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Iron release from haemosiderin and production of iron-catalysed hydroxyl radicals *in vitro*

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Isolated haemosiderin contained iron and nitrogen in a weight ratio of 6.75, with phosphorus and no detectable haem. Considerably more iron was released from haemosiderin under acidic conditions than under neutral conditions in the presence of ascorbate, nitrilotriacetate or dithionite. Unlike the situation with ascorbate, chelators such as citrate, ADP or succinate induced the release of only some iron, with almost no pH-dependence. Dehydroascorbate (the oxidized form of ascorbate with no reducing capacity) behaved like citrate, ADP, succinate or desferal, rather than like ascorbate itself, in releasing iron. GSH had less effect on the release of iron than these chelators, but in the presence of a small amount of chelator the release of iron increased, especially under acidic conditions. Thus reduction, chelation and pH were all found to be important factors involved in the release of iron from haemosiderin. Investigation by e.p.r. of hydroxyl-radical production by the released iron showed high radical productivity at an acidic pH. However, at a physiological pH, almost no radical formation was detected, except in the presence of nitrilotriacetate. These findings suggested that, under physiological conditions, haemosiderin was not an effective iron donor and was almost not involved in radical production. Under acidic conditions, however, such as in inflammation, hypoxia and in a lysosomal milieu, it could possibly be an iron donor and is thought to be implicated in radical production and tissue damage in iron-overloaded conditions.

INTRODUCTION

Haemosiderin is defined as a water-insoluble iron-containing protein which forms comparatively large cytoplasmic aggregates known to store iron as ferric oxyhydroxide inside the protein shell and, at times, outside. This iron-storage protein is thought to be produced as a consequence of lysosomal modification of ferritin (Fishbach *et al.*, 1971; Wixon *et al.*, 1980) through degradation and polymerization (Hoy & Jacobs, 1981). In normal tissues, ferritin accounts for the major part of the stored iron, with small quantities as haemosiderin deposits. However, in iron-loaded conditions, haemosiderin becomes the predominant iron-storage protein, and, in iron-overloaded patients, tissue damage occurs where this protein is deposited heavily (Richter, 1978).

Haemosiderin accumulates first in the lysosomes, especially in those in the liver, and a close positive correlation has been shown between enhanced lysosomal fragility and liver haemosiderin content (Seymour & Peters, 1978; Selden & Peters, 1979). Iron-mediated peroxidative injury to isolated hepatic lysosomes has also been reported (Mak & Weglicki, 1985). *In vivo*, Bacon *et al.* (1983) showed hepatic membrane lipid peroxidation in rats subjected to chronic iron-loaded conditions. Bannister *et al.* (1984) reported that incubation of human ferritin with a superoxide-anion (O_2^-)-generating system induced production of hydroxyl radicals ($OH^\cdot$), which could be identified by e.p.r. spin trapping, and O'Connell *et al.* (1986*a*,*b*) compared $OH^\cdot$ productivity associated with haemosiderin iron and ferritin iron, and suggested that haemosiderin formation was a biological-protection mechanism. It was also shown that iron was released from haemosiderin at pH 4.5 and that this release stimulated liposomal lipid peroxidation, and that, even at pH 7.4, peroxidation could be induced by the addition of ascorbate (O'Connell *et al.*, 1985). Thus haemosiderin appeared to be implicated in the cellular damage caused during iron-overloading.

Several studies on the isolation and characterization of haemosiderin have shown it to have higher iron/protein weight ratios than ferritin, to have almost no haem and to contain lipids of various classes (Shoden & Sturgeon, 1960; McKay & Fineberg, 1964*a*,*b*). Weir *et al.* (1984) isolated, by centrifugation through concentrated KI solutions, haemosiderin from a human spleen taken from an individual who had had multiple transfusions, and they presented some analytical data and characteristics (Weir *et al.*, 1984).

In the present study, human spleen haemosiderin was isolated by the method of Weir *et al.* (1984) and its iron-release characteristics were examined. In addition, induction of $OH^\cdot$ production by the released iron was studied by a spin-trapping/e.p.r. method. Moreover, the possibility of haemosiderin-related $OH^\cdot$ production in acidic tissue is discussed.

EXPERIMENTAL

Materials

ADP, nitrilotriacetic acid (NTA) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were purchased from Sigma Chemical Co. Sodium hydrosulphite and succinic acid were obtained from Nakarai Chemicals Co. (Kyoto, Japan), GSH from Kojin Co. (Tokyo, Japan), and desferal from CIBA–Geigy. DMPO was diluted and

Abbreviations used: $OH^\cdot$, hydroxyl radical; NTA, nitrilotriacetic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide.

passed in a vacuum through charcoal; then, after degassing it with N_2, it was stored at −80 °C. All other chemicals were of analytical grade and were used without further purification.

Isolation and analysis of haemosiderin

Human haemosiderin was isolated by the method of Weir *et al.* (1984) from iron-overloaded spleens removed from patients who had received multiple blood transfusions as treatment for leukaemia. Isolated haemosiderin was then freeze-dried and stored desiccated at 4 °C. For the determination of iron content, 250 μg of haemosiderin was wet-ashed with concentrated H_2SO_4, neutralized with aq. NH_3, then diluted to a volume of 100 ml with Chelex-treated iron-free distilled water. The sample solution was subjected to colorimetric analysis with a bathophenanthroline reagent, the absorbance at 535 nm being measured in a spectrophotometer. For nitrogen analysis, 200 μg of haemosiderin was wet-ashed and diluted to a volume of 2.0 ml, after which the nitrogen content was colorimetrically determined with Nessler's reagent. For phosphorus analysis, 1.0 mg of haemosiderin was wet-ashed, diluted to a volume of 3.6 ml, and mixed with 0.2 ml of 5.0% ammonium molybdate and 0.2 ml of amidol reagent. The absorbance of this solution was measured at 830 nm. For lipofuscin analysis, 1.0 mg of haemosiderin was suspended in 5 ml of methanol/chloroform (1:2, v/v) and shaken for 24 h at room temperature. After the addition of 5 ml of distilled water, the sample was kept at 4 °C for 3 h, then centrifuged at 3000 rev./min ($r_{av.}$ 18 cm) for 10 min. A 2 ml portion of the resultant chloroform layer was taken and the fluorescence spectrum recorded with a Hitachi 650-10S spectrophotofluorometer, after standardization with quinine sulphate at a concentration of 1 μg/ml in 0.05 M-H_2SO_4 at an excitation wavelength of 380 nm and an emission wavelength of 460 nm (Fletcher *et al.*, 1973; Taubold *et al.*, 1975).

Iron release from haemosiderin

The effectiveness of a series of reducing agents and chelators on the release of iron from haemosiderin was compared in the following way; 100 mM-Pipes buffer was used for buffer solutions above pH 6.5, and 100 mM-sodium acetate buffer was used for solutions below pH 6.0. In each experiment, 20 ml of buffer solution (100 mM final concn.) contained 10 mM reducing agent and/or 10 mM chelator. Reactions were initiated by addition of 200 μg of haemosiderin (57.12 μg of iron) to the solution, and samples were incubated at 37 °C for 48 h. At suitable time intervals, portions were removed and centrifuged at 5000 rev./min ($r_{av.}$ 18 cm) for 15 min. The iron concentration of the supernatant was determined by reaction with a bathophenanthroline reagent, after reduction with thioglycollate (1.0%, w/v). Experiments using reducing agents were carried out under N_2 atmosphere.

Detection of iron-catalysed radical formation

For each supernatant containing released iron at pH 4.0, 5.0, 6.0 and 7.4, the iron concentration was determined by the method described above, and equalized by addition of buffer. Aliquots (200 μl) of e.p.r. spin-trapping reaction mixture contained 0.1% H_2O_2, 153 mM-DMPO (spin-trapping agent) and 160 μl of sample solution (28.6 μM-iron solution). The reaction was started by addition of 20 μl of 1% H_2O_2, and each sample was immediately transferred to an e.p.r. flat cell and subjected to e.p.r. E.p.r. spectra were recorded chronologically at room temperature with a JEOL JES-1XG e.s.r. spectrometer equipped with 100 kHz magnetic-field modulation (Janzen, 1971; Harbour *et al.*, 1974).

Table 1. Analysis of isolated haemosideirn (Hs)

Analytical data are shown for haemosiderin (means ± S.E.M.) isolated from iron-loaded human spleen. After wet-ashing haemosiderin, the iron content was determined colorimetrically using bathophenanthroline reagent, nitrogen content with Nessler's reagent and phosphorus content with amidol reagent. For lipofuscin analysis, haemosiderin suspended in methanol/chloroform (1:2, v/v) was shaken for 24 h at room temperature and, after addition of 5 ml of distilled water, was kept at 4 °C for 3 h and centrifuged at 3000 rev./min ($r_{av.}$ 18 cm) for 10 min. The chloroform layer was taken and fluorescence spectra were recorded with $\lambda_{excitation}$ 380 nm and $\lambda_{emission}$ 460 nm (1 unit ≡ quinine sulphate at a concentration of 1 μg/ml in 0.05 M-H_2SO_4). Values in parentheses indicate the number of preparations analysed.

Component	Content
Iron	28.6±2.0% (5)
Nitrogen	4.2±0.2% (4)
Phosphorus	0.15±0% (5)
Lipofuscin	9.4±1.1 unit/mg of Hs (3)

RESULTS

Analysis of haemosiderin

Analytical data for haemosiderin are given in Table 1. The iron and nitrogen content agreed closely with values obtained for horse spleen haemosiderin (McKay & Fineberg, 1964*a*,*b*); however, there was slightly more iron and slightly less phosphorus than was found by Weir *et al.* (1984) in human spleen haemosiderin. Lipofuscin is defined as a yellow–brown lipid-soluble autofluorescent pigment (Scarpelli & Chiga, 1985) having a fluorescence maximum in the region 430–470 nm (Taubold *et al.*, 1975). It is found in cytoplasm often around haemosiderin deposits. The fluorescent chromophore R-N=CH-CH=CH-NH=R is believed to be responsible for the spectral properties of these pigments, which are thought to be formed from various unsaturated lipids, malonaldehyde and free amino groups from amino acids, proteins and nucleic acids (Taubold *et al.*, 1975). The present study showed that 1 mg of haemosiderin contained about 10 units of lipofuscin, with one standard unit defined as fluorescence equal to 1 μg of quinine sulphate/ml in 0.05 M-H_2SO_4. The absorption spectrum of haemosiderin dissolved in 80% (v/v) mercaptoacetic acid compared with that of haemoglobin showed that it contained no haem. Electron micrographs of unstained haemosiderin (Fig. 1) showed large aggregates of numerous small and highly dense particles, supporting the claim that haemosiderin is derived from ferritin through lysosomal modification.

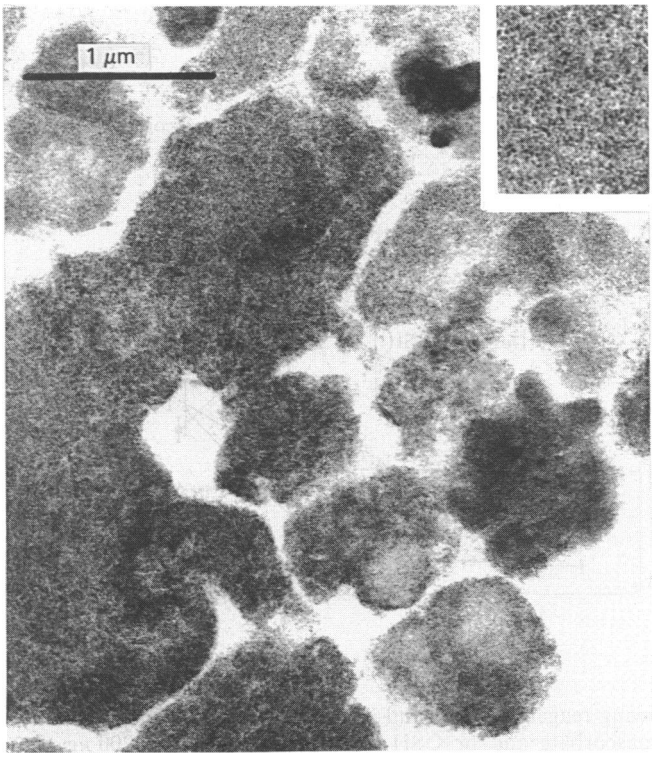

Fig. 1. Electron micrograph of unstained isolated haemosiderin

Note the large heterogeneous aggregates consisting of numerous small highly dense particles.

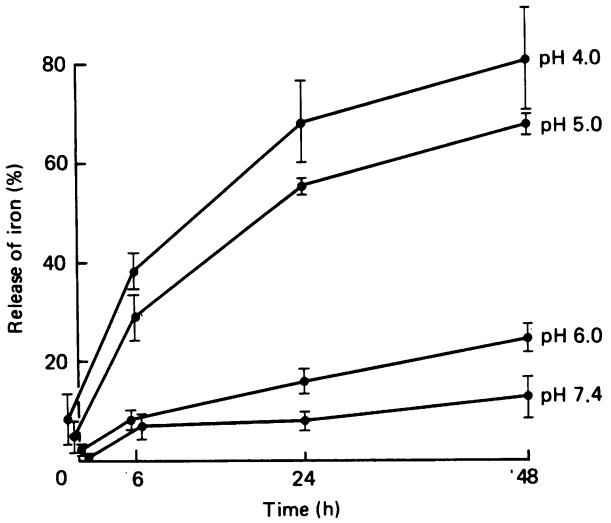

Fig. 2. Iron release from haemosiderin at various pH values

Reaction mixtures contained, in a total volume of 20 ml, the following reagents at the final concentration stated: haemosiderin (200 μg), containing 57.12 μg of iron, ascorbate (10 mM) and a buffer system (100 mM-Pipes buffer, pH 7.4; 100 mM-sodium acetate buffer, pH 6.0, 5.0 and 4.0). Reaction mixtures were incubated at 37 °C for 48 h. After 0.1, 6, 24 and 48 h samples were taken and centrifuged at 5000 rev./min ($r_{av.}$ 18 cm) for 15 min. The supernatant iron concentration was measured colorimetrically with bathophenanthroline reagent after reduction with thioglycollate (1.0%, w/v). The percentage release of iron is shown as the mean ± S.E.M. for five preparations at each pH.

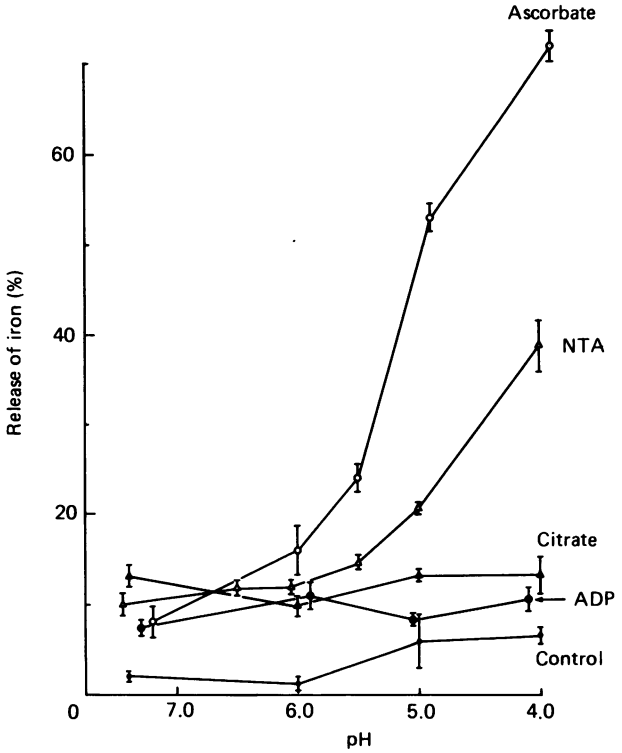

Fig. 3. Effects of chelation on the release of iron from haemosiderin

Reaction mixtures contained, in a total volume of 20 ml, the following reagents at the final concentrations stated: one of the following chelators: ascorbate, NTA, citrate, desferal, ADP or succinate (10 mM); haemosiderin (200 μg), containing 57.12 μg of iron; and a buffer system (100 mM-Pipes buffer for pH values higher than 6.5; 100 mM-sodium acetate buffer for pH values lower than 6.0). The control group contained no chelators. The reaction mixtures were incubated at 37 °C for 24 h. Samples were taken after 24 h and centrifuged at 5000 rev./min ($r_{av.}$ 18 cm) for 15 min. Supernatant iron concentrations were measured colorimetrically with bathophenanthroline reagent after reduction with thioglycollate (1.0%, w/v). The percentage release of iron is shown as the mean ± S.E.M. for five preparations at each pH.

Iron release from haemosiderin

The release of iron from haemosiderin in the presence of 10 mM-ascorbate at various pH values was monitored for 48 h. Much more iron was released from haemosiderin at pH 4.0 and 5.0 than at pH 6.0 and 7.4 (Fig. 2). Further monitoring up to 120 h showed almost no additional release of iron. There was a significant change in the release of iron between pH 5.0 and 6.0, indicating that, under physiological conditions, only small amounts of iron were released from haemosiderin, whereas under acidic conditions iron release was significant.

Effects of chelation on the release of iron from haemosiderin

To examine the effects of chelation on the release of iron from haemosiderin, a range of chelators, as well as ascorbate, were used (Fig. 3). Ascorbate and NTA induced release of large amounts of iron from haemosiderin under acidic conditions, whereas at a physio-

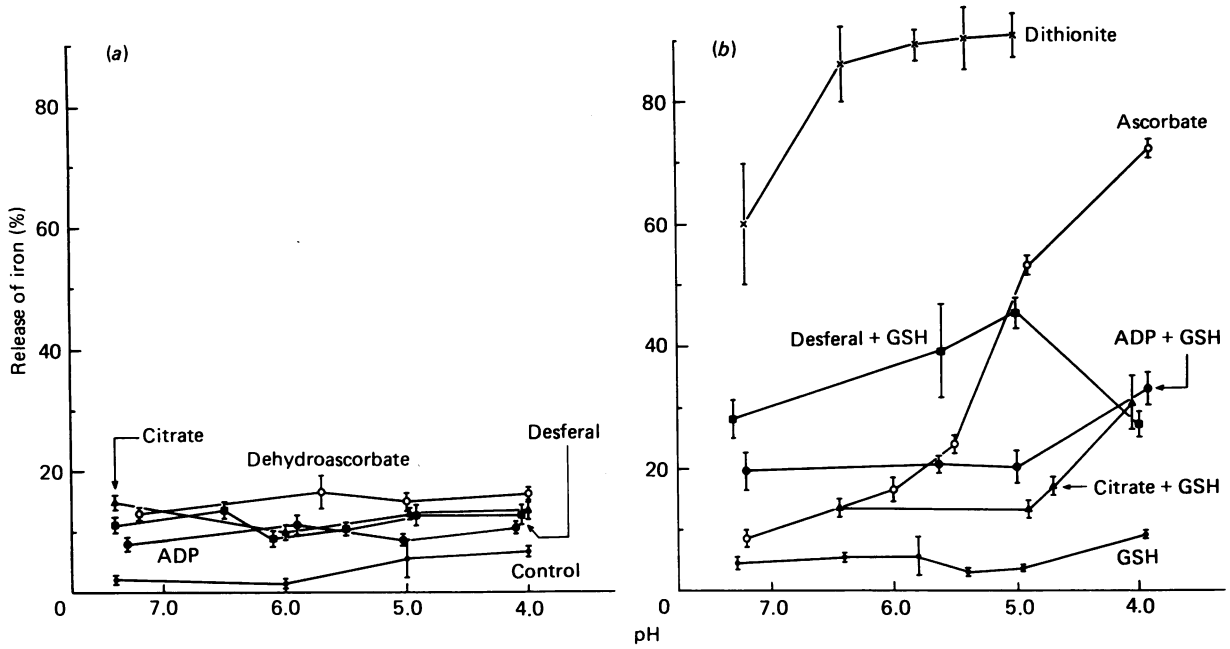

Fig. 4. Effects of reduction on the release of iron from haemosiderin

Reaction mixtures contained, in a total volume of 20 ml, the following reagents at the final concentrations stated: one of the following chelators: citrate, desferal, ADP, ascorbate or dehydroascorbate and/or GSH or dithionite (10 mM); 200 μg of haemosiderin, containing 57.12 μg of iron; and a buffer system (100 mM-Pipes buffer for pH values higher than 6.5; 100 mM-sodium acetate buffer for pH values lower than 6.0). Reaction mixtures were incubated at 37 °C for 24 h. Aliquots were taken and centrifuged at 5000 rev./min ($r_{av.}$ 18 cm) for 15 min. Supernatant iron concentrations were measured colorimetrically with bathophenanthroline reagent after reduction with thioglycollate (1.0%, w/v). (*a*) No reducing agent added; (*b*) reducing agent added. The percentage release of iron is shown as the mean ± S.E.M. for five preparations at each pH.

logical pH, less than 20% was released, that is, almost the same as that achieved by the other chelators. Chelators such as citrate, ADP and succinate released less than 20% of the total iron, independently of pH. In the control group without chelators, slightly more iron was released under acidic than under neutral conditions. Desferal did not strongly induce release of iron at any pH.

Effects of reduction on the release of iron from haemosiderin

To examine the effects of reduction on the release of iron from haemosiderin, we used dithionite as a strong reducing agent, ascorbate, dehydroascorbate (the oxidized form of ascorbate) and the other chelators (Fig. 4). Dithionite induced release of iron very strongly even at a physiological pH, whereas release induced by citrate, ADP or desferal was increased by addition of GSH, especially under acidic conditions. Dehydroascorbate, which has no reducing capacity, induced release of iron to the same degree as the above chelators. However, ascorbate, which has both reducing and chelating capacities, induced release of large amounts of iron under acidic conditions. These observations suggest that reduction is the key factor in the release of iron from haemosiderin and that reduction and chelation have not additive, but synergistic, effects on that release.

Comparison by e.p.r. spin trapping of OH$^{\cdot}$ production by released iron

We investigated released-iron-catalysed OH$^{\cdot}$ production in an OH$^{\cdot}$-production system containing H_2O_2 and DMPO. OH$^{\cdot}$ is produced from H_2O_2 in the presence of Fe^{2+} by the Fenton reaction:

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

and is trapped by DMPO, forming a comparatively stable DMPO–OH$^{\cdot}$ complex (DMPO spin adduct). E.p.r. was used to measure the spectra of DMPO–OH$^{\cdot}$ chronologically; a 1:2:2:1 quartet pattern (Janzen, 1971; Harbour *et al.*, 1974) was observed.

Under neutral conditions, almost no e.p.r. spectra were detected, other than in haemosiderin samples treated with NTA (Figs. 5 and 6). However, under acidic conditions, e.p.r. spectra of the DMPO spin adduct were detected in samples of iron–ascorbate, iron–NTA, iron–dithionite, iron–ADP, and iron–citrate, with the first three giving especially intense e.p.r. spectra (Figs. 5 and 6). Dithionite is a stronger reducing agent than ascorbate and, at pH 4.0, iron–dithionite produced about 25% as much OH$^{\cdot}$ as iron-ascorbate (Fig. 6). However, under neutral conditions, no OH$^{\cdot}$ production by either was detectable. Ascorbate is known to be an effective scavenger for OH$^{\cdot}$ at neutral pH (Bielski *et al.*, 1975) and has also been found to induce lipid peroxidation in the presence of Fe ions even at neutral pH (Leung *et al.*, 1981). The present data indicated (i) that under neutral conditions iron–ascorbate induced lipid peroxidation not through OH$^{\cdot}$ formation, but through direct effects on the lipid and (ii) that reduction of iron alone was not enough to produce OH$^{\cdot}$ in this system. Chelation of NTA with Fe ions has been reported to be cytotoxic (Yamanoi *et al.*, 1982, 1984; Nakamoto *et al.*, 1986), and in the present study iron–NTA showed comparatively

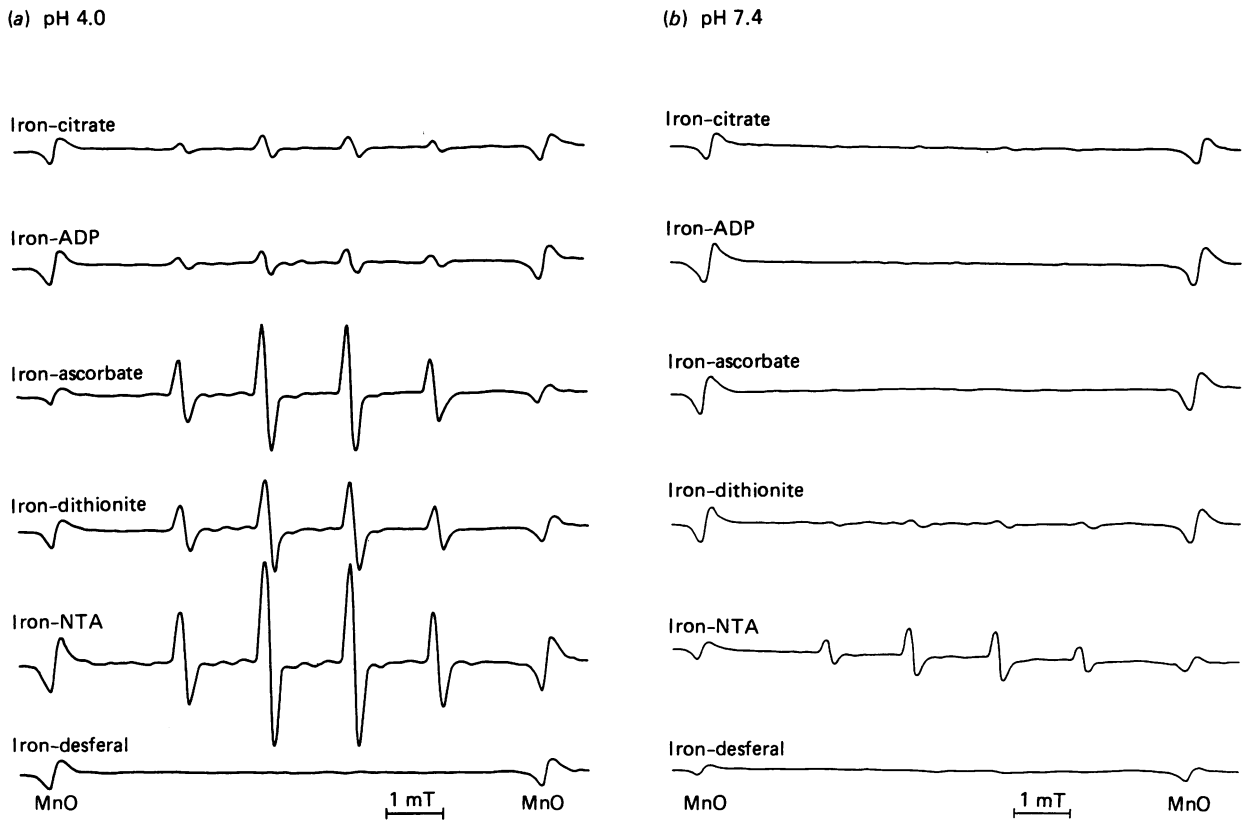

Fig. 5. E.p.r. spectra of the DMPO spin adduct formed in iron solutions containing H_2O_2 and DMPO 3 min after the reaction at pH 4.0 (*a*) and 7.4 (*b*)

Reaction mixtures contained, in a total volume of 200 μl, the following reagents at the final concentrations stated: iron (28.6 μM), citrate, ADP, ascorbate, dithionite, NTA or desferal (8.0 mM), DMPO (153 mM), H_2O_2 (0.1%) and a buffer system (100 mM-Pipes buffer, pH 7.4; 100 mM-sodium acetate buffer, pH 6.0, 5.0 and 4.0). Reactions were initiated by the addition of H_2O_2, and the samples were immediately transferred to e.p.r. flat cells and subjected to e.p.r. E.p.r. spectra were recorded with an external standard of manganous oxide (MnO) at room temperature under the following conditions; microwave power, 4.0 mW; modulation amplitude, 0.125 mT; scanning field, 336.3 ± 5 mT; amplitude, 6.3×100; sweep time, 30 s.

strong e.p.r. spectra of DMPO spin adduct even at neutral pH, suggesting that it would be cytotoxic enough to induce tissue damage, even under physiological conditions, through $OH^{\cdot}$ formation. Desferal is an iron-chelating agent used for the therapy of iron-overloaded patients (Keberle, 1964). It was shown, in the release of iron from haemosiderin, to behave like the other chelators, and although it was found to be ineffective in inducing iron release, the iron–desferal formed produced no $OH^{\cdot}$ at any pH. Haemosiderin suspensions containing the same amount of iron in the absence of both reducing agents and chelators also produced no $OH^{\cdot}$ at any pH. O'Connell *et al.* (1986*c*) also suggested that it is the released iron, not native haemosiderin, that mediates lipid peroxidation in liposomes.

DISCUSSION

Some of the constituents of human spleen haemosiderin isolated by the method of Weir *et al.* (1984) were analysed, and the data obtained for the iron, nitrogen and phosphorus content were found to be similar to those of others (McKay & Fineberg, 1964*a*,*b*; Weir *et al.*, 1984). Lipofuscin has often been observed located around the haemosiderin deposits in iron-overloaded tissues and was shown by fluoroscopy to accompany isolated haemosiderin. O'Connell *et al.* (1986*b*) also showed fluoroscopically the presence of Schiff's-base adducts of the type found in lipofuscin, and they suggested that haemosiderin is a product of oxidative reactions occurring intralysosomally in iron-overloaded conditions. Thus intracellular lipid peroxidation may be induced or accelerated around the heaviest sites of haemosiderin deposition.

Considerable amounts of iron were released from haemosiderin in the presence of ascorbate under acidic conditions, whereas at pH values higher than 6.0, less than 20% of the iron was released. In normal tissues the intracellular pH seems to be neutral, but to be about 6.0 in inflammatory and hypoxic conditions (Ambrosio *et al.*, 1987). The lysosomal pH, on the other hand, is thought to be about 5.0 (Reijngoud & Tager, 1977), suggesting that haemosiderin could be a rather effective intralysosomal iron donor in the presence of certain chelators and also be a possible iron donor in the cytoplasm of inflammatory and hypoxic tissues. Furthermore, e.p.r. spin trapping showed strong $OH^{\cdot}$ formation from H_2O_2 in the presence of iron–ascorbate at acidic pH. This may be related to enhanced lysosomal fragility in tissue containing heavy deposits of haemosiderin (Seymour & Peters, 1978; Selden & Peters, 1979).

In the absence of chelators, iron was not effectively

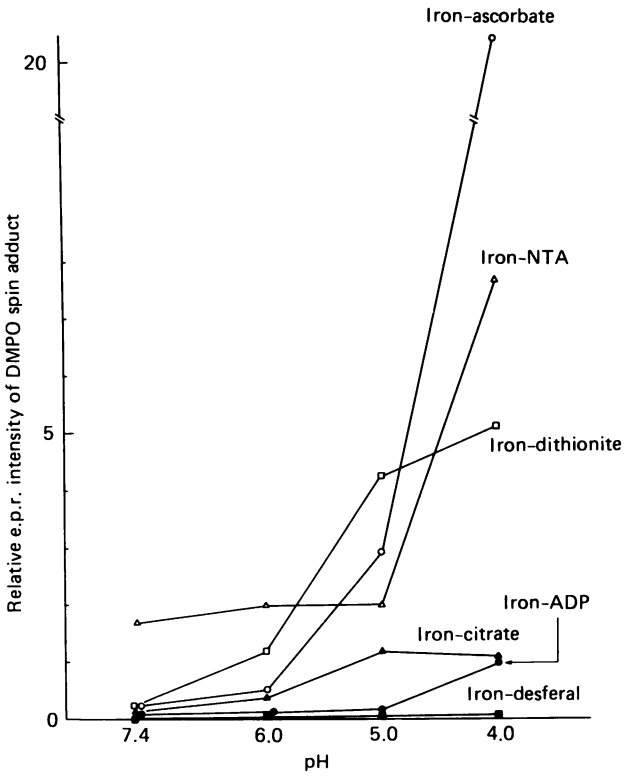

Fig. 6. OH· production catalysed by released iron

For each e.p.r. spectrum of the DMPO spin adduct, obtained as described in Fig. 5, intensity was plotted relative to the external standard of manganous oxide (MnO). Reaction mixtures contained the following reagents at the final concentrations stated; iron (28.6 μM), ascorbate, NTA, dithionite, citrate, ADP, desferal (8.0 mM), DMPO (153 mM), H_2O_2 (0.1%) and a buffer system (100 mM-Pipes buffer, pH 7.4; 100 mM-sodium acetate buffer, pH 6.0, 5.0 and 4.0).

released from haemosiderin, except at an acidic pH where some release did occur. In their presence, however, about 10% of the total iron was constantly released independently of the pH. Iron release induced by either citrate or ADP also showed some radical production at an acidic pH, but not at a neutral pH. It is known that ADP-stabilized Fe^{2+} induces lipid peroxidation of microsomal membranes even under neutral conditions (Högberg *et al.*, 1975*a*,*b*), and is thought to be involved in the propagation step of lipid peroxidation (Kappus, 1985). This is supported by the results of the present study, which found that iron–ADP did not produce hydroxyl radicals at pH 7.4.

Almost the same amount of iron was released from haemosiderin in the presence of GSH alone as by control samples without chelators. However, addition of some chelator caused iron to be effectively released at an acidic pH. Dehydroascorbate, in its effects on the release of iron, behaved as the chelators other than ascorbate. The release induced by ascorbate was much greater than that caused by GSH with a chelator, even though the reduction potential of the former was weaker, with redox potentials of 0.08 at pH 6.4 and −0.23 at pH 7.0 respectively (White *et al.*, 1978). This stronger releasing activity may be due to ascorbate having both reducing and chelating capacities. Furthermore, similar results on the release of iron and on OH· formation were obtained for chelators alone and for GSH in the presence of chelators (results not shown). These results may indicate that reduction of haemosiderin iron weakens the iron–protein links, thus making them more susceptible to chelation. NTA induced a greater release of iron and formation of radicals at acidic than at neutral pH, but shows high radical productivity even at pH 7.4. The cytotoxic effects of iron–NTA have already been reported (Yamanoi *et al.*, 1982, 1984; Nakamoto *et al.*, 1986), and the present data showed that it may be cytotoxic even under physiological, as well as under pathological, conditions. Desferal's capacity to induce iron release from haemosiderin was no different from those of the other chelators. On the other hand, O'Connell *et al.* (1986*c*) reported that desferal released comparatively large amounts of iron from haemosiderin. Desferal's inefficiency in releasing iron, despite its greater capacity for chelation of iron, may be explained by its large M_r and positive charge (Kontoghiorghes *et al.*, 1987). However, radical formation was not detected at any pH. Therefore it may be especially effective in lessening the cytotoxicity of chelated Fe ions.

From these data, three factors (reduction and chelation of iron and pH) seem to be important. In addition, in these experiments, reduction and chelation of iron had no additive, but synergistic, effects, both on the release of iron from haemosiderin and on the released-iron-catalysed production of OH·. It can be speculated that, in normal tissues, haemosiderin is not an effective iron donor and may not be related to iron-mediated cytotoxicity and membrane lipid peroxidation. However, it may be an effective iron donor in lysosomes in the presence of certain chelators and/or reducing agents. Intracellular levels of GSH and ascorbate in mammalian cells are known to be in the millimolar range (Meister & Anderson, 1983; Bernheim, 1961) and they are candidates for iron releaser from haemosiderin *in vivo* in acidic conditions. In pathological tissues, such as in inflammation and severe hypoxia, where the tissue pH is at times lower than 6.0, haemosiderin may be an iron donor, inducing and/or accelerating tissue damage. In other words, haemosiderin may offer 'a specific site' for membrane lipid peroxidation in tissues suffering acidic conditions.

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