*Polish J. Chem.*, **78**, 261–271 (2004)

# Oxidation of Hexacyanoferrate(II) by Peroxynitrite. A Mechanistic and Kinetic Study

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*(Received September 26th, 2003)*

A kinetic study of the oxidation of hexacyanoferrate(II) by peroxynitrite has been carried out, using a stopped-flow technique. The variation of the experimental rate constant over a wide concentration range of $Fe(CN)_6^{4-}$ has been investigated under different experimental conditions in terms of pH, ionic strength and temperature. A correlation between $k_{exp}$ and the substrate concentration has been obtained and this shows first-order behavior in substrate at high concentrations of $Fe(CN)_6^{4-}$. A reaction mechanism is proposed, in which the hexacyanoferrate(II) reacts in competitive pathways with the peroxynitrous acid (direct oxidation) and with HO· and $\cdot NO_2$ radicals formed in the homolysis of the peroxide bond of HOONO (indirect oxidation). Moreover, two individual constants, $k_c$ (the formation constant of the germinated radical pair $[HO\cdot\ \cdot NO_2]$) and $k_7$ (direct oxidation constant), have been evaluated together with the thermodynamic parameters of these elementary steps.

**Key words**: oxidation, peroxynitrite, hexacyanoferrate(II), stopped-flow

Peroxynitrite [1] is an important inorganic species and has been subject of numerous studies in recent years, due to its prominent role in several areas of molecular science [2]. In atmospheric chemistry, for example, the photoisomerization of nitrate to peroxynitrite with UV radiation [3] must be considered as a chemical change, that can occur in the Earth's atmosphere. In this way peroxynitrite, due to its strong oxidizing properties, will react with chlorine-containing species (*e.g.* chlorofluorocarbons) to produce Cl atoms that may contribute to the destruction of the ozone layer [4].

In biology it is well known that activated macrophages and neutrophils produce two free radicals, nitrogen monoxide and superoxide, that form the peroxynitrite anion [5,6] under physiological conditions. This anion has free access to cell interiors because of its ability to pass through phospholipid membranes [7]. Therefore, peroxynitrite may play an important role in different human diseases such as strokes, myocardial ischemia and pulmonary edema [8].

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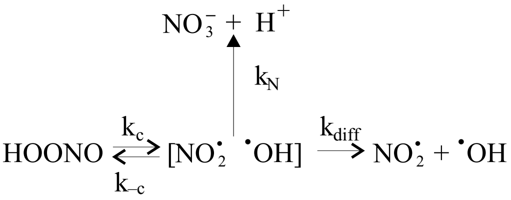
The peroxynitrite anion is relatively stable and can be preserved for weeks in strongly alkaline media at –18°C. Its conjugate acid, peroxynitrous acid ($pK_a = 6.8$) [2,9–11], is very unstable since it has a half-life of less than 1 s at 37°C and isomerizes to nitrate [12] or decomposes to nitrite and molecular oxygen [13–15]. Isomerization to nitrate is the main pathway in acidic media.

Peroxynitrous acid can oxidize numerous substrates such as methionine [10], ascorbate [16], DNA [17] and lipids [18], and can act on aromatic compounds as a nitrating [19,20] or a hydroxylating [21] agent. The oxidation reactions can take place by an indirect pathway, which is first order in peroxynitrite and zero order in the oxidizable species [5,22], or a direct pathway that is first order in both peroxynitrite and substrate [2,10,11,23]. However, many substrates appear to react by both pathways simultaneously [16]. The indirect oxidation involves the decomposition of HOONO into a highly reactive intermediate species. The exact nature of the intermediate species has been the subject of great debate [2,5,14,21,24,25,26], but numerous studies have clearly demonstrated the radical nature of reactions involving peroxynitrite [14,21,24,25]. Thus, the reactivity of the intermediate species has mainly been attributed to the germinated radical pair $[HO\cdot\ \cdot NO_2]$, which is formed by homolysis of the peroxide bond. The germinated radical pair can either isomerize to nitrate, regenerate HOONO or escape outside of the cage to give free radicals that are capable of oxidizing the substrate.



$$\begin{tikzcd} & NO_3^- + H^+ & \\ HOONO \arrow[r, shift left, "k_c"] & {[NO_2^{\bullet}\ {}^{\bullet}OH]} \arrow[l, shift left, "k_{-c}"] \arrow[u, "k_N"'] \arrow[r, "k_{diff}"] & NO_2^{\bullet} + {}^{\bullet}OH \end{tikzcd} \qquad (1)$$

Certain authors [27,28] have studied the reaction of cyano complexes with peroxynitrite, assuming that the oxidation rate is independent of substrate concentration and therefore there is only indirect oxidation. These studies were carried out with sub-millimolar concentrations and only Czapski *et al.* [29] and Lymar *et al.* [30] have studied $Fe(CN)_6^{4-}$ at high concentrations. Under these conditions they observed an increase in the oxidation rate and the second-order rate constant was determined to be 5.3 and 8.2 $M^{-1}\,s^{-1}$, respectively. The reason for the discrepancy between these values is unclear [30].

The purpose of this work was to study in detail the oxidation of hexacyanoferrate(II) by peroxynitrite over a wide concentration range and in different experimental conditions in terms of pH, ionic strength and temperature. Furthermore, significant effort was made to find a complete experimental rate equation that includes all of these experimental conditions.

## EXPERIMENTAL

*Materials*: All chemicals were of analytical grade and were used as received. Solutions were prepared with water obtained from a SETA Osmo B6 purification system. Buffer solutions were prepared from salts and acids, never from hydroxides, in order to avoid contamination with $HCO_3^-$.

*Synthesis of peroxynitrite*: Most of the synthetic processes are associated with various problems: low yield of $^-OONO$, excess of $H_2O_2$ and the presence of nitrate and nitrite ions in the final solution [31,32]. In order to overcome these difficulties, the method described by Leis [33] was employed. This method is based on the *in situ* formation of the peroxynitrite anion in alkaline media, as a result of the strong nucleophilicity of $HOO^-$ towards the nitrous group in alkyl nitrites.

$$RONO + HOO^- \longrightarrow ROH + ONOO^- \tag{2}$$

2-Ethoxyethyl nitrite (0.016 M) was used as a moderately reactive alkyl nitrite and equimolecular amounts of $H_2O_2$ were employed. The yield of $^-OONO$ in the final solution was greater than 94%. The residual nitrite concentration is capable of reacting with the peroxynitrite, although given the low rate constant (3.2 $M^{-1}$ $s^{-1}$) this reaction is negligible [34]. The solutions used in each experiment were prepared by dilution of a stock solution, and the peroxynitrite concentration were determined spectrophotometrically at 302 nm ($\varepsilon$ = 1670 $M^{-1}$ $cm^{-1}$) [31].

*Kinetics*: Kinetic measurements were carried out using an SX-18MV Sequential Stopped-Flow apparatus (Applied Photophysics) with a mixing time of less than 2 ms. The oxidation of hexacyanoferrate(II) by peroxynitrite was followed by observing the appearance of hexacyanoferrate(III) at 420 nm ($\varepsilon$ = 1000 $M^{-1}cm^{-1}$) [35]. This reaction was initiated by mixing equal volumes of peroxynitrite with solutions of the substrate in the appropriate buffer (0.1 M) at different pHs. The substrate concentration was maintained in excess of the peroxynitrite concentration. The pH of the reaction mixture was measured at the end of the process. In order to avoid any catalytic effect of $CO_2$ on the peroxynitrite reactions [28,29,36], the buffers were purged with Ar for at least 30 minutes prior to each experiment. Experimental rate constants were determined by non-linear least-squares fitting of absorbance/time data to single exponential functions, $A_{420} = (A_{420})_\infty(1 - e^{-k_{exp}t})$. The values reported are the averages of at least five independent determinations. The selected temperature was kept constant within ±0.05°C. Release of $O_2$ through oxidation of the substrate by peroxynitrite was measured with a YSI-5000 dissolved oxygen meter in conjunction with a YSI-5010 BOD probe. The electrode was calibrated in air by placing the probe in air at 100% relative humidity and a temperature of 21°C. The determination of nitrate and nitrite as products of the hexacyanoferrate(II) oxidation was performed by HPLC techniques using a Perkin-Elmer series 4 liquid chromatograph (column: MFE®-Sil amino 5 $\mu$m 150 ×4.6 mm). The solvent isotope effect was studied by preparing all solutions in 99.9% $D_2O$ – except for the stock peroxynitrite solution, which was prepared in $H_2O$. In this way the solvent in the reaction mixture contained approximately 95% $D_2O$.

## RESULTS AND DISCUSSION

The oxidation of hexacyanoferrate(II) by peroxynitrite was studied over the pH range 4–10. The experimental first-order rate constant, $k_{exp}$, as a function of pH showed a sigmoid dependence (Figure 1) and can be described by the equation proposed by Keith and Powell [37]

$$k_{exp} = k_{HA}^{S}\left(\frac{[H^+]}{K_a + [H^+]}\right) \tag{3}$$

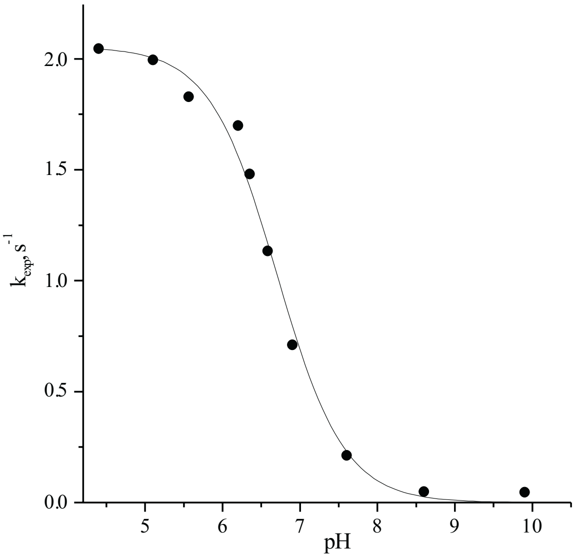


**Figure 1.** Influence of pH on the experimental rate constant of hexacyanoferrate(II) (0.01 M) oxidation by peroxynitrite (8 mM) at 300 K.

where $k^{S}_{HA}$ is the first order rate constant for hexacyanoferrate oxidation by peroxynitrite and $K_a$ is the ionization constant for peroxynitrous acid. By fitting the $k_{exp}/[H^+]$ data to equation (3), we obtained the values $pK_a = 6.8$ and $k^{S}_{HA} = 2.05\ s^{-1}$ at 300 K and 10 mM of $Fe(CN)_6^{4-}$. The $pK_a$ value is in good agreement with others [11,38]. The sigmoid shape in Figure 1 is due to the existence of the protonation equilibrium of peroxynitrite and the decrease in $k_{exp}$ with pH suggests that HOONO rather than $ONOO^-$ is the reactive species.

$$H^+ + {}^-OONO \rightleftarrows HOONO \tag{4}$$

The variation in the ionic strength over the range 0.1–3 M upon addition of sodium acetate or sodium chloride did not influence $k_{exp}$. The value of the experimental rate constant was also found to be independent of the initial concentration of peroxynitrite over the concentration range 0.2 mM to 1 mM. However, the plot of $k_{exp}$ *versus* $[Fe(CN)_6^{4-}]_0$ over the range 0.01 M to 0.3 M showed a first order dependence at pH 5.0, 7.3 and 8.6 (Figure 2). The oxidation yield, defined as the ratio $[Fe(CN)_6^{3-}]/[HOONO]_0$, increased with the hexacyanoferrate(II) concentration (Figure 3) and was independent of the peroxynitrite concentration and pH. Similar results have been reported [14,30].

In accordance with Scheme 1, the free radicals HO· and $\cdot NO_2$ diffunded in the medium can oxidize the substrate (indirect oxidation) [14,30]

$$Fe(CN)_6^{4-} + \cdot OH \longrightarrow Fe(CN)_6^{3-} + OH^- \tag{5}$$

$$Fe(CN)_6^{4-} + \cdot NO_2 \longrightarrow Fe(CN)_6^{3-} + NO_2^- \tag{6}$$

where $k_5 = 1 \times 10^{10}\ M^{-1}\ s^{-1}$ [39] and $k_6 = 2.1 \times 10^{6}\ M^{-1}\ s^{-1}$ [40].

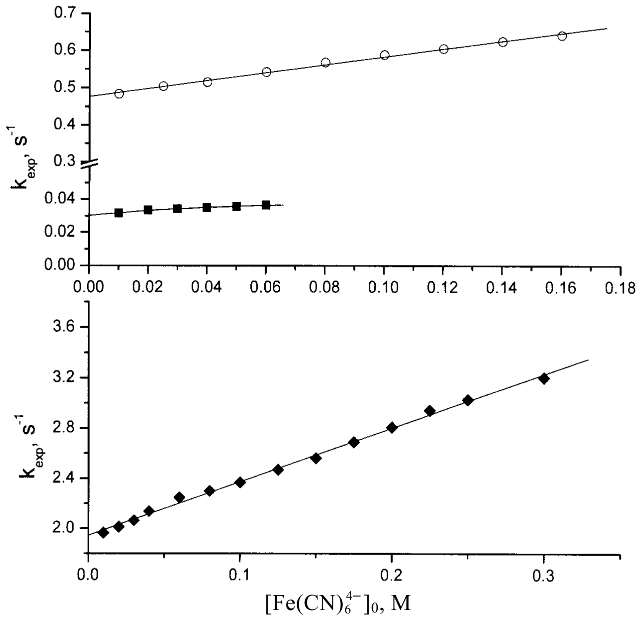


**Figure 2.** Variation of the experimental rate constant as a function of hexacyanoferrate(II) concentration. The peroxynitrite concentration (0.8 mM) and temperature (300 K) were kept constant, at (◆) pH = 5.0, (o) pH = 7.3 and (■) pH = 8.6.

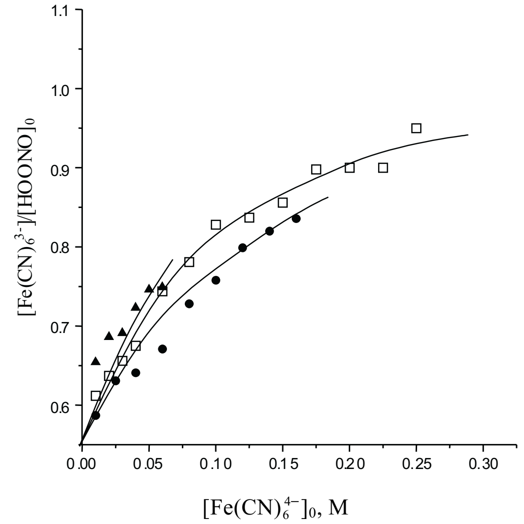


**Figure 3.** Influence of the oxidation yield on the hexacyanoferrate(II) concentration at 300 K, [HOONO] = 8 mM and (□) pH = 5.0, (●) pH = 7.3 and (▲) pH = 8.6.

In our case, the increase in $k_{exp}$ upon increasing the hexacyanoferrate(II) concentration also suggests the existence of a direct oxidation, (7), followed the rapid reaction 6, and therefore a mixture of both oxidation pathways appears to take place.

$$\mathrm{Fe(CN)_6^{4-} + ONOOH \xrightarrow{k_7} Fe(CN)_6^{3-} + OH^- + \cdot NO_2} \tag{7}$$

Reactions (1) and (5)–(7) predict the dependence of the oxidation yield on the hexacyanoferrate(II) concentration.

The $\cdot NO_2$ radical produced in reaction (1), besides reacting with hexacyanoferrate(II), can dimerize and hydrolyze through the following reactions:

$$\mathrm{\cdot NO_2 + \cdot NO_2 \rightleftarrows N_2O_4} \tag{8}$$

$$\mathrm{N_2O_4 + H_2O \longrightarrow NO_2^- + NO_3^- + 2H^+} \tag{9}$$

for which $k_8 = 4.6\times10^8$ $M^{-1}$ $s^{-1}$, $k_{-8} = 6.9\times10^3$ $s^{-1}$ and $k_9 = 1\times10^3$ $M^{-1}$ $s^{-1}$ [41].

Moreover, $\cdot OH$ is also produced in reaction (1) and can oxidize $NO_2^-$ as follows:

$$\mathrm{\cdot HO + NO_2^- \longrightarrow HO^- + \cdot NO_2} \tag{10}$$

with the rate constant $k_{10} = 1\times10^{10}$ $M^{-1}$ $s^{-1}$ [42]. This scheme explains the presence of the nitrate and nitrite observed by HPLC. In basic media the peroxynitrite anion can react with hydroxyl radicals to produce the superoxide ion. This ion is reduced to $\cdot NO_2$ and produces $O_2$

$$\mathrm{HO\cdot + {}^-OONO \longrightarrow H^+ + NO_2^- + \cdot O_2^-} \tag{11}$$

$$\mathrm{\cdot O_2^- + H^+ \rightleftarrows \cdot HO_2} \tag{12}$$

$$\mathrm{\cdot O_2^- + \cdot NO_2 \longrightarrow O_2 + NO_2^-} \tag{13}$$

for which $k_{11} = 4.8\times10^9$ $M^{-1}$ $s^{-1}$ [43], $pK_{12} = 4.8$ and $k_{13} = 4.5\times10^9$ $M^{-1}$ $s^{-1}$ [44]. Furthermore, the hexacyanoferrate(III) formed in reactions (5), (6) and (7) can oxidize $\cdot O_2^-$ and produce $O_2$.

$$\mathrm{Fe(CN)_6^{3-} + \cdot O_2^- \longrightarrow Fe(CN)_6^{4-} + O_2} \tag{14}$$

where $k_{14} = 3\times10^2$ $M^{-1}$ $s^{-1}$ [40].

The presence of $\cdot O_2^-$ in the medium was confirmed by studying the hexacyanoferrate(II) oxidation by peroxynitrite in the presence of tetranitromethane, TNM. A characteristic absorption band at 360 nm, due to the $C(NO_2)_3^-$ formed in reaction (15), has been observed at basic pH values.

$$\mathrm{C(NO_2)_4 + \cdot O_2^- \longrightarrow C(NO_2)_3^- + NO_2 + O_2} \tag{15}$$

for which $k_{15} = 2\times10^9$ $M^{-1}$ $s^{-1}$ [45]. At acidic pHs, however, the concentration of $ONOO^-$ is very low, so the formation of $\cdot O_2^-$ through reaction (11) is negligible and $C(NO_2)_3^-$ will not be formed at all – a situation that has been observed experimentally.

Moreover, the variation in the $O_2$ concentration in the reaction medium was measured with a YSI-5000 dissolved oxygen meter in conjunction with a YSI-5010 BOD probe. When peroxynitrite (0.8 mM) was added to the substrate (10 mM) at 295.5 K and pH = 8.6, the yield of $O_2$ was 7.7 mg/L. In contrast, at pH = 5.0 the release of $O_2$ from the solution was insignificant.

By applying the steady state approximation to the short lived cage, $[NO_2\cdot \cdot OH]$, we obtained the experimental rate constant for the first-order disappearance of peroxynitrite.

$$k_{exp} = \left[\frac{k_c(k_N + k_{diff})}{(k_{-c} + k_N + k_{diff})} + k_7[Fe(CN)_6^{4-}]_0\right]\left(\frac{[H^+]}{K_a + [H^+]}\right) \tag{16}$$

where the disappearance of peroxynitrite was the sum of the anion and its conjugate acid. The experimental rate constant for the oxidation of hexacyanoferrate(II) by peroxynitrite would be

$$k_{HA}^S = \frac{k_c(k_N + k_{diff})}{(k_{-c} + k_N + k_{diff})} + k_7[Fe(CN)_6^{4-}]_0 \tag{17}$$

where the first addend is the rate constant of the indirect reaction, $k_{HA}$.

The values of $k_{HA}^S$ were obtained by dividing $k_{exp}$ by 0.980, 0.241 and 0.015, which are the values of $[H^+]/(K_a + [H^+])$ at pH 5.0, 7.3 and 8.6, respectively. The rate constants for both the direct reaction, $k_7$, and indirect reaction, $k_{HA}$, were obtained from the slope and y-intercept of the linear plot $k_{HA}^S$ *vs.* $[Fe(CN)_6^{4-}]_0$, Table 1. At low concentrations, in which the main pathway is the indirect oxidation, the oxidation yield is 30% and therefore $k_N \approx 2.33k_{diff}$. The combination reaction of $HO\cdot + \cdot NO_2$ yields approximately equal amounts of HOONO and $NO_3^- + H^+$, therefore $k_N/k_{diff} \approx k_{-c}/k_{diff} = 2.33$ [42] and hence $k_{HA} = 0.6\, k_c$ (18), Table 1. It can be observed that the rate constants for the three processes are independent of the pH. A value of $k_c = 1.6\ s^{-1}$ (295 K) has been reported [30]. The discrepancy with our data could be due to the different experimental conditions, since these constants are strongly temperature dependent. The value of $k_7$ obtained is close to that reported by Czapski *et al.* [29] (5.2 $M^{-1}\ s^{-1}$ at 294.3 K), and the slight difference could again be due to the variation in the temperature. These data differ significantly from the value obtained by Lymar *et al.* [30] (8.2 $M^{-1}\ s^{-1}$ at 295 K). An explanation for these results is unclear, since the experimental conditions used in the studies [29–30] are similar.

Further information can be obtained by considering the influence of the temperature on $k_{exp}$ at different initial concentrations of hexacyanoferrate(II), Figure 4. The experimental activation energy decreased from 19.96±0.46 to 17.44±53 kcal/mol on increasing the initial concentration of substrate from 0 to 0.3 M. Fitting the data

$k_{exp}$/[S], at eight different temperatures, to (16) and (17) by non-linear regression gives the variation of $k_c$ and $k_7$ with temperature. These constants obey Arrhenius (Figure 5) and Eyring laws and from these plots the activation parameters of both elemental steps can be calculated (Table 2) for the first time. The Arrhenius preexponential factor, A, found for the homolysis of HOONO is close to $6\times10^{15}$ $s^{-1}$, which is the approximate value calculated for unimolecular O–O bond cleavage in HOONO, in the gas phase, using thermokinetic properties [15]. Moreover, A is in agreement with the expected value of $10^{16\pm1}$ for unimolecular homolysis reactions [2]. This result supports the presence of radicals in the medium.

**Table 1.** Rate constants for the different pathways of hexacyanoferrate(II) oxidation by peroxynitrite (0.8 mM) at 300 K.

| pH | $k_{HA}$, $s^{-1}$ | $k_c$, $s^{-1}$ | $k_7$, $M^{-1}$ $s^{-1}$ |
|---|---|---|---|
| 5.0 | 1.9±0.2 | 3.4±0.2 | 4.4±0.4 |
| 7.3 | 1.9±0.2 | 3.4±0.3 | 4.5±0.3 |
| 8.6 | 2.1±0.3 | 3.7±0.2 | 4.7±0.5 |

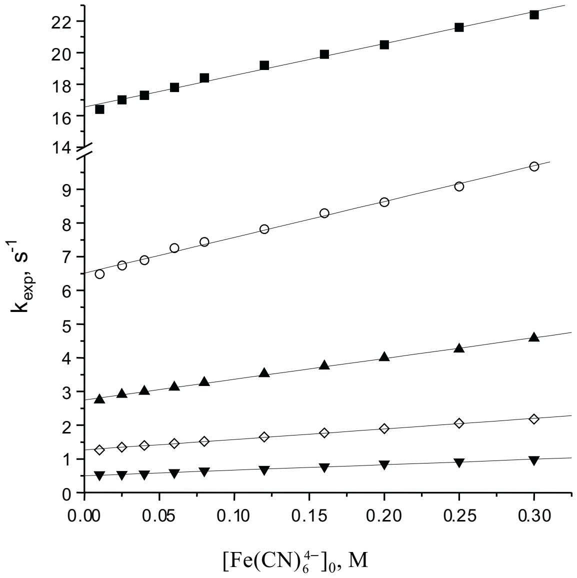


**Figure 4.** Variation of the experimental rate constant as a function of the temperature and hexacyanoferrate(II) concentration. Peroxynitrite (0.8 mM) was added to the substrate in 0.1 M acetate buffer, pH 5.0, at (■) 320 K, (o) 311 K, (▲) 304 K, (◇) 296 K and (▼) 287 K.

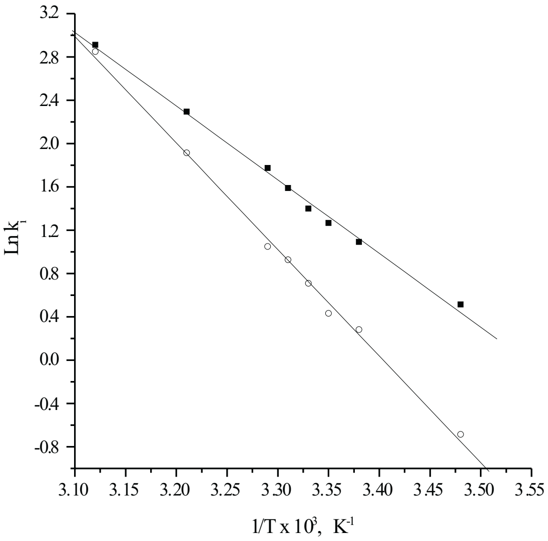


**Figure 5.** Arrhenius plot of (o) the formation constant of the germinated radical pair [HO· ·$NO_2$], i = c, and (■) direct oxidation constant, i = 7.

**Table 2.** Thermodynamic parameters of both elemental pathways for hexacyanoferrate(II) oxidation by peroxynitrite (0.8 mM): the formation of a germinated radical pair [HO· ·$NO_2$] and direct oxidation.

| | [HO· ·$NO_2$] formation | direct oxidation |
|---|---|---|
| A, $s^{-1}$ or $M^{-1}$ $s^{-1}$ | $(2.8\pm1.8)\times10^{14}$ | $(7.9\pm0.5)\times10^{10}$ |
| Ea, kcal $mol^{-1}$ | $19.4\pm0.3$ | $14.1\pm0.4$ |
| $\Delta H^{\#}$, kcal $mol^{-1}$ | $18.8\pm0.2$ | $13.5\pm0.2$ |
| $\Delta S^{\#}$, cal $mol^{-1}$ $K^{-1}$ | $7.8\pm1.2$ | $-7.4\pm1.0$ |

Additional support for the free radical mechanism comes from the results obtained on studying the solvent isotope effect. The average secondary isotope effect reported in a C–CH homolysis reaction is 1.05 [47]. Since the theoretical maximum values for secondary OH and CH effects are 2.02 and 1.74 [48], a secondary isotope effect in the O–OH homolysis reaction would be expected to be a slightly greater than 1.05. The secondary isotope effect obtained for the hexacyanoferrate(II) oxidation by peroxynitrite is $k_{exp}/k^{D}_{exp} = 1.17$, which would lend support to the existence of the homolysis reaction.

Direct oxidation of $Fe(CN)_8^{4-}$ by peroxynitrous acid may occur by an outer-sphere electron transfer mechanism. In this case, the value of the enthalpy of activation and the small negative entropy of activation (Table 2) would support the formation of an encounter pair without an interaction close enough for electron transfer. Similar $\Delta H^{\neq}$ and $\Delta S^{\neq}$ data have been reported for different reactions of organic and inorganic species with outer-sphere electron-transfer mechanisms [48–50]. Besides, the activation energy is within the range 13–18 Kcal mol$^{-1}$ calculated theoretically for the concerted oxidation of amines, sulfides and alkenes by peroxynitrite [51].

## CONCLUSIONS

We have obtained a correlation between $k_{exp}$ and $[Fe(CN)_6^{4-}]_0$ for the oxidation of hexacyanoferrate(II) by peroxynitrite. This relation indicates that the kinetics are first-order in substrate at high $Fe(CN)_6^{4-}$ concentrations. From this relationship we have proposed a mechanism, in which the substrate is oxidized simultaneously by HOONO (direct oxidation) and $HO\cdot \cdot NO_2$ radicals (indirect oxidation). Moreover, we have calculated the formation constant of a germinated radical pair ($k_c$), the direct oxidation constant ($k_7$) and the thermodynamic parameters of both elemental steps. These data have not been reported previously in the literature. These parameters are compatible with an outer-sphere electron-transfer reaction between $Fe(CN)_6^{4-}$ and HOONO.

### Acknowledgment

The authors acknowledge the financial support of the Consejería de Educación y Cultura of the Junta de Comunidades de Castilla-La Mancha (Spain).

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