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The Competition between Enterobactin and Glutathione for Iron

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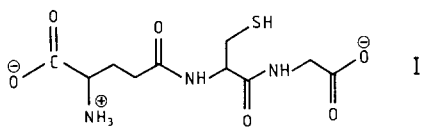
The competition for iron between thiol and catechol compounds is described. At pH values < 7.0, both mercaptoethanol and glutathione are able to completely remove iron from catechol. Although it is more difficult to abstract iron from enterobactin, a tris-catecholato ligand, glutathione in contrast to mercaptoethanol, is able to compete effectively with this siderophore at pH values < 6.0. As a result of being a multidentate ligand, glutathione forms stable complexes with iron(II). These findings offer a realistic explanation for the in vivo *removal of iron from enterobactin without necessitating its destruction.*

Introduction

In a recent study [1] of the solution chemistry of phenolic and catecholic iron(II) and iron(III) complexes, it was suggested that the siderophore ferric enterobactin donates iron to *Escherichia coli* by the reduction of the valence state of the coordinated iron. If the siderophore is to be re-utilised, then the enterobactin radical must be reduced. In principle, this reduction can be achieved by either ascorbic acid or glutathione both of which are known to be present in the cytoplasm of *Escherichia coli*. This concept of iron removal from an intact enterobactin molecule contradicts the thermodynamic arguments presented by O'Brien *et al.* [2] and Cooper *et al.* [3]. In order to clarify the apparent contradiction, we have studied the direct competition of enterobactin and thiol containing molecules (glutathione and mercaptoethanol) for iron.



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*This paper is part 2 of a series concerning Model Compounds for Microbial Iron Transport.

The predominant glutathione isomer (1) at physiological pH values possesses eight potential binding sites: two carboxylic acid groups, an amino group, a sulfhydryl group and two amide functions. As all binding sites can not be simultaneously coordinated to a single metal ion, the coordination chemistry of glutathione is characterised by the formation of protonated and polynuclear complexes [4]. Glutathione complexation of iron(III) in the pH range 1 to 3 has been reported [5] and we have made extensive studies of the interaction of both iron(III) and iron(II) with glutathione in both oxidation states. As demonstrated by Mössbauer spectroscopy, glutathione reduces iron(III) readily in aqueous solution to yield stable Fe(II) glutathione complexes (Hamed, Silver and Wilson, *unpublished*). In view of the high affinity of glutathione for iron(II) and its ability to reduce iron(III) we reasoned that it might be able to remove iron from enterobactin. As iron-catechol compounds have been used extensively as models for the catecholato siderophores [1, 6, 7], the initial thiol competition studies were run with such complexes.

Experimental

Materials

Anhydrous glutathione [reduced crystalline (Sigma)], anhydrous iron(III) chloride (SLR, Fisons), and mercaptoethanol (Sigma), were used without further purification. Catechol (SLR, Fisons) was recrystallised before use. Enterobactin was kindly provided by J. B. Neilands (Berkeley).

Aqueous solutions of iron-enterobactin were prepared by dissolving a weighed amount in methanol, evaporating the solution under reduced pressure then dissolving the residue in a known volume of deionized degassed water. The pH of the resulting solution was adjusted to 7.1 and stored at –20 °C.

Spectrophotometric Characterisation of Complex Ions in Solution

Solutions were prepared by dissolving weighed amounts of the reagents in conductivity water, and

were stored under oxygen-free nitrogen. The nitrogen was passed through a B.T.S. copper catalyst column to remove oxygen traces and dried by passage through sulphuric acid.

Spectra were recorded with a Perkin Elmer coleman 575 spectrophotometer. All measurements were recorded at 25 °C. The colourless solutions were extremely oxygen sensitive and the spectra were taken under nitrogen immediately after mixing. The solutions were pulled into a syringe under positive pressure of nitrogen, transferred to a super-sealed spectrophotometer cell supplied with nitrogen.

All necessary precautions were taken to eliminate effects due to dilution.

pH Titration Curves

Iron (10^{-3} mol dm^{-3}) was used throughout with different ratios of ligand. Additions of NaOH (1 *M*) or HCl (1 *M*) were achieved under nitrogen and monitored by a Philips (PW-9409) digital pH meter. The values of C_β/C_M, where C_β is the concentration of the base (titrant) and C_M is the analytical metal concentration, were corrected for increasing volume during titrations.

Mossbauer Spectroscopy

An aqueous solution of recrystallised catechol (0.6 *M*), anhydrous iron(III) chloride (0.2 *M*) and 0.074 mol of mercaptoethanol was added in a total volume of (40 ml). The pH of the catechol iron solution was adjusted to pH 9.0 prior to mercaptoethanol addition and then adjusted to 5.0 to facilitate the reduction of iron(III). The pH was then returned to 7.8. All experiments were carried out under oxygen-free nitrogen. The solution was transferred to a liquid cell, frozen in liquid nitrogen and transferred to a pre-cooled Harwell MNC 200 cryostat. The Mössbauer spectra were obtained at 80 °K using a Harwell spectrometer (Waveform, generator MWG 200, servo-amplifier MSA 200, proportional counter MPC 200, vibrator MV 200), and Canberra (multichannel analyser series 30, HV power supply 3105 Amplifier 2012, pre amplifier 200 BE). The source was cobalt-57 (5 mCi) in rhodium (Radiochemical Centre, Amersham). The spectrometer was operated in a 'saw tooth' mode and the spectra computer fitted. The spectrometer was calibrated with a 25 μm thick natural iron reference absorber. All isomer shifts are referred to this as zero shift.

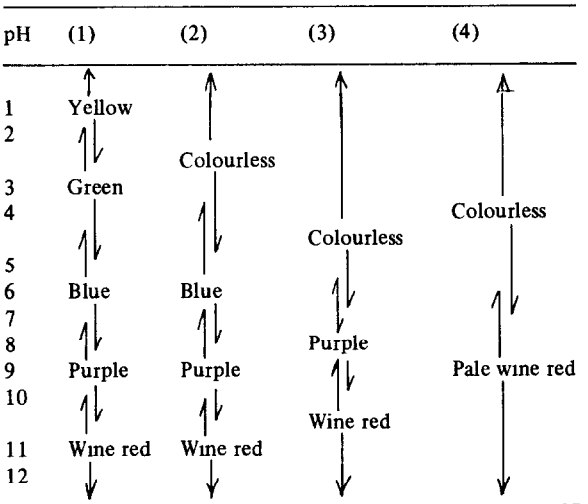
Results

Catechol–$FeCl_3$–Glutathione System

In the presence of GSH, the acid stable blue and green iron(II) complexes reported for the $FeCl_3$–catechol system [1, 6] were not observed (Table I, Fig. 1). Similar behaviour was found in the presence of ascorbic acid for the green complexes although not for the blue. This observation was explained in terms

TABLE I.

The pH dependence of the colour of (1) Fe^{III}–catechol, (2) Fe^{III}–Ascorbic acid–catechol, (3) Fe^{III}–glutathione–catechol, (4) Fe^{III}– mercaptoethanol. No precipitate was observed under any of these conditions.



Complexes established for catechol–iron system (1)

$Cat \cdot Fe^{II} \cdot (H_2O)_4 \rightleftharpoons Cat_3 \cdot Fe^{II} \rightleftharpoons Cat_4 \cdot Fe_2^{III} \rightleftharpoons Cat_3 \cdot Fe^{III}$

(green) (blue) (purple) (red)

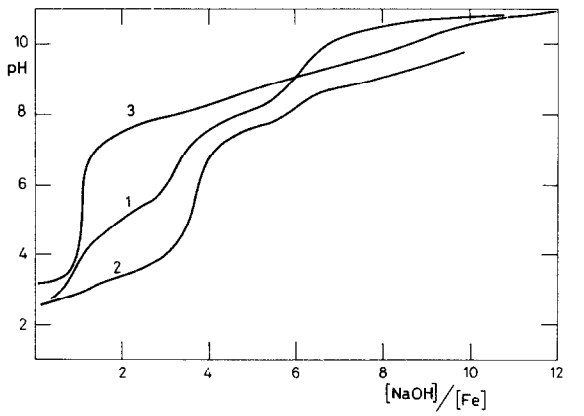


Fig. 1. pH titration curves of (1) catechol–Fe^{III} (3:1), (2) glutathione–catechol–Fe^{III} (3:3:1), (3) mercaptoethanol–catechol–Fe^{III}, $[Fe^{III}] = 6.65 \times 10^{-4}$ *M*.

of the relative abilities of ascorbic acid and catechol to reduce Fe(III) below pH 4.5 [1]. In view of the high reducing potential of the thiol function, a similar explanation is possible for the loss of the green and blue species in the presence of GSH. Indeed when solutions containing $FeCl_3$ and glutathione are freeze dried, Mössbauer spectra with parameters typical of iron(II) (δ = 1.18(8) mm s^{-1} and Δ = 2.06(2) mm s^{-1}) are observed.

Above pH 7.5 trace quantities of a purple complex were observed and on increasing the pH to 8.5 a red species was generated. Although these complexes appeared to be identical to those found in the catechol–$FeCl_3$ system [1], the pale purple species

reported here has a different spectrum. Presumably this complex contains both catechol and GSH. In contrast the red complex observed in the glutathione–catechol–$FeCl_3$ system has an identical spectrum to that found in the $FeCl_3$–catechol system (Fig. 2) and therefore is identified as $[Fe(Cat)_3]^{3-}$ [1, 6, 7]. This red complex forms at a lower pH in the presence of GSH, namely pH 8.8 as compared to 9.75 in the iron–catechol system (Fig. 1, Table I). This observation is in keeping with the finding that the purple species are different in the two systems under study; the glutathione containing purple species being apparently less stable with respect to tris-catechol iron(III) at high pH.

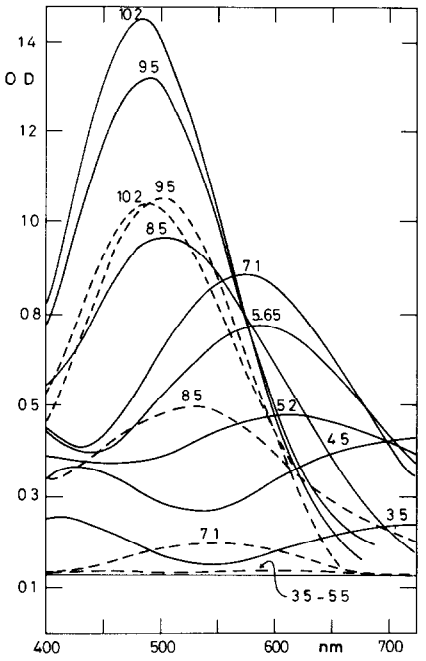


Fig. 2. Visible absorption spectra of iron–catechol (1:3) solutions as a function of pH, in the absence of glutathione (——) and in the presence of glutathione (– – – –). [Iron] = 2×10^{-4} *M*, glutathione = 1×10^{-3} *M*.

The rate of formation of the purple species in the presence of glutathione under nitrogen is much slower than in the corresponding thiol-free system. However, this slow rate can be considerably enhanced by the introduction of oxygen. Furthermore the concentration of the red tris-catecholato iron(III) is lower in presence of glutathione than in its absence when the sytem is maintained under a nitrogen atmosphere (Fig. 2). Again the introduction of oxygen dramatically influences the system, increasing the concentration of $[Fe(Cat)_3]^{3-}$ to that found in the absence of the thiol. In the thiol-free system the purple species which contains iron(III) is directly generated from a blue iron(II) catechol radical complex [1]. In the presence of GSH no such species could be detected and therefore the most likely explanation for the conversion of iron(II) to iron(III) is reaction with trace amounts of oxygen. It is clear from the studies outlined in Fig. 1 and 2 that in solutions with pH <7.5, glutathione has greater affinity than catechol for iron(II).

Catechol–$FeCl_3$–Mercaptoethanol System

The presence of mercaptoethanol induces a similar phenomenon to that observed in the presence of GSH, namely that the green and blue iron(II)–catechol complexes were not observed. Furthermore mercaptoethanol totally suppressed the formation of a purple complex (Fig. 1, Table I). In contrast to the GSH experiments, even the formation of the red $[Fe(Cat)_3]^{3-}$ species was severely retarded and appreciable concentrations of this complex only appeared after standing for several hours at pH values above 10. Open access to oxygen was found to greatly enhance the development of this red complex. Clearly, in the absence of oxygen mercaptoethanol retains the iron in the reduced state throughout the entire pH range under study. A Mössbauer spectrum of a frozen solution of catechol–iron–mercaptoethanol (3:1:10) at pH 7.8 indicated the presence of two iron(II) sites, (Fig. 3). We are currently investigating the Mössbauer properties of iron mercaptoethanol and iron glutathione systems in detail.

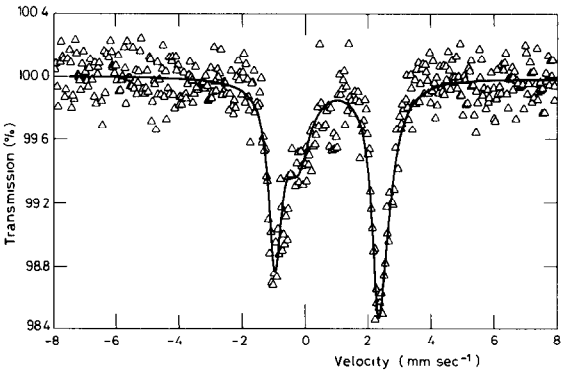


Fig. 3. Mössbauer spectrum of a solution of iron(III) chloride–catechol (1:3), with ten fold excess mercaptoethanol pH 7.8, at 80 K. The spectrum shows two iron(II) sites, one with isomer shift (δ) of 0.68(1) mm s^{-1} and a quadrupole splitting (Δ) of 3.30(2) mm s^{-1}, and another with δ = 1.15(5) mm s^{-1} and Δ = 2.76(7) mm s^{-1}. The first site is similar to that found in $FeIn_2S_4$ [8, 9] (δ = 0.62 mm s^{-1}, Δ = 3.23 mm s^{-1}) and therefore is likely to result from an octahedral Fe(II) site in a hexacoordinated sulphur environment (*i.e.* Fe(II) coordinated to six mercaptoethanol ligands). The second site is also an Fe(II) site although not similar to any found in the Fe–catechol system at this pH [1]. It is possible that this site corresponds to an octahedral Fe(II) site generated from both oxygen and sulphur ligands.

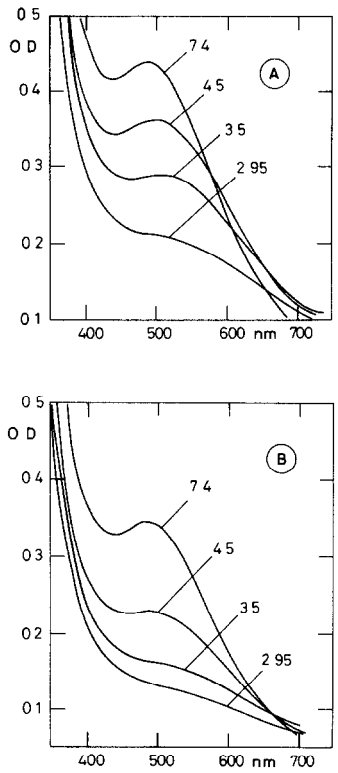


Fig. 4. Visible absorption spectra of enterobactin–Fe(III) chloride solutions (1:1) as a function of pH (A) in the absence of glutathione, (B) in presence of glutathione. [Iron] = 1×10^{-4} *M*, [glutathione] = 1×10^{-3} *M*.

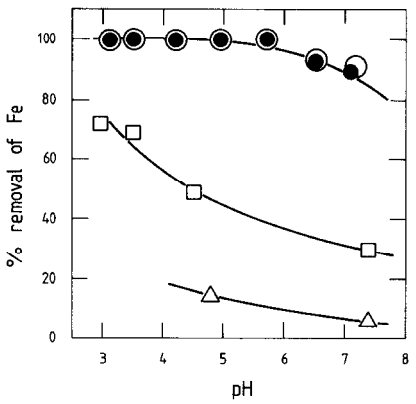


Fig. 5. Percentage removal of iron from catecholato ligands by a tenfold excess of thiol as a function of pH. (●), catechol–Fe^{III} (3:1)–glutathione; (○), catechol–Fe^{III} (3:1)–mercaptoethanol; [iron] [2×10^{-4} *M*]. (□), Enterobactin–Fe^{III} (1:1)–glutathione; (△), enterobactin–Fe^{III}–mercaptoethanol; [iron] = 1×10^{-4} *M*.

Enterobactin–$FeCl_3$–Thiol Systems

Glutathione in a tenfold excess was found to compete with enterobactin for iron in the pH range 3–7 (Fig. 4). However, the degree of competition was

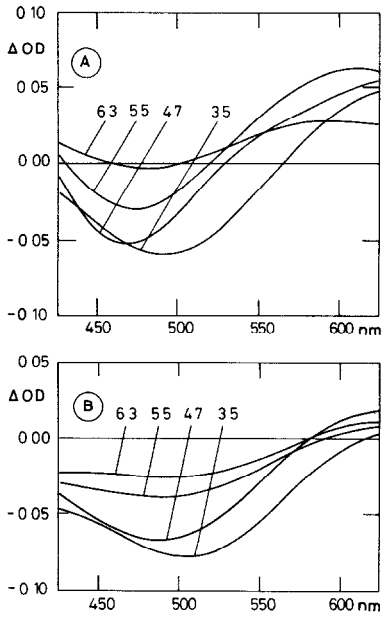


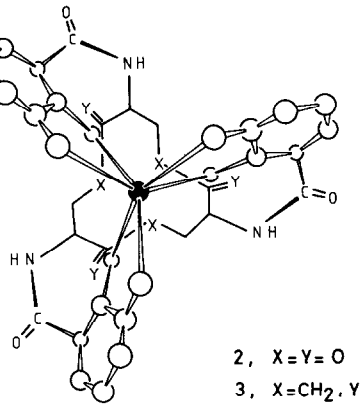
Fig. 6. The pH dependent spectral difference of iron–enterobactin (A) in the presence (B) in the absence of glutathione, in 50% methanol; the difference spectra were based on the spectrum of iron enterobactin at pH 7.1. [Iron] = 1×10^{-4} *M*.

less than that observed with catechol (Fig. 5). When such competition experiments are run in 50% aqueous methanol a marked contrast in the difference spectra is observed in the presence and absence of glutathione (Fig. 6). In the absence of glutathione the absorbance above 550 nm increased as the pH decreases. This increase is associated with the formation of the blue iron(II) enterobactin species [10]. In contrast no such reduced species is detected in the presence of glutathione, the thiol forming a colourless iron(II) complex and so preventing the formation of the blue iron(II) enterobactin species.

Glutathione is more efficient at the removal of iron from enterobactin than mercaptoethanol (Fig. 5) demonstrating the involvement of additional ligands in the coordination of iron.

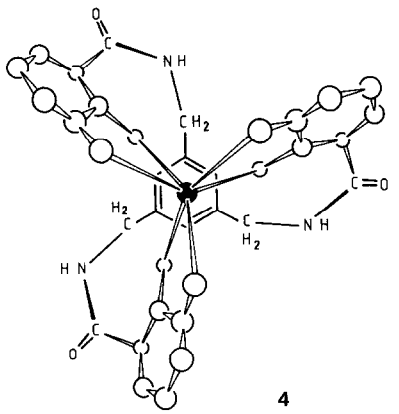
Discussion

It is established that iron enterobactin complexes (2) enter bacteria and subsequently render the complexed iron to the cytoplasm of the organism [2, 11], How this donation is achieved is not clear. Ferrichrome, a hydroxamate siderophore has been shown to be reutilised by *Ustilago sphaerogena* clearly demonstrating that the iron is removed without ligand destruction [12]. A similar situation exists for the uptake of iron(III) aerobactin by *Aerobacter aerogenes* [13]. In these examples the release of iron



2, X=Y=O
3, $X=CH_2$, $Y=H_2$

probably occurs via the reduction of iron(III), hydroxamates possessing only a weak affinity for iron(II). The redox potential of tris-hydroxamato iron falls within the range of known physiological redox systems and thus can be reduced *in vivo*. In contrast, the redox potential of iron enterobactin is reported to be much lower than the range of physiological reductants [2, 3]. It has been argued that for bacteria to remove iron from enterobactin, the hexadentate ligand must be converted to 3 bidentate ligands by hydrolysis of the ester links of the macrocyclic compound (2) [2, 14]. These corresponding bidentate ligands possess a higher redox potential [2]. Indeed the existance of such an esterase has been reported [2]; however, there is some controversy as to whether the substrate for the enzyme is the free ligand or the iron(III) complex [15, 16]. Emery [17] has shown in a related study with fusarinine that a specific esterase does exist, but that it is only able to lyse the free ligand and not the iron(III) complex. Studies with the enterobactin analogues (3) and (4)



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have shown that the tri ester structure of enterobactin is not essential for iron release *in vivo* as neither of the analogue ring structures can be readily hydrolysed and yet they are both able to provide iron to bacteria [18, 19]. Thus it would appear that the esterase reported to hydrolyse enterobactin is not essential for iron uptake and thus in principle the siderophore can be recycled.

Although the redox potential of iron enterobactin is reported to be low, it is possibly significant that these measurements were made at or above pH 7.0 [2, 3]. Under such conditions iron enterobactin is completely in the iron(III) form (2). However at pH values lower than 7.0 this is not the case. Indeed in solutions of low dielectric (50% aqueous methanol, a medium reported to be similar to membrane surface layers of structured water [20]) appreciable concentrations of the blue iron(II) enterobactin species are present at pH values <6.0 [1]. The protonation of one or more of the catechol oxygen atoms would change the ligand field of the coordinated iron, facilitating its reduction to the iron(II) state and on the basis of the results reported in this work (Figs. 4, 5 and 6) such complexes would loose their iron to thiol functions. Thus the thermodynamic arguments based on redox potentials, which are outlined above [2, 3], do not apply at pH values <6.0. Equivalent protonation could be readily achieved at an enzyme active site. Furthermore, on accepting iron(II), the thiol could simultaneously reduce the enterobactin radical so regenerating the native siderophore.

As indicated in this work the multidentate glutathione is superior to mercaptoethanol at removing iron from enterobactin (Fig. 5). Glutathione is present in most gram-negative bacteria but is replaced by another water soluble thiol in gram-positive organisms [21]. High glutathione levels are found in *E. Coli* [22] and two glutathione-deficient mutants of *E. Coli* have been characterised [23, 24]. No significant differences in growth were reported for these mutants and their parent strains and on the basis of this finding it was suggested that glutathione has a protective role [24]. However, growth studies were not performed under rigorously low iron conditions. Preliminary experiments have shown that one of these mutants BH400 derived from *E. Coli* K12 has a markedly reduced growth rate under low iron conditions (Hider and Neilands, unpublished), thus strongly supporting the major conclusion of this work.

It should be noted that evidence for the removal of iron by reduction has been previously reported for hydroxamate containing siderophores [25–27] and the catechol containing siderophore, agrobactin excreted by *Micrococcus denitrificans* [28]. The high affinity of glutathione for iron(II) renders it likely to be a low molecular weight intracellular iron transport compound. Such a role is not likely to be restricted to bacteria [29] and indeed may offer a realistic reason for the almost universal distribution of glutathione.

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