Iron speciation at physiological pH in media containing ascorbate and oxygen

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The stability of iron ascorbate solutions was investigated, under both anaerobic and aerobic conditions, with the Fe^{2+} and Fe^{3+} indicators, respectively ferrozine and mimosine, at different pH values. The species present under the differing conditions were investigated by electron paramagnetic resonance (EPR) and Mössbauer spectroscopy and by gel-filtration chromatography. At physiological pH (6·8–7·4) iron ascorbate solutions rapidly form mononuclear chelatable Fe^{3+} species as reflected by indicator studies and EPR. Mössbauer spectroscopy fails to detect any Fe^{2+} species. EPR studies show a time-dependent decrease in rhombic Fe^{3+}, particularly in oxygenated buffers, consistent with a conversion to polynuclear Fe. O_2 uptake studies show that the conversion of Fe^{2+} to Fe^{3+} in Fe–ascorbate solutions at pH > 7·0 was accompanied by rapid O_2 consumption but preceded depletion of ascorbate. Addition of high concentrations of mannitol (50–200 mM) reduces the O_2 consumption and partly stabilizes the rapidly chelatable Fe form. Gel filtration studies show that the oxidation of Fe–ascorbate solutions at pH 7·4 is accompanied by an increase in the apparent relative molecular mass of the Fe, presumably due to Fe polymer formation. These studies indicate the inherent instability of Fe–ascorbate solutions above neutral pH and clearly have important implications in the use of ascorbate in studies of Fe physiology.

Iron: Ascorbate solutions: Speciation of iron

Fe-containing media, incorporating both O_2 and ascorbate, have been widely used in studies of intestinal Fe absorption (Hyde, 1957; Brown & Justus, 1958; Manis & Schachter, 1962; Pearson & Reich, 1965; Hopping & Ruliffson, 1966; Jacobs *et al.* 1966; Helbock & Saltman, 1967; Thomson and Valberg, 1971; Acheson & Schultz, 1972; Howard & Jacobs, 1972; Sheehan, 1976; Savin & Cook, 1978; Cox & O'Donnell, 1982; Johnson *et al.* 1983; Simpson *et al.* 1986). Ascorbate and/or other dietary reducing agents are present with Fe and O_2 in the intestinal lumen. In addition, ascorbate is an ingredient of many pharmacological Fe preparations and experimental diets and is present in rodent bile (Conrad & Schade, 1968) and rodent and human gastric juice (Breidenbach *et al.* 1952; Rathbone *et al.* 1989). Ascorbate is known to have a profound enhancing effect on Fe

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absorption as measured by several different techniques (Brise & Hallberg, 1962; Conrad & Schade, 1968; Forth & Rummel, 1973; Hungerford & Linder, 1983; Marx & Stiekman, 1985). This effect has usually been attributed to a combination of reducing and chelating effects of ascorbate (Gorman & Clydesdale, 1983). Reduction is believed to prevent inhibitory effects of Fe^{3+} ligands such as phytic acid and hydroxides while chelation is thought to maintain Fe in a soluble and, therefore, available form. Recently, it was suggested that separate mechanisms of intestinal absorption might exist for different luminal Fe species (Peters *et al.* 1988; Simpson *et al.* 1989; Simpson & Peters, 1990).

We have recently investigated Fe species present in mouse stomach and duodenal lumen (Simpson & Peters, 1990). Others have investigated the importance of luminal reduction of Fe for Fe absorption (Wien & Van Campen, 1991). Attempts were made to relate the species observed to model Fe solutions, Fe–nitrilotriacetate (NTA) or Fe–ascorbate, as used in many studies of Fe absorption mechanisms. These studies also noted apparent instability of Fe–ascorbate solutions, not associated with Fe precipitation, at physiological pH. In the present paper we have further investigated and characterized the Fe species present in Fe–ascorbate solutions over a pH range relevant to Fe absorption in the small intestine. Knowledge of ageing and disposition of Fe in these solutions is essential for the interpretation of past and future studies of Fe absorption from Fe–ascorbate solutions as well as the understanding of dietary Fe chemistry in the intestinal lumen.

METHODS AND MATERIALS

Materials

$FeSO_4$, L-ascorbic acid (sodium salt), HEPES, mannitol, ferrozine and mimosine were all obtained from Sigma Chemical Co. Ltd. $FeCl_3$. $6H_2O$, NaCl and sodium acetate were Analar grade obtained from BDH Chemicals Ltd, Poole, Dorset. Sephadex G50 was from Pharmacia, $^{59}FeCl_3$ was from Amersham International (UK) and $^{57}FeCl_3$ was from The Isotope Separation Unit, AERE, Harwell, Oxon.

Spectrophotometric studies

Fe–ascorbate (1:20, v/v) solutions (100 μmol Fe/l, 2 mmol ascorbate/l), at pH 6·4–7·4 (20 mM-HEPES buffer containing 100 mmol NaCl/l and 100 mmol mannitol/l) and at pH 4·1–5·5 (100 mmol acetate buffer/l) were prepared by mixing 10 mmol $FeCl_3$/l in 10 mmol HCl/l or fresh 10 mmol $FeSO_4$/l in water and double strength HEPES–mannitol–NaCl buffer and were incubated at 37° for up to 60 min. At various time intervals, portions were removed and mixed with an equal volume of either 2 mmol ferrozine/l or 2 mmol mimosine/l and the absorbance (562 and 450 nm respectively) determined immediately in a Perkin Elmer 557 dual-beam spectrophotometer.

Electron paramagnetic resonance (EPR) spectroscopy

X Band EPR spectra (9300 MHz, using 100 kHz field modulation) were obtained with a Varian E9 spectrometer or a Bruker ESP300 spectrometer. Samples were gas-cooled to about 100 K. Spectra were baseline-corrected by subtraction of a cavity spectrum or water–buffer under identical conditions. Spectrometer settings are indicated in individual figure legends.

Mössbauer spectroscopy

Spectra were recorded at 77 K using 90% enriched $^{57}FeCl_3$–ascorbate (1:20, v/v) solutions at pH 6·8 and 7·4, as described previously, in a conventional constant-acceleration spectrometer, using a ^{57}Co in Rh source.

Gel-filtration analysis of Fe–ascorbate solutions

The apparent relative molecular mass of the species in fresh and aged Fe–ascorbate solutions was determined with a Sephadex G50 (10 × 125 mm) column, equilibrated and eluted with mannitol–NaCl–HEPES, pH 7·4 or pH 6·4 at 21°. Fractions (1 ml) were collected and radioactivity measured for ^{59}Fe in an LKB gamma-7000 counter. The column was calibrated with ferric-EDTA, vitamin B_{12}, aprotinin and cytochrome c.

Rate of O_2 consumption

O_2 consumption was measured with a Rank Pt/Ag electrode, with 0·6 V voltage applied across the half-cells. After calibration, 1·94 ml buffer (mannitol–NaCl–HEPES, pH 6·4–7·4) was saturated to 95% with O_2 and 60 μl Fe–ascorbate (1:20, v/v; pH 6·4–7·4) added with a Hamilton syringe. The subsequent consumption of O_2 was recorded as a function of time.

RESULTS

Visible spectroscopic studies

The reaction between the chelator ferrozine (which gives a strong absorbance (562 nm) with Fe^{2+} but not Fe^{3+} (Gibbs, 1976)) and $FeCl_3$ and Fe^{2+}–ascorbate (1:20, v/v) solutions shows an instantaneous formation of Fe^{2+}–ferrozine complex (Fig. 1). This initial phase is followed by a slower formation of Fe^{2+}–ferrozine suggesting that the Fe in the Fe–ascorbate mixture is present in more than one species, and that these forms can be discriminated by their rate of delivery of Fe to ferrozine. As Fe–ascorbate solutions are allowed to age, the amount of Fe rapidly available for chelation by ferrozine declines. Rapidly-available Fe may be Fe^{2+} or an easily-reduced Fe^{3+} complex (Simpson & Peters, 1990), slowly available Fe may be a more slowly reducible Fe^{3+} species (Simpson & Peters, 1990).

Fig. 2 shows the extent of the initial, rapid phase as a function of incubation time of the Fe–ascorbate mixture at different pH, before addition of ferrozine. At pH > 7·0 there is a pronounced decline in this species suggesting that, with incubation, the Fe is converted to another less-available species. Equivalent results were obtained when 10 mM-$FeSO_4$ was substituted for $FeCl_3$ in the preparation of the mixtures.

Fig. 3 shows that omitting NaCl or mannitol accelerates the ageing of the Fe–ascorbate mixture. Omission of mannitol had a particularly marked effect (Fig. 3). These and previously published results (Simpson & Peters, 1987) suggest that HEPES, mannitol, NaCl or ferrozine are not responsible for the ageing of the solutions. The pH dependence of the ferrozine-available Fe^{2+}, measured after 5 min, shows a third-order dependence on OH^- concentration, suggesting that the formation of iron hydroxide may be important (Simpson & Peters, 1987). Changing the ascorbate concentration (Fig. 4) at pH 7·4 indicates that a higher ascorbate concentration results in a higher end-point level of ferrozine-available Fe. Omission of ascorbate (fresh $FeSO_4$ solutions used as Fe source) resulted in complete disappearance of ferrozine-available Fe by approximately 30 min. The initial rate of loss of ferrozine-available Fe is similar to that with ascorbate present. However, the time-courses (as in Fig. 1) show no slowly ferrozine-available Fe.

Similar experiments to those shown in Fig. 1, substituting the Fe^{3+} chelator mimosine for ferrozine, reveal quantitatively comparable observations, suggesting that Fe^{2+} and Fe^{3+} are in rapid equilibrium in these solutions. Studies of the ferrozine- and mimosine-chelatable Fe in Fe–ascorbate solutions pre-incubated at pH values in the range 4·1–5·5 showed that Fe remains 100% ferrozine-available at pH 4·5 and 5·5 as well as at pH 6·8. Fe rapidly available to mimosine, however, decreases from 100% at pH 6·8 to 0% at pH 4·5, indicating a change in the nature of the readily chelatable Fe, from Fe in equilibrium between oxidation states Fe^{2+} and Fe^{3+}, to Fe in oxidation state Fe^{2+} only. Below pH 4·5,

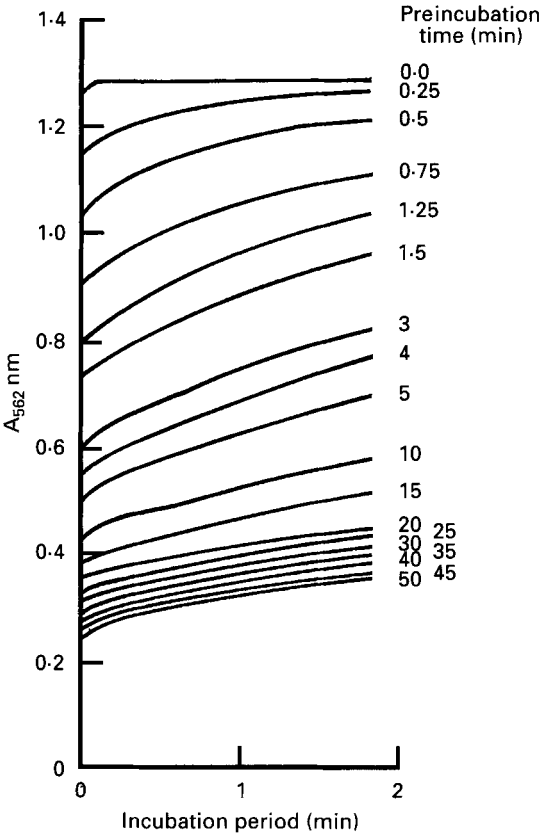

Fig. 1. Ferrozine–ferrous ion complex formation in an iron–ascorbate solution. $FeCl_3$–ascorbate mixtures were prepared and at preincubation time t 0, prewarmed (37°) buffer solutions were added to give a solution of 100 μmol Fe/l, 2 mmol sodium ascorbate/l, 0·1 mol mannitol/l, 20 mmol HEPES/l, pH 7·4 (air equilibrated). After various preincubation times at 37° (as indicated), 400 μl of the mixture was added to 400 μl ferrozine (2 mmol/l) and absorbance ($A_{562\,nm}$) recording commenced. For details of procedures, see p. 158.

Fe^{2+} and Fe^{3+} can be completely distinguished by the two chelators; above pH 5·9, they are indistinguishable on the time-scale of this method.

Gel filtration

Studies of fresh $FeCl_3$–ascorbate (1:20, v/v) solutions at pH 7·4 and solutions at pH 7·4 incubated at 37° for 1 h (Fig. 5) show that the ageing of Fe–ascorbate solutions at pH 7·4 involves an apparent increase in the relative molecular mass of the Fe-containing species from approximately 500 to 1500. The lower recovery of Fe at short incubation periods may be due to binding of Fe^{3+} ions to the multiple OH^- groups on the column matrix or to trace amounts of acid groups which are present in Sephadex columns. Fresh solutions prepared at pH 6·4 show similar apparent relative molecular masses of those of fresh solutions at pH 7·4.

EPR spectra

$FeCl_3$–ascorbate (1:20, v/v) solutions show that rhombic Fe^{3+} is present in these solutions as indicated by the g_{eff} 4·3 signal (Fig. 6). Studies of spectra of a variety of Fe^{3+} complexes (NTA, EDTA, citrate, albumin, hydroxypyridinone complexes, transferrin and

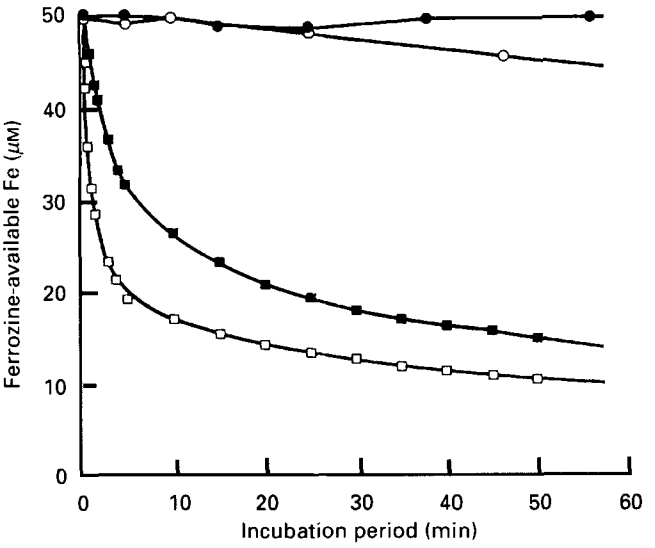

Fig. 2. Rapidly ferrozine-chelatable iron in Fe–ascorbate solutions. Initial ferrozine–ferrous ion concentrations were obtained from experiments shown in Fig. 1 and are plotted for incubations performed at various pH values: (●), 6·8; (○), 7·0; (■), 7·2; (□), 7·4. For details of procedures, see p. 158.

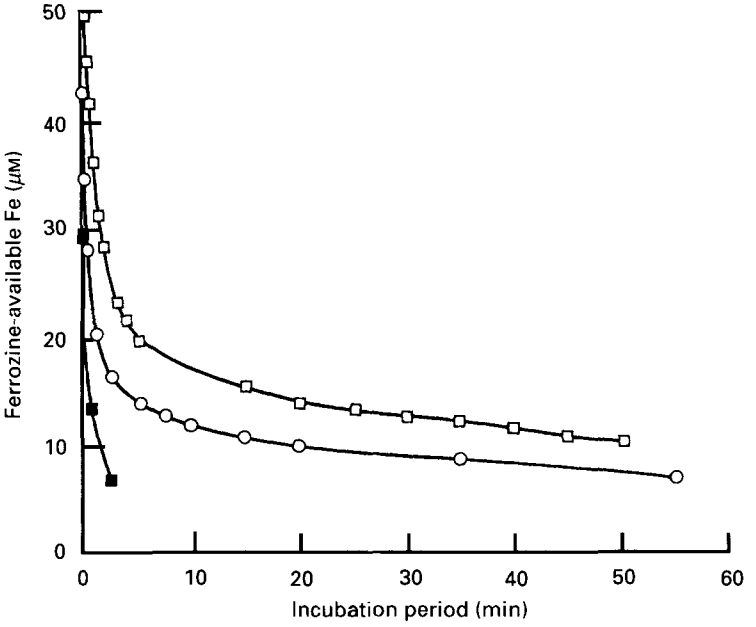

Fig. 3. Effect of medium composition on loss of ferrozine-available iron from Fe–ascorbate solutions. Rapidly ferrozine-chelatable Fe was determined as shown in Fig. 2 (pH 7·4) in Fe–ascorbate solutions shown in Fig. 2 (□) or lacking mannitol (■) or lacking NaCl (○). For details of procedures, see p. 158.

ferrioxamine) revealed g_{eff} 4·3 signals with varying line shapes. Double integration of these spectra showed a constant relationship between peak area and complex concentration for these many complexes, provided that chelator was present in excess. Peak height could also be used as an estimate of concentration where line shape variations were negligible.

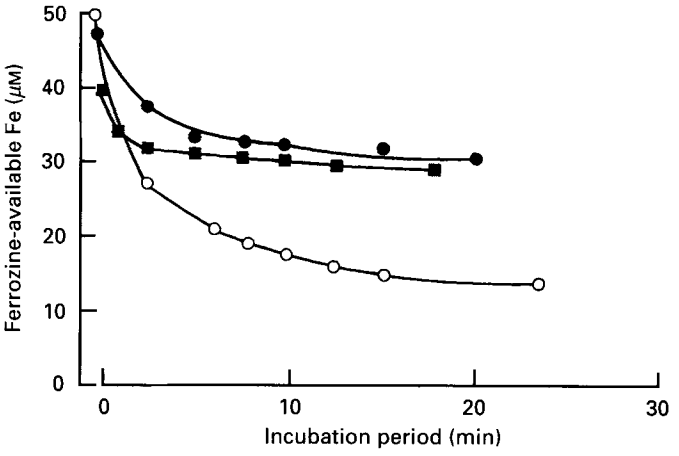

Fig. 4. Effect of medium ascorbate concentrations on rapidly ferrozine-available iron in Fe–ascorbate solutions. Ascorbate concentrations were (mmol/l): 1 (○), 5 (■), 10 (●). For details of procedures, see p. 158 and Fig. 1.

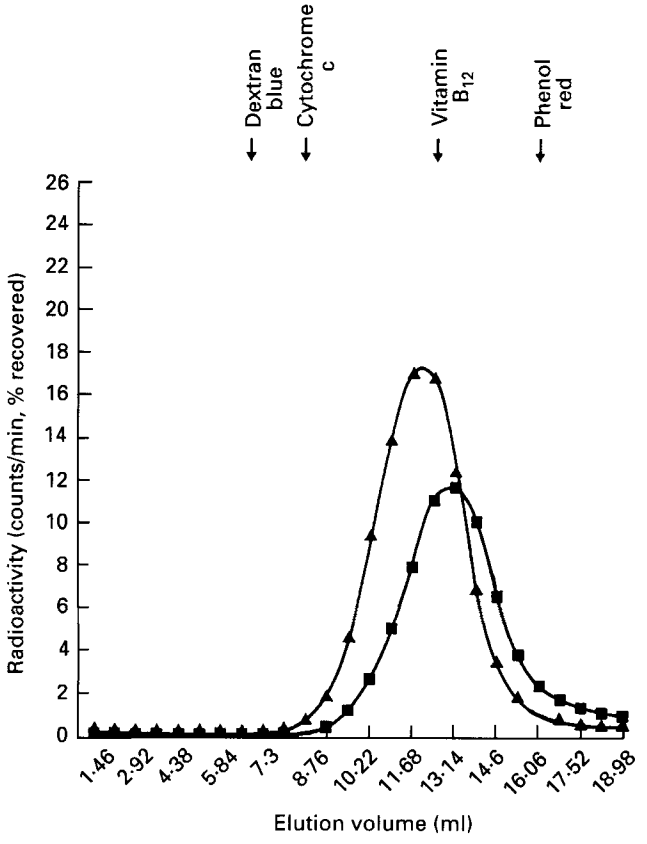

Fig. 5. Gel filtration analysis of iron in Fe–ascorbate mixtures. ^{59}Fe–ascorbate mixture, 0·5 ml, was prepared as in Fig. 1 except that $^{59}FeCl_3$ in 10 mmol HCl/l was mixed with $FeCl_3$ before addition of ascorbate, and was applied to a Sephadex G50 column equilibrated and eluted as described on p. 159. Fe–ascorbate solutions were preincubated at pH 7·4 for 4·5 min (▲) or for 2 h (■). ^{59}Fe recoveries from the column were 78 and 115%, respectively.

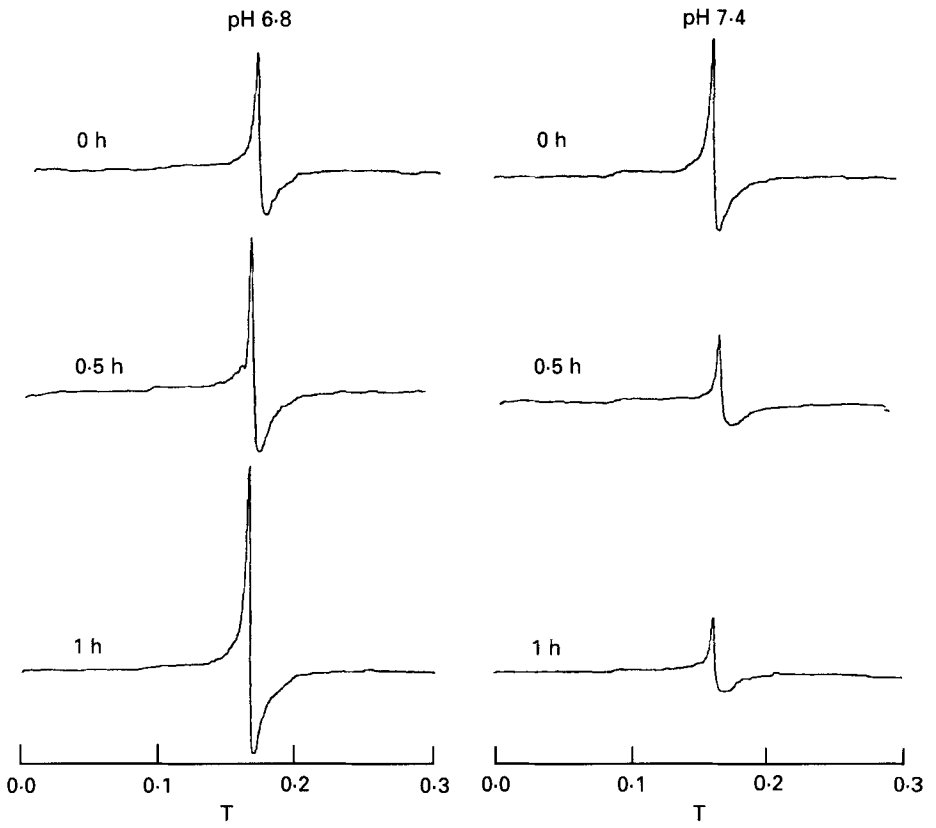

Fig. 6. Electron paramagnetic resonance (EPR) spectra of $FeCl_3$–ascorbate (1:20, v/v) solutions. Fe–ascorbate samples, 0·3 ml (in EPR tubes) were prepared as in Fig. 1, incubated at 37° and frozen at the times indicated. Spectrometer settings were microwave frequency 9·11 GHz, microwave power 20 mW, 100 kHz modulation, modulation amplitude 8, sweep time 32 min, temperature 103 K, time constant 0·3 s.

Close examination of the spectra from Fe–ascorbate mixtures revealed only minor line shape changes during incubations such as those shown in Fig. 6. Fig. 7 shows how the peak-to-peak height of this signal varies with period of incubation in a variety of Fe–ascorbate mixtures and incubation conditions. This is presumed to reflect changes in the concentration of rhombic Fe in oxidation state Fe^{3+} within the mixtures.

An estimate of the proportion of rhombic Fe in oxidation state Fe^{3+} can be made by comparing g_{eff} 4·3 peak heights with the signal from a standard solution of $FeNTA_2$, and this indicated that the maximal peak height observed (pH 6·8 after 1 h incubation with air present) represented approximately 66% of total Fe. A similar value is obtained if the double integral of the g_{eff} 4·3 signal is compared with the double integral of the other mononuclear Fe^{3+} standards (transferrin, mimosine). The decline in signal (pH 7·0–7·4) indicates either reduction to Fe^{2+}, or formation of an Fe^{3+} polymeric species with strong coupling between the centres (Weir *et al.* 1985). The observation that the decline corresponds with Fe being no longer available for rapid chelation by ferrozine suggests that the latter possibility is correct. At pH 6·8, steady formation of rhombic Fe^{3+} is observed. A similar study of Fe–ascorbate solutions at pH 7·4 and 6·8, but using degassed solutions incubated under N_2, shows a marked decrease in the size of the g_{eff} 4·3 peak; presumably the Fe remains as Fe^{2+} under these conditions. Subsequent exposure to air of solutions

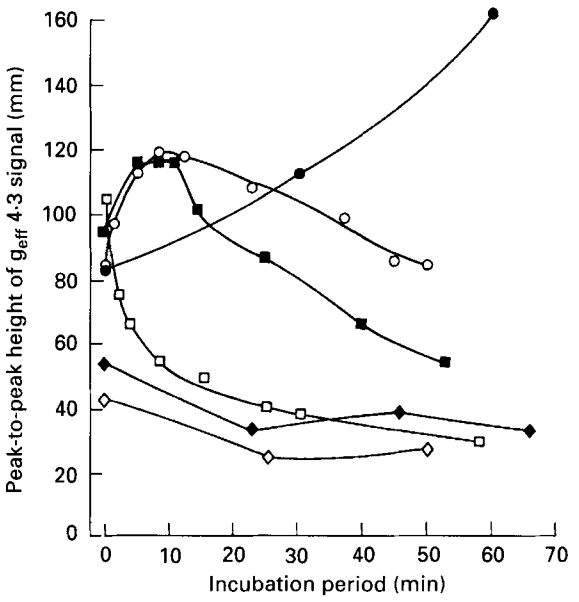

Fig. 7. Electron paramagnetic resonance (EPR) variables of Fe–ascorbate solutions. Experiments performed as in Fig. 6. Background corrected peak-to-peak height of g_{eff} 4·3 (0·17 T) signal in spectra as a function of incubation time at 37°. Samples were initially air-equilibrated (□, ■, ○, ●) or bubbled with nitrogen (◇, ◆). During the incubation sample pH was 6·8 (●, ◆), 7·0 (○), 7·2 (■) or 7·4 (□, ◇).

incubated at 37° for 1 h resulted in an increase in the peak of the signals to a value equivalent to that observed without N_2. EPR of Fe–ascorbate in HEPES buffer lacking mannitol at pH 7·4 or 6·8 revealed only a small g_{eff} 4·3 peak, compared with those shown in Fig. 6, even at 1 min after preparation (values not shown).

Mössbauer spectroscopy

The 77 K Mössbauer spectra of ^{57}Fe–ascorbate solutions, prepared with 90 %-enriched ^{57}Fe, and incubated for 1 h at pH 6·8 and 7·4 are shown in Fig. 8. The spectra have a relatively poor signal:noise value, as a result of the low Fe concentration. Both spectra are quadrupole split doublets and have been computer-fitted to obtain the Mössbauer variables. The fitted values of these variables are the same, within experimental error, for both samples, being 0·70 (SE 0·05) mm/s for the quadrupole splitting and 0·46 (SE 0·03) mm/s for the chemical shift. These variables indicate that the Fe is Fe^{3+} and are consistent with oxide or hydroxide ligands. There is no evidence for the presence of any significant Fe^{2+} component, which would show a much larger quadrupole splitting.

O_2 consumption studies

The initial rate of consumption of O_2 by (1:20, v/v) $FeSO_4$–ascorbate solutions declines markedly with decrease in pH from 7·4 to 6·4 (Fig. 9). Experiments in which $FeSO_4$ was varied show an increase in the rate of consumption with increased $FeSO_4$ (0–400 μM) concentration at constant (2 mM) ascorbate concentration, and similarly for varying ascorbate (0–10 mM) concentrations at constant $FeSO_4$ (100 μM) concentration. Increase in either Fe or ascorbate concentrations both lead to an increase in the initial rate of O_2 consumption, suggesting that an Fe–ascorbate complex is responsible. This is consistent with previous studies at low pH (Taqui Khan & Martell, 1967). The rate of O_2 consumption

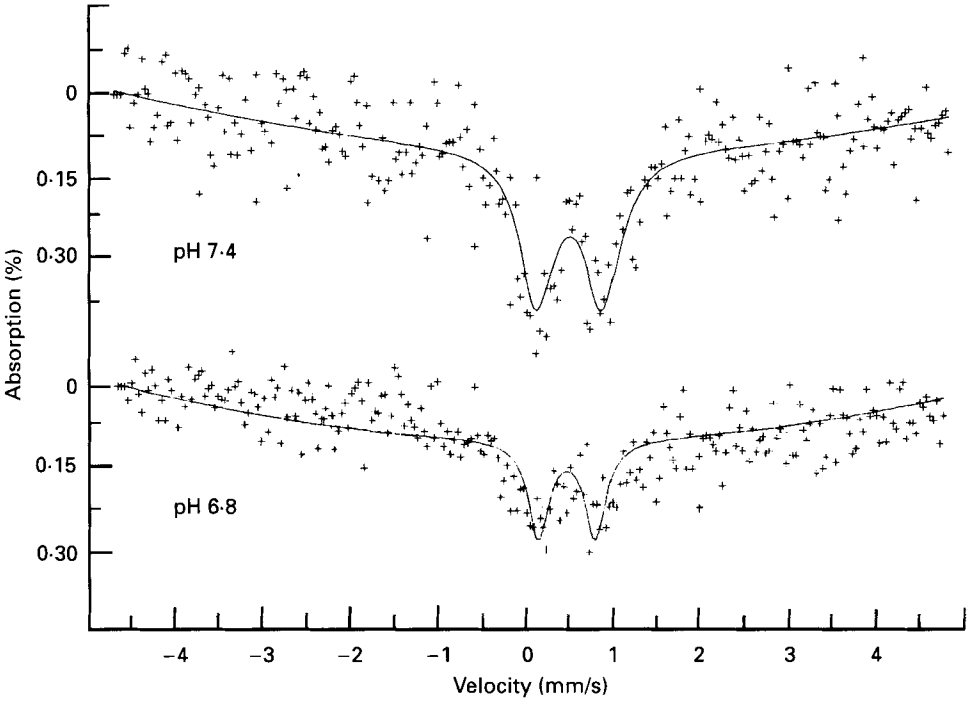

Fig. 8. The 77 K Mössbauer spectra of ^{57}Fe–ascorbate solutions prepared as described in Fig. 1 at pH 6·8 and 7·4. Incubation was for 1 h at 37°, then samples were frozen for analysis.

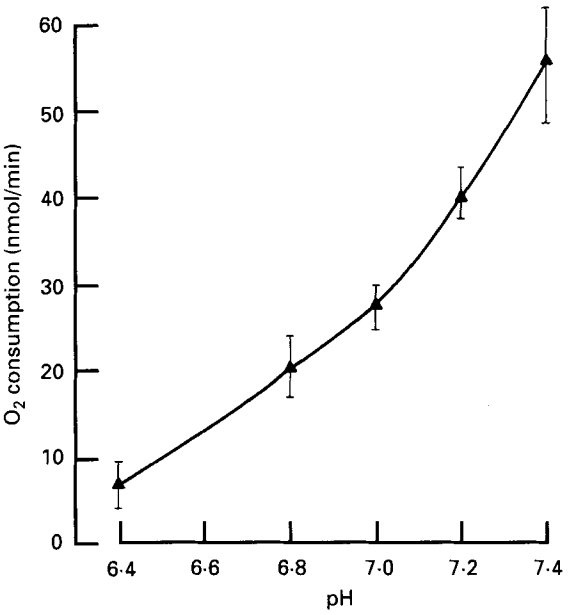

Fig. 9. Oxygen consumption by iron–ascorbate solutions. media were prepared, incubated at 37° and initial rate of O_2 consumption measured as described on pp. 158–159. Medium pH was varied and determined in solutions after O_2 consumption determination; Fe concentration was 100 μmol/l, ascorbate 2 mmol/l.

suggests that ascorbate depletion is not responsible for the Fe oxidation for short incubation periods (up to 10–20 min). The addition of superoxide dismutase (*EC* 1.15.1.1) appears to have little effect on O_2 consumption. Decreasing the initial degree of O_2 saturation of the solutions decreases the consumption rate, but this does not appear to be a first-order effect, as was previously observed (Taqui Khan & Martell, 1967). Increasing the mannitol concentration (50–200 mM) results in a decrease in the rate of consumption, which might suggest that a free radical intermediate is involved in the reaction, although mannitol may also act by binding to the Fe.

DISCUSSION

Iron species in Fe–ascorbate–O_2 mixtures

Ascorbic acid is widely used in Fe absorption studies because it is a physiological Fe reductant–chelator and it is effective at maintaining Fe in a soluble and bioavailable form (Conrad & Schade, 1968). We have concerned ourselves with changes in Fe speciation after an acid Fe–ascorbate mixture (presumed to contain Fe^{2+} ions) is neutralized with buffer to pH values in the range 6·8–7·4. This represents a model for changes occurring in the proximal regions of the small intestine and also represents changes occurring after preparation of Fe–ascorbate solutions for *in vitro* studies. Thermodynamic considerations show that the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid is favoured below pH values of approximately 6 but not at pH 7 or greater (Hungerford & Linder, 1983; Schneider, 1987), and consideration of Fe–ascorbate as an Fe^{2+}–ascorbate complex is unsatisfactory at neutral pH (Plug *et al.* 1984). Unfortunately we cannot think of Fe–ascorbate mixtures in terms of an ascorbate–dehydroascorbate couple because the dehydroascorbate ring opens irreversibly and is, therefore, not in equilibrium with its precursor (Dryhurst *et al.* 1982). Understanding the species present in Fe–ascorbate mixtures is, therefore, not possible from thermodynamic considerations alone.

Our experiments, based on kinetics of reaction with an Fe^{2+} chelator (ferrozine) (Simpson & Peters, 1990), have indicated that incubation for up to 60 min in buffered solutions (pH 6·8–7·4) causes significant changes in the nature of the Fe such that the Fe^{2+} concentration diminishes, the Fe being progressively converted to ferric species which are re-reduced on addition of the strong Fe^{2+} chelator, ferrozine. The loss is slow or negligible at pH 7 or below but is rapid and extensive at pH 7·2 or above. Rates of O_2 consumption show that in oxygenated solutions, as used frequently in *in vitro* studies of Fe absorption, depletion of ascorbate would only be a significant factor in longer incubations (approximately 1 h).

Chloride and mannitol are often used in physiological media to satisfy osmotic requirements of cells. These ligands are likely to coordinate and stabilize the Fe in its ferric form. It would appear that such Fe is still ferrozine-available, however. In the absence of chloride and of mannitol, the rate at which Fe converts to some unavailable form accelerates markedly. Likewise, the presence of ascorbate partly protects from the loss of ferrozine-available Fe.

In positive attempts to comment on the forms of Fe present in these complex solutions we have performed some gel filtration and some spectroscopic measurements. The former give a clear indication that the molecular size of one form of Fe present increases with the passage of time while the latter indicate the presence of Fe^{3+} and the absence of Fe^{2+}. The rhombic EPR signal at g_{eff} 4·3 is characteristic of monomeric high-spin Fe^{2+} (Gibson, 1980). Note, however, that it represents only a proportion of the Fe present and that its behaviour parallels that of the ferrozine-available Fe at pH 7·4. At pH 6·8 this parallelism is not present and it would appear that some form of Fe other than that represented by the

EPR is also ferrozine-available. The Fe seen by the Mössbauer experiment is also Fe^{3+}, but is not variable with pH. This is consistent with the fact that the Mössbauer spectrum results from high-spin Fe^{3+} atoms in both cases, with the coupling which affects the EPR spectrum having essentially no effect on the Mössbauer spectrum. Thus, our studies suggest that ferric ascorbate is stable as a mononuclear complex at pH 6·8 or less but at higher pH polynuclear species of limited size are rapidly formed. The data presented in the present paper demonstrate that Fe^{3+} is a major Fe species in Fe–ascorbate mixtures at neutral pH values. The demonstration of Fe^{2+} in such mixtures by addition of a powerful Fe^{2+} chelator is misleading unless the kinetics of formation of the Fe^{2+}–chelator complex are taken into account.

Implications for previous studies of intestinal Fe absorption

These investigations of iron ascorbate solutions were prompted by our previous work into the mechanism of mucosal Fe uptake using *in vitro* systems. We had attempted to interpret those studies on the assumption that Fe^{2+} ions are the principle Fe species in Fe–ascorbate solutions. Studies of liposome Fe uptake had, however, demonstrated ageing of Fe–ascorbate solutions (Simpson & Peters, 1987). The present work necessitates a re-interpretation of those studies, as well as work by other groups. It would appear that oxygenated Fe–ascorbate (at pH 7·4 and ascorbate:Fe values of 20:1 or less) contains predominantly Fe^{3+} species as mononuclear chelates and small polynuclear species. This mixture probably resembles the species present in other weak Fe^{3+}chelates, e.g. dilute Fe–NTA at low NTA:Fe values ($< 2:1$) or Fe–citrate (citrate:Fe $< 20:1$). This in turn provides an explanation for the similarity in uptake of ^{59}Fe from Fe–ascorbate solutions and Fe^{3+} chelates by *in vitro* incubated intestinal tissue (Sheehan, 1976; Cox & O'Donnell, 1982; Simpson *et al.* 1989). This similarity in uptake by tissue incubated *in vitro* may be contrasted with differences in uptake seen with Fe–ascorbate compared with Fe–NTA solutions incubated with brush-border membrane preparations or tied-off intestinal segments *in vivo*. We originally suggested that these differences could be due to differences in Fe valency (Simpson *et al.* 1986). This suggestion was clearly premature and a better explanation would be that the differences arise from differences in stability of Fe^{3+} complexes (Simpson *et al.* 1989). The fact that *in vitro* intestine sees Fe–ascorbate differently from *in vivo* intestine or isolated brush-border membrane vesicles may be attributable to the use of freshly neutralized Fe–ascorbate in the latter experiments (Simpson *et al.* 1986), while the *in vitro* studies employed somewhat aged, O_2-bubbled Fe–ascorbate solutions.

It is more difficult to re-interpret studies with Fe–ascorbate by other groups of workers as the age of the solutions was not controlled. It is tempting to suggest, however, that some of the contradictory results reported (e.g. the failure of Sheehan (1976) to demonstrate regulated uptake, in contrast to other workers) may have been due to differences in the preparation and pretreatment of the Fe–ascorbate mixtures, leading to different ^{59}Fe species being present during the experiments.

Some studies supplied evidence for persistence of significant Fe^{2+} ions in long periods of incubation (Rulifsson & Hopping, 1963). The present work shows that such evidence is misleading due to conversion of Fe^{3+} to Fe^{2+} after addition of an Fe^{2+} chelator. Given a lack of special attempts to use short incubation periods and freshly neutralized solutions (as used in our own membrane studies), the Fe present in those studies probably was predominantly small polynuclear Fe^{3+}. The findings suggest, therefore, that small polynuclear Fe species are available for uptake by intestinal mucosa, perhaps by reductive release of Fe^{2+} ions at the brush-border membrane, as has been proposed recently (Raja *et al.* 1992). This latter suggestion would seem confusing, given the presence of ascorbate,

but our work shows clearly that, at pH > 7·0, ascorbate is not an effective reducing agent for Fe^{3+} (unless a powerful Fe^{2+} chelator is present). This finding disagrees with the observations of Gorman & Clydesdale (1983) but agrees with the work of Hungerford & Linder (1983) and Schneider (1987).

Implications for Fe availability from foods and pharmaceuticals

Ascorbate is very well known for its promotion of Fe absorption from foods and pharmaceutical Fe preparations (Baynes & Bothwell, 1990). The attribution of this effect to chelation and solubilization of Fe is supported by our work, as is the reduction of Fe^{3+} to Fe^{2+} at the low pH of the stomach. Many foods that contain ascorbate also have lower pH values (e.g. fruit juices), therefore Fe^{2+} may be expected to be stabilized by ascorbate, as observed by Nojeim & Clydesdale (1981). The present work suggests that rapid re-oxidation of Fe^{2+} to Fe^{3+} occurs on neutralization, even in the presence of ascorbate, at the ascorbate:Fe values often seen in foods and pharmaceuticals (< 20:1). The detailed kinetics of Fe^{2+} oxidation could not be investigated at pH > 7·0 due to the rapidity of the process. In foods, however, other stronger reducing agents may be present (e.g. cysteine) and these may be able to maintain Fe^{2+} in the intestinal lumen (Wein & Van Campen, 1991). O_2 concentrations may also be low in the gastrointestinal lumen. Further work is needed to determine whether Fe^{2+} really persists in the intestinal lumen during digestion and absorption, although earlier studies (Simpson & Peters, 1990) have provided evidence that oxidation of Fe^{2+} to Fe^{3+} accompanies the neutralization of stomach contents in the duodenal lumen.

Conclusions

When O_2 is present, Fe in ascorbate mixtures remains rapidly chelatable only at pH < 7·0. Fe in such mixtures is predominantly Fe^{3+} at physiological pH when O_2 is present.

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