Competitive Measurement of Rate Constants for Hydroxyl Radical Reactions Using Radiolytic Hydroxylation of Benzoate

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Hydroxyl radicals were generated from N_2O-saturated phosphate-buffered saline solution (pH 7.5) by irradiation with a 3.7 TBq ^{137}Cs source. The radicals attacked benzoate to form highly fluorescent products. The induction of fluorescence was tested in 0.002 to 2 mM of benzoate solution; the fluorescence intensity was approximately constant at benzoate concentrations more than 0.1 mM during irradiation, and increased linearly with irradiation dose up to 53 Gy tested. The major primary products detected by high-performance liquid chromatography were three monohydroxybenzoates, 2-, 3- and 4-hydroxybenzoate (HOBZ). The *G*-values obtained from γ-irradiation for 60 min of a 0.2 mM benzoate solution were *G*(2-HOBZ) = 0.97, *G*(3-HOBZ) = 0.48 and *G*(4-HOBZ) = 0.45. As the fluorescence of irradiated benzoate solution arose mostly from 2- and 3-HOBZ and the intensity of 2-HOBZ is 30 times that of 3-HOBZ, 98% of the fluorescence intensity induced was ascribed to 2-HOBZ. A hydroxyl radical scavenger competed with benzoate for hydroxyl radicals produced, and diminished the fluorescent products. A rate constant for the reaction of a scavenger with hydroxyl radical could be determined from the reduction in fluorescence intensity, using 0.2 mM benzoate and various concentrations of the scavenger. The fluorescence intensity was detectable down to 0.15 μM of 2-HOBZ produced. For various compounds, rate constants obtained in this way were similar to those measured by pulse-radiolysis.

Keywords hydroxyl radical; gamma-irradiation; rate constant; benzoate; fluorescence; hydroxybenzoate

The hydroxyl radical is known to be a powerfully active oxidizing agent. It reacts with most organic and biological substances rapidly at almost a diffusion-controlled rate.[1] When it is formed in biological systems, the reaction causes serious damage. Many rate constant have therefore been measured by pulse-radiolysis, γ-irradiation, and Fenton-type reaction systems. The pulse-radiolysis method is most accurate and extensive rate constants obtained in this way have been published.[1] The γ-irradiation method using bleaching of *p*-nitrosodimethylaniline (PNDA) as a detector molecule was proposed by Kralijič and Trumbore,[2] and was applied for the determination of rate constants of commonly occurring amino acids and several enzymes.[3] The bleaching mechanism of PNDA by γ-irradiation is ambiguous, however and the difference in optical density at 440 nm between the initial and bleached samples was small, whereas, it has been reported[4] that the radiolysis of benzoate solution induced hydroxylation, decarboxylation with the release of CO_2, and formation of phenol. The strong fluorescences of hydroxybenzoates were used to measure the conversion of superoxide to hydroxyl radical.[5] Further, using the reaction of hydroxyl radicals generated by Fenton reaction with benzoate as a detector molecule, rate constants for scavengers were determined.[6] Though it was a simple test-tube method, several values were lower than those obtained by pulse-radiolysis.

In the present paper, we exposed benzoate to hydroxyl radicals generated by γ-irradiation in N_2O-saturated aqueous solution, and ascertained the primary fluorescent products by high-performance liquid chromatography (HPLC). Then, the rate constants for different hydroxyl radical scavengers were determined through competition with benzoate and compared with those obtained by pulse-radiolysis.

Experimental

Materials Sodium benzoate, 2-hydroxybenzoate (2-HOBZ) and 4-hydroxybenzoate (4-HOBZ) were purchased from Nakalai Tesque, Inc. (Kyoto, Japan). 3-Hydroxybenzoate (3-HOBZ) was obtained from Tokyo Kasei Co. (Tokyo, Japan). 1-Methyl-2-mercaptoimidazole was purchased from K & K Laboratories and was used after recrystallization from methanol. All other chemicals used were of the highest purity available. Deionized water (18 MΩ/cm) generated from a Millipore Milli Q purification system was used in all experiments. All reaction mixtures were prepared in Chlex 100-treated Dulbecco phosphate-buffered saline solution without calcium and magnesium ions, pH 7.5.[7]

Irradiation Gamma-irradiation was carried out using a 3.7 TBq ^{137}Cs source with the dose rate of 32 Gy/h, as determined by a Fricke dosimeter. The concentration of benzoate solution used on irradiation was 0.2 mM. Each benzoate solution (1.0 ml) containing various concentrations of a scavenger was put into a polypropylene tube (75 × 12 mm), bubbled with N_2O for 1.5 min and sealed, since the effect of bubbling time was the same at more than 1 min. Irradiation was performed for 60 min at room temperature (20—25 °C), after which 2.0 ml of phosphate buffer was added

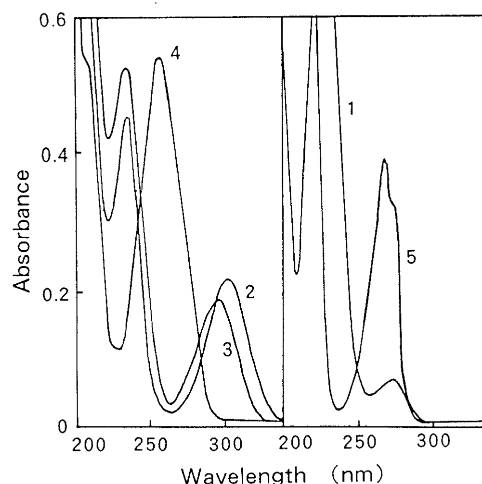

Fig. 1. Absorption Spectra of Benzoate, Monohydroxybenzoates and Phenol at pH 2.5

Authentic benzoate (1; BZ), 2-HOBZ (2), 3-HOBZ (3), 4-HOBZ (4) and phenol (5) were dissolved in 10% (v/v) acetonitrile–10 mM phosphate buffer, pH 2.5. The concentrations were 10 μg/ml for benzoate, 2-HOBZ and 3-HOBZ, and 5 μg/ml for 4-HOBZ and phenol.

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to each solution, and then the fluorescence intensity was measured at 407 nm emission after excitation at 305 nm. The intensity of 98% was converted into a concentration of 2-HOBZ as described below. Three separate experiments were performed in duplicate.

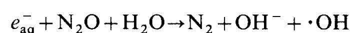
Chromatography Hydroxylated products of benzoate were analyzed by HPLC. Hydroxylated products were extracted as described by Gutteridge.[6] The products formed by irradiation for 60 min were extracted from solution adjusted to pH 2.5 by adding 1 N H_3PO_4 to ethyl acetate and the solvent was removed under a stream of N_2. The residue was dissolved in HPLC eluent, giving a 20-fold concentration of products. Chromatography was carried out on a LC-6A HPLC instrument (Shimadzu, Kyoto, Japan) equipped with a SPD-6AV spectrophotometer and a SPD-M6A photodiode array detector. Separations were achieved on a Lichrosphere 100 RP-18 (5 μm) 250 × 4.0 mm i.d. column and a guard column (4 × 4 mm i.d.) in a column oven at 40 °C using a gradient elution.[6] The initial solvent was 10% (v/v) acetonitrile–10 mM phosphate buffer, pH 2.5. The acetonitrile concentration was raised linearly to 60% (v/v) over 20 min. The injection volume of sample was 20 μl and the flow rate was 1.0 ml/min. Peak elutions were detected at 240 nm for 3-HOBZ, 4-HOBZ and benzoate, at 280 nm for phenol, and at 300 nm for 2-HOBZ and 3-HOBZ, since benzoate and the expected products, 2-HOBZ, 3-HOBZ, 4-HOBZ and phenol, absorb in the region of 200 to 340 nm at pH 2.5 as shown in Fig. 1.

Results and Discussion

During the irradiation of aqueous solutions, the following reaction takes place:

$$H_2O \rightarrow e^-_{aq},\ \cdot OH,\ H_2O_2,\ H_2,\ H_3O^+$$

When the solution is saturated with N_2O, hydrated electron (e^-_{aq}) is converted into hydroxyl radical ($\cdot$OH)



$$e^-_{aq} + N_2O + H_2O \rightarrow N_2 + OH^- + \cdot OH$$

and hydroxyl radical then comprises more than 90% of all the radicals present in the solution.[8] After N_2O saturated benzoate solution was irradiated, the solution was highly fluorescent (Fig. 2). The detector molecule, benzoate, is poorly fluorescent. It has been reported[4] that, when the benzoate molecule is attacked by hydroxyl radical, monohydroxybenzoates, dihydroxybenzoates and phenol are formed. As shown in Fig. 2, 2-HOBZ shows the highest fluorescence among the three monohydroxybenzoates and 4-HOBZ is weakly fluorescent. Further, when dihydroxybenzoates and phenol are excited at 305 nm, they scarcely show the fluorescence. Hence, the highly fluorescent irradiation-products may be 2-HOBZ and 3-HOBZ.

Thus, the irradiated benzoate solution was analyzed by HPLC separation (Fig. 3). The retention times of peaks 1, 2, 3 and 4 corresponded to those of authentic 4-HOBZ, 3-HOBZ, benzoate and 2-HOBZ, respectively. Each peak fraction was treated with 1 N NaOH to make it basic, and then only peaks 2 and 4 appeared to be fluorescent. Further, the four peaks were analyzed using a photodiode array detector. The spectra of peak fractions 1, 2, 3 and 4 agreed well with those of 4-HOBZ, 3-HOBZ, benzoate and 2-HOBZ, respectively (data not shown). As shown in Fig. 3, no major peaks corresponding to phenol at 280 nm and dihydroxybenzoates at 240 nm were observed, since authentic phenol and dihydroxybenzoates are eluted near 12 min and earlier than 9 min, respectively. Accordingly, the major primary products formed during the irradiation were identified as 2-HOBZ, 3-HOBZ and 4-HOBZ.

Benzoate solutions were irradiated for 60 min at various concentrations of 2 μM to 2 mM. The fluorescence intensities induced were similar in more than 0.1 mM of solution (Fig. 4). In the irradiation of 0.2 and 1.0 mM benzoate solutions, the fluorescences increased linearly with irradiation time up to 100 min (53 Gy); the intensity induced in 1.0 mM benzoate solution was only 1.35 times that of 0.2 mM solution (data not shown). Accordingly, 0.2 mM benzoate solution was used to measure the rate constants for hydroxyl radicals.

The 0.2 mM benzoate solutions were irradiated for 60 and

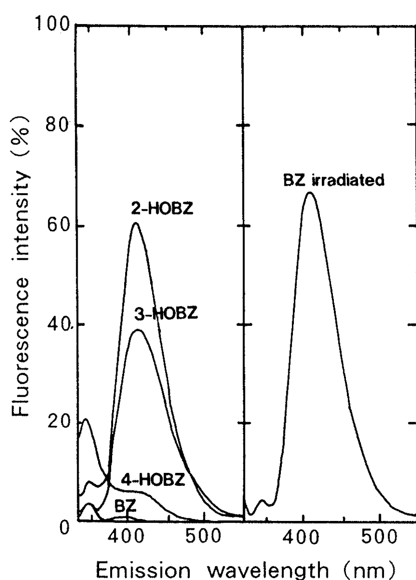


Fig. 2. Fluorescence Spectra of γ-Irradiated Benzoate Solution and Authentic Hydroxybenzoates

Benzoate (BZ, 0.2 mM), 2-HOBZ (1 μM), 3-HOBZ (20 μM) and 4-HOBZ (200 μM) were in phosphate-buffered saline solution, pH 7.5. Benzoate solution was γ-irradiated for 60 min (32 Gy). Emission was scanned at an excitation of 305 nm.

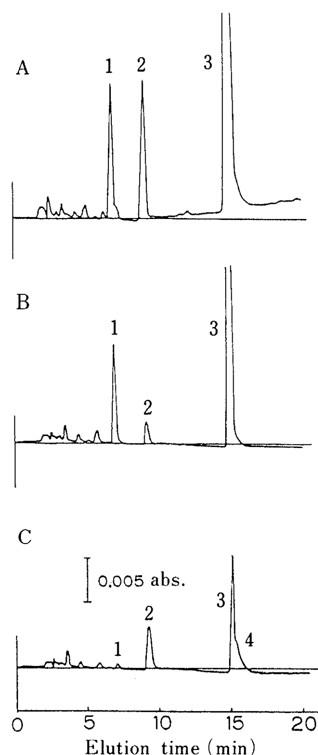


Fig. 3. Chromatograms of Benzoate Hydroxylation Products

The hydroxylation products were extracted from 0.2 mM benzoate solution irradiated for 60 min. HPLC separation was carried out as described in Experimental. A, 240 nm; B, 280 nm; C, 300 nm. Authentic benzoate, 2-HOBZ, 3-HOBZ and 4-HOBZ were eluted in about 15, 15.6, 9 and 7 min, respectively. Peaks were assigned to 4-HOBZ for peak 1, 3-HOBZ for peak 2, BZ for peak 3 and 2-HOBZ for peak 4.

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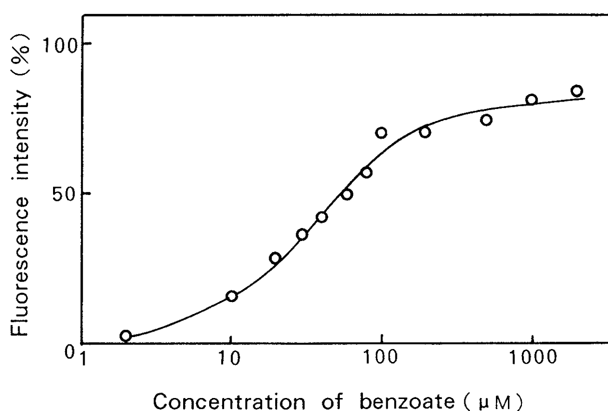

Fig. 4. Fluorescences of Benzoate Solution γ-Irradiated at Various Concentrations

Benzoate solutions of 0.002 to 2 mM were γ-irradiated in phosphate-buffered saline solution, pH 7.5, for 60 min (32 Gy).

Table I. Yields of Radiolytic Products from Benzoate Solution

Product	Yield/μM (*G*-value)	
	60 min	180 min
2-HOBZ	3.2 (0.97)	10.3 (1.0)
3-HOBZ	1.6 (0.48)	5.1 (0.51)
4-HOBZ	1.5 (0.45)	4.5 (0.45)

G-value: molecules/100 eV. Benzoate solutions of 0.2 mM were irradiated for 60 and 180 min at a dose rate of 32 Gy/h with a 3.7 TBq ^{137}Cs source.

180 min to learn the yields of products. The yields of the major products, 2-HOBZ, 3-HOBZ and 4-HOBZ, were determined using HPLC separations for 3-HOBZ and 4-HOBZ and fluoresence measurements for 2-HOBZ. Table I shows three yeidls and the *G*-values. The *G*-values represent the number of HOBZ molecules formed per 100 eV of adsorbed energy. The concentrations of 3-HOBZ and 4-HOBZ were determined using their calibration curves of the peak areas detected at 240 nm *versus* the concentrations (curves not shown). The yields of 3-HOBZ and 4-HOBZ were 1.6 and 1.5 μM for 60 min-irradiation, and 5.1 and 4.5 μM for 180 min-irradiation, respectively. The fluorescence intensities of 1.6 and 5.1 μM of 3-HOBZ were estimated using the fluorescence calibration curve as shown in Fig. 5. 4-HOBZ was little fluorescent at concentrations of less than 10 μM. Accordingly, the fluorescence intensities of 2-HOBZ produced were obtained by subtracting the fluorescence intensities of 3-HOBZ from the intensities of irradiated benzoate solutions, and then were converted into concentrations using the fluorescence calibration curve as shown in Fig. 5. The yields of 2-HOBZ were 3.2 μM for 60 min-irradiation, and 10.3 μM for 180 min-irradiation. The *G*-values of 2-HOBZ, 3-HOBZ and 4-HOBZ obtained by 60 min-irradiation were 0.97, 0.48 and 0.45, respectively; these were similar to the *G*-values for 180 min-irradiation as shown in Table I. The results indicate that *G*-values are independent of irradiation time up to 180 min under these experimental conditions. The ratio of 2-HOBZ : 3-HOBZ : 4-HOBZ yields was 2 : 1 : 0.9, similar to those reported previously.[4a,b] Further, the fluorescence intensity of 2-HOBZ was 30 times that of 3-HOBZ, based on their calibration curves as shown in Fig. 5. In the irradiated

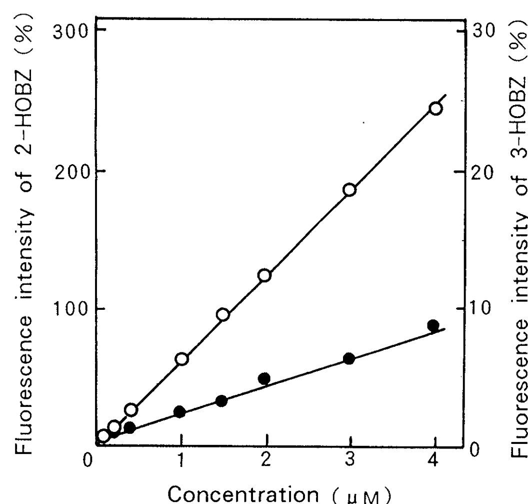

Fig. 5. Fluorescence Calibration Curves of 2-HOBZ and 3-HOBZ

—○—, 2-HOBZ; —●—, 3-HOBZ. 2-HOBZ and 3-OHBZ were in phosphate-buffered saline solution, pH 7.5. The fluorescence intensity was measured at 407 nm excited at 305 nm. Calibration curves were obtained as $y=0.95+61.6x$, $r=1.000$, for 2-HOBZ and $y=0.38+2.07x$, $r=0.998$, for 3-HOBZ.

benzoate solution, therefore, 98% of the fluorescence intensity induced arose from 2-HOBZ and the residual percent from 3-HOBZ.

If N_2O-saturated benzoate solution is irradiated in the presence of another substrate, benzoate (BZ) and a scavenger (S) are competitively attacked by hydroxyl radicals (·OH) as given by

$$\mathrm{BZ} + \cdot\mathrm{OH} \xrightarrow{k_{BZ}} \mathrm{BZOH} \quad (1)$$

$$\mathrm{S} + \cdot\mathrm{OH} \xrightarrow{k_S} \mathrm{P} \quad (2)$$

and monohydroxybenzoates (BZOH) and products of scavenger (P) are formed. The concentrations of monohydroxybenzoates induced, [BZOH], and the products of scavenger, [P], are given by

$$[\mathrm{BZOH}] = K_{BZ}[\mathrm{BZ}][\cdot\mathrm{OH}] \quad (3)$$

$$[\mathrm{P}] = K_S[\mathrm{S}][\cdot\mathrm{OH}] \quad (4)$$

In the absence of scavenger, the concentrations of monohydroxybenzoates induced, $[\mathrm{BZOH}]_0$, and hydroxyl radicals, [·OH], are given by

$$[\mathrm{BZOH}]_0 = k_{BZ}[\mathrm{BZ}][\cdot\mathrm{OH}] + k_S[\mathrm{S}][\cdot\mathrm{OH}] \quad (5)$$

$$[\cdot\mathrm{OH}] = \frac{[\mathrm{BZOH}]}{k_{BZ}[\mathrm{BZ}]} \quad (6)$$

These equations are shown as follows:

$$\frac{1}{[\mathrm{BZOH}]} = \frac{1}{[\mathrm{BZOH}]_0} + \frac{k_S[\mathrm{S}]}{k_{BZ}[\mathrm{BZ}][\mathrm{BZOH}]_0} \quad (7)$$

The [BZOH] can be calculated from the induced fluorescence using their yields (Table I) and fluorescence calibration curves (Fig. 5). A plot of 1/[BZOH] against [S] should give a straight line of slope *m* with an intercept on the *y*-axis *a*

$$m = \frac{k_S}{k_{BZ}[\mathrm{BZ}][\mathrm{BZOH}]_0} \quad (8)$$

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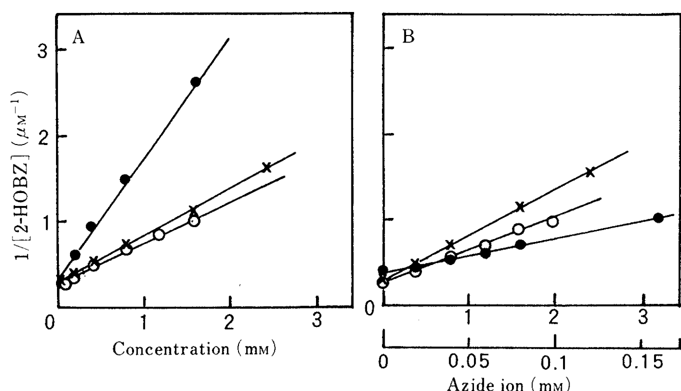

Fig. 6. Reciprocal of the Concentration of 2-HOBZ Produced as a Function of Scavenger Concentration

A: —●—, histidine; —○—, ethanol; —×—, deoxyribose. B: —●—, azide ion; —○—, glucose; —×—, mannitol. Benzoate solutions of 0.2 mM were γ-irradiated for 60 min in the presence of various concentrations of a scavenger.

$$a=\frac{1}{[\mathrm{BZOH}]_0} \tag{9}$$

and the rate constant for reaction of S with hydroxyl radical, k_S, can be given by

$$k_S=\frac{mk_{BZ}[\mathrm{BZ}]}{a} \tag{10}$$

In this paper, [BZOH] is represented as [2-HOBZ], since 98% of the fluorescnece intensity was due to 2-HOBZ as described above. The rate constant used, k_{BZ}, was $5.9 \times 10^9\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}$, which is the value selected from multiple measurements by several investigators using various pulse-radiolysis experiments,[1] and the concentration of benzoate, [BZ], was $2 \times 10^{-4}\,\mathrm{M}$.

Figure 6 shows the results of several experiments in which the scavengers were histidine, deoxyribose, ethanol, mannitol, glucose and azide ion; such linear plots were also obtained in similar experiments with other scavengers. Table II summarizes the results obtained. For most scavengers except thiourea there is close agreement between the data of this work and pulse-radiolysis study. The benzoate hydroxylation by Fenton reaction gave lower values with mannitol, ethanol, histidine and formate ion. In the case of thiourea, it was noted[1] that the rate constant by pulse-radiolysis, $3.9 \times 10^9\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}$, could be 2 to 3 times smaller than that expected as a sulfur compound. Further, the rate constant for 1-methyl-2-mercaptoimidazole (MMI), which in an aqueous solution exists as a thione form[9] as dose thiourea, was reported to be $1.4 \times 10^{10}\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}$ by pulse-radiolysis.[10] In our benzoate method, we obtained a similar value, $1.13 \times 10^{10}\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}$, for MMI, and $9.8 \times 10^9\,\mathrm{M}^{-1}\,\mathrm{s}^{-1}$ for thiourea.

In this study, irradiation of benzoate was carried out by a small gamma source, and the major primary fluorescent product was 2-HOBZ, which was rather stable and very easily detected. The strong fluorescence of 2-HOBZ made it possible to sensitively determine the rate constants of different compounds for hydroxyl radicals.

Table II. Rate Constants for ·OH Scavengers Determined by Benzoate Hydroxylation Using γ-Irradiation

Scavenger	Rate constant ($\times 10^{-9}\,\mathrm{M}^{-1}\cdot\mathrm{s}^{-1}$)		
	This work[a]	Benzoate hydroxylation[b]	Pulse-radiolysis[c]
Deoxyribose	2.3	2.6	2.5
Glucose	1.5	1.5	1.5
Mannitol	1.9	1.1	1.7
Methanol	0.83	—	0.97
Ethanol	2.2	0.7	1.9
tert-Butyl alcohol	0.48	—	0.60
2-Propanol	1.6	1.5	1.9
Histidine	4.8	2.7	5.0
Formate ion	3.2	2.0	3.2
Thiocyanate ion	12.1	—	11
Iodide ion	10.6	—	11
Azide ion	13.9	—	12
Thiourea	9.8	8.5	3.9[d]
MMI	11.3	—	14[e]

MMI: 1-methyl-2-mercaptoimidazole. *a*) The rate constants were calculated using $5.9 \times 10^9\,\mathrm{M}^{-1}\cdot\mathrm{s}^{-1}$ for benzoate. The values shown represent the results obtained in at least three duplicate experiments and have deviations of less than ±5% except for deoxyribose, glucose and mannitol, which have errors of up to ±10%. *b*) From ref. 6 (by Fenton reaction). The original data determined using $3.3 \times 10^9\,\mathrm{M}^{-1}\cdot\mathrm{s}^{-1}$ for benzoate were corrected for $5.9 \times 10^9\,\mathrm{M}^{-1}\cdot\mathrm{s}^{-1}$. *c*) From ref. 1. *d*) It was commented in ref. 1 that this k value could be 2 to 3 times larger. *e*) From ref. 10.

References

1) G. V. Buxton, C. L. Greenstock, W. P. Helman, A. B. Ross, *J. Phys. Chem. Ref. Data*, **17**, 513 (1988).
2) I. Kralijič, C. N. Trumbore, *J. Am. Chem. Soc.*, **87**, 2547 (1965).
3) T. Masuda, S. Nakano, M. Kondo, *J. Radiat. Res.*, **14**, 339 (1973).
4) *a*) W. A. Armstrong, B. A. Black, D. W. Grant, *J. Phys. Chem.*, **64**, 1415 (1960); *b*) I. Loeff, A. J. Swallow, *ibid.*, **68**, 2470 (1964); *c*) G. W. Klein, K. Bhatia, V. Madhavan, R. H. Schuler, *ibid.*, **79**, 1767 (1975).
5) M. S. Baker, J. M. Gebicki, *Arch. Biochem. Biophys.*, **234**, 258 (1964); W. H. Melhuish, H. C. Sutton, *J. Chem. Soc., Chem. Commun.*, **1978**, 970.
6) J. M. C. Gutteridge, *Biochem. J.*, **243**, 709 (1987).
7) R. Dulbecco, M. Vogt, *J. Exp. Med.*, **99**, 167 (1954).
8) V. S. Rao, S. Goldstein, G. Czapski, *Free Rad. Res. Commun.*, **12—13**, 67 (1991).
9) A. L. Hayden, M. Maienthal, *J. Assoc. Offic. Agr.*, **48**, 596 (1965).
10) J. J. Taylor, R. L. Willson, P. Kendall-Taylor, *FEBS Lett.*, **176**, 337 (1984).

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