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Ferrous-salt-promoted damage to deoxyribose and benzoate

The increased effectiveness of hydroxyl-radical scavangers in the presence of EDTA

John M. C. GUTTERIDGE
Division of Antibiotics and Chemistry, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, U.K.

Hydroxyl radicals ($OH^{\cdot}$) in free solution react with scavengers at rates predictable from their known second-order rate constants. However, when $OH^{\cdot}$ radicals are produced in biological systems by metal-ion-dependent Fenton-type reactions scavengers do not always appear to conform to these established rate constants. The detector molecules deoxyribose and benzoate were used to study damage by $OH^{\cdot}$ involving a hydrogen-abstraction reaction and an aromatic hydroxylation. In the presence of EDTA the rate constant for the reaction of scavengers with $OH^{\cdot}$ was generally higher than in the absence of EDTA. This radiomimetic effect of EDTA can be explained by the removal of iron from the detector molecule, where it brings about a site-specific reaction, by EDTA allowing more $OH^{\cdot}$ radicals to escape into free solution to react with added scavengers. The deoxyribose assay, although chemically complex, in the presence of EDTA appears to give a simple and cheap method of obtaining rate constants for $OH^{\cdot}$ reactions that compare well with those obtained by using pulse radiolysis.

INTRODUCTION

Radiolysis of water produces a substantial yield of hydroxyl radicals ($OH^{\cdot}$) in free solution. Any molecules present in radiolysed water will react with $OH^{\cdot}$ radicals at rates predictable from their established second-order rate constants. Some of these molecules react with $OH^{\cdot}$ in aqueous solution rapidly at almost diffusion-controlled rates, and are popularly known as 'hydroxyl-radical scavengers'. These include simple substances such as mannitol, thiourea, glucose, histidine, butan-1-ol, propan-2-ol, formate, benzoate and many others (for a compilation see Anbar & Neta, 1967).

In biological systems, not subjected to high-energy radiation, the majority of $OH^{\cdot}$ radicals, or oxidants with similar activity, such as ferryl ions (FeO^{2+}), are probably produced when certain transition-metal ions react with H_2O_2 in a Fenton-type reaction. The reactions leading to the formation of $OH^{\cdot}$ radicals from ferrous ions are shown in eqns. (1)–(3):

$$Fe^{2+} + O_2 \rightleftharpoons Fe^{3+}{-}O_2^{-} \rightleftharpoons Fe^{3+} + O_2^{-} \quad (1)$$

$$2O_2^{-} + 2H^{+} \rightarrow H_2O_2 + O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\cdot} \quad (3)$$

Biology, however, does not usually allow reactive metal ions to exist in free solution, they are bound with varied affinities to ligands. In the case of iron, the most abundant biological transition metal, binding affinity may be high (as occurs with the proteins transferrin or lactoferrin) or may be relatively low (as seen with albumin, carbohydrates and phosphate esters). If the low-affinity ligand-bound metal ion is able to react with H_2O_2, then $OH^{\cdot}$ radicals will be formed at the point of metal binding, giving rise to site-specific damage to the ligand (Van Hemmen & Meuling, 1975; Czapski, 1978). Damage caused at a specific site will be difficult to arrest or prevent by adding $OH^{\cdot}$ scavengers unless they have the special ability to approach the metal-binding sites closely. However, if a scavenger molecule has a higher binding affinity for the metal than the ligand, then the scavenger can protect the ligand, transferring the damage to itself (Gutteridge, 1984*b*). In a similar way the metal chelator EDTA can remove iron from a detector molecule and transfer damage elsewhere.

In the studies reported in the present paper, deoxyribose and benzoate were used to detect damage by $OH^{\cdot}$ in the presence and in the absence of EDTA. Damage to the carbohydrate deoxyribose by a hydrogen-abstraction mechanism results in the release of thiobarbituric acid-reactive material (Bucknall *et al.*, 1978; Von Sonntag, 1980; Gutteridge, 1981), whereas benzoate is hydroxylated to hydroxybenzoates by an addition reaction (Armstrong *et al.*, 1960) as well as decarboxylated to phenol (Loeff & Swallow, 1964; Winston & Cederbaum, 1982). By using the hydrogen-abstraction and addition reactions it was found that in most cases EDTA increased the effectiveness of the $OH^{\cdot}$ scavengers.

MATERIALS AND METHODS

Materials

2-Deoxy-D-ribose, 3-hydroxybenzoate and 4-hydroxybenzoate were from the Sigma Chemical Co., Poole, Dorset, U.K. All other chemicals used were of the highest purity available from BDH Chemicals, Poole, Dorset, U.K.

Degradation of deoxyribose

The deoxyribose reaction summarized below:

$$\xrightarrow[O_2]{Fe^{2+}} OH^{\cdot} + \text{deoxyribose} \longrightarrow \text{fragment} \xrightarrow[\text{Acid}]{\text{Heat}}$$

Malondialdehyde-like thiobarbituric acid-reactive adduct absorbing at 532 nm

was carried out in 30 mM-sodium phosphate buffer, pH 7.4, containing 40 mM-NaCl. The concentrations of deoxyribose present in the reaction mixtures are shown in the appropriate Figures and Table. The final concentration of ferrous salt, freshly prepared in Chelex-resin-treated distilled water, pH 7.0, in all reactions was 30 μM. Where indicated, EDTA was also added to the reaction before the addition of the ferrous salt at a final concentration of 43 μM. Tube contents were incubated at 37 °C for 1 h, and thiobarbituric acid-reactivity was developed by adding 0.5 ml of thiobarbituric acid reagent (1%, w/v, in 50 mM-NaOH) and 0.5 ml of 2.8% (w/v) trichloroacetic acid followed by heating at 100 °C for 10 min. When the mixture was cool the absorbance was measured at 532 nm against appropriate blanks.

Hydroxylation of benzoate

The buffer concentrations were as described above for deoxyribose degradation. However, the iron salt was added at a final concentration of 200 μM to sodium benzoate at reaction concentrations shown in appropriate Figures and Table. EDTA, when added before the iron salt, was present at a final concentration of 30 μM. The mixtures were incubated at 37 °C for 1 h, and fluorescence was measured at 407 nm emission after excitation at 305 nm. Results were expressed in relative fluorescence intensity units against a standard of 1,1,4,4-tetraphenylbuta-1,3-diene (10 μM). The rates of deoxyribose degradation and benzoate hydroxylation were constant during incubation. Results shown are the means for three separate experiments in which the results differed by less than $\pm 5\%$.

H.p.l.c. separation of hydroxylation products

Hydroxylated products were extracted from acidified solution into equal volumes of ethyl acetate. The solvent was removed under a stream of N_2, and the residue was dissolved in 0.5 ml of ethanol, giving a 20-fold concentration of the material. Separations were achieved

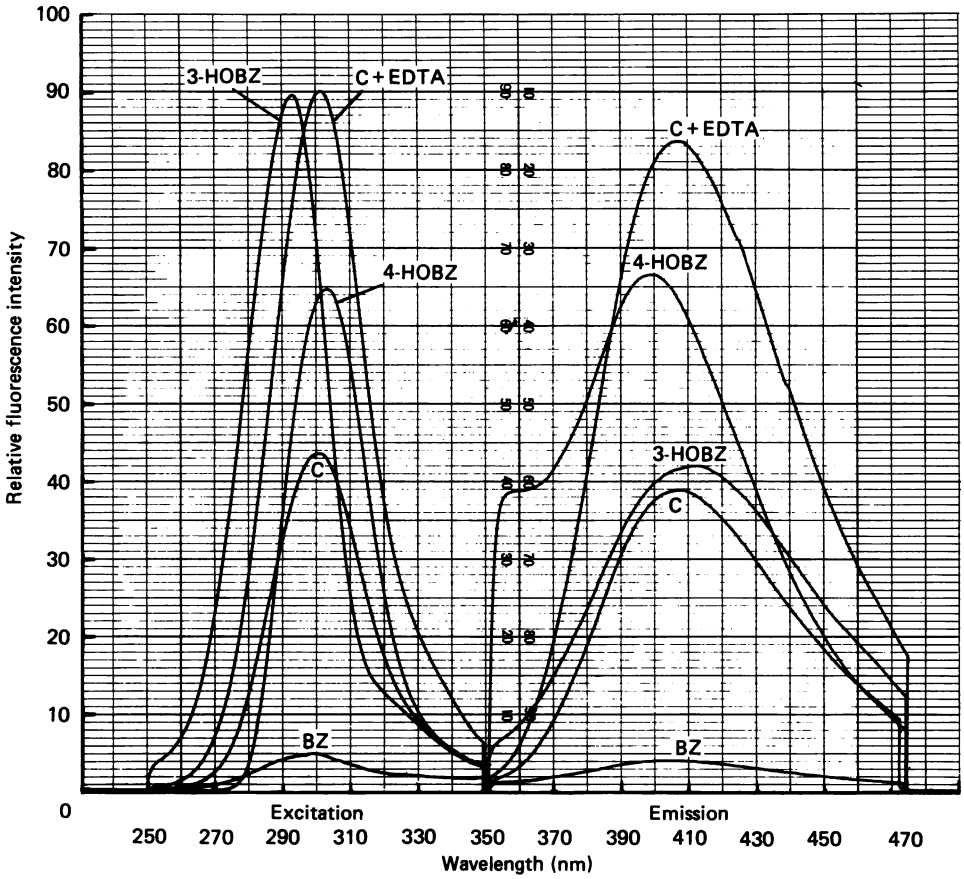

Fig. 1. Fluorescence scans of benzoate that had reacted with a ferrous salt

The Figure shows fluorescence scans of benzoate (100 μg/ml) (BZ), of benzoate that had been incubated at 37 °C for 1 h at pH 7.4 with a ferrous salt (0.20 mM) (C) and of benzoate that had been incubated at 37 °C for 1 h at pH 7.4 with a ferrous salt + EDTA (0.03 mM) (C + EDTA). Standards 4-hydroxybenzoate (800 μg/ml) (4-HOBZ) and 3-hydroxybenzoate (5 μg/ml) (3-HOBZ) were in phosphate buffer, pH 7.4. Variable excitation was scanned at 407 nm emission, and variable emission was scanned at an excitation of 305 nm. Relative fluorescence intensity is shown with 100 units equivalent to a standard of tetraphenylbutadiene (10 μM).

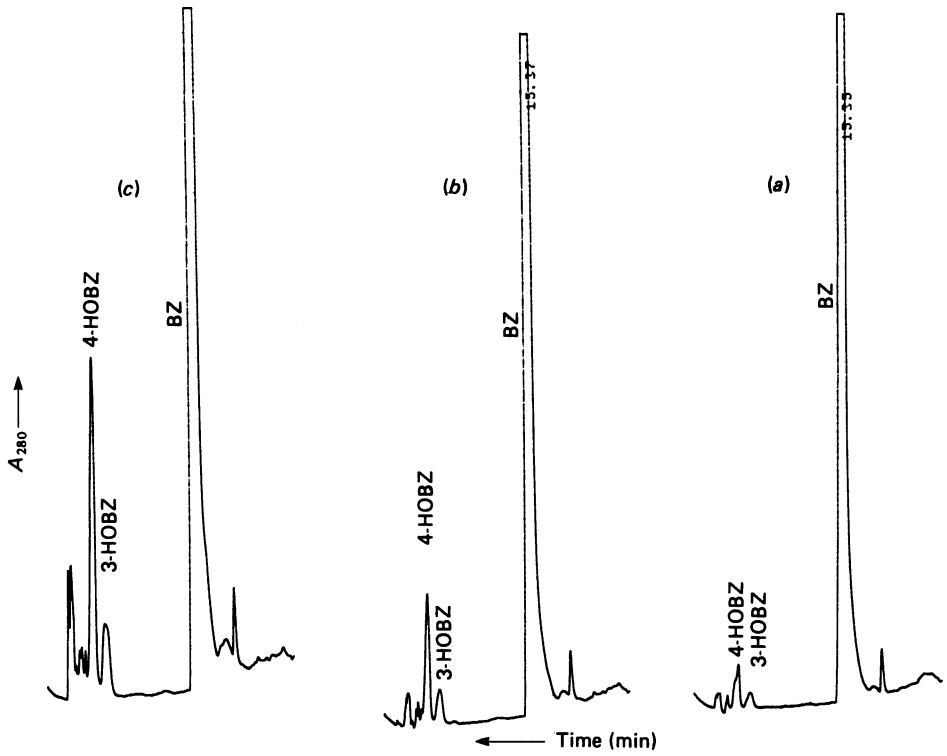

Fig. 2. H.p.l.c. separation of benzoate hydroxylation products

H.p.l.c. separation of hdyroxylation products extracted from benzoate (1 mM) incubated at pH 7.4 with a ferrous salt (0.2 mM) for 1 h at 37 °C and monitored at 280 nm. (*a*) Benzoate control; (*b*) benzoate + ferrous salt; (*c*) benzoate + ferrous salt + EDTA (0.03 mM). The retention times were benzoate (BZ) 15.36 min, 3-hydroxybenzoate (3-HOBZ) 6.02 min, and 4-hydroxybenzoate (4-HOBZ) 4.70 min.

on porous silica gel with bonded octadecyl groups (ODS 5 μm-pore-size Hypersil; Shandon Southern, Runcorn, Cheshire, U.K.) by using a gradient elution with detection at 280 nm. The initial solvent composition was 10% (v/v) acetonitrile in 10 mM-sodium phosphate buffer,

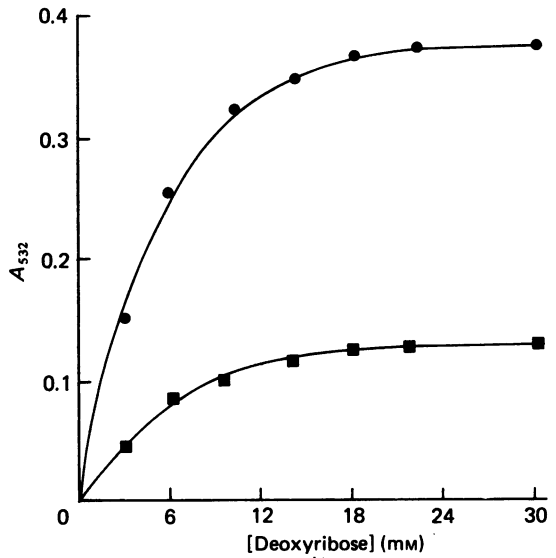

Fig. 3. Deoxyribose degradation by a ferrous salt as a function of deoxyribose concentration

The Figure shows the degradation of deoxyribose in phosphate buffer, pH 7.4, with the release of thiobarbituric acid-reactive material (A_{532}) by a ferrous salt (29 μM) (●) and by a ferrous salt + EDTA (43 μM) (■).

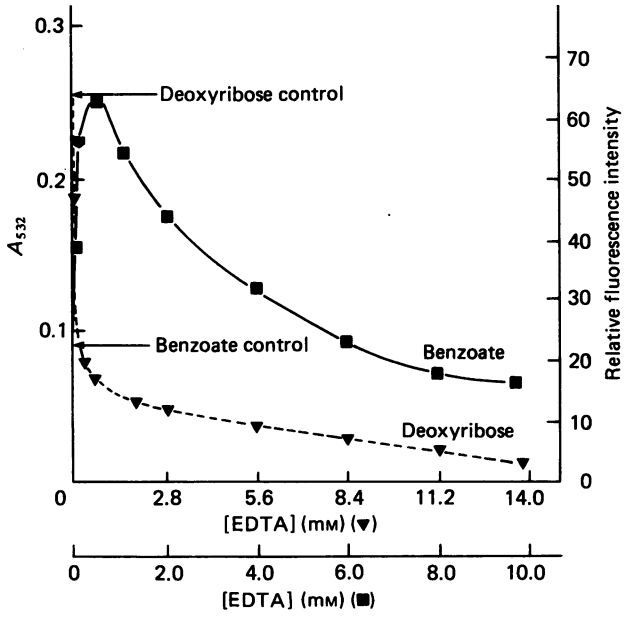

Fig. 4. Effect of EDTA on damage to deoxyribose and benzoate

The Figure shows the effect of EDTA (final concentration in the reaction mixture) on release of thiobarbituric acid-reactivity (A_{532}) from deoxyribose (9 mM) (▼) and of EDTA on hydroxylation (relative fluorescence intensity) of benzoate (1 mM) (■). Both reactions were carried out in phosphate buffer, pH 7.4, for 1 h at 37 °C and stimulated by the addition of a ferrous salt (final reaction concentrations were 200 μM-Fe^{2+} to benzoate and 29 μM-Fe^{2+} to deoxyribose). Relative fluorescence intensity is as described in the legend to Fig. 1.

Table 1. Rate constants for OH· scavengers in the presence and in the absence of EDTA determined by damage to deoxyribose and sodium benzoate

Approximate rate constants were determined by using the techniques described in the Materials and methods section with values corresponding to 1.9×10^9 $M^{-1} \cdot s^{-1}$ for deoxyribose and 3.3×10^9 $M^{-1} \cdot s^{-1}$ for benzoate. Results shown are the means for three separate experiments that differed by less than $\pm 5\%$. Pulse-radiolysis rate constants are taken from the following references: (a) Goldstein & Czapski (1984); (b) Bors *et al.* (1982); (c) Anbar & Neta (1967).

Scavenger	Deoxyribose degradation rate constant ($M^{-1} \cdot s^{-1}$)	Benzoate hydroxylation rate constant ($M^{-1} \cdot s^{-1}$)	Pulse-radiolysis rate constant ($M^{-1} \cdot s^{-1}$)
Mannitol	2.65×10^9	4.7×10^8	1.8×10^9 (a) 1.0×10^9 (b)
Mannitol + EDTA	1.87×10^9	6.1×10^8	
Formate	Interference	8.25×10^8	2.5×10^9 (c) 2.8×10^9 (b)
Formate + EDTA	Interference	1.1×10^9	
Histidine	2.0×10^9	3.58×10^8	3.0×10^9 (c)
Histidine + EDTA	2.59×10^9	1.53×10^9	
Glucose	1.08×10^9	8.15×10^8	8.0×10^8 (c) 1.0×10^9 (c)
Glucose + EDTA	2.28×10^9	8.57×10^8	
Butan-1-ol	$< 1.14 \times 10^8$	1.29×10^9	2.2×10^9 (c)
Butan-1-ol + EDTA	3.08×10^9	1.65×10^9	
Thiourea	2.07×10^9	4.1×10^9	4.7×10^9 (c)
Thiourea + EDTA	2.33×10^9	4.78×10^9	
Ethanol	4.19×10^6	5.78×10^8	1.1×10^9 (c) 1.8×10^9 (b)
Ethanol + EDTA	4.75×10^7	3.88×10^8	
Benzoate	$< 5.7 \times 10^7$		3.3×10^9 (c) 5.7×10^9 (b)
Benzoate + EDTA	$\sim 8.14 \times 10^7$		
Propan-2-ol	$< 5.7 \times 10^6$	6.6×10^8	3.9×10^9 (c)
Propan-2-ol + EDTA	$\sim 1.14 \times 10^7$	8.25×10^8	
Deoxyribose		6.95×10^8	1.9×10^9 (c)
Deoxyribose + EDTA		1.43×10^9	

pH 2.5, at a flow rate of 1.0 ml/min. The acetonitrile concentration was raised linearly to 60% (v/v) within 20 min.

RESULTS

Measurement of spectrofluorimetric changes in the detector molecule benzoate was found to be a simple and convenient way to measure OH· formation (Fig. 1). Benzoate is weakly fluorescent under the conditions described, but after monohydroxylation forms highly fluorescent products (Fig. 1). Two major products could be identified by h.p.l.c. separation, corresponding to 3-hydroxybenzoate and 4-hydroxybenzoate (Fig. 2). Of these two products the 3-hydroxy compound is considerably more fluorescent than the 4-hydroxy product (Fig. 1). No major peak corresponding to phenol was observed.

The maximum rate of deoxyribose degradation, dependent on OH· radicals generated from the ferrous salt, was determined in the presence and in the absence of EDTA as a function of deoxyribose concentration (Fig. 3). This reaction was not inhibitable with superoxide dismutase, as previously shown (Halliwell & Gutteridge, 1981). At a final concentration of 17 mM deoxyribose appeared to be intercepting essentially all the OH· radicals generated in the reaction. By use of the same approach, the maximum rate of benzoate hydroxylation occurred at a benzoate concentration of 1 mM both in the presence and in the absence of EDTA (results not shown). The addition of EDTA, at concentrations ranging from 1 to 5 mM, to the benzoate reaction stimulated the rate of hydroxylation in the lower concentration range but inhibited at higher values (Figs. 1 and 4). Generation of thiobarbituric acid-reactivity from deoxyribose, however, was inhibited by EDTA when this exceeded the concentration of iron salt present (Fig. 4).

Under the standard conditions of substrate (detector molecule), iron salt and EDTA described above, different concentrations of OH· scavengers were added to determine the amount of scavenger required to inhibit damage to the detector molecule by 50%. At this concentration it is assumed that half the OH· radicals generated will be intercepted by the scavenger. By using published rate constants for the OH· scavengers, derived from radiolysis experiments, it is possible to calculate approximate rate constants in the two different detector systems. Values derived from these calculations are shown in Table 1. For most scavengers there was a close agreement between the deoxyribose and benzoate methods, with only glucose, histidine and mannitol showing somewhat lower values with the hydroxylation technique. EDTA usually increased the apparent rate

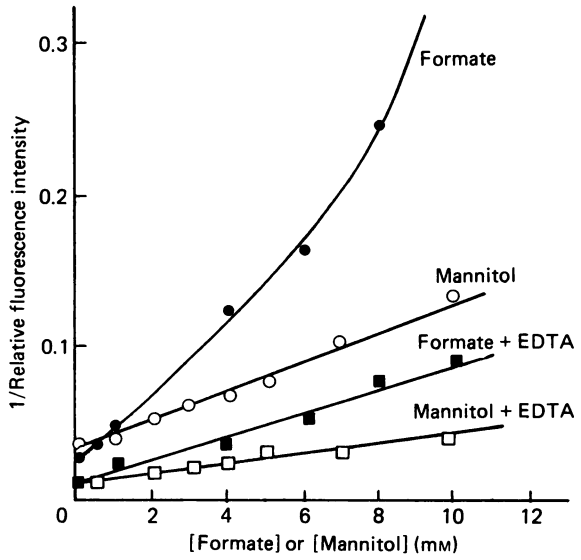

Fig. 5. Effects of formate and mannitol on hydroxylation of benzoate

Formate and mannitol were added, to give the final reaction concentrations shown, and the reaction mixture was incubated at 37 °C for 1 h. ●, Formate, no EDTA; ■, formate+EDTA (30 μM); ○, mannitol, no EDTA; □, mannitol+EDTA (30 μM). Relative fluorescence intensity is as described in the legend to Fig. 1.

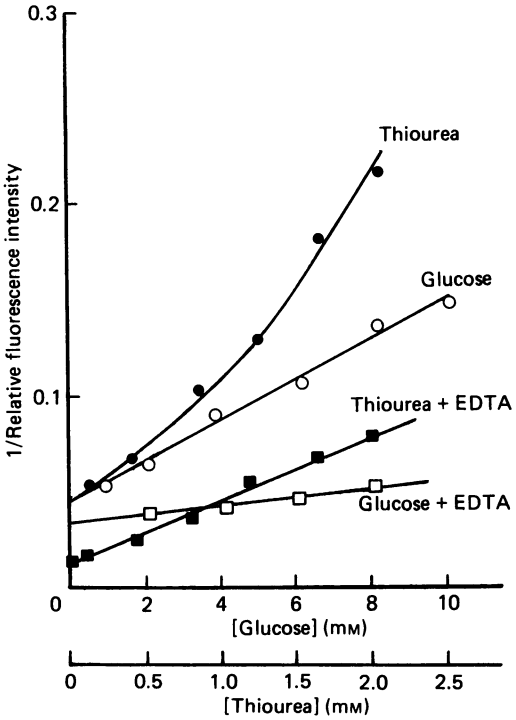

Fig. 6. Effects of glucose and thiourea on hydroxylation of benzoate

Glucose and thiourea were added, to give the final reaction concentrations shown, and the reaction mixture was incubated at 37 °C for 1 h. ●, Thiourea, no EDTA; ■, thiourea+EDTA (30 μM); ○, glucose, no EDTA; □, glucose+EDTA (30 μM). Relative fluorescence intensity is as described in the legend to Fig. 1.

constant, and this was most pronounced for formate, ethanol, propan-2-ol and butan-1-ol in the deoxyribose system. Competition kinetic plots were made for the detector molecule and OH$^\cdot$ scavengers, since OH$^\cdot$ scavengers may well react with other intermediates of the reaction mixture. These are shown in Figs. 5 and 6 for thiourea, glucose, mannitol and formate. Thiourea and formate in the absence of EDTA deviated from simple competition kinetics (Figs. 5 and 6), whereas glucose and mannitol (Figs. 5 and 6) and also Tris, histidine, butan-1-ol, propan-1-ol and deoxyribose (results not shown) did not.

DISCUSSION

Simple molecules reacting with OH$^\cdot$, and undergoing a detectable change characteristic of such radical attack, can be used as 'detectors' for OH$^\cdot$ or the formation of oxidants with similar properties such as the ferryl ion. Two such detectors have been used here for comparing rate constants of OH$^\cdot$ scavengers and for competitive-inhibition studies. Iron salts damage deoxyribose with the release of thiobarbituric acid-reactive material (Gutteridge, 1981), and this damage can be related to the formation of OH$^\cdot$ radicals (Halliwell & Gutteridge, 1981), since peroxyl and alkoxyl radicals formed from iron chelates and t-butyl hydroperoxide do not release thiobarbituric acid-reactive material from deoxyribose (results not shown). Damage to this carbohydrate by OH$^\cdot$ occurs mainly by hydrogen abstraction, resulting in the release of fragments with thiobarbituric acid-reactivity indistinguishable from that of malondialdehyde (Gutteridge & Toeg, 1982). The reaction of benzoate with OH$^\cdot$ is more complex, since at least three different radical mechanisms are known to be involved. Each mechanism has been used as the basis of a method for the detection of OH$^\cdot$ radicals. Thus decarboxylation with the release of CO_2 and the formation of phenol (Matthews & Sangster, 1965; Sagone *et al.*, 1980), hydroxylation to increase fluorescence (Melhuish & Sutton, 1978; Baker & Gebicki, 1984) and release of thiobarbituric acid-reactive material (Gutteridge, 1984*b*) can be applied to benzoate. No evidence to support benzoate decarboxylation as a major pathway after damage by OH$^\cdot$ was found, in agreement with previous studies (M. Grootveld, personal communication).

Recent studies have shown that OH$^\cdot$ scavengers do not always protect the detector molecule from OH$^\cdot$ radicals, generated in a Fenton system, to the extent expected from their established rate constants (Gutteridge, 1984*b*; Moorehouse *et al.*, 1985; Gutteridge & Halliwell, 1986). These discrepancies can be partly explained by assuming that iron ions bind, with various affinities, to components of the reaction mixture. When they bind to the detector molecule, then damage will be directed to this molecule in a site-specific way. However, when they bind to the scavenger, the detector will be efficiently protected. When EDTA is present in the reaction, OH$^\cdot$ scavengers appear to act with efficiencies closely related to their rate constants derived from radiolysis experiments (Gutteridge, 1984*b*; Gutteridge & Halliwell, 1986). This 'radiomimetic' effect of EDTA is probably due to the 'open' structure of its iron chelates, which allows sufficient OH$^\cdot$ radicals to escape reaction with EDTA and to be released into free solution, where they are

equally accessible to the detector molecule and to any scavenger added.

The formation of $OH^{\cdot}$ in the presence of EDTA does not necessarily mean that damage by $OH^{\cdot}$ will be increased. Indeed, here it is shown that when EDTA exceeds the concentration of iron salt present it does not increase formation of thiobarbituric acid-reactive material from deoxyribose, suggesting that the binding of iron to the deoxyribose molecule is advantageous to the release of thiobarbituric acid-reactive fragments. Hydroxylation of benzoate, however, was increased by the presence of EDTA, suggesting that the site of metal binding was not advantageous to hydroxylation leading to fluorescence. EDTA has several unique properties when present in radical reactions; these include its ability to alter the redox potential of iron ions, to solubilize iron at physiological pH values, to accelerate the autoxidation of Fe^{2+} ions and to eliminate the inhibitory effects of Cu^{2+} ions (Gutteridge, 1984*a*; Cohen, 1985; Grootveld & Halliwell, 1986).

In Fenton reactions it is likely that $OH^{\cdot}$ scavengers with higher iron-binding affinities than the detector molecule will always appear to be more efficient scavengers of $OH^{\cdot}$ than those that bind iron less tightly. When this occurs, the addition of EDTA can restore the effectiveness of an $OH^{\cdot}$ scavenger. Molecules such as glucose, mannitol, histidine and thiourea are known to complex metal ions, and this may explain why their apparent rate constants were not significantly increased by the addition of EDTA. The reasons why ethanol, propan-2-ol and benzoate are not so greatly affected by the addition of EDTA and show low values in the deoxyribose method are at present unclear. One possibility is that the radical formed after $OH^{\cdot}$ attack on the scavenger interacts with the iron salt.

Competition kinetics between the detectors and $OH^{\cdot}$ scavengers showed a linear response for most of the scavengers tested. However, thiourea and formate deviated from linearity, suggesting that they were acting in some other way with components of the reaction mixture (Moorehouse *et al.*, 1985). It is known that thiourea reacts with H_2O_2, an important intermediate in the formation of $OH^{\cdot}$ (Cederbaum *et al.*, 1979). Mannitol is one of the most widely used $OH^{\cdot}$ scavengers because it is effective in Fenton reactions when other scavengers are not. Reasons for this other than metal binding (Bourne *et al.*, 1959; Spiro & Saltman, 1969) remain unclear. The presence of phosphate in both the deoxyribose and the benzoate reactions may complicate the interpretation of iron-binding reactions, but phosphate is an important biological constituent that should be taken into account in experiments involving Fenton chemistry.

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REFERENCES

Anbar, M. & Neta, P. (1967) Int. J. Appl. Radiat. Isot. **18**, 493–523
Armstrong, W. A., Black, B. A. & Grant, D. W. (1960) J. Phys. Chem. **64**, 1415–1419
Baker, M. S. & Gebicki, J. M. (1984) Arch. Biochem. Biophys. **234**, 258–264
Bors, W., Saran, M. & Michel, C. (1982) in Superoxide Dismutase (Oberley, L. W., ed.), vol. 2, pp. 31–62, CRC Press, Boca Raton
Bourne, E. J., Nery, R. & Weigel, H. (1959) Chem. Ind. (London) 998–999
Bucknall, T., Edwards, H. E., Kemsley, K. G., Moore, J. S. & Phillips, G. O. (1978) Carbohydr. Res. **62**, 49–59
Cederbaum,, A. I., Dicker, E., Rubin, E. & Cohen, G. (1979) Biochemistry **18**, 1187–1191
Cohen, G. (1985) in Handbook of Methods for Oxygen Radical Research (Greenwald, R. A., ed.), pp. 55–64, CRC Press, Boca Raton
Czapski, G. (1978) Photochem. Photobiol. **28**, 651–653
Goldstein, S. & Czapski, G. (1984) Int. J. Radiat. Biol. **46**, 725–729
Grootveld, M. & Halliwell, B. (1986) Free Radical Res. Commun. **1**, 243–250
Gutteridge, J. M. C. (1981) FEBS Lett. **128**, 343–346
Gutteridge, J. M. C. (1984*a*) Biochem. J. **224**, 697–701
Gutteridge, J. M. C. (1984*b*) Biochem. J. **224**, 761–767
Gutteridge, J. M. C. & Halliwell, B. (1986) in Oxygen and Sulfur Radicals in Chemistry and Medicine (Breccia, A., Rodgers, M. A. J. & Semerano, G., eds.), pp. 47–52, Lo Scarabeo, Bologna
Gutteridge, J. M. C. & Toeg, D. (1982) Int. J. Biochem. **14**, 891–893
Halliwell, B. & Gutteridge, J. M. C. (1981) FEBS Lett. **128**, 347–352
Loeff, L. & Swallow, A. J. (1964) J. Phys. Chem. **68**, 2470–2475
Matthews, R. W. & Sangster, D. F. (1965) J. Phys. Chem. **69**, 1938–1946
Melhuish, W. H. & Sutton, H. C. (1978) J. Chem. Soc. Commun. 970–971
Moorehouse, C. P., Halliwell, B., Grootveld, M. & Gutteridge, J. M. C. (1985) Biochim. Biophys. Acta **843**, 261–268
Sagone, A. L., Decker, M. A., Wells, R. M. & Democko, C. (1980) Biochim. Biophys. Acta **628**, 90–97
Spiro, Th. G. & Saltman, P. (1969) Struct. Bonding (Berlin) **6**, 116–156
Van Hemmen, J. J. & Meuling, W. J. A. (1975) Biochim. Biophys. Acta **402**, 133–141
Von Sonntag, C. (1980) in Advances in Carbohydrate Chemistry (Tipson, R. S. & Horton, D., eds.), pp. 7–77, Academic Press, New York
Winston, G. W. & Cederbaum, A. I. (1982) Biochemistry **21**, 4265–4270

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