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IRON COMPLEXES AND THEIR REACTIVITY IN THE BLEOMYCIN ASSAY FOR RADICAL-PROMOTING LOOSELY-BOUND IRON

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The sensitivity of the bleomycin assay for loosely-bound iron depends on the concentration of bleomycin and ascorbic acid and the pH of the reaction. The non-haem-iron proteins transferrin, conalbumin and ferritin release iron at an acid pH value, whereas the haem-iron proteins release iron more readily at an alkaline pH. In addition, haem proteins are liable to release iron when peroxides are present. Organic peroxides and hydrogen peroxide can be produced during the bleomycin reaction leading to iron release from haem proteins. However, this can be prevented from reacting with bleomycin by adding zinc ions to the reaction following addition of the sample. Iron already bound to bleomycin is not displaced by zinc whereas zinc bound to bleomycin is not displaced by iron allowing 'free' and 'released' iron to be discriminated.

KEY WORDS: Bleomycin-detectable iron, oxygen radicals, iron binding proteins, hydroperoxides, zinc ion binding, catatylic transition metals.

INTRODUCTION

Apart from exposure of tissues to high energy radiation, the hydroxyl radical (OH·) is probably formed in biological material only when transition metals promote a Fenton-type reaction.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH\cdot \qquad (1)$$

Iron (equation 1) is the most likely biological promotor of OH· formation, although other transition metal ions such as copper, cobalt and titanium also react with hydrogen peroxide (H_2O_2) to produce OH·. In biological solutions metal ions, do not exist 'free' in solution but as complexes with other molecules present. Binding may be strong, e.g. between ferric ions and the protein transferrin at pH 7.4, or may be weak as seen with ferric ions and albumin, phosphates and carbohydrates. Metal binding is an important protective antioxidant function of certain proteins in extracellular fluids.[1,2] Thus, binding of iron may prevent redox cycling leading to OH· formation or, alternatively, binding may direct OH· formation on to a protein like albumin whose damage may be biologically insignificant in view of the large amount present.

Disturbances in iron metabolism resulting from cellular damage, or excessive iron uptake, can result in 'mal-placed' iron promoting OH· formation at critical sites on membrane lipids, on DNA or at the active sites of enzymes, leading to damage seen as lipid peroxidation, strand scissions and enzyme inactivation respectively. An approach to the detection and measurement, in biological fluids, of loosely-bound

iron able to promote radical formation was recently described.[3] This method uses the absolute requirement for iron of the antitumour drug bleomycin for the release of thiobarbituric acid (TBA)-reactive material from DNA. When applied to a variety of body fluids, bleomycin-detectable iron was found to be increased in several pathological and physiological conditions.[4–7]

In order to better understand the possible origins of 'mal-placed' iron, as detected by the bleomycin assay, the following experiments were carried out.

MATERIALS AND METHODS

DNA (calf thymus), dichlorophenolindophenol, deoxyribose, haemin, haemoglobin, (human) catalase, conalbumin, albumin (human) and peroxidase were from the Sigma Chemical Co., Poole, Dorset. Transferrin and ferritin were from the Boehringer Corporation Ltd., East Sussex. The smaple of haemosiderin was a gift from Dr T. Peters, CRC, Northwick Park, London. All other chemicals were of the highest purity available from BDH Chemicals Ltd., Poole, Dorset.

1. The bleomycin assay

DNA (1 mg/ml) was prepared in Chelex resin treated distilled water and allowed to stand overnight to effect solution. This solution was treated with 0.3 g Chelex resin and centrifuged before use. Bleomycin sulphate was dissolved in Chelex resin treated distilled water to give a stock solution of 1.5 units/ml (approx. 0.6 mM). Magnesium chloride (50 mM) was treated with 0.3 g Chelex resin. Reagents at the concentrations indicated in appropriate Figures and Tables were added to new clean plastic tubes in the order indicated.

(A) Standard iron solutions. When ferric salts are used to prepare iron standards care must be taken to avoid precipitation of iron from solution at neutral to alkaline pHs. A BDH iron standard, for atomic absorption, containing 1 mg iron/ml in 1 M HCl was found to be suitable for use as a stock standard in the bleomycin assay providing the acid present is adequately neutralised in the final reaction mixture.

(B) Standardisation of ascorbate solutions. 0.7 g Analar ascorbic acid were dissolved in 10 ml of Chelex resin-treated water. 0.4 g Chelex resin were added and shaken well before centrifuging at 2000 g to deposit the resin. A solution of dichlorophenolindophenol (DICPIP) containing 40 mg/100 ml was titrated against a freshly prepared standard of ascorbic acid. 1.0 ml of the DICPIP solution was found to be equivalent to 0.2 mg of ascorbic acid). The Chelex treated working solution of ascorbic acid was then carefully standardised with the DICPIP solution to a final concentration of 7.5 mM.

A working-solution containing 7.5 mM ascorbic acid can be directly prepared in Chelex resin treated distilled water but this should not be further treated with solid Chelex resin.

(C) Preparation of Buffer solutions. A Tris buffer (1 M) was found to be suitable for use with bleomycin. Contaminating iron was removed from the buffer by placing sealed dialysis tubing (containing 5% conalbumin together with a small amount of

bicarbonate), inside the reagent. The buffer was left for 48 hours at 4°C before use, during which time the conalbumin turned pink as iron was removed.

2. Incubation and TBA-reactivity

New clean plastic tubes containing DNA, bleomycin, $MgCl_2$, sample, buffer and ascorbate were incubated along with appropriate controls for 2 hours at 37°C. 0.1 ml of EDTA (0.1 M), 1.0 ml TBA (1% in 0.05 M NaOH) and 1.0 ml of HCl (25% v/v) were added to each tube. Tube contents were mixed, transferred to clean glass tubes and heated for 10 min at 100°C to develop TBA-reactivity. When cool, the tubes were centrifuged at 2000 g for 10 minutes and the absorbance of the supernatant read at 532 nm. If turbidity is experienced, the TBA chromogen can be extracted into butan-1-ol and read at A 532 nm.

RESULTS

The optimal amount of ascorbic acid required for use in the bleomycin reaction is 7.5 mM and Figure 1 shows the effect of ascorbate concentration on colour development using 12.5 μM $FeCl_3$. The concentration of bleomycin present in the reaction is important in determining the sensitivity of iron detection (Figure 2) as is the pH of the reaction (Figure 3). The bleomycin reaction appears under certain conditions to

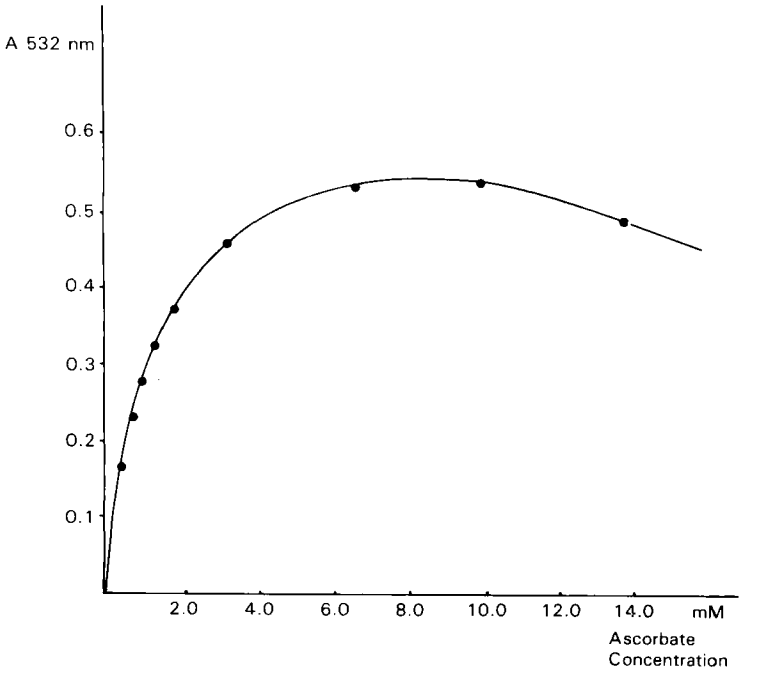

FIGURE 1 Bleomycin-dependent release of TBA-reactivity from DNA at pH 7.4, in the presence of 12.5 μM ferric chloride, as a function of the ascorbate concentration added to the reaction. Reaction conditions were as described in Tables 1 and 2.

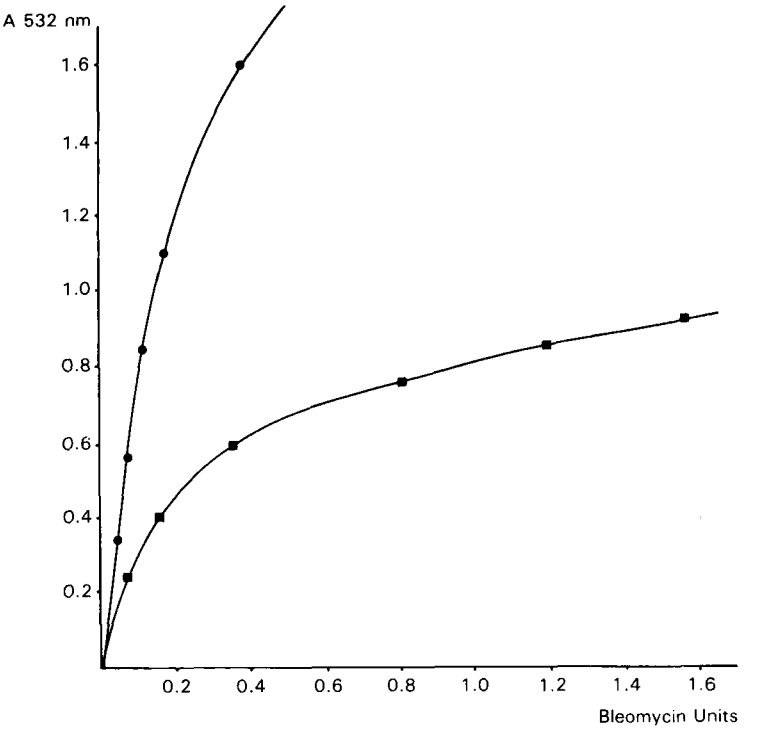

FIGURE 2 Release of TBA-reactivity from DNA as a function of the initial concentration of bleomycin added. The reactions were carried out at pH 7.4 in the presence of 12.5 μM ferric chloride. Reaction conditions were as described in Tables 1 and 2 with and without the addition of zinc ions. ●● without zinc ions; ■■ with zinc ions.

be able to release iron, complexable by bleomycin, from haem-containing proteins. Iron release as a function of haemoglobin concentration is shown in Figure 4. The binding of this iron, released during the bleomycin reaction, to bleomycin can be prevented by adding a zinc salt after addition of the sample or iron standard. It appears that if iron ions first complex with bleomycin they are not displaced by zinc ions and conversely zinc ions bound to bleomycin are not displaced by iron ions. It is therefore possible to differentiate 'free' loosely-bound iron salts from those 'released' from more complex molecules during the assay procedure. Figure 5 shows the effect of zinc ions on the concentration of iron salt in the bleomycin assay. When zinc ions are added before the iron ions all the bleomycin activity is blocked (Figure 5). However, a dose response curve is obtained for iron when zinc is added after the iron and this is unaffected by the presence of haemoglobin (Figure 5). Blank values are substantially reduced (0.005–0.010 A532 nm) in the presence of zinc ions.

In the absence of added zinc ions the release of iron from non-haem iron proteins is pH dependent with transferrin conalbumin and ferritin releasing iron at an acid pH value (Table 1). Haem-containing complexes, however, in the presence of zinc ions showed a reverse pattern of iron release, increasing with pH (Table 1).

The contribution of 'free' iron salt and 'released' iron in the bleomycin assay was

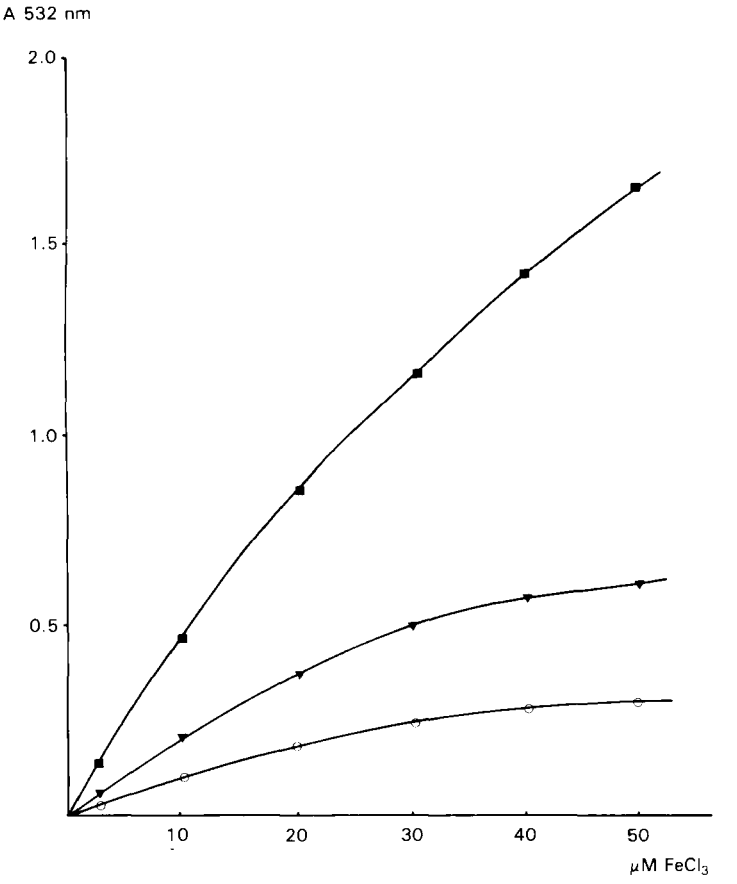

FIGURE 3 Release of TBA-reactive material from DNA as a function of the iron concentration at different pH values. Reaction conditions were as described in Tables 1 and 2 without the addition of zinc ions. ■■■ pH 7.4; ▲▲▲ pH 5.2; ○○○ pH 8.7.

tentatively re-examined in several biological samples (Table 2). These showed the presence of 'free' loosely-bound iron salts when zinc ions were added. Even in the absence of added zinc ions haemoglobin did not give bleomycin detectable iron when added at a concentration of 3 mg/ml to normal human serum (Table 2).

DISCUSSION

Previous studies have shown that haem and non-haem iron-containing proteins do not release iron to bleomycin.[3] However, this depends on the pH at which the assay is carried out and the ability of the reaction system to release iron from complexes. Haem-proteins readily release iron in the presence of peroxides,[14] and peroxides are

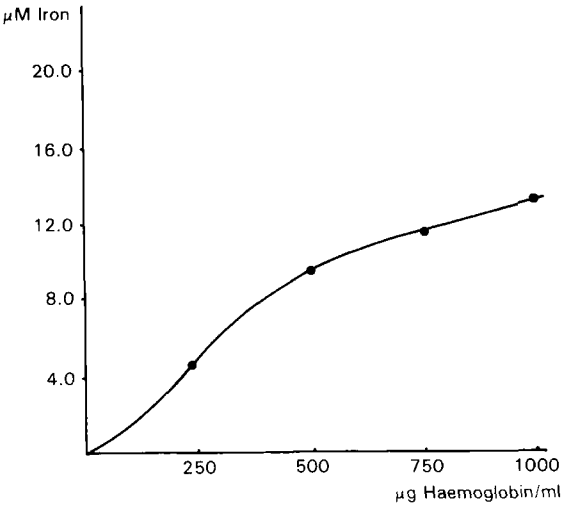

FIGURE 4 Release of iron to bleomycin from haemoglobin during the assay procedure. Reaction conditions were at pH 7.4 as described in Tables 1 and 2 without the addition of zinc ions.

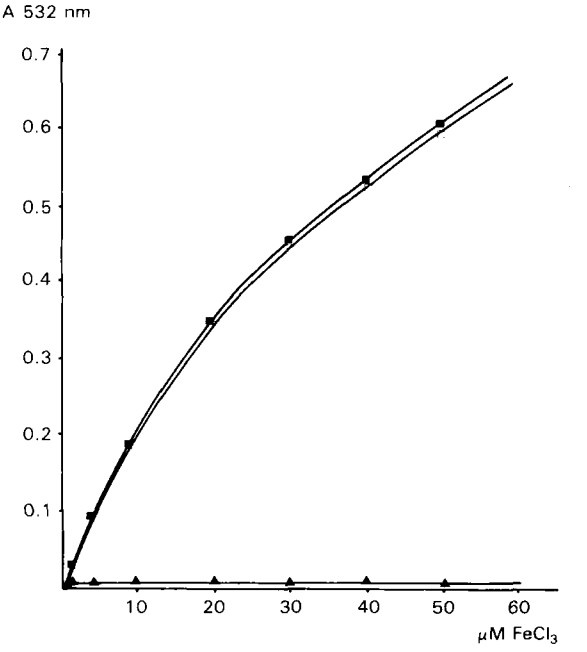

FIGURE 5 The effect of zinc ions on the detection of iron in the bleomycin assay; ■■■Ferric chloride added to the reaction before the addition of zinc ions (final reaction concentration 0.1 mM); ▲▲▲ Zinc ions (0.1 mM) added before the addition of $FeCl_3$; □□□ Ferric chloride + Haemoglobin (1 mg/ml) added before the addition of zinc ions (0.1 mM). Reaction conditions, with and without, zinc were as described in Tables 1 and 2.

TABLE I
The effect of pH on the release of bleomycin-detectable iron from iron-containing complexes

Iron complexes	pH 5.2 Bleomycin-detectable iron (μM)	Ph 7.4 Bleomycin-detectable iron (μM)	pH 8.7 Bleomycin-detectable iron (μM)
Apotransferrin	0	0	0
Transferrin 30% iron stautrated	5.0	0	
Transferrin 100% iron saturated	25.0	0	0
Conalbumin	0	0	0
Conalbumin 30 % iron saturated	6.0	1.0	0
Conalbumin 100% iron saturated	21.0	3.1	0.3
Albumin	0.5	0.6	0.1
Albumin + ferric chloride (12.5 μM)	8.5	7.1	2.0
Haemosiderin	0	0	0
Zinc salt added after the sample			
Ferritin	46.5	0.17	0
Peroxidase (horseradish)	0	0.49	1.2
Haemoglobin	0	0.18	0.55
Haemin	0.2	0	0
Catalase	0	0.23	0.64

Reaction mixture contained 0.5 mg DNA, 0.005 units bleomycin, 5 mM $MgCl_2$, 0.1 ml of sample or standard, 0.1 M Tris buffer pH 7.4 and 0.75 mM ascorbate. Where indicated 0.1 mM zinc salt was added to the reaction after the sample or standard and the amount of bleomycin increased to 0.01 units. Reagents were added to the reaction in the order listed above.

TABLE II
Bleomycin-detectable iron in biological samples at pH 7.4 when zinc ions are added to the reaction after the sample

	A532 nm	μM Iron
Blank	0.005	
Standard $FeCl_3$ (10 μM)	0.215	10 μM
Pooled normal human serum	0.005	0
Pooled normal human serum + 25 μM $FeCl_3$	0.005	0
Pooled normal human serum + 50 μM $FeCl_3$	0.255	11.9
Serum from patent with idiopathic haemochromatosis	0.152	7.0
Ultrafiltrate of above serum	0.085	3.8
CSF fluid (normal)	0.026	1.0
Sweat fluid (back)	0.072	3.2
Synovial fluid from iron dextran treated patient	0.047	2.0
Normal human pooled serum without added zinc irons		
+ Haemoglobin 1 mg/ml	less than blank	0
+ Haemoglobin 2 mg/ml	less than blank	0
+ Haemoglobin 3 mg/ml	less than blank	0

The above reactions contained, 0.5 mg DNA, 0.01 units of bleomycin, 5 mM $MgCl_2$, 0.1 ml of sample or standard, 0.1 M Tris pH 7.4, 0.75 mM ascorbate and 0.1 mM zinc salt.

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known to be produced during the bleomycin reaction.[15,16] Peroxides associated with solutions of DNA *in vitro* or lipid peroxides in biological samples may therefore influence the availability of iron to bleomycin in the assay. Zinc, which also binds to bleomycin, does not appear to displace iron already complexed to bleomycin allowing it to undergo its normal redox cycles. In contrast, when zinc ions are first bound to bleomycin they are not displaced by iron and the reaction is inhibited. Using zinc in this way it is possible to delineate 'free' and 'released' iron in samples. Previous assays on biological samples[4–7] in which loosely-bound iron was detected are further supported by the studies here. Biological fluids like serum appear to have a protective activity towards iron release without the need to add zinc. Whether zinc irons are active when added to biological samples or inactivated by protein binding remains to be established.

Trace-iron detected in some commercial samples of metal-proteins probably arises from impurities or fragments not removable by Chelex resin. This also applies to the blank which is extremely low when zinc is added, suggesting that most of the iron reacting in the blank is released from complexes during the reaction. Possible sources of this iron are bacterial endotoxin or the DNA. When ferritin was purified and fractionated according to its iron loading[17] no bleomycin detectable iron was then found.[3] As expected, the iron-binding proteins transferrin and conalbumin and the iron-storage protein ferritin release iron at pH 5.2 in the presence of ascorbate. Interestingly, the non-haem protein haemosiderin does not release iron to bleomycin over the pH range tested. This finding supports the proposal of O'Connell *et al.* that conversion of ferritin to haemosiderin may be a protective event to reduce iron-promoted reactions.

The site location of iron in biological systems, clearly, plays a major part in determining the severity of oxygen radical damage and the extent to which protection by scavengers can be afforded.

The iron-specific release of TBA-reactive material from DNA by bleomycin provides a novel technique for detecting and measuring trace amounts of loosely-bound iron in biological samples by catalytic redox cycling of the iron. The kinetics of the reaction are extremely complex[8–10] and have been suggested to proceed by an enzyme-like mechanism.[11] This is further supported by the stoichiometry of bleomycin, iron and zinc used in these studies which indicate a catalytic reaction. The site-specific nature of the bleomycin-iron-oxygen reaction on DNA substantially reduces interference by naturally occurring antioxidants and radical scavengers[12] which also influence the reaction of peroxides, produced during the assay, on haem-proteins. Compared with other metal chelating agents, such as EDTA, bleomycin only weakly binds iron and readily donates this iron to proteins like transferrin and lactoferrin when they themselves are poorly iron-loaded. Indeed, this iron-binding antioxidant function *in vivo* explains why 'free' iron salts are not normally present to catalyse oxidative damage and arise only from an 'overwhelming' of protective binding.[1,5]

The interpretation of the presence of bleomycin-detectable iron in biological fluids is more complex than always implying the presence of 'free' iron salts. Indeed, only in cases of iron-overload when the transferrin is fully saturated would bleomycin-detectable iron be expected to appear in human serum; an observation substantiated by experimentation.[5] In other fluids, such as synovial fluid from patients with rheumatoid arthritis, the transferrrin is not fully iron-loaded yet bleomycin-detectable iron is sometimes present.[4] This suggests that iron complexes may release iron to bleomycin

in a form not available to transferrin such as may occur when there are changes in the levels of citrate and bicarbonate (13). Alternatively, released iron may first have to saturate the transferrin before iron appears in the assay. If ferric ions are released from an iron complex in synovial fluid they would, in the presence of ascorbate, be reduced to the ferrous state and presumably bind more readily to bleomycin than to transferrin. The nature of these biological iron complexes is at present unknown but under detailed investigations.

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