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Studies on the Reactions of Ferric Iron with Ascorbic Acid. A Study of Solution Chemistry using Mössbauer Spectroscopy and Stopped-flow Techniques

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(Received March 7, 1986)

Abstract

We report studies on the interaction of iron(III) and ascorbic acid as a function of pH in pure water, pure methanol and mixtures of these solvents.

Mössbauer data indicates the iron(III) is reduced in water at low pH to iron(II). Rapid mixing studies and pH jump investigations using stopped flow spectrophotometry have been used to follow the reactions and show evidence for blue intermediates in the reduction pathway of iron at low pH values. A scheme is proposed to account for the complex reaction between iron and ascorbate in aqueous solvent. Binding constants between iron(III) and ascorbate are given.

Introduction

Over the past few years we have undertaken a study of the chemistry of glutathione with ferric iron [1–5]. In the course of these studies we reported the occurrence of intense blue intermediate transient species [1–3] in the reduction of iron(III) to iron(II). Similar blue intermediates have been reported by ourselves and others for other thiols [6]. In addition we have been interested in iron(III) catechol solution chemistry [7], and noted blue transients in these systems also [10]. In both the thiol and catechol systems these blue intermediates have been assigned to the presence of transient complexes of Fe(II) and radicals. It is of interest, therefore, to see how widespread is the presence of such transient complexes during iron(III) to iron(II) reduction, using organic reducing agents.

A number of papers studying the reaction of iron(III) with ascorbic acid [7, 11–18] have appeared. These studies have ranged from stopped-flow investigations [15], ESR [18], and Mössbauer spectroscopy [16]. However, there remains disagreement both in the nature of the end products and of the intermediates. As several of these papers refer to blue transient species, we thought it useful to study this system and compare it with the thiol and catechol systems.

In addition, the reaction of ascorbic acid with ferric iron is of some biomedical importance [19–21]. Reduction of the single electron acceptor, Fe(III), by the two electron donor ascorbic acid and reoxidation of the resulting Fe(II) by molecular oxygen will certainly involve radicals and species such as H_2O_2. Both of these are considered to be inflammatory agents and are suspected as agents in initiating the inflammation response in arthritis. Indeed, in arthritic patients it has been found that the ratio of dehydro ascorbate to ascorbate is elevated [19] suggesting the involvement of abnormal redox or radical quenching processes in this condition. Also, as the level of iron in the synovial fluid is raised in arthritic patients, it seems possible that this metal may be involved in redox reactions leading to inflammation.

Although the metal ion catalysed oxidation of ascorbate has been studied previously, the reaction is of sufficient importance to warrant further investigation. In this paper we report our investigation of the reaction between ascorbate and ferric iron under anaerobic conditions, using rapid kinetic methods, visible spectroscopy, pH titrations and Mössbauer spectrocopy. All studies performed in the pH range 2 to 8.

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Experimental

Material

Ascorbic acid (Aldrich) and sodium salts of ascorbic acid (Sigma), anhydrous iron(III) chloride SLR (Fisons) were used without further purification.

pH Titration and Static Spectrophotometric Measurements

Iron(III) was used throughout with different ratios of ligand. Additions of sodium hydroxide (2 M dm^{-3}) were achieved under nitrogen in 80% methanol/water and monitored by Philips (pw-9409) digital pH meter. All static spectra and spectrophotometric titrations were carried out in pure methanol or 80% methanol/water using Perkin-Elmer 575 spectrophotometer.

Stopped-flow

A weighed amount of iron(III) chloride and ligands (ascorbic acid or sodium salt of ascorbic acid) were dissolved in conductivity water and absolute methanol or a mixture of methanol/water, the pH of the iron(II) salt was constant (2–2.5) and the pH of the ascorbate was adjusted to the value required by the addition of sodium hydroxide or hydrochloric acid. The solutions were degassed and each was transferred to a syringe under positive pressure of nitrogen. Stopped-flow experiments were performed in a Durrum–Gibson instrument with a 2 cm light path and a dead time of 3 ms. All experiments were performed anaerobically in the pH range 2 to 8 at a temperature of 20 °C; the outflow from all stopped-flow experiments was collected and the final pH monitored.

Mössbauer Spectroscopy

Aqueous or methanolic solutions of ascorbic acid or sodium ascorbate were mixed with iron(III) chloride solutions under anaerobic conditions and the pH adjusted; syringes were used to transfer the solutions to a polythene cell and quench frozen in liquid nitrogen. The cell was then transferred to a precooled Harwell MNC-200 cryostat. Mössbauer spectra were recorded at 80 K using the system described in a previous paper [8] the resulting data was computer fitted.

Results and Discussion

Mössbauer Spectroscopy

The Mössbauer data are presented in Table I. Spectra were obtained from both frozen solutions (numbers 1–4) and from solids (numbers 5 and 6). In excess ascorbic acid (number 2 of Table I) in water around pH 2, only iron(II) is observed. This is in keeping with earlier reports of the reduction of iron(III) by ascorbic acid. When only a limited amount of ascorbate is used (as in number 1 of Table I) then some residual iron(III) remains in 95% methanol/H_2O at pH 4. In this case two ascorbate molecules were present for each atom of iron and around 13% of the iron was iron(III). Number 3 is the data for a 1:5 iron to ascorbate solution in absolute methanol at pH 10. Here no reduction has occurred as only iron(III) is present. When the pH of this solution is lowered using concentrated hydrochloric acid only iron(II) is found (number 4). Thus, in pure methanol at high pH ascorbic acid does not reduce iron(III) whereas at low pH it does. This experiment was not repeated in pure water as iron(III) does not dissolve at high pH values.

The fact that at high pH ascorbic acid does not reduce iron(III) is in keeping with earlier work from these laboratories [7].

The solid material (number 5) obtained from the solution used for spectrum number 1 after exposure

TABLE I. [57]Fe Mössbauer Spectroscopic Data on Solids and Frozen Solution Materials Prepared from $FeCl_3$ and Ascorbic Acid

	Phase	δ (mm s^{-1})	Δ (mm s^{-1})	Γ (mm s^{-1})	Absorption (%)
	Frozen solutions				
1	$FeCl_3$ (0.1 M) + H_2Asc (0.2 M) pH 4	1.37(1)	3.34(1)	0.20(1)	86.7(2.4)
	95% methanol/H_2O	0.51(2)	0.59(3)	0.19(3)	13.3(2.4)
2	$FeCl_3$ (0.1 M) + H_2Asc (1 M) pH ~2	1.39(1)	3.33(2)	0.21(2)	100
3	$FeCl_3$ (0.2 M) + $5Na_2Asc$ (0.2 M) pH 10.0	0.49(2)	0.81(2)	0.22(2)	100
	(methanol)				
4	As (3) (but + HCl conc) at pH 1.0	1.36(1)	3.55(1)	0.20(1)	100
	Solids				
5	Solids from (1) + methanol	1.40(2)	3.12(5)	0.27(4)	18.3(3.3)
	(violet blue)	0.55(1)	0.66(1)	0.21(1)	81.7(2.8)
6	Same as (5) + HCl	1.37(1)	3.27(1)	0.25(1)	28.1(1.4)
	(methanol) (violet blue)	0.55(1)	0.67(1)	0.22(1)	71.8(1.3)

to air gave both iron(II) and iron(III) Mössbauer parameters. This solid on analyses gave 1 iron to 1.2 ascorbates. Approximately 80% iron(III) was present from the Mössbauer data. When HCl was added to this material the percentage of iron(II) increased by around 10%. This small increase was probably due to most of the ascorbate being already oxidised.

Overall, the Mössbauer data shows that at pH values below 4 iron(II) is the only product of the reaction of iron(III) with ascorbic acid. In methanol at higher pH iron(III) species are stable.

Static and Kinetic Studies in the Visible Spectral Region

Anaerobic solutions of $FeCl_3$ (maintained at $\simeq pH$ 2.5) were mixed in water with 10-fold excess of ascorbic acid, the pH of which was controlled by addition of NaOH. The final reaction mixture was faintly pink at pH values above 6.5, indicative of ferric ion being in hydroxy or μ-oxy complexes. At lower pH values the resulting solutions were completely colourless.

When similar experiments were performed in the stopped-flow apparatus, clear evidence of highly coloured intermediates in the reaction was obtained. At all pH values explored, we observed, following mixing, a biphasic progress curve leading to a loss of absorption. Typical examples are given in the Fig. 1a.

It is clear that both the rate and the extent of the reaction are pH dependent. It is also apparent that as we are monitoring a decrease in absorption at a wavelength at which the reactants do not themselves absorb, then some highly coloured transient intermediate must have formed within the dead-time (3 ms) of the apparatus. By performing this experiment at a number of wavelengths, we were able to construct the overall kinetic difference spectrum between the intermediate(s) and the final products. As the products do themselves not absorb in this wavelength range, the spectra given in Fig. 1b must be the absolute spectra of the intermediate-(s) 3 ms after initiation of the reaction.

At pH 6.0 the intermediate(s) are characterised by an intense absorption at 560–580 nm (if all the iron is in the absorbing species then $\epsilon_{max} \simeq 200$ M^{-1} cm^{-1}) and a shoulder at around 650 nm. As the pH is lowered the intensity of the 560 nm band decreases until at around pH 2.0 the spectrum consists of a broad band in the blue spectral region centred at 650 nm. We may draw attention to the fact that glutathione and catechol iron complexes at low pH also exhibit bands in this region. The two kinetic phases seen in Fig. 1a may be separated and the amplitude of each plotted as a function of wavelength (Fig. 2).
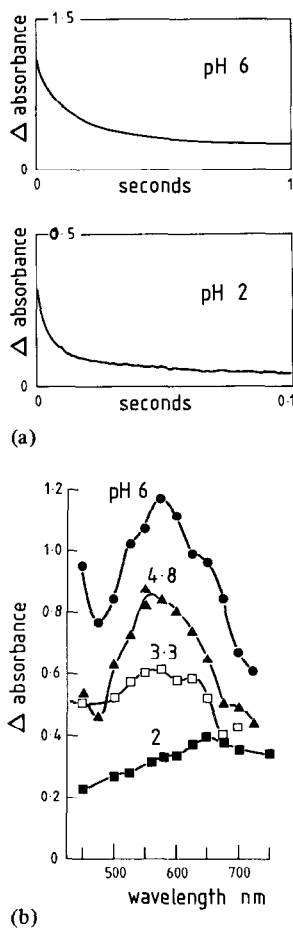

(a)

(b)

Fig. 1. (a) Stopped-flow traces of the reaction between anaerobic aqueous solutions of ascorbic acid and iron(III) chloride. Upper: the ferric chloride and ascorbic acid concentrations were 1.25×10^{-2} M and 0.1 M respectively. The monitoring wavelength was 560 nm. Lower: the ferric chloride and ascorbic acid concentrations were 2.5×10^{-3} M and 2.5×10^{-2} respectively. The monitoring wavelength was 560 nm. All reactant concentrations given are those after mixing. $T = 20$ °C. (b) Kinetic difference spectra for the reaction between anaerobic aqueous solutions of ascorbic acid and iron(III) chloride at different pH values. Conditions: Ferric chloride concentration 1.25×10^{-3} M; ascorbate concentration 1.25×10^{-2} M; pH 6.0. Ferric chloride concentration 1.25×10^{-3} M; ascorbic acid concentrations 1.2×10^{-3} M; pH 4.8. Ferric chloride concentration 1.25×10^{-3} M; ascorbic acid concentration 1.2×10^{-3} M; pH 3.3. Ferric chloride concentration 2.5×10^{-3} M; ascorbic acid concentration 2×10^{-1} M; pH 2.0. All reactant concentrations given are those before mixing.

At higher pH values the two kinetic phases display similar spectral features and each is similar to the overall difference spectrum, *i.e.* both display a peak $\simeq 580$ nm (Fig. 2). At low pH values the faster process reflects the decay of the blue intermediate, peak at 650 nm, while the slower phase reflects

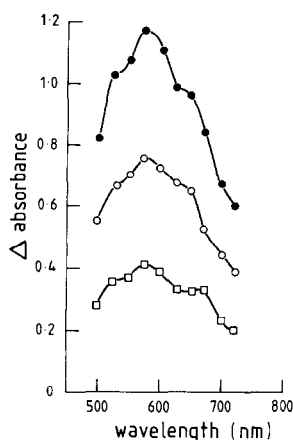Fig. 2. Kinetic difference spectra for the reaction between anaerobic aqueous solutions of iron(II) chloride (1.25 × 10^{-3} M) and ascorbic acid (1.25 × 10^{-2} M) at pH 6.0. Overall kinetic difference spectrum (●). Kinetic difference spectrum of the fast phase (completed within 0.5 s) (○). Kinetic difference spectrum of the slow phase (□). Reactant concentrations given are those before mixing.
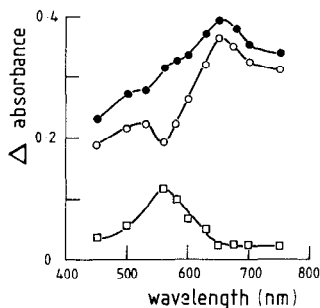

Fig. 3. Kinetic difference spectra for the reaction between anaerobic aqueous solutions of iron(III) chloride (2.5 × 10^{-2} M) and ascorbic acid (2 × 10^{-1} M) at pH 2.0. Overall kinetic difference spectrum (●). Kinetic difference spectrum of the fast phase (completed within 20 m s) (○). Kinetic difference spectrum of the slow phase (□). Reactant concentrations given are those before mixing.

the decay of an intermediate spectrallly similar to those found at high pH (Fig. 3).

Our interpretation of this data is as follows.

(i) At pH values ~6.0 (after mixing) iron rapidly coordinates to ascorbate (mainly as the mono anion) [12, 20] and to solvent generated ligands, i.e. predominantly OH ions, forming a complex of ascorbate and iron(III) with the iron also complexed to hydroxyl ligands or as μ-oxy complexes. These species are formed within the dead-time of our measurements and absorb in the 560—580 nm region. Although rapidly formed, they are not stable in water at this pH. The ascorbate is replaced by water leading to the loss of absorbance that we monitor in the stopped-flow spectrophotometer. In these reactions the majority of the iron remains in the ferric form

(within the time of our experiment). (See later also). At these relatively low concentrations of iron the insoluble iron hydroxide species form outside the time of our stopped flow observation.

(ii) At low pH values ~pH 2.0 the majority of the iron is free (as [Fe(H$_2$O)$_5$Cl]$^{2+}$, i.e. not coordinated to hydroxyl ions). Ascorbate when free is largely in the neutral form, (see also for pK_a values in discussion of titration data). On mixing, transient complexes are rapidly formed (again within the dead-time of the apparatus). These complexes are between iron and the mono anion of ascorbate (iron having replaced a proton), and between iron and some neutral ascorbate.

The decay of these complexes is through two routes. The majority of the iron, coordinated to the mono anion of ascorbate, forms an iron(II)/ascorbate radical complex which is blue ($\lambda_{max} \sim 630$ nm) and which rapidly decays (see Figs. 1 and 3) by replacement of the radical by solvent. A relatively small fraction remains iron(III) either because the iron is coordinated in a hydroxyl/aquo and hydroxyl/oxy complexes as at pH 6.0 or the iron is coordinated to the neutral ascorbate species. This fraction slowly goes to iron(II), rate limited either by hydroxyl replacement/μ-oxy dimer dissociation or by deprotonation of the neutral form. In this case any blue radical intermediate is not visible because its rate of production is much less than its rate of disappearance due to radical displacement.

Several lines of evidence can be used to substantiate our above interpretation.

(a) Separate experiments showed that the reaction of the μ-oxy dimer ([Cl$_3$Fe–O–FeCl$_3$]$^{2-}$) [22, 23] in water with ascorbate at pH 2.6, leads to the formation of a species (stable over a period of >10 min) absorbing around 560 nm. In this pH range this band of $\lambda = 560$ nm is most likely to be composite of complex μ-oxy species $\lambda = 530$ nm and some other iron complex such as the intermediates shown in Scheme 1. The formation is in the seconds time range (Fig. 4). These results are very similar to those reported for the reaction of ascorbate with μ-3-oxyacetate at pH 7.0 (ref. 17) which led to a ferric ion complex. The complex we observe here is also spectrally similar to that transiently formed by free iron and ascorbate at pH 6.0 (see Fig. 1).

(b) We have undertaken experiments in which we have reacted FeCl$_3$ with sodium ascorbate in either pure methanol or in methanol/water mixtures in order to reduce ligand replacement by water and thus possibly to stabilise coloured intermediates which exist only transiently in water.

Figure 5 shows a typical spectrum of the complex formed in pure methanol at an apparent pH of 6.15 between FeCl$_3$ and sodium ascorbate. The resulting stable species absorbed at 530—580 nm, similar to the transient shown in Fig. 1, and to the μ-oxy
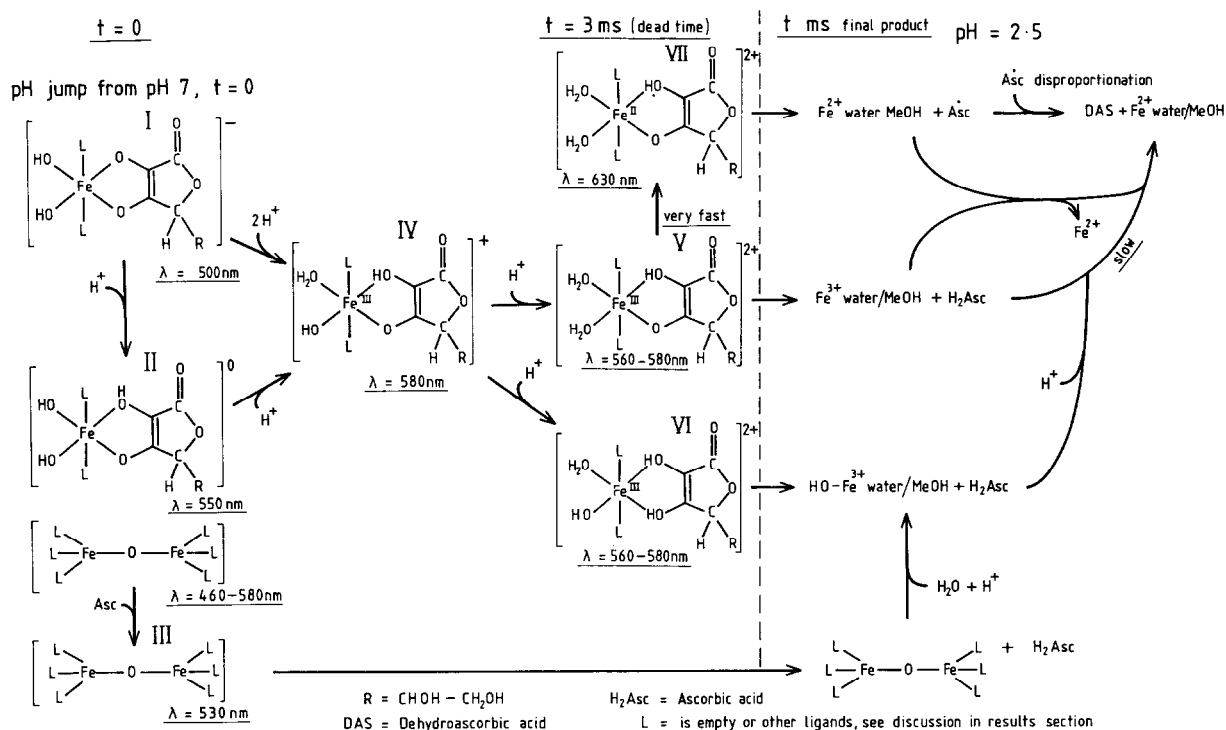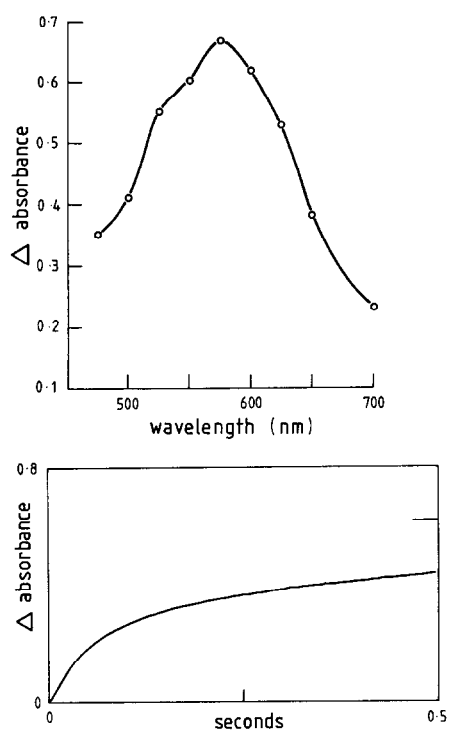
Scheme 1.

Fig. 4. Kinetic difference spectrum for the reaction between anaerobic aqueous solutions of ascorbic acid (0.25 M) and iron μ-oxy chloride ($[Cl_3Fe-O-FeCl_3]^{2-}$ concentration 1.25×10^{-3} M) at pH 2.6. The inset shows a typical progress curve followed at 550 nm. Reactant concentrations given are those before mixing.
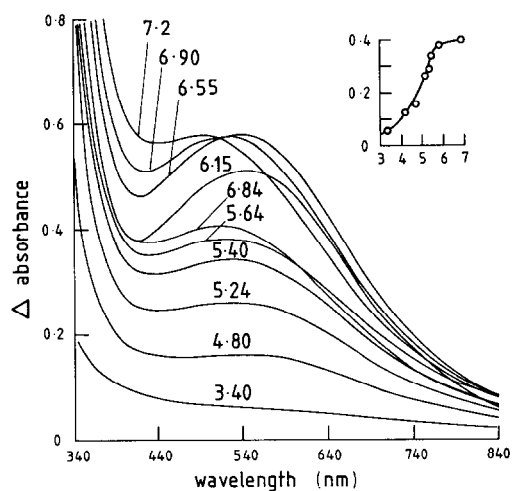

Fig. 5. Static spectrum of the complex formed between ascorbic acid or the sodium salt of ascorbic acid (1×10^{-2} M) with iron(III) chloride (1×10^{-3} M) in pure methanol at different pH values as indicated. The titration was performed from high pH to low pH. The inset shows a spectrophotometric titration curve ($pK \sim 5.2$) monitored at 540 nm.

ascorbate complex shown in Fig. 4. This latter comparison is pertinent as it has been reported [22, 23] that μ-oxy dimers also form and are stable in methanol.

On lowering the pH of the complex formed in pure methanol at pH 7.20, by addition of HCl, the
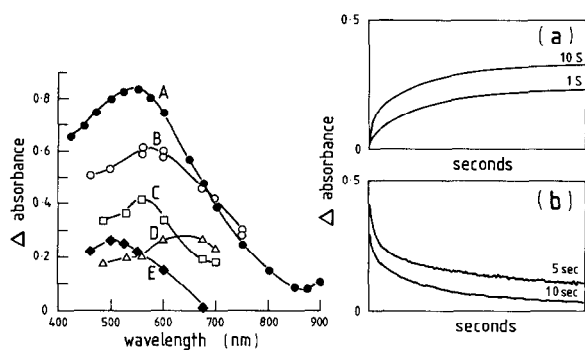
Fig. 6. Spectrum A. Is the static spectrum of the complex formed by the reaction between anaerobic methanolic solution (MeOH 80% water 20%) of iron(III) chloride (1×10^{-3} M) and sodium ascorbate (5×10^{-2} M) at pH 6.9. Spectrum B. Is the overall kinetic difference spectrum, *i.e.* the sum of spectrum C and spectrum D for the bleaching of this complex on mixing with acidified methanolic solution (MeOH 80%/ H_2O 20%) at pH 1.0 (final pH 2.5). Spectrum C. Is the kinetic difference spectrum of the fast phase of the bleaching reaction. Spectrum D. Is the kinetic difference spectrum of the slow phase. Spectrum E. Is the difference between the static spectra and the overall difference spectrum, *i.e.* spectrum A minus spectrum B. Inset (a) Progress curve, taken at two sweep times, for the formation of the complex (Spectrum A) on mixing iron(III) chloride (concentration 3.3 × 10^{-4} M) and ascorbate (concentration 1.5×10^{-2} M) monitored at 560 nm. Inset (b) Progress curve, taken at two sweep times for the disappearance of the complex (of Spectrum B) at pH 2.5.

maximum absorbance at ~ 530 nm decreases following a simple titration curve (see also Fig. 6).

This bleaching of the absorbance on lowering pH to 3.0 is reversible provided the titration is carried out quickly, *i.e.* within a few tens of minutes and the pH does not drop below $\sim$pH 1.0. The explanation for this behaviour is found in the kinetic stability of the μ-oxo dimer formed in methanol. The dimers bind mono protonated ascorbate to form coloured complexes and these complexes dissociate on protonation of the ascorbate anion. The μ-oxo dimer breaks down, especially at low pH, but this process is slow compared to the protonation and ligand exchange reactions. If the titration to low pH goes to pH $\sim$1.0 or the solution is held at pH 2–3 for some time, $\sim$1 h, then the μ-oxo dimer splits and at these pH values ascorbate binds and reduces iron (see above). In these circumstances the reaction is not reversible by reverse titration. The proportion of the μ-oxo dimer ascorbate in methanol depends on the starting pH of the medium in which sodium ascorbate and ferric chloride were mixed, the higher the pH the larger the fraction of iron which is μ-oxo bridged.

In 80% methanol, 20% water, the appearance of a stable species ($\lambda_{max} \sim 550$), could be observed in rapid mixing experiments, as in pure methanol (see

trace (a) Fig. 6). Also the bleaching of a transient intermediate could be followed at low pH ($\sim$2–3) around 630 nm similar to the behaviour in pure water. Thus the mixed medium (*i.e.* methanol and water) showed properties exhibited by the individual components singly.

pH Jump Experiments

An alternative way to study the intermediates which occur at low pH in the reactions of iron with ascorbate is to perform pH jump experiments. Instead of mixing ascorbate and iron at low pH one may make iron/ascorbate complexes at high pH and rapidly increase the proton concentration by mixing such complexes with acid. For the reasons discussed above, we cannot carry out experiments of this type in water because Fe(III)/ascorbate complexes are unstable (see Fig. 1). However, it is possible to prepare complexes in methanol or methanol/water mixtures (see above). Figure 6 shows a stopped-flow trace of an experiment in which Fe(III)/ascorbate complexes in methanol/water (80:20), apparent pH 6.9, were mixed with methanol/water HCl having an apparent pH $\sim$1.0; the final pH after mixing was $\sim$2.5.

At all wavelengths a biphasic loss of absorbance was recorded (see for example progress curve b, Fig. 6), and the final solution was colourless; Mössbauer spectroscopy of comparable solutions, but at higher reagent concentrations, shows that on completion of the reaction, the iron is as in the ferrous state. Figure 6 also compares the kinetic difference spectrum of the overall reaction and of each of the separated phases with the static difference spectrum obtained in a spectrophotometer.

Several points may be made:

(i) Although similar, there are distinct differences between the static spectrum (A) and the overall kinetic difference spectrum (B). This implies that within the dead-time of the apparatus (3 ms) a rapid protonation has occurred leading to a loss of absorbance. By subtracting spectrum (B) from spectrum (A) we may display the difference spectrum (E) of this rapid protonation; this spectrum has a peak around 500 nm.

(ii) The faster of the decay processes seen in Fig. 6, (inset b) has a spectrum peaking around 560 nm (spectrum C), while the slower phase is most prominent in the 600–700 nm region (spectrum D). This slow process is spectrally identical (within our errors) to that observed in the mixing experiments reported in Fig. 3 in which water was the medium. Thus, once again, we see that reactions leading to reduction of ferric iron by ascorbate involves an intermediate absorbing maximally around 625–650 nm.

pH Titrations

Figure 7 reports the results of pH titrations of sodium ascorbate with HCl in the absence and pres-
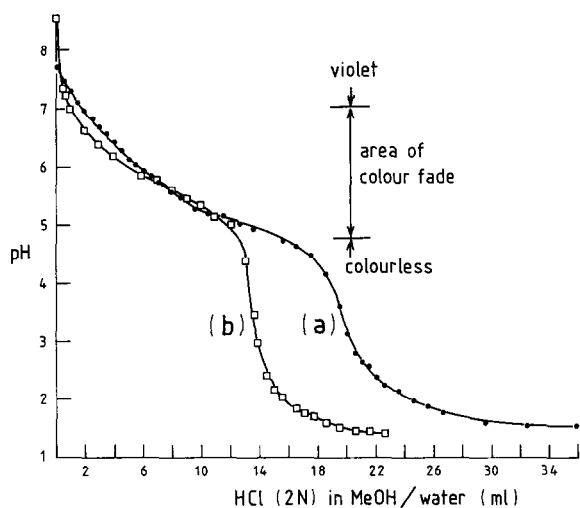
Fig. 7. pH Titration curves of sodium ascorbate with hydrochloric acid (2 N) in (a) the presence and (b) the absence of iron(III) chloride. (a) 0.1 M Ascorbate (Na salt) in 200 cm³ 80% methanol/water together with 0.1 M iron(III) chloride in 100 cm³ 80% methanol/water. (b) 0.1 M Ascorbate (Na salt) in 200 cm³ 80% methanol/water.

ence of iron (Asc:Fe = 2:1), and in 80% methanol: water. In the absence of iron (curve b) and over the pH range 8 to 3, we observe a simple titration curve ($pK \approx 5.7$), involving a single proton per ascorbate ion. The identical pK_a value was obtained if ascorbic acid was substituted for sodium ascorbate. We interpret this titration as the protonation of the ascorbate mono anion to the fully protonated form; the pK_a for this transition in water is ≈ 4.2 [24] and the elevation of this value to 5.7 is consistent with the change in medium. In the presence of iron (curve a), we note two changes to this pattern. Firstly, we require more acid for the titration, close to one extra proton per iron atom. Secondly, we observe a more complex curve comprising at least two and possibly three overlapping transitions which are accompanied by a distinct colour change from violet at pH ~6 and above to colourless at around pH 4 and below.

Bearing in mind the complex nature of the possible interactions in solution between iron and the other species present, we must interpret this data with caution. Using the spectral titration in methanol (Fig. 6) we offer the following mechanism, which is consistent with the pH titrations and the other experimental data discussed in the other sections.

At pH ~8 and in 80% MeOH/water two main species are present:

(i) the predominant complex is one which if four coordinate involves ferric iron complexed with the monoanion of ascorbate and two hydroxyl ions, alternatively if six coordinate two addition ligands which fill the 5th and 6th coordination sites are likely

to be solvent molecules or possible Cl⁻ ions, (the latter possibility does not change our conclusions).

(ii) a complex of the μ-oxo dimer with one ascorbate monoanion per iron atom. This species is the minor component of the mixture under these conditions but forms the major complex in pure methanol, *i.e.* in the absence of water (and is likely to be four coordinate as for example found in [Cl₃Fe−O−FeCl₃]⁻²) [22].

On addition of acid the major species accepts a proton and one iron-bound hydroxyl is titrated with a pK_a ~6.5 (see Fig. 7), the iron remains ferric and the complex is violet in colour. On further addition protonation of a second hydroxyl of this species leads to reduction of iron and loss of colour pK_a ~5.1. The remaining uncomplexed ascorbate also titrates in this region (pK ~5.7) and this transition overlaps that involving the iron reduction transition. The minor component, the μ-oxo dimer, undergoes slow hydrolysis catalysed by low pH forming ferric iron/ascorbate hydroxyl complexes which then undergo the titrations outlined above. Incubation of iron with excess (10-fold) ascorbate in 80% MeOH/ H₂O at high pH (~12) leads to a more reddish solution containing higher proportions of this μ-oxo dimer. Titration of such solutions follow the pattern described above with the addition that we observed a transition in the pH region 1−2 which we take to reflect the proton catalysed rupture of the μ-oxo bridge and subsequent titration of the hydroxyls.

The reverse titrations from low pH ~1.5 to high pH do not exhibit the colourless to violet colour change, thus indicating that the iron redox transition is not reversible. This may be expected as the process of dismutation of ascorbate radicals to form dehydroascorbate is difficult to reverse once it has occurred, especially as iron(II) will coordinate poorly to the oxygen atoms of dehydroascorbate. Where reduction does not occur rapidly on acid addition, *i.e.* in the complex of the μ-oxo dimer and ascorbate, the colour transition is reversible (see Fig. 5).

Stoichiometry of Iron Ascorbate Complexes and their Affinitiy Constants

In an attempt to measure both stoichiometry and binding constants of iron to ascorbic acid, we have undertaken experiments in which iron(III), at constant concentration, was mixed with ascorbate at known but variable concentrations in the stopped flow apparatus. The total absorbance change due to complex formation (or decay) was monitored, thus giving a measure of the complex concentration as a function of ascorbate concentration.

In order to examine binding of ascorbate to ferric iron, uncomplicated by redox processes and ligand exchange, we performed experiments by mixing iron in 80% MeOH (pH apparent = 2 to minimise hydroxy and μ-oxo formation) with sodium ascorbate in 80%
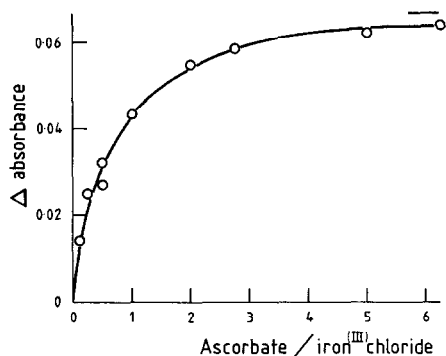
Fig. 8. Absorbance changes in a series of stopped-flow experiments obtained by mixing different concentrations of sodium ascorbate (varied from 0.1 to 25 fold that of iron(III) concentration with iron(III) chloride (4 × 10⁻⁴ M at pH 2) in 80% methanol/water. The final pH after mixing was 6.9. Experimental points (o). Theoretical line (–).
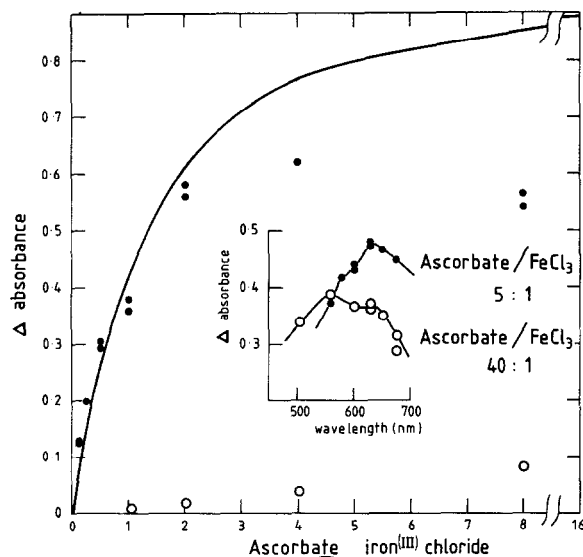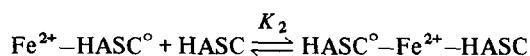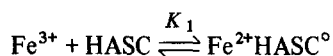

Fig. 9. The absorbance change in a series of stopped-flow experiments obtained by mixing a series of different sodium ascorbate concentrations (varied from 0.125 to 20 fold that of iron(III) concentration at pH 2) and a constant concentration of iron(III) chloride (0.025 M at pH 2). Both were prepared in aqueous solution and the final pH after mixing remained at pH 2. (●) Total maximum absorption obtained from stopped-flow experiment as a function of ascorbate concentrations. The solid line conforms to a theoretical curve for a formation of a 1:1 complex of ascorbate and iron with apparent binding constant, of 10^2 M⁻¹. (o) Absorption obtained from slow phase. Inset: Kinetic difference spectra for the reaction between ascorbic acid and iron(III) chloride in anaerobic aqueous solution at pH 2.0. (o) Ascorbic acid concentration (1 M); iron(III) chloride concentration (0.025 M). (●) Ascorbic acid concentration (0.125 M); iron(III) chloride concentration (0.025 M). Concentrations given are those before mixing.

MeOH at higher pH, so the final pH was 6.9 (apparent). Under these conditions and at low iron concentration, we observed a single kinetic progress curve leading to the formation of the violet species absorbing at approximately 560 nm, discussed above (curve a, Fig. 6). This species we believe, on the basis of Mössbauer and optical spectroscopy, to contain only iron(III). The progress curve for the production of this complex, followed at 560 nm, conformed to a first order process when the ascorbate was in excess over the iron. The pseudo-first-order rate constant was linearly dependent on the ascorbate concentration and the second-order rate constant for complex formation was found to be 1.5×10^3 M⁻¹s⁻¹.

Figure 8 shows the absorbance change at 560 nm in a series of stopped-flow experiments in which the ascorbate concentration was varied from 0.1 to 25-fold that of the Fe(III) concentration. The experimental points are displayed together with a theoretical curve for the formation of a one to one complex of ascorbate and iron(III) and with a binding constant of 7.5×10^3 M⁻¹. The coincidence of experiment and simulation suggests that under these conditions the violet complex contains iron and ascorbate in a ratio of 1:1 and the simplest conclusion would be that at this pH each iron atom is coordinated to a single mono-anion of ascorbate. The binding constant of 7.5×10^3 M⁻¹ between the mono ion of ascorbate and iron(III) is lower than for catecholato and hydroxamate complexes and explains why the complex with ascorbate dissociates in pure water through ligand competition [7, 9].

To examine binding of ascorbate to ferric iron at low pH however, is more complicated because reduction of the metal also occurs. In this case, we performed experiments in which ferric iron and ascorbic acid, both in water at pH 2, were mixed.

The final pH of the solution after mixing remained at pH 2. Under these conditions we observed biphasic kinetic progress curves leading to the disappearance of a blue transient intermediate(s) (see Fig. 2). This compound, (λ_{max} = 630 nm), we believe is a complex of Fe(II) and an ascorbate radical. The lose of absorbance is related to the dissociation of this Fe(II)-radical complex by solvent replacement. Figure 9 shows the absorbance change at 650 nm in a series of stopped-flow experiments in which the ascorbate concentration was varied from 0.125 to 20-fold that of the Fe(III)-concentration. In contrast to the experiment at high pH, the binding curve shows not only the expected increase of the complex as ascorbate concentration increases, but also an additional phase in which the overall absorbance decreases on further addition of ascorbate. An examination of the spectra (see inset to Fig. 9) of the transient intermediate(s) determined at ascorbate/iron ratios of 5:1 and 40:1 showed that the predominant intermediate species was different under these two con-

ditions. The species formed at low ascorbate:iron ratio absorbed more at 650 nm while the species formed at high ratio absorbed slightly more at around 550 nm. There is an isosbestic point between these two spectra at 550 nm. It appears therefore that at high ascorbate concentrations, binding of this ligand occurs leading to a complex with lowered extinction coefficient at 625 nm. There are a number of mechanisms one may propose to account for this behaviour. One possibility is illustrated as follows:

$$Fe^{3+} + HASC \underset{}{\overset{K_1}{\rightleftharpoons}} Fe^{2+}HASC^{\circ}$$

$$Fe^{2+}-HASC^{\circ} + HASC \underset{}{\overset{K_2}{\rightleftharpoons}} HASC^{\circ}-Fe^{2+}-HASC$$

In this mechanism the addition of a single mono-protonated ascorbate anion to ferric iron leads to binding and reduction of the metal to form the blue intermediate (λ_{max} 630 nm) within the dead-time (3 ms) of our apparatus. At low ascorbate concentrations this complex decays by solvent replacement of the radical on the iron(II) and it is this process we observe in the stopped-flow apparatus. At high ascorbate concentrations a second monoprotonated ascorbate ion may bind, also within the dead-time, leading to an iron(II) complex containing both an ascorbate radical and an anion. This complex is proposed to have a lower extinction at 625 nm. At intermediate ascorbate concentrations a mixture of these two complexes form and subsequently decay. However, it may be considered unlikely that iron(II) complexes containing two ascorbate molecules at low pH exist, especially in the presence of chloride ions [25]. An alternative mechanism is one in which at high concentrations of ascorbate (and at this low pH), di-protonated ascorbate molecules coordinate a greater proportion of the iron and stabilizes this in the ferric form for a period of time. This leads to a decrease in the absorbance at 625 nm, as this absorbance results from the formation of an iron(II)-radical complex. Examination of the progress curves for the bleaching reaction shows that it is biphasic (see Fig. 9). The slower phase being more prominent at higher ascorbate concentrations; it is possible that this slower phase reflects the increased binding of the diprotonated ascorbate at high ascorbate concentrations. The diprotonated species on loosing a proton then undergoes reduction of the iron atom.

On either mechanism, the first part of the curve shown in Fig. 9 conforms to a simple binding process between iron and ascorbate in a ratio of 1:1 and with an apparent binding constant (which includes both true binding and the redox equilibrium) of approximately 10^2 M^{-1}.

Discussion

The reactions of iron in aqueous solutions, iron-(III) in particular, are known to be exceedingly complicated because of the reaction of the metal with solvent or solvent-derived ligands [2]. The reactions of ascorbic acid with iron(III) reported here proved no exception to this rule. In our case, this is in part due to the relatively low affinity of ascorbate for iron(III)/(II) allowing solvent replacement reactions to dominate the mechanism. This is less so for tighter binding ligands such as catechol, adriamycin *etc.* [7, 25].

Through investigation of the reactions of ascorbic acid with iron(III) by rapid kinetic methods in a variety of solvents, we have been able to rationalise the main routes of reaction and possible intermediates and these are summarised in Scheme 1. The details of this scheme will now be described. On reacting ascorbate with iron(III) at pH values between 6 and 7, the main species initially present are I, II, and III. The proportions of these species depends on the exact pH value, but all contain iron(III) and absorb maximally between 500 and 600 nm. In water these species dissociate, the ascorbate being replaced by water or hydroxyl ions leading to loss of colour toward that of dilute iron(III) solutions at these pH values. In methanol or methanol/water (80:20) mixtures, these species are stable and the dihydroxy form I and μ-oxo form III are favoured.

On lowering the pH rapid protonation (within the dead-time of our experiments) leads, via species IV to species V and VI. Species V is a complex of a singly protonated ascorbate molecule and iron(III) coordinated to water molecules (solvent and/or chloride ions present) but not to hydroxyl ions which strongly stabilise iron(III). It is known that the redox behaviour of iron is strongly pH dependent [26]. The coordinated monoprotonated ascorbate may now rapidly reduce the iron(III) via intramolecular electron transfer. This internal redox reaction leads to species VII which is an iron(II) ascorbate radical complex absorbing at 630 nm. It is this species which we observe as a transient blue intermediate in the reduction of iron(III) by ascorbate. The position of the absorption band, at 630 nm, is consistent with the postulated nature of species VII, the absorption band presumably arising from a metal to ligand charge transfer complex [21, 27]. The assignment of this complex to an iron(II) oxidation state is confirmed by Mössbauer spectroscopy of the product formed by its dissociation (Scheme 1). It is not possible to observe the equivalent spectrum for the transient blue species VII. Similar species are seen in the iron–catechol reaction [9]. Prior to electron transfer (i.e. in species I–VI) the absorbance is due to a ligand to metal charge transfer in which

the transition is between orbitals which are mainly ligand to those which are mainly metal in character. After electron transfer, the situation is reversed, that is the absorbance is due to transitions from mainly metallic character orbitals to orbitals which are made up of predominantly ligand character. The relative extinction coefficients and the position of λ_{max} of the species before and after electron transfer are compatible with this view [21, 27].

As both the protonation and electron transfer reactions are fast compared with other steps, the major mechanistic route is via species VII which then dissociates to Fe^{2+} (coordinated by solvent molecules) and ascorbate radicals, *i.e.* to colourless products. It is this dissociation process we observe in the stopped-flow apparatus at low pH values.

Minor routes (because they are relatively slower than that via species VII), in leading to iron(II) are also indicated in the scheme. The route from the μ-oxo dimer may be important in methanol and methanol/water mixture because species III is significantly propulated under these conditions. The reduction pathway now exhibits no clear intermediates because the rate is limited by the slow rupture of the bridge which must precede reduction of the iron atoms.

Acknowledgement

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