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Iron-induced ascorbate oxidation in plasma as monitored by ascorbate free radical formation

No spin-trapping evidence for the hydroxyl radical in iron-overloaded plasma

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A study was made of the interaction of plasma ascorbate and ascorbate free radical (AFR) with exogenously added iron. The quantitative determination of AFR has the advantage that transient increases in ascorbate oxidation can be directly monitored by e.p.r. spectroscopy. An AFR signal was found in the plasma of all donors and was unaffected by superoxide dismutase, catalase and the strong iron chelator deferoxamine. These findings and the rapid decrease in AFR under a nitrogen atmosphere suggest that plasma AFR is probably a result of air auto-oxidation. Iron loading of plasma did not affect the intensity of the AFR signal until the iron concentration approached or exceeded the plasma latent iron-binding capacity. In iron-overloaded plasma, the intensity of the AFR signal increased to about 10 times the normal level before decreasing rapidly to undetectable levels after 15–20 min. Determination of plasma ascorbate showed that the disappearance of AFR was due to a complete loss of the vitamin. When 50 μM-ascorbate was loaded with iron in iso-osmotic phosphate buffer there was an increase in the AFR signal, independent of the iron concentration, which was stable at least for 15 min. Thus the rate of ascorbate loss in the iso-osmotic phosphate buffer was considerably lower than in iron-overloaded plasma. The addition of different iron chelators produced comparable effects on the intensity of the AFR signal in both iron-overloaded plasma and ascorbate solution. These results suggest that the characteristic behaviour of plasma AFR after iron loading is due to its specific iron-binding capacity and to plasma ferroxidase activity. The ferroxidase activity of plasma is important to promote the transfer of Fe^{2+} into transferrin without a transient ascorbate oxidation. Spin-trapping studies with 5,5-dimethyl-1-pyrroline *N*-oxide and *N*-t-butyl-α-phenylnitrone revealed that iron-overloaded plasma was unable to produce spin-trap adducts even in the presence of 50–300 μM-hydrogen peroxide or 100 μM-azide. Evidence of $OH^{\cdot}$ radical formation was obtained only after the addition of EDTA. Therefore, iron-overloaded plasma itself does not produce a Fenton reaction and, if ascorbate does indeed have a free-radical-mediated pro-oxidant role, it is not detectable in plasma by spin-trapping experiments.

INTRODUCTION

Antioxidants such as ascorbate, which are taken up in the diet and are easily modifiable by dietary supplementation, have received particular attention on account of their possible action as beneficial agents in a number of human diseases [1,2]. Notwithstanding the general agreement that ascorbate acts as an antioxidant *in vivo* [3], its high reactivity with transition metals that are known to promote metal-dependent oxidative damage has suggested that, at least under certain conditions, it may act as a pro-oxidant [4,5].

In biological systems most transition metals are bound to specific proteins, which prevent any metal-dependent catalysis of free radical reactions. However, experiments *in vitro* have shown that reactive oxygen species are able to release transition metals from binding proteins [6,7] and, when this occurs, ascorbate can act as a pro-oxidant [5]. This may be the case in some pathologies, but an adverse effect of ascorbate supplementation has still not been proved. Still less clear is the role of ascorbate during transient iron mobilization in extracellular and intracellular compartments where a mobile pool of iron is unavoidable. The metal is continuously shifted from one ligand to another, and it is not known whether this mobile pool may transiently react with ascorbate and increase its utilization. The ability of iron to promote radical damage led to the concept that the antioxidant potential of extracellular fluids is largely due to their ability to prevent metal complexes from participating in radical reactions [8].

Haematological disorders leading to iron overloading are frequently accompanied by a decrease in plasma and leucocyte ascorbate, a finding suggestive of increased vitamin utilization [9–11]. Further, adverse effects of ascorbate have been described in heavily iron-overloaded patients [11]. When the iron concentration overloads the binding capacity of specific proteins, the excess metal can participate in free radical reactions, which could be exacerbated by the presence of ascorbate. Ascorbate seems to be the only cellular reducing agent which can replace the superoxide anion to sustain the production of $OH^{\cdot}$ via a Fenton reaction [4,12,13].

One significant site at which the ascorbate–metal interaction can occur is blood plasma. Ascorbate is the most important antioxidant in the defence of human plasma against oxygen radicals [3], but if the iron does react with ascorbate, it could both increase vitamin utilization and possibly promote oxidative damage to the plasma.

The present studies address these questions by measuring the

Abbreviations used: AFR, ascorbate free radical; LIBC, latent iron-binding capacity; TIBC, total iron-binding capacity; DFO, deferoxamine mesylate; DTPA, diethylenetriaminepenta-acetic acid; SOD, superoxide dismutase; DMSO, dimethyl sulphoxide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; PBN, *N*-t-butyl-α-phenylnitrone.

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levels of ascorbate and ascorbate free radical (AFR) in plasma exposed to exogenous iron loading, and also consider whether iron-overloaded plasma can support the production of oxygen radicals by use of spin-trapping experiments.

MATERIALS AND METHODS

Reagents

Deferoxamine mesylate (DFO), diethylenetriaminepentaacetic acid (DTPA), catalase, superoxide dismutase (SOD), dimethyl sulphoxide (DMSO), EDTA and 4-amino-tempo nitroxide radical were purchased from Sigma (St. Louis, MO, U.S.A.). Citrate and Chelex 100 were obtained from BDH. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) and *N*-t-butyl-α-phenylnitrone (PBN) were obtained from Aldrich Chemicals (Milano, Italy). DMPO was purified on activated charcoal before use [14].

Preparation of plasma and treatments

Fresh heparinized human blood was obtained from healthy subjects with informed consent. The samples were analysed within 1–2 h of collection. To minimize ascorbate auto-oxidation, the blood was centrifuged immediately before analysis. Reactions were initiated via the addition of a small volume (typically 1–2 μl) from a concentrated solution of $(NH_4)_2Fe(SO_4)_2$ to 100 μl of plasma. Iso-osmotic phosphate buffer (0.15 M-NaCl/5 mM-sodium phosphate) was treated with Chelex 100 to remove iron contamination [15] and the pH was adjusted to 7.4. The following precautions were taken to minimize the oxidation of ascorbate: (i) a concentrated solution of 100 mM-ascorbate was prepared in 0.15 M-NaCl and treated with Chelex 100; (ii) the working solution was prepared immediately before use by dilution in Chelex-treated phosphate buffer, pH 7.4; (iii) solutions were freshly prepared and nitrogen-purged; and (iv) adventitious catalytic metals present in laboratory equipment were removed by washing with 0.1 M-HCl followed by repeated washing in metal-free de-ionized water [15].

Determination of ascorbate and iron-binding capacity

The loss of ascorbate in the iso-osmotic phosphate buffer was determined by measuring the decrease in its absorbance at 265 nm (ϵ_{265} 14700 $mM^{-1} \cdot cm^{-1}$) according to Buettner's method [15]. All spectra were obtained with a Lambda 17 UV/VIS Spectrophotometer (Perkin–Elmer, Norwalk, CT, U.S.A.). The kinetics of ascorbate loss were linear (at least for the first 15 min) and the slope was used to calculate the rate of loss. Plasma ascorbate determination was performed by h.p.l.c. and u.v. detection [16] with the following modification: cold methanol, used to precipitate plasma proteins, contained 0.1 mM-DFO in order to prevent post-incubation ascorbate oxidation. Plasma transferrin is normally one-third iron-saturated and the portion that can bind additional iron represents the latent iron-binding capacity. Determination of plasma total and latent iron-binding capacity (TIBC and LIBC respectively) was performed using the IBC and Iron FZ tests (Roche, Basel, Switzerland). The IBC test is based on the precipitation of non-transferrin-bound iron with magnesium hydroxide carbonate, and iron determination in the FZ test is based on ferrozine reaction [17].

E.p.r. measurements

All spectra were measured on a Bruker ESP 300 spectrometer equipped with an ESP 1600 Data System. Unless noted otherwise, all measurements were carried out at 37 °C. The gas flow was air or pure N_2 as indicated. Immediately after mixing, the samples were drawn into a gas-permeable Teflon tube with 0.81 mm internal diameter and 0.05 mm wall thickness (Zeus Industrial Products, Raritan, NJ, U.S.A.). The Teflon tube was folded 4 times and inserted into a quartz e.p.r. tube open at both ends. The spectrometer was equipped with a TE_{102} standard cavity. AFR was identified in plasma by its characteristic doublet at $g = 2.0054$ and $a_H = 1.88$ G, which is superimposable on that of ascorbate solutions. The AFR concentration was determined by using 4-amino-tempo nitroxide radical as a standard. Since many factors affect e.p.r. signal area measurements, the standard and AFR spectra were obtained using the Teflon tube and identical instrument settings. In experiments requiring a more rapid sampling, the Teflon tube was loaded by a peristaltic pump soon after the addition of iron.

RESULTS

AFR is present in plasma exposed to molecular oxygen but not in whole blood

Fig. 1(*a*) shows a typical e.p.r. spectrum of AFR in plasma. The mean value ± S.D. of the AFR concentration in 20 normal donors was 0.10 ± 0.03 μM.

It has been reported that AFR can easily be observed in human plasma at physiological temperatures [18]. AFR is the intermediate during the oxidation of ascorbate to dehydroascorbate [19]. Further, two molecules of AFR disproportionate [19] to yield the oxidized form (dehydroascorbate) and the reduced form (ascorbate). This oxidation of ascorbate may result from: (i) a reaction with reactive oxygen species [20,21]; (ii) interaction with transition metals present in plasma or released from laboratory equipment [15,21]; or (iii) air autoxidation [21]. To test which mechanism was operating, plasma was treated with 1 mM-DFO, 1 mM-DTPA, SOD (60 units/ml) or catalase (100 units/ml) added either singly or simultaneously. DFO and DTPA are chelators of transition metals known to inhibit metal-dependent ascorbate oxidation [21–23]; DFO is very effective at slowing iron-catalysed oxidation, and DTPA is effective against iron- and copper-catalysed oxidation [22]. None of these treatments was able to modify the concentration of plasma AFR. Only the change of gas flow from air to pure N_2 was able to decrease the AFR signal. After 5 min under N_2, the AFR signal was decreased by 50% (Fig. 1*b*). It should be noted that, in the absence of catalytic transition metals, or in the presence of DFO and/or DTPA, the concentration of AFR was not zero in plasma

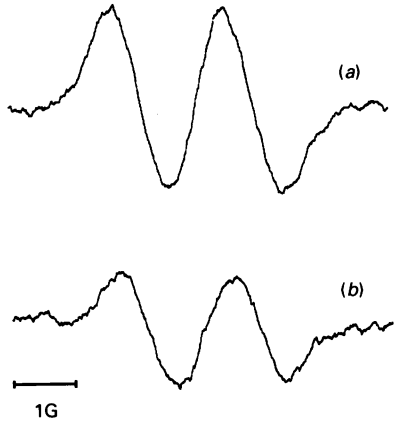

Fig. 1. E.p.r. spectrum of AFR in human plasma in air (*a*) and after 5 min under pure N_2 (*b*)

Plasma was drawn into a gas-permeable Teflon tube and spectra were recorded at 37 °C with air or N_2 as flowing gas. Spectrometer conditions were: frequency, 9.4 GHz; field modulation, 100 kHz; centre field, 3360 G; modulation amplitude, 1.0 G; power, 18 mW; time constant, 0.6 s; scan time, 0.5 min; scan range, 8 G; gain, 2.5×10^6; number of scans, 6.

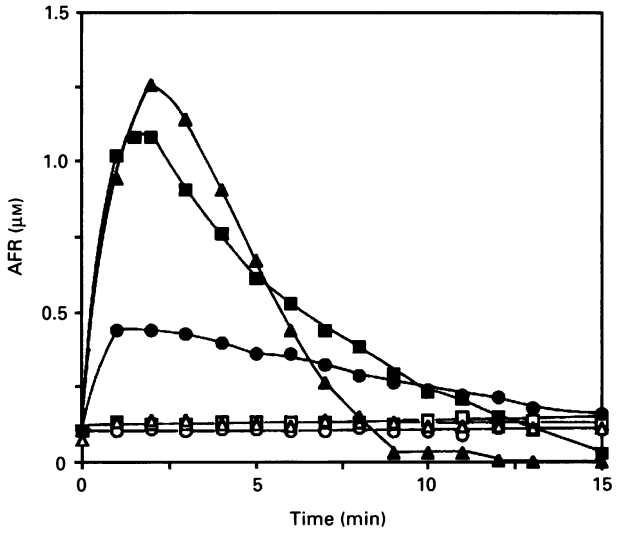

Fig. 2. Time course of AFR in human plasma after iron loading

Plasma was treated with 0 (○), 10 (△), 30 (□), 45 (●), 50 (■) and 70 (▲) μM-Fe^{2+}. The sample was drawn into a gas-permeable Teflon tube and spectra were recorded at 37 °C with air as flowing gas. Spectrometer conditions were as in Fig. 1. The figure shows a typical result from plasma with 60 μM-ascorbate and an LIBC of 43 μM-Fe.

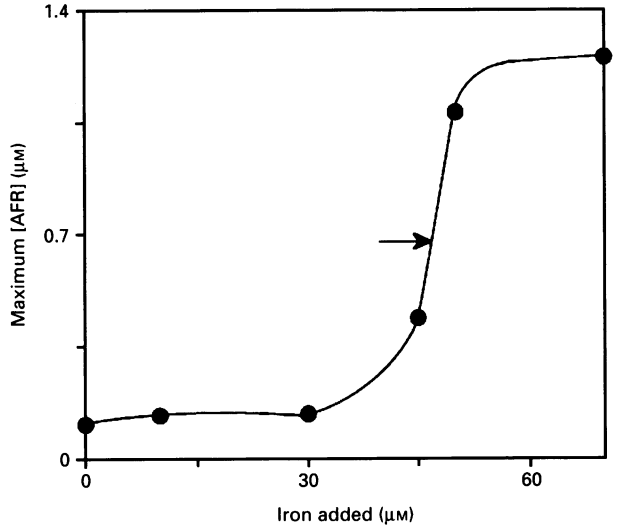

Fig. 3. Maximum concentration of AFR as a function of iron concentration

The plot was obtained from data of Fig. 2. The maximum concentration of AFR was generally achieved 2 min after the addition of iron. The arrow indicates the iron concentration producing 50% of the increase in AFR, referred to as the critical iron concentration.

or in metal-free solutions at neutral pH. The question of whether ascorbate auto-oxidizes at neutral pH in the absence of transition metals has been a matter of debate [15,21,24]. However, owing to the presence of the AFR signal even in the presence of metal ion chelators, ascorbate oxidation has been attributed by Buettner [21] to true air auto-oxidation. In plasma, the inability of SOD, catalase and metal chelators to eliminate the AFR signal suggests a similar hypothesis, although the complexity of plasma makes it impossible to exclude other oxidative reactions completely. The hypothesis of air auto-oxidation is consistent with the observation that plasma AFR was unaffected by DFO, DTPA, SOD and catalase, but was consistently decreased by N_2.

Table 1. Comparison between plasma iron-binding capacity and critical iron concentration as determined by the rise in AFR

The critical [Fe^{2+}] is the concentration of added Fe^{2+} producing 50% of the maximal AFR rise.

Donor	TIBC (μM)	LIBC (μM)	Transferrin saturation (%)	Critical [Fe^{2+}] (μM)
A	67.7	56.2	17	51.0
B	51.7	33.9	34	35.0
C	61.0	48.5	20	44.6
D	53.6	43.0	20	45.0
E	58.2	36.4	37	45.0
F	57.8	37.3	35	40.0
Mean ± S.D.	58.3 ± 5.2	42.6 ± 7.7	27.2 ± 8.3	43.4 ± 4.9

Since dehydroascorbate can be reduced by the action of dehydroascorbate reductase, an enzyme that is present in blood cells [25], we studied the effects on the plasma AFR signal of different amounts of erythrocytes. The addition of erythrocytes at a concentration exceeding 40% (v/v) was able to eliminate the AFR signal completely.

Iron increases the level of plasma AFR only when its concentration exceeds the plasma LIBC

Fig. 2 shows the effects of Fe^{2+} treatment on the plasma AFR concentration. Iron added to the plasma did not affect the AFR level up to 30 μM-Fe^{2+}. At 45 μM-Fe^{2+}, AFR rose to about 0.40 μM and, at higher concentrations, increased rapidly to reach about 10–12 times the control value in 2–3 min. After the addition of 50 μM-Fe^{2+} the maximum AFR concentration was 1.4 ± 0.5 μM (mean ± S.D., $n = 20$). As shown in Fig. 2, when plasma was treated with 50 μM- or 70 μM-Fe^{2+} the AFR decreased immediately after the initial rise and was undetectable after 10–20 min. Fig. 3 plots the maximum concentration of AFR as a function of exogenous added iron. This clearly indicates that the AFR concentration rises rapidly above 40 μM-Fe^{2+} and reaches a constant value above 50 μM-Fe^{2+}. The iron concentration producing 50% of the AFR increase (indicated by the arrow in Fig. 3) was chosen to describe ascorbate reactivity in iron-loaded plasma (hereafter referred to as the critical iron concentration).

Any iron added to plasma may initially be expected to be bound by transferrin [26,27], a carrier molecule that is usually only 20–30% iron-saturated in healthy subjects. Transferrin is the only plasma component which is able to store iron in a safe form (i.e. in a form that cannot catalyse free-radical-mediated oxidative processes [27]). To test whether plasma LIBC was related to the critical iron concentration, these two parameters were measured simultaneously in six normal donors. Interestingly, as Table 1 shows, the LIBC and critical iron concentration were comparable.

Comparison of iron-induced ascorbate oxidation in plasma and in an iso-osmotic phosphate buffer

In this study we chose to compare the effects of Fe^{2+} on plasma ascorbate with those produced in an iso-osmotic phosphate buffer containing 50 μM-ascorbate. The iron form added was Fe^{2+}, but in both media it is rapidly oxidized to Fe^{3+}. Plasma can oxidize iron to Fe^{3+} by ceruloplasmin ferroxidase activity [28,29], and phosphate buffers can catalyse the auto-oxidation of Fe^{2+}. As reported by Lambeth *et al.* [30], about 90% of Fe^{2+} is oxidized to Fe^{3+} after 5 min in 10 mM-sodium phosphate, pH 7.4, buffer.

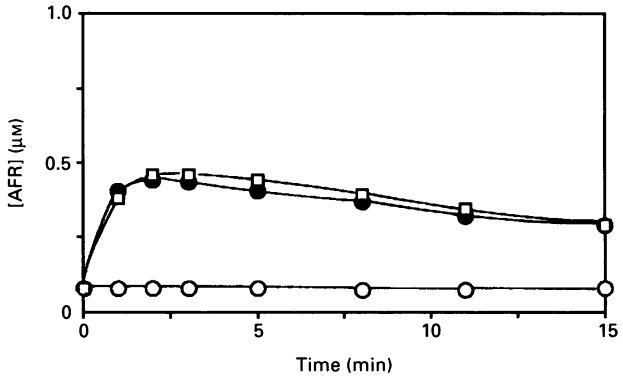

Fig. 4. Time course of AFR production in ascorbate/iso-osmotic phosphate buffer after iron loading

At the beginning of the experiment the ascorbate concentration was 50 μM. Spectra were recorded at 37 °C after the addition of 0 (○), 10 (●) or 50 (□) μM-Fe^{2+}. Samples were drawn into a gas-permeable Teflon tube and spectra were recorded with air as flowing gas. Spectrometer conditions were as in Fig. 1.

Table 2. Effect of iron loading on the rate of ascorbate oxidation in plasma and in 50 μM-ascorbate/iso-osmotic phosphate buffer

Results are mean values ± S.D. (n = 5).

Ascorbate	[Fe^{2+}] (μM)	Rate of ascorbate loss (μM/min)	Residual ascorbate after 10 min (%)
Plasma	0	0.27±0.14	99±3
	10	0.29±0.22	96±3
	50	3.60±0.33	45±6
Ascorbate solution	0	0.04±0.01	99.2±0.2
	10	0.18±0.05	96.4±1.0
	25	0.31±0.06	93.8±1.2
	50	0.63±0.05	87.4±1.2
	75	0.69±0.05	86.2±1.0
	100	0.75±0.05	85.0±1.0
	50+1 mM-EDTA	10.30±0.10	0

The concentration of AFR in the iso-osmotic phosphate buffer in the absence of contaminating metal ions or in the presence of metal chelators (DFO, DTPA) was about 80 nM, in good agreement to that previously found by Scarpa *et al.* [24]. Whereas the concentrations of AFR in plasma and in the iso-osmotic phosphate buffer were comparable, the behaviour of AFR after iron loading was completely different in the two media. As shown in Fig. 4, the level of AFR after the addition of 10 μM-Fe^{2+} to iso-osmotic phosphate buffer was identical to that obtained when 50 μM-Fe^{2+} was added. For both iron concentrations, the AFR level did not decrease to zero in 15 min as observed in iron-overloaded plasma (compare Figs. 2 and 4). These results suggest that iron added to an iso-osmotic phosphate buffer produces a steady-state level of AFR that is independent of the metal concentration. Since the rate constant of disproportionation [10] is sufficiently high (5.0×10^5 $M^{-1} \cdot s^{-1}$), the steady-state level of AFR observed in Fig. 4 probably reflects an equilibrium between slow ascorbate oxidation and the disproportionation reaction.

Recently, Burkitt & Gilbert [23] reported that Fe^{3+} complexes were able to rapidly oxidize the ascorbate in buffer solutions, as shown by the increase in the AFR concentration. In their study the maximum concentration of the radical was reached within 10–20 ms of mixing. The rapid formation of AFR reflects the high rate of ascorbate oxidation when iron is added in a complexed form. In our study, the increase in AFR concentration in plasma and in iso-osmotic phosphate buffer after iron loading was less than that reported by Burkitt & Gilbert [23]. This may reflect the slower oxidation rate of ascorbate owing to the different forms of iron ligands (i.e. plasma iron-binding components and phosphate in our study, and strong iron chelators in that of Burkitt & Gilbert [23]). When we used a peristaltic pump to obtain a more rapid sampling we did not detect high levels of AFR in plasma, and the time course of AFR formation was similar to that reported in Fig. 2. Rapid sampling allowed spectra to be collected 30 s after mixing.

In order to investigate ascorbate–iron reactivity further, ascorbate loss was measured in plasma and in iso-osmotic phosphate buffer. Table 2 shows the rates of plasma ascorbate loss after the addition of 0, 10 and 50 μM-iron. These concentrations were chosen because the iron in excess of the LIBC was about 10 μM. As shown in Table 2, the addition of 10 μM-Fe^{2+} increased the rate of ascorbate oxidation only very slightly, whereas the addition of 10 μM-Fe^{2+} above the LIBC produced massive loss and the plasma ascorbate was exhausted after 20 min. The complete oxidation of ascorbate in iron-overloaded plasma can explain the loss of AFR observed in Fig. 3.

The addition of iron to the ascorbate in iso-osmotic phosphate buffer increased the rate of ascorbate oxidation above that due to air auto-oxidation (Table 2). Although at higher iron concentrations ascorbate oxidation increased further, even at 100 μM-Fe^{2+} the oxidation rate was considerably less than in iron-overloaded plasma (Table 2).

To explain the rapid ascorbate oxidation in iron-overloaded plasma, it should be remembered that iron is susceptible to ascorbate reduction, but ceruloplasmin ferroxidase activity can promote its oxidation. The rapid Fe^{2+}/Fe^{3+} cycling may be a reason for the rapid loss of ascorbate observed. A second reason for the rapid ascorbate loss in iron-overloaded plasma may be the catalytic activity of iron ligands. It is known that any ligand can alter the metal's reactivity with ascorbate [23]. Lastly, it should be considered that in the reaction of complexed iron with ascorbate, reactive oxygen species (for example the superoxide anion and/or H_2O_2) can be produced, which can further increase ascorbate loss.

Iron chelators produce similar effects on AFR in iron-overloaded plasma and in phosphate buffer

Fig. 5 shows the effects of iron chelators on the concentration of AFR in iron-overloaded plasma (Fig. 5*a*) and in 50 μM-ascorbate/iso-osmotic phosphate buffer (Fig. 5*b*). Chelators were added at 1 mM concentration before the addition of 50 μM-Fe^{2+}. As shown in Fig. 5, DFO and DTPA completely prevented the iron-dependent AFR increase in both iron-overloaded plasma and iron-treated ascorbate solution. By contrast, EDTA promoted very rapid ascorbate oxidation in both media (Table 2), as suggested by the disappearance of AFR. In Fig. 5, the maximum level of AFR is underestimated on account of the slow sampling technique employed in this study (for this reason, the initial AFR rise in samples containing EDTA is not marked in Fig. 5). The rapid ascorbate loss both in iron-overloaded plasma and in ascorbate solution in the presence of EDTA can be explained by the ability of Fe–EDTA complex not only to interact with ascorbate but also to promote $OH^{\cdot}$ generation even in the absence of exogenously added H_2O_2 [23]. Since ascorbate reacts with $OH^{\cdot}$ [20], its production can further promote the loss of ascorbate.

The citrate–iron complex can be reduced by ascorbate but, unlike EDTA, it is a poor producer of $OH^{\cdot}$ unless exogenous

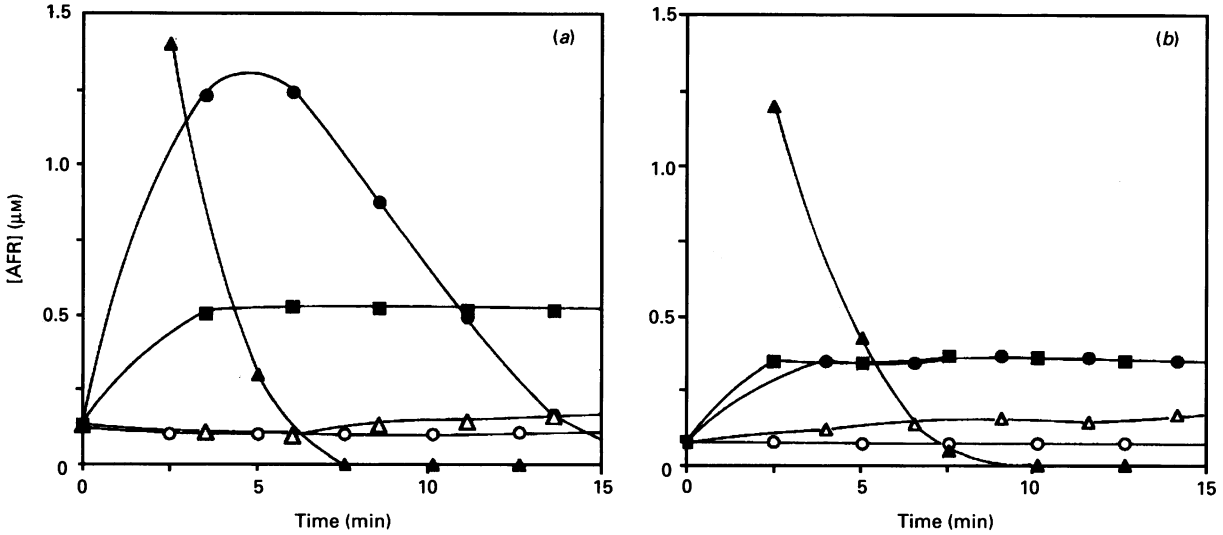

Fig. 5. Effects of iron chelators on the time course of AFR production in iron-overloaded plasma (*a*) and in 50 μM-Fe^{2+}/ascorbate/iso-osmotic phosphate buffer (*b*)

The AFR concentration was measured at 37 °C in the absence of chelators (●), and in the presence of DFO (○), DTPA (△), citrate (■) or EDTA (▲). Plasma was iron-overloaded with 50 μM-Fe^{2+}. The chelator was added at 1 mM, before the addition of iron. Samples were drawn into a gas-permeable Teflon tube and spectra were recorded with air as the flowing gas. Spectrometer conditions were as in Fig. 1.

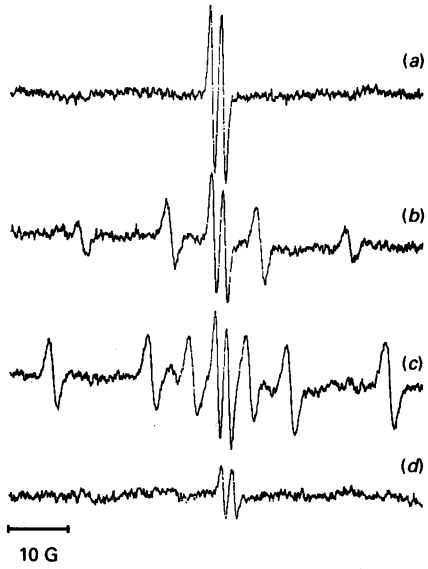

Fig. 6. E.p.r. spectra of DMPO radical adducts from iron-overloaded plasma at 37 °C

Reagent concentrations in plasma were: (*a*) 50 μM-Fe^{2+} and 0.1 M-DMPO; (*b*) 50 μM-Fe^{2+}, 0.1 M-DMPO and 1 mM-EDTA; (*c*) 50 μM-Fe^{2+}, 0.1 M-DMPO, 1 mM-EDTA and 0.14 M-DMSO; (*d*) 50 μM-Fe^{2+}, 0.1 M-DMPO and 300 μM-H_2O_2. Samples were drawn into a gas-permeable Teflon tube and spectra were recorded with air as the flowing gas. Spectrometer conditions were as in Fig. 1, except: scan time, 3 min; scan range, 70 G; gain, 8×10^5; number of scans, 1.

H_2O_2 is added [23]. In iron-overloaded plasma, citrate produced a smaller increase in AFR than that observed in plasma not loaded with iron, and the signal did not decrease rapidly (Fig. 5*a*). The behaviour of AFR indicates that the presence of citrate produces less ascorbate oxidation than does iron alone and suggests that iron is shifted from the physiological ligands to form a citrate–Fe^{3+} complex. As shown in Fig. 5(*b*), the behaviour of AFR in the ascorbate/iso-osmotic phosphate buffer containing 50 μM-Fe^{2+} was not modified by the addition of citrate.

A comparison of Figs. 5(*a*) and 5(*b*) clearly indicates that the different behaviour of AFR in plasma and in ascorbate/iso-osmotic phosphate buffer was eliminated after both iron overloading and the addition of iron chelators. These results suggest that exogenous chelators successfully compete for iron with plasma ligands and confirm that the peculiar reactivity of ascorbate/iron in plasma is due mainly to endogenous ligands.

There is no spin-trapping evidence for hydroxyl radical formation in iron-overloaded plasma

It is known that the reduction of chelated iron by ascorbate can catalyse the formation of OH• [20,23]. The reaction leading to the production of OH• is known as the Fenton reaction, and the occurrence in biological systems of this reaction is a matter of debate [31]. OH• is highly reactive (diffusion-controlled reactivity) and for this reason is believed to be an important cause of damage in several diseases [2,20,31]. Although plasma that is not loaded with iron cannot catalyse the Fenton reaction [4], iron-overloaded plasma has some of the prerequisites for being a source of OH•. This is because: (i) iron in excess of the transferrin-binding capacity is complexed with plasma components in a reactive form (e.g. ascorbate reactivity, Fig. 2 and Table 2), and (ii) plasma ferroxidase activity, in combination with ascorbate reduction, supplies continuous Fe^{3+}/Fe^{2+} cycling.

We used the spin-trapping technique to test if OH• could be formed in iron-overloaded plasma. Under appropriate conditions this technique confirms OH• formation [32]. We utilized two spin traps: water-soluble DMPO and lipid-soluble PBN. Fig. 6(*a*) shows the e.p.r. spectrum of plasma treated with 0.1 M-DMPO and 50 μM-Fe^{2+}. AFR was detected without any evidence of trapped radical species. Nor was the formation of any adduct observed when PBN was used as spin-trapping agent.

The lack of detectable OH• production in iron-overloaded plasma could be due to an insufficient production of H_2O_2 (possibly removed by catalase present in plasma), or to plasma ferroxidase activity, which could oxidize Fe^{2+} before it is able to participate in a Fenton reaction. In order to test these hypotheses, plasma was treated with H_2O_2 and/or with azide. Azide can inhibit both ferroxidase and catalase activities. H_2O_2 was added to iron-overloaded plasma in the 50–300 μM range in the presence

of DMPO or PBN. Even this did not lead to the formation of radical adducts, and when high concentrations of H_2O_2 were added, the effect was to bring about a more rapid decrease in the concentration of AFR (Fig. 6*d*). The treatment of plasma with azide (100 μM) with or without the addition of 300 μM-H_2O_2 also failed to produce any spin-trap adduct.

On the other hand, the lack of detectable OH$^\cdot$ production by the spin traps even in the presence of azide and/or exogenous H_2O_2 suggests that any iron in excess of the amount bound by transferrin is not in a chelated form that allows the formation of OH$^\cdot$. One consequence of this hypothesis is that the addition of a chelator, such as EDTA, could shift iron from the physiological ligands to form EDTA–Fe^{3+}, thus permitting the Fenton reaction. To test this possibility, EDTA was added to iron-overloaded plasma containing DMPO. As shown in Fig. 6(*b*), 1 mM-EDTA induced the formation of a DMPO adduct with hyperfine splitting constants ($a_H = a_N = 14.9$ G) characteristic of 5,5-dimethyl-2-hydroxyl-1-pyrrolidinyloxyl (DMPO-OH) [33]. This adduct may be due either to the trapping of a true OH$^\cdot$ or to the decomposition of DMPO-OH [33]. In the presence of 0.14 M-DMSO, OH$^\cdot$ leads to the formation of methyl radical, which can be spin-trapped as 5,5,2-trimethyl-1-pyrrolidinyloxyl (DMPO-CH_3). When the concentration of DMSO exceeds that of DMPO, DMPO-CH_3 is formed and this adduct is a more specific OH$^\cdot$ detector [33]. As shown in Fig. 6(*c*), a DMPO-CH_3 adduct ($a_H = 23.2$ and $a_N = 16.4$ G) was detected in iron-overloaded plasma when 0.14 M-DMSO and EDTA were present. In the absence of EDTA, the addition of 0.14 M-DMSO to iron-overloaded plasma did not produce the DMPO-CH_3 adduct (spectrum not shown). Figs. 6(*b*) and 6(*c*) show the simultaneous presence of DMPO adducts and AFR, thus showing that the OH$^\cdot$ can be trapped by DMPO when ascorbate is still present in plasma.

DISCUSSION

This study shows that the AFR signal can be a convenient and non-invasive tool for monitoring ascorbate oxidation in complex biological systems. In healthy subjects AFR can easily be detected in plasma, but it is undetectable in whole blood (bearing in mind the e.p.r. sensitivity, about 10^{-8} M). This is supported not only by the known dehydroascorbate reductase activity of blood cells [25], but also by the consideration that circulating plasma is exposed to oxygen partial pressure which is below that experienced in laboratory conditions.

Iron mobilization is an important physiopathological process that is expected to occur during iron transport or as a consequence of tissue damage [2,11,20]. Our results on plasma AFR after iron supplementation at concentrations below the LIBC indicate that iron is stored in a form that does not promote ascorbate oxidation. Transient ascorbate oxidation should be detectable by a rise in AFR and by a decrease in plasma ascorbate, but we did not detect any increase in the intensity of AFR (Figs. 2 and 3), and ascorbate loss was not greater than that caused by the auto-oxidation in air of untreated plasma (Table 2). The plasma iron-binding protein mainly responsible for keeping released iron in a non-reactive form is transferrin. This is suggested by a comparison between the plasma LIBC and the critical iron concentration inducing a rise in AFR. Our results agree with those on purified transferrin reported by Aruoma & Halliwell [34], who showed that apo-transferrin can give protection against an ascorbate-promoted iron-catalysed production of free radicals, but iron-loaded transferrin cannot. Moreover, our data also suggest a role for ceruloplasmin ferroxidase activity. The process of loading iron in transferrin requires 10–15 min, but ceruloplasmin has been shown [35] to accelerate the process considerably (transferrin saturation accomplished in less than 30 s). The rapid storage of iron in transferrin may explain our finding that the process occurs without transient ascorbate oxidation.

Since ascorbate is an outstanding antioxidant [3], the ability of human plasma to preserve this vitamin during iron mobilization is an important factor in defence against oxidation. This statement is in agreement with the proposal of Gutteridge [8] that the antioxidant potential of extracellular fluids is due to their ability to prevent metal complexes from participating in radical reactions.

We observed that an excess of iron in plasma as low as 5–7 μM over the LIBC produced massive ascorbate oxidation. Iron overloading and low ascorbate levels occur in a number of haematological disorders [8–11] and it would be useful to know whether a pro-oxidant role of ascorbate may be partially responsible for iron-mediated tissue damage. Iron-overloaded plasma may be ready to catalyse OH$^\cdot$ production, at least until the complete exhaustion of ascorbate. The presence of ascorbate is necessary to reduce iron and sustain the production of OH$^\cdot$ via a Fenton reaction [4,12,13]. Nevertheless, when the spin-trapping technique was used, no evidence was found in the present study for the production of OH$^\cdot$ or other free radical species. It should be stressed, however, that spin traps cannot identify OH$^\cdot$ radicals possibly formed in specific iron-binding sites where the spin-trap concentration may be low owing to the low partition of the spin trap. For this reason, the absence of trapped radicals does not conclusively prove that no free radicals are produced. This limitation is common to all of the available OH$^\cdot$ detection methods applied to complex human body fluids, because the high reactivity of OH$^\cdot$ prevents its detection far from the sites of formation. On the other hand, these postulated specific iron-binding sites do not allow very tight binding, nor are they completely inaccessible to hydrophilic compounds as suggested by the possibility of iron being detached during ultrafiltration [36] and by the accessibility to this form of iron of a very hydrophilic compound such as ascorbate.

To circumvent the problem of spin-trap availability into hydrophilic/hydrophobic sites we used two spin traps with different solubility (i.e. DMPO is water-soluble and PBN is lipid-soluble), but no evidence of adduct formation was obtained with either compound. Notably, Gutteridge *et al.* [36], using a different OH$^\cdot$ detection method, found no evidence of OH$^\cdot$ radical formation in iron-overloaded plasma from a patient with idiopathic haemochromatosis. In the present study the DMPO-OH adduct was observed only after the addition of a suitable iron chelator such as EDTA, which shifts iron from the physiological ligands to form EDTA–Fe^{3+} (Fig. 6). We conclude that, with the limitations discussed above, the form of iron present in iron-overloaded plasma seems to be unable to participate in a Fenton reaction, at least in a manner detectable by spin-trapping experiments.

The precise chemical nature of non-transferrin-bound iron in iron-overloaded plasma is not completely clear [37,38]. It has recently been suggested that the presence of this non-transferrin-bound iron may be largely due to citrate or possibly to iron–citrate–acetate complexes [37]. Our experiments addressed this question indirectly, i.e. by looking at the reactivity of ascorbate/iron in the absence or in the presence of a large excess of citrate (1 mM). The reactivity of non-transferrin-bound iron in normal plasma was found to be higher than that in citrate-loaded plasma. This complex was, in fact, a relatively poor promoter of ascorbate oxidation (Fig. 5). Thus our studies suggest that the ascorbate reactivity of iron in excess of the transferrin-binding capacity is not identical to that of the iron–citrate complex. It should be noted, however, that iron overloading in this study was obtained by adding exogenous iron, whereas in the work of

Grootveld *et al.* [37], plasma was from patients with idiopathic haemochromatosis showing iron overloading. Notably, these patients present citrate levels (0.3–0.4 mM) that are 3–6 times higher than normal values [37]. Further, even in idiopathic haemochromatosis patients, at least 50–70 % of non-transferrin-bound iron appears to be bound to plasma proteins [37], and this iron may account for the ascorbate reactivity found in our study.

In conclusion, one interesting finding arising from the present investigation is that iron-dependent damage to human plasma involves the rapid utilization of ascorbate, which occurs when iron exceeds the transferrin-binding capacity. E.p.r. spectroscopy of AFR in fresh plasma samples may provide useful information about clinical disorders of metal ion metabolism involving increased ascorbate utilization.

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