Biochem J. (1987) 241, 273–278 (Printed in Great Britain)

Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals from hydrogen peroxide in the presence of iron

Are lactoferrin and transferrin promoters of hydroxyl-radical generation?

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Apo-lactoferrin and apo-transferrin protect against iron-ion-dependent hydroxyl-radical (·OH) generation from H_2O_2 in the presence of superoxide radicals or ascorbic acid at pH 7.4, whether the necessary iron is added as ionic iron or as ferritin. Iron-loaded transferrin and lactoferrin [2 mol of Fe(III)/mol] show no protective ability, but do not themselves accelerate ·OH production unless chelating agents are present in the reaction mixture, especially if the proteins are incorrectly loaded with iron. At acidic pH values, the protective ability of the apoproteins is diminished, and the fully iron-loaded proteins can release some iron in a form able to accelerate ·OH generation. The physiological significance of these observations is discussed.

INTRODUCTION

It now seems clearly established that formation of hydroxyl radical (·OH), or a similar highly oxidizing species, accounts for much of the damage done to biological systems by increased generation of superoxide radicals ($O_2^{\cdot-}$) and H_2O_2 (for reviews see Halliwell & Gutteridge, 1985, 1986; Czapski & Goldstein, 1986). ·OH formation in systems generating $O_2^{\cdot-}$ and H_2O_2 requires the presence of a suitable metal-ion promoter. Although a number of metal ions, including Cu(II), Ti(III), Co(II) and Cr(II), are effective promoters of ·OH formation *in vitro*, most attention has been given to iron ions as potential promoters *in vivo* (Halliwell & Gutteridge, 1986). Thus complexes of iron ions with organic acids (Bannister *et al.*, 1983; Baker & Gebicki, 1986), adenine nucleotides (Floyd, 1983; Flitter *et al.*, 1983), sulphide (Berglin & Carlsson, 1985) and DNA (Floyd, 1981) are all effective in accelerating ·OH generation at physiological pH. Exposure of the protein ferritin to $O_2^{\cdot-}$ (Biemond *et al.*, 1984), ascorbic acid (Gutteridge *et al.*, 1983) or organic peroxides (Gutteridge, 1985) can release iron ions in a form capable of promoting free-radical reactions. A similar 'decompartmentalization' (Willson, 1978) of redox-active iron occurs when haemoglobin is treated with peroxides (Gutteridge, 1986).

Transferrin is the iron-transport protein of human plasma. In the presence of a suitable anion (usually HCO_3^-), it binds 2 mol of Fe(III)/mol of protein with a stability constant of about 10^{20} at physiological pH. There is a dispute in the literature concerning the ability of transferrin-bound iron to accelerate ·OH formation and lipid peroxidation. Thus McCord & Day (1978) reported that iron-loaded transferrin promoted ·OH formation from $O_2^{\cdot-}$ and H_2O_2 at pH 7.4, an observation apparently confirmed by Bannister *et al.* (1982*a*), Motohashi & Mori (1983) and Burton *et al.* (1984). Maguire *et al.* (1982) and Baldwin *et al.* (1984) reported that iron-loaded transferrin was not active in accelerating ·OH formation at pH 7.8. Gutteridge *et al.* (1981) found that partially iron-loaded transferrin was an inhibitor of iron-dependent lipid peroxidation at pH 7.4, and suggested (also see Halliwell & Gutteridge, 1986) that transferrin is an important extracellular antioxidant because of its ability to bind iron and stop it participating in free-radical reactions (Stocks *et al.*, 1974). Halliwell *et al.* (1985) also suggested that iron ions might become released from transferrin at low pH values (e.g. in ischaemic tissues or in the microenvironment of activated phagocytic cells) in a form able to promote radical reactions, since iron ions can be mobilized from the protein under acid conditions. Indeed, this is the mechanism of intracellular iron unloading from transferrin (Rao *et al.*, 1983).

There is also a debate in the literature concerning the protein lactoferrin, an iron-binding protein similar to transferrin that is released from activated phagocytic cells. Partially iron-loaded lactoferrin inhibits iron-dependent lipid peroxidation (Gutteridge *et al.*, 1981), but the ability of the fully iron-loaded protein [2 mol of Fe(III)/mol of lactoferrin] to accelerate ·OH formation from $O_2^{\cdot-}$ and H_2O_2 has variously been reported as good (Ambruso & Johnston, 1981; Bannister *et al.*, 1982*b*), excellent (Vercellotti *et al.*, 1985), poor (Winterbourn, 1983) and nil (Baldwin *et al.*, 1984). Winterbourn (1983) has suggested that the procedures used to load iron on to lactoferrin, and the presence of chelating agents in the reaction mixture, can account for the variability of the results obtained, and that iron on lactoferrin is not active in accelerating ·OH generation at pH 7.4.

In the present paper, we report a detailed study of the ability of both transferrin and lactoferrin to accelerate ·OH formation in systems containing H_2O_2 and $O_2^{\cdot-}$, or H_2O_2 and ascorbic acid (Winterbourn, 1979), at different pH values. The effect of these proteins on ·OH formation accelerated by addition of simple iron salts or of ferritin was also examined.

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MATERIALS AND METHODS

Reagents

Horse spleen ferritin was from Calbiochem, desferrioxamine (as Desferal) was from CIBA–Geigy and deoxyribose, lactoferrin, transferrin, catalase, xanthine oxidase and bovine superoxide dismutase (copper–zinc enzyme) were from Sigma Chemical Co. All other reagents were of the highest quality available from BDH Chemicals. Units of catalase are defined as μmol of H_2O_2 decomposed/min, and units of xanthine oxidase as μmol of urate formed/min, under the assay conditions given in the Sigma catalogue. Units of superoxide dismutase are as defined by McCord & Fridovich (1969).

Iron proteins

Human milk lactoferrin and human plasma transferrin were purified, loaded with iron by using Fe(III)-nitrilotriacetate and purified by two cycles of gel filtration on Sephadex G-25 as described by Bates & Schlabach (1973). Where indicated in the text, the proteins were also loaded by mixing them with a fresh solution of $FeCl_3$ on a 1:2 molar basis.

Deoxyribose assay

The deoxyribose assay was carried out as described by Gutteridge (1981); full details are given in Table legends. The results shown are generally the means of duplicates that differed by 10% or less and have been corrected for the low rate of deoxyribose degradation seen in the absence of added iron promoter, which is due to iron contamination of the reagents.

RESULTS

Effect of apo-lactoferrin and apo-transferrin on 'OH generation

The sugar deoxyribose is attacked by 'OH to give a product that reacts on heating with thiobarbituric acid to form a chromogen (Gutteridge, 1981). This has been made the basis of a sensitive assay to measure iron-dependent 'OH formation from H_2O_2/ascorbate

Table 1. Deoxyribose degradation by iron-salt-dependent 'OH formation: effects of apo-transferrin and apo-lactoferrin

All reaction mixtures contained, at the final concentrations stated, ascorbate (100 μM), H_2O_2 (1.44 mM), KH_2PO_4/KOH buffer, pH 7.4 or pH 4.5 (10 mM in phosphate), and deoxyribose (2.8 mM). They were incubated with gentle shaking at 37 °C for 1 h, and colour was developed as described by Halliwell & Gutteridge (1981). Solutions of iron salts were made up in double-distilled water immediately before use. The rate of deoxyribose degradation was approximately constant over the time of incubation. In a series of experiments, 15 μM-$FeCl_3$ restored the deoxyribose degradation inhibited by 5 μM-apo-transferrin or -apo-lactoferrin to 80–102% of the control containing 5 μM-$FeCl_3$ only. Abbreviations: ATF, apo-transferrin; ALF, apo-lactoferrin.

Addition to reaction mixture (final concn.)	Extent of deoxyribose degradation at pH 7.4		Extent of deoxyribose degradation at pH 4.5	
	A_{532}	Inhibition by ATF or ALF (%)	A_{532}	Inhibition by ATF or ALF (%)
5 μM-$FeCl_3$	0.200	—	0.355	—
5 μM-$FeCl_3$ + 5 μM-ATF	0.055	72	0.253	29
5 μM-$FeCl_3$ + 5 μM-ALF	0.033	84	0.294	17
15 μM-$FeCl_3$ + 5 μM-ATF	0.170	—	0.349	—
15 μM-$FeCl_3$ + 5 μM-ALF	0.186	—	0.360	—
5 μM-$FeCl_3$ + 1 mM-$NaHCO_3$	0.196	—	0.342	—
5 μM-$FeCl_3$ + 1 mM-$NaHCO_3$ + 5 μM-ATF	0.055	72	0.198	42
5 μM-$FeCl_3$ + 1 mM-$NaHCO_3$ + 5 μM-ALF	0.028	86	0.186	46

Table 2. Deoxyribose degradation by ferritin-dependent 'OH formation: effects of apo-transferrin and apo-lactoferrin

Reaction mixtures were identical with those given in Table 1, except that iron was added in the form of horse spleen ferritin. The micromolar concentrations of ferritin quoted refer to the iron content of the protein. Abbreviations: ATF, apo-transferrin; ALF, apo-lactoferrin.

Addition to reaction mixture (final concn.)	Extent of deoxyribose degradation at pH 7.4		Extent of deoxyribose degradation at pH 4.5	
	A_{532}	Inhibition by added ATF or ALF (%)	A_{532}	Inhibition by added ATF or ALF (%)
20 μM-Ferritin iron	0.515	—	0.700	—
20 μM-Ferritin + 5 μM-ATF	0.192	63	0.384	45
20 μM-Ferritin + 10 μM-ATF	0.118	77	0.287	59
20 μM-Ferritin + 10 μM-ALF	0.034	94	0.259	63
20 μM-Ferritin + 1 mM-$NaHCO_3$	0.482	—	0.685	—
20 μM-Ferritin + 1 mM-$NaHCO_3$ + 10 μM-ATF	0.079	84	0.193	72
20 μM-Ferritin + 1 mM-$NaHCO_3$ + 10 μM-ALF	0.029	94	0.171	75

(O'Connell *et al.*, 1986) or $H_2O_2/O_2^{\cdot-}$ (hypoxanthine + xanthine oxidase) mixtures (Halliwell & Gutteridge, 1981).

Table 1 shows the effects of human milk apo-lactoferrin and human plasma apo-transferrin on deoxyribose degradation in the presence of ascorbate, H_2O_2 and a simple iron salt ($FeCl_3$). Both proteins inhibited the deoxyribose degradation at pH 7.4, and addition of sufficient $FeCl_3$ to overcome the iron-binding capacity of the proteins [2 μmol of Fe(III)/mol of protein] restored the degradation. Inclusion of 1 mM-$NaHCO_3$ in the reaction mixture had little effect on either the iron-dependent deoxyribose degradation or the action of the iron-binding proteins upon it. If assays were performed at pH 4.5, the protective effects of the proteins on deoxyribose degradation were markedly decreased, but could be restored to some extent by inclusion of 1 mM-$NaHCO_3$ in the reaction mixture (Table 1). Neither protein was as protective as was observed at pH 7.4. Higher concentrations of $NaHCO_3$ could not be tested, since they began to diminish the control rate of deoxyribose degradation (HCO_3^- is a scavenger of 'OH radicals). Statistical analysis of the results of ten experiments similar to those in Table 1 showed that the protective effects of lactoferrin and transferrin were not significantly different. Deoxyribose degradation was

Table 3. Deoxyribose degradation by hypoxanthine + xanthine oxidase: effects of apo-transferrin and apo-lactoferrin

The reaction mixture contained, in a final volume of 1.2 ml, the following reagents at the final concentrations stated: deoxyribose (2.8 mM), KH_2PO_4/KOH buffer, pH 7.4 (10 mM in phosphate), hypoxanthine (330 μM) and, where indicated, iron as $FeCl_3$ or as horse spleen ferritin. Reaction was started by adding 40 μl of xanthine oxidase (diluted to 1.0 enzyme unit/ml in buffer immediately before use), and tubes were incubated at 37 °C for 60 min. Colour was developed as described by Halliwell & Gutteridge (1981). Inclusion of $NaHCO_3$ to a final concentration of 1 mM had no significant effect on any of the results obtained. Abbreviations: ATF, apo-transferrin; ALF, apo-lactoferrin.

Addition to reaction mixture (final concn.)	Extent of deoxyribose degradation (A_{532})	Inhibition of deoxyribose degradation (%)
10 μM-$FeCl_3$	0.203	—
10 μM-$FeCl_3$ + 5.2 μM-ATF	0.092	55
10 μM-$FeCl_3$ + 10 μM-ATF	0.088	57
10 μM-$FeCl_3$ + 5.2 μM-ALF	0.079	61
20 μM-Ferritin iron	0.215	—
20 μM-Ferritin + 5.2 μM-ATF	0.102	53
20 μM-Ferritin + 10 μM-ATF	0.097	55
20 μM-Ferritin + 10 μM-ALF	0.088	59

Table 4. Deoxyribose degradation by hypoxanthine + xanthine oxidase: effects of iron-loaded transferrin

The hypoxanthine + xanthine oxidase system was as described in the legend to Table 3 except that no ferritin or $FeCl_3$ was added. The amount of iron added with the transferrin is twice the molar concentration of the protein. Where indicated, EDTA and/or other reagent were added to give the final concentrations stated. Transferrin A was iron-loaded as described by Bates & Schlabach (1973); transferrin B was iron-loaded by pre-mixing the apoprotein with a freshly made solution of $FeCl_3$ on a 1:2 molar basis. For comparison, inclusion of 10 μM-$FeCl_3$ in the reaction mixture instead of transferrin gave an absorbance of 0.266.

Final concn. of protein added (μM)	Other reagent added	Extent of deoxyribose degradation (A_{532})					
		Transferrin A			Transferrin B		
		No EDTA	0.1 mM-EDTA	0.2 mM-EDTA	No EDTA	0.1 mM-EDTA	0.2 mM-EDTA
0	—	0	0	0	0	0	0
2.6	—	0	0	0.004	0	0.024	0.054
3.7	—	0	0.006	0.008	0	0.050	0.094
5.2	—	0	0.015	0.024	0	0.078	0.105
10.9	—	0	0.018	0.033	0	0.121	0.137
15.6	—	0	0.024	0.056	0	0.103	0.128
18.3	—	0	0.049	0.078	0	0.100	0.126
22.0	—	0	0.046	0.054	0	0.141	0.139
10.0	—	0	—	—	0	0.120	—
10.0	Catalase (10^3 units/ml)	0	—	—	0	0.007	—
10.0	Mannitol (10 mM)	0	—	—	0	0.006	—
10.0	Desferrioxamine (250 μM)	0	—	—	0	0.000	—
10.0	Superoxide dismutase (10^2 units/ml)	0	—	—	0	0.010	—

inhibited by catalase (10^3 units/ml), mannitol (10 mM final concentration) and desferrioxamine (equimolar to the $FeCl_3$), confirming that it was due to $^{\cdot}OH$ generation (results not shown; see Halliwell & Gutteridge, 1981).

Table 2 shows experiments in which the iron source for $^{\cdot}OH$ generation is ferritin. Again, catalase, mannitol and desferrioxamine inhibited the deoxyribose degradation (results not shown). Both apo-lactoferrin and apo-transferrin were inhibitory at pH 7.4. They were also more inhibitory at pH 4.5 than in the $FeCl_3$ system (Table 1), presumably because the amount of iron released from the ferritin is smaller. Their protective effect at acid pH was further increased in the presence of $NaHCO_3$ (1 mM final concentration). Again, no significant difference in the protective effects of apo-lactoferrin and apo-transferrin at either pH value was observed.

Similar protective effects of these iron-binding proteins at pH 7.4 were observed when $^{\cdot}OH$ was generated by a

Table 5. Deoxyribose degradation by ascorbate and H_2O_2: effects of iron-loaded proteins

Reaction mixtures contained the following reagents at the final concentrations stated: KH_2PO_4/KOH buffer of the pH stated (10 mM in phosphate), deoxyribose (2.8 mM), ascorbate (100 μM), H_2O_2 (1.44 mM) and iron protein (6.5 μM) containing 13 μM-iron. They were incubated with gentle shaking at 37 °C for 1 h. $NaHCO_3$ (1 mM) had no significant effect on deoxyribose degradation promoted by $FeCl_3$ in this system at any pH value. The pH values of the incubation mixtures were measured at the end of the reaction. Abbreviation: DETAPAC, diethylenetriaminepenta-acetic acid.

pH	Other reagent added to reaction mixture	Extent of deoxyribose degradation (A_{532}) With Fe(III)-transferrin	With Fe(III)-lactoferrin
4.08	—	0.196	0.195
4.40	—	0.167	0.165
4.40	1 mM-$NaHCO_3$	0.067	0.049
5.01	—	0.041	0.000
6.50	—	0.000	0.000
7.40	—	0.000	0.000
7.40	0.1 mM-EDTA	0.084	0.082
7.40	1.5 mM-EDTA	0.288	—
7.40	0.1 mM-DETAPAC	0.014	—
7.40	0.2 mM-DETAPAC	0.029	—

Table 6. Possible experimental artifacts in testing the ability of transferrin and lactoferrin to accelerate $^{\cdot}OH$ production in systems producing $O_2^{\cdot -}$ and H_2O_2

Reference	Source of $O_2^{\cdot -}/H_2O_2$	Method used for iron-loading protein	Conclusion of cited reference
Section A			
McCord & Day (1978)	Xanthine oxidase (Sigma) + xanthine	Not stated	Transferrin accelerates $^{\cdot}OH$ formation at pH 7.4
Ambruso & Johnston (1981)	Activated neutrophils/ xanthine oxidase (Sigma) + xanthine	Addition of ferric citrate, followed by dialysis	Lactoferrin accelerates $^{\cdot}OH$ formation at pH 7.3 or 7.8
Bannister *et al.* (1982*a*)	Activated neutrophil NADPH oxidase	Reconstituted with $FeCl_3$	Transferrin accelerates $^{\cdot}OH$ formation
Bannister *et al.* (1982*b*)	Xanthine oxidase (source not stated) + xanthine	Not stated	Lactoferrin accelerates $^{\cdot}OH$ formation at pH 7.8
Motohashi & Mori (1983)	Hypoxanthine + xanthine oxidase (Sigma)	Not stated	Transferrin accelerates $^{\cdot}OH$ formation at pH 7.4
Burton *et al.* (1984)	Purine + xanthine oxidase (prepared)	$FeCl_3$ + $NaHCO_3$, then dialysis	Transferrin accelerates $^{\cdot}OH$ formation at physiological pH
Section B			
Winterbourn (1983)	Xanthine + xanthine oxidase (Sigma) or ascorbate + H_2O_2	Fe(III)–nitrilotriacetate and prolonged dialysis	Lactoferrin is, at best, a poor catalyst of $^{\cdot}OH$ formation at pH 7.4
Maguire *et al.* (1982)	Xanthine + xanthine oxidase (Sigma)	Bates & Schlabach (1973)	Transferrin not a promoter of $^{\cdot}OH$ formation at pH 7.8
Baldwin *et al.* (1984)	Xanthine + xanthine oxidase (Sigma)	Fe(III)–nitrilotriacetate and prolonged dialysis	Neither lactoferrin nor transferrin active at pH 7.4

mixture of hypoxanthine, xanthine oxidase and $FeCl_3$ or ferritin (Table 3). Degradation of deoxyribose by this system was also inhibited by catalase, mannitol, desferrioxamine and superoxide dismutase (100 units/ml), confirming that the ·OH generation was dependent on both $O_2^{\cdot-}$ and H_2O_2 (results not shown).

Effect of iron-loaded proteins on $O_2^{\cdot-}$-dependent ·OH generation

Iron-loaded [2 mol of Fe(III)/mol of protein] transferrin and lactoferrin were prepared as described by Bates & Schlabach (1973), by using two cycles of gel filtration to remove unbound iron from the proteins. If these proteins were added to an $O_2^{\cdot-}$-generating system, no deoxyribose degradation was detected, nor did they inhibit deoxyribose degradation caused by added $FeCl_3$. Table 4 shows some results for transferrin; lactoferrin gave similar results. If EDTA was included in the reaction mixture, a small rate of deoxyribose degradation was observed (Table 4).

Some authors have prepared iron-loaded proteins by simply pre-mixing protein with $FeCl_3$ on a 1:2 molar basis (see the Discussion section). Iron-loaded transferrin or lactoferrin prepared in this way again failed to stimulate deoxyribose degradation in the hypoxanthine + xanthine oxidase system. However, inclusion of EDTA in the reaction mixture produced a significant rate of deoxyribose degradation, which could be inhibited by superoxide dismutase, catalase, mannitol and desferrioxamine. Table 4 shows a typical set of results for transferrin; those for lactoferrin were very similar.

Effect of iron-loaded proteins on ascorbate-dependent ·OH generation

In the presence of ascorbic acid and H_2O_2, iron-loaded lactoferrin or transferrin did not accelerate deoxyribose degradation at pH values above 5, but did so at lower pH values (Table 5). Inclusion of 1 mM-$NaHCO_3$ in the reaction mixture decreased the deoxyribose degradation but did not prevent it. Deoxyribose degradation was inhibited by catalase, mannitol and desferrioxamine, as expected (results not shown), or by omission of ascorbate or H_2O_2 from the reaction mixture. However, if EDTA was included in the reaction mixture, both iron-loaded proteins could acclerate degradation at pH 7.4 (Table 5). The chelating agent diethylenetriaminepenta-acetic acid also had some effect, but much less so than EDTA.

DISCUSSION

At pH 7.4, apo-lactoferrin and apo-transferrin are able to bind iron ions and protect against ·OH generation promoted by iron added as $FeCl_3$ or by iron released from ferritin in the presence of ascorbate or an $O_2^{\cdot-}$-generating system (Tables 1–3). Since neither of these proteins is likely to be even approaching full iron loading *in vivo*, except under conditions of iron overload, these results support the proposal (Stocks *et al.*, 1974; Gutteridge *et al.*, 1981; Halliwell & Gutteridge, 1986) that iron-binding proteins function as antioxidants *in vivo*. The inhibitory effects listed in Tables 1–3 are not a property of the protein molecules themselves, since they are lost when the proteins are saturated with iron.

We could not find any stimulation of ·OH formation by iron-loaded lactoferrin or transferrin at pH 7.4 in the presence of either ascorbate plus H_2O_2 or an $O_2^{\cdot-}$-generating system, with the use of proteins iron-loaded by correct procedures (Bates & Schlabach, 1973) and carefully freed of extraneous iron by two cycles of gel filtration. However, inclusion of EDTA in the reaction mixtures did produce some ·OH generation (Tables 4 and 5), probably because this chelator can slowly mobilize iron from the proteins (Morgan, 1979). Diethylenetriaminepenta-acetic acid had a much smaller effect, possibly because complexes of iron with this chelator are much less active than are iron–EDTA complexes in promoting ·OH formation (Halliwell, 1978; Butler & Halliwell, 1982). Nevertheless, the small effect seen suggests that iron mobilized from the proteins and bound by diethylenetriaminepenta-acetic acid is still more active than iron bound to lactoferrin or transferrin. If the proteins were iron-loaded by mixing with $FeCl_3$, which does not permit complete tight binding of Fe(III) to correct sites on the proteins (Bates & Schlabach, 1973), this effect of EDTA was much more marked (Table 4). The summary in Table 6 (section B) shows that previous authors who used techniques similar to ours to load iron on to lactoferrin or transferrin also concluded that these proteins were ineffective, or only poorly effective, in promoting ·OH formation at pH values at or near 7.4. In papers claiming otherwise (Table 6, section A), the method used to iron-load the proteins is usually inadequate (Bates & Schlabach, 1973) or undescribed. Some authors also had chelating agents present in their reaction mixtures, such as diethylenetriaminepenta-acetic acid (Bannister *et al.*, 1982*b*) or 2-oxo-4-thiomethylbutanoate (Ambruso & Johnston, 1981). This compound is often used to detect ·OH production, but it is also an iron-complexing agent (Winston *et al.*, 1986). It might act similarly to EDTA and diethylenetriaminepenta-acetic acid in releasing iron from the proteins, especially if they have been incorrectly iron-loaded. Our studies, and those listed in section B of Table 6, suggest that iron correctly bound to the two sites on each protein *in vivo* does not cause formation of ·OH radicals that can be detected in free solution at pH 7.4. Indeed, several authors have concluded that iron bound to transferrin is not even accessible to ascorbate (Carver & Frieden, 1978; Morgan, 1979; Ankel & Petering, 1980) and that its redox potential is so low that it could not be reduced by ascorbate (Harris *et al.*, 1985).

As pH falls, the iron becomes more easily mobilized from the binding sites of each protein. Table 5 shows that iron is released in a form able to promote ·OH formation under our reaction conditions at pH values less than 5. That the iron is actually released from the protein is shown by the inhibitory effect of desferrioxamine, a chelating agent that inhibits iron-dependent ·OH production (Gutteridge *et al.*, 1979; Halliwell, 1985). pH values of 5 or less may exist in the microenvironment of activated phagocytic cells (Etherington *et al.*, 1981), and iron mobilized from transferrin at low pH has been suggested to promote ·OH formation that contributes to cartilage damage in the inflamed rheumatoid joint (Halliwell *et al.*, 1985). It is possible that ischaemic tissues might create microenvironments of sufficiently low pH to release iron in this way, iron that might then be available to mediate damaging ·OH formation on re-perfusion (McCord, 1985). Inclusion in the reaction mixture of HCO_3^-, which stabilizes iron–transferrin or

iron–lactoferrin complexes (Morgan, 1979), decreases, but does not prevent, this release of iron.

However, the apoproteins still appear to bind some iron at acidic pH values, especially in the presence of HCO_3^-, in that they diminish deoxyribose degradation promoted by $FeCl_3$ or by iron released from ferritin (Table 2). Presumably at pH 4.5 the proteins exist in equilibrium with a given concentration of 'free' iron, under our reaction conditions. Hence added iron will still bind to proteins of low iron loading, but iron will be released from proteins of high iron loading. Thus, even under acid conditions, the precise iron loading of the protein will be a critical factor in determining whether it is a promoter of damage or a protector against it. One unexpected feature of our results is that lactoferrin and transferrin behaved very similarly in all the systems studied (e.g. Tables 1 and 5), even though it is often stated that iron is released from lactoferrin at acid pH values much more slowly than from transferrin (Morgan, 1979; Lonnerdal *et al.*, 1981). However, our results are highly reproducible.

We conclude that iron bound to lactoferrin or transferrin *in vivo* is not active in accelerating oxidative damage at pH 7.4 in the absence of a strong chelating agent. At acidic pH values, these proteins may still be protective against, or may promote, oxidative damage, depending on their degree of iron loading and on the localized concentration of iron ions released from other sources, such as ferritin and haemoglobin.

We thank the Arthritis and Rheumatism Council, the Sports Council and the Wellcome Trust for financial support. B.H. is a Lister Institute Research Fellow.

REFERENCES

Ambruso, D. R. & Johnston, R. B. (1981) J. Clin. Invest. **67**, 352–360
Ankel, E. & Petering, D. H. (1980) Biochem. Pharmacol. **29**, 1833–1837
Baker, M. S. & Gebicki, J. M. (1986) Arch. Biochem. Biophys. **246**, 581–588
Baldwin, D. A., Jenny, E. R. & Aisen, P. (1984) J. Biol. Chem. **259**, 13391–13394
Bannister, J. V., Bellavite, P., Davoli, A., Thornalley, P. J. & Rossi, F. (1982*a*) FEBS Lett. **150**, 300–302
Bannister, J. V., Bannister, W. H., Hill, H. A. O. & Thornalley, P. J. (1982*b*) Biochim. Biophys. Acta **715**, 116–120
Bannister, W. H., Bannister, J. V., Searle, A. J. F. & Thornalley, P. J. (1983) Inorg. Chim. Acta **78**, 139–142
Bates, G. W. & Schlabach, M. R. (1973) J. Biol. Chem. **248**, 3228–3232
Berglin, E. H. & Carlsson, J. (1985) Infect. Immun. **49**, 538–543
Biemond, P., van Eijk, H. G., Swaak, A. J. G. & Koster, J. F. (1984) J. Clin. Invest. **73**, 1576–1579
Burton, K. P., McCord, J. M. & Ghai, G. (1984) Am. J. Physiol. **246**, H776–H783
Butler, J. & Halliwell, B. (1982) Arch. Biochem. Biophys. **218**, 174–178
Carver, F. J. & Frieden, E. (1978) Biochemistry **17**, 167–172
Czapski, G. & Goldstein, S. (1986) Free Radical Res. Commun. **1**, 157–161
Etherington, D. J., Pugh, G. & Silver, I. A. (1981) Acta Biol. Med. Ger. **40**, 1625–1631
Flitter, W., Rowley, D. A. & Halliwell, B. (1983) FEBS Lett. **158**, 310–312
Floyd, R. A. (1981) Biochem. Biophys. Res. Commun. **99**, 1209–1215
Floyd, R. A. (1983) Arch. Biochem. Biophys. **225**, 263–270
Gutteridge, J. M. C. (1981) FEBS Lett. **128**, 343–346
Gutteridge, J. M. C. (1985) Biochim. Biophys. Acta **869**, 119–127
Gutteridge, J. M. C. (1986) FEBS Lett. **201**, 291–295
Gutteridge, J. M. C., Richmond, R. & Halliwell, B. (1979) Biochem. J. **184**, 469–472
Gutteridge, J. M. C., Paterson, S. K., Segal, A. W. & Halliwell, B. (1981) Biochem. J. **199**, 259–261
Gutteridge, J. M. C., Halliwell, B., Treffry, A., Harrison, P. M. & Blake, D. (1983) Biochem. J. **209**, 557–560
Halliwell, B. (1978) FEBS Lett. **92**, 321–326
Halliwell, B. (1985) Biochem. Pharmacol. **34**, 229–233
Halliwell, B. & Gutteridge, J. M. C. (1981) FEBS Lett. **128**, 347–352
Halliwell, B. & Gutteridge, J. M. C. (1985) Mol. Aspects Med. **8**, 89–193
Halliwell, B. & Gutteridge, J. M. C. (1986) Arch. Biochem. Biophys. **246**, 501–514
Halliwell, B., Gutteridge, J. M. C. & Blake, D. (1985) Philos. Trans. R. Soc. London Ser. B **311**, 659–671
Harris, D. C., Rinehart, A. L., Hereld, D., Schwartz, R. W., Burke, F. P. & Salvador, A. P. (1985) Biochim. Biophys. Acta **838**, 295–301
Lonnerdal, B., Keen, C. L. & Hurley, L. S. (1981) Annu. Rev. Nutr. **1**, 149–174
Maguire, J. J., Kellogg, E. W. & Packer, L. (1982) Toxicol. Lett. **14**, 27–34
McCord, J. M. (1985) N. Engl. J. Med. **312**, 159–163
McCord, J. M. & Day, E. D. (1978) FEBS Lett. **86**, 139–142
McCord, J. M. & Fridovich, I. (1969) J. Biol. Chem. **244**, 6049–6055
Morgan, E. H. (1979) Biochim. Biophys. Acta **580**, 312–326
Motohashi, M. & Mori, I. (1983) FEBS Lett. **157**, 197–199
O'Connell, M., Halliwell, B., Moorhouse, C. P., Aruoma, O. I., Baum, H. & Peters, T. J. (1986) Biochem. J. **234**, 727–731
Rao, K., van Renswoude, J., Kempf, C. & Klausner, R. D. (1983) FEBS Lett. **160**, 213–216
Stocks, J., Gutteridge, J. M. C., Sharp, R. J. & Dormandy, T. L. (1974) Clin. Sci. **47**, 223–233
Vercellotti, G. M., Sweder van Asbeck, B. & Jacob, H. S. (1985) J. Clin. Invest. **76**, 956–962
Willson, R. L. (1978) Ciba Found. Symp. **51**, 331–354
Winston, G. W., Eibschutz, O. M., Strekas, T. & Cederbaum, A. I. (1986) Biochem. J. **235**, 521–529
Winterbourn, C. C. (1979) Biochem. J. **182**, 625–628
Winterbourn, C. C. (1983) Biochem. J. **210**, 15–19

Received 4 July 1986/11 August 1986; accepted 16 September 1986