate constant, considering more than three half-lives, was $k_{obs}$ = (2.42 ±

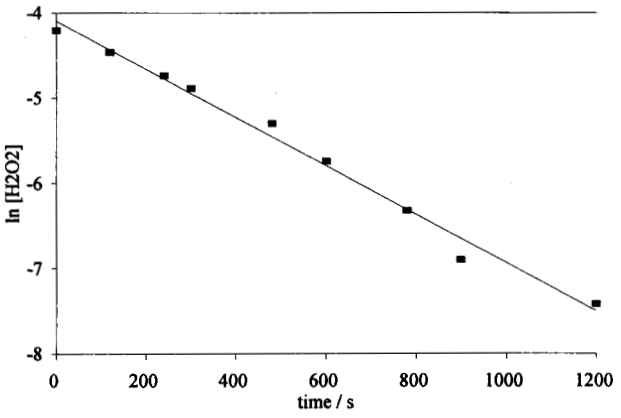


**Figure 1.** Plot of ln $[H_2O_2]$ *vs.* time. $[H_2O_2] = 1.50 \times 10^{-2}$ mol $dm^{-3}$, $[Mn]_T = 7.53 \times 10^{-4}$ mol $dm^{-3}$, $[Gluconate]_T = 0.100$ mol $dm^{-3}$, and [Ethylene glycol] = 1.00 mol $dm^{-3}$. Reaction at (303 ± 0.5) K, under nitrogen, and pH = 10 (bicarbonate buffer 0.016 mol $dm^{-3}$).

0.12) $10^{-3}$ $s^{-1}$, in a 0.016 mol $dm^{-3}$ carbonate-bicarbonate buffer (pH = 10), at (303.0 ± 0.5) K. The corresponding value in the absence of bicarbonate ions was (0.99 ± 0.05) $10^{-3}$ $s^{-1}$.

A second-order rate constant of (3.94 ± 0.20) $mol^{-1}$ $dm^3$ $s^{-1}$ was determined through the dependence of $k_{obs}$ on the total manganese concentration, $[Mn]_T$ (Fig. 2). This value is *ca.* 3 times higher than the corresponding value observed without bicarbonate ions[11], k = (1.41 ± 0.07) $mol^{-1}$ $dm^3$ $s^{-1}$.

An inhibitory effect with the increasing [gluconate] concentration was also observed, as shown in Fig. 3. By plotting the reciprocal of the rate constant *vs.* [gluconate] a straight line was obtained, according to the expression: $1/k_{obs} = 1.34 \times 10^2 + 3.55 \times 10^3 [Gluc]_T$. A similar inhibitory effect was recently observed in a synthetic mimic of the Mn-catalase enzyme, where a μ-carboxylate ligand dissociates in an initial hydration equilibrium[18].

In addition, a pseudo-first order dependence on the bicarbonate buffer concentration was observed (Fig. 4), followed by a saturation effect at $[HCO_3^-] > 0.02$ mol $dm^{-3}$. An analogous influence was verified in the concentration of the formaldehyde product, formed by the oxidative cleavage of carbon-carbon bonds in the gluconate ligand, and in the added ethylene glycol substrate, as shown in Table 1. An appreciable increase in the formation of formaldehyde was observed in the presence of bicarbonate ions. In the absence of these ions, a concentration of (16.1 ± 0.5) $10^{-6}$ mol $dm^{-3}$ formaldehyde was determined. A maximum concentration of this product was observed at 30 min of reaction (Table 2), probably due to further oxidation to formate, although these ions were not detected in the reaction solution[11].

The reaction rate was observed to be independent of the substrate concentration as shown in Fig. 5, with a rate constant value $k_{obs}$ = (2.3 ± 0.2) $10^{-3}$ $s^{-1}$, in the range of [ethylene glycol] = 0 to 1.00 mol $dm^{-3}$. On the contrary, a

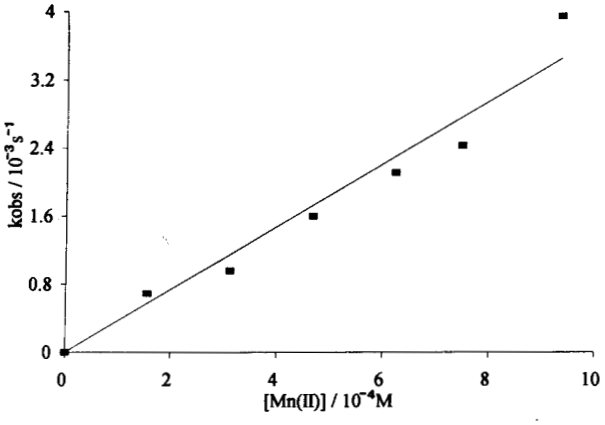


**Figure 2.** Dependence of $k_{obs}$ on the total manganese concentration. Reaction at (303 ± 0.5) K, under nitrogen, and pH = 10 (bicarbonate buffer 0.016 mol $dm^{-3}$). $[H_2O_2] = 3.00 \times 10^{-2}$ mol $dm^{-3}$, $[Gluconate]_T$ = 0.100 mol $dm^{-3}$, and [Ethylene glycol] = 1.00 mol $dm^{-3}$.

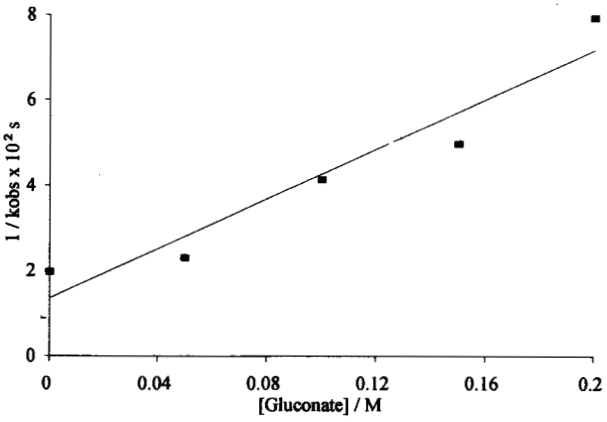


**Figure 3.** Curve of the rate constant reciprocal with the total gluconate concentration. Reaction at (303 ± 0.5)K, under nitrogen, and pH = 10 (bicarbonate buffer 0.016 mol $dm^{-3}$). $[H_2O_2] = 3.00 \times 10^{-2}$ mol $dm^{-3}$, $[Mn]_T = 7.53 \times 10^{-4}$ mol $dm^{-3}$, and [Ethylene glycol] = 1.00 mol $dm^{-3}$.

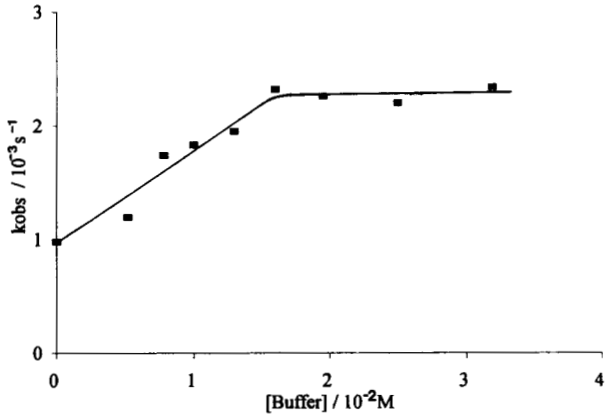


**Figure 4.** Influence of the bicarbonate ion concentration on the rate constant of hydrogen peroxide consumption. Reaction at (303 ± 0.5) K, under nitrogen, and pH = 10 (bicarbonate buffer). $[H_2O_2] = 3.00 \times 10^{-2}$ mol $dm^{-3}$, $[Mn]_T = 7.53 \times 10^{-4}$ mol $dm^{-3}$, $[Gluconate]_T$ = 0.100 mol $dm^{-3}$, and [Ethylene glycol] = 1.00 mol $dm^{-3}$.

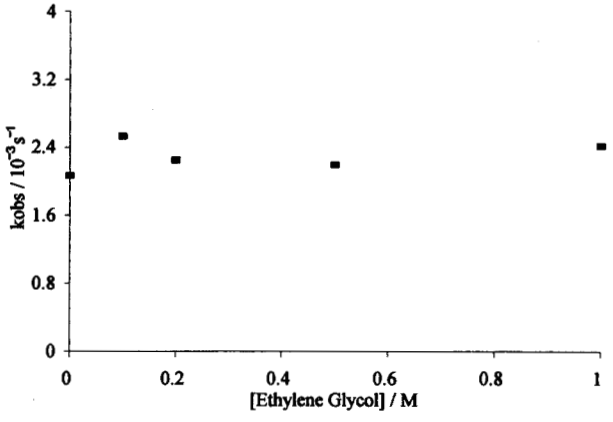


**Figure 5.** Dependence of $k_{obs}$ on the concentration of ethylene glycol. Reaction at (303 ± 0.5)K, under nitrogen, and pH = 10 (bicarbonate buffer 0.016 mol $dm^{-3}$). $[H_2O_2] = 3.00 \times 10^{-2}$ mol $dm^{-3}$, $[Mn]_T = 7.53 \times 10^{-4}$ mol $dm^{-3}$, and $[Gluconate]_T$ = 0.100 mol $dm^{-3}$.

**Table 1.** Dependence of the determined formaldehyde on the concentration of the used carbonate-bicarbonate buffer. Reaction at (303 ± 0.5) K, under nitrogen, and pH = 10 (bicarbonate buffer 0.016 mol $dm^{-3}$). $[H_2O_2] = 9.00 \times 10^{-2}$ mol $dm^{-3}$, $[Mn]_T = 7.53 \times 10^{-4}$ mol $dm^{-3}$, $[Gluconate]_T$ = 0.100 mol $dm^{-3}$, and [Ethylene glycol] = 1.00 mol $dm^{-3}$.

| [formaldehyde], μ mol $dm^{-3}$ | $[HCO_3^-]$, mol $dm^{-3}$ |
|---|---|
| 16.1 ± 0.5 | 0 |
| 22.8 ± 1.0 | 0.016 |
| 22.7 ± 1.0 | 0.032 |
| 22.8 ± 1.0 | 0.050 |

**Table 2.** Determination of formaldehyde at different times of reaction. Reaction at (303 ± 0.5) K, under nitrogen, and pH = 10 (bicarbonate buffer 0.016 mol $dm^{-3}$). $[H_2O_2] = 9.00 \times 10^{-2}$ mol $dm^{-3}$, $[Mn]_T = 7.53 \times 10^{-4}$ mol $dm^{-3}$, $[Gluconate]_T$ = 0.100 mol $dm^{-3}$, and [Ethylene glycol] = 1.00 mol $dm^{-3}$. Formaldehyde analyzed spectrophotometrically (= 412 nm), by the Hantsch reaction[17].

| [formaldehyde], μ mol $dm^{-3}$ | time, min |
|---|---|
| 0 | 0 |
| 45.3 ± 3.0 | 30 |
| 32.1 ± 2.0 | 60 |
| 29.3 ± 1.5 | 90 |
| 22.8 ± 1.0 | 120 |
| 25.9 ± 1.0 | 180 |

pseudo-first order dependence on the ethylene glycol concentration was verified in the absence of bicarbonate.

*Detection of radical intermediates and identification of products*

By using the spin-trapping method, it was possible to detect the presence of hydroxyl radicals in the reaction solution, through the observation of the EPR spectra of the DMPO-OH adduct (Fig. 6) under strictly anaerobic conditions. The signals corresponding to this adduct increased in the first 20 minutes, and then rapidly decreased. They were detected as broad lines, probably indicating the simultaneous presence of a superoxide anion, or of carbon-centered radicals, whose adduct parameters are larger than those of the hydroxyl derivative[19]. For the $\bullet CH_2OH$ radical, for example, $A_N$ = 16.0, and $A_H$ = 22.7 G, while for hydroxyl radicals $A_N = A_H$ = 15.0 G.

In contrast, similar experiments in the absence of bicarbonate ions showed only traces of oxygen reactive radicals[11], although they could be detected if oxygen was admitted into the reaction mixture.

By precipitation of the corresponding 2,4-DNP hydrazones, after 2 hours of reaction, it was possible to identify formaldehyde, and predominantly glycolaldehyde as products of the oxidation of ethylene glycol. Formaldehyde is also formed from the oxidative degradation of the ligand

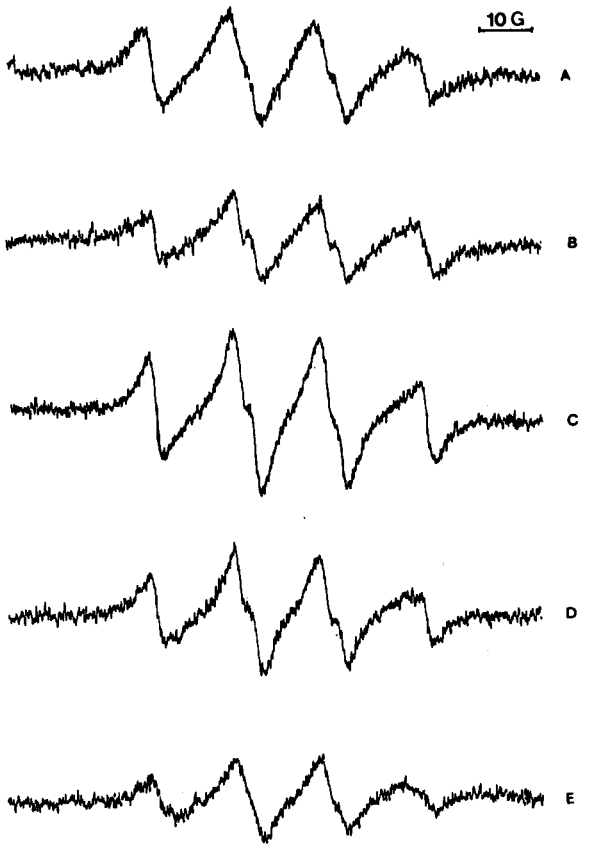


**Figure 6.** EPR spectra of the DMPO-OH adduct formed at the earlier stages of reaction. $[H_2O_2] = 3.00 \times 10^{-2}$ mol $dm^{-3}$, $[Mn]_T = 7.53 \times 10^{-4}$ mol $dm^{-3}$, $[Gluconate]_T$ = 0.100 mol $dm^{-3}$, and [Ethylene glycol] = 1.00 mol $dm^{-3}$. Reaction at (303 ± 0.5) K, under nitrogen, and pH = 10 (bicarbonate buffer 0.016 mol $dm^{-3}$). Spectra after: (A) 3, (B) 15, (C) 20, (D) 30, and (E) 40 min of reaction.

gluconate, as previously detected[11], and was determined by the specific Hantsch reaction, through the formation of a chromophore derivative[17] (Tables 1 and 2).

Gas chromatograms of acetone-solutions of the obtained hydrazone precipitate exhibited peaks at 8 and 17 min, corresponding to molecular weights of 210 and 238 g/mol, identified with the help of mass spectrometry as the 2,4-DNP derivatives of formaldehyde, and glycolaldehyde, respectively.

## Discussion

In contrast to previous results[11], the kinetic data obtained in the carbonate-bicarbonate buffer indicate a radicalar mechanism, with the generation of hydroxyl radicals in the earlier stages of the reaction. In this medium, the formation of formaldehyde, a product resulting from the cleavage of carbon-carbon bonds in the gluconate ligand and in the ethylene glycol substrate, was considerably increased.

An alternative mechanism was therefore suggested, to explain the bicarbonate-mediated catalytic activity of the manganese(II)-gluconate complex. In addition to the pre-

viously proposed nonradical mechanism[11], involving two-electron transfer reactions, and the formation of a peroxo-Mn(III) complex:

$$[Mn^{II}_2L_4(H_2O)_2]^{4-} + H_2O_2 \xrightarrow{\text{rapid}} [Mn^{III}_2L_4(OH)_2]^{4-} + 2\,H_2O$$

$$[Mn^{III}_2L_4(OH)_2]^{4-} + 2\,H_2O \overset{K1}{\rightleftharpoons} [Mn^{III}_2L_3(OH)_3(H_2O)]^{3-} + HL^-$$

$$[Mn^{III}_2L_3(OH)_3(H_2O)]^{3-} + H_2O_2 \xrightarrow{k} [Mn^{III}_2L_3(OOH)(OH)_2(H_2O)]^{3-} + H_2O$$

$$[Mn^{III}_2L_3(OOH)(OH)_2(H_2O)]^{3-} \xrightarrow{k1} O_2 + [Mn^{II}_2L_3(OH)(H_2O)_2]^{3-}$$

$$[Mn^{III}_2L_3(OOH)(OH)_2(H_2O)]^{3-} + RH \xrightarrow{k2} R_{ox} + [Mn^{II}_2L_3(OH)(H_2O)_2]^{3-}$$

$$[Mn^{II}_2\,L_3(OH)(H_2O)_2]^{3-} + HL^- \xrightarrow{\text{rapid}} [Mn^{II}_2L_4(H_2O)_2]^{4-} + H_2O$$

(where L = gluconate dianion, RH = gluconate or ethylene glycol, and $R_{ox}$ = oxidized products) and leading to the rate law:

$$\frac{-d[H_2O_2]}{dt} = \frac{(k_1 + k_2[RH])\,k\,K_1[H_2O_2]\,[Mn]_T}{2\,([L]_T + K_1)}$$

a simultaneous radicalar mechanism may be operating. In this case, the observed generation of oxygen radical species, and probably also of carbon-centered radicals, should occur by one-electron transfer steps. Therefore, this would lead to the mixed-valence $Mn^{II}Mn^{III}$ state, which has been characterized in model systems[20], as well as in the dimanganese catalase enzyme[21], although in the enzymatic system only the totally reduced $Mn^{II}Mn^{II}$ and the oxidized $Mn^{III}Mn^{III}$ states seem to be implicated in the catalase mechanism.

Schematically, the following one-electron transfer steps are suggested:

$$[Mn^{II}_2L_4(H_2O)_2]^{4-} + H_2O_2 \longrightarrow [Mn_2II,IIIL_4\,(OH)(H_2O)]^{4-} + \bullet OH + H_2O$$

$$\bullet OH + HCO_3^- \longrightarrow CO_3^{\bullet-} + H_2O \qquad k' = 3.65 \times 10^8\ mol^{-1}\ dm^3\ s^{-1}$$

$$CO_3^{\bullet-} + H_2O_2 \longrightarrow HCO_3^- + O_2^{\bullet-} + H^+ \qquad k'' = 8 \times 10^5\ mol^{-1}\ dm^3\ s^{-1}$$

$$CO_3^{\bullet-} + [Mn_2II,IIIL_4(OH)(H_2O)]^{4-} \longrightarrow [Mn^{III}_2L_4(OH)_2]^{4-} + HCO_3^-$$

$$O_2^{\bullet-} + H^+ + [Mn_2II,IIIL_4(OH)(H_2O)]^{4-} \longrightarrow [Mn^{II}_2L_4(H_2O)_2]^{4-} + O_2$$

The generation of oxygen radicals in this system is certainly facilitated by the high concentrations of bicarbonate ions present in the solution. The ability of •OH radicals to react with $HCO_3^-$ ions is firmly established[22]. The rate constants for the radicalar steps (k', and k''), determined by flash photolysis and pulse radiolysis, are much higher than those for the rate determining steps involving the manganese species. Similar to the observed oxidation of amino acids catalyzed by the Mn(II)-bicarbonate complex[23], "caged" hydroxyl radicals could be formed in close proximity to the coordinated gluconate or to a substrate moiety, and would preferentially abstract a hydrogen atom to form a carbon-centered radical, increasing the peroxidase activity of the system. Therefore, induced monoelectronic transfers would lead to the oxidation of the substrates, in a concerted mechanism, depending on Mn(III), and facilitated by the presence of bicarbonate ions:

$$Mn^{3+}(HO\text{-}C\text{-}C\text{-}OH) \longrightarrow Mn^{2+}(HO\text{-}C^{\bullet}\text{-}C\text{-}OH) + H^+$$

$$Mn^{2+}(HO\text{-}C^{\bullet}\text{-}C\text{-}OH) + CO_3^{\bullet-}\ (^{\bullet}OH) \longrightarrow Mn^{2+} + HOCH_2\text{-}CHO + HCO_3^-\ (H_2O)$$

$$\text{or } Mn^{2+}(HO\text{-}C\text{-}C\text{-}OH) + CO_3^{\bullet-} \longrightarrow Mn^{2+} + H_2C{=}O + -\bullet C\text{-}OH + HCO_3^-$$

$$-\bullet C\text{-}OH + {}^{\cdot}OH\,. \longrightarrow H_2C{=}O + H_2O$$

These steps occur as a substitute for the previously suggested nonradical reaction:

$$[Mn^{III}_2L_3(OOH)(OH)_2(H_2O)]^{3-} + RH \longrightarrow R_{ox} + [Mn^{II}_2L_3(OH)(H_2O)_2]^{3-}$$

This mechanism can explain the verified dependence on $HCO_3$- concentration, and the independence of the rate law on the concentration of ethylene glycol. The radical mechanism is not predominant, as attested to by the low yield of oxidized products. However, in the presence of bicarbonate ions, one-electron transfer reactions are favored, and a significant increase in the oxidation of both the ligand and the added substrate was observed.

## Conclusions

The peroxidase activity of the dimeric manganese(II)-gluconate complex was shown to be facilitated, in the presence of bicarbonate ions, by a simultaneous radicalar mechanism. In contrast to previous results obtained in the absence of these ions, the generation of oxygen reactive species was detected by EPR measurements, using DMPO as a spin-trap. An increase in the rate constant and in the formaldehyde formation, as a result of the oxidative cleav-

age of both the ligand and the ethylene glycol, was also verified.

The catalytically active species are a dimeric Mn(III)-complex, and probably "caged" hydroxyl radicals formed in a rate determining step. By interaction with the excess bicarbonate ions in solution, carbonate radical anions $CO_3^{\bullet-}$ are formed, which propagate the chain reaction.

## Acknowledgments

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