A Re-investigation of the Reactions between Superoxide Anion and Metal Picolinate Complexes

S. M. TROTMAN, J. WEBB*

School of Mathematical and Physical Sciences, Murdoch University, Murdoch, W.A. 6150, Australia

and D. F. SANGSTER

CSIRO, Division of Materials Science and Technology, Lucas Heights Research Laboratories, Private Mailbag 7, Menai, N.S.W. 2234, Australia

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Abstract

Rate constants for the reactions of superoxide with the α-picolinate ion and its complexes with copper(II), iron(III) and zinc(II), and for the reaction of α-picolinate with the hydrated electron, were measured using pulse radiolysis. The rate constant for the reaction of superoxide with copper(II)picolinate at pH 9 [$(4.1 \pm 0.4) \times 10^7$ l mol^{-1} s^{-1}] was an order of magnitude higher than that determined previously (W. H. Bannister, J. V. Bannister, A. J. F. Searle and P. J. Thornally, *Inorg. Chim. Acta, 78,* 139 (1983)) using a less direct competitive inhibition method. The corresponding rate constant for iron(III)picolinate [$(7.5 \pm 1.5) \times 10^3$ l mol^{-1} s^{-1}] was an order of magnitude lower than a previous pulse radiolysis determination (same reference as above). We are not able to reconcile these two values for iron(III)-picolinate, although a possible source of spuriously high results is contamination with the kinetically active copper(II) complex. The likely roles of iron(III)picolinate and other low molecular weight iron complexes as potential catalysts of an *in vivo* superoxide-driven Fenton reaction are discussed in the light of present measurements.

Introduction

The superoxide-driven Fenton reaction (sometimes called the iron-catalysed Haber–Weiss reaction) has been widely accepted [1–13] as a major factor contributing to the toxicity of the superoxide anion in biological systems. It has been suggested [14] that low molecular weight iron complexes catalyse the *in vivo* reaction of O_2 with H_2O_2 in a two step mechanism. Thus O_2^- reduces iron(III) to iron(II),

$$O_2^- + \text{Fe(III)complex} \longrightarrow O_2 + \text{Fe(II)complex} \quad (1)$$

which then reacts with H_2O_2 (the Fenton reaction) to give reactive OH radicals,

$$H_2O_2 + \text{Fe(II)complex} \longrightarrow OH + OH^- + \text{Fe(III)complex} \quad (2)$$

According to reactions (1) and (2) the major role for superoxide in the *in vivo* generation of toxic OH radicals is simply to act as a source of reduced iron species.

Although the Fenton reaction is invoked with increasing frequency to explain both *in vivo* and *in vitro* biological damage, there are serious unanswered questions [15–20] about its relevance to *in vivo* superoxide toxicity. In particular, although there now seems little doubt [21–27] that *in vitro* mixtures of O_2^-, H_2O_2 and biological iron complexes can produce detectable quantities of OH, it is unclear whether the rate of reaction (1) is sufficient for it to compete effectively with other reactions of O_2^- and Fe(III) complexes *in vivo*.

Widespread investigation [28] has revealed only one 'biological' iron complex, iron(III)picolinate, which reacts with superoxide with a rate constant approaching that for the uncatalysed dismutation of O_2^- at biological pH. Bannister *et al.* [22], in a study of the role of metal picolinates as possible catalysts for the Haber-Weiss reaction, reported a second-order rate constant, k, for the reaction of O_2^- with iron(III) picolinate of 9.3×10^4 l mol^{-1} s^{-1} at pH 8.5. This is comparable with the k value of 8×10^4 l mol^{-1} s^{-1} for the uncatalysed dismutation of O_2^- at pH 7.8 [28, 29], but between 10 and 100 times smaller than the values commonly reported [28] for abiological iron complexes.

In the present pulse radiolysis studies of the reactions of O_2^- with a variety of metal complexes, we re-examined the reactivities of iron and copper picolinates and found a rate constant of only $(7.5 \pm 1.5) \times 10^3$ l mol^{-1} s^{-1} for the reaction of O_2^- with iron(III)picolinate at pH 9, which is an order of

*Author to whom correspondence should be addressed.

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magnitude smaller than that determined by Bannister *et al.* [22]. In the light of these results the role of iron–picolinate complexes as potential catalysts for the Haber–Weiss reaction may have to be reconsidered.

Experimental

The pulse radiolysis experiments were carried out using the 1.3 MeV accelerator facility† described in detail elsewhere [30]. In brief, solutions were irradiated with 3.4 μs electron pulses of energy (2.0 ± 0.4) × 10^{-2} J, along the short axis of a 2.0 cm × 1.0 cm × 0.3 cm Suprasil cell (dose range 26–40 Gy per pulse). Absorbing transients were detected perpendicular to the electron beam using an Osram XBO450 Xenon lamp, a Bausch and Lomb high intensity monochromator fitted with a UV–Vis grating (bandpass 7 nm), and an RCA 1P28 photomultiplier tube. When measurements of the hydrated electron absorption at 580 nm were carried out, a glass filter was placed between the light source and the irradiation cell to eliminate second-order interference from species absorbing at wavelengths shorter than 320 nm. Transient signals were captured on a Biomation 610B digitizer and processed on a PDP 11/23 computer. The output of the accelerator was monitored periodically throughout the experiments using thiocyanate dosimetry [31]. All measurements were carried out at a temperature of (18 ± 1) °C.

Superoxide was generated in oxygen-saturated 0.01 M solutions of sodium formate buffered at pHs of 7.5 or 9 by NaH_2PO_4/Na_2HPO_4 or Na_2HPO_4/Na_3PO_4, respectively. Metal picolinate complexes were prepared *in situ* immediately prior to irradiation by mixing appropriate volumes of 0.01 M α-picolinic acid (pyridine-2-carboxylic acid) solution and 0.01 M solutions of $CuSO_4$, $ZnSO_4$ or $FeNH_4(SO_4)_2$. Sufficient picolinic acid was added to ensure that the concentration of uncomplexed picolinate ion in the final reaction mixture was 100 μM. The metal ion/picolinic acid mixtures were allowed to stand for about 20 min before the formate and buffer solutions were added. The final mixture was bubbled with washed industrial grade oxygen for 15 min before irradiation. Water was distilled under an atmosphere of oxygen in a well-leached five stage pyrex glass still, which included a distillation from alkaline permanganate and a distillation from acidified dichromate. All other chemicals were AR grade and used as supplied. Stock solutions of picolinic acid, sodium formate and buffer were stored over Chelex 100 resin to minimize contamination by impurity transition metal ions. An apparent reaction of zinc(II)picolinate with O_2^- which was unexpectedly observed in preliminary experiments was eliminated by this resin treatment. Table I shows the concentrations of species in a typical oxygen-saturated test solution. Test solutions containing metal picolinates in the concentration ranges given in Table I varied less than 0.05 pH units from the nominal working pHs of 7.5 and 9, and had ionic strengths, I, in the range 0.12–0.15 mol l^{-1}.

TABLE I. Concentrations of Species in a Typical Test Solution[a]

Species	Concentration ($\times 10^{-6}$ M^{-1})
($H_2PO_4 + HPO_4^{2-} + PO_4^{3-}$)	45000
Formate	10000
Free picolinate	100
$Fe(picolinate)_2OH$	30–150
or $Cu(picolinate)_2$	20–100
or $Zn(picolinate)_2$	50–250
O_2	~1300
O_2^-	15–7

[a]The complexes listed are the predominant form of each metal picolinate under the prevailing conditions [22]. For each complex the table lists the concentration range over which the effects of the complex on the O_2^- decay were examined. The concentration of dissolved O_2 was calculated from its solubility in water under 1 atmosphere of oxygen gas [31]. The concentration of O_2^- produced by a single radiation pulse was calculated from the maximum transient absorbance at 250 nm, assuming [28] $E_{250} = 2260$ l mol^{-1} cm^{-1}. The post-pulse concentration of O_2^- was smallest at the highest added concentration of metal picolinate.

The reactions of O_2^- were monitored by measuring the decay of absorption at 250 nm. At the concentrations of metal complex shown in Table I the decay of O_2^- absorption was always pseudo-first order, and the decay constant was obtained from a weighted linear least squares analysis [32] of the transient over a period of 2–3 half-lives. In the absence of added metal complex the measured lifetimes of O_2^- were about 0.2 s at pH 7.5 and 2 s at pH 9. The rate constant for the reaction of e_{aq}^- with picolinate was obtained from a similar analysis of the pseudo-first-order decay of absorption at 580 and 620 nm in a nitrogen saturated solution of pH 9 buffer.

All quoted errors in this work are determined at the 95% level of confidence.

Results

The sequence of reactions which leads to the formation of O_2^- from the primary radiolysis

†The facility is located at the Lucas Heights Research Laboratories, New South Wales, Australia, and is operated by the Australian Institute of Nuclear Science and Engineering.

products (e_{aq}^-, H, OH, H_2O^+) in electron-irradiated saturated formate solution is well documented in other publications [28, 33–35].

Although picolinate ion and its complexes all absorb in the 200–300 nm region, blank experiments containing N_2-saturated formate solutions with picolinate and metal picolinates showed no significant transients in the region of 250 nm over the time scale during which O_2^- decays were normally examined.

In the concentration ranges studied (Table I) picolinate competed effectively for e_{aq}^- with dissolved O_2. In a picolinate-free solution, the concentration of O_2^- produced by a single radiation pulse was typically 17 μM. At the highest concentrations of picolinate/picolinate complex examined, the immediate post-pulse concentration of O_2^- was typically 7 μM. Measurement precision in these solutions was substantially reduced, as the O_2^- concentration was approaching the detection limit, but the effect on measured rate constants due to loss of metal picolinate by direct reaction with e_{aq}^- was calculated to be less than 5%.

Table II shows second-order rate constants, k, for three of the five different reactions examined in this study. The rate constants were determined from the slopes of the plots shown in Figs. 1–3. Figures 1 and 2 show the effect of increasing concentrations of Fe(picolinate)$_2$OH and Cu(picolinate)$_2$ on the pseudo-first-order decay of the transient O_2^- absorption at 250 nm. Figure 3 is a similar plot showing the effect of increasing concentrations of free picolinate ion on the pseudo-first-order decay of transient e_{aq}^- absorption at 580 nm. Each point in Figs. 1–3 is the mean of four independent determinations of the rate constant, obtained from duplicate measurements on each of two separately prepared test solutions. Each rate constant was obtained from analysis of a decay curve constructed by averaging at least five independent pulse transients. The error bars in all Figures represent the 95% confidence limits in these measurements.

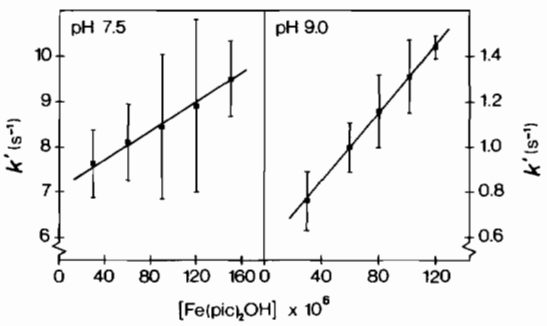

Fig. 1. The effects of increasing concentrations of iron(III)-picolinate on the rate constants, k^1, at pHs 7.5 and 9.0 for the pseudo-first order decay of superoxide absorption at 250 nm. At each pH the second order rate constant for the reaction of iron(III)picolinate with the superoxide anion is obtained from the slope of the weighted least squares line of best fit, which is (16 ± 10) × 10^3 l mol^{-1} s^{-1} at pH 7.5 and (7.5 ± 1.5) × 10^3 l mol^{-1} s^{-1} at pH 9.0.

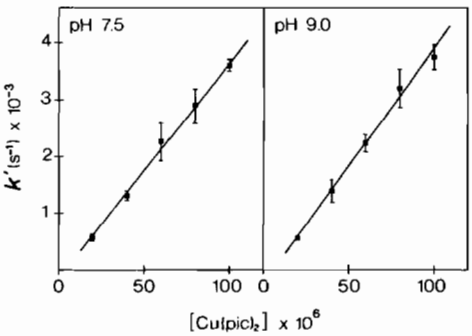

Fig. 2. The effects of increasing concentrations of copper(II)-picolinate on the rate constants, k^1, at pHs 7.5 and 9.0 for the pseudo-first order decay of superoxide absorption at 250 nm. At each pH the second order rate constant for the reaction of copper(II)picolinate with the superoxide anion is obtained from the slope of the weighted least squares line of best fit, which is (3.8 ± 0.4) × 10^7 l mol^{-1} s^{-1} at pH 7.5 and (4.1 ± 0.4) × 10^7 l mol^{-1} s^{-1} at pH 9.0.

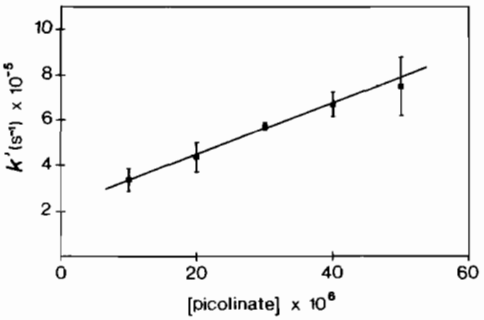

Fig. 3. The effect of increasing concentrations of picolinate ion on the rate constant, k^1, at pH 9.0 for the pseudo-first order decay of the hydrated electron absorption at 580 nm. The second order rate constant for the reaction of picolinate with the hydrated electron is obtained from the slope of the weighted least squares line of best fit, and is (1.14 ± 0.12) × 10^{10} l mol^{-1} s^{-1}.

Free picolinate ion and zinc(II)picolinate did not significantly affect the decay of O_2^- absorption at concentrations up to 500 and 250 μM respectively (Figs. 4 and 5). A reaction with a second order rate constant as low as 2 × 10^3 l mol^{-1} s^{-1} would have been detected under the conditions of measurement, which therefore represents an upper limit to k for either of reaction (4) or (7) in Table II.

Unfortunately the natural decay rate of O_2^- at pH 7.5 was sufficiently fast that the effects of added iron(III)picolinate were only just detectable, and the measurement precision was so low that any significant pH dependence in the k's listed in Table II would not have been detected.

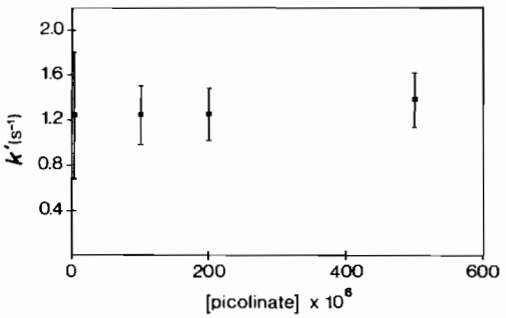

Fig. 4. The effect of increasing concentrations of picolinate ion on the rate constant, k^1, at pH 9.0 for the pseudo-first order decay of superoxide absorption at 250 nm.

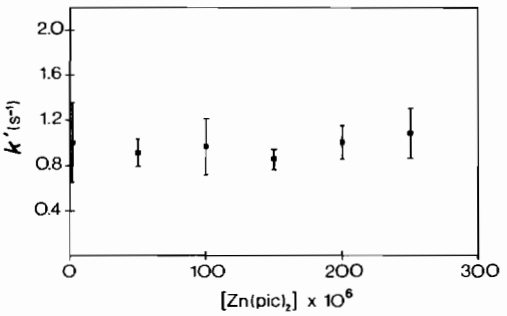

Fig. 5. The effect of increasing concentrations of zinc(II)-picolinate on the rate constant, k^1, at pH 9.0 for the pseudo-first order decay of superoxide absorption at 250 nm.

TABLE II. Second Order Rate Constants for Selected Reactions of O_2^- and e_{aq}^- [a]

Reaction	k at pH 7.5 (1 mol^{-1} s^{-1})	k at pH 9 (1 mol^{-1} s^{-1})
(3) e_{aq}^- + pic		$(1.14 \pm 0.12) \times 10^{10}$
(4) O_2^- + pic		no reaction
(5) O_2^- + $Fe(pic)_2OH$	$(16 \pm 10) \times 10^3$	$(7.5 \pm 1.5) \times 10^3$
(6) O_2^- + $Cu(pic)_2$	$(3.8 \pm 0.4) \times 10^7$	$(4.1 \pm 0.4) \times 10^7$
(7) O_2^- + $Zn(pic)_2$		no reaction

[a]Pic, picolinate ion. Errors are the 95% confidence limits in the slopes of Figs. 1–3. Ionic strengths were 0.12 (pH 7.5) and 0.15 (pH 9) for reactions (5) and (6), and 0.068 for reaction (3). For reactions (4) and (7) the minimum detectable value of k was *ca.* 2×10^3 1 mol^{-1} s^{-1}.

Discussion

The rate constant in Table II for the reaction of O_2^- with iron(III)picolinate at pH 9, $(7.5 \pm 1.5) \times 10^3$ 1 mol^{-1} s^{-1}, is an order of magnitude lower than the value of 9.3×10^4 1 mol^{-1} s^{-1} determined by Bannister *et al.* [22] at pH 8.5. Both rate constants were determined by a direct pulse radiolysis method. The present value is similar to that for other 'biological' iron complexes [28] and is a factor of 10 lower than that for the non-enzymic dismutation of O_2^- at pH 7.8 [28, 29].

The rate constant in Table II for the reaction of O_2^- with copper(II)picolinate of $(4.1 \pm 0.4) \times 10^7$ 1 mol^{-1} s^{-1} is an order of magnitude greater than the value of 2×10^6 1 mol^{-1} s^{-1} determined by Bannister *et al.* [22] using a less direct competitive inhibition method. Those workers [22] also quoted a pulse radiolysis derived rate constant of 1.4×10^8 1 mol^{-1} s^{-1} for the copper(II)picolinate catalysed bimolecular decay of O_2^-. This was presumably an overall decay constant for O_2^- in the presence of a particular concentration of copper(II)picolinate, but the concentration was not recorded, and no attempt was made to reconcile the two values quoted. The present value of k for the reaction of copper(II)picolinate with O_2^- is within the range of values found for other copper complexes [28], and is about 100 times lower than that for the reaction of O_2^- with natural copper zinc superoxide dismutase.

The rate constant for the reaction of free picolinate with the hydrated electron is in good agreement with the only other [36] published determination, of 1.1×10^{10} 1 mol^{-1} s^{-1}.

The origins of the differences between the present study and that by Bannister *et al.* [22] are unclear, since those authors provided few details of their rate constant measurements. Differences in the pH of measurement cannot account for the disagreement, since present results indicate negligible pH dependence for k (Table II).

The most probable source of error in work of this type is incomplete control of the compositions and concentrations of the reacting species. Thus, if species such as formate or phosphate, which are present in solution in millimolar quantities, formed complexes with the micromolar concentrations of iron or copper in competition with picolinate, the nature of the catalysing complex may not be well-defined. If the iron or copper picolinates hydrolysed to form metastable hydroxides at any stage during the preparation of the alkaline test solutions, the complex concentration may not be definitely known. Finally, since the rate of O_2^- dismutation is pH dependent [28], any systematic drift in pH during the addition of the metal complex may produce spurious changes in the

rate of O_2^- decay, which are incorrectly interpreted as resulting directly from the effect of the complex.

In the present study, care was taken to ensure that all test solutions were well-defined. Computer simulation of the equilibrium species' concentrations using known [37] complex formation constants showed that at the concentrations given in Table I all (>98%) of the added metal ion existed as the specified picolinate complex. Complexes were prepared in slightly acid conditions by *in situ* mixing of solutions of the appropriate metal ion and picolinic acid. Complex formation was rapid: when 100 μM solutions of Cu^{2+} and picolinic acid were mixed, the deep blue complex was visible within the time of mixing. At least twenty minutes then elapsed before formate was added and the solution buffered to its final alkaline pH. There was no visible evidence of hydroxide formation in any of the test solutions before or after irradiation, although there was ample evidence that hydrolysis did occur if due care was not taken. For example, fine cloudy precipitates often formed when the residues from the preparation of test solutions were mixed in an uncontrolled manner. Similar precipitates formed during attempts to prepare *ca.* 1000 μM stock solutions of iron(III)-picolinate from the purified solid complex. Finally, the pH of all test solutions was determined before use and, although a slight systematic decrease of about 0.1 pH units was observed in moving from zero to 150 μM added metal picolinate, this could not account for the differences between the present work and that of Bannister *et al.* [22]. The effects of a pH change of 0.1 unit on the rate of O_2^- dismutation can be readily calculated [28] and were shown to be undetectable at the decay rates observed in this work. Certainly there are no significant pH effects evident in the data of Figs. 4 and 5, in which similar pH drifts occurred. In any event, a systematic decrease in pH would, if anything, give a spuriously *high* rate constant, and could not explain the presently observed *smaller* values.

Any contribution to superoxide toxicity in a normal biological system by the superoxide-driven Fenton reaction depends on reaction (1) occurring to a significant extent in the presence of all other competitors for O_2^- and iron(III) complexes. The present re-evaluation of the superoxide/iron(III)picolinate reactions means that despite a large amount of work over several years, no biological iron complex has yet been identified with a reactivity towards O_2^- comparable to, or greater than, that of normal uncatalysed dismutation. The prospects of isolating some uniquely reactive low molecular weight complex seem remote. On the other hand, although superoxide is generally regarded as biologically unreactive, it has been shown [28] to react with more than twenty biological organic compounds with a rate const $> 10^5$ l mol^{-1} s^{-1}.

It is important to remember that simply comparing rate constants is insufficient to establish whether or not reaction (1) plays a role in superoxide toxicity. The local concentrations of O_2^-, any iron(III)complex, and their competitors, are equally critical in determining the importance of reaction (1), and high local concentrations may compensate for a poor specific reactivity. There is evidence [15] to suggest that the concentration of O_2^- will be insufficient for it to compete for iron(III) with other reductants such as ascorbate, at least in the early stages of a directly competitive reaction.

The total concentration of available iron(III), rather than its particular complexed form, may therefore be the most critical factor in determining the effectiveness of reaction (1). It seems reasonable to suggest that cellular regions which are rich in ferritin, a likely source of high concentrations of mixed iron(III)complexes, are the most probable sites for an *in vivo* superoxide driven Fenton reaction to occur.

Although the contribution of reactions (1) and (2) to superoxide toxicity in a normally functioning organism remains unresolved, there is an impressive body of evidence [38] suggesting, at least indirectly, that the superoxide-driven Fenton reaction is important in certain pathological conditions. Therefore it seems increasingly desirable to focus attention on the reactivities with superoxide of complexes of pharmacological metal chelators. Such complexes may act as inhibitors of Fenton-like reactions, if especially unreactive, or as promoters if particularly reactive. The combination of a high specific reactivity with abnormally high complex concentration in patients undergoing chelation therapy for iron overload may induce sensitivity to a superoxide-driven Fenton reaction which is not apparent in normal circumstances.

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