molecular and electronic structure in the triplet state is very similar for all three σ-bonded $[TCNQ]_2^{2-}$ dimers.

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Registry No. $[Cu(DMP)_2]_2[TCNQ]_2$, 85479-82-9; $Cu(DMP)_2I$, 85479-83-0; LiTCNQ, 1283-90-5.

Supplementary Material Available: Anisotropic thermal parameters (2 pages). Ordering information is given on any current masthead page.

Coordination Chemistry of Microbial Iron Transport Compounds. 22. pH-Dependent Mössbauer Spectroscopy of Ferric Enterobactin and Synthetic Analogues[1]

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Abstract: Iron complexes of the siderophore enterobactin (ent) and the synthetic analogue *N,N',N''*-tris(2,3-dihydroxybenzoyl)-1,3,5-tris(aminomethyl)benzene (MECAM) have been shown through Mössbauer spectroscopy to remain as Fe(III) complexes from pH 2 to 10 in aqueous solution. At high pH and low temperature these two complexes and *N,N',N''*-tris(2,3-dihydroxybenzyl)-1,3,5-tricarbamoylbenzene (TRIMCAM) exhibit both Mössbauer and EPR spectra indicative of high-spin ferric iron in a low-symmetry environment: a broad "4.3" type EPR signal is observed and the 4.2 K Mössbauer spectra are magnetic six-line patterns. At low pH, the magnetic Mössbauer spectrum collapses to a broad quadrupole pair (ΔE_Q = 0.8 mm/s, δ = 0.5 mm/s), indicating that the spin relaxation rate is fast compared with the nuclear precession frequency. At low pH ($\geq$2) titration data show that $[Fe(ent)]^{3-}$ and its close analogue $[Fe(MECAM)]^{3-}$ undergo stepwise protonation in aqueous solution. The ultimate products of this protonation are the insoluble compounds $[Fe(H_3ent)]^0$ and $[Fe(H_3MECAM)]^0$, in which protonation of one catechol oxygen results in a shift to a "salicylate" mode of coordination involving the ortho carbonyl of the 2,3-dihydroxybenzoyl rings. Chemical analysis of the supernatant solutions from these solids show no iron is present. From pH 10 to 2, in solution and the solid state, the complexes remain intact and the iron atom remains as high-spin Fe(III) in a six-coordinate, rhombic environment (g = 4.3). For $[Fe(TRIMCAM)]^{3-}$, a structural isomer of the MECAM complex which does not have a carbonyl group attached to the catechol ring and hence is not capable of the salicylate coordination mode, lowering the pH results in stepwise dissociation of the complex, in contrast to the corresponding enterobactin or MECAM complexes. While $[Fe(ent)]^{3-}$ in neutral aqueous solution cannot readily release its iron by reduction, the potential drops rapidly with pH and reductive release of iron under acid conditions is feasible. In the absence of a specific reductase enzyme, either the internal reduction of ferric ion by enterobactin ligand (to give Fe^{2+} annd semiquinone) in *nonaqueous* solution or the reduction in an acidic, *aqueous* environment of the Fe(III) tris(salicylate) species $Fe(H_3ent)$, which forms below pH 4, provides an alternative to the esterase mechanism of iron acquisition by microbes which use enterobactin in iron uptake. In contrast to the aqueous complex, Fe(ent) *in methanol* at low pH exhibits a quasi-reversible redox chemistry; at a pH meter reading of 1, approximately 45% of the iron is in the ferrous form (ΔE_Q = 3.44 mm/s, δ = 1.38 mm/s).

Iron is an essential element in the chemistry of living systems. Although the metal is relatively abundant in the earth's crust, it is inaccessible to microorganisms under normal conditions due to the formation of insoluble hydroxides. As an evolutionary response to this stress, microbes produce low molecular weight organic ligands (siderophores), which effectively solubilize ferric ion for transport into the cell.[4,5] One such compound is enterochelin[6] (called here enterobactin[7]), synthesized by the enteric bacteria *Salmonella typhimurium*, *Escherichia coli*, and *Klebsiella pneumoniae*. The structure of this cyclic triester of (2,3-dihydroxybenzoyl)serine is diagrammed in Figure 1.

Enterobactin (ent) coordinates ferric ion octahedrally with six oxygens from three catechoyl moieties.[6-12] The resulting molecule possesses the largest formation constant (approximately 10^{52}) of any known ferric complex.[11] It has been proposed that release of iron from $[Fe(ent)]^{3-}$, once inside the cell, proceeds by ligand degradation through enzymatic hydrolysis of the ester linkages.[13] Much of the research into the structure and bonding of enterobactin has been devoted to elucidating the properties of tris(catecholate) coordination in $[Fe(ent)]^{3-}$ at high or neutral pH. Since

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(2) Department of Chemistry, University of California, Berkeley, CA. 94720.
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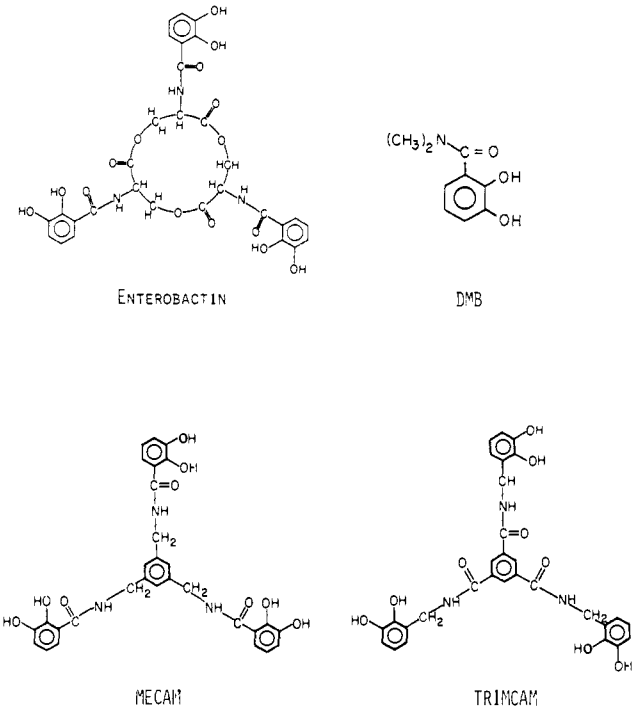

Figure 1. Structural formulas of enterobactin and its analogues: DMB, MECAM, and TRIMCAM.

study of enterobactin and its coordination compounds is complicated by the susceptibility of the ester linkages to base hydrolysis, a large portion of this work has been done with synthetic analogues (Figure 1) which mimic the enterobactin coordination site and possess an experimental advantage in being resistant to ring hydrolysis.

Recent literature has demonstrated that there are pH and potential gradients associated with biological membranes.[14] Since ferric enterobactin could encounter these gradients, a protonated species may be an important intermediate in the process of membrane transport and possibly in the iron-release mechanism, as has been suggested by Hider et al.[15] Although the very negative $[Fe(ent)]^{3-/4-}$ redox couple (−1.0 V at pH 10) is inaccessible to known biological reductants, the observed $[Fe(H_nent)]^{(n-3)/(n-4)}$ potential increases rapidly as the pH is lowered;[11,12] extrapolation of the potential vs. pH curve for Fe(ent) or estimation of the potential for the tris(salicylate)[16] form of the Fe(ent) complex from the known thermodynamic constants of this system[11] leads to the conclusion that complete protonation of the complex would shift the potential sufficiently to permit physiological reduction and release of iron as Fe(II).[17]

Investigations of the low pH coordination chemistry of iron enterobactin complexes have begun only recently. The iron complex undergoes a series of three one-step protonations to form a neutral blue solid at pH 3.0.[11] A model proposed[11] for the observed protonation equilibria predicts the sequential shift from catecholate to salicylate[16] coordination. The resultant tris(salicylate) complex $[Fe(H_3ent)]^0$ is a neutral compound which precipitates from aqueous solution but is soluble in methanol and diethyl ether. One of the synthetic catechoylamides, MECAM, may, like enterobactin, exhibit salicylate bonding through the amide carbonyl.[18] The structural isomer TRIMCAM lacks a carbonyl oxygen adjacent to the catechol moiety and, therefore, should not display salicylate coordination. Predictions for the chemistry of ferric MECAM and ferric TRIMCAM complexes, based on the salicylate model, have been verified by a variety of techniques.[17-19]

There are two underlying tenets of the salicylate coordination model. First, in order to form a neutral triprotonated species the ferric oxidation level must be preserved. Second, the catechoyl moiety is required to behave as a redox-innocent ligand which merely shifts its mode of coordination during the protonation reaction.

Spartalian et al.[10] reported the Mössbauer spectrum of ferric enterobactin in methanol at pH 7 and demonstrated the fully formed chelate contained Fe(III) exclusively. More recently Hider et al. have reported Mössbauer data of Fe(ent) at low pH in methanol[15] and of simple catecholates in aqueous solution.[20] These authors proposed that at low pH the ferric complex undergoes protonation by *four* H^+ ions with concomitant reduction (Fe^{3+} to Fe^{2+}) by catechol (to semiquinone).[20] Such reduction of the iron by a catechol group to give a semiquinone radical would normally exhibit a sharp $g = 2.0$ signal. However, the proximity of the high-spin ferrous iron could severely broaden the free radical signal.

Here we report Mössbauer studies of ferric enterobactin in both aqueous and methanolic solution. The reported Mössbauer spectra were recorded over a wide range of pH.[21] In agreement with Hider et al.,[15] we observed an Fe^{2+} Mössbauer signal in *methanol* at low pH. In *aqueous* solution, however, we find the iron remains in the ferric oxidation state over the *entire* pH range studied. We also report Mössbauer data of the aqueous Fe(III) complexes of MECAM and DMB over a similar pH range and of TRIMCAM at high pH.

Materials and Methods

Preparation of Compounds. Anaerobic manipulations were performed on a Schlenk line. Chemical analyses were done by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley.

Enterobactin. Enterobactin was obtained from *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*) by modification of a literature procedure.[7] Phosphate salts were freed of iron by passage through a K^+-form Chelex-100 (Bio Rad) resin column. After addition of the remaining inorganic salts, the pH was adjusted to 6.8. Supernatant from 5-L batch cultures, grown at 35–37 °C, was acidified to pH 5 and extracted three times with ethyl acetate. The combined ethyl acetate layers were washed with water (pH 4) and dried with $MgSO_4$. After concentration to 20–50 mL, slow addition of hexane precipitated enterobactin as a hygroscopic powder, which was washed well with hexane, dried in vacuo overnight at 25 °C, and stored under inert atmosphere; yield ~40 mg/L. Further purification was effected after formation of the iron complex.

***N,N',N''*-Tris(2,3-dihydroxybenzoyl)-1,3,5-tris(aminomethyl)benzene (MECAM).** Preparation of MECAM followed the published procedure[22] with the following changes. The coupling reaction between 1,3,5-tris(aminomethyl)benzene trihydrochloride and 2,3-dimethoxybenzoyl chloride was carried out in refluxing $CHCl_3$. Evaporation of the washed (NaOH; HCl; H_2O) and dried ($MgSO_4$)·$CHCl_3$ layer yielded an oil which eventually crystallized. The methyl-protected MECAM was recrystallized from hot MeOH. After deprotonation, MECAM in ethyl acetate solution was washed (0.1 M, pH 7, phosphate buffer; H_2O) and dried before precipitation with petroleum ether and drying in vacuo.

***N,N',N''*-Tris(2,3-dihydroxybenzyl)-1,3,5-tricarbamoylbenzene (TRIMCAM).** The methyl-protected derivative[22] was recrystallized from a concentrated DMF solution to which four to five volumes of MeOH were added. After deprotection, crude TRIMCAM was recrystallized from hot DMF (1 g/2 mL, 100 °C) to which five volumes of hot MeOH

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and one volume of water were added. The white microcrystalline powder was washed with 50% MeOH/H_2O and dried in vacuo for several hours at 50 °C.

***N,N*-Dimethyl-2,3-dihydroxybenzamide (DMB).** The DMB was prepared by the published procedure.[22] Crude DMB was recrystallized from hot H_2O to yield a white compound with a melting point of 184–186 °C.

Iron Complexes. All manipulations were performed under nitrogen unless noted otherwise. Samples were prepared not more than 3 days (usually 1 day) in advance and were stored dry under nitrogen until utilized.

To a methanol solution of a small excess of enterobactin was added $^{57}Fe(NO_3)_3$ in MeOH/H_2O. Addition of 6 equiv of aqueous KOH produced a color change from purple to purplish red. After removal of solvent in vacuo at room temperature, the residue was dissolved in a small amount of H_2O. The solution was adjusted to pH 7.5–8 in the air, diluted with 0.25 volume of MeOH, and passed through a 15 × 1 cm neutral alumina column previously equilibrated with 20% MeOH/H_2O (pH 8). Fractions containing the red $[Fe(ent)]^{3-}$ were evaporated in vacuo. Purified $K_3[Fe(ent)]$ displayed a single reduction wave when measured by cyclic voltammetry.[12] This technique has been shown to be a very sensitive measure of ferric enterobactin purity.

Both $[^{57}Fe(MECAM)]^{3-}$, $[^{57}Fe(TRIMCAM)]^{3-}$, and $[^{57}Fe(DMB)_3]^{3-}$ were prepared by adding $^{57}Fe(NO_3)_3$ in MeOH/H_2O to a 10% excess of the appropriate ligand deprotonated with 6 equiv of aqueous KOH in MeOH. Solvent from the red solution was removed in vacuo. The Mössbauer samples were prepared by dissolving the solid residue in H_2O and adjusting the pH with HNO_3 or NaOH.

Physical Measurements. Infrared spectra of samples in KBr pellets were obtained on a Perkin-Elmer Model 597 spectrophotometer. Visible spectra were recorded on a Cary 110 spectrophotometer. The EPR data (U.M.) in Figure 5 were collected on a Varian E-109 spectrometer fitted with an Oxford Instruments low-temperature cavity. Additional EPR data (Berkeley) on frozen aqueous or methanol solutions (Figures 6 through 8) are included as supplementary material.

Titrations. The pH measurements for Mössbauer samples were made under an argon atmosphere using a Fisher Accamel 620 pH meter and a Sigma combination electrode. The pH meter was standardized by using Mallinckrodt pH 4.01 and 7.00 buffers. Three milliliters of ca. 5 mM $[^{57}Fe(ent)]^{3-}$, $[^{57}Fe(MECAM)]^{3-}$, $[^{57}Fe(TRIMCAM)]^{3-}$, or $[^{57}Fe(DMB)_3]$ solution containing excess ligand was initially adjusted with 2 M KOH to ensure formation of the completely deprotonated complex. The pH was decreased incrementally by addition of 3 M HNO_3 directly into the Mössbauer sample cell. When present, solid was separated from supernatant by contrifugation, washed well with degassed pH 2–3 H_2O (from added HNO_3), and resuspended in degassed H_2O. The iron in the supernatant solution of comparable, unenriched samples was assayed by adding an excess of MECAMS to the solution and adjusting the pH to 7. At neutral pH and aerobic conditions any iron in the supernatant would be converted to the Fe^{III} MECAMS complex. Since the extinction coefficient for this complex is 5250 M^{-1} cm^{-1}, very small amounts of iron can be detected. This assay demonstrated that less than 0.2% of the iron from the protonated ferric enterobactin precipitate was in the supernatant solution.

We found that the redox behavior of titration solutions observed at low pH in methanol was not reversible for long time periods (1 day). Therefore a solution of $[^{57}Fe(ent)]^{3-}$ in a basic methanol medium was adjusted to pH 1[21] in methanol, a sample was removed, and the solution was then immediately brought back to pH 8. The Mössbauer spectra of both high pH solutions, before and after acidification, were identical.

Mössbauer Experiments. All Mössbauer samples were in the frozen solution form, 6 mm thick, in DELRIN sample holders. All sample volumes were ~0.3 mL. The iron complexes were made by using 95% enriched ^{57}Fe (New England Nuclear). The Mössbauer spectra were recorded in the horizontal transmission geometry using a constant acceleration spectrometer operated in conjunction with a 512 channel analyzer in the time scale mode.[23] The source velocity waveform was symmetric and data were collected during both positive and negative acceleration. The source was at room temperature and consisted of 30 mCi of ^{57}Co diffused in rhodium foil. The spectrometer was calibrated against a metallic iron foil, and zero velocity was taken as the centroid of its room-temperature Mössbauer spectrum. In these calibrations spectra line widths of about 0.24 mm/s were normally observed.

Theory

The paramagnetic properties of a high-spin ferric complex may be described by a $S = {}^5/_2$ spin Hamiltonian:

$$H_e = 2\beta_e \vec{H}^{app}\cdot\vec{S} + H_{CF} \quad (1)$$

The first term is the electronic Zeeman interaction, and H_{CF} describes the splitting of the sextet into three doublets via spin–orbit coupling with excited multiplets. In a previous Mössbauer investigation of $^{57}Fe(ent)$ in pH 7 methanol solution, Spartalian et al.[10] expressed H_{CF} as

$$H_{CF} = D\left\{S_z^2 - \frac{35}{12} + \lambda(S_x^2 - S_y^2) + \frac{1}{6}\mu\left(S_x^4 + S_y^4 + S_z^4 - \frac{707}{16}\right)\right\} \quad (2)$$

The Mössbauer spectrum is determined by the hyperfine interactions between the unpaired electron spin and the nuclear spin I.

$$H_N = -g_N\beta_N\vec{H}^{app}\cdot\vec{I} + A_0\vec{S}\cdot\vec{I} + H_Q$$

The first term is the direct interaction of the nuclear moment with the externally applied field. The second is the magnetic hyperfine interaction which in the case of high-spin ferric ions arises almost entirely from the isotropic Fermi contact term[24] (i.e., A_0 is a scaler). H_Q is the interaction of the electric quadrupole moment of the $I = {}^3/_2$ excited Fe^{57} nucleus and the electric field gradient (EFG) produced by extra nuclear charges. If the EFG tensor is diagonal in the xyz frame defined by H_{CF}, then H_Q is given by

$$H_Q = \frac{QV_{zz}}{4}\left[I_z^2 - \frac{5}{4} + \frac{1}{3}\eta(I_x^2 - I_y^2)\right] \quad (3)$$

$$\eta = (V_{xx} - V_{yy})/V_{zz}$$

For temperatures below 30 K, the electronic relaxation rates of the complexes studied here are slow compared with the ^{57}Fe nuclear precession frequency. At such low temperatures the observed Mössbauer spectrum will be a weighted sum of the spectra produced by each populated electronic state. The intensity of any given spectral component is proportional to the thermal population of the corresponding electronic state.

The shape of the Mössbauer spectrum of a slowly relaxing, high-spin ferric complex is dominated by the magnetic hyperfine interaction. In applied fields of ~0.02 T and larger, the expectation value of $\vec{S}$ will be determined completely by H_{CF} and $\vec{H}^{app}$. Hence, H_N may be written as

$$H_N = -g_N\beta_N(\vec{H}^{app} + \vec{H}^{int})\cdot\vec{I} + H_Q \quad (4)$$

where $\vec{H}^{int}$ is the internal field:

$$\vec{H}^{int} = -\frac{A_0}{g_N\beta_N}\langle\vec{S}\rangle_i \quad (5)$$

Typically $A_0/g_N\beta_N$ is −22 T. The subscript i refers to the ith state of the sextet. In applied fields such that $\beta_e H^{app}$ is small compared with the zero field splittings between the doublets, $\langle\vec{S}\rangle_i$ for one member of a Kramers doublet will have equal magnitude but opposite direction as that of the other member of doublet. If $H^{app} \ll H^{int}$, both members of the doublet will produce identical Mössbauer spectra. The internal field may be expressed in terms of the EPR g values of the appropriate doublet

$$\vec{H}^{int} = \frac{A_0}{4g_N\beta_N g_h}(h_x g_x^2\hat{x} + h_y g_y^2\hat{y} + h_z g_z^2\hat{z}) \quad (6)$$

where $g_h = [h_x^2 g_x^2 + h_y^2 g_y^2 + h_z^2 g_z^2]^{1/2}$; h_x, h_y and h_z are the direction cosines of $\vec{H}^{app}$ and $\hat{x}$, $\hat{y}$, and $\hat{z}$ are unit vectors.[24]

Results

Mössbauer Results. All the spectra shown in Figures 2–4 are comprised of varying amounts of four spectral components. These are the following: (1) A well-defined magnetic pattern with outer lines at −8 and +9 mm/s. This component arises from slow

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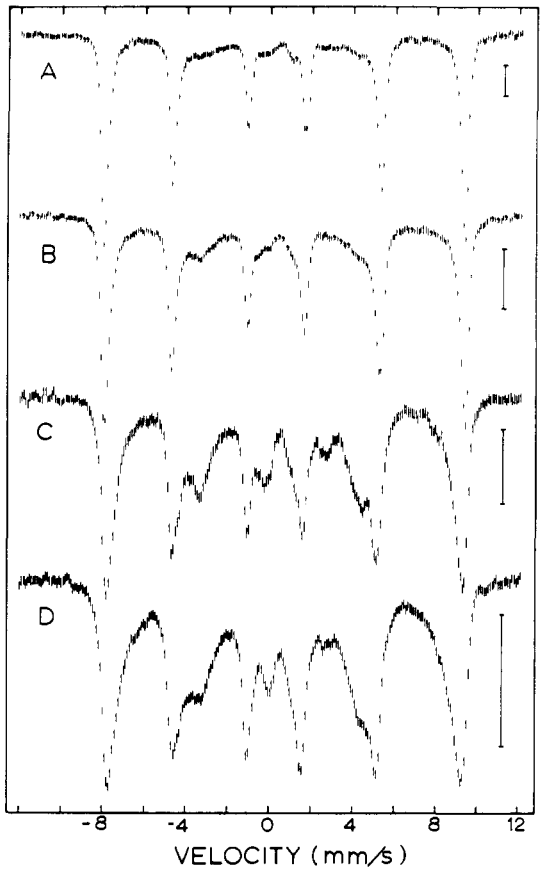

Figure 2. Mössbauer spectra of the aqueous ferric complexes of (A) enterobactin, pH 9, (B) MECAM, pH 9, (C) TRIMCAM, pH 10, and (D) DMB, pH 10, at 1.5 K (H^{app} = 60 mT parallel to γ beam). The vertical bars indicate 1% absorption.

relaxing high-spin Fe(III). (2) A broad, ill-defined magnetic component stretching from −8 to +10 mm/s which arises from intermediate relaxing Fe(III). (3) A quadrupole pair with ΔE_Q = 0.6–0.8 mm/s and δ = 0.5 mm/s which represents fast relaxing high-spin Fe(III). (4) A quadrupole pair with ΔE_Q = 3.4 mm/s and δ = 1.4 mm/s which reflects high-spin Fe(II). The fraction of total iron in each sample was quantitated by analyzing the areas of the two quadrupole pairs. This analysis was achieved by using computer techniques developed at the University of Minnesota by E. Münck.[24] The absorption not assigned to the Fe(II) or the fast relaxing Fe(III) was attributed to the sum of the slow and intermediate Fe(III). The recoil-free fractions of all the different species at 4.2 K were assumed to be equal. This assumption is supported by the studies of Debrunner[25] and others[24] which show that at 4.2 K the recoil-free fractions are essentially the same for a variety of iron sites in proteins and model compounds.

In aqueous solution the ^{57}Fe complexes of ent and the three analogues all display Mössbauer spectra typical of high-spin ferric complexes. Over the pH range studied, little or no ferrous material was observed. Figure 2A shows the 1.5 K, high-pH spectrum of ^{57}Fe(ent). The six-line pattern with intensity ratios of approximately 3:2:1 arises from the ground doublet. This sharp pattern indicates that the internal field at the Fe nuclei has a magnitude of 53.9 T and is along a fixed direction relative to the molecule, i.e., the induced electronic moment is primarily along a single direction. The electric field gradient (EFG) component along $\vec{H}^{int}$ is +0.38 mm/s, and the isomeric shift δ is 0.51 mm/s.

High-pH spectra of the aqueous ^{57}Fe complexes of MECAM, TRIMCAM, and DMB at 1.5 K are also shown in Figure 2. The absorption patterns arising from the ground doublets of these complexes are very similar to that of ent (see also Table I).

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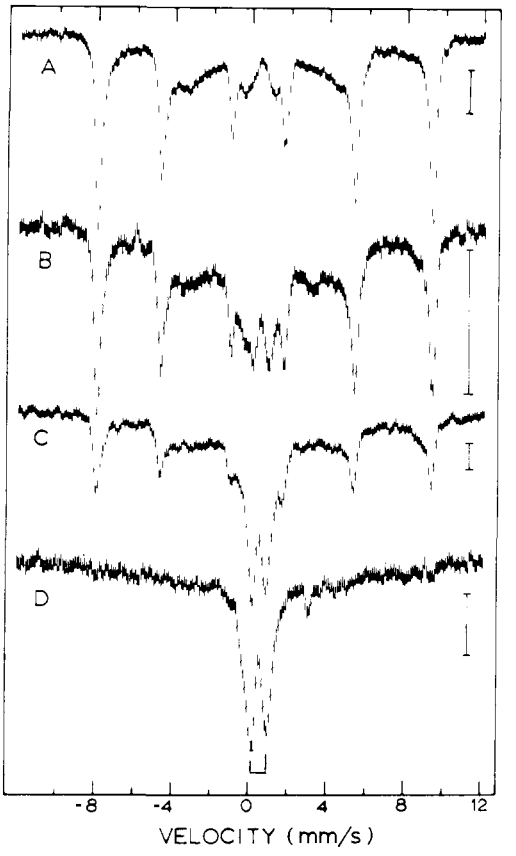

Figure 3. Mössbauer spectra at 4.2 K of ferric enterobactin in water as a function of pH: (A) pH 9, (B) pH 6, (C) pH 5, (D) pH 2 (H^{app} = 60 mT parallel). The vertical bars indicate 1% absorption.

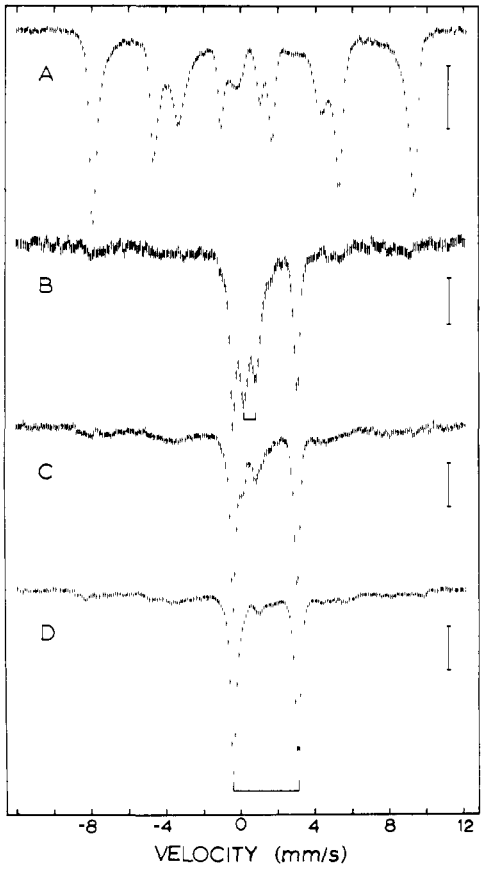

Figure 4. Mössbauer spectra at 4.2 K of ferric enterobactin in methanol as a function of "pH": (A) pH′ 9.2, (B) pH′ 4.0, (C) pH′ 2.3, (D) pH′ 1.2 (H^{app} = 60 mT parallel). The vertical bars indicate 1% absorption.

Table I. Mössbauer and EPR Parameters for Iron Enterobactin and Analogues at High pH

	ent	ent	MECAM	TRIMCAM	DMB	uncertainty
solvent	H_2O	MeOH	H_2O	H_2O	H_2O	
pH	9	"9"	9	10	10	
δ, mm/s	0.51	0.51	0.51	0.50	0.49	±0.03
V_{ii},[a] mm/s	+0.37	+0.37	+0.43	+0.52	0.49	±0.03
H^{int}, T	53.9	53.6	53.7	53.0	52.7	±0.03
$\Gamma_{4,3}$,[b] mT	13	5	13	10	12	

[a] V_{ii} is the EFG component along the internal field observed for electronic ground doublet. [b] $\Gamma_{4,3}$ is the peak to peak line width of the EPR derivative signal at 1.5 kG.

However, significant differences are observed at Doppler velocities +4 mm/s and −3.5 mm/s. The absorption in these regions is roughly proportional to the thermal population of the middle "4.3" doublet. The MECAM complex exhibits a middle doublet population slightly greater than that of ent. The spectra of the TRIMCAM and DMB complexes show appreciable population of the "4.3" doublet, indicating that the zero field energies of these doublets are comparable to $kT = 1$ cm^{-1}.

The 4.2 K spectrum of aqueous Fe(ent) (Figure 3A) shows the absorption associated with its middle doublet is broad. This shape is not compatible with the electronic model postulated previously for Fe(ent) in methanol.[10] However, the intensity is compatible with the 2.5 cm^{-1} energy level quoted for the methanol sample. A 4.2 K spectrum of DMB (not shown) exhibits an absorption peak at −8.3 mm/s, evidence of the uppermost doublet being populated. Preliminary analysis indicates that about 10% of the total absorption is associated with this component; hence the energy of the most excited doublet of Fe–DMB is about 5 cm^{-1} above the ground doublet.

The outermost lines of the TRIMCAM and DMB 1.5 K spectra are broad and indicate that within the samples there are substantial variations in the internal field produced by the ground doublets. The EPR data discussed below suggest that this range of internal fields is produced by a heterogeneous iron environment and not by a homogeneous sample with a well-defined ground doublet. The sharper outer lines of parts A and B of Figure 2 may seem to imply that the ent and MECAM samples are homogeneous. However, the spectral components associated with their excited doublets are very broad and suggest the same order of heterogeneity as observed for TRIMCAM and DMB. (See the 4.2 K ent spectrum in Figure 3A. The middle doublet yields absorption again in the +4 and −3.5 mm/s regions, and the third doublet yields absorption at −6 and +7 mm/s.)

Figure 3 illustrates the pH dependence of the aqueous ^{57}Fe(ent) Mössbauer spectra. As the pH is lowered, the magnetic spectrum collapses into a quadrupole pair with $\Delta E_Q = 0.8$ mm/s and $\delta = 0.5$ mm/s. This quadrupole splitting and isomer shift are characteristic of high-spin ferric ions and not of ferrous material. The small peak at +3 mm/s in spectrum D of Figure 3 *does* arise from high-spin ferrous material and represents about 5% of the total iron in that sample, consistent with previous observations for monomeric catechol ligands such as DMB.[11] The fraction of fast relaxing ferric material at each pH is given in Table II. A similar pH dependence was observed for MECAM and DMB.

The Fe(ent) complex precipitates as the pH is lowered (<4). A possible explanation of the appearance of the fast-relaxing species is that a decrease in solubility of the Fe(ent) causes a decrease in the iron–iron distance. This could lead to an increase in the spin relaxation rate due to spin–spin interactions. However, no change was observed in the fast relaxing fraction of the pH 5 sample upon addition of 0.2 mL of 0.1 M $NaNO_3$. Also, a decrease in the Fe(ent) concentration by a factor of 8 caused no observable difference.

Mössbauer spectra were also recorded of the ^{57}Fe(ent) complex in methanol. Figure 4A shows the high pH spectrum is very similar to the aqueous sample; however, the spectral component arising from the middle Kramers doublet is more distinct. The sharper features of this component correlate well with the EPR data presented below. As the pH is lowered, two things occur. First, the magnetic spectrum of the ferric ions collapses into a quadrupole pair as in the aqueous samples ($\Delta E_Q = 0.6$ mm/s, $\delta = 0.5$ mm/s, see bracket in Figure 4B). Second, a significant fraction of the iron is reduced to high-spin Fe^{2+}. [Our ^{57}Fe-enriched samples yielded spectra with signal-to-noise ratios an order of magnitude greater than the published spectrum of Hider et al.[15] These better quality data allowed us to quantitate the relative amounts of Fe(III) and Fe(II). The Fe(III) signal stretching from −8 to +10 mm/s is resolved in Figure 4D but would be lost in the noise if the sample had not been enriched.] The ferrous material exhibits a quadrupole pair with $\Delta E_Q = 3.44$ mm/s and $\delta = 1.38$ mm/s (indicated by the bracket in Figure 4D). The fractions of reduced material in each methanol sample are given in Table II. This redox behavior is reversible if the pH is raised immediately after acidification, but irreversible if allowed to stand for several hours. A mechanism which explains this behavior is presented below.

Table II. Solvent and pH Dependence of Iron Electronic State and Relaxation Rates

chelating agent	solvent	pH	% Fe^{3+}	% Fe^{3+} fast relaxing	% Fe^{2+}
enterobactin	H_2O	9	>95	<5	<5
enterobactin	H_2O	6	>95	15 ± 5	<5
enterobactin	H_2O	5	>95	30 ± 5	<5
enterobactin	H_2O	2	~95	40 ± 5	~5
enterobactin	methanol	9[a]	>98		<2
enterobactin	methanol	4	70 ± 5		30 ± 5
enterobactin	methanol	2	60 ± 5		40 ± 5
enterobactin	methanol	1	55 ± 5		45 ± 5
MECAM	H_2O	9	>95	<5	<5
MECAM	H_2O	6	>95	42 ± 5	<5
MECAM	H_2O	4	>95	50 ± 5	<5
MECAM	H_2O	<2.5	>95	45 ± 5	<5
TRIMCAM	H_2O	10	>95	<5	<5
DMB	H_2O	10	>95	<5	<5
DMB	H_2O	4	>95	45 ± 5	~5

[a] See Materials and Methods section and ref 21.

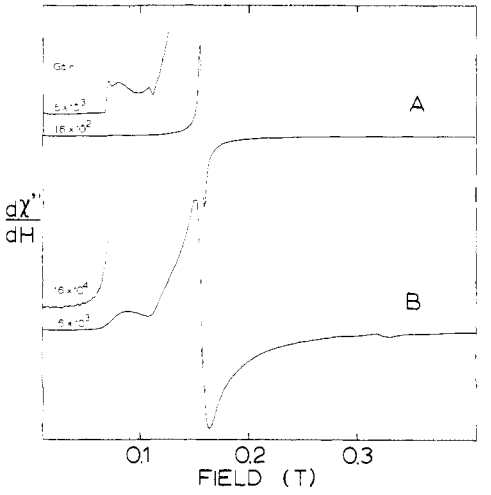

Figure 5. EPR spectra of Iron-57 enterobactin at high pH in methanol (A) and in water (B). The Mössbauer spectrum of the sample used for A is shown in Figure 4A. Mössbauer spectra of the sample used for B are shown in Figures 2A and 3A. EPR conditions: microwave frequency 9.22 GHz and temperature 3 K.

EPR Results. The EPR spectra of two samples used in the Mössbauer studies of the high-pH complexes of aqueous and methanolic ^{57}Fe(ent) are shown in Figure 5. A striking contrast is observed between the extremely broad EPR features of the aqueous sample and the comparatively sharp signal of the methanol sample. The 5-mT peak-to-peak width of the "4.3" signal of the methanol sample compares favorably with the 4 mT width observed by O'Brien et al.[6] Compared with the 13-mT width

observed for the aqueous sample, the 5 mT width suggests a much more isotropic and homogeneous induced moment for the iron atoms of the methanol sample. This difference is observed in the Mössbauer data as well. The features of the spectral component of the middle Kramers doublet are much sharper in Figure 4A than in Figure 3A. The EPR spectra of high-pH aqueous samples of MECAM, TRIMCAM, and DMB were also recorded and are very similar to the aqueous ent spectrum of Figure 5B. The widths of the "4.3" features of all the high-pH complexes are given in Table I.

A number of other EPR spectra of the ferric complexes of enterobactin, MECAM, and TRIMCAM as these complexes are protonated at low pH were recorded, and some of these spectra are included as supplementary material (Figures 6 through 8).

Discussion

The broad EPR and Mössbauer spectral components associated with the aqueous Fe(ent) middle Kramers doublet suggest a significant variation in the iron environment within the sample. Such heterogeneity has been termed "g strain".[26] The EPR spectrum of Figure 5B indicates significant absorption from g = 6 to 4.3; and the corresponding Mössbauer absorption around +4 and −3.5 mm/s is broad and featureless. However, the Mössbauer spectral component arising from the ground doublet is just as sharp as that of the ground doublet of Fe(ent) in methanol (see Figures 3A and 4A). If we assume an intrinsic Mössbauer line width of 0.3 mm/s, the 0.5 mm/s line widths of the outermost lines of Figure 2A imply that H^{int} varies by less than 2% throughout the sample. Thus the nature of the heterogeneity of the iron environment is such that the ground doublet changes little but the middle doublet is greatly affected.

Spartalian et al.[10] determined a set of values for D, λ, μ, V_{zz}, Γ, and A_0 for Fe(ent) in methanol. Their model was constrained to yield an isotropic 4.3 g value for the middle doublet as observed by O'Brien.[6] Our high pH methanol Fe(ent) spectrum is very similar to that observed earlier[10] except for significant improvement in the signal to noise ratio. Our data indicate that a model with $g_x = g_y = g_z = 4.3$ for the middle doublet will not suffice. As can be seen from eq 6, the isotropic $g = 4.3$ will produce an internal field of constant magnitude. Thus, such a doublet would produce sharper absorption features than are observed in Figure 3A. Hence, the Mössbauer data of the methanol sample also suggest "g strain" but to a much smaller extent than in the aqueous samples. The $\Gamma_{pp}^{4.3}$ = 5 mT EPR line width for the methanol samples is also broader than some other high-spin ferric complexes. For example, 3,4-dioxygenase from *B. fuscum* exhibits a $\Gamma_{pp}^{4.3}$ < 2 mT line width.[27]

The fast relaxing Fe^{3+} spectral component of the methanol sample at pH 4 has ΔE_Q = 0.62 mm/s, significantly larger than the 0.38 mm/s reported earlier.[10] However, the sensitive quantity in the slowly relaxing spectrum is V_{ii}, the EFG component along the internal field. The two reported values of V_{ii} for the ground doublet of Fe(ent) in methanol agree well.

The structures of the ground multiplet of the MECAM, TRIMCAM, and DMB complexes in aqueous solution appear to be similar to that of ent. The same effects of "g strain" are observed in both the EPR and Mössbauer data. However, the middle doublets of TRIMCAM and DMB are significantly lower in energy than those of ent and MECAM. The similar EPR spectra and the different energy levels could be explained in terms of the fourth-order Hamiltonian of eq 2.

At low pH the magnetic spectrum of the aqueous Fe(ent) was observed to collapse into a quadrupole pair characteristic of high-spin Fe^{3+}. This collapse could be explained by at least two separate schemes: (1) At low pH the Fe(ent) molecules could dimerize, to form diamagnetic pairs. Such a dimer would yield the observed quadrupole pattern. However, such a dimerization process can be ruled out from the solution titration data presented previously.[8,9] (2) An increase in the spin relaxation rate would lead to such a collapse. The data suggest the second mechanism is correct since the presence of Fe sites having an intermediate relaxation rate would explain the observed broad absorption in Figure 3D.

We have previously proposed that, as the acidity of an aqueous solution of ferric enterobactin is increase, three stepwise single protonations of the metal complex occur;[11,17-19] and the ferric ion coordination sphere is filled by the ortho-phenolic and carbonyl oxygens of three catecholylamide moieties in what has been called a "salicylate" bonding mode.[16] Recently, two papers[15,20] have appeared which claim that, at low pH, ferric enterobactin (in methanol) and the ferric complexes of simple bidentate catecholates (in water) undergo spontaneous electron transfer to form ferrous semiquinone complexes. If such a redox mechanism were operating for enterobactin in aqueous solution, the proton stoichiometries and physical properties of the iron enterobactin complex could not be explained by the salicylate bonding hypothesis.

However, the data presented here prove that in aqueous solution the iron enterobactin complex remains predominantly in the high spin ferric oxidation state at *all* observed pH values. Therefore, we conclude that in aqueous solution the salicylate bonding mode is still the best explanation of all these data. The following paper (a Fourier transform infrared study) discusses this argument in greater detail.

Our experiments have also confirmed that, in methanolic solution, ferric enterobactin undergoes an internal electron transfer under acidic conditions. However, we have quantified the amount of ferrous ion present and demonstrated that a substantial proportion of the iron remains as ferric ion. (The quantification is very important, as will be discussed further in the following report.) The mechanism shown below explains both the redox chemistry as well as the potential irreversibility of the reaction.

$$[Fe^{III}(ent)]^{3-} \underset{\text{methanol}}{\overset{H^+}{\rightleftharpoons}} [Fe^{III}(H_n ent)]^{n-3} \rightleftharpoons [Fe^{II}(H_n ent)]^{n-3} \rightarrow \text{polymeric quinones} + Fe^{2+}$$

As the acidity of a methanolic solution is increased, the expected highly negative redox potential for $[Fe(ent)]^{3-/4-}$ becomes more positive, thus stabilizing the ferrous complex. Mentasti[28] has shown that for monomeric catechol ligands under very acidic conditions in water, $[Fe^{III}(cat)]^+$ will form ferrous ion and the semiquinone radical via an internal iron–catechol redox couple. If the solution is kept strictly anaerobic and the pH raised rapidly, the ferric catecholate can be regenerated. However, if the solution is allowed to stand, the seniquinone radicals may couple, yielding stable polymeric quinones. Formation of these polymers would push the reaction further toward Fe^{2+}.

In microbial iron uptake via enterobactin, a decrease in charge upon protonation of ferric enterobactin may facilitate the passage of the complex through the inner membrane: two different structures for this protonated complex have been proposed, as discussed in the following paper. Hider et al.[15] have suggested, based on their Mössbauer data of the iron–ent system; that the mechanism of iron release may be via the internal redox reaction of Fe(III)/catechol to Fe(II)/semiquinone in the nonaqueous environment of a membrane. Under such conditions the Fe(II) would be released. However, the results shown here show that it is not valid to extend this model to low pH aqueous solutions, since the complexes remain as Fe(III) to pH 2. There is however a large change in redox potential in acidic aqueous solution, such that reduction of the protonated ferric enterobactin complex becomes feasible.[17]

The earlier model for enterobactin iron release[11,12] involves cleavage of the ester ring of ferric enterobactin, which causes the redox potential to move within the accessible range of physiological reductants.[14] Bacterial growth studies[29] using synthetic catecholate

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analogues such as MECAM indicate that the esterase model may be incorrect (these analogues have similarly negative redox couples as enterobactin, without susceptibility to central ring cleavage), since some of them act as effective sources of iron. In the absence of a specific cytoplasmic ferrireductase, there are now three thermodynamically feasible mechanisms for iron release by enterobactin within the organism. These are ligand hydrolysis and complex reduction, protonation and internal electron transfer within a low pH nonaqueous environment, or protonation and reduction in a low pH aqueous environment. Which is operative remains an open question.

Summary

Mössbauer, EPR, and titration experiments have demonstrated that in aqueous solution the iron complexes of enterobactin and synthetic analogues remain in the ferric oxidation state between the pH range 2 and 10. In the Mössbauer spectra, the overall zero field splittings of the sextets of the four complexes appear to be roughly 5 cm^{-1}. However, the energy of the middle doublet, E^m, varies as 2.5 $cm^{-1} \simeq E^m_{ent} \geq E^m_{MECAM} \geq E^m_{TRIMCAM} \simeq E^m_{DMB} \simeq 1$ cm^{-1}. As the hydrogen ion concentration is increased, a quadrupole doublet indicative of a fast-relaxing, high-spin, ferric complex is observed.

In contrast to our aqueous results, there appears to be a quasi-reversible redox reaction which occurs at low pH′ in methanol.[21] However, if the acidic solution is allowed to stand for a prolonged period, all of the ferric species cannot be regenerated. A mechanism for the low pH′ methanolic solution chemistry would involve protonation of the ferric enterobactin complex, followed by subsequent reduction of the metal by the coordinated catechol moiety. The semiquinone radical which is formed may either couple to another semiquonine to form a polymeric quinone or, if the pH is rapidly raised, can allow regeneration of the ferric catecholate complex.

The redox chemistry observed in nonaqueous solvents is consistent with a mechanism for cellular iron removal from catecholate siderophores which utilizes large pH gradients. When the dielectric constant and/or pH of the medium is changed, it may be possible to reduce the ferric enterobactin complex without prior hydrolysis of the ester ring.

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Registry No. K_3[Fe(ent)], 85760-76-5; [^{57}Fe(MECAM)]$^{3-}$, 85710-20-9; [^{57}Fe(TRIMCAM)]$^{3-}$, 85710-21-0; [^{57}Fe(DMB)$_3$]$^{3-}$, 85710-22-1; MECAM, 69146-59-4; TRIMCAM, 71353-09-8; enterobactin, 28384-96-5.

Supplementary Material Available: The pH- and temperature-dependent EPR spectra of ferric complexes of enterobactin, MECAM, and TRIMCAM in Figures 6, 7, and 8 (4 pages). Ordering information is given on any current masthead page.

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Coordination Chemistry of Microbial Iron Transport Compounds. 23. Fourier Transform Infrared Spectroscopy of Ferric Catechoylamide Analogues of Enterobactin[1]

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Abstract: Infrared spectroscopy has been used to investigate the mode of coordination of ferric ion by catechoylamide complexes in the solid state and in acidic D_2O media. Shifts in the carbonyl stretching frequency during protonation of ferric enterobactin model complexes are consistent with a "salicylate" bonding mode that has been postulated for the enterobactin system and rule out a two-proton stoichiometry in a mechanism involving stepwise dissociation of individual catechol rings. The synthetic catecholates MECAMS [*N,N′,N″*-tris(2,3-dihydroxy-5-sulfobenzoyl)-1,3,5-tris(aminomethyl)benzene], 3,4-LICAMS [*N,N′,N″*-tris(2,3-dihydroxy-5-sulfobenzoyl)-1,5,10-triazadecane], and TRIMCAMS [*N,N′,N″*-tris(2,3-dihydroxy-5-sulfobenzyl)-1,3,5-tricarbamoylbenzene] have been investigated. Of special interest are MECAMS and TRIMCAMS, since these structural isomers differ only in the location of a carbonyl group. Examination of a bis(catecholate) complex, cupric MECAMS, has shown that a single uncoordinated ligand arm can be detected even when the other two arms are coordinated to the metal. The synthetic tris(salicyloyl) ligand MESAM [*N,N′,N″*-tris(2-hydroxybenzoyl)-1,3,5-tris(aminomethyl)benzene] has been synthesized as a model for the "salicylate" bonding mode. The bands (KBr) at 1640 cm^{-1} for the free ligand and 1606 cm^{-1} for the ferric complex are consistent with published IR (KBr) data for the related ferric MECAM and ferric enterobactin complexes. These results are consistent with the previous thermodynamic data for ferric enterobactin, which predict a reduction potential of +170 mV (NHE) at pH 4.

The solution chemistry of enterobactin (Figure 1) and its metal complexes has been extensively studied.[1,5-15] At high pH the six phenolic oxygens derived from catecholate groups form a high-spin, octahedral complex around the metal ion.[8,13] The redox potential

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