THIOBARBITURIC ACID-REACTIVITY FOLLOWING IRON-DEPENDENT FREE-RADICAL DAMAGE TO AMINO ACIDS AND CARBOHYDRATES

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1. Introduction

The thiobarbituric acid reaction has been widely used during the last 40 years for detecting oxidative deterioration in bulk lipids, and more recently it has been increasingly applied to complex biological materials. Lipid peroxidation is a free-radical-mediated event in which metal ions play important catalytic roles both during the initiation stage and in subsequent reactions. The primary products of such damage are a complex mixture of peroxides, which then break down to produce carbonyl compounds. The 3-carbon compound malondialdehyde (MDA) is one such carbonyl, which forms a characteristic chromogenic adduct with 2 molecules of thiobarbituric acid (TBA) [1]. This has led to the widespread belief that the TBA test detects MDA formation in biological systems, which was never claimed in the early studies using the TBA test as a measure of lipid peroxidation. Indeed, several workers showed that 'free' MDA is but a minor component of peroxidised lipid [1–3]. Most of the MDA detected by the TBA test is in fact produced from peroxidic precursors under the acid-heating conditions of the assay [4,5].

Malondialdehyde is not exclusively derived from polyunsaturated fatty acid intermediates. Recently, two structurally different anti-tumour antibiotics have been shown to release an intermediate from DNA which gives rise to MDA during the TBA test [6–8]. High-energy irradiation of carbohydrates and amino acids has also been reported to liberate TBA-reactive products as well as 'malondialdehyde' [9–13]. High-energy radiation, like the autoxidation of reduced metal ions, will result in the formation of hydroxyl radicals ($OH^{\cdot}$). These oxygen free-radicals are indiscriminate in their damage to biomolecules [14]. This study was undertaken to assess iron-dependent free-radical damage to carbohydrates and amino acids and to characterise the resulting TBA chromogen.

2. Methods and materials

2.1. *Reagents*

Amino acids, D-galactose, sucrose, D-glucose and lactose were obtained from BDH. D-Ribose, 2-deoxy-D-ribose, β-D-fructose and D-glucuronic acid were obtained from Sigma. 1,1,3,3-Tetramethoxypropane was from Aldrich. Other chemicals were of AnalaR grade where available and obtained from BDH.

2.2. *Iron damage to amino acids*

Amino acid solution (0.5 ml, 5 mM) was added to 0.5 ml 0.1 M phosphate buffer (pH 7.4) in 0.15 M NaCl. The reaction was started by the addition of 0.1 ml solution of Fe^{2+} (10 mM). The tubes were incubated at 37°C for 15 min with appropriate blanks to which iron had not been added.

2.3. *Iron damage to carbohydrates*

Carbohydrate solution (0.5 ml, 5 mM) was added to either 0.1 M phosphate buffer (pH 7.4) in 0.15 M NaCl or to a 0.5 M borate buffer (pH 9.5). Fe^{2+} (0.1 ml, 10 mM) were added to start the reaction, which was carried out for 15 min at 37°C. Appropriate blanks were included for each sample in the different buffers.

2.4. *Measurement of TBA-reactivity*

TBA (1.0 ml of 1% (w/v)) in 0.05 M NaOH was added to the incubated samples of amino acids and carbohydrates, followed by 1 ml glacial acetic acid.

The tubes were heated for 30 min at 100°C, cooled and the absorbance read against a paired blank in the spectrophotometer. Spectrophotometric scans were recorded as scan-difference spectra using a Perkin-Elmer 402. TBA fluorescence was measured using a Perkin Elmer MPF-4 spectrofluorimeter and expressed as relative fluorescence units against a block standard containing 3×10^{-6} M rhodamine B [13]. This standard was set to 100 units at excitation 480 nm, emission 580 nm, slits 10 nm and sensitivity × 1. TBA fluorescence was measured using excitation at 532 nm, emission 553 nm slits 10 nm. Samples with high fluorescence were diluted where necessary, to avoid inner-filter effects.

3. Results

After treatment with low concentrations of Fe^{2+}, 6 different amino acids yielded a TBA chromogen upon heating at acid pH, viz., glutamic acid, 2-amino-butyric acid, proline, arginine, homocysteine and methionine (table 1). Development of the chromogen required the presence of acetic acid and heating at 100°C for 30 min. However, when the products of Fe^{2+} damage to these amino acids were steam distilled from acid solution [7] no volatile aldehydes could be detected in the distillate either by TBA-reactivity or UV-spectrophotometry.

The TBA chromogen was characterised by its visible and fluorescent properties. The fluorescence spectra for all the reactive amino acids were indistinguishable from that of a TBA–MDA adduct, showing excitation maxima at 532 nm and emission at 553 nm. Scan difference spectra of the TBA-reactive chromogens from the amino acids, showed that they all had an absorbance maximum of 532 nm in acid solution and 555 nm at an alkaline pH, again characteristic of the TBA–MDA adduct (fig.1).

Fe^{2+} damage to a selection of carbohydrates at pH 7.4 in the presence of phosphate and at pH 9.5 in borate showed that 2 sugars produced TBA-reactive material, namely deoxyribose and glucuronic acid (table 2). Several of the carbohydrates reacted with the TBA reagent in glacial acetic acid to give high blank values in the absence of iron damage. At high iron concentrations (100–200 mM) both galactose and sucrose gave a faintly detectable TBA chromogen absorbing at 532 nm. In the presence of phosphate, iron damage to glucose, galactose and ribose resulted in the formation of a 'blue' chromogen with TBA and glacial acetic acid, having a peak absorbance maximum at 635 nm. This did not occur when borate buffers were used. Of the carbohydrates tested only deoxyribose yielded a volatile TBA-reactive product after acid steam distillation [7] of the reaction mixture, following iron damage to the molecule. This had UV absorption peaks at 245 nm in acid solution and 270 nm in alkaline solution, characteristic of the MDA standard similarly treated. The TBA chromogens from iron-damaged deoxyribose or glucuronic acid showed fluorescence spectra and pH-dependent visible absorption spectra indistinguishable from those of an MDA–TBA adduct treated similarly (fig.1).

Table 1
Fe^{2+} damage to amino acids (TBA reactivity)

Amino acids	As A_{532}	As fluorescence $E_{x,532}$ $E_{m,553}$
D,L-Alanine	0.022	16
D,L-2-Amino-*n*-butyric acid	0.200	420
L-Arginine	0.106	210
D,L-Aspartic acid	0.028	18
L-Cysteine	0.022	15
L-Cysteine[a]	0.024	15
D,L-Dihydroxyphenylalanine	0.098	12
L-Glutamic acid	0.300	500
Glycine	0.022	21
L-Histidine	0.046	19
L-Hydroxyproline	0.044	18
L-Leucine	0.024	17
D,L-iso-leucine	0.040	16
D,L-nor-leucine	0.030	47
L-Lysine	0.023	21
D,L-Methionine	0.130	240
D,L-Ornithine	0.060	110
D,L-β-phenylalanine	0.071	110
L-Proline	0.111	200
D,L-Serine	0.050	21
D,L-Threonine	0.036	25
D,L-Tryptophan[a]	0.048	31
L-Tyrosine[a]	0.040	26
D,L-Valine	0.024	15
D,L-Homocystine[a]	0.100	210

[a] Ultra-sonicated aqueous suspensions

Amino acids at a final concentration of 2.3 mM were incubated with 0.9 mM ferrous ammonium sulphate in phosphate buffer at pH 7.4. TBA reactivity was measured both as fluorescence and as A_{532}

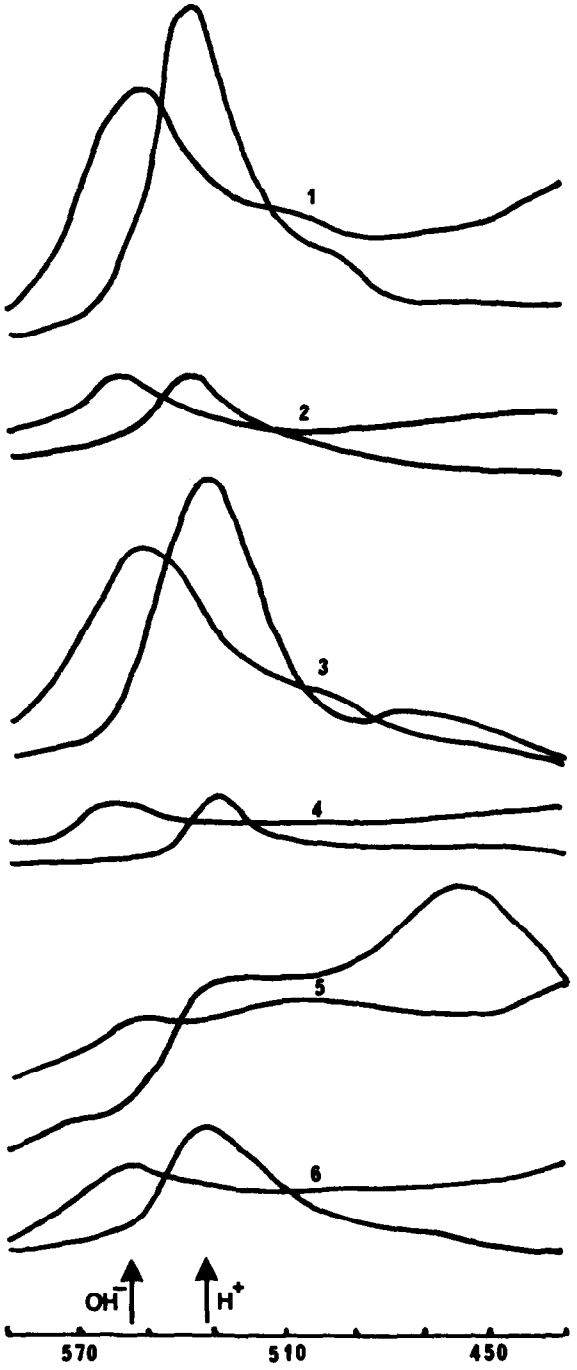

Fig.1. Scan-difference spectra of TBA-reactive amino acids and carbohydrates. Glucuronic acid (5), Methionine (4), glutamic acid (1), and aminobutyric acid (6) were present as 2.3 mM solutions; deoxyribose (3) as a 0.45 mM solution. Iron damage was brought about by 0.9 mM Fe^{2+}. The MDA standard (2) was prepared by adding 20 μl 20 μM solution of 1,1,3,3-tetramethoxypropane in the same reaction volume as the test samples. pH was adjusted by the addition of NaOH.

Table 2
Fe^{2+} damage to carbohydrates (TBA reactivity)

Carbohydrates	As A_{532}		As fluorescence $E_{x,532}$ $E_{m,553}$	
	pH 7.4	pH 9.5	(pH 7.4)	(pH 9.5)
Sucrose	0.066	0.035	46	37
Lactose	0.055	0.024	20	16
β-D-Fructose	0.042	0.024	16	23
D-Glucose	0.047	0.034	15	15
D-Ribose	0.083	0.046	22	14
D-Galactose	0.068	0.035	16	16
D-Glucuronic acid	0.170	0.170	91	98
Deoxyribose	1.270	0.734	1280	441

Sugars at a final reaction concentration of 2.3 mM were incubated with 0.9 mM ferrous ammonium sulphate at pH 7.4 (phosphate buffer) or at pH 9.5 (borate buffer). TBA-reactivity is shown both as fluorescence and A_{532}. Further details are in section 2

4. Discussion

When water is irradiated, a variety of reactive radicals are formed

$$H_2O \rightarrow OH^{\cdot}, H^{\cdot}, e^-_{aq}, H_3O^+ \quad (1)$$

$$H^{\cdot} + O_2 \rightarrow HO_2^{\cdot} \quad (2)$$

$$HO_2^{\cdot} \rightarrow H^+ + O_2^{\cdot -} \quad (3)$$

$$O_2^{\cdot -} + O_2^{\cdot -} + 2\,H^+ \rightarrow H_2O_2 + O_2 \quad (4)$$

$$O_2 + e^-_{aq} \rightarrow O_2^{\cdot -} \quad (5)$$

including the hydroxyl radical ($OH^{\cdot}$) which is known to be formed in living systems by iron salt–oxygen interactions [14]. Radiation damage to amino acids has been shown to result in the formation of TBA-reactive products [12] and radiation damage to carbohydrates has been claimed to yield 'malondialdehyde' [10,11]. However, it has been shown here that Fe^{2+} themselves in the presence of oxygen can damage both amino acids and carbohydrates, with the formation of an intermediate breakdown product which gives an apparent MDA–TBA adduct under the conditions of the TBA assay. In all test systems in which the TBA reaction is used as a measure of oxidative damage to molecules, most, if not all, of the MDA is

derived from precursor molecules during the acid-heating stage of the TBA test itself. In vivo these same intermediates can also be broken down to MDA, although the mechanisms are still poorly understood. Except in the case of deoxyribose, the TBA-reactive intermediates, both carbohydrates and amino acids, were relatively stable and did not yield a volatile aldehyde upon acid distillation. This stability, as well as the short incubation time, may also explain why polar auto-fluorescent Schiff base complexes could not be detected following iron damage to the amino acids.

A mechanism involving decarboxylation, oxidative deamination and oxidation has been proposed for the formation of malondialdehyde following radiation damage to amino acids [12]. Iron damage does not appear to result in the direct formation of MDA. If, as appears, the TBA-reactive chromogen is an MBA–TBA adduct, then these reactions must be divided between the iron-damage and the acid-heating stage of the TBA test.

An important implication of iron damage to amino acids and carbohydrates is that it extends the range of toxic and potentially damaging free-radical-derived intermediates that can be formed from biomolecules in vivo. Also, although lipid antioxidants are commonly added to our foods to protect fats against peroxidation, they may not protect the susceptible polar compounds, described here, against iron damage. Finally, the use of the TBA test to detect 'lipid peroxidation' induced in complex biological systems by addition of ferrous salts, or ferric salts plus reducing agents such as ascorbate, is open to many artefacts.

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