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Kinetic studies on the manganese(II) complex catalyzed oxidation of epinephrine

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ABSTRACT

The manganese complex $[Mn_2(HL)_2](BPh_4)_2$ was found to be a selective catalyst for the oxidation of epinephrine (a catecholamine derivative) to adrenochrome at room temperature in sodium carbonate–bicarbonate buffer. The epinephrine auto-oxidation rate significantly increases upon the addition of manganese complex. The kinetics of reaction was studied by spectrophotometric method, monitoring the increase in concentration of adrenochrome product. According to the proposed mechanism, O_2 binding to the manganese complex is followed by the formation of a ternary catalyst–dioxygen–substrate complex as active intermediate, which decomposes in a rate-determining step, generating the adrenochrome.

1. Introduction

The catalytic activation of dioxygen by transition metal complexes has been attracting interest because of its relevance to biological oxidation and elucidation of the reaction mechanism *via* structural or functional modelling of oxidoreductase enzymes. One of the most important substrate groups are catechol and phenol derivatives. Catechol oxidase (EC 1.10.3.1) catalyzes the oxidation of catechol to *o*-quinone and it is involved in the formation of melanin pigments and in the enzymatic browning of fruits [1–3]. Extensive research was done to elucidate the reaction mechanism of copper, iron, cobalt and manganese complexes as functional models systems [4–9].

Related to catecholase activity is the ability of tyrosinase (EC 1.14.18.1) to promote the oxidation of epinephrine (also known as adrenaline, H_4A) to adrenochrome (AC). Adrenaline is produced in the medulla of the adrenal glands and plays an important role as neurotransmitter in the central nervous system. The reduction of catecholamine level caused by the exposure of cells to reactive oxygen species (ROS) contributes to Alzheimer's, Parkinson's and inflammation diseases. The catecholase oxidation reaction is applied in medical diagnosis for the determination of the hormonally active catecholamines [10]. In order to understand their physiological function, it is necessary to develop small molecular weight metal complexes for catechol oxidase as models, and different methods for determining catecholamines.

Various studies have assessed the acidity constants of the catecholamine derivatives by different methods such as potentiometric [11] or spectrophotometric titrations and point-by-point analysis [12]. Several metal–adrenaline complexes and its chemical behavior have been studied by many research groups. It was found that the coordination of catecholamines (adrenaline and noradrenaline) with copper-, nickel-, manganese-, and zinc(II) ions is toward the phenolic groups in aqueous solution [13]. Gergely et al. [14] have determined the stability constants for the above mentioned catecholamine–metal complexes. At a metal ion:ligand ratio of 1:1 and at higher pH, the participation of the side-chain donor atoms in complex formation increase and a polynuclear complex may also be formed. In the presence of excess ligand, mainly the phenolic hydroxyl groups take part in the coordination [15].

Methods, such as chromatography [16], fluorescence [17], chemiluminescence [18], and capillary electrophoresis [19] are used to distinguish catecholamine derivatives. Chen and co-workers [20] have developed a rapid and sensitive new chemiluminescence method for the determination of catecholamines. Reactive oxygen species generated in potassium periodate – luminol system by gallic acid, acetaldehyde and manganese(II) were scavenged by adrenaline and dopamine. Another chemiluminescence method was proposed for the determination of adrenaline by Kohji et al. [21]. The light emission of the oxidation was increased after the addition of a manganese(II) catalyst.

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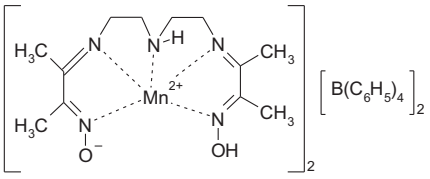
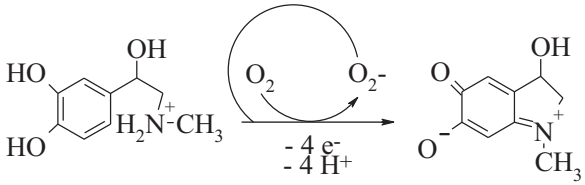


Scheme 1. Structure of the $[Mn_2(HL)_2](BPh_4)_2$ complex.



Scheme 2. Auto-oxidation reaction of adrenaline.

The determination of adrenaline and other catecholamines have been also investigated by numerous electroanalytical techniques [22–24]. The stability constants of lanthanum(III) [25] and yttrium(III) [26]–adrenaline complexes have been determined by potentiometric and spectrophotometric studies. In all the investigated La(III)–catecholamine systems, metals have been found to interact with the phenolic group, but one of the phenolate protons does not dissociate, as in the Y(III) complexes. The yttrium–catecholamine complexes show similar behavior as the calcium analogues, making them useful *in vitro* studies. Oxovanadyl(IV) can catalyze the oxidation of adrenaline [27] by forming a vanadyl-tris(adrenaline) species.

Under physiological conditions the auto-oxidation of adrenaline is very slow, however, its rate can be increased with pH or by catalysts [28,29]. Oxidation of adrenaline by different metal salts in phosphate buffer at pH 7.0 was studied by Chaix et al. [30]. They have observed that the formation of adrenochrome as product depends upon the formation of an adrenaline–metal complex. The maximal velocity of oxygen consumption in case of copper(II)-ion was observed at a ratio of Cu(II)/adrenaline of the order of ½, while manganese showed maximum at a concentration equal to that of adrenaline. Copper possessed the greatest catalytic activity in the oxidation of catecholamines among nickel and manganese; and iron possessed the least [31]. Futhermore manganese(III) was found more efficient catalyst than manganese(II) or manganese(IV) [32]. Iron(III) gives much more stable complexes with adrenaline, than with dopamine, especially the $[Fe(H_2L)]^{3+}$ species in acidic medium [33]. Intermediates of adrenaline oxidation catalyzed by ceric-, iron ions, permanganate, ferricyanide and ceruloplasmin were detected by electron paramagnetic resonance [34] and fluorescence spectrometric analysis [35].

Recently, new type of catalysts (mesoporous silica nanoparticles [36], metal porphyrin-immobilized anion-exchange resin [37]) and different transition metal complexes [38] have been developed as functional catecholase enzyme models for oxidation of adrenaline.

According to our recent findings, the dioximato-manganese(II) dimer $[Mn_2(HL)_2](BPh_4)_2$ (**1**) {where H_2L is the quinquedentate dioxime ligand $HON{=}C(CH_3)C(CH_3){=}N{-}CH_2CH_2)_2NH$} acts as a catecholase mimic in methanol, converting the 3,5-di-*tert*-butylcatechol to the corresponding *o*-quinone [39]. In MeOH solution the dimer **1** dissociates to monomeric form $[Mn(HL)]^+$ (**2**). We have now found that complex **1** (see Scheme 1) is also a functional model for catechol oxidase, converting adrenaline to adrenochrome. In the present paper the results of the kinetic study on this functional model system are presented.

2. Experimental

2.1. Materials

The ligand 3,3'-(3-aza-pentanediyldiimino)-bis-butan-2-one dioxime (H_2L) was prepared according to ref. [40]. The octahedral complex $[Mn_2(HL)_2](BPh_4)_2$ (**1**) was synthesized and characterized by a procedure developed by us [39].

Adrenaline and superoxide dismutase were purchased from Sigma–Aldrich (Hungary). A stock solution of adrenaline (9 mM) was prepared in 0.01 M hydrochloric acid and stored at 4 °C. 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO) was purchased from Sigma–Aldrich. Analytical reagent-grade chemicals were used for buffer preparation.

2.2. Methods

All experiments were done at 25 °C in 0.05 M sodium carbonate–bicarbonate buffer at pH 10.2. The spectrophotometric studies were performed using a Hewlett-Packard 8453 diode array UV–vis spectrophotometer thermostated at 25 °C with Grant LTD 6G circulating water bath. Kinetic measurements were carried out by spectrophotometric monitoring the increase in the concentration of the strongly absorbing adrenochrome product (λ = 480 nm, ε = 4020 M^{-1} cm^{-1}) [41] in a quartz cuvette with a 1 cm optical path.

In a typical experiment 0.1 ml of 5×10^{-4} M solution of manganese complex and 0.15 ml of 9×10^{-3} M solution of adrenaline substrate were mixed in carbonate buffer, for a final volume of 3 ml. Directly after addition of adrenaline, the UV–vis spectra were recorded.

EPR spectra of reacting solutions were recorded on a Bruker ELEXYS E500 CW-EPR spectrometer. Spectra were accumulated over the initial 30 min of the reaction time. Hydroxyl radical produced was identified by means of spin-trapping method with EPR detection. Simulations of the spectra were carried out using the program developed by Rockenbauer et al. [42].

3. Results and discussion

3.1. Oxidation of adrenaline

The auto-oxidation of adrenaline to adrenochrome is known to take place with increasing pH, as well as the conversion is a multistep process [43–45]. Misra et al. [28] have observed that at higher pH the mechanism involves a superoxide-mediated free radical chain reaction. Scheme 2 illustrates the oxidation process of adrenaline to adrenochrome.

The oxidation product adrenochrome exhibits a strong absorption band at 480 nm. The course of the auto-oxidation reaction was followed by UV–vis spectroscopy over the first 6 min. Fig. 1 shows the time evolution of the absorbance spectra of the oxidation of adrenaline to adrenochrome in carbonate–bicarbonate buffer.

The spectra reveal that, in the beginning of the reaction, the solution presents a band with maximum absorbance at 294 nm characteristic for adrenaline solution. The reactive species in this process is the superoxide ($O_2^{-\cdot}$), which occurs at high pH. It binds adrenaline affording the adrenaline semiquinone (245 nm), which then oxidizes further to the *o*-quinone (340 nm). The cyclization reaction of adrenaline-quinone is fast and the leucochrome oxidizes quickly to adrenochrome (480 nm). An additional band appears at 375 nm after 240 s corresponding to the adrenochrome tautomer.

The catalytic activity of the dioximato-manganese complex was examined in carbonate–bicarbonate buffer at 25 °C. The catalytic solution was prepared by mixing 5.00×10^{-4} M methanol solution of **1** with 0.05 M carbonate buffer (methanol gave 3.3% of the final volume). When 1.67×10^{-5} M solution of complex was added to a

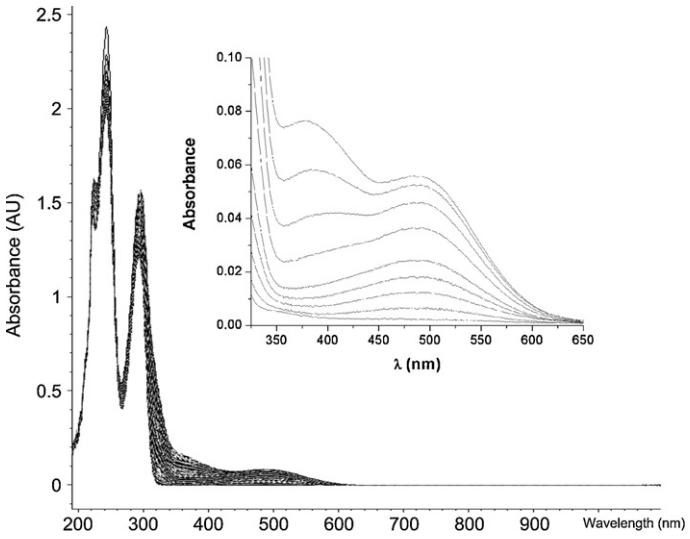


Fig. 1. Auto-oxidation of adrenaline. Time dependent spectra of adrenaline auto-oxidation at pH 10.2, $[H_4A]_o = 3.00 \times 10^{-4}$ M, $T = 25\,^{\circ}C$. Spectra 1–5 (from bottom up) were taken at 30 s and spectra 5–9 at 60 s intervals.

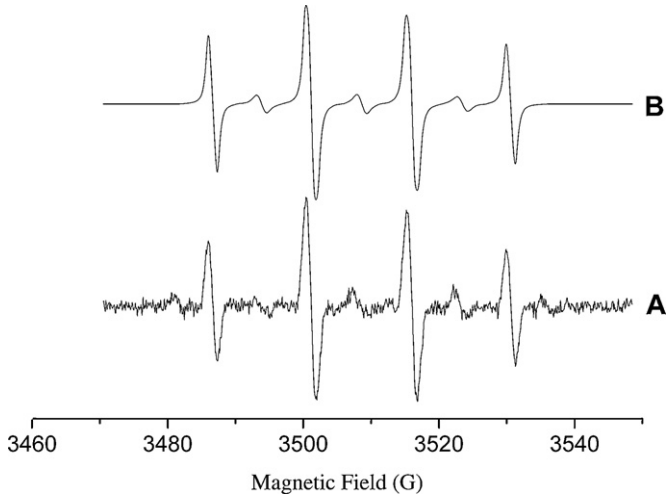


Fig. 3. EPR spectra EPR spectra of the DMPO-OH adduct, (a) experimental spectrum, and (b) simulated spectrum using the parameters $g = 2.0055$, $a_H = 14.25$ G, $a_N = 14.84$ G in carbonate buffer, $T = 25\,^{\circ}C$, $[Mn]_o = 2.50 \times 10^{-5}$ M, $[H_4A]_o = 9.00 \times 10^{-4}$ M.

solution in which the auto-oxidation of adrenaline was in progress, it brings about a remarkable increase (*ca.* 5-fold) in the oxidation rate. This acceleration is illustrated by the absorbance measured at 480 nm displayed in Fig. 2 as a function of time. An introduction time (25 s) was required to generate active oxygen radicals, as can be seen at the beginning of Section 1.

The same experiment done without adrenaline has shown that the slope of absorbance at 480 nm *vs.* time is essentially zero (data not shown). These results clearly demonstrate the catalytic effect of the manganese complex.

The monitoring of the EPR spectra during the reaction provides information upon the nature of manganese species present and on the reaction intermediates. After methanol solution of manganese complex was added to the carbonate buffer (total volume 3 ml) at room temperature, the typical six-line pattern of monomeric Mn(II) disappeared. Using DMPO as spin-trapping agent, the catalytic solution exhibited a typical ESR signal of hydroxyl radical–DMPO

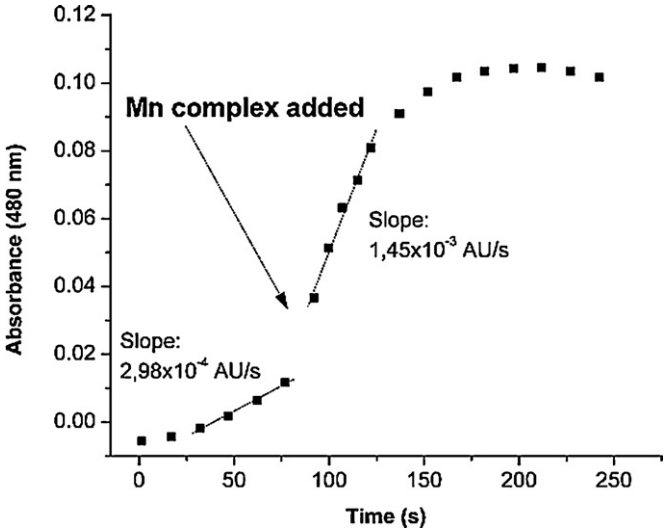


Fig. 2. Manganese catalyzed oxidation of adrenaline. Rate of the auto-oxidation (Section 1) and its increase upon the addition of Mn complex (Section 2). Rate measured spectrophotometrically by monitoring the increase of absorbance at 480 nm (1 cm cell). Section 1: $[H_4A]_o = 4.50 \times 10^{-4}$ M, $T = 25\,^{\circ}C$. Section 2: same as 1 but with $[Mn]_o = 1.67 \times 10^{-5}$ M added.

(DMPO–OH) adduct, shown in Fig. 3. Improvement in the signal to noise ratio by the accumulation of 200 successive spectra provided a good quality spectrum. The ESR parameters obtained from the simulation of the spectrum was compared with the literature values [46].

3.2. Kinetic measurements

The kinetics of the adrenaline oxidation were determined by the method of initial rates through monitoring the increase in the characteristic adrenochrome absorption band at 480 nm as a function of time. The initial rates of adrenochrome formation were determined as a function of the catalyst and substrate concentration. The data were taken at an interval of 2 s and the conversion was measured up to 10 min in each case. Observed initial rates were expressed as $M\,s^{-1}$ by taking the extinction coefficient of adrenochrome into account and calculated from the initial slope of the curve describing the evolution of product *versus* time. The results are shown in Tables S1–S2 (see Supplementary information), where each value shown is the average of three individual runs (reproducible to within ±3%). To determine the dependence of the rates on the catalyst concentration, solutions of the substrate were treated with increasing amounts of **2**. A second order dependence was found for the initial rate *vs.* the catalyst concentrations (Fig. 4).

The dependence of initial rate of the adrenochrome formation on the initial concentration of substrate at fixed catalyst concentration ($[Mn]_o = 2.50 \times 10^{-5}$ M) is illustrated in Fig. 5.

Under this experimental condition, saturation type curve was observed for the initial rate *vs.* adrenaline concentration. A kinetic treatment on the basis of the Michaelis–Menten model, originally developed for enzyme kinetics, was applied. The kinetic parameters were calculated using the Lineweaver–Burk plot (or double reciprocal plot, Fig. 6).

The calculated Michaelis constant (K_M) and V_{max} are 1.04×10^{-3} M and $6.84 \times 10^{-7}\,M s^{-1}$, $k_{cat} = 2.74 \times 10^{-2}\,s^{-1}$ and $(k_{cat}/K_M) = 26.35\,M^{-1}s^{-1}$. Our result is comparable to those reported for other model systems [30–32,43], but lower than in the case of silica nanoparticles [36].

Based on results from the kinetic measurements shown in Figs. 3 and 4 and the Lineweaver–Burk plot (Fig. 6) we suggest a general reaction mechanism shown in Scheme 3.

According to the proposed mechanism (1)–(6) in Scheme 3, step (1) is a pre-equilibrium dimerization of the monomeric

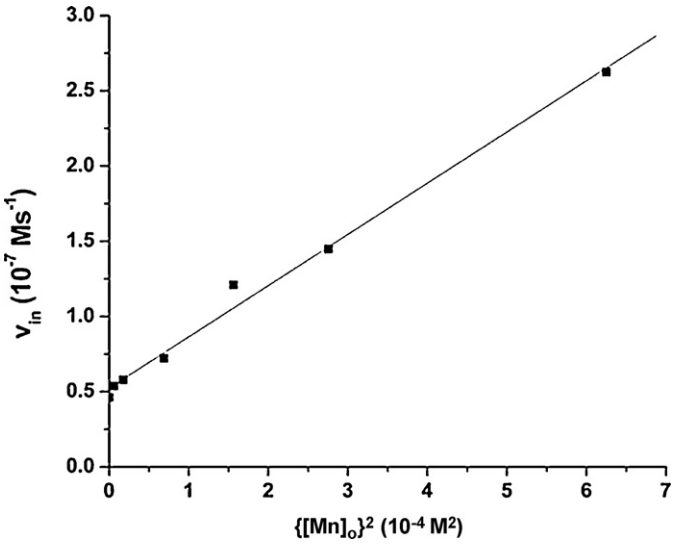


Fig. 4. Initial rate *vs.* catalyst concentration. Dependence of the initial rate on the square of concentration of catalyst. $[H_4A]_o = 3.00 \times 10^{-4}$ M, $T = 25\,^{\circ}C$, in air.

$Mn^{II}(HL)(MeOH)$, in which the dimeric Mn^{II}/Mn^{II} catalyst is generated. This is followed by the reversibly binding dioxygen molecule to the dimer, affording the superoxomanganese(III) complex. The latter is capable to bind to the substrate in step (3), by cleavaging the nitrogen bond from the pseudo axial position (N5) in the complex. The Mn-substrate-O_2 active ternary complex (**X**) decomposes in rate-determining step (4) to hydrogen peroxide and leucochrome, which is rapidly oxidized to the product adrenochrome and manganese dimer complex regenerates. Final fast step (6) describes how intermediate O_2^- is converted to OH^-.

In this catalytic process, steps (2) and (3) can be regarded as pre-equilibria and (4) as rate determining step. To derive a kinetic equation corresponding to the proposed mechanism (Scheme 3), one needs the mass balance for the manganese species and for H_4A, which can be expressed by Eqs. (1) and (2).

$$[Mn]_o = [Mn^{II}] + [Mn^{II}{}_2] + [Mn^{III}{}_2(O_2)] + [X] + [Mn^{II}{}_2(H_2A)] \quad (1)$$

$$[H_4A]_o = [H_4A] + [X] + [Mn^{II}{}_2(H_2A)] + AC \quad (2)$$

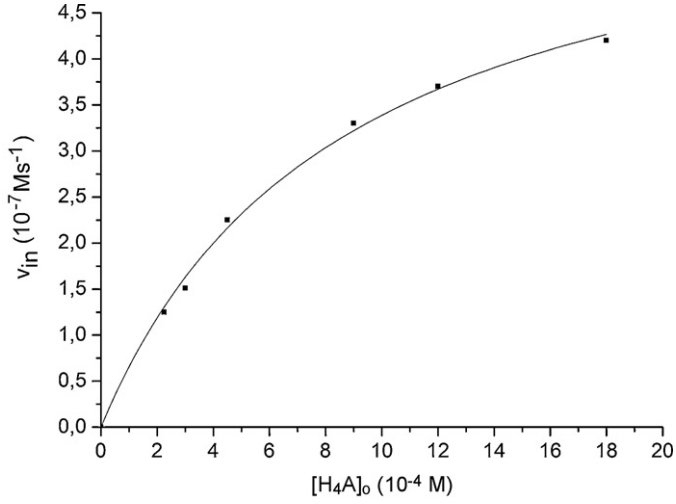


Fig. 5. Initial rate *vs.* substrate concentration. Dependence of the initial rate of adrenochrome formation on the substrate concentration. $[Mn]_o = 2.50 \times 10^{-5}$ M, $T = 25\,^{\circ}C$, in air.

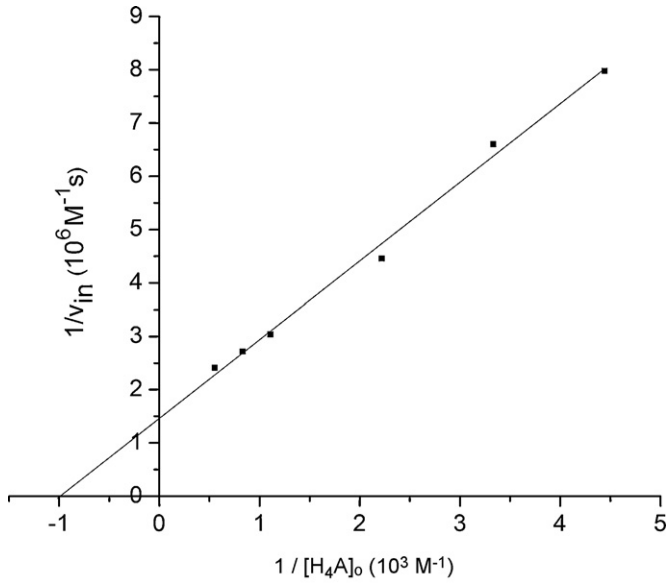


Fig. 6. (Initial rate)$^{-1}$ *vs.* (substrate)$^{-1}$ concentration. Lineweaver–Burk plot (all investigations were carried out three times with constant catalyst concentration).

The concentrations of $[Mn^{II}]$, $[Mn^{II}{}_2(H_2A)]$ species and substrate consumption are negligible. Combination of the mass balance with the expressions for equilibria in steps (2) and (3) leads to the kinetic equation (Eq. (3)) for the mechanism (1)–(6)

$$V_{in} = \frac{k_4 K_1 K_2 K_3 [Mn]_0^2 [O_2]_o [H_4A]_o}{1 + K_3 [H_4A]_o} \quad (3)$$

where $[Mn]_o$ and $[H_4A]_o$ are the initial concentrations of added dioximato-manganese(II) and adrenaline, respectively.

In our experiments the dioxygen concentration was held constant, but Nachtman and co-workers [47] have shown that the rate of oxidation was increased in 95% dioxygen relative to air. The mechanism found consistent with the observed kinetic behaviour of dioximato-manganese(II) is in several aspects similar to the mechanism of dioxygen activation by divalent transition-metal complexes [48].

3.3. Superoxide dismutase activity

Superoxide dismutase (EC 1.15.1.1) (SOD), is an enzyme capable to protect living organisms from oxidative damage by dismutation of superoxide radical into oxygen and hydrogen peroxide. It is possible to test the involvement of O_2^- in the oxidation of adrenaline. This popular assay of superoxide and its variations have been attracting interest in the last few decades [49–51]. The superoxide ion is generated indirectly in the oxidation at alkaline pH, to serve as substrates for SOD. Because SOD slows down the rate of adrenochrome formation, it is considered as a potent inhibitor of this oxidation. This assay does not work if dopamine is used instead of adrenaline, since the quinone cyclization reaction is much slower and the reduction of the quinone by superoxide predominates.

$2\,[Mn(HL)]^+ \rightleftharpoons [Mn_2(HL)_2]^{2+}$	K_1	(1)
$[(HL)_2Mn_2]^{2+} + O_2 \rightleftharpoons [(HL)_2Mn_2{}^{III}(O_2)]^{2+}$	K_2	(2)
$[(HL)_2Mn_2{}^{III}(O_2)]^{2+} + H_4A \rightleftharpoons [(HL)_2Mn_2{}^{III}(O_2)(H_4A)]^{2+}$	K_3	(3)
$[(HL)_2Mn_2{}^{III}(O_2)(H_4A)]^{2+} \longrightarrow [(HL)_2Mn_2{}^{II}(H_2A)]^{2+} + H_2O_2$	k_4	(4)
$[(HL)_2Mn_2(H_2A)]^{2+} + O_2 \longrightarrow [(HL)_2Mn_2]^{2+} + AC + O_2^- + H^+$	fast	(5)
$H_2O_2 + O_2^- \longrightarrow O_2 + OH^- + OH^\cdot$	fast	(6)

Scheme 3. Proposed reaction mechanism of the adrenaline oxidation catalyzed by **2**.

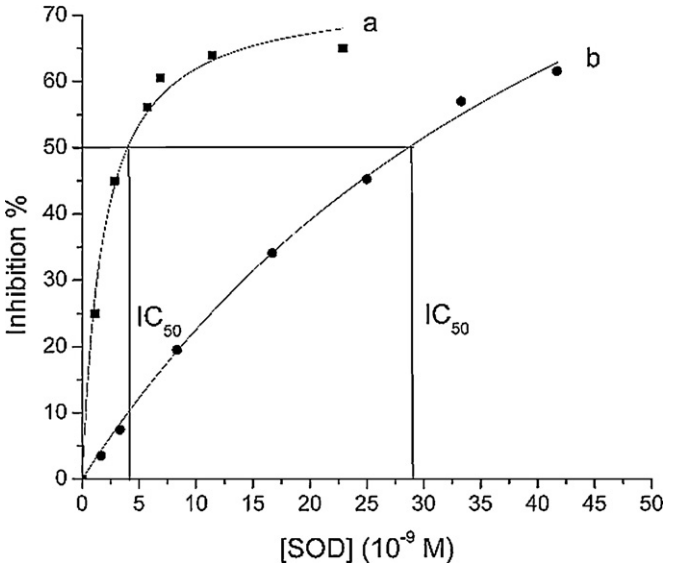


Fig. 7. IC_{50} *vs.* SOD concentration. Inhibition percentage as a function of superoxide dismutase concentration. Conditions: $[H_4A]_o = 3.00 \times 10^{-4}$ M; curve a: 136.8 ng/ml superoxide dismutase; curve b: 912.0 ng/ml superoxide dismutase, $[Mn]_o = 1.67 \times 10^{-5}$ M, $T = 25^\circ$C, in air.

Increasing amounts of SOD was added to adrenaline solution (3×10^{-4} M), and the effect on the rate of adrenochrome formation was recorded at 480 nm. The auto-oxidation of adrenaline at pH 10.2 was inhibited by SOD as shown in Fig. 7a. The SOD-activity was characterized by determining the IC_{50} value (the concentration where inhibition of adrenochrome formation is 50%). The IC_{50} can be obtained from the plot of $(v_0 - v_{SOD})/v_0 \times 100\%$ *vs.* enzyme concentration, where v_0 is the slope of the absorbance *vs.* time in the absence of enzyme, whereas v_{SOD} is the slope in the presence of enzyme. The percent inhibition (%I) shows hyperbolic dependences as a function of SOD concentration, as long as the maximum of inhibition does not exceed 70%. This is probably due to the presence of interfering intermediates which activated the alternative oxidative pathways [28].

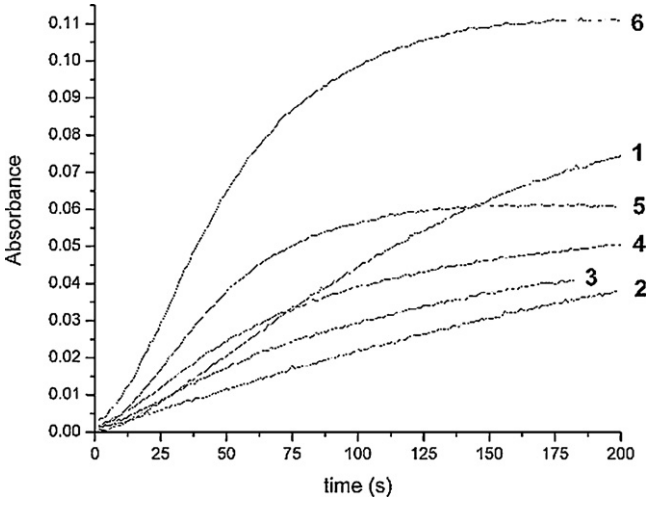


Fig. 8. Absorbance *vs.* time Effects of manganese complex on the 50% inhibited rate of oxidation. $[H_4A]_o = 3.00 \times 10^{-4}$ M; curve (1): $[SOD]_o = 0$, $[Mn]_o = 0$; (2): $[SOD]_o = 4.28 \times 10^{-9}$ M, $[Mn]_o = 0$; (3): $[SOD]_o = 4.28 \times 10^{-9}$ M, $[Mn]_o = 8.33 \times 10^{-6}$ M, (4): $[SOD]_o = 4.28 \times 10^{-9}$ M, $[Mn]_o = 1.67 \times 10^{-5}$ M; (5): $[SOD]_o = 4.28 \times 10^{-9}$ M, $[Mn]_o = 2.50 \times 10^{-5}$ M; (6): $[SOD]_o = 4.28 \times 10^{-9}$ M, $[Mn]_o = 3.33 \times 10^{-5}$ M, $T = 25^\circ$C, in air.

In the presence of manganese complex we have investigated the above mentioned inhibition reaction. To determine the IC_{50}, a solution containing adrenaline (3×10^{-4} M) and manganese complex (1.67×10^{-5} M) was treated with increasing amounts of SOD enzyme (Fig. 7b). As expected, the 50% inhibition was achieved at higher SOD concentration, due to the catalytic effect of manganese on the oxidation of adrenaline.

The manganese catalytic effects on the oxidation of adrenaline to adrenochrome inhibited by SOD were measured at a constant SOD concentration (corresponding to 50% inhibition). Under this condition the oxidation rate increases with addition of manganese as shown in Fig. 8.

We have found that the auto-oxidation of adrenaline was inhibited by superoxide-dismutase in 0.05 M sodium carbonate–bicarbonate buffer (pH 10.2) and $IC_{50} = 4.28 \times 10^{-9}$ M. In the presence of manganese complex the 50% inhibition of adrenaline oxidation was observed at 28.5×10^{-9} M SOD. These results are consistent with the suggested reaction mechanism (Scheme 3) and it can be conclude that the manganese complex has no SOD-like activity.

4. Conclusion

In this work, we have carried out detailed kinetic studies on functional catechol oxidase model based on manganese complex, using adrenaline as model substrate. The catalytic effect is due to the formation of a reactive ternary complex intermediate ($[(HL)_2Mn_2^{III}(O_2)(H_4A)]^{2+}$), which decomposes to adrenochrome. The kinetic measurements have revealed second-order dependence on the catalyst concentration and saturation type behaviour with respect to the substrate concentration, as well as the dioximato-manganese complex does not catalyze the dismutation of superoxide, so it cannot be considered as a functional model of superoxide dismutase.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcata.2013.02.003.

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