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Reactivity of hydroxyl and hydroxyl-like radicals discriminated by release of thiobarbituric acid-reactive material from deoxy sugars, nucleosides and benzoate

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Hydroxyl radicals ($OH^{\bullet}$) can be formed in aqueous solution by a superoxide ($O_2^{\bullet -}$)-generating system in the presence of a ferric salt or in a reaction independent of $O_2^{\bullet -}$ by the direct addition of a ferrous salt. $OH^{\bullet}$ damage was detected in the present work by the release of thiobarbituric acid-reactive material from deoxy sugars, nucleosides and benzoate. The carbohydrates deoxyribose, deoxygalactose and deoxyglucose were substantially degraded by the iron(II) salt and the iron(III) salt in the presence of an $O_2^{\bullet -}$-generating system, whereas deoxyinosine, deoxyadenosine and benzoate were not. Addition of EDTA to the reaction systems producing radicals greatly enhanced damage to deoxyribose, deoxyinosine, deoxyadenosine and benzoate, but decreased damage to deoxygalactose and deoxyglucose. Further, $OH^{\bullet}$ scavengers were effective inhibitors only when EDTA was present. Inhibition by catalase and desferrioxamine confirmed that H_2O_2 and iron salts were essential for these reactions. The results suggest that, in the absence of EDTA, iron ions bind to the carbohydrate detector molecules and bring about a site-specific reaction on the molecule. This reaction is poorly inhibited by most $OH^{\bullet}$ scavengers, but is strongly inhibited by scavengers such as mannitol, glucose and thiourea, which can themselves bind iron ions, albeit weakly. In the presence of EDTA, however, iron is removed from these binding sites to produce $OH^{\bullet}$ in 'free' solution. These can be readily intercepted by the addition of $OH^{\bullet}$ scavengers.

The toxicity of O_2, when supplied at concentrations only slightly greater than those of normal air, has been known for a considerable time (see citations in Balentine, 1982). Among the biochemical mechanisms responsible for O_2 toxicity is an increased formation of the superoxide radical, $O_2^{\bullet -}$, *in vivo* at elevated O_2 concentrations (Fridovich, 1975; Halliwell, 1981). However, $O_2^{\bullet -}$ itself is not sufficiently reactive in aqueous solution to account for the type of damage observed in $O_2^{\bullet -}$-generating systems, so this damage must be due to the $O_2^{\bullet -}$-dependent formation of more-reactive species (for a review see Halliwell & Gutteridge, 1984*a*). The most likely reactive species is the hydroxyl radical, $OH^{\bullet}$ (McCord & Day, 1978; Halliwell, 1978). Indeed, damage inflicted by $O_2^{\bullet -}$-generating systems is often prevented by 'scavengers' of $OH^{\bullet}$, such as glucose, mannitol, formate, thiourea, butan-1-ol and ethanol (Anbar & Neta, 1967). If the damage is due to $OH^{\bullet}$ generation, then one would expect any compound that reacts with this species at high rate constants to offer protection, unless, of course, the radical formed as a result of the reaction of $OH^{\bullet}$ with the scavenger molecule is itself a damaging species.

The $O_2^{\bullet -}$-dependent formation of $OH^{\bullet}$ requires the presence of transition-metal ions, of which iron and copper ions are known to be effective (McCord & Day, 1978; Halliwell, 1978; Halliwell & Gutteridge, 1981; Rowley & Halliwell, 1983). However, iron and copper ions *in vivo* cannot exist free in solution; they must be bound, with various affinities, to a wide range of ligands, including albumin, ATP, citrate, DNA and membrane lipids (for a review see Halliwell & Gutteridge, 1984*b*), as well as to specific metalloproteins such as ferritin or caeruloplasmin (Gutteridge & Stocks, 1981; Halliwell & Gutteridge, 1984*a*). Iron ions complexed to DNA (Floyd, 1981; Gutteridge & Toeg, 1982*a*) and to ATP (Flitter *et al.*, 1983; Floyd,

1983) are capable of stimulating the production of $OH^{\cdot}$. The $OH^{\cdot}$ formed in this way would seem to be especially likely to attack the ligand binding the metal ion. Indeed, direct evidence for a 'site-specific' formation of $OH^{\cdot}$ by copper ions directly bound to proteins has been presented (Samuni *et al.*, 1981; Gutteridge & Wilkins, 1983; Rowley & Halliwell, 1983). The concept of 'site-specific' damage has been extended to include iron salts (Youngman & Elstner, 1981; Youngman *et al.*, 1982), although no direct evidence for it has been presented.

In the present paper I have attempted to distinguish the damage done by $OH^{\cdot}$ formed in 'free' solution from that brought about by such radicals bound to ligands. For this, the chelating agent EDTA provides several unique properties. EDTA binds iron ions very tightly and removes them from low-M_r biological complexes (Gutteridge *et al.*, 1981), but the iron–EDTA chelates are effective catalysts of the formation of 'free' $OH^{\cdot}$ (McCord & Day, 1978; Halliwell, 1978). Damage done by $OH^{\cdot}$ to a variety of biological molecules under these conditions was detected by the formation of thiobarbituric acid-reactive material (Gutteridge, 1981; Halliwell & Gutteridge, 1981; Gutteridge, 1982).

Materials and methods

Materials

Xanthine oxidase (grade I), hypoxanthine, 2-deoxy-D-ribose, 2-deoxy-D-glucose, 2-deoxy-D-galactose, 2′-deoxyadenosine, 2-deoxyinosine, catalase (bovine liver, thymol-free), superoxide dismutase (bovine erythrocyte) and albumin (human, fatty acid-free) were from Sigma Chemical Co., Poole, Dorset, U.K. Desferrioxamine (Desferal) was from Ciba–Geigy, Horsham, Sussex, U.K. All other chemicals were of the highest purity available from BDH Chemicals, Poole, Dorset, U.K.

Radical-generating systems

A solution of ferrous salt (2mM) was prepared in Chelex-resin-treated distilled water gassed with N_2 to remove O_2. Phosphate/saline buffer pH 7.4 contained 0.1M-phosphate (Na_2HPO_4/NaH_2PO_4) in 0.15M-NaCl. A portion of this buffer was saturated with hypoxanthine as a substrate for xanthine oxidase. Xanthine oxidase (0.5 units/mg of protein) was diluted 1:40 with water and passed through a column of Sephadex G-25 to remove salts and salicylate. A 0.1ml portion of the resulting column eluate was added to each reaction tube. Substrates ('detector' molecules) were prepared from deoxyribose, deoxyglucose, deoxygalactose, deoxyadenosine, deoxyinosine and sodium benzoate as 5mM solutions in Chelex-resin-treated distilled water. All reaction mixtures had a final volume of 0.9ml and contained 44.4mM-phosphate in 56.7mM-NaCl. In all cases inhibitors or scavengers were added to the reaction before iron salt or xanthine oxidase. Values shown in Tables 1–4 are final reaction concentrations. Results are the mean of three separate experiments, in which results differed by not more than 5%.

Thiobarbituric acid-reactivity

The xanthine oxidase reaction mixture was incubated for 2h at 37°C; all other reactions were incubated at 37°C for 1h. At the end of the incubation period, 0.5ml of 1% (w/v) thiobarbituric acid in 0.05M-NaOH was added to each tube with 0.5ml of 2.8% (w/v) trichloroacetic acid. The glass tubes were heated for 10min at 100°C to develop the colour. When cool, the absorbance was read at 532nm against appropriate blanks.

Results

Damage by an $O_2^{\cdot-}$-generating system

A mixture of hypoxanthine and xanthine oxidase generates $O_2^{\cdot-}$, which can, in the presence of iron complexes, interact with H_2O_2 to form $OH^{\cdot}$. Iron–EDTA is an especially effective catalyst (McCord & Day, 1978; Halliwell, 1978). Table 1 shows that the hypoxanthine/xanthine oxidase/iron–EDTA system, to which iron was not added but was present as a contaminant of all reagents used (Wong *et al.*, 1981), was able to degrade deoxyribose, deoxygalactose, deoxyglucose, deoxyadenosine, deoxyinosine and benzoate with the formation of thiobarbituric acid-reactive material. Degradation of these molecules was strongly inhibited by all the $OH^{\cdot}$ scavengers tested (formate, ethanol, butan-1-ol, thiourea, glucose and mannitol) and the buffer Tris, but not by urea, which reacts only slowly with $OH^{\cdot}$ (Table 2). Damage was also inhibited by superoxide dismutase, catalase and desferrioxamine (Table 3), a chelating agent that binds iron in a form unable to catalyse $OH^{\cdot}$ production (Gutteridge *et al.*, 1979; Hoe *et al.*, 1982).

When EDTA was omitted from the reaction mixture (i.e. a hypoxanthine/xanthine oxidase/trace-iron-salt system) formation of thiobarbituric acid-reactive material from deoxyribose, deoxyadenosine, deoxyinosine and benzoate was significantly decreased, but that to deoxygalactose and deoxyglucose was increased. As previously, the damage was prevented by superoxide dismutase, desferrioxamine and catalase (Table 3), but the pattern of inhibition by scavengers of $OH^{\cdot}$ changed markedly. Table 2 shows data for deoxyribose, deoxygalactose and deoxyglucose.

Table 1. *Effect of different radical-generating systems on the release of thiobarbituric acid-reactive material from deoxy sugars, deoxynucleosides and benzoate*
Final reaction concentrations are shown. The substrate molecules, i.e. deoxy sugars, deoxy nucleosides and benzoate, were at a final concentration of 1.1 mM in 44.4 mM-phosphate buffer, pH 7.4. Blank values (0.005) have been subtracted from the values shown. Abbreviations: XOD, xanthine oxidase; HXn, hypoxanthine.

	Thiobarbituric acid-reactivity (A_{532})					
Reaction system	Deoxyribose	Deoxygalactose	Deoxyglucose	Deoxyadenosine	Deoxyinosine	Benzoate
Fe^{2+} (0.22 mM)	0.282	0.237	0.248	0.034	0.038	0.006
Fe^{2+} (0.22 mM)+EDTA (0.22 mM)	0.332	0.101	0.093	0.334	0.334	0.060
Fe^{2+} (0.11 mM)+H_2O_2 (0.22 mM)	0.292	0.205	0.178	0.092	0.093	0.026
$O_2^{\cdot-}$ (XOD/HXn)	0.257	0.173	0.187	0.116	0.136	0.040
$O_2^{\cdot-}$ (XOD/HXn)+EDTA (0.11 mM)	0.400	0.103	0.129	0.407	0.341	0.230

Table 2. *Effect of OH· scavengers on damage to deoxy sugars, deoxy nucleosides and benzoate by a $O_2^{\cdot-}$-generating system*
Final reaction concentrations are shown. The substrate molecules were at a final concentration of 1.1 mM in phosphate buffer, pH 7.4 (44.4 mM). Inhibition (%) was calculated after the subtraction of appropriate blanks. The reaction was started by the addition of hypoxanthine and xanthine oxidase as described in the text.

	Thiobarbituric acid-reactivity													
	$O_2^{\cdot-}$						$O_2^{\cdot-}$+EDTA (0.11 mM)							
	Deoxyribose		Deoxygalactose		Deoxyglucose		Deoxyribose		Deoxyadenosine		Deoxyinosine		Benzoate	
	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition
Blank (no xanthine oxidase)	0.005		0.004		0.002		0.005		0.002		0.005		0.006	
Control (xanthine oxidase+ hypoxanthine)	0.257		0.173		0.187		0.400		0.407		0.341		0.230	
+Formate (11.1 mM)	0.205	20%	0.161	9%	0.162	13%	0.171	57%	0.221	46%	0.181	47%	0.154	33%
+Ethanol (11.1 mM)	0.221	14%	0.173	0%	0.178	5%	0.220	45%	0.248	39%	0.227	33%	0.173	25%
+Butan-1-ol (11.1 mM)	0.208	19%	0.193	0%	0.182	3%	0.120	70%	0.161	60%	0.144	58%	0.126	45%
+Urea (11.1 mM)	0.198	23%	0.189	0%	0.171	9%	0.354	12%	0.423	0%	0.347	0%	0.238	0%
+Thiourea (11.1 mM)	0.164	36%	0.116	33%	0.092	51%	0.203	49%	0.178	56%	0.148	57%	0.161	30%
+Glucose (11.1 mM)	0.129	50%	0.092	47%	0.094	50%	0.199	50%	0.229	44%	0.202	41%	0.177	23%
+Mannitol (11.1 mM)	0.110	57%	0.079	54%	0.079	58%	0.206	49%	0.273	33%	0.236	31%	0.195	15%
+Tris (11.1 mM)	0.132	49%	0.073	58%	0.069	63%	0.211	47%	0.209	49%	0.181	47%	0.146	37%

Table 3. *Effect of superoxide dismutase, catalase and desferrioxamine on deoxyribose degradation in different radical-generating systems*

Final reaction concentrations are shown. Deoxyribose was present at a concentration of 1.1 mM in 44.4 mM-phosphate buffer, pH 7.4. Inhibition (%) was calculated after subtraction of appropriate blanks. The reaction was started by the addition of hypoxanthine and xanthine oxidase as described in the text.

	Thiobarbituric acid-reactivity									
	Fe^{2+} (0.22 mM)		Fe^{2+} (0.22 mM) + EDTA (0.22 mM)		Fe^{2+} (0.11 mM) + H_2O_2 (0.22 mM)		$O_2^{\cdot-}$		$O_2^{\cdot-}$ + EDTA (0.11 mM)	
	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition
Control (xanthine oxidase + hypoxanthine)	0.282		0.332		0.292		0.257		0.400	
+ Catalase (0.06 mg/ml)	0.138	51%	0.126	62%	0.097	67%	0.015	94%	0.020	95%
+ Catalase (denatured)	0.290	0%	0.356	0%	0.324	0%	0.267	0%	0.492	0%
+ Superoxide dismutase (0.06 mg/ml)	0.290	0%	0.363	0%	0.414	0%	0.001	100%	0.014	97%
+ Albumin (0.06 mg/ml)	0.290	0%	0.375	0%	0.302	0%	0.255	1%	0.454	0%
+ Desferrioxamine (0.44 mM)	0.010	96%	0.082	75%	0.031	89%	0.003	99%	0.008	98%

Fig. 1. *Fluorescence scans of emission following excitation at 532 nm and of the excitation spectrum giving emission at 553 nm of thiobarbituric acid-reactive material released after iron-dependent free-radical damage to: benzoate (A), deoxyglucose (B), deoxygalactose (C), malondialdehyde standard (6 μM) (prepared from hydrolysed 1,1,3,3-tetramethoxypropane) (D) and deoxyribose (E)*

The deoxyribose-containing nucleosides deoxyadenosine and deoxyinosine gave fluorescence scans identical with those of deoxyribose and the other reactants.

Thiourea, glucose, mannitol and Tris still showed significant inhibition, but formate, ethanol or butan-1-ol had little, if any, more effect than did urea.

The thiobarbituric acid-reactive material released from all substrates used had fluorescence characteristics indistinguishable from those formed by reaction of thiobarbituric acid with a malondialdehyde standard (Fig. 1).

Table 4. *Effect of OH· scavengers on damage to deoxy sugars and nucleosides initiated by a ferrous salt*

Final reaction concentrations are shown. Substrate molecules were added at a final concentration of 1.1 mM in 44.4 mM-phosphate buffer, pH 7.4. Inhibition (%) was calculated after the subtraction of appropriate blank values.

Scavenger added	Thiobarbituric acid-reactivity																			
	Fe^{2+} (0.22 mM)						Fe^{2+} (0.11 mM) + H_2O_2 (0.22 mM)						Fe^{2+} (0.22 mM) + EDTA (0.22 mM)							
	Deoxyribose		Deoxygalactose		Deoxyglucose		Deoxyribose		Deoxygalactose		Deoxyglucose		Deoxyribose		Deoxyadenosine		Deoxyinosine	
	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition	A_{532}	Inhibition
Blank (no Fe^{2+})	0.005		0.004		0.001		0.005		0.004		0.001		0.005		0.001		0.005	
Control	0.282		0.237		0.248		0.290		0.205		0.178		0.332		0.334		0.334	
+ Formate (11.1 mM)	0.250	11%	0.208	12%	0.231	7%	0.234	19%	0.184	10%	0.191	0%	0.049	85%	0.058	83%	0.083	75%
+ Ethanol (11.1 mM)	0.249	12%	0.227	4%	0.244	2%	0.248	15%	0.198	3%	0.175	2%	0.070	79%	0.083	75%	0.104	69%
+ Butan-1-ol (11.1 mM)	0.233	17%	0.239	0%	0.235	5%	0.204	30%	0.189	8%	0.185	0%	0.054	83%	0.039	88%	0.057	83%
+ Urea (1.11 mM)	0.263	7%	0.239	0%	0.251	0%	0.275	5%	0.211	0%	0.198	0%	0.310	4%	0.297	11%	0.305	9%
+ Thiourea (1.11 mM)	0.144	49%	0.144	39%	0.143	42%	0.181	38%	0.140	32%	0.176	1%	0.068	80%	0.122	64%	0.130	61%
+ Glucose (11.1 mM)	0.071	75%	0.064	73%	0.070	72%	0.152	48%	0.134	36%	0.126	29%	0.072	78%	0.098	71%	0.104	69%
+ Mannitol (11.1 mM)	0.030	89%	0.027	89%	0.030	88%	0.085	71%	0.082	60%	0.067	62%	0.056	83%	0.088	74%	0.101	70%
+ Tris (11.1 mM)	0.016	94%	0.011	95%	0.013	95%	0.100	66%	0.058	72%	0.051	71%	0.052	84%	0.083	75%	0.083	75%

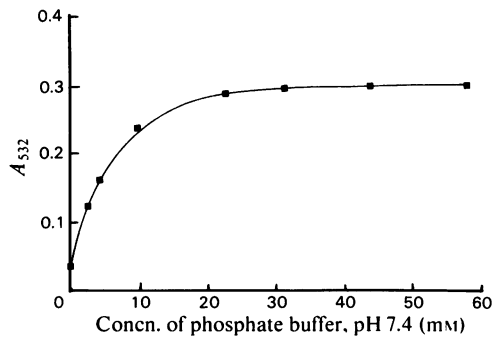

Fig. 2. *Effect of phosphate ions (final reaction concentration) on the degradation of deoxyribose (1.1 mM) in the presence of Fe(II) ions (0.22 mM)*

Damage by iron(II) salts

OH· can also be generated, in a reaction independent of $O_2^{\cdot -}$, by the direct addition of iron(II) salts to a reaction mixture containing phosphate buffer (Halliwell, 1978; Wong *et al.*, 1981; Halliwell & Gutteridge, 1981). Such addition of an iron(II) salt to reaction mixtures containing deoxyribose, deoxygalactose or deoxyglucose produced substantial degradation of these molecules to thiobarbituric acid-reactive material. Little effect was observed with deoxyadenosine, deoxyinosine or benzoate (Table 1). Inclusion of H_2O_2 in the reaction mixtures did not markedly change the result (Table 1). Damage was inhibited by catalase and desferrioxamine, but not by superoxide dismutase, as expected (Wong *et al.*, 1981; Halliwell & Gutteridge, 1981). Damage was also inhibited by thiourea, glucose, mannitol or Tris, but not by formate, ethanol, butan-1-ol or the control, urea. Again, similar results were obtained whether or not H_2O_2 had been added to the reaction mixture (Table 4).

Addition of EDTA to iron(II) salts is complicated by the fact that EDTA accelerates their oxidation to the iron(III) state (Cohen & Sinet, 1980; Halliwell & Gutteridge, 1981). Nonetheless, addition of EDTA increased damage by iron(II) salts to deoxyribose, deoxyadenosine, deoxyinosine and benzoate, but not to deoxyglucose or deoxygalactose (Table 1). Damage in the presence of EDTA was still decreased by catalase and desferrioxamine (Table 3), but was now decreased by every one of the OH· scavengers tested (Table 4).

The different radical-generating systems were present in a phosphate buffer, pH 7.4. In the presence of Fe(II) ions and the absence of phosphate little deoxyribose degradation was observed (Fig. 1).

Discussion

In the presence of EDTA, both an iron(II) salt and an iron(III) salt with an $O_2^{\cdot-}$-generating system caused damage to a range of biological molecules. Damage as measured by release of thiobarbituric acid-reactive material was diminished by all the scavengers of $OH^{\cdot}$ tested. A logical explanation of these results would be that $OH^{\cdot}$ is formed by the following series of reactions for Fe(II)–EDTA:

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

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

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

and in the $O_2^{\cdot-}$-generating system:

$$Fe^{3+}\text{–EDTA} + O_2^{\cdot-} \rightarrow Fe^{2+}\text{–EDTA} + O_2 \quad (4)$$

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

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

Catalase inhibits the $OH^{\cdot}$ formation in both cases, since H_2O_2 is required (eqn. 3), but superoxide dismutase inhibits only the latter reaction (eq. 4).

$OH^{\cdot}$ produced by these EDTA-containing systems is probably formed in 'free' solution and has to migrate a minute distance before it attacks the substrate molecule being damaged ('detector' molecule). Hence there would be a free competition between the 'detector' molecule being damaged and any other compound present that can readily react with $OH^{\cdot}$. This would explain why all the $OH^{\cdot}$ 'scavengers' tested inhibited damage in systems containing EDTA (Tables 1–4). The fact that they did indeed inhibit damage to the detector molecule suggests that, in these cases, the secondary radicals produced by attack of $OH^{\cdot}$ on the 'scavengers' were insufficiently reactive to damage the 'detector' molecules used here, although this need not always be the case (Schuessler & Freundl, 1983; Miller & Raleigh, 1983).

Iron ions will not exist 'free' in aqueous solution, since they will bind readily in a loose association to some components of the reaction mixture. In pure chemical terms the reaction has been complicated by the addition of a phosphate buffer, which binds metal ions. This buffer was chosen for two reasons. Firstly, other common laboratory buffers such as Tris (Paschen & Weser, 1975; Halliwell & Ahluwalia, 1976) and Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] (B. Halliwell, unpublished work) are powerful scavengers of $OH^{\cdot}$. Secondly, it is important to assess the likely physiological importance of iron-dependent radical reactions. Phosphate at pH 7.4 is an important buffer *in vivo* both intra- and extra-cellularly, and its presence should be considered.

In the absence of added EDTA, iron salts will bind to the buffer, to the 'detector' molecule or to some other component of the reaction mixture. Iron–phosphate complexes are weakly active in producing 'free' $OH^{\cdot}$ (Flitter *et al.*, 1983) as compared with iron–EDTA complexes. Iron bound to the 'detector' molecules would probably catalyse a 'site-specific' production of $OH^{\cdot}$. The decreased damage to deoxyribose, deoxyadenosine, deoxyinosine and benzoate in the absence of EDTA could in theory be attributed to less-efficient formation of 'free' $OH^{\cdot}$ in the absence of EDTA, whereas the accelerated damage to deoxygalactose and deoxyglucose could be attributed to iron binding and site-specific damage. However, these explanations seem unlikely, since if the first were true then all the $OH^{\cdot}$ scavengers should still inhibit damage (but formate, ethanol and butan-1-ol do not do so), and in the second case it is difficult to see why any of them should scavenge under these circumstances since very high concentrations of a scavenger would probably be necessary to scavenge $OH^{\cdot}$ being formed at a specific site. Given the ability of iron ions to bind to many carbohydrates and other compounds (Spiro & Saltman, 1969), it suggests that the target molecules ('detector') bind iron ions with differing affinities in the absence of EDTA. If the binding site of the metal ion is at, or near, the site that can lead to formation of thiobarbituric acid-reactive material, then the formation of $OH^{\cdot}$ at the site on the 'detector' molecule should produce more efficient damage than the formation of $OH^{\cdot}$ in 'free' solution. If iron is not bound at that site, then less damage should be apparent, i.e. the molecule might well be attacked somewhere else, but not in a way that would show up in these measurements. This point can be illustrated by the reaction of iron and iron–bleomycin with DNA (Gutteridge & Toeg, 1982*b*). It would seem unlikely that thiourea, glucose, mannitol and Tris protect against damage in the absence of EDTA by scavenging $OH^{\cdot}$ formed at specific sites, since formate, ethanol and butan-1-ol should do the same, yet they have little or no effect. Thiols are known to bind metals avidly (Willson, 1983), as do carbohydrates (Spiro & Saltman, 1969). This may suggest that protection by thiourea, glucose and mannitol is simply due to their competitive binding of iron ions, so removing them from the detector molecules and preventing damage. These compounds do not of course bind iron strongly enough to remove it from EDTA. The failure of ethanol, formate and butan-1-ol to inhibit damage suggests that iron-dependent formation of $OH^{\cdot}$ radical at a specific site cannot be effectively protected against by the classical '$OH^{\cdot}$' scavengers, as is also the case for copper-dependent damage (Samuni *et al.*, 1981, 1983; Gutteridge & Wilkins, 1983).

Patterns of inhibition in which formate and certain other OH· scavengers do not protect against damage to deoxyribose, mediated by anti-tumour antibiotics and paraquat, have been reported (Gutteridge & Toeg, 1982*b*; Sutton & Winterbourn, 1984; Gutteridge *et al.*, 1984; Gutteridge, 1984).

The data presented here cannot exclude the possibility that the reactive radical formed in the absence of EDTA is, not OH·, but some 'crypto-OH·' radical (Elstner *et al.*, 1980) or ferryl species (FeO^{2+}). However, the data on which these species have been postulated (e.g. inhibition of reaction by some scavengers of OH· but not by others) can still be explained by OH· formation (see above). It has also been implied (e.g. Czapski, 1978) that 'site-specific' attack of a molecule by OH· will be more damaging than attack of it by externally generated 'free' OH· radical. Data summarized here suggest that whether or not this is so depends on the exact binding site of the metal catalysing OH· formation. Indeed, Rosen & Klebanoff (1981) found that EDTA greatly accelerated the bactericidal effect of an $O_2^{\cdot-}$-generating system, which suggests that the iron ions involved were 'safely bound' somewhere until removed by added EDTA.

Some technical points arise from these data. Exposure of benzoate to iron salts or $O_2^{\cdot-}$-generating systems in the presence of EDTA causes release of thiobarbituric acid-reactive material. Care should therefore be used in interpreting the effects of benzoate added as an OH· scavenger in lipid peroxidation and other thiobarbituric acid-reactive systems. Secondly, the failure of such OH· scavengers as formate or ethanol to inhibit oxidative damage in systems not containing EDTA does not rule out the involvement of OH· in such damage. Conversely, inhibition of damage by mannitol or thiourea does not prove that it is mediated by 'free' OH· radicals: a wide range of scavengers should be tested to prove this.

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