FORMATION OF A THIOBARBITURIC-ACID-REACTIVE SUBSTANCE FROM DEOXYRIBOSE IN THE PRESENCE OF IRON SALTS

The role of superoxide and hydroxyl radicals

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1. Introduction

Oxygen is essential for the survival of aerobic cells, but it has long been known to be toxic to them when supplied at concentrations greater than those in normal air [1–3]. The biochemical mechanisms responsible for O_2 toxicity include lipid peroxidation and the generation of H_2O_2 plus the superoxide radical, $O_2^{\cdot-}$. In biochemical systems, $O_2^{\cdot-}$ and H_2O_2 react together to form the hydroxyl radical, $OH^{\cdot}$, which can attack and destroy almost all known biomolecules [1–3]. Neither lipid peroxidation nor generation of $OH^{\cdot}$ from $O_2^{\cdot-}$ occurs in vitro, however, unless traces of transition metal ions, especially iron, are present [4–7]. Hydroxyl radicals can also be generated in living systems by ionising radiation [8].

Traces of iron salts are present in all biological systems, and any increase in the normal concentration will potentiate the toxic effects of oxygen [3]. The preceding paper [11] showed that treatment of several amino acids or carbohydrates with iron (II) salts in phosphate buffer causes formation of a product that reacts with thiobarbituric acid (TBA) to produce a chromogen. Since the TBA-test is widely used in studies of lipid peroxidation [9] and several papers have suggested that the hydroxyl radical can apparently induce lipid peroxidation and DNA damage [1–3,10], we have examined the role of this radical in iron-dependent damage to the sugar deoxyribose.

2. Materials and methods

2.1. *Reagents*

Xanthine oxidase, superoxide dismutase (spec. act. 2900 units/mg protein), catalase (bovine liver, thymol free), albumin (bovine, fatty acid free) and deoxyribose were obtained from Sigma. The catalase was further purified on Sephadex G-25. Desferrioxamine was from Ciba. All other reagents were of the highest quality available from BDH.

2.2. *Measurement of iron (II)-dependent damage to deoxyribose*

The reaction mixture, in a total volume of 1.2 ml, contained the following reagents at the final concentrations stated: potassium phosphate buffer (pH 7.4) (10 mM); NaCl (63 mM); deoxyribose (0.8 mM). The reaction was initiated by adding ferrous ammonium sulphate (21 μM) and the mixture incubated at 37°C for 15 min. TBA (1 ml of 1% (w/v)) was then added, plus 1 ml 2.8% (w/v) trichloroacetic acid. The whole was heated at 100°C for 10 min, cooled, and the chromogen determined by its fluorescence at 553 nm as in [11]. Solutions of iron salts were made up fresh just before use.

2.3. *Assay of damage to deoxyribose in the presence of iron (III) salts plus a superoxide-generating system*

The reaction mixture contained, in a total volume of 1.4 ml, the following reagents at the final concentrations stated: potassium phosphate buffer (pH 7.4) (9 mM); NaCl (54 mM); deoxyribose (0.7 mM); xanthine (0.07 mM); xanthine oxidase (1.25 μl); and ferric ammonium sulphate (36 μM). After incubation at 37°C for 15 min, TBA-reactive material was measured as in section 2.2. Solutions of iron salts were made up fresh just before use.

3. Results

3.1. *Damage to deoxyribose by iron (II) salts*

In agreement with [11], addition of Fe(II) salts at μM levels to a solution of deoxyribose in phosphate buffer at pH 7.4 caused rapid degradation of the sugar to a product that reacts with thiobarbituric acid to form a chromogen. By contrast, iron (III) salts at similar concentrations did not affect the deoxyribose.

If hydroxyl radicals are involved in the degradation of deoxyribose by iron(II) salts, the reaction should be inhibited by scavengers of such radicals, e.g., mannitol, thiourea and catechol. Table 1 shows that this was the case. Urea, which reacts only slowly with $OH^{\cdot}$ radicals [12], had little effect. The iron chelators diethylenetriamine pentaacetic acid (DETAPAC) and desferrioxamine, which prevent generation of hydroxyl radicals from iron salts [4,5,13,14], also inhibited degradation. Damage was also prevented by including catalase in the reaction mixture, which suggests that H_2O_2 was the source of the $OH^{\cdot}$ radicals responsible for the degradation. Heat-denaturation of catalase abolished its effect. Superoxide dismutase, by contrast, did not prevent deoxyribose degradation, and so $O_2^{\cdot-}$ radicals cannot be necessary for the generation of $OH^{\cdot}$.

Several workers have investigated the effect of EDTA on iron-dependent hydroxyl radical-generating systems, but results have ranged from stimulation through no effect to inhibition [4–7,10,13,14,17,18]. Table 2 provides some indication of the reasons for such variability. When EDTA was present in the reaction mixture before Fe^{2+} was added, it had a partial inhibitory effect on subsequent deoxyribose degradation. A fresh solution of EDTA had less effect than a solution that had been stored in the laboratory in a sealed container (either glass or plastic) for 24 or 48 h. Further, if deoxyribose degradation was initiated by adding EDTA pre-mixed with the Fe^{2+} salt, the apparent inhibitory effect of EDTA was greatly increased, especially if the EDTA–Fe^{2+} mixture was allowed to stand for 5 min before adding it to the reaction mixture. The results with DETAPAC and desferrioxamine (table 1) were independent of the mode of addition of the chelator or the age of the solution, however.

3.2. *Damage to deoxyribose by a $O_2^{\cdot-}$-generating system*

A mixture of xanthine and xanthine oxidase generates $O_2^{\cdot-}$ and H_2O_2 [15] and, in the presence of μM levels of iron salts, these can react together to form $OH^{\cdot}$ radicals [6,7]. All chemical reagents and biological fluids are contaminated with iron salts at concentrations sufficient to produce $OH^{\cdot}$ [5,7,17,18].

Table 3 shows that a xanthine–xanthine oxidase system was able to degrade deoxyribose to produce a

Table 1
Effect of inhibitors on damage to deoxyribose induced by iron (II) salts

Inhibitor added	Amount of TBA-reactive product formed (fluorescence units)	% Inhibition of deoxyribose degradation
None	115	0
Mannitol (10 mM)	13	89
Thiourea (0.5 mM)	78	32
Catechol (0.1 mM)	35	70
Urea (0.5 mM)	114	1
DETAPAC (0.2 mM)	38	67
Desferrioxamine (0.2 mM)	12	90
Superoxide dismutase (50 μg/ml)	115	0
Catalase (50 μg/ml)	30	74
Albumin (100 μg/ml)	134	0
Denatured catalase (50 μg/ml)	116	0

Damage to deoxyribose by Fe^{2+} (21 μM) was measured as in section 2.2. The concentrations stated above were the final concentrations of each reagent in the reaction mixture. The results of a typical experiment are presented, but they were highly reproducible. The superoxide dismutase contained 2900 units/mg protein (units defined as in [15]) and the catalase 25 000 units/mg protein (units defined as in [16]). Where indicated, catalase was denatured by heating at 100°C for 10 min and cooling before use. The albumin used was free of fatty acids; DETAPAC, diethylenetriamine pentaacetic acid

Table 2
Effect of EDTA on damage to deoxyribose induced by iron (II) salts

Experiment	Amount of TBA-reactive product formed (fluorescence units)	% Inhibition of deoxyribose degradation
No EDTA	115	0
EDTA present in reaction mixture before Fe^{2+} was added:		
– fresh EDTA solution	52	55
– solution 24 h old	9	92
– solution 48 h old	35	70
EDTA (fresh solution) mixed with Fe^{2+} salt and the mixture added at once to the assay	4	97
EDTA (fresh solution) mixed with Fe^{2+} salt, the mixture allowed to stand for 5 min and then added to the assay	0	100

The results of a typical experiment are presented, but they were highly reproducible. Storage of the EDTA solution in either glass or plastic containers did not alter the results of the experiment. In all cases the final concentration of EDTA in the reaction mixture was 0.2 mM and that of Fe^{2+} was 21 μM, and assays were done as in section 2.2. Pre-incubation of Fe^{2+} solutions with EDTA before addition to the reaction mixture greatly decreased subsequent deoxyribose damage

Table 3
Effect of inhibitors on damage to deoxyribose in the presence of a superoxide-generating system and iron (III) salts

Inhibitor added	Amount of TBA-reactive product formed (fluorescence units)	% Inhibition of deoxyribose degradation
None	148	0
None (omit Fe^{3+})	68	(54)
Mannitol (10 mM)	10	93
Thiourea (0.5 mM)	80	46
Urea (0.5 mM)	140	5
Catechol (0.1 mM)	15	90
DETAPAC (0.2 mM)	33	78
Desferrioxamine (0.2 mM)	24	84
Catalase (50 μg/ml)	6	96
Denatured catalase (50 μg/ml)	127	14
Albumin (100 μg/ml)	149	0
Superoxide dismutase (50 μg/ml)	6	96
Denatured superoxide dismutase (50 μg/ml)	9	94

Damage to deoxyribose by a xanthine–xanthine oxidase system in the presence of Fe^{3+} was measured as in section 2.3. All reaction mixtures contained Fe^{3+} except where indicated. Concentrations stated are the final concentrations of each reagent in the reaction mixture. The results of a typical experiment are presented, but they were highly reproducible. Where indicated, superoxide dismutase was denatured by heating at 100°C for 10 min. For further details see the legend to table 1

TBA-reactive chromogen. Addition of low concentrations of iron (III) salts, which themselves do not affect deoxyribose, accelerated the rate of reaction. Deoxyribose degradation was inhibited by mannitol, thiourea, catechol, DETAPAC, desferrioxamine and catalase. By contrast, heat-denatured catalase, urea or bovine serum albumin had little or no effect.

Superoxide dismutase was also a powerful inhibitor of deoxyribose degradation, suggesting that not only H_2O_2 but also $O_2^{\cdot-}$ radicals are required for formation of $OH^{\cdot}$ in this system. Heat denaturation of the SOD did not, however, abolish its inhibitory activity (table 3). The superoxide dismutase used in these experiments is a copper protein [1,15] and it is possible that Cu^{2+} released from it upon denaturation are responsible for this effect, since free Cu^{2+} and copper–amino acid complexes can scavenge $O_2^{\cdot-}$ [19,20]. However, the dismutation of $O_2^{\cdot-}$ by Cu^{2+} is prevented by EDTA [21] whereas the action of superoxide dismutase is not [15]. Table 4 shows that EDTA itself had some inhibitory effect on deoxyribose degradation

Table 4
Effect of EDTA and superoxide dismutase on damage to deoxyribose by a superoxide-generating system plus iron (III) salt

Addition to reaction mixture	Amount of TBA-reactive product formed (fluorescence units)
None	148
$CuCl_2$ (50 μM)	4
EDTA (100 μM)	80
EDTA + $CuCl_2$ (50 μM)	80
EDTA + superoxide dismutase	
– 5 μg/ml	26
– 10 μg/ml	8
– 50 μg/ml	7
EDTA + denatured superoxide dismutase	
– 5 μg/ml	65
– 10 μg/ml	72
– 50 μg/ml	72

Eperiments were carried out as in table 3. Cu^{2+} salts, superoxide dismutase and heat-denatured enzyme inhibit deoxyribose degradation strongly (see above and table 3). The presence of EDTA in the reaction mixture decreased deoxyribose damage by 46%, but the inhibitory effect of Cu^{2+} or denatured superoxide dismutase was prevented by EDTA. Inhibition by native enzyme was not affected by EDTA (compare tables 3 and 4). Where indicated, superoxide dismutase was denatured by heating at 100°C for 10 min

by xanthine–xanthine oxidase plus Fe^{3+} salts. The inhibitory action of superoxide dismutase was still seen in the presence of EDTA, whereas that of heat-denatured enzyme was almost completely abolished. Table 4 further shows that Cu^{2+} inhibited deoxyribose degradation in this system, an effect that was prevented by EDTA. It may be concluded that the action of heat-denatured superoxide dismutase shown in table 3 is due to the release from it of free Cu^{2+}, which can also catalyse the dismutation of $O_2^{\cdot-}$.

4. Discussion

Addition of low concentrations of Fe^{2+} salts to deoxyribose causes degradation of the sugar into a 'malondialdehyde-like' compound which forms a chromogen with thiobarbituric acid. Iron (II) salts in aqueous solution slowly autoxidise to form $O_2^{\cdot-}$ [13]:

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

$O_2^{\cdot-}$ undergoes non-enzymic dismutation rapidly at pH 7.4:

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

and H_2O_2 interacts with Fe^{2+} salts to form $OH^{\cdot}$ radicals, the 'Fenton reaction':

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

Evidence that reactions (1–3) generate the hydroxyl radical, which degrades deoxyribose, is provided by the inhibition of degradation by catalase, by scavengers of $OH^{\cdot}$ and by chelators (DETAPAC and desferrioxamine) which inhibit reactions (1) and (3) [4,7,14]. Superoxide dismutase does not inhibit, however, since it merely accelerates reaction (2) and does not therefore interfere with generation of OH. Fe^{3+} salts are ineffective because, by themselves, they cannot produce $O_2^{\cdot-}$ in aqueous solution. Production of oxygen radicals in solutions of Fe^{2+} salts has also been detected by their ability to hydroxylate aromatic compounds [7,13] and to nick closed-circular viral DNA [22].

A superoxide-generating system, xanthine + xanthine oxidase, also converted deoxyribose into the TBA-reactive material. The rate of reaction was greatly accelerated by addition of Fe^{3+} salts, some of which would already have been present in the reaction mix-

ture because of contamination of the reagents used [5,17,18]. Degradation was again inhibited by $OH^{\cdot}$ scavengers, desferrioxamine, DETAPAC and catalase, indicating that reaction (3) was important in producing the chromogen. Superoxide dismutase or Cu^{2+} salts, which are powerful scavengers of $O_2^{\cdot -}$, also inhibited the reaction. It seems likely that $O_2^{\cdot -}$ was acting to reduce contaminating iron (III) into iron (II), so as to allow reaction (3) to occur:

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2 \qquad (4)$$

Superoxide dismutase, by blocking reaction (4), would stop production of $OH^{\cdot}$ radicals. Evidence for a reduction of iron salts by $O_2^{\cdot -}$ has been provided by indirect assay systems [23], pulse radiolysis [24] and stopped-flow experiments [25] and the occurrence of reaction (4) is supported by much indirect evidence [6,26]. A recent claim that superoxide does not interact with iron salts [27] is thus unlikely to be true, although it might be that the reactions are more complex than shown in reaction (4).

Two technical points also arise from our data:

(i) The dismutating ability of denatured superoxide dismutase, due to the release of copper from it (table 4). This action must be critically dependent on the exact mode and time of denaturation used, since it has not been observed previously in our laboratory [4,7,28].

(ii) The inhibitory action of EDTA varies with the exact experimental conditions used (table 2) and, for an unknown reason, with the age of the solution. Whereas autoxidation of Fe^{2+} salts at pH 7.4 is fairly slow, Fe^{2+}–EDTA autoxidises more rapidly [30] and so, in pre-mixing experiments, what is probably being added to the reaction mixture is Fe^{3+}–EDTA, which is inactive in generating $OH^{\cdot}$ unless a $O_2^{\cdot -}$-generating system is present. Hence EDTA pre-mixed with Fe^{2+} salts has an apparently more powerful inhibitory effect than when pre-mixing is not carried out. Even when EDTA is present in the reaction mixture before Fe^{2+} is added, the accelerated autoxidation may, by quickly removing Fe^{2+}, decrease the rate of reaction (3).

Given that iron–EDTA chelates interact with $O_2^{\cdot -}$ faster than do free iron salts [21,23–26] it becomes possible to see the reasons for the wide variation in the reported effects of EDTA upon systems in which oxygen radicals are generated in the presence of iron salts.

Finally, the simple colorimetric assay described in this paper provides an extremely convenient way of detecting $OH^{\cdot}$ radicals.

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