Free Rad. Res., Vol. 20, No. 6, pp. 345-363
Reprints available directly from the publisher
Photocopying permitted by license only

KINETICS OF THE COMPETITIVE DEGRADATION OF DEOXYRIBOSE AND OTHER BIOMOLECULES BY HYDROXYL RADICALS PRODUCED BY THE FENTON REACTION

M.J. ZHAO and L. JUNG*

Laboratoire de Chimie Thérapeutique, Faculté de Pharmacie, 74, route du Rhin, 67401 Illkirch France

C. TANIELIAN and R. MECHIN

Laboratoire de Photochimie, Ecole Européenne des Hautes Etudes des Industries de Strasbourg, 1, rue Blaise Pascal, 67000 Strasbourg France

(Received November 11th, 1993; in revised form December 13th, 1993)

The objective of this work is to reexamine the competitive degradation of deoxyribose by hydroxyl radicals (·OH) produced by the reaction between H_2O_2 and Fe^{2+}-EDTA. The ·OH radicals produced attack deoxyribose (D, rate constant k_D) and eventually an ·OH scavenger (S, rate constant k_S). First, we examined the effect of [D], [H_2O_2], [Fe^{2+}-EDTA], [EDTA]/[Fe^{2+}] ratio and reaction time on the rate of D degradation, measured as the absorbance of the chromogen formed between the product of the reaction D + ·OH (malondialdehyde) and thiobarbituric acid. In particular, it was showed that under our experimental conditions ([D] = 3 mM, [H_2O_2] = 0.85 mM, [Fe^{2+}] = 0.13 mM), the rate of overall process is first order in Fe^{2+}, zero order in H_2O_2 and is maximal for a ratio [EDTA]/[Fe^{2+}] = 1.1. Second, the kinetics of ·OH radical reaction in competition experiments between D and S (mannitol) was investigated. The results show that the ratio of the rates of D degradation in the absence (V_D) and in the presence (V_D^S) of S should be represented by $V_D/V_D^S = 1 + k_S[S]/(k_D[D] + k_x)$ where k_x accounts for the rate of ·OH reactions with other reagents such as Fe^{2+}-EDTA, H_2O_2 etc . . . After having determined k_x for each set of experimental conditions, we obtained the values of k_S/k_D by determining the variations of V_D/V_D^S as a function of [S] and [D]. By taking $k_D = 1.9 \times 10^9\ M^{-1}s^{-1}$, a value of $k_S = 1.9 \times 10^9\ M^{-1}s^{-1}$ was obtained, very close to that obtained by pulse radiolysis. Finally, the validity of the established relation was confirmed for other biomolecules (methionine, $k = 5.6 \times 10^9 M^{-1}s^{-1}$ and alanine, $k = 3.3 \times 10^8\ M^{-1}s^{-1}$). By constrast, it was not applicable to cysteine, thiourea and mercaptoethanol which was attributed to an interaction of the latter scavengers with Fe^{2+} and/or H_2O_2.

KEY WORDS: Hydroxyl radical, Fenton reaction, Deoxyribose, Mannitol, Kinetics.
Abbreviations EDTA: ethylenediaminetetraacetic acid, MDA: malondialdehyde, TBA: thiobarbituric acid.

INTRODUCTION

The hydroxyl radical (·OH) is known to be a powerful active oxidizing agent. It reacts very rapidly with most organic and biological substances and causes serious damage.[1,2] It is consequently very important to know the rate constants for the reactions of ·OH radicals with potential biological targets and with the substances that

*Author to whom correspondence should be addressed. Tel: 033-88 67 69 66.

may protect such targets (·OH scavengers).[3,4] To determine such rate constants, it is first necessary to produce ·OH radicals. Besides the technique of pulse radiolysis,[5] the Fenton reaction is widely used to generate ·OH radicals:[6-8]

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \cdot OH$$

Whenever the ·OH radicals are formed, they will be revealed by reaction with a substrate leading to a product that can be quantitatively detected. When another substance is added to the reaction mixture and if its reaction with ·OH does not give the same product, the competition method can be used and gives the ratio of the rate constants for the reactions of ·OH with the substrate and the substance. This is the basis of Halliwell's method[9] which uses deoxyribose (2-deoxy-D-ribose) as substrate. In this method, deoxyribose (D) is degraded by ·OH radicals generated by the reaction between H_2O_2 and Fe^{2+}-EDTA in the presence of ascorbate. One of the D degradation products is malondialdehyde (MDA)[10] which is quantitatively detected by its ability to react with thiobarbituric acid (TBA) to form a pink chromogen.[11,12] Any ·OH scavenger (S) added to the reaction mixture should compete with deoxyribose for ·OH to an extent depending on its rate constant for reaction with ·OH (k_S) and its concentration relative to deoxyribose; hence, it will decrease the rate of deoxyribose degradation (Figure 1).

In practice, the extent of deoxyribose degradation is measured as the absorbance (A) of the chromogen at 532 nm at a given reaction time.

Whilst investigating a new class of sunscreens, we were particularly interested in their capacity to protect skin against the reactive oxygen species such as singlet oxygen, superoxide anion and hydroxyl radical, and then determined the rate constants of the corresponding reactions. With regard to the ·OH radicals, Halliwell's method is extremely fascinating due to its simplicity. However, we found this method restrictive, for example the deoxyribose concentration used should be greater than 2.8 mM. This requirement renders impossible any competition between deoxyribose and ·OH scavengers whose solubility in aqueous solution is very low. This prompted us to reexamine the kinetics of the Fenton reaction and to define the role of ascorbate in Halliwell's method. In this paper, the results obtained in the absence of ascorbate are presented.

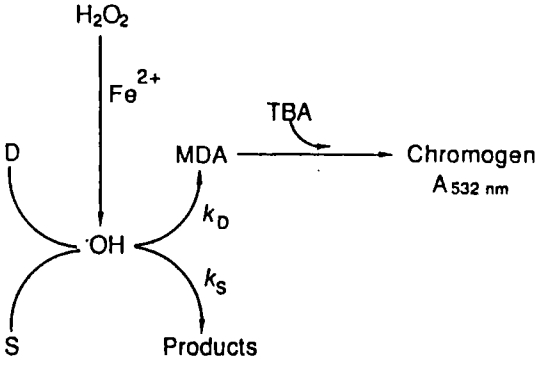

FIGURE 1 Schematic representation of the competition reaction of ·OH generated by Fenton reaction between deoxyribose (D) and a scavanger (S).

MATERIALS AND METHODS

Reagents

2-Deoxy-D-ribose was from Aldrich. All other chemicals used were of the highest grade available.

Degradation of Deoxyribose

Reaction mixtures contained, in a final volume of 3 ml, the following reagents added in the order stated and at the final concentrations noted in the appropriate figures: phosphate-saline buffer, pH 7.4 (24 mM NaH_2PO_4-Na_2HPO_4 in 15 mM NaCl); deoxyribose; hydrogen peroxide (H_2O_2); EDTA and $(NH_4)_2Fe(SO_4)_2$. $(NH_4)_2Fe(SO_4)_2$ and EDTA were premixed just before addition to the reaction mixture. Solution of $(NH_4)_2Fe(SO_4)_2$ was made up immediately before use in de-aerated water. EDTA-$(NH_4)_2Fe(SO_4)_2$ was added to start the reaction. Reaction mixtures were incubated at 37°C for a given time noted in each experiment. 1.5 ml of 2.8% cold trichloroacetic acid were then added and 1.0 ml of the mixture obtained was drawn. Thiobarbituric acid (TBA) reactivity was developed by adding 1 ml of TBA reagent (1%, w/v, in 0.05 M NaOH), followed by heating at 100°C for 15 min. When the mixture was cool, the absorbance at 532 nm was measured against appropriate blanks.

RESULTS AND DISCUSSION

Kinetics of Deoxyribose Degradation: Effect of Experimental Conditions

In order to determine a range of experimental conditions which may be used for the study of various kinds of ·OH scavengers, we first examined the main parameters which may influence the production of ·OH radicals or deoxyribose degradation, such as incubation time, the concentrations of deoxyribose, H_2O_2, Fe^{2+}-EDTA and the EDTA/Fe^{2+} ratio. The kinetics of deoxyribose degradation can be schematized as follows:

$$H_2O_2 + Fe^{2+} \longrightarrow \cdot OH \xrightarrow{D} \text{Chromogen (A)}$$

Fe^{2+} is frequently used in complex form with EDTA[13,14] which prevents iron from binding to deoxyribose. This complexion between iron ions and EDTA of course also prevents the possibility of complex formation between the scavengers and iron ions which should modify the kinetic scheme and the analysis of the results obtained.[12,15,16] Moreover, EDTA maintains iron ions in solution in the presence of phosphate buffer usually used as reaction medium to simulate physiological conditions.[4]

In general, the concentration of H_2O_2 used in the Fenton reaction is in excess over that of Fe^{2+} (10 times or more),[17,18] in order to ensure that the rate of ·OH radical production depends essentially on $[Fe^{2+}]$, and not on $[H_2O_2]$.

Under these conditions, the rates of chromogen formation, dA/dt, of deoxyribose degradation, $-d[D]/dt$, of ·OH radical production, $d[\cdot OH]/dt$, and of Fe^{2+} consumption, $-d[Fe^{2+}]/dt$, are proportional:

$$\frac{dA}{dt} \propto -\frac{d[D]}{dt} \propto \frac{d[\cdot OH]}{dt} \propto -\frac{d[Fe^{2+}]}{dt}$$

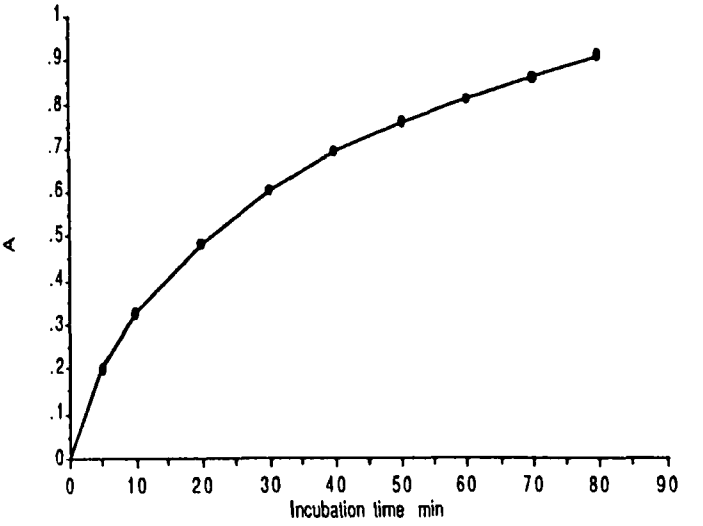

FIGURE 2 Deoxyribose degradation by ·OH radicals vs incubation time. [D] = 3 mM, [H_2O_2] = 0.85 mM, [Fe^{2+}] = 0.13 mM and [EDTA] = 0.143 mM. The reaction was carried out in phosphate buffer (pH7.4) at 37°C. A represents the absorbance at 532 nm of the chromogen formed between deoxyribose degradation product (malondialdehyde) and thiobarbituric acid.

Effect of Incubation Time

The effect of incubation time on deoxyribose degradation was evaluated by using [Fe^{2+}] 0.13 mM, [EDTA] 0.143 mM, [H_2O_2] 0.85 mM and [D] 3 mM (Figure 2). Although [H_2O_2]/[Fe^{2+}] only appeared to be 6.5, in fact, [H_2O_2] greatly exceeded [Fe^{2+}] at the beginning of the reaction, since the Fe^{2+}-EDTA complex oxidizes extremely quickly and by the time it is added to the reaction mixture, most will have formed Fe^{3+}-EDTA[4,19,20] (see later Figure 5). For this reason all the experiments were carried out under strictly identical conditions. The problem of autoxidation of Fe^{2+}-EDTA is, in fact, one reason why the use of Fe^{2+}-EDTA/ascorbate is preferable. As expected the rate of deoxyribose degradation decreased regularly as a consequence of the Fe^{2+}-EDTA content decrease with reaction time.

In an attempt to determine the reaction order of the Fenton reaction in Fe^{2+}, we drew the plot $\ln[(A_\infty/A_\infty - A)]$ as a function of reaction time (Figure 3), in which A_∞ is the absorbance at 532 nm at infinite reaction time and was determined to be 1.016 by the least square method. The fact that Figure 3 gives a straight line signifies that the Fenton reaction or ·OH radical production is first order in Fe^{2+} under our experimental conditions since $dA/dt \propto -d[Fe^{2+}]/dt$.

Effect of Deoxyribose Concentration

The deoxyribose degradation (A) as a function of its initial concentration is shown in Figure 4 using the other experimental conditions similar to those described above (Figure 2). It is observed that the absorbances at 532 nm for a reaction time of 30 min increase sharply as a function of the initial deoxyribose concentration, when the latter is lower than 1 mM. However, when the deoxyribose concentration is higher

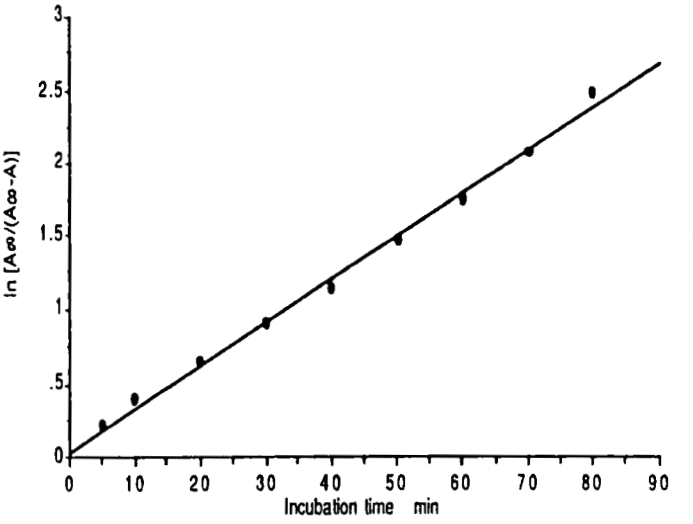

FIGURE 3 Relationship between $\ln(A_\infty/A_\infty - A)$ and incubation time from the data shown in Figure 2.

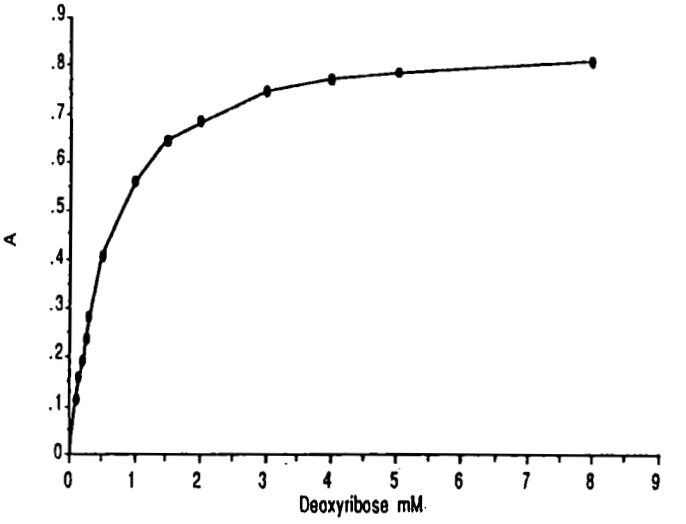

FIGURE 4 Effect of initial deoxyribose concentration on its degradation by ·OH radicals. The other conditions were similar to those of Figure 2 and the reaction time was 30 min.

than 3 mM, the absorbance at 532 nm remains relatively constant. This result suggests that under the latter experimental conditions, deoxyribose intercepts almost all ·OH radicals produced.

Effect of H_2O_2 Concentration

Figure 5 shows that deoxyribose degradation increases with an increasing concentration of H_2O_2 until a plateau is reached at a concentration of about 0.8 mM. This

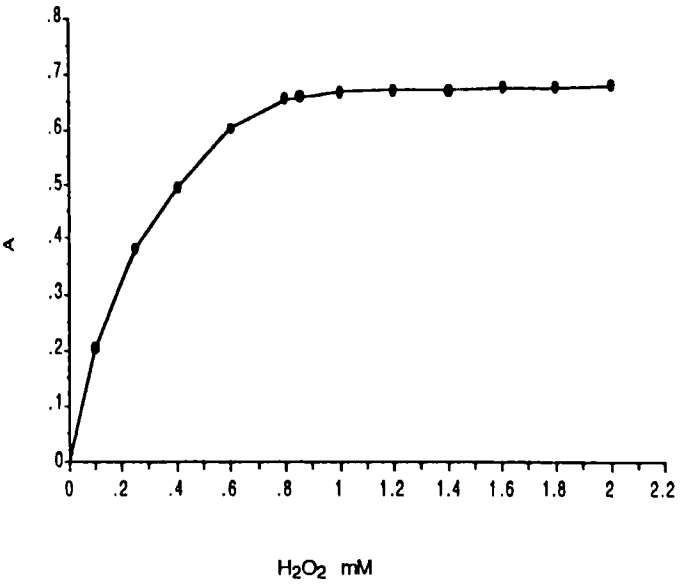

FIGURE 5 Effect of H_2O_2 concentration on deoxyribose degradation. The other conditions were similar to those of Figure 2 and the reaction time was 30 min.

confirms that under our experimental conditions ($[Fe^{2+}] = 0.13$ mM and $[H_2O_2] = 0.85$ mM), $[H_2O_2]$ is far in excess over $[Fe^{2+}]$.

Effect of EDTA/Fe^{2+} Ratio

Deoxyribose degradation was studied by using a fixed concentration of Fe^{2+} ions (0.13 mM) and different concentrations of EDTA (Figure 6). A correlation between the absorbance at 532 nm and the amounts of EDTA was observed at a low concentration, but at a high concentration, the absorbance at 532 nm actually dropped. These results clearly show that a critical EDTA concentration is required for the maximal production of ·OH radicals. In fact, EDTA added to the reaction mixture, thus preventing iron from binding to deoxyribose, increases H_2O_2 decomposition and then ·OH radical production, since the Fe^{2+}-EDTA complex has a redox potential lower than that of free Fe^{2+}: E° (Fe^{3+}/Fe^{2+}) = 0.71 V (pH 3) and E° (Fe^{3+}-EDTA/ Fe^{2+}-EDTA) = 0.12 V (pH 7)[21]. But at the same time, it intercepts ·OH radicals.[22] These two effects are opposed and there exists a concentration of EDTA for a fixed concentration of Fe^{2+}, or a ratio [EDTA]/[Fe^{2+}], for which the concentration of ·OH radicals or deoxyribose degradation is maximal. This ratio is 1.1 under our experimental conditions.

Effect of Fe^{2+}-EDTA Concentration

Figure 7 displays a proportionality between deoxyribose degradation and Fe^{2+}-EDTA concentration up to about 0.13 mM. Above this concentration a deviation from the linearity was observed. This confirms the proportionality $dA/dt \propto d[Fe^{2+}]/dt$. These results are consistent with the fact that under our experimental

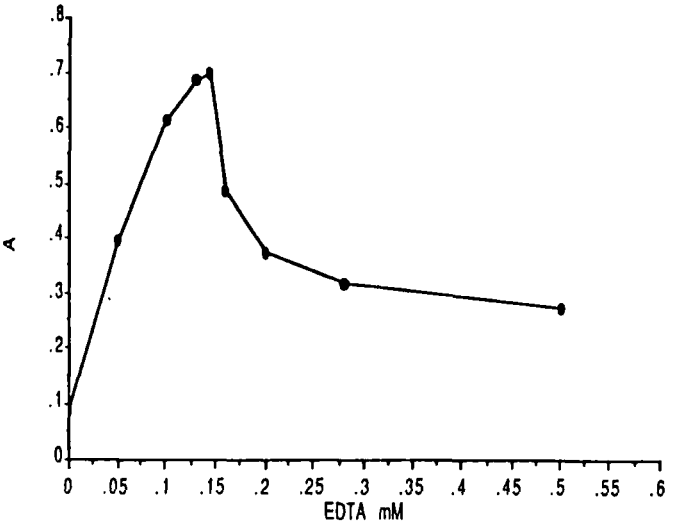

FIGURE 6 Effect of EDTA concentration on deoxyribose degradation. The other conditions were similar to those of Figure 2 and the reaction time was 30 min.

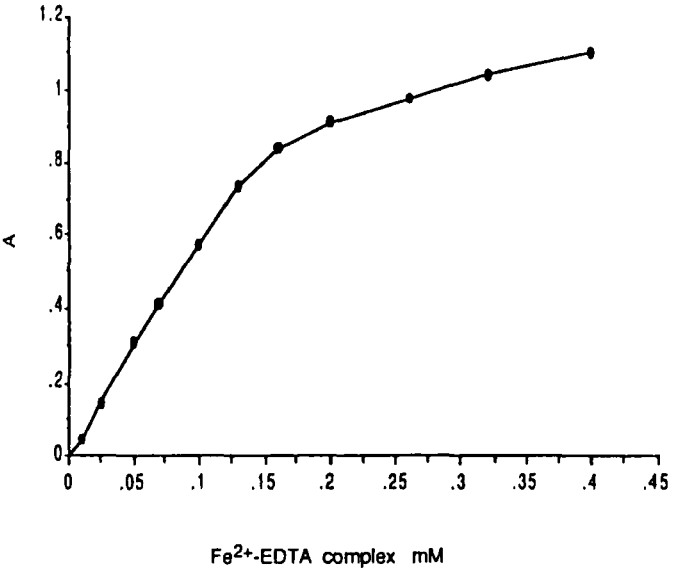

FIGURE 7 Effect of Fe^{2+}-EDTA concentration on deoxyribose degradation. Variable EDTA concentrations were used to hold up the ratio [EDTA]/Fe^{2+}] 1.1. The other conditions were similar to those of Figure 2 and the reaction time was 30 min.

conditions the production of ·OH radicals is first order in Fe^{2+} and that the rate of ·OH production which corresponds to the deoxyribose degradation is proportional to Fe^{2+}-EDTA concentration.

According to the results obtained and the analysis made above, the basic experimental conditions to be used in later experiments are: [D] 3 mM, [H_2O_2] 0.85 mM, [Fe^{2+}] 0.13 mM, [EDTA] 0.143 mM and reaction time 30 min.

Investigation of the Competition Method. Rate Constant for the Reaction of Mannitol with ·OH Radicals

Mannitol is frequently used as a reference scavenger of ·OH radicals for studying the ability of a substance to intercept ·OH radicals.[23,24] In order to examine the kinetic approach which will be developed below, we also chose mannitol as a reference substance, owing to the agreement on the values of its rate constant determined by different methods.[25]

The expected proportionality between the absorbance at 532 nm (A°) and the rate of deoxyribose degradation (V_D) in the absence of a scavenger can be expressed as:

$$A^\circ \propto V_D = V_{\cdot OH} \times \phi_D \tag{1}$$

where $V_{\cdot OH}$ is the rate of ·OH radical production and ϕ_D the probability that D reacts with ·OH radicals. In a similar way, the following equation can be obtained:

$$A \propto V_D{}^S = V^S{}_{\cdot OH} \times \phi_D{}^S \tag{2}$$

where $V_D{}^S$ and $V^S{}_{\cdot OH}$ are the rates of the deoxyribose degradation and the ·OH production in the presence of the scavenger S respectively, and $\phi_D{}^S$ is the probability that D reacts with ·OH in the presence of S. If k_D and k_S are the rate constants for the reactions of D and S with ·OH radicals respectively, the simplest expression that can be drawn from equations (1) and (2) is equation (3):

$$\frac{A^\circ}{A} = 1 + \frac{k_S[S]}{k_D[D]} \tag{3}$$

proposed by Halliwell[9] by making the following implicit hypothesis:

$$V_{\cdot OH} = V^S{}_{\cdot OH},$$

$$\phi_D = 1$$

and $\phi_D{}^S = \dfrac{k_D[D]}{k_D[D] + k_S[S]}$.

According to equation (3), both the plots A°/A vs [S] and A°/A vs 1/[D] should give straight lines with a slope (*sl*) of $k_S/k_D[D]$ and $k_S/[S]k_D$ respectively. Thus, in order to examine these relations, we determined the variations of A°/A with the concentration of mannitol (chosen as a reference scavenger) and that of deoxyribose.

Figure 8 shows the linear relation between A°/A and mannitol concentration for four different deoxyribose concentrations of 0.5, 2, 3 and 4 mM. The corresponding values of ($sl \times k_D[D]$) are not constant (and equal to 0.74×10^9, 1.52×10^9, 1.54×10^9 and 1.58×10^9 $M^{-1}s^{-1}$ respectively, calculated using a rate constant value of 1.9×10^9 $M^{-1}s^{-1}$ for deoxyribose).[26] Consequently equation (3) is not satisfied.

Figure 9 shows the plots A°/A vs 1/[D] for three different mannitol concentrations (1, 3 and 5 mM). It is observed that neither of these plots give a straight line. This means that equation (3) is not satisfied and that, either mannitol interferes with the ·OH formation ($V_{\cdot OH} \neq V^S{}_{\cdot OH}$), or that other processes responsible for the disappearance of ·OH radicals besides its reaction with deoxyribose occur ($\phi_D \neq 1$).

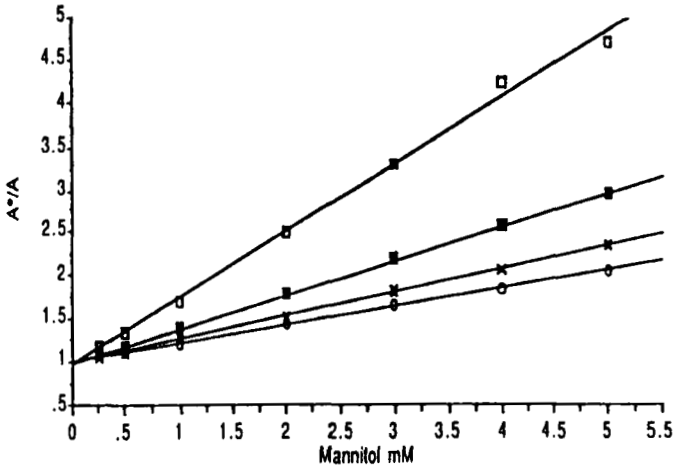

FIGURE 8 Competition experiment between ·OH reactions with deoxyribose (D) and mannitol (S). Variation with [S] of the relative extent A°/A of deoxyribose degradation in the absence (A°) and in the presence (A) of mannitol. □, [D] = 0.5 mM; ■, [D] = 2 mM; ×, [D] = 3 mM and ○, [D] = 4 mM. $[H_2O_2]$ = 0.85 mM, $[Fe^{2+}]$ = 0.13 mM, [EDTA] = 0.143 mM. The reaction was carried out in phosphate buffer (pH 7.4) at 37°C for 30 min.

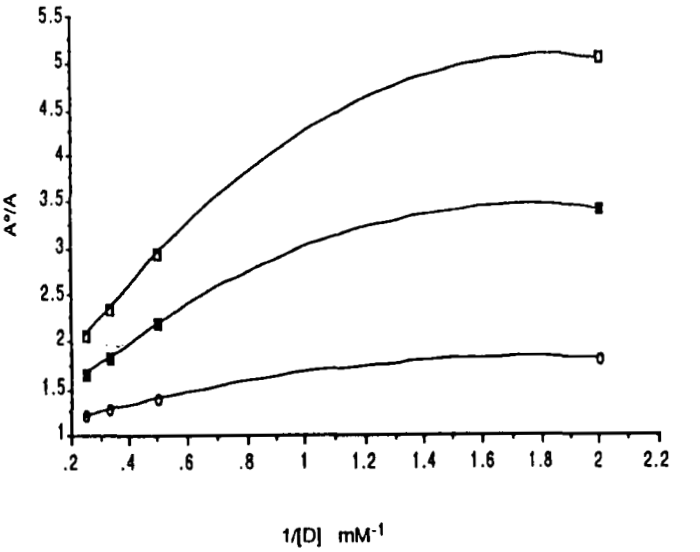

FIGURE 9 Competition experiment between ·OH reactions with deoxyribose (D) and mannitol (S). Variation of the ratio A°/A with [D], ○, [S] = 1 mM; ■, [S] = 3 mM and □, [S] = 5 mM. $[H_2O_2]$ = 0.85 mM, $[Fe^{2+}]$ = 0.13 mM, [EDTA] = 0.143 mM. The reaction was carried out in phosphate buffer (pH 7.4) at 37°C for 30 min.

Thus, taking the latter processes into consideration leads to the modified kinetics of deoxyribose degadation (Figure 10):

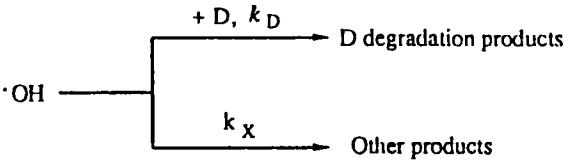

FIGURE 10 Schematic representation of the modified kinetics of deoxyribose degradation.

and to the modified expression for ϕ_D:

$$\phi_D = \frac{k_D[D]}{k_D[D] + k_x} \tag{4}$$

where k_x represents the part of ·OH reacting with other reagents except deoxyribose, such as Fe^{2+}-EDTA complex, H_2O_2 etc . . . In fact, k_x can be tentatively written as:

$$k_x = k_{Fe^{2+}\text{-EDTA}}[Fe^{2+}\text{-EDTA}] + k_{H_2O_2}[H_2O_2] + \ldots \tag{5}$$

where $k_{Fe^{2+}\text{-EDTA}}$ and $k_{H_2O_2}$ represent respectively the rate constants of reactions of ·OH with the corresponding species.

Then k_x depends on experimental conditions such as the concentrations of Fe^{2+}-EDTA complex, H_2O_2 etc . . ., especially on that of Fe^{2+}-EDTA since $k_{Fe^{2+}\text{-EDTA}}$ is nearly two orders of magnitude greater than $k_{H_2O_2}$.[22,27]

The rate of deoxyribose degradation will be

$$V_D = V_{\cdot OH}\frac{k_D[D]}{k_D[D] + k_x} \tag{6}$$

and

$$\frac{1}{V_D} \propto \frac{1}{A^\circ} \propto \frac{1}{V_{\cdot OH}}\left(1 + \frac{k_x}{k_D[D]}\right) \tag{7}$$

Figure 11 represents the linear variations of $1/A^\circ$ vs $1/[D]$ for different concentrations of Fe^{2+}-EDTA. The corresponding k_x/k_D values are 0.47, 0.76 and 1.02 mM for Fe^{2+}-EDTA concentrations of 0.08, 0.13 and 0.20 mM respectively. According to equation (5) which can be expressed as

$$\frac{k_x}{k_D} = \frac{k_{Fe^{2+}\text{-EDTA}}}{k_D}[Fe^{2+}\text{-EDTA}] + \frac{k_{H_2O_2}}{k_D}[H_2O_2] + \ldots,$$

the plot k_x/k_D vs $[Fe^{2+}$-EDTA] should give a straight line, as shown in Figure 12. From this line, $k_{Fe^{2+}\text{-EDTA}}$ is calculated as $8.6 \times 10^9\ M^{-1}s^{-1}$ if a value of $1.9 \times 10^9\ M^{-1}s^{-1}$ for k_D is used. The observed value is slightly greater than that reported for EDTA itself ($2.7 \times 10^9\ M^{-1}s^{-1}$).[22]

Insertion of the processes represented by k_x, into the probability that ·OH radicals react with D in the presence of S, gives:

$$V_D^S = V^S_{\cdot OH} \times \phi_D^S = V^S_{\cdot OH}\frac{k_D[D]}{k_D[D] + k_S[S] + k_x} \tag{8}$$

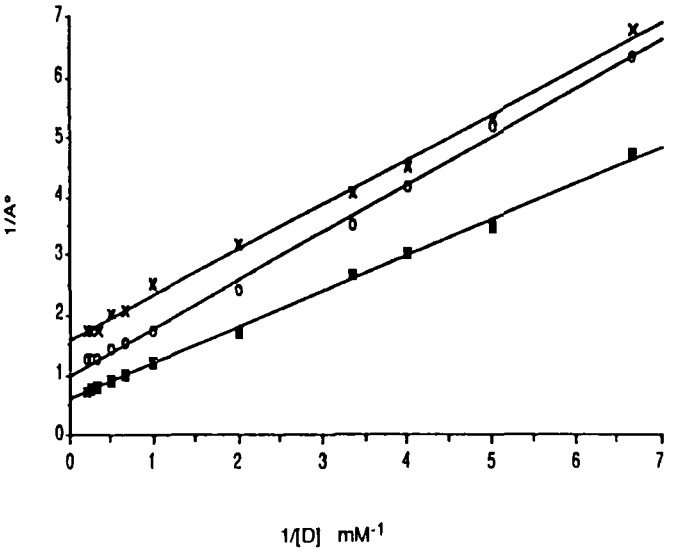

FIGURE 11 Representation of the variations of deoxyribose degradation with its concentration according to equation (7). Determination of parameter k_x, x, $[Fe^{2+}]=0.08$ mM; ○, $[Fe^{2+}]=0.13$ mM and ■, $[Fe^{2+}]=0.20$ mM. Ratio $[EDTA]/[Fe^{2+}]=1.1$ for the three concentrations of Fe^{2+} used. $[H_2O_2]=0.85$ mM. The reaction was carried out in phosphate buffer (pH 7.4) at 37°C for 30 min.

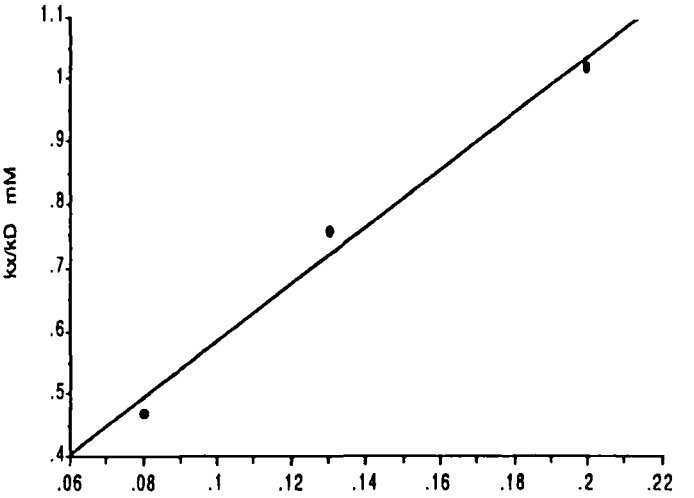

FIGURE 12 Tentative interpretation of the parameter k_x: relationship between k_x and Fe^{2+}-EDTA complex concentration. The values of k_x were obtained from the data shown in Figure 11.

From equations (6) and (8) we deduce:

$$\frac{V_D}{V_D^S} = \frac{A^\circ}{A} = \frac{V_{\cdot OH}}{V^S_{\cdot OH}}\left(1 + \frac{k_S[S]}{k_D[D] + k_x}\right) \tag{9}$$

Since all the lines A°/A vs [S] cut closely across 1 on *y*-axis (Figure 8), the term $V_{\cdot OH}/V^S_{\cdot OH}$ should be equal to 1, in other words, $V_{\cdot OH} = V^S_{\cdot OH}$. This result suggests that although mannitol is known to form complexes with metal ions and hydrogen peroxide,[28,29] it has no effect on the production of ·OH radicals under our experimental conditions. Thus equation (9) can be simplified as:

$$\frac{A^\circ}{A} = 1 + \frac{k_S[S]}{k_D[D] + k_x} \tag{10}$$

According to equation (10), there are two possibilities to determine k_S. The first is to draw the line A°/A vs [S] using a fixed [D], and the second the line A°/A vs $1/(k_D[D] + k_x)$ using a fixed [S].

The first possibility was verified by calculating the rate constant of mannitol from the *sl* displayed on Figure 8. The rate constant values obtained from

$$k_S = sl\,(k_D[D] + k_x)$$

are 1.88×10^9 ([D] = 0.5 mM), 2.09×10^9 ([D] = 2 mM), 1.93×10^9 ([D] = 3 mM) and $1.88 \times 10^9\ M^{-1}s^{-1}$ ([D] = 4 mM) respectively. We observe that this time the k_S value is independent of deoxyribose concentration used. The mean value of the k_S obtained is $(1.94 \pm 0.10) \times 10^9\ M^{-1}s^{-1}$.

The second possibility to determine k_S from equation (10), was examined by drawing the kinetic plots A°/A vs $1/(k_D[D] + k_x)$ according to the results shown in Figure 9. The introduction of the parameter k_x into the kinetic equation resulted in,

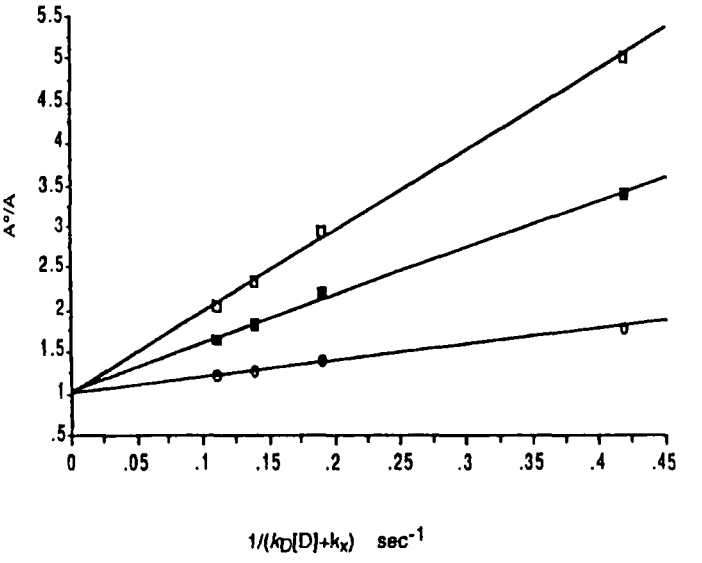

FIGURE 13 Relationship between A°/A and $1/k_D[D] + k_x$, from the data shown in Figure 9. ○, [S] = 1 mM; ■, [S] = 3 mM and □, [S] = 5 mM.

as shown in Figure 13, the linear lines A°/A vs $1/(k_D[D]+k_x)$ for three different mannitol concentrations. The rate constant values of mannitol calculated from the slopes of the lines are 1.88×10^9 ([S] = 1 mM), 1.89×10^9 ([S] = 3 mM) and 1.94×10^9 $M^{-1}s^{-1}$ ([S] = 5 mM) respectively; the mean value is $(1.90\pm0.05)\times10^9\ M^{-1}s^{-1}$. The good concordance between the latter values and the preceding ones demonstrates the autocoherence of the two approaches which consist in varying either [S] or [D].

As a general rule, equation (10) may be used to determine, without experimental condition limitation, the rate constant of substances which do not influence the rate of ·OH radical production. In contrast, equation (3) can be used only when the deoxyribose concentration is sufficiently high in such a way that $k_D[D] \gg k_x$.

TABLE 1
Effect of different experimental conditions on the measurement of the rate constant of mannitol. The reaction was carried out in phosphate buffer (pH 7.4) at 37°C

Mannitol (mM)	[D] (mM)	[Fe^{2+}] (mM)	[EDTA] (mM)	[H_2O_2] (mM)	Incubation time (min)	Rate constants ($M^{-1}s^{-1}$)
variable	3	0.13	0.143	0.85	30	1.93×10^9
variable	3	0.08	0.088	0.85	30	1.91×10^9
	3	0.2	0.22	0.85	30	1.87×10^9
variable	3	0.13	0.143	0.40	30	2.00×10^9
	3	0.13	0.143	1.40	30	1.92×10^9
variable	3	0.13	0.143	0.85	10	1.88×10^9
	3	0.13	0.143	0.85	20	1.81×10^9
	3	0.13	0.143	0.85	60	1.91×10^9
variable	0.5	0.13	0.143	0.85	30	1.88×10^9
	2	0.13	0.143	0.85	30	2.09×10^9
	4	0.13	0.143	0.85	30	1.88×10^9
1	variable	0.13	0.143	0.85	10	1.88×10^9
3		0.13	0.143	0.85	20	1.89×10^9
5		0.13	0.143	0.85	60	1.94×10^9

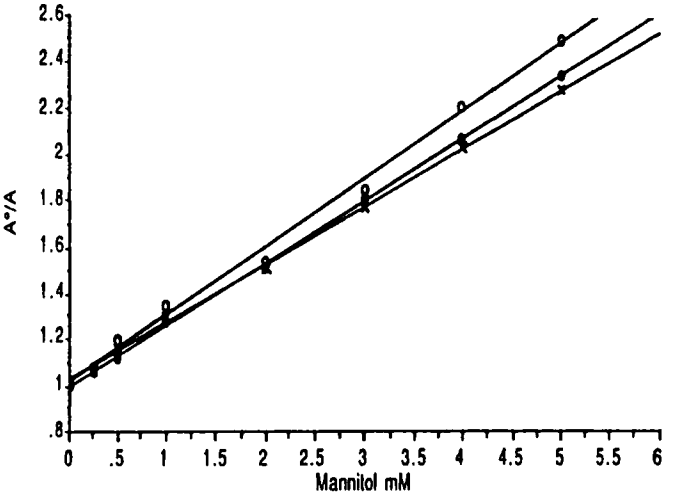

FIGURE 14 Relationship between A°/A and mannitol concentration for three concentration of Fe^{2+}: ○, 0.08 mM, ●, 0.13 mM and *x*, 0.20 mM. The ratio [EDTA]/[Fe^{2+}] was equal to 1.1. [D] = 3 mM, [H_2O_2] = 0.85 mM. The reaction was carried out in phosphate buffer (pH 7.4) at 37°C for 30 min.

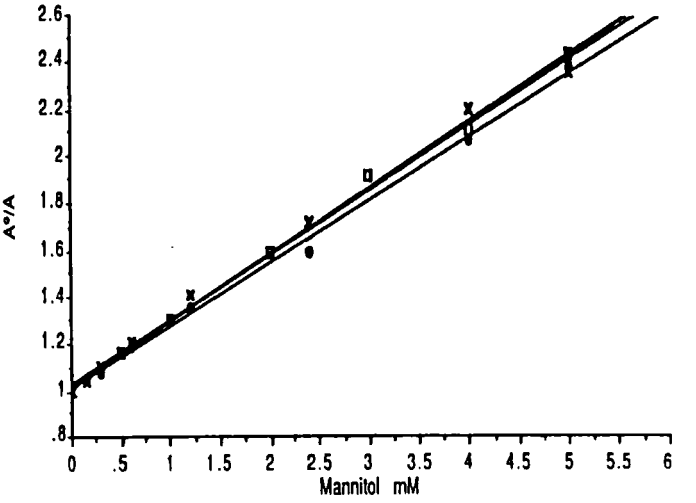

FIGURE 15 Relationship between A°/A and mannitol concentration for three concentrations of H_2O_2: *x*, 0.4 mM, □, 0.85 mM and ●, 1.4 mM. [D] = 3 mM, [Fe^{2+}] = 0.13 mM, [EDTA] = 0.143 mM. The reaction was carried out in phosphate buffer (pH 7.4) at 37°C for 30 min.

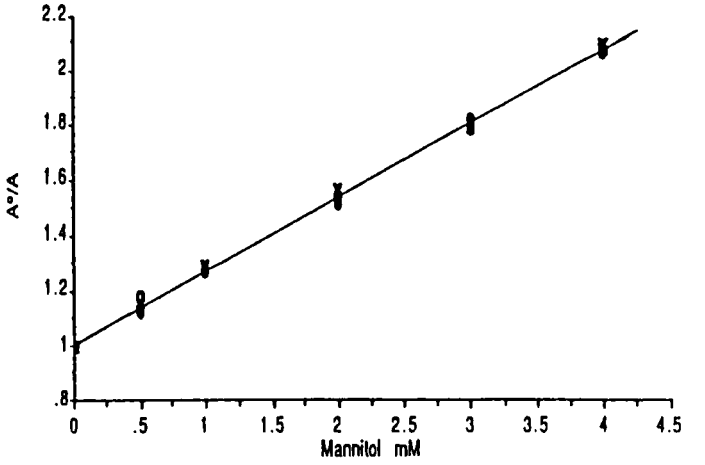

FIGURE 16 Relationship between A°/A and mannitol concentration for four incubation times: *x*, 10 min., ○, 20 min, ●, 30 min, and □, 60 min. [D] = 3 mM, [Fe^{2+}] = 0.13 mM, [EDTA] = 0.143 mM. [H_2O_2] = 0.85 mM. The reaction was carried out in phosphate buffer (pH 7.4) at 37°C.

Next, we studied the validity of the preceding conclusions by extending the range of experimental conditions. Thus, the rate constant of mannitol was determined at different concentrations of Fe^{2+}-EDTA and H_2O_2 and for different incubation times (Figures 14, 15 and 16). We observe that all the plots A°/A vs mannitol concentration give straight lines. The rate constant values of mannitol obtained under these various conditions, as well as those determined above, are summarized in Table 1. We find that these values are in close ageement with each other [the mean value is

$(1.91 \pm 0.07) \times 10^9\,M^{-1}s^{-1}$ calculated taking a value of $1.9 \times 10^9\,M^{-1}s^{-1}$ for deoxyribose][26] and with the value of literature ($1.8 \times 10^9\,M^{-1}s^{-1}$).[25] These results confirm that the hypothesis made and the corresponding analysis, are valid for a wide scale of experimental conditions.

Restriction in the Applicability of the Competition Method: Interaction of Biomolecules with the Fenton Reagents

In order to extrapolate our kinetic analysis, we tried to determine the rate constants of some substances of biological interest, such as alanine, methionine, thiourea, cysteine and mercaptoethanol. It is observed that only the two plots A°/A vs [S] for alanine (Figure 17) and methionine (Figure 18) give straight lines. The rate constants are calculated to be 3.3×10^8 for alanine and $5.6 \times 10^9\,M^{-1}s^{-1}$ for methionine by using a value of $1.9 \times 10^9\,M^{-1}s^{-1}$ for deoxyribose.[26] These values are close to those of the literature, i.e., 1.2×10^8 for alanine[26] and $5.1 \times 10^9\,M^{-1}s^{-1}$ for methionine.[26] Thus, for the three substances investigated up to this point, we obtain the reactivity ratios 2.95/1.00/0.17 for methionine, mannitol and alanine with respect to deoxyribose. The selected literature values obtained by pulse radiolysis lead to the relative values 2.68/0.95/ 0.06 respectively. This suggests that the proposed kinetic approach may provide reliable results within a wide range of reactivities. By contrast, the plots A°/A vs [S] for thiourea (Figure 19), cysteine and mercaptoethanol (Figure 20) demonstrated complex kinetic plots. The fact that the behaviour of these three substances derives from linearity, suggests that they are reacting either with Fenton reagents,[30] or with the intermediate radicals derived from the ·OH attack. It is known that thiourea reacts with H_2O_2 and reduces the rate of ·OH generation,[31,32] whereas cysteine and mercaptoethanol regenerate Fe^{2+} from Fe^{3+} and increase the rate of ·OH generation.[33,34] In these two cases, the rates of ·OH production differ

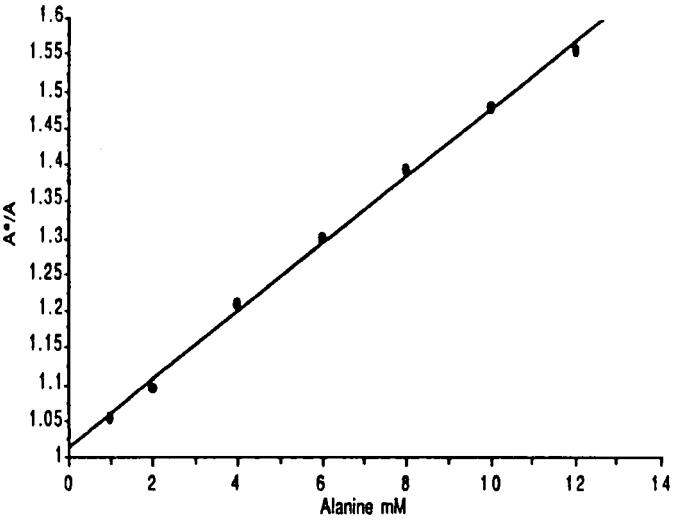

FIGURE 17 Relationship between A°/A and alanine concentration. Validity of the equation (10). [D] = 3 mM, $[Fe^{2+}]$ = 0.13 mM, [EDTA] = 0.143 mM. $[H_2O_2]$ = 0.85 mM. The reaction was carried out in phosphate buffer (pH 7.4) at 37°C for 30 min.

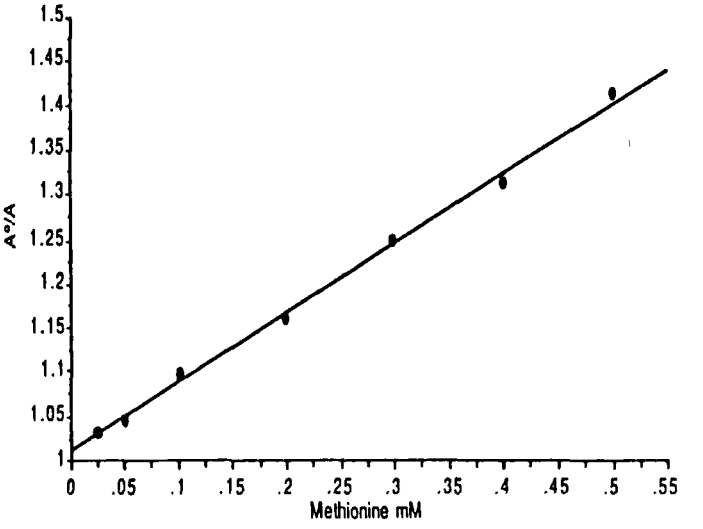

FIGURE 18 Relationship between A°/A and methionine concentration. Validity of the equation (10). The same conditions as for Figure 17 were used.

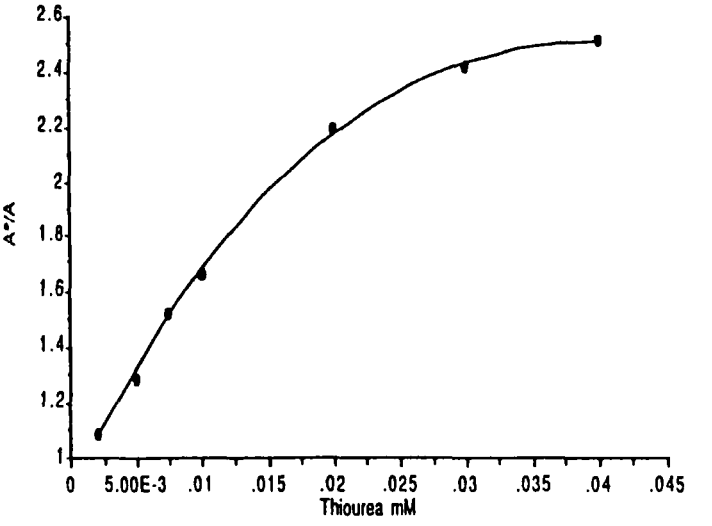

FIGURE 19 Relationship between A°/A and thiourea concentration. Non applicability of the equation (10). The same conditions as for Figure 17 were used.

according to whether the scavenger is present or not, i.e., $V_{\cdot OH} \neq V^{S}_{\cdot OH}$.[30] Thus the competition for ·OH radicals between deoxyribose and the scavenger is not a simple one and equation (9) cannot be simplified to become equation (10). In other words, the interference of the scavenger with the Fenton reagents will be revealed in a nonlinear competition plot. However, the inclusion of ascorbate in the reaction mixture may overcome, in certain cases, the problem of interference owing to a large increase in the rate of ·OH generation.[9,35,36]

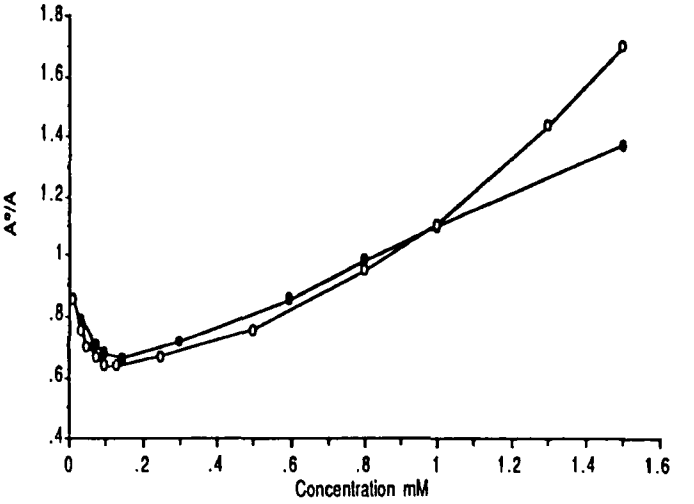

FIGURE 20 Relationship between A°/A and concentrations of cysteine (○) and of mercaptoethanol (●). Non applicability of the equation (10). The same conditions as for Figure 17 were used.

CONCLUSION

We studied the competition method to determine rate constants for reactions of ·OH radicals with biomolecules using the Fenton reaction and deoxyribose. It was found that the equation (3) can be used only when the deoxyribose concentration is relatively high. This condition does not prevent the normal use of equation (3) for water-soluble compounds. However, this is not the case for the compounds whose solubility in water is low. In order to make equation (3) applicable to all conditions, we therefore introduced a parameter k_x to represent the part of ·OH radicals which reacts with other reagents except deoxyribose, such as Fe^{2+}-EDTA, H_2O_2 etc . . . This modified equation (10) was justified by the linearity of the two type plots (A°/A vs [S] and A°/A vs $1/(k_D[D]+k_x)$ and the consistence between k_S of mannitol obtained in this study under different experimental conditions and that obtained by pulse radiolysis. The advantage of the latter equation is that we can use any concentration of deoxyribose to determine the rate constant of scavengers. This allows us to optimize the competition conditions ($k_S[S]$ and $k_D[D]+k_x$ have a same order of magnitude). However, it should be noted that some substances such as thiourea, cysteine and mercaptoethanol give more complex results due to their interference with the Fenton reagents. In all cases, this is translated by nonlinear kinetic competition plots.

References

1. G.V. Buxton, C.L. Greenstock, W.P. Helman and A.B. Ross (1988) Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals (·OH/·O⁻) in aqueous solution. *Journal of Physical and Chemical Reference data*, **17**, 676–759.
2. B. Halliwell and M. Grootveld (1987) The measurement of free radical reactions in humans. *FEBS Lettrs*, **213**, 9–13.
3. K.O. Hiller, P.L. Hodd and R.L. Willson (1983) *Chemico-Biological Interactions*, **47**, 293–305.

4. M. Grootveld and B. Halliwell (1986) An aromatic hydroxylation assay for hydroxyl radicals utilizing high-performance liquid chromatogaphy (HPLC). Use to investigate the effect of EDTA on the Fenton reaction. *Free Radical Reseach Communications*, **1**, 243–250.
5. M. Ebert, J.P. Keene, A.J. Swallow and J.H. Baxendale (1965) *Pulse Radiolysis*. Academic Press. London.
6. W.T. Dixon and R.O.C. Norman (1962) Free radicals formed during the oxidation and reduction of peroxides. *Nature (London)*, **196**, 891–892.
7. B.C. Gilbert and M. Jeff (1988) Oxidative damage and radical repair. One-electron transfer reactions involving metal ions, peroxides and free radicals. In *Free Radicals in Chemistry, Biology and Medicine* (Eds. C. Rice-Evans and T. Dormandy), Richlieu press, 25–49.
8. B.C. Gilbert, J.K. Stell and M. Jeff (1988) Electron-spin resonance studies of the effect of copper (II) and copper (I) on the generation and reactions of organic radicals formed from the Fenton reaction and the Ti^{III}-H_2O_2 and Ti^{III}-$S_2O_8^{-2}$ redox couples. *Journal of the Chemical Society, Perkin Transactions*, **2**, 1867–1873.
9. B. Halliwell, J.M.C. Gutteridge and O.I. Aruoma (1987) The deoxyribose method: a simple "test-tube" assay for determination of rate constant for reaction of hydroxyl radicals. *Analytical Biochemistry*, **165**, 215–219.
10. K.I. Cheeseman, A. Beavis and H. Esterbauer (1988) Hydroxyl-radical-induced iron-catalysed degradation of 2-deoxyribose. *Biological Journal*, **252**, 649–653.
11. J.M.C. Gutteridge (1981) Thiobarbituric acid-activity following iron-dependent free radical damage to amino acids and carbohydrates. *FEBS Letters*, **128**, 343–346.
12. J.M.C. Gutteridge (1987) Ferrous-salt-promoted damage to deoxyribose and benzoate. *Biochemical Journal*, **243**, 709–714.
13. B. Halliwell (1978) Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelators. Is it a mechanism for hydroxyl radical production in biochemical systems? *Chemico-Biological Interaction*, **15**, 77–89.
14. S. Croft, B.C. Gilbert, J.R. Lindsay Smith and A.C. Whitwood (1992) An E.S.R. investigation of the reactive intermediate generated in the reaction between Fe^{II} and H_2O_2 in aqueous solution. Direct evidence for the formation of the hydroxyl radical. *Free Radical Reseach Communications*, **17**, 21–39.
15. J.M.C. Gutteridge (1984) Reactivity of hydroxyl and hydroxyl-like radicals discriminated by release of thiobarbituric acid-reactive material from deoxy sugars, nucleosides and benzoate. *Biochemical Journal*, **224**, 761–767.
16. O.I. Aruoma, M. Grootveld and B. Halliwell (1987) The role of iron in ascorbate-dependent deoxyribose degradation. Evidence consistent with a site-specific hydroxyl radical generation caused by iron bound to the deoxyribose molecule. *Journal of Inorganic Biochemistry*, **29**, 289–299.
17. O.I. Aruoma and B. Halliwell (1988) The iron-banding and hydroxyl radical scavenging action of anti-inflammatory drugs. *Xenobiotic*, **18**, 459–470.
18. J.M.C. Gutteridge (1990) Superoxide-dependent formation of hydroxyl radical from ferric-complexes and hydrogen peroxide: an evaluation of fouteen iron chelators. *Free Radical Reseach Communications*, **9**, 119–125.
19. B. Halliwell and J.M.C. Gutteridge (1981) Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts: the role of superoxide and hydroxyl radicals. *FEBS Letters*, **128**, 347–352.
20. D. Yin, H. Lingnert, B. Erstrand and U.T. Brunk (1992) Fenton reagents may not initiate lipid peroxidation in an emulsified linoleic acid model system. *Free Radical Biology and Medicine*, **13**, 543–556.
21. W.H. Koppenol and J. Butler (1985) Energetics of interconversion of oxyradicals. *Advance in Free radicals in Biology and Medicine*, **1**, 91–131.
22. C. Walling, R.E. Partch and T. Weil (1975) Kinetics of the decomposition of hydrogen peroxide catalysed by ferric ethylenediaminetetraacetate complex. *Proceeding of the National Academy of Sciences USA*, **72**, 140–142.
23. H.P. Misra and I. Fridovich (1976) Superoxide dismutase and the oxygen enhancement of radiation lethality. *Archives of Biochemistry Biophysics*, **176**, 577–581.
24. J.M.C. Gutteridge and B. Halliwell (1982) The role of superoxide and hydroxyl radicals in the degradation of DNA and deoxyribose induced by a copper-phenanthroline complex. *Biochemical Pharmacology*, **31**, 2801–2805.
25. S. Goldstein and G. Czapski (1984) Mannitol as an ·OH scavenger in aqueous solution and in biological systems. *International Journal of Radiation Biology*, **46**, 725–729.
26. M. Anbar and P. Neta (1967) A compilation of specific biomolecular rate constants for the reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals with inorganic and organic compounds in aqueous solution. *International Journal of Applied Radiation Isotopes*, **18**, 493–523.
27. C. Willing (1975) Fenton's reagent revisited. *Accounts of Chemical Research*, **8**, 125–131.

28. E.J. Boume, R. Nery and H. Weigel (1959) Metal chelators of polyhydroxy compounds. *Chemical Industry (London)*, 998-999.
29. The *Merck Index* 11th Edition. Merck and Co., Inc. Rahway N.J., USA (1989).
30. B. Halliwell (1990) How to characterize a biological antioxidant. *Free Radical Reseach Communications*, **9**, 1-32.
31. A.J.F. Searle and A. Tomasi (1982) Hydroxyl free radical production in iron-cysteine solution and protection by zinc. *Journal of Inorganic Biochemistry*, **17**, 161-166.
32. A.I. Cederbaum, E. Dicker, E. Rubin and G. Cohen (1979) Effect of thiourea on microsomal oxidation of alcohols and associated microsomal functions. *Biochemistry*, **18**, 1187-1191.
33. D.A. Rowley and B. Halliwell (1982) Superoxide-dependent formation of hydroxyl radicals in the presence of thiol compounds. *FEBS Letters*, **138**, 33-36.
34. D.A. Rowley and B. Halliwell (1982) Superoxide-dependent formation of hydroxyl radical from NADH and NADPH in the presence of iron salts. *FEBS Letters*, **142**, 39-41.
35. J.R. Mahoney and E. Graf (1986) Role of alpha-tocopherol, ascorbic acid, citric EDTA as oxidants in the model systems. *Journal of Food Science*, **51**, 1293-1296.
36. M. Scarpa, R. Slevanato, P. Viglino and A. Rigo (1983) Superoxide ion as active intermediate in the autoxidation of ascorbate by molecule oxygen. *Journal of Biological Chemistry*, **258**, 6695-6697.

Accepted by Professor B. Halliwell