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A REINVESTIGATION OF THE REACTION OF DESFERRIOXAMINE WITH SUPEROXIDE RADICALS. A PULSE RADIOLYSIS STUDY

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The reaction of desferrioxamine with superoxide has been studied using the pulse radiolysis technique. The decay of O_2^- was not accelerated in the presence of up to 4×10^{-4} M desferrioxamine at physiological pH. The rate constant was found to be lower than 2×10^4 $M^{-1}s^{-1}$. In acid solutions the rate constant of the reaction between desferrioxamine and $HO_2^\cdot$ was found to be lower than 10^5 $M^{-1}s^{-1}$. The reaction was not studied in alkaline solutions due to the high absorbance of desferrioxamine in the U.V. region. The pK of desferrioxamine was determined to be 9.2 ± 0.05.

KEY WORDS: Desferrioxamine, pulse radiolysis, O_2^-, $HO_2^\cdot$, dismutation.

INTRODUCTION

The iron chelator desferrioxamine (DFO), produced by *Streptomyces pilosus*,[1] is widely used in the treatment of iron poisoning as well as in inhibiting the iron catalyzed production of $OH^\cdot$ radicals.[2-5] The last process is believed to take place in many biological systems, e.g. lipid peroxidation, DNA damage, reperfusion and ageing.[2-6] Therefore, it is assumed that DFO, by chelating iron, inhibits the damage caused by O_2^-.

It has also been suggested that DFO can scavenge oxy-radicals. The rate constants of the reaction of DFO with $OH^\cdot$ and O_2^- were determined to be 1.3×10^{10} $M^{-1}s^{-1}$ [7] and $< 2 \times 10^5$ $M^{-1}s^{-1}$,[8] respectively, with the use of the pulse radiolysis technique. Since, with the pulse radiolysis technique, it is not possible to measure low reaction rates, the reaction of DFO with O_2^- was studied indirectly using the cytochrome *c* and NBT assays, and the rate constant was determined to be $(0.9–1.1) \times 10^3$ $M^{-1}s^{-1}$ at physiological pH.[9-10] According to this low rate constant, it was concluded that this reaction can be ignored in most applications of this chelating agent in biological processes.[3]

Recently, an extensive study by Sabourault *et al.*[11] on the reaction of DFO with O_2^-, using the pulse and gamma radiolysis techniques, confirmed earlier results of this group, presented in the SOD IV meeting in Rome in 1985,[12] that the rate constant of this reaction is 1.3×10^6 $M^{-1}s^{-1}$ at physiological pH, and thus DFO can scavenge O_2^-.

In view of the widespread use of DFO *in vivo* and *in vitro*, it was thought important to reinvestigate this reaction, especially as the results of Sabourault *et al.*[11] and Ribiere *et al.*[12] seem to be inconsistent, and in contrast to earlier results from the same group.[3]

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MATERIALS AND METHODS

All chemicals were of analytical grade and were used as received: Desferrioxamine B methanesulphonate (Desferal) was a generous gift from CIBA-Geigy, bovine superoxide dismutase (SOD) was from Sigma. All other reagents were of the highest quality available from Merck. All solutions were prepared with distilled water that had been passed through a Millipore ultrapurification system. Unless otherwise stated, solutions for irradiations contained 0.1 M formate, and were saturated with oxygen. The pH of the solutions was adjusted using 2 mM phosphate buffer (pH 4.5–8.2) or NaOH and $HClO_4$ for the other pH's. The concentrations of DFO were calculated assuming a Mr of 657. We confirmed that desferrioxamine obeys the Beer-Lambert law over the concentration range studied (50–500 μM).

Pulse radiolysis experiments were conducted using a Varian 7715 linear accelerator with a 200 mA current of 5 MeV electrons. Irradiations were carried out in a 4 cm spectrosil cell with an optical path length of 12.1 cm. The optical detection system consisted of 150 W Xe–Hg lamp, a single Bausch & Lomb grating monochromator model D330/D331 Mk. II and an IP28 photomultiplier. Appropriate filters were placed in front of the analyzing light to minimize photochemical effects. The signals from the photomultiplier were transferred through a SONY/TEXTRONIX 390AD programmable digitizer to a micro PDP-11/24 computer, which operated the whole pulse radiolysis system. All the experiments were carried out at room temperature, and each point represents an average of at least four measurements.

The pH of the solutions was measured with a Corning pH meter (Model 240).

Absorption spectra were recorded with a HP 8452A diode array spectrophotometer.

On pulsing O_2-saturated solution containing formate, the following reactions take place:[13]

$$H_2O \rightarrow e_{aq}^-, OH^\cdot, H^\cdot, H_2O_2, H_2, H_3O^+ \quad (1)$$

$$e_{aq}^- + O_2 \rightarrow O_2^- \quad k_2 = 2 \times 10^{10} \ M^{-1}s^{-1} \quad (2)$$

$$e_{aq}^- + H^+ \rightarrow H^\cdot \quad k_3 = 2.2 \times 10^{10} \ M^{-1}s^{-1} \quad (3)$$

$$OH^\cdot/H^\cdot + HCO_2^- \rightarrow H_2O/H_2 + CO_2^- \quad (4)$$

$$k_{OH} = 3.5 \times 10^9 \ M^{-1}s^{-1} \quad k_H = 1.3 \times 10^8 \ M^{-1}s^{-1}$$

$$CO_2^- + O_2 \rightarrow CO_2 + O_2^- \quad k_5 = 2.4 \times 10^9 \ M^{-1}s^{-1} \quad (5)$$

$$H^\cdot + O_2 \rightarrow HO_2^\cdot \quad k_6 = 2 \times 10^{10} \ M^{-1}s^{-1} \quad (6)$$

$$HO_2^\cdot \rightleftharpoons H^+ + O_2^- \quad pK_a = 4.69 \quad (7)$$

Because of the high rate constants of these reactions, all the primary radicals are converted into O_2^- before the end of the pulse, and the yield of O_2^- is $G = 6.05$ 6.05 (G-values represent the number of molecules formed per 100 eV of energy absorbed

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TABLE I

The observed rate constant for the decay of the absorbance at 265 nm and pH 6.9 ± 0.1 under various conditions

EDTA, μM	DFO, μM	k, s^{-1}	2k, $M^{-1}s^{-1}$
-	-	70	-
2	-	-	1.94×10^6
-	50	-	2.34×10^6
-	100	-	2.00×10^6
2	100	-	1.88×10^6
-	200	-	1.81×10^6
-	400	-	2.28×10^6
2	400	-	1.96×10^6
† -	200	-	2.48×10^6
‡ -	200	-	2.56×10^6

All solutions were saturated with oxygen and contained 0.1 M formate and 2mM phosphate buffer.
$\varepsilon_{265} = 1725\ M^{-1}cm^{-1}$, $[O_2^-]_o = 17\,\mu M$, the optical pathlength 12.1 cm.
†0.16 M formate
‡0.16 M formate and 60 mM phosphate buffer.

by the solution). The initial concentrations of O_2^- thus generated was determined using $\varepsilon_{265} = 1725\ M^{-1}cm^{-1}$ at pH 8,[13] and was about 17 μM. The decay of O_2^- was followed at 265 nm.

RESULTS

When oxygen-saturated aqueous solutions were irradiated in the presence of formate, the decay of the absorbance of O_2^- was first order under all experimental conditions because of traces of metal ion impurities, which catalyze O_2^- dismutation. When 2 μM EDTA was added, this decay obeyed a second order rate law, and the half life of the superoxide radicals increased (Table I).

TABLE II

The dependence of the observed rate constant of the decay of the absorbance at 265 nm on pH and desferrioxamine

pH	DFO, μM	2k, $M^{-1}s^{-1}$ (†)	$T_{1/2}$, ms
2.3	-	$2.9 \times 10^6 (2.5 \times 10^6)$	20
2.3	200	3.5×10^6	16
4.5	-	$4.7 \times 10^7 (4.9 \times 10^7)$	1.6
4.5	100	5.0×10^7	1.5
4.5	400	5.4×10^6	1.2
6.9	-	$1.9 \times 10^6 (1.2 \times 10^6)$	35
6.9	100	1.8×10^6	34
6.9	400	1.9×10^6	35
7.4	-	$5.4 \times 10^5 (3.9 \times 10^5)$	113
7.4	200	5.2×10^5	125

All solutions were saturated with oxygen and contained 0.1 M formate, 2 μM EDTA and 2 mM phosphate buffer except for pH 2.3.
$\varepsilon_{265} = 390$ (pH 2.3), 800 (pH 4.5) and $1725\ M^{-1}cm^{-1}$ (pH 6.9–7.4), $[O_2^-]_o = 17\,\mu M$, the optical pathlength 12.1 cm.
(†) Literature values obtained from ref. 13 according to the equation obtained for k as a function of pH.

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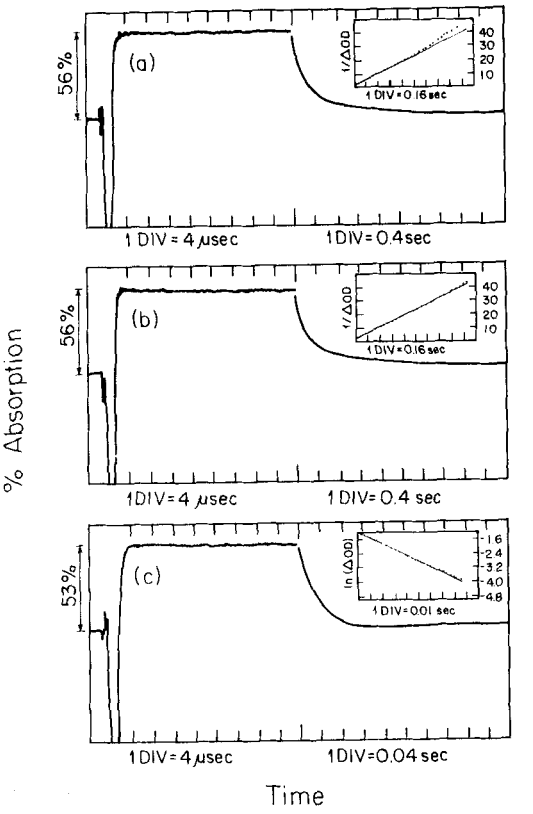

FIGURE 1 Kinetic plots obtained at 265 nm in oxygenated aqueous solutions containing 0.1 M formate and 2 μM EDTA at pH 7.4 (2 mM phosphate buffer).

(a) [DFO] = 0; The inset contains a fit of the decay to a second order reaction, where $2k/\varepsilon l = 25.5\,s^{-1}$.

(b) [DFO] = 200 μM; The inset contains a fit of the decay to a second order reaction, where $2k/\varepsilon l = 26.2\,s^{-1}$.

(c) [DFO] = 200μM, [SOD] = 0.016 μM; The inset contains a fit of the decay to a first order reaction, where $k = 33.5\,s^{-1}$. The optical path length was 12.1 cm.

No acceleration of O_2^- decay was observed in a 2 μM EDTA solution at pH 6.9 $\pm$ 0.1 in the presence of 50–400 μM DFO (Table I). Moreover, in the absence of EDTA the half life of O_2^- increased in the presence of DFO, and the kinetics changed from first to second order (Table I). From the data in Table II it can be calculated that the half life of O_2^- varies between 30–110 ms at pH 6.9–7.4, respectively. In the presence of 400 μM DFO, the half life of O_2^- remained unchanged and the second order rate constant for the reaction between DFO and O_2^- at the physiological pH is less than 2×10^4 $M^{-1}s^{-1}$. The results did not indicate any reaction between DFO and O_2^- at any pH in the range 2.3–7.4 (Table II). From the data at pH 2.3 it can be calculated that the rate constants for the reaction between DFO and $HO_2^\cdot$ is less than 1×10^5 $M^{-1}s^{-1}$. In order to demonstrate that the reaction observed in the presence of DFO is really the spontaneous dismutation of O_2^-, superoxide dismutase (SOD) was added at a concentration that would decrease the half life of the

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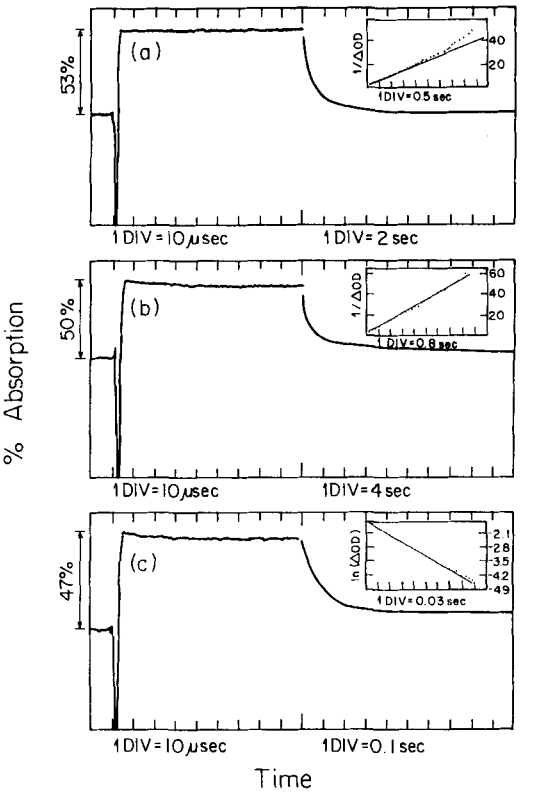

FIGURE 2. Kinetic plots obtained at 265 nm in oxygenated aqueous solutions containing 0.1 M formate and 2 μM EDTA at pH 8.15 (2 mM phosphate buffer).

(a) [DFO] = 0; The inset contains a fit of the decay to a second order reaction, where $2k/\varepsilon l = 8.2\,s^{-1}$.

(b) [DFO] = 100 μM; The inset contains a fit of the decay to a second order reaction, where $2k/\varepsilon l = 7.9\,s^{-1}$.

(c) [DFO] = 100μM, [SOD] = 4.7 nM; The inset contains a fit of the decay to a first order reaction, where $k = 11.7\,s^{-1}$. The optical path length was 12.1 cm.

reaction by about 10-fold, assuming that the 'turnover' rate constant k_{cat} is 2.3×10^9 $M^{-1}s^{-1}$.[13] Indeed, at pH 7.4 in the presence of 0.016 μM SOD, the kinetics changed from a second to a first order reaction. The observed first order rate constant was found to be $35 \pm 2\,s^{-1}$ in agreement with k_{cat} of $(2.2 \pm 0.1) \times 10^9\,M^{-1}s^{-1}$. In Figure 1 typical kinetic plots at pH 7.4 are given: (a) In the absence of DFO and SOD; (b) In the presence of 200 μM DFO; (c) In the presence of 200 μM DFO and 0.016μM SOD.

At pH > 7.5 DFO absorbs at 265 nm, and undergoes photochemistry as was seen from the bleaching of DFO at these pH values. Nevertheless, we tested the ability of DFO to scavenge O_2^- at pH 7.7 and 8.15. The kinetics of the decay of the absorbance measured at 265 nm were similar at both pH's and were somewhat different from those observed at pH $\leqslant$ 7.4. Typical kinetic plots at pH 8.15 are given in Figure 2: (a) In the absence of DFO and SOD; (b) In the presence of 100 μM DFO; (c) In the presence of 100μM DFO and 4.7 nM SOD. In the presence of DFO as well as in the presence of DFO and SOD the initial absorbance 10 μs after the end of the pulse was

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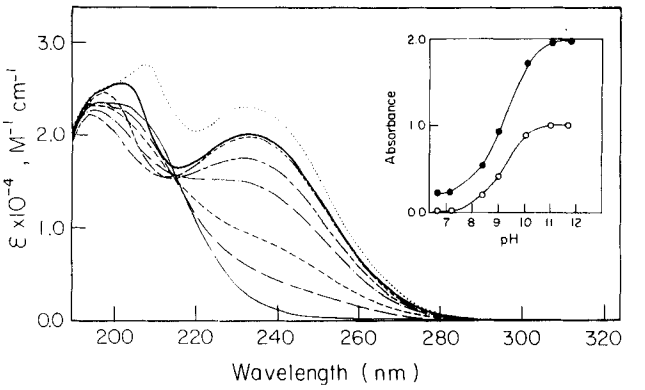

FIGURE 3 The absorbance spectrum of 100 μM DFO at various pH values: pH 6.7; ▬ ▬ ▬ pH 8.4; – – – – – – – – pH 9; ®.®.® pH 9.6; ▬ – ▬ – ▬ pH 10.1; ®– – –®– – – pH 11.2; pH 11.7; ····· pH 12.2.
The inset contains a plot describing the dependence of the absorbance on pH as determined at ● – 234 nm; ○ – 254 nm. The optical path length was 1 cm.

lower than that obtained in their absence, depending inversely on [DFO]. The decay of the adsorbance consisted of three subsequent reactions which were well separated. The last reaction, which is not shown in Figure 2b and 2c, was a very slow reaction mixed with photochemistry, caused by the analyzing light. We were able to determine the rate constant of this slow reaction by preventing the photochemistry through the use of appropriate light filters. At both pH values the reaction obeyed a first order rate law, and a rate constant of $0.06 \pm 0.01\,s^{-1}$ was calculated. The first reaction was completed within 100 μs after the end of the pulse. As the change in the absorbance was only about 10% of the total absorbance, and the rate of the reaction was very fast, it was impossible to determine the kinetics of the first fast process. However, the main process was the spontaneous dismutation of O_2^-, as the second order rate constant of this reaction was the same as in the absence of DFO, and the addition of 4.7 nM SOD changed only the kinetics of the second process from a second to a first order reaction, with $k_{obs} = 11.5 \pm 0.2\,s^{-1}$, yielding a k_{cat} value of $(2.45 \pm 0.05) \times 10^9\ M^{-1}s^{-1}$, which is the value of the 'turnover' rate constant of SOD.

In alkaline solutions, DFO has an intense absorption in the U.V. region, and it undergoes photochemistry. Therefore, under our experimental conditions it was impossible to study the reaction between DFO and O_2^- at pH > 8.2. In Figure 3 the spectra of DFO at pH 6.7–12.2 are given, from which a pK = 9.25 ± 0.05 was calculated for DFO (inset of Figure 3). It seems that as pH > 11.5 a different form of DFO is obtained (Figure 3).

DISCUSSION

Both $HO_2^\cdot$ and O_2^- disappear by second order processes with rates that vary with pH in aqueous solutions.[13] The data in Tables I and II in the presence of EDTA and in the absence of DFO are in agreement with the literature values (Table II).[13] In the absence of EDTA the decay of the absorbance followed a first order rate law, and the

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half life of O_2^- was shorter, as compared to that where EDTA was present, due to metal ion impurities in the solutions. DFO behaved similarly to EDTA, but much higher concentrations of DFO, as compared to EDTA, had to be used in order to change the kinetics from a first to a second order reaction. At pH $\leqslant$ 7.4 DFO did not scavenge superoxide radicals, and upper limits for the rate constants of the reactions between DFO and O_2^- and $HO_2^\cdot$ of 2×10^4 and 1×10^5 $M^{-1}s^{-1}$, respectively, have been determined.

At pH $\geqslant$ 7.7, in the presence of DFO, the results differed from those obtained at pH $\leqslant$ 7.4, where only one decay, the spontaneous dismutation of superoxide radicals, was observed. At pH $\geqslant$ 7.7 three subsequent reactions were observed. The main process among the three is the second reaction, which obeyed a second order rate law. The observed second order rate constant was, within experimental error, identical to that of the spontaneous dismutation of O_2^- at the same pH. Furthermore, the addition of SOD changed only the kinetics of this process from a second to a first order decay. The rate constant of the first order reaction was identical to that of O_2^- with SOD (Figure 2). Therefore, there is no doubt that the second reaction is the spontaneous dismutation of O_2^-. The yield of O_2^-, which decayed by spontaneous dismutation, decreased as the concentration of DFO increased (about 70% and 50% of the total radical yield at 100 μM and 200 μM, respectively). We could not interpret the first and third processes, as we were unable either to conduct the experiments above pH 8.2, or to raise DFO concentration above 200 μM due to the high absorbance obtained under these conditions at the wavelength studied. One of the possible explanations for such behaviour is that the basic form of DFO competes with oxygen for CO_2^- radicals to form a transient which absorbs in the U.V. region, and decomposes slowly. The absorbance of such a species must be lower than that of O_2^- at 265 nm, otherwise the initial absorbance after the end of the pulse would not have decreased as the concentration of DFO increased. This is also supported by the results showing that the contribution of the first and third processes increased with [DFO] (data not shown).

Sabourault *et al.*[11] and Ribiere *et al.*[12] have conducted pulse radiolysis experiments at pH 7.4[11] (very similar, if not identical, graphs and data in an earlier publication were supposed to be at pH 7.2[12]) in the presence of 0.16 M formate and 60 mM phosphate buffer[11,12] and also at pH 8.2[11] in the absence of a buffer. In both studies 100 and 200 μM DFO were used. They claimed that the decay of the absorbance at both pH's consisted of three subsequent reactions. The first reaction was completed within 25 ms after the pulse:

$$O_2^- + DFO + 2H^+ \rightarrow H_2O_2 + DFO^\cdot \quad (8)$$

where

$k_8 = (1.3 \pm 0.1) \times 10^6\ M^{-1}s^{-1}$ at pH 7.4[11] (7.2)[12]

$k_8 = (4.25 \pm 0.1) \times 10^6\ M^{-1}s^{-1}$ at pH 8.2

The second reaction was completed 600–900 ms after the pulse:

$$2DFO^\cdot \rightarrow DFO + DFO'' \quad (9)$$

$2k_9 = (9.7 \pm 0.05) \times 10^4\ M^{-1}s^{-1}$ at pH 7.4[11] (7.2)[12]

$2k_9 = 7.4 \times 10^4\ M^{-1}s^{-1}$ at pH 8.2

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The third process was completed 24 s after the pulse:

$$DFO'' \rightarrow \text{products} \qquad (10)$$

$$k_{10} = 0.07\ s^{-1}$$

They showed that the spectrum of DFO$^{\cdot}$ was pH dependent: ε_{250} = 1000 and 1900 $M^{-1}cm^{-1}$ at pH 7.4 (7.2) and 8.2, respectively. Surprisingly, the species obtained 600–900 ms after the pulse had a higher absorbance than either O_2^- or DFO$^{\cdot}$ (as shown in Figure 2 in reference 11, which appears the same as Figure 3 in reference 12) although they described that the absorption decays with time. Moreover, after 24 s they found a species that also absorbs in the U.V. region. In addition, it is notable that the OD scale on the figures does not fit the ε scale in the same figures, using the dose and the optical path length given. Moreover, the shape and the maxima of all three spectra given for the different species are the same as that of O_2^-.[13]

We have repeated some of their experiments under their conditions. The results in Table I show that the decay of the absorbance did not depend on formate and phosphate concentrations.

Sabourault *et al.*[11] described the first and the second decays, which were over 25 ms and about 1 sec, respectively, after the pulse. We can show that both decays are one process, the dismutation of O_2^-. The absorption after 25 ms, which they attributed to DFO$^{\cdot}$, is that predicted for O_2^- remaining 25 ms after the end of the pulse, where only the dismutation of O_2^- is assumed. The pH dependence of the "ε attributed for DFO$^{\cdot}$" is just the yield of O_2^- at 25 ms, where the pH dependendce of ε originates from the pH dependence of the second order rate constant of O_2^- dismutation (k_D). At pH 7.4 (7.2), taking for O_2^- dismutation $2k_D = 3.9 \times 10^5$ (6.2×10^5) $M^{-1}s^{-1}$, ε_{250} = 2260 $M^{-1}cm^{-1}$,[13] and using their dose where $[O_2^-]_o = 8 \times 10^{-5}$ M, 25 ms after the pulse $[O_2^-] = 4.5 \times 10^{-5}$ (3.6×10^{-5}) M. Similar calculations at pH 8.2 for $[O_2^-]_o = 5 \times 10^{-5}$ M ($2k_D = 6.3 \times 10^4$ $M^{-1}s^{-1}$)[13] yield 25 ms after the pulse $[O_2^-] = 4.6 \times 10^{-5}$ M. With these values one can calculate the OD at 25 ms, which should be observed, and it agrees with those observed by Sabourault *et al.*,[11] who wrongly attributed it to DFO$^{\cdot}$ formation and calculated accordingly for this radical ε_{250} = 1000 and 1900 $M^{-1}cm^{-1}$ at pH 7.4 and 8.2, respectively.

According to the mechanism, suggested by Sabourault *et al.*,[11] $G(H_2O_2) = G(O_2^-) = 2G(DFO)$. They calculated from the slope of the lines in their Figures 4A and 4B values of G(DFO) = 0.31 $\mu molJ^{-1}$ and $G(H_2O_2)$ = 0.69 μolJ^{-1}, respectively.[11] We calculated from the slope of their line in Figure 4A a value which is three times smaller than the value they reported. The lines in Figure 4B seemed to be drawn in an arbitrary way, especially the line in the absence of DFO, the slope of which could be wrong by up to 3-fold, giving different values for $G(H_2O_2)$. Moreover, by using different methods for determining the concentration of H_2O_2, different results were obtained by the same group.[9,11,12]

The use of indirect methods for determining the ability of a compound to catalyse O_2^- dismutation may lead to misinterpretations of the results, especially due to the low concentrations of the compound that had to be used.[14–15] In the case where high concentrations of the compound are used in order to determine its ability to scavenge O_2^-, most of the pitfalls of the method disappear. Thus, there is no reason to believe, as Sabourault *et al.*[11] suggested, that the reaction of the products resulting from the decay of DFO$^{\cdot}$ with the reduced and oxidized forms of cytochrome C is significant. Moreover, Davies *et al.*[16] have determined the half life of DFO$^{\cdot}$ to be about 10 min,

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so that during the irradiation DFO· would be quite stable, and it can easily be detected after 5–10 minutes of radiation. The experimental conditions used by Sabourault *et al.*[11] are not the ideal ones. They used 3.5 mM cytochrome *C*, without the addition of EDTA. Thus, catalytic metal impurities, which did not interfere with their measurements, using the pulse radiolysis method, in the absence of EDTA, may interfere in the gamma radiolysis due to the very low concentration O_2^- produced by this method. Indeed, in the absence of DFO, the yield of the reduced cytochrome *C* was lower than that expected for their dose, although under their conditions the spontaneous dismutation of O_2^- did not compete with the reaction of O_2^- with cytochrome *C*. The fact that 100 μM DFO did not cause any decrease in the initial yield of the reduced cytochrome C, which should have been the case even if $k_8 = 10^3\ M^{-1}s^{-1}$, suggests that their system is different from the method used by Sinaceur *et al.*[9] and Halliwell,[10] where O_2^- was produced by the xanthine/xanthine oxidase in the presence of 10 μM cytochrome C and 1 mM DETAPAC[9] or 10 μM EDTA.[10]

Sabourault *et al.*[11] thought that their results are in agreement with those obtained by Davies *et al.*,[16] who generated DFO· at pH 7 with gamma radiolysis, and determined its half life to be about 10 min. Davies *et al.*[16] did not claim that the yield of DFO· was 100%, and a value of $k_8 = 1 \times 10^3\ M^{-1}s^{-1}$ would have yielded about 50% DFO· under their conditions. They determined the half life of DFO· by taking the e.s.r. spectra 2 and 16 min after the irradiation. Under their conditions, [DFO·] after irradiation is in the range of $1\ \mu M \leqslant [DFO^{\cdot}] \leqslant 10\ \mu M$, and hence the concentration is only about one order of magnitude lower than that obtained by pulse radiolysis, and not several orders of magnitude as thought to be the case by Sabourault *et al.*,[11] Therefore, the species observed by Sabourault *et al.*,[11] for which they determined $2k_9 = 9.7 \times 10^4\ M^{-1}s^{-1}$, is not identical to that observed by Davies *et al.*,[16] where $2k_9 \leqslant 2 \times 10^3\ M^{-1}s^{-1}$. This supports our conclusion that the decay which was followed by Sabourault *et al.*[11] is the spontaneous dismutation of superoxide radical and not the decay of DFO· and the decay of the products produced by the last reaction.

Using the cytochrome *C* and NBT assays, it was found that k_8 is about three times lower at pH 10.2 than that obtained in physiological pH.[10] This may be explained by assuming that O_2^- reacts slowly with the basic form of DFO as compared to the acid form as the pK was determed to be 9.25.

We conclude that under pulse radiolysis conditions DFO does not scavenge O_2^- at physiological pH, and an upper limit for the rate constant for the reactions between DFO and O_2^- of $2 \times 10^4\ M^{-1}s^{-1}$ can be determined.

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