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Hydroxyl-radical-induced iron-catalysed degradation of 2-deoxyribose

Quantitative determination of malondialdehyde

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The degradation of 2-deoxyribose to thiobarbituric acid-reactive material was investigated with two hydroxyl-radical-generating systems: (i) a defined γ-radiolysis method and (ii) incubation with $FeSO_4$ in phosphate buffer. In each case the thiobarbituric acid-reactive material can be accounted for by malondialdehyde, as measured by an h.p.l.c. method for free malondialdehyde. In the radiolysis system there is a large post-irradiation increase in free malondialdehyde if iron ions are added to the samples. It is proposed that this is due to iron ions catalysing the formation of hydroxyl radicals from radiolytically generated H_2O_2 as well as stimulating the breakdown of an intermediate deoxyribose degradation product. A mechanism for the formation of malondialdehyde during deoxyribose degradation is proposed.

INTRODUCTION

The dicarbonyl compound malondialdehyde (MDA) is well known as one of the many carbonyl products of lipid peroxidation: its reaction with thiobarbituric acid (TBA) to form an intensely coloured adduct is the most frequently used test for lipid peroxidation in biological systems (Slater, 1984). MDA is also formed during the degradation of certain carbohydrates: indeed, the TBA assay was used for the determination of deoxy sugars nearly 30 years ago (Waravdekar & Saslaw, 1959). More recently the oxidative degradation of deoxy sugars to TBA-reactive material in free-radical-mediated cell injury has stimulated considerable interest. Kuo & Haidle (1973) have shown that the strand breakage of DNA induced by the anti-tumour antibiotic bleomycin [a reaction believed to involve attack by hydroxyl radicals ($HO^{\bullet}$) on deoxyribose moieties] is accompanied by the release of TBA-reactive material. Although this product was originally identified as MDA (Gutteridge, 1979), later work (Burger *et al.*, 1980; Giloni *et al.*, 1981) showed that four different 'base propenals' were formed (from the four nucleoside bases) and that the MDA is produced from these by hydrolysis (e.g. during the TBA assay). Gutteridge (1981) studied the iron-ion-dependent degradation of various carbohydrates and amino acids to TBA-reactive material: by this test, 2-deoxyribose was the most sensitive of the compounds tested. The $HO^{\bullet}$ radical appeared to be the damaging species in this degradation of 2-deoxyribose, and the release of TBA-reactive material from 2-deoxyribose was proposed as a convenient detection system for $HO^{\bullet}$ radicals (Halliwell & Gutteridge, 1981; Gutteridge *et al.*, 1984; Gutteridge, 1984, 1987; Gutteridge & Quinlan, 1985).

In the present paper we report studies of two systems of 2-deoxyribose degradation: the $FeSO_4$-dependent system described by Gutteridge (1984) and a defined $HO^{\bullet}$-generating system based on γ-irradiation, in which the TBA-reactive product has been characterized as MDA by a direct h.p.l.c. assay (Esterbauer & Slater, 1981; Esterbauer *et al.*, 1984). The use of the h.p.l.c. assay for free MDA reveals the actual amount of free MDA formed rather than the amount of MDA precursor, which is not distinguishable from free MDA in the TBA test. We have confirmed that the TBA-reactive material formed during deoxy sugar degradation is MDA and have examined the role of iron ions in mediating its formation.

MATERIALS AND METHODS

Deoxy sugars and deoxyadenosine were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) and were dissolved in double-distilled MilliQ (Millipore)-filtered water for the irradiation experiments. All other reagents were of the highest standard available. A 10 mM stock solution of MDA was prepared by incubating malonaldehyde bisdimethylacetal in 1% (v/v) H_2SO_4 for 2 h at room temperature. This was suitably diluted (to 5–10 μM) and used to calibrate the h.p.l.c. assay. The concentration of this standard solution was checked by performing the TBA assay (Slater & Sawyer, 1971) and using a molar absorption coefficient at 535 nm of 1.49×10^5 $M^{-1} \cdot cm^{-1}$: 1 ml of test solution was mixed with 0.5 ml of 20% (w/v) trichloroacetic acid and 1.5 ml of 0.67% (w/v) TBA. The mixture was heated (at 100 °C for 10 min), then cooled, and the absorbance was measured at 535 nm. The direct h.p.l.c. assay for free MDA was performed essentially as described previously (Esterbauer & Slater, 1981): separations were made with a Waters carbohydrate analysis column (3.5 mm × 30 cm; part no. 84035) with a mobile phase of 0.03 M-Tris/HCl buffer (pH 7.4)/acetonitrile (9:1, v/v) at a flow rate of 0.8 ml/min and the detector at 270 nm.

The $HO^{\bullet}$-induced degradation of 2-deoxyribose and other compounds was studied in two systems. The first

Abbreviations used: MDA, malondialdehyde; TBA, thiobarbituric acid.

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was essentially that described by Gutteridge (1984): deoxy sugars (2 mM) in 0.1 M-sodium phosphate buffer, pH 7.4, were incubated in the presence and in the absence of $FeSO_4$ (usually 0.2 mM) at 37 °C for 15 min. The second procedure utilized γ-radiolysis of water to produce $HO^\cdot$ radicals: aqueous solutions of substrate (1 mM; pH 7.0) were saturated with N_2O/O_2 (4:1) and exposed to γ-irradiation from the Brunel University ^{60}Co source (up to 130 Gy at 6.5 Gy/min). Under these conditions the major radical species present is $HO^\cdot$ ($G = 5.4$, where the G value corresponds to μM concentration of product per 10 Gy dose):

$$H_2O \rightarrow e^-_{aq.}\ (G = 2.7) + H^\cdot\ (G = 0.5) + HO^\cdot\ (G = 2.7)$$

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

At the end of the irradiation period the sample was assayed either immediately or after adding $FeSO_4$ (5 or 10 μM) and/or leaving at room temperature for up to 70 min. In all cases a 1 ml sample was taken for the TBA assay described above, and a 50 μl sample was used for the h.p.l.c. assay. The iron-ion-catalysed production of MDA from irradiated 2-deoxyribose was also monitored by u.v. spectroscopy with a Cary 219 spectrophotometer. In all cases where $FeSO_4$ was used it was prepared immediately before use in N_2-purged water.

RESULTS

When the deoxy sugars 2-deoxyribose, 2-deoxyglucose and 2-deoxygalactose (2 mM) were incubated with $FeSO_4$ (usually 0.2 mM) in 0.1 M-phosphate buffer, pH 7.4, TBA-reactive material was observable, as previously reported (Halliwell & Gutteridge, 1981; Gutteridge, 1984). By analysing the incubation medium by the h.p.l.c. method it was possible to detect free MDA (Fig. 1), and in each case the amount of free MDA detected was sufficient to account for all of the TBA-reactive material (Table 1). In fact, in 16 separate determinations of 2-deoxyribose degradation with various $FeSO_4$ concentrations (0.1–1.0 mM) a range of MDA concentrations (2–12 μM) was measured with the ratio of MDA determined by the TBA test to that determined chromatographically equal to 1.0 ± 0.1 (mean $\pm$ S.D.).

TBA-reactive material was also formed during the γ-irradiation of deoxy sugars and deoxyadenosine (Fig. 2). 2-Deoxyribose yielded the most TBA-reactive material ($G = 0.56$), deoxyadenosine a little less ($G = 0.42$) and 2-deoxyglucose and 2-deoxygalactose yielded similarly low amounts ($G = 0.1$ approx.). 2-Deoxyribose was subsequently chosen as the most suitable substrate for further studies.

When γ-irradiated 2-deoxyribose was assayed by the h.p.l.c. method free MDA could be detected (Fig. 1), but the close agreement with the TBA test that had been found in the $FeSO_4$-dependent system was not apparent; indeed, the discrepancies were large and rather variable. It was noted that the amount of free MDA determined chromatographically increased slowly if the irradiated 2-deoxyribose solutions were allowed to stand for a long period of time. The generation of free MDA in irradiated 2-deoxyribose solutions could be accelerated by the addition after the irradiation period of $FeSO_4$ in concentrations not sufficient to produce a significant amount of MDA from non-irradiated 2-deoxyribose. In fact, when a low concentration of iron ions was added

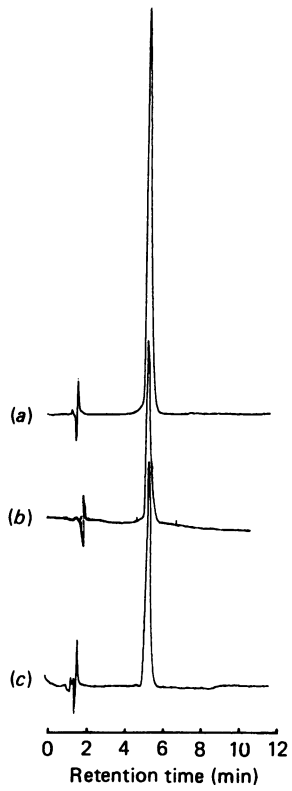


Fig. 1. Typical h.p.l.c. chromatograms of (*a*) authentic MDA standard, (*b*) irradiated 2-deoxyribose and (*c*) 2-deoxyribose incubated in the Fe^{2+}/phosphate buffer system

For methods see the text. Absorption units full scale = 0.05 throughout.

Table 1. MDA production from the $FeSO_4$-dependent oxidation of 2-deoxyribose, 2-deoxyglucose and 2-deoxygalactose

Substrates (each 2 mM in 0.1 M-phosphate buffer, pH 7.4) were incubated with $FeSO_4$ (0.2 mM) for 15 min at 37 °C. MDA was assayed by the TBA test and by the h.p.l.c. method. Values are means $\pm$ S.E.M. for three determinations.

	[MDA] (μM)	
Substrate	TBA assay	H.p.l.c. assay
2-Deoxyribose	3.49 ± 0.01	3.31 ± 0.02
2-Deoxyglucose	2.10 ± 0.08	2.61 ± 0.09
2-Deoxygalactose	2.26 ± 0.09	2.64 ± 0.21

and the samples were allowed to stand for about an hour a reasonably good agreement was found between the TBA test and the h.p.l.c. test for free MDA. As well as the large increase in free MDA, the addition of iron ions also caused a smaller but significant increase in the amount of TBA-reactive material. The data are summarized in Table 2.

If catalase was added to the irradiated deoxyribose solution before adding the iron, the slight increase in TBA-reactive material and the large increase in free MDA were both prevented. This suggested that the post-irradiation effect of iron ions was due to H_2O_2 present in

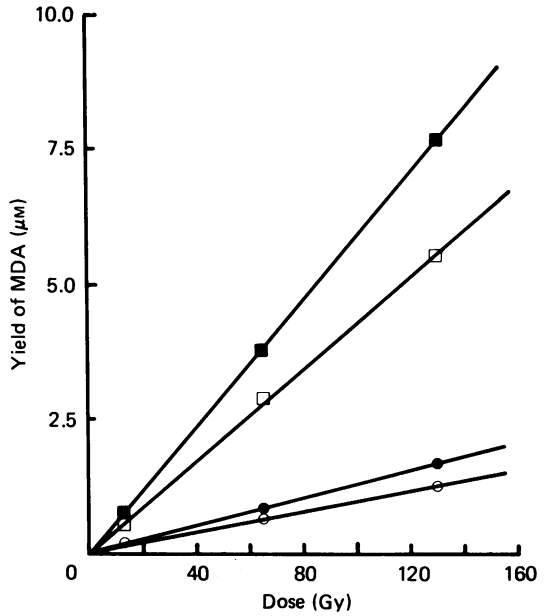


Fig. 2. Typical relationship between the production of MDA (measured as TBA-reactive material) and the radiation dose for solutions of 2-deoxyribose (□), deoxyadenosine (■), 2-deoxygalactose (○) and 2-deoxyglucose (●) γ-irradiated at 6.5 Gy/min

All solutions were 1 mM, saturated with N_2O/O_2 (4:1) and at pH 7.0.

Table 2. Yield of MDA obtained after the γ-radiolysis of 2-deoxyribose, determined as TBA-reactive material or by the direct h.p.l.c. method

The yields are expressed as the G value (μM-MDA per 10 Gy radiation). 2-Deoxyribose [1 mM in water; pH 7.0; saturated with N_2O/O_2 (4:1)] was exposed to 130 Gy of γ-radiation at 6.5 Gy/min. Where appropriate $FeSO_4$ was added at the final concentration indicated immediately after the cessation of γ-irradiation. Samples were then assayed 2 min and 70 min later. In each assay the yield of MDA due to addition of iron ions in the absence of irradiation (which was never more than 3% of that in the presence of irradiation) was subtracted for the calculation of G values. Data are expressed as means ± S.E.M. for the numbers of experiments in parentheses.

Post-irradiation time (min)	$[Fe^{2+}]$ (μM)	G_{MDA} TBA assay	G_{MDA} H.p.l.c. assay
2	0	0.56±0.04 (3)	0.14±0.04 (3)
	5	1.07 (2)	0.51 (2)
	10	1.36±0.12 (3)	0.64±0.18 (3)
70	0	0.67±0.07 (6)	0.14±0.06 (6)
	5	1.13±0.04 (5)	0.07±0.19 (5)
	10	1.38±0.14 (3)	1.51±0.26 (3)

the irradiated samples, and we performed experiments to test what contribution radiolytically generated H_2O_2 was making to the iron ion-dependent increase in MDA. Table 3 shows that catalase prevents the iron ion-dependent increase in TBA-reactive material formation

Table 3. Production of TBA-reactive material from deoxyribose after γ-irradiation on addition to irradiated water: effect of Fe^{2+} and catalase

Water and 1 mM-deoxyribose were adjusted to pH 7.0, saturated with N_2O/O_2 (4:1) and irradiated with 130 Gy at 11 Gy/min. At the end of the irradiation samples (1 ml) were taken and mixed with 0.1 ml of either $FeSO_4$ (final concn. 5 μM), catalase (100 units), native deoxyribose (final concn. 1 mM) or combinations thereof. After catalase addition the sample was left for 10 min at room temperature before further additions or before the TBA test. A representative experiment of several is shown, and the data are means for duplicate samples that were within 5% of each other.

Post-irradiation addition	Yield of MDA (μM) Irradiated water	Irradiated 1 mM-deoxyribose
Water control	–	8.52
$FeSO_4$	–	11.34
Catalase	–	7.76
Catalase + $FeSO_4$	–	7.94
Deoxyribose	0	–
Deoxyribose + $FeSO_4$	3.48	–
Deoxyribose + catalase + $FeSO_4$	0.28	–

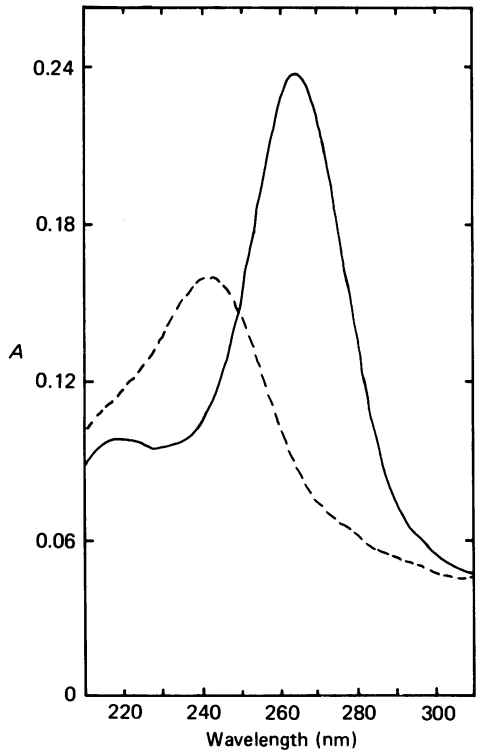
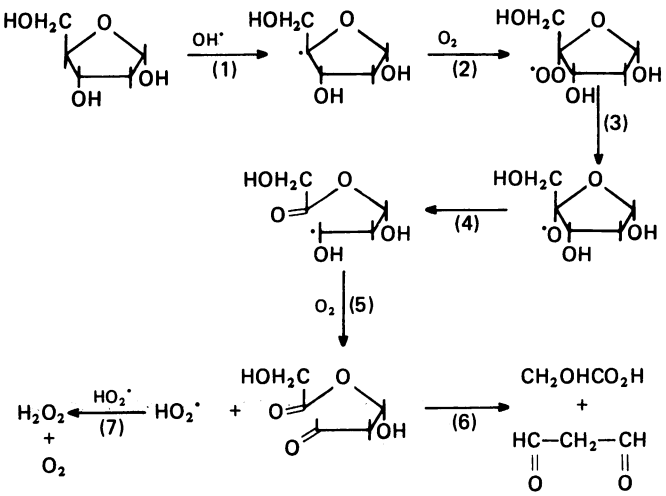


Fig. 3. Spectrum of irradiated deoxyribose 30 min after addition of $FeSO_4$ at pH 7.0 (——) and at pH 2.0 (----)

Deoxyribose (1 mM) was saturated with N_2O/O_2 (4:1) at pH 7.0 and γ-irradiated with 120 Gy. $FeSO_4$ (0.1 ml to 3.0 ml of deoxyribose; final concn. 10 μM) was added and the spectra were recorded 30 min later. The reference cuvette contained non-irradiated deoxyribose.

from irradiated deoxyribose. Heat-inactivated catalase or albumin at an equivalent protein concentration does not prevent the increase (results not shown). Moreover, if untreated deoxyribose is added to irradiated water, and $FeSO_4$ is then added, TBA-reactive material is produced in an amount sufficient to account for the iron ion-dependent increase in formation of TBA-reactive material from irradiated deoxyribose. This phenomenon can also be inhibited by catalase, but not by heat-inactivated catalase or an equivalent amount of albumin. No TBA-reactive material is produced when untreated deoxyribose is added to irradiated water without $FeSO_4$ addition or if the $FeSO_4$ is added after the TBA reagent.

When the iron ion-catalysed generation of MDA from irradiated 2-deoxyribose was monitored spectrophotometrically the spectrum obtained soon after irradiation showed a broad peak with maxima at 266 nm and 260 nm. The maximum absorption at 266 nm increased with time upon addition of $FeSO_4$ (5 μM or 10 μM) until the final spectrum agreed closely with that of MDA. If the solution was made acidic (pH 2.0) the spectrum changed such that the peak wavelength became 245 nm (Fig. 3). Identical spectra were obtained with solutions of authentic MDA under the same conditions (results not shown), as would be expected (Saunders & May, 1963). An identical spectrum was obtained when $FeSO_4$ was added to 2-deoxyribose in phosphate buffer, under the same conditions as described above, provided that the spectrum was corrected for the absorbance changes due to the addition of $FeSO_4$ to phosphate buffer alone (spectrum not shown).



Scheme 1. Proposed mechanism for the formation of MDA from carbon atoms C-1, C-2 and C-3 of 2-deoxyribose following HO$^\cdot$ attack in the presence of O_2

Only HO$^\cdot$ attack at C-4 is indicated.

DISCUSSION

It is well established that the oxidative degradation of deoxyribose yields a product reacting with TBA to give a pink chromogen. Doubt over the exact identity of this product is indicated by it being referred to as 'TBA-reactive product' or 'MDA-like material'. In each of the systems we have used here the degradation of deoxyribose yields a product that is apparently MDA as evidenced by the following: (i) it reacts with TBA to give an adduct whose spectrum is identical with that obtained with authentic MDA (Gutteridge, 1981); (ii) its u.v. spectrum is identical with that of authentic MDA at both pH 7.0 and pH 2.0; (iii) it co-chromatographs with authentic MDA in the h.p.l.c. system described in the present paper; (iv) the yields of 'MDA' determined by the TBA test agree with those determined by h.p.l.c. so long as the experimental conditions allow full development of free MDA. In the Fe^{2+}-dependent system described by Gutteridge (1984) the TBA test and the h.p.l.c. system agree exactly not only with deoxyribose as substrate but also with deoxyglucose and deoxygalactose.

In the radiolysis system the agreement between the TBA test and the h.p.l.c. system depends on the presence of catalytic amounts of Fe^{2+}. The inhibition of the iron ion-stimulated increase in MDA yield from irradiated deoxyribose suggests that H_2O_2 is involved. H_2O_2 is a known product of the radiolysis of water ($G = 0.7$; Swallow, 1973), and more H_2O_2 may be produced during the decomposition of deoxyribose by the release and dismutation of $HO_2^\cdot$ radicals (Schulte-Frohlinde & von Sonntag, 1985; Scheme 1). The addition of Fe^{2+} to samples containing H_2O_2 will result in further HO$^\cdot$ radical formation by the Fenton reaction:

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

That this is a possible and significant route to further HO$^\cdot$-dependent deoxyribose degradation is shown by adding deoxyribose to irradiated water and adding iron ions (Table 3). The fact that the addition of iron ions causes a larger increase in free MDA detectable by h.p.l.c. than in the amount detected by the TBA test suggests that Fe^{2+} also catalyses the breakdown of an MDA precursor. Thus this MDA precursor, possibly a deoxyribose hydroperoxide, may be relatively stable at room temperature in the absence of metal ions but may break down under the harsh conditions of the TBA test or in the presence of iron ions (e.g. when iron ions are added to irradiated deoxyribose and in the Fe^{2+}/phosphate buffer system). This would explain the observation made by Langfinger (1984), who noted (as we have done) that the TBA-reactive product derived from HO$^\cdot$ attack on deoxyribose reacted more slowly with TBA than did authentic MDA. It would also mean that in the Fe^{2+}/phosphate buffer system of Gutteridge (1984) the iron ions play two roles: first, the generation of HO$^\cdot$ radicals in an iron ion-catalysed Fenton reaction and, secondly, the catalysis of the breakdown of the MDA precursor.

The products of HO$^\cdot$/O_2-mediated deoxyribose degradation have not been fully characterized, though a thorough characterization has been made of the products formed in the absence of O_2 (Schulte-Frohlinde & von Sonntag, 1972). These authors show that H$^\cdot$ abstraction is possible at all the carbon atoms of the sugar molecule. Langfinger (1984) noted the necessity of the deoxy function at C-2 for the formation of TBA-reactive material: with ribose the G value was only 0.01. It seems likely therefore that the MDA is derived from carbon atoms C-1, C-2 and C-3 of the deoxyribose molecule after formation of a peroxyl radical at C-4. This is shown in Scheme 1, which is based on the mechanism proposed by Schulte-Frohlinde & von Sonntag (1985) for thymidine breakdown.

This situation would thus be analogous to that described to the thymidine peroxyl radical (Schulte-Frohlinde & von Sonntag, 1985). The lower *G* values for the formation of TBA-reactive material from 2-deoxyglucose and 2-deoxygalactose are probably due to a greater number of possible sites of attack open to the HO· radical; thus a greater proportion of non-TBA-reactive products would be formed. It may be noted, however, that these sugars produce as much MDA as does deoxyribose in the Fe^{2+}/phosphate buffer system of Gutteridge (1984) (Table 1), possibly implicating a 'site-specific' attack directed by iron ions bound to the target molecule (see Gutteridge, 1987).

Schulte-Frohlinde & von Sonntag (1972) suggest that approx. 20% of the HO· radicals produced will attack the C-4 position of deoxyribose. Taking this value into account, along with the known *G* values for HO· and H_2O_2 production the yield of MDA obtained is consistent with the mechanism shown in Scheme 1. Further work is required to identify intermediate products in that reaction pathway and the role of metal ions in their breakdown. For example, the mechanism of alkoxyl radical formation (step 3 in Scheme 1) is not clear, but may involve iron ion-catalysed breakdown of a hydroperoxide or tetroxide intermediate. It is clear that the use of h.p.l.c. to measure free MDA may provide a better understanding of deoxyribose degradation and related reactions. Moreover, having established that the TBA-reactive material produced in the system of Gutteridge (1984, 1987) is, in fact, MDA, then one can, by using the known molar absorption coefficient for the MDA–TBA complex, obtain more quantitative data from such experiments.

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