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.

Acknowledgment. We thank Dr. Melvin P. Klein (U.C.B.) for access to his laboratory and helpful discussions and Dr. H. Frank and Mr. J. McCracken (U.M.) for experimental assistance. Discussions with Drs. B. H. Huynh, C. J. Carrano, and W. R. Harris are acknowledged with pleasure. This research was supported by the NIH (Grant AI 11744) and in part (T.K.), the NSF (Grant PCM 80-05610). An American Cancer Society postdoctoral fellowship to G.B.W. is gratefully acknowledged.

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

(1) Previous paper in this series: Pecoraro, V. L.; Wong, G. B.; Kent, T. A.; Huynh, B. H.; Raymond, K. N., preceding paper in this issue.

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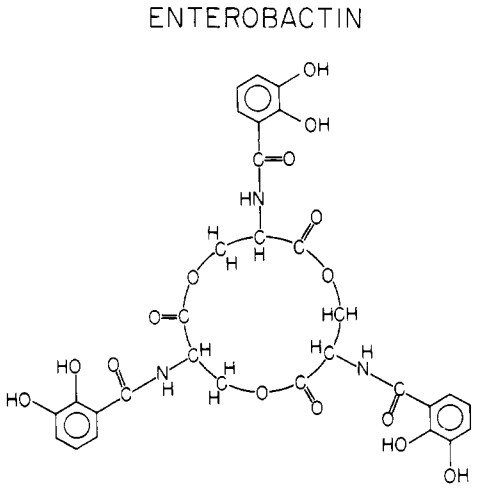

Figure 1. Structural formula of the catecholate siderophore enterobactin.

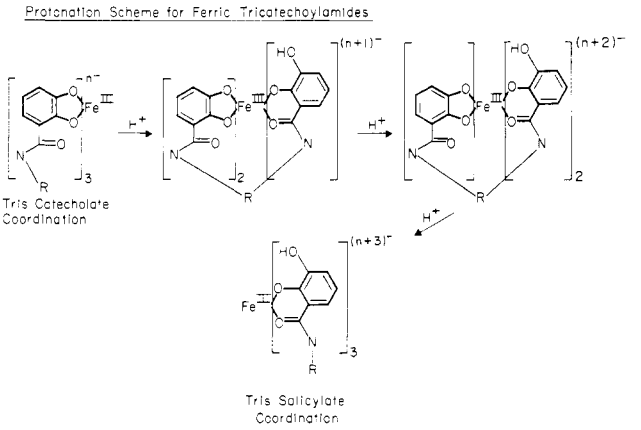

Figure 2. Proposed protonation scheme for ferric tricatechoylamide complexes which possess a carbonyl group adjacent to the catecholate ring. The resultant triply protonated species engages in the "salicylate" coordination mode which is described in the text.

for $[Fe(ent)]^{3-}$[10] is probably far too negative (estimated ~ -750 mV at pH 7) to allow reduction by known biological reductants in neutral aqueous solution. In contrast, the tris[2,3-(dihydroxybenzoyl)serine]iron(III) complex can be reduced within the range of known physiological reductants. It has been proposed[16] that the microbial mechanism for iron release from enterobactin includes degradation of the ester ring by an esterase, followed by reduction of the ferric complex. However, it has been shown recently that heart[17] and microbial[18] cells also can acquire iron from synthetic catecholate complexes[19] that closely mimic the enterobactin coordination site. These compounds possess similar thermodynamic stability and cyclic voltammetric behavior (unpublished results) but lack ester backbones which could be enzymatically hydrolyzed.[20-23] Thus, it appears that an alternative mechanism of iron release may be operative.

A previous investigation of ferric enterobactin has shown that the complex undergoes a series of three, overlapping, single-protonation steps to form a water-insoluble purple solid.[8] The solid-state IR spectrum of $H_3Fe(ent)^0$ in KBr suggested that the carbonyl oxygen adjacent to the catecholate ring was directly coordinated to the metal.[8] This mode of bonding, summarized in Figure 2, in which the ortho-hydroxyl and α-carbonyl oxygens are ligating atoms has been called "salicylate bonding" by analogy to the complexes formed by salicylic acid.

Experimentally, the study of enterobactin is complicated by rapid hydrolysis of the central ester linkages, the water insolubility of the triprotonated ferric complex, and the air sensitivity of the catechol ring in basic solution. For these reasons, much of our work is done with synthetic analogues which closely mimic the enterobactin coordination site, while being functionalized to allow study in aqueous solution and prevent air oxidation. A number of synthetic enterobactin analogues[24] are shown in Figure 3. These compounds comprise three anion types: MECAMS, which potentially can engage in salicylate bonding; TRIMCAM, which cannot undergo this bonding mode; and MESAM, which bonds only via the salicylate mode.

In this report, we directly probe the coordination environment of the metal ion as a function of pH. We have obtained data from these synthetic ligands, which support a salicylate mode of bonding, by specifically monitoring the stretching frequency of the α-carbonyl.

An alternative mechanism whereby iron may be released from enterobactin at low pH has been put forward by Hider et al.[12,25] These authors propose that an internal redox reaction between ferric ion and catechol generates a blue, ferrous semiquinone species. In the preceding paper, we have demonstrated by Mössbauer spectroscopy that iron–enterobactin complexes at pH 2 or greater generate only trace amounts of Fe(II); the predominant species in both solution and the solid state remain complexes of Fe(III). In this paper we present additional evidence which demonstrates that the conclusions of Hider et al.[12,25] are incorrect.

Materials and Methods

Preparation of Compounds. Chemical analyses were performed by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. Enterobactin was isolated from cultures of *Kleb-*

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(24) Abbreviations used: 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; 3,4,3-CYCAMS, 1,5,9-tris(2,3-dihydroxy-5-sulfobenzoyl)-1,5,9-cyclotriazadecane; TRIMCAMS, *N,N',N''*-tris(2,3-dihydroxy-5-sulfobenzyl)-1,3,5-tricarbamoylbenzene; MESAM, *N,N',N''*-tris(2-hydroxybenzoyl)-1,3,5-tris(aminomethyl)benzene; TRENSAM, *N,N',N''*-tris(2-hydroxybenzoyl)-tris(2-aminoethyl)amine; TREN, tris(2-aminoethyl)amine; ent, enterobactin; MECAM, *N,N',N''*-tris-(2,3-dihydroxybenzoyl)-1,3,5-tris(aminomethyl)benzene; TRIMCAM, *N,N',N''*-(2,3-dihydroxybenzyl)-1,3,5-tricarbamoylbenzene.

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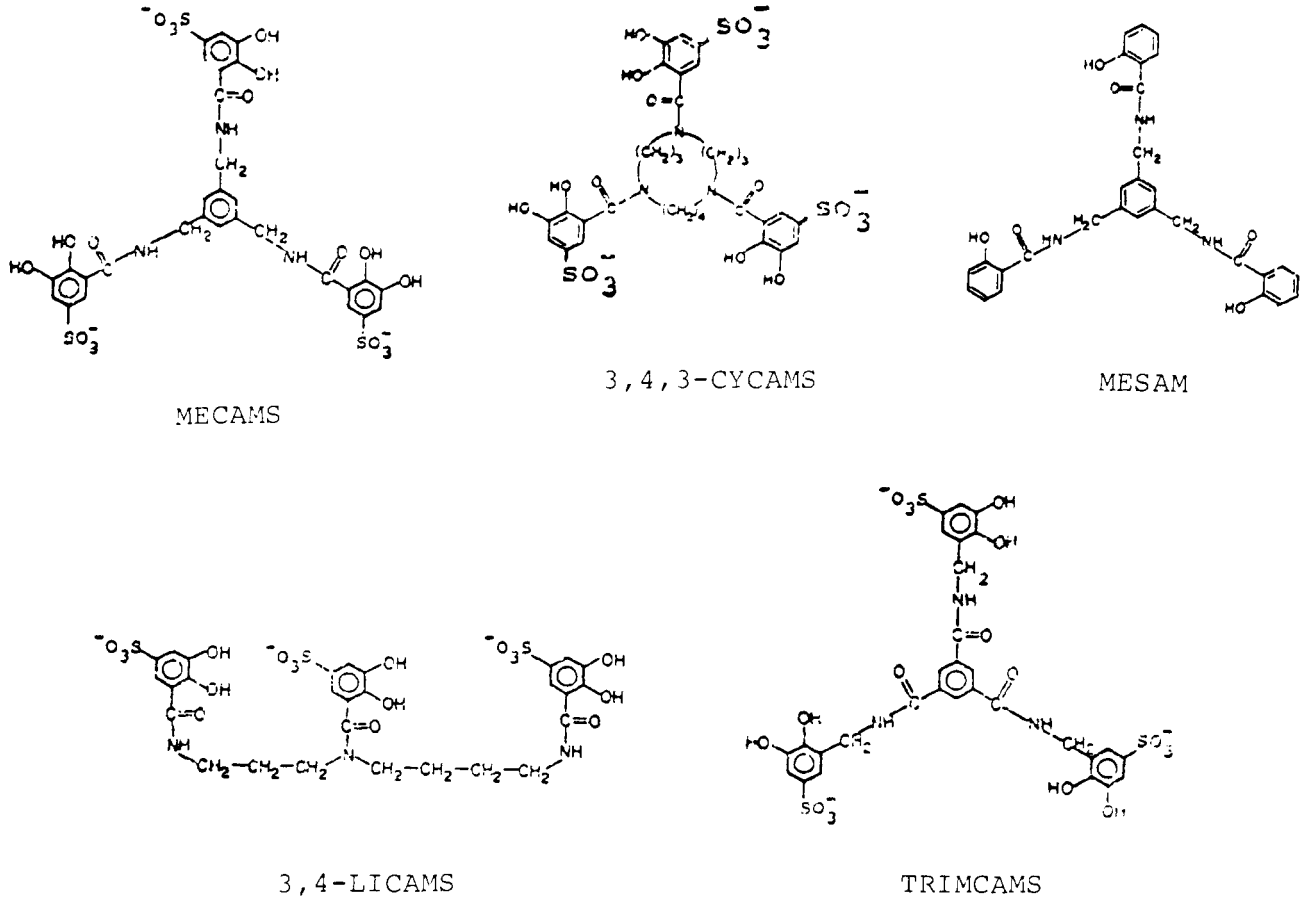

Figure 3. Synthetic tricatechoylamide sequestering agents used in the study.

siella pneumoniae by modification[1] of a literature procedure[7] and further purified after formation of the iron complex.[1] The preparation of MECAM has been previously described.[1,19] Sulfonated catechoylamides MECAMS, TRIMCAMS, 3,4-LICAMS, and 3,4,3-CYCAMS were generously provided by Dr. Frederick L. Weitl.

The 2-methoxybenzoyl chloride was prepared by refluxing 2-methoxybenzoic acid in excess $SOCl_2$. After removal of $SOCl_2$ by coevaporation with CCl_4, the product was used without further purification.

***N,N',N''*-Tris(2-methoxybenzoyl)-1,3,5-tris(aminomethyl)benzene (Me_3MESAM).** A solution of 1,3,5-tris(aminomethyl)benzene trihydrochloride (7.3 mmol, 2.0 g),[19] 2-methoxybenzoyl chloride (0.03 mol), and triethylamine (0.03 mol) in 30 mL of $CHCl_3$ was refluxed overnight. The reaction mixture was washed (dilute NaOH, dilute HNO_3), dried ($MgSO_4$), and evaporated to an oil. Crude product was dissolved in $CHCl_3$ and applied to a silica gel column (Baker analyzed reagent). After the solution was washed with $CHCl_3$ to remove some impurities, the product was eluted with $CHCl_3$/hexane/EtOH (9:5:1) and detected by TLC (Baker silica gel G/HR, eluting solvent, I_2). The purest fractions were combined, concentrated to a pale yellow oil, and used in the next step.

***N,N',N''*-Tris(2-hydroxybenzoyl)-1,3,5-tris(aminomethyl)benzene (MESAM).** To an ice-cooled CH_2Cl_2 (70 mL) solution of Me_3MESAM (2.6 g, 3.9 mmol) under N_2 was slowly added 4 mL of BBr_3 in 50 mL of CH_2Cl_2. The resulting yellow suspension was stirred at room temperature overnight. After slow addition of 50 mL of water, any lumps were broken to afford a fine powder which was collected by filtration, washed well with water, and air-dried. The powder was dissolved in boiling ethanol, and water was added gradually to precipitate the crude product, yield 50%. This was recrystallized twice from a minimum of boiling methanol to yield white microcrystals: mp (uncorrected) 166–167 °C; ^{1}H NMR (Me_2SO-d_6) δ 4.4 (d, 6 H, Ch_2), 7.1 (s, 3 H, central high H), 6.5–8.0 (m, 12 H, ring H), 9.1 (br, 3 H, NH). Anal. Calcd for $C_{30}H_{27}N_3O_6$: C, 68.56; H, 5.18; N, 8.00. Found: C, 68.34; H, 5.31; N, 7.91.

Preparation of Iron Complexes. Ferric Enterobactin. To a methanol solution of enterobactin was added a small excess of aqueous ferric nitrate solution. Neutralization to pH 7 with aqueous KOH produced a burgundy color. After removal of solvent in vacuo at room temperature, the residue was dissolved in a small amount of water (pH 8) and diluted with a 0.25 volume of methanol. This solution was passed through a 15 × 1 cm neutral alumina column previously equilibrated with 20% methanol/water (pH 8). Fractions containing the red $[Fe(ent)]^{3-}$ were evaporated in vacuo and then dissolved in D_2O just prior to the IR measurements.

Synthetic Ferric Catechoylamides. The $[Fe(MECAM)]^{3-}$, $[Fe(MECAMS)]^{6-}$, $[Fe(TRIMCAMS)]^{6-}$, $[Fe(3,4\text{-LICAMS})]^{6-}$, and $[Fe(3,4,3\text{-CYAMS})]^{6-}$ complexes were all prepared by adding a 50% excess of $Fe(NO_3)_3$ to a D_2O solution of the appropriate ligand, which had been deprotonated with NaOD in D_2O solution.

$[Fe(MESAM)]^0$. The $[Fe(MESAM)]^0$ was prepared by adding a 5% excess of $Fe(NO_3)_3$ aqueous solution to a methanol solution of MESAM. Neutralization with aqueous KOH produced an amorphous orange solid, only slightly soluble in MeOH or water. After the mixture was washed with methanol, the solid was dried in vacuo. Protonated Fe–MESAM was prepared by the addition of a small amount of acid to $[Fe(MESAM)]^0$ in methanol, causing it to dissolve to form a purple solution.

Cupric MECAMS. A 5% excess of $Cu(NO_3)_2$ in aqueous solution was added to MECAMS in D_2O. By use of NaOD or DCl solutions, pH was adjusted in order to produce the species desired, $[Cu(H\text{-MECAMS})]^{6-}$ or $[Cu(MECAMS)]^{7-}$, as determined from published titration data.[26]

Titrations. All pH measurements were made by using a Beckman Instruments pH-102 Metrohm pH equipped with a Sigma combination electrode. The pH meter was standardized by using Mallinckrodt pH 4.01 and 7.00 buffers. The pH values reported here for D_2O solutions were obtained by taking pH meter readings in the usual manner and correcting the readings for D_2O using the method of Perrin.[27] The pH was decreased incrementally by addition of approximately 3 M DCl to each solution. To disprove possible degradation of the ligands and ferric complexes, IR spectra were taken on a sample before acidification and on a base-neutralized sample after acidification. There were no spectral differences.

IR and Visible Spectra. Solid-state spectra were taken on KBr pellets with a Perkin-Elmer Model 597 spectrophotometer. Fourier transform infrared spectra of D_2O solutions were obtained on a Nicolet 7199 FT IR spectrometer at 0.5-cm^{-1} resolution. Perkin-Elmer AgCl cells were

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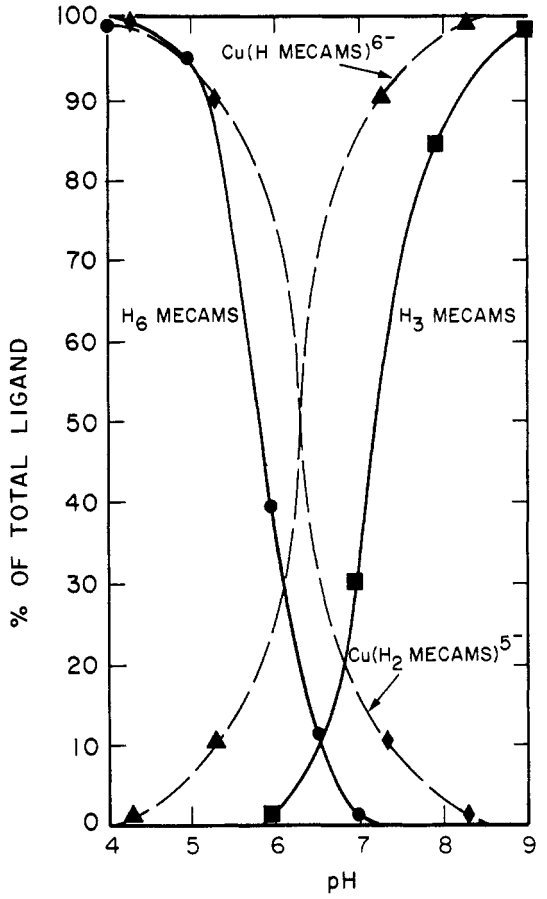

Figure 4. Species distribution curves for the protonation equilibria of $H_nMECAMS$ and $CuH_nMECAMS$: $H_6MECAMS^{3-}$ (—); $H_3MECAMS^{6-}$ (---); $Cu(HMECAMS)^{6-}$ (-·-); $Cu(H_2MECAMS)^{5-}$ (-···-).

used for all spectra. Visible spectra were recorded on a Hewlett-Packard 8450A or Cary 118 spectrophotometer.

Results

Unlike enterobactin, MECAMS and 3,4-LICAMS are water soluble. This allows one to work in pH regions that would have resulted in precipitation of enterobactin or its iron complexes.

Cu^{II}–MECAMS. Species distribution curves for Cu–MECAMS and MECAMS free ligand are shown in Figure 4. Below pH 5.4 copper(II) forms a complex by binding two of the catechol arms of MECAMS: the third arm remains free.[26] The IR spectra at pH 5 (Table I) show a CO band at 1601 cm^{-1}, assigned to the metal-coordinated catechol arms, and another band at 1629 cm^{-1}. Since the metal complex is completely formed at pH 5 and metal is in slight excess, free ligand cannot be present. Therefore, the 1629 cm^{-1} band does not arise from free ligand but must be due to an unbound protonated catechol arm on a metal complex. This observation demonstrates that this sort of ligand arm configuration can be identified in the IR spectra. At higher pH this arm becomes monodeprotonated and shows up as a shoulder at ~1604 cm^{-1}, the same band position as for a mono-deprotonated arm on free ligand.

TRIMCAMS. The carbonyl stretching frequency of TRIMCAMS did not shift significantly from 1638 cm^{-1} upon formation of the iron complex (Table I). Protonation of ferric TRIMCAMS produced no significant shifts in ν_{CO}, which is not surprising in light of the previous observation.

MESAM. An orange solution of $[Fe(MESAM)]^0$ in methanol absorbs at λ_{max} = 468 nm, which shifts to ~492 nm when a purple protonated complex is formed. Infrared data (Table II, Figure 5) show that the ligand ν_{CO} shifts from 1640 cm^{-1} in the free ligand to 1606 cm^{-1} in the Fe(MESAM) complex; when this complex is protonated, the 1606 cm^{-1} band assigned to metal-bound arms remains, but an additional band at 1640 cm^{-1} appears which we assign to a dissociated ligand arm.

Ferric MECAMS and 3,4-LICAMS. Infrared data (Table I, Figures 6 and 7) for the metal complexes show that Fe–MECAMS and Fe–LICAMS behave similarly. Published[21] equilibrium constants for the first three metal complex protonation reactions (log K = 5.74, 4.10, and 3.46 and 6.16, 5.3, and 3.10, respectively) allow determination of proton-to-complex stoichiometries at any given pH. The resultant species distribution curves for Fe–MECAMS have been calculated and are shown in Figure 8. In the pH region corresponding to the addition of zero to three protons, infrared bands are present near 1605 or 1608 cm^{-1} for the two complexes, respectively, which are assigned to metal-bonded catechol arms. there is a band at 1629 cm^{-1} which would indicate

Table I. Perturbation of the Amide I Band as a Function of pH for Metal Catechoylamide Complexes

		ν, cm^{-1}		
ligand	pH[a]	free arm[b] carbonyl	catechol/ salicylate[c] carbonyl	solv[d]
MECAM		1635		KBr
$Fe(MECAM)^{3-}$			1605	D_2O
$Fe(H_3MECAM)^0$			1610 (sh)	KBr
MECAMS	3	1629		D_2O
MECAMS	9	1604		D_2O
Fe(MECAMS)	8.0		1605	D_2O
	5.1		1608	
	4.5		1608	
	3.4		1610	
	2.3	1624 (sh)	1610	
	1.0	1627		
Ga(MECAMS)	8.5		1609	D_2O
	5.4		1611	
	4.6	1629 (sh)	1612	
	3.0	1629 (sh)	1613 (sh)	
	0.8	1628		
Cu(MECAMS)	8.9	1611 (sh)	1602	D_2O
	6.6	1614 (sh)	1600	
	5.9	1629 (sh), 1616 (sh)	1601	
	4.9	1629		
3,4-LICAMS	1.0	1625		D_2O
Fe(3,4-LICAMS)	7.1		1603	D_2O
	6.0		1602	
	4.1		1604	
	3.2		1604	
	1.2	1628	1605	
TRICAM		1638		KBr
Fe(TRIMCAM)			1640	KBr
protonated Fe(TRIMCAM)			1636	KBr
TRIMCAMS		1638		
Fe(TRIMCAMS)	9.2	1636		
	6.6	1635		
	3.7	1638		
	2.5	1638		

[a] pH calculated by using method described in ref 27. [b] Peak assignment for amide I band of a noncoordinated catechol moiety. [c] Peak assignment for amide I band of a catechol moiety coordinated to a metal ion. This may either be via a salicylate or catecholate bonding mode. See text for further discussion. [d] Spectra were recorded as described in Materials and Methods.

Table II. Comparison of the Amide I Stretching Frequencies for Catechoylamide and Salicylamide Complexes[a]

		$\nu_{C=O}$, cm^{-1}
ligand	enterobactin	1640
	MECAM	1635
	MESAM	1640
catecholate complexes	$[Fe(ent)]^{3-}$	1593
	$[Fe(MECAM)]^{3-}$	1605
salicylate complexes	$[Fe(H_3ent)]^0$	1640, 1620
	$[Fe(H_3MECAM)]^0$	~1610
	$[Fe(MESAM)]^0$	1606

[a] Spectra were taken in KBr.

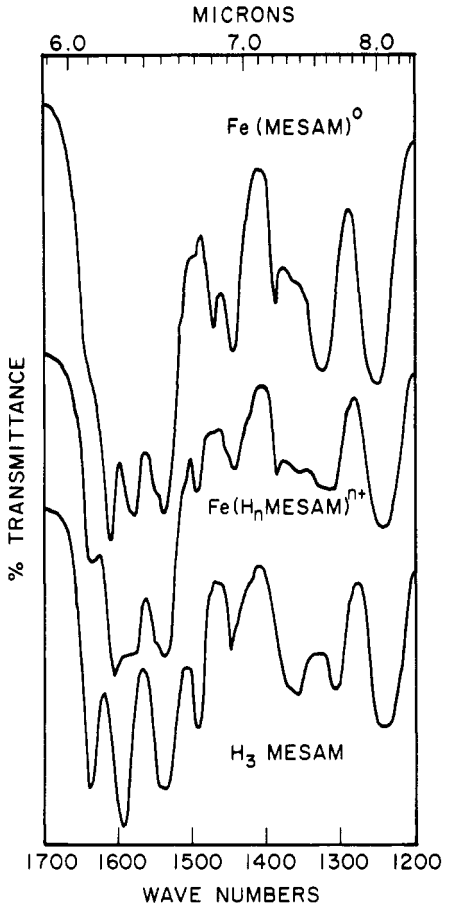

Figure 5. Solid-state IR spectra (KBr) of the ferric complexes of MESAM. Curves: (a) Fe(MESAM)^0; (b) Fe(H_nMESAM)$^{n+}$; (c) H_3MESAM.

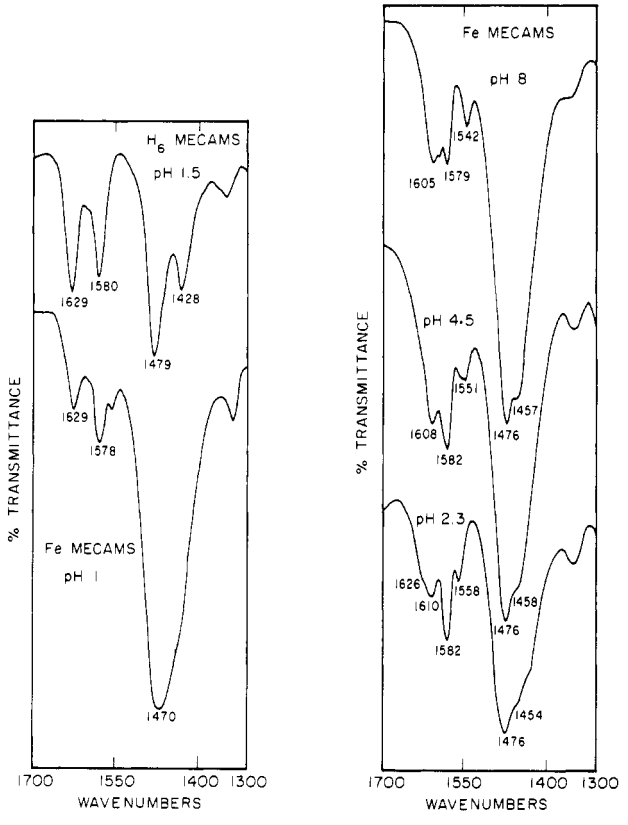

Figure 6. FT IR solution spectra (a) of ferric MECAMS as a function of pH in D_2O solution and (b) of MECAMS and ferric MECAMS product in acidic solution.

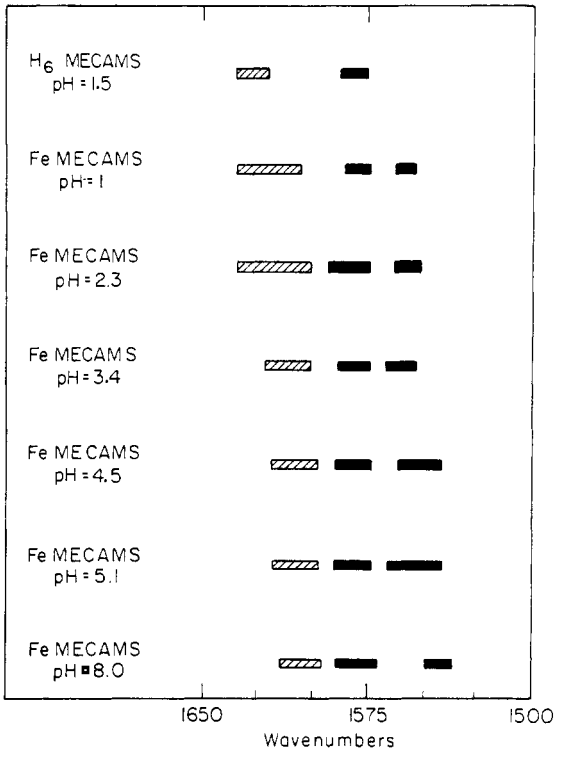

Figure 7. A schematic representation of the shift in the carbonyl stretching frequency as a function of pH for the ferric MECAMS complexes.

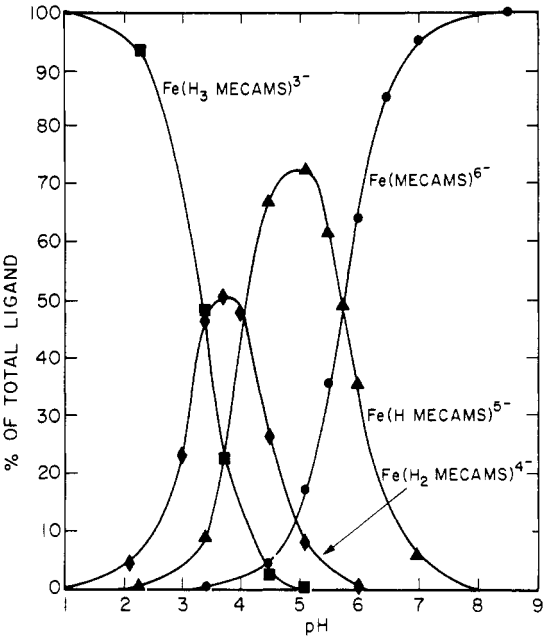

Figure 8. Species distribution curves for the protonation equilibria of Fe(H_nMECAMS): Fe(MECAMS)$^{6-}$ (-·-); Fe(HMECAMS)$^{5-}$ (-▲-); Fe(H_2MECAMS)$^{4-}$ (—): Fe(H_3MECAMS) (-■-).

catechol dissociation. Only at lower pH values does a band grow in at 1626 cm^{-1}, which appears near the same place as the free ligand value of 1629 cm^{-1} and corresponds to the dissociation of one or more catechol arms from the metal complex. Ferric complexes of 3,4-LICAMS and 3,3,4-CYCAMS[21] also were examined and showed the same behavior. Infrared studies on complexes of Ga^{3+}, an ion of similar size and charge to Fe^{3+}, have provided another example where the complex apparently does not dissociate until at least three protons have been added.[28]

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Table III. Summary of Infrared Spectral Features for Enterobactin and Its Ferric Complexes

	enterobactin		band assignments[a]
solvent	KBr pellet	D_2O mull	$\nu_{C=O}$ ester
band positions	1750	1740	$\nu_{C=O}$ amide I
(cm^{-1})	1640	1626	$\nu_{C=O}$ catecholate and salicylate coordinated
	1590		$\nu_{C=C}$ ring (8a or 8b)
	1540		$\nu_{C=O}$ amide II
	1460	ν	$\nu_{C=C}$ ring (19b)
	1343		$\nu_{C=C}$ ring (14)
	1265		α_{CH} in plane deformation (3)
	1240		ν_{CO} phenol (13)
	1175		ν_{CO} phenol (7a)

	$Fe(ent)^{3-}$	protonated Fe-ent[b]			$[H_3Fe(ent)]^0$
solvent	D_2O	D_2O	D_2O	D_2O	KBe
pH	9.7	5.3	4.0	2.0	
band	1750	1750	1750		1750
positions			1623 (sh)	1624	1640
(cm^{-1})	1593	1600 (sh)	1600	1600 (sh)	1620
	1583	1585	1584	1577	1587
	1540	1550	1551	1548	1540
	1483, 1445	1480, 1446	1482, 1458, 1448	1483, 1463, 1452	1450

[a] Assignments based on data reported in ref 29-32. Numbers in parentheses refer to the normal vibrational modes of benzene. [b] At these pH values overlapping protonation equilibria are present; therefore, IR spectra reflect a mixture of protonated ferric enterobactin complexes.

Enterobactin. The solution and solid-state IR spectral data for enterobactin and its ferric complexes as a function of pH are shown in Tables II and III. Assignment of the free lgand and iron complex bands are based on data in ref 29–32. The band at 1750 cm^{-1}, which is seen in the spectra of the free ligand and metal complexes, is assigned to the carbonyl stretching frequency of the ring esters. The amide I band (mainly $\nu_{C=O}$) for the free ligand is observed at 1640 cm^{-1} in KBr and 1626 cm^{-1} at low pH in D_2O. Benzene ring C=C deformations appear at 1590, 1460, and 1343 cm^{-1} and probably correspond to the 8a or 8b, 19b, and 14 skeletal modes, respectively. The features at 1240 and 1175 cm^{-1} are a result of ν_{CO} with the corresponding benzene equivalents 13 and 7a. The prominent peak at 1265 cm^{-1} is due to a C–H in-plane (9a) bending mode which is IR active. The assignment of the bands for the ferric complexes are given in detail in Table III.

The species $[Fe(ent)]^{3-}$ becomes protonted via overlapping *one-proton* steps to form $[Fe(Hent)]^{2-}$ and $[Fe(H_2ent)]^-$ with metal chelate protonation constants of $10^{-4.8}$ and $10^{-3.15}$, respectively.[8] In the pH range 6–4.5, the fully formed tris(catechol) chelate and the monoprotonated $[Fe(Hent)]^{2-}$ are the predominant species in solution. As the acidity is increased, the complex is further protonated, which results in precipitation of a dark purple solid. At pH values greater than 4 there is no evidence for an uncoordinated catechol arm. However, at pH 2, the band at 1626 cm^{-1} indicates that a significant fraction of the catechol arms have dissociated from the metal, either in the form of a pendant, uncoordinated catechol group of a partially coordinated ligand or a completely dissociated free ligand.

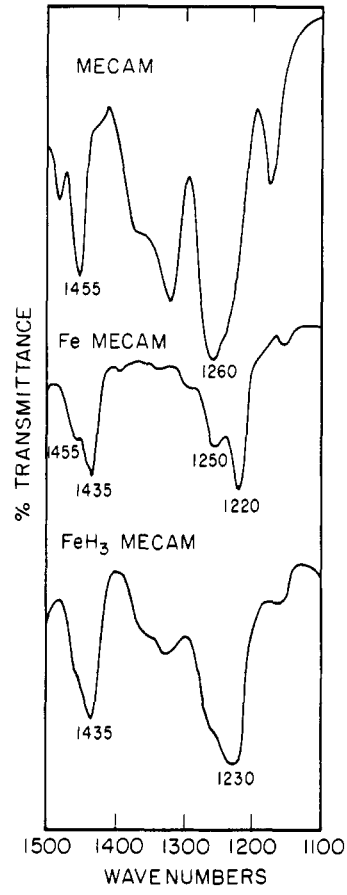

Figure 9. The solid-state FT IR spectra of MECAM (top), the red $[Fe(MECAM)]^{3-}$ complex (middle), and blue $Fe(H_3MECAM)$ complex (bottom).

IR Identification of Semiquinone Coordination. Catechol is an electrochemically active ligand, and it is well established that the reaction of catechol with high-valent metal ions can produce semiquinone complexes with concomitant reduction of the metal ion.[31,38,39] Indeed, ferric ion can be reduced by catechol, but only at low pH, where the 1:1 ferric catechol species predominates.[40,41] The sequential change from catechol to semiquinone to quinone changes the carbon–oxygen bond order from a single bond to a formal double bond in the quinone. This causes substantial shifts in the carbon–oxygen stretching frequencies; in catechol the singlet-bond C–O stretching bands appear near 1250 and 1170 cm^{-1}, while the C–O stretching frequencies in semiquinones are significantly higher.[42] The alkali-metal salts of bromanil and chloranil semiquinones have bands at 1515 and 1540 cm^{-1}, respectively.[42] Coordination of a transition-metal ion to the semiquinone oxygens should shift the peaks to lower wavenumbers, and indeed intense bands at 1440 and 1460 cm^{-1} have been assigned to C–O stretches of semiquinones coordinated to chromium[38] and iron,[39] respectively. Therefore, we have examined the 1400–1500 cm^{-1} region of solid-state and solution spectra of the ferric complexes of both enterobactin and the synthetic tricatechoylamide ligands to determine if ferrous semiquinones are being formed in these systems.

The solid-state spectra of MECAM, the red Fe(MECAM) complex, and the blue $Fe(H_3MECAM)$ complex are shown in

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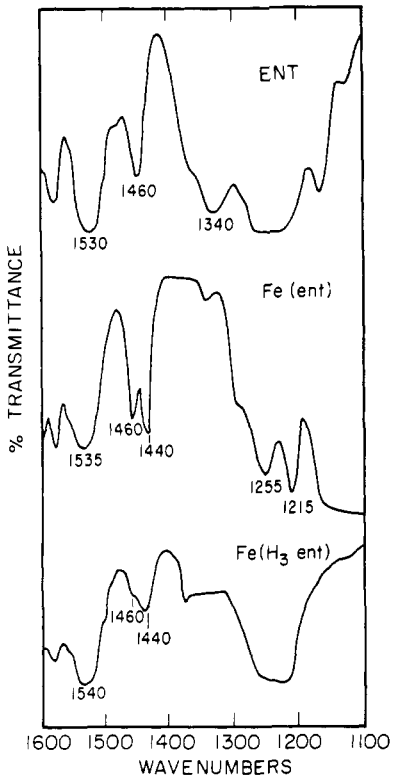


Figure 10. The solid-state FT IR spectra of enterobactin (top), red $[Fe(ent)]^{3-}$ (middle), and blue $[Fe(H_3ent)]^0$ (bottom).

Figure 9. The free ligand has two bands at 1485 and 1455 cm^{-1}, which shift upon formation of the red complex to 1455 and 1435 cm^{-1}. Although protonation of this species to give the blue ferric complex broadens these bands somewhat, no new bands appear, nor do the relative intensities of these bands increase. In fact, the intensities in the blue complex appear to decrease relative to the ring mode at 1540 cm^{-1}. Thus there is no evidence that the blue, protonated ferric MECAM species has any significant fraction of ferrous semiquinone character. Analogous results were obtained for ferric enterobactin, as shown in Figure 10. The free ligand has a single band at 1460 cm^{-1}, which splits into two bands at 1460 and 1440 cm^{-1} in the red ferric complex. In the spectrum of the blue, protonated ferric enterobactin complex, these bands have broadened, but there are no new bands and no increase in relative intensity. Again, this argues strongly against formulating this species as a ferrous semiquinone.

The ferric enterobactin system was also studied in D_2O solution, and the spectra are shown in Figure 11. At pH 10, where the fully-deprotonated, tris(catecholato) form of ferric enterobactin is the only species present, there are two, well-resolved bands at 1483 and 1445 cm^{-1}. In contrast to the solid-state spectra, a small shoulder appears at 1458 cm^{-1} at pH 4, which corresponds to the addition of about 1 equiv of acid to the system. At lower pH the blue species precipitates; a spectrum was recorded on the aqueous slurry at pH 2. This spectrum has an intense new band at 1463 cm^{-1} which could be assigned to a ferrous semiquinone. However, since we were unable to prepare a pure semiquinone species, it is very difficult to estimate what fraction of the iron has been reduced at pH 2.

Solution spectra have also been recorded for the ferric complexes of the sulfonated tricatechoylamide ligands. Sulfonation of the catechol ring appears to widen the gap between the two bands in the free ligand, which appear at about 1480 and 1420 cm^{-1} for MECAMS, CYCAMS, and LICAMS. Formation of the red, fully-deprotonated species increases the intensity of the 1480 cm^{-1} band and shifts the lower band to about 1450 cm^{-1}. These bands generally broaden and lose intensity as the pH is lowered, as shown in Figure 6 for MECAMS. This pH range easily encompasses the red to blue transition caused by protonation of the ferric MECAMS complex. However, when the pH was lowered to

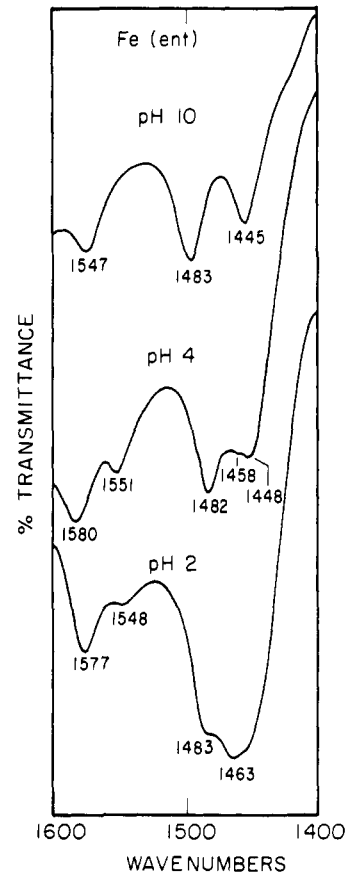


Figure 11. The FT IR spectra in D_2O solutions of ferric enterobactin: pH 10 (top), pH 4 (middle), and pH 2 (bottom).

approximately 1, very intense bands were observed at 1470 and 1450 cm^{-1} for ferric MECAMS (Figure 6b) and ferric LICAMS, respectively.

The changes observed in the ferric CYCAMS system were similar to those for ferric MECAMS but tended to occur at higher pH values. Two bands at 1480 and 1450 cm^{-1} were retained in the solution spectra over the pH range 5–12. At pH 4, a shoulder appears at 1490 cm^{-1}, and at pH 2.5 a clear, though rather weak band was observed at this position. No spectra were recorded on solutions at pH below 2.5.

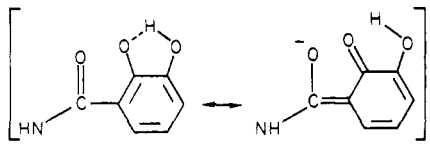
Discussion

A model we have proposed for the protonation equilibria of ferric catechoylamides involves the sequential shift from catecholate to salicylate coordination, using the ortho-hydroxyl and α-carbonyl oxygens.[8,21,22] Potentiometric titrations and pH-dependent visible spectroscopy have given proton stoichiometries which are consistent with a salicylate bonding model and have ruled out formation of a simple bis(catecholato)iron(III) species.[8,21] Further support comes from systems that should not exhibit salicylate bonding, such as ferric TRIMCAMS and ferric TRIMCAM; indeed, the two-proton step observed upon protonation and a visible spectrum resembling bis(catecholato)iron(III) supports a bis(catecholate) formulation for these protonated complexes.[21,22] Until now evidence for this bonding scheme has been indirect, since none of the methods above specifically monitor the functional groups involved in metal coordination. In order to prove the amide carbonyl involvement, these solution infrared spectral studies have been carried out over the pH range where protonation of the catechoylamide complexes occurs.

The carbonyl stretching frequency is affected by both ligand deprotonation and metal ion coordination. To interpret the infrared results over a pH range of 1–10, it is necessary to assign stretching frequencies for at least six different types of α-carbonyls. Two of these forms are the fully protonated and the monodeprotonated catechoyl arms of a free ligand molecule. There are also the analogous fully protonated and monoprotonated form

of a pendant (uncoordinated) catechoyl side group in a metal complex. Finally, there are the catecholate and salicylate coordinated catechoyl side arms. We have prepared compounds representing each catecholate type and measured the carbonyl stretching frequencies. These data can then be used to interpret the pH-dependent infrared spectra of the tricatechoylamide ion complexes.

Free Ligand IR Properties. The carbonyl stretching frequencies for a wide range of fully protonated dihydroxybenzoyl ligands fall in the relatively narrow range of 1625–1645 cm^{-1}. This band is shifted to ~1605 cm^{-1} by the first deprotonation of the catechoyl group. This is due to the conjugation of the carbonyl into the catechol ring, as shown by resonance forms a + b. In the fully protonated form of the ligand the contribution of form *b* will be relatively unimportant.



a b

Pendant Catecholato IR Properties. It is possible that the carbonyl stretching frequency of a single dihydroxybenzoyl group of a tricatecholate ligand could be shifted by coordination of the two remaining catecholate groups of the ligand. We have previously shown[26] that one of the three catecholate groups of MECAMS is not coordinated in the copper(II) MECAMS complex. Furthermore, this pendant side group undergoes normal deprotonation with a pK_a of 6.3. The relative contribution of each form as a function of pH is shown in Figure 4.

At pH 8.5 only Cu(HMECAMS) is present in solution and we see carbonyl stretching frequencies at 1611 and 1602 cm^{-1} without a corresponding peak at ~1630 cm^{-1}. As the pH is decreased to 5.5, bands at 1629, 1616, and 1601 cm^{-1} are evident and at pH 4.5 the intermediate carbonyl stretch disappears leaving only absorptions at 1629 and 1599 cm^{-1}. The three band assignments in increasing frequency, are metal-bound catechoyl, deprotonated free catechoyl, and protonated free catechoyl. Thus, where one phenolic oxygen of the catechol ring is deprotonated, the carbonyl stretching frequency is nearly indistinguishable from a salicylate or catecholate coordinated arm; however, when both oxygens are protonated (>95% at pH 5) the band at 1629 cm^{-1} becomes evident.

These data on the cupric MECAMS complex indicate that a band near 1630 cm^{-1} is indicative of a fully protonated dihydroxybenzoyl group and that it does not matter whether the carbonyl group is contained in a free ligand or in the pendant (uncoordinated) group of a metal–ligand complex. Thus a pendant dihydroxybenzoyl group can be readily detected in the infrared spectrum as long as the solution pH is sufficiently low to ensure complete protonation of both catechoyl oxygens.

At a pH high enough to deprotonate the pendant group, the carbonyl stretch shifts to ≃1610 cm^{-1}, which is difficult to distinguish from that for groups involved in "catecholate" type coordination to a metal ion. This is indicated by the high-pH infrared spectra of a series of ferric tricatechoylamide complexes, listed in Table I, which all show strong carbonyl stretching bands in the range 1600–1605 cm^{-1}. Since it has been clearly demonstrated[8,21,33] that at high pH these complexes all involve coordination via the six phenolic oxygens, this band at ≃1605 must represent a carbonyl group from a dihydroxybenzoyl moiety which is involved in catecholate bonding to ferric ion.

For catechoylamide ligands containing a carbonyl adjacent to the catecholate ring a shift in $\nu_{C=O}$ is seen upon metal binding by the catechols due to carbonyl conjugation to the catechol ring. In contrast, as shown in Table I, the carbonyl stretching frequency of TRIMCAMS (a structural isomer of MECAMS in which the carbonyl and methylene units have been interchanged) is not significantly perturbed when a metal complex is formed. Therefore, conjugation of the catechol ring and carbonyl appears to be responsible for the shift in $\nu_{C=O}$ observed in the IR of Fe(MECAMS) and Fe(3,4-LICAMS).

Salicylate Coordination IR Properties. In order to determine the approximate frequency for a carbonyl oxygen coordinated to ferric ion and the magnitude of the difference between the unassociated (free) ligand and the salicylate metal complex, we synthesized a compound which could only engage in metal complexation via three carbonyl and three phenol oxygens. Reaction of ferric ion with MESAM yields the neutral, red orange solid, $[Fe(MESAM)]^0$, which is insoluble in aqueous solution. Ferric ion is coordinated via the thre salicylamide moieties in this complex. This formulation is preferable to one in which the three phenols are bound to a metal without concomitant coordination of the carbonyl oxygens for the following reason: First, although the carbonyl is formally neutral, resonance structures which are analogous to those of acetylacetonate can be drawn which place negative charge on the carbonyl oxygen making it a much more effective ligand for Fe(III). Second, salicylate coordination results in the formation of a very stable six-membered chelate ring.

Carbonyl stretching frequencies of MESAM and its metal complexes are given in Table II and are shown in Figure 5. The $\nu_{C=O}$ for the ligand (1640 cm^{-1}) is at slightly higher energy than for the sulfonated catechoyl amides. Upon complexation of ferric ion to form $[Fe(MESAM)]^0$, this band shifts to 1606 cm^{-1}; at 34-cm^{-1} decrease in the stretching frequency of a salicylate coordinate arm. As the acidity of the solution is increased, a purple water-soluble protonated species is formed, which may be represented as $[Fe(H_nMESAM)]^{n+}$. This species is expected to have one or more uncoordinated salicylamide arms with the remaining moieties bound to the metal. The infrared spectra, with bands at 1640 and 1606 cm^{-1}, respectively, corroborate this formulation.

pH Dependence of IR Spectra. The species distribution curves for Fe–MECAMS in Figure 8 clearly show that at pH 3.5 all Fe–MECAMS species are protonated, yet IR data of Fe–MECAMS at pH ~3.5 do not exhibit a carbonyl infrared band (in the region 1625–1630 cm^{-1}) which can be associated with uncoordinated catechoyl arms. There is no evidence for a dissociated catechoyl arm until very low pH, where a shoulder at 1624 cm^{-1} is observed at pH 2.4, and only at even lower pH does the 1610 cm^{-1} band of coordinated catechoyl diminish significantly. Moreover, even in the case of Ga–MECAMS, dissociation of a catechoyl arm is observed only at low pH, in spite of the fact that the Ga–MECAMS complex is less stable than Fe–MECAMS.[28] *These results indicate that all three catechoyl arms remain associated with the metal ion when ferric MECAMS is protonated by up to three protons.*

The relationship between charge and coordination differences of these ligands can be explored in the three ligands MESAM, MECAMS, and MECAM. The ligand MECAM is a tricatechol which shows the same coordination chemistry[8,21] as MECAMS but is unsulfonated, which allows a better comparison with the salicylate ligand analogue, MESAM. According to our proposed mechanism,[8] the insoluble triply protonated $[Fe(H_3MECAM)]^0$ complex should possess a "tris(salicylate)" type of coordination and, therefore, would be essentially a substituted derivative of $[Fe(MESAM)]^0$. Infrared data on MECAM[33] in Table II show a shift from 1635 cm^{-1} in the free ligand to 1610 cm^{-1} upon formation of $[Fe(H_3MECAM)]^0$, a behavior similar to that of MESAM and its ferric complex. Also, the vibrational frequencies of the "salicylate" complexes $[Fe(H_3MECAM)]$ and [Fe(MESAM)] are not markedly different from those of the catecholate complexes. This supports the assignment that bands due to a salicylate-bonded arm and to a catecholate-bonded arm almost overlap. Therefore, one does not expect a marked shift in the carbonyl band upon change in coordination from a catecholate-type complex to a triply protonated salicylate type. Since the solution chemistry of MECAM and MECAMS are very similar until the onset of precipitation of the unsulfonated compound, it is reasonable to assume that the conclusions from MECAM and MESAM studies apply to the sulfonated derivatives also.

Results of the experiments described above have all been consistent with the bonding scheme in Figure 2 which involves

salicylate-type coordination. Infrared data show that initial protonation of the ferric complexes of the sulfonated tricatechoyl ligands MECAMS and 3,4-LICAMS (as well as 3,4,3-CYAMS) does not dissociate a catechoyl arm. Not until very low pH, corresponding to the addition of more than three protons, do bands associated with a dissociated arm appear. We observe that both the absolute frequencies of the tris(salicylate) metal complexes as well as the frequency difference between the unassociated ligand vs. the complexes are similar for all species believed to be tris(salicylate) types. These results are best explained by the salicylate model, which accounts also for the sequential *one-proton* steps and the lack of a bis(catecholato)iron(III) visible spectrum at any point during protonation titrations.[21]

Relevance of Model Compound IR Spectra to Enterobactin. The synthetic ligand MECAM is known[8,23] to mimic enterobactin in that (1) spectral features of both ferric complexes are similar (including their corresponding protonated species), (2) protonation of these complexes proceeds by discrete *one-proton* steps, (3) insoluble compounds precipitate when approximately three protons are added, and (4) Fe(MECAM) is recognized by the enterobactin receptor in *E. coli*.[18] The study of both MECAM and enterobactin solution chemistry has been hampered by the insolubility of both the ligands and some of the protonated ferric complex species. In addition, enterobactin is sensitive to hydrolysis of the central triester ring. Therefore use of the analogues shown in Figure 3 and previous IR data[29-32] has allowed us to study the chemistry of catechoylamides in solution over a larger pH range than is accessible for ferric enterobactin. Because of the many similarities between the chemistry of MECAM and enterobactin and of the sulfonated tricatechoylamides, we can correlate the spectral results for enterobactin and the synthetic models.

The IR spectra of $[Fe(ent)]^{3-}$ in D_2O show a carbonyl band at 1593 cm^{-1}, assigned to metal-bound catechoyl moiety, and none ascribed to an uncoordinated arm near 1626 cm^{-1}. This is as expected for a tris(catecholate)-type of complex. The first two metal chelate protonation constants[8] for ferric enterobactin are $10^{4.80}$ and $10^{3.15}$; therefore at pH 5.3 and 4.0 approximately 25% and 85%, respectively, of Fe–ent is in a protonated form. At pH 5.3 there is no evidence for dissociation and the same comments made for the model compounds apply here. At pH 4.0 a band at 1623 cm^{-1} appears. It is not clear whether this band is due to dissociation of a single catechoyl arm or to complete dissociation of the complex; however, the insolubility of metal-free enterobactin possibly could shift the equilibrium toward dissociation. Below pH 2–2.5, the precipitation of the mixture of free ligand and iron complex is complete. An iron analysis of this material consistently gave values lower than expected, which is consistent for a mixture of an iron species ($[Fe(H_3ent)]^0$) and ligand. From the IR data we believe that in at least the initial stages of protonation, ferric enterobactin does not dissociate a catechoyl arm and, on the basis of other data[8] and by analogy to chemistry of the model compounds, that the latter stages of protonation are similar to protonation of the synthetic analogues.

Although the IR data on the carbonyl stretching frequencies rule out the presence of any uncoordinated side groups in the $Fe(H_3ent)$ complex, they do not directly prove salicylate bonding. A second protonation scheme has been suggested by Hider et al.,[12] in which the three meta-phenolate oxygens are protonated but remain coordinated to the iron, although no direct evidence for such a species was presented. As discussed above, the carbonyl stretching frequencies appear to be sensitive only to the protonation and deprotonation of the ortho-phenolate oxygen. Since this group is deprotonated in the species proposed by Hider et al., we would expect the carbonyl bands to be essentially indistinguishable from those of our proposed salicylate mode of bonding. Hider's alernative protonation scheme is discussed in more detail in the following sections.

On Enterobactin Semiquinone Formation. Hider et al.[12] have claimed that the blue, protonated complex of ferric enterobactin is in fact a ferrous complex in which one of the coordinated catecholato groups of the enterobactin has been oxidizes to a semiquinone. It is well established that coordinated semiquinones have intense bands in the 1440–1460 cm^{-1} region corresponding to the C–O stretching mode when the oxygen is coordinated.[38,39,42] We have examined the solution and solid-state spectra of the ferric complexes of enterobactin and several of the synthetic tricatechoylamide ligands and have shown that the addition of one to three protons to the ferric complexes of these ligands does not produce any significant new absorbances in the 1400–1500 cm^{-1} region. The solid-state spectra of $Fe(H_3ent)$ and $Fe(H_3MECAM)$ are particularly important in this discussion because they represent essentially pure samples of the very enterobactin species which Hider et al.[12] claim to be semiquinone complexes. Yet we find no indication at all of a semiquinone band in the spectra of these complexes.

Similar observations can be made on the ferric CYCAMS system. Because of the relatively low stability of this complex, the chelate protonation reactions occur at relatively high pH.[21] The ferric CYCAMS titration curve shows a clear break at 3 equiv of base, so that one can be certain that only the $Fe(H_3CYAMS)^{3-}$ complex is present in solution over the pH range 4–5.[21] Spectra at pH 5.2 show no indication of a semiquinone species, and spectra at pH 3.7 have only a small shoulder at 1490 cm^{-1} which might be due to a trace of ferrous semiquinone, although it is outside the frequency range previously reported for semiquinones.[38,39,43] These data prove that the blue species formed by protonation of ferric CYCAMS is not a semiquinone complex.

The solution spectra of MECAMS and LICAMS are somewhat more difficult to evaluate because the protonation reactions overlap to such an extent that it is impossible to obtain a spectrum of a single, pure protonated species. However, no semiquinone bands are observed until the pH is ~1, which is well below the pH necessary to produce the triply protonated complexes of these ligands. In fact, possible semiquinone bands are only observed at pH values so low that the presence of free catechoylamide carbonyl stretching bands indicates that the ferric complexes are partially dissociated. It has been reported by Mentasti[41] and later by ourselves[40] that ferric ion will oxidize catechol in acidic solution under conditions where the maximum degree of complexation would be a 1:1 complex and hence the chemical activity of free ferric ion becomes significant. At higher pH this reaction stops because the addition of successive catechol ligands shifts the reduction potential of the ferric ion from a very positive to a very negative potential as the concentration of free ferric ion rapidly decreases. Thus as the ferric complexes of enterobactin and the synthetic hexadentate ligands begin to dissociate in very acidic solution, we would expect the catechoylamide side groups to react essentially as a simple bidentate ligand, and it is not at all surprising that some semiquinone formation is observed at very low pH. However, since these reactions occur only in strongly acid solutions, they have little bearing on the complexation chemistry which occurs at higher pH or the biological chemistry of enterobactin.

The crucial data on which Hider et al.[12] based their identification of the blue, protonated iron enterobactin species as a ferrous semiquinone complex come from their Mössbauer experiments, which indicated the presence of ferrous ion in a methanol solution of iron enterobactin. However, our own Mössbauer data (in the preceding paper[1]) have shown: (1) no significant amount of ferrous ion is present in aqueous solution at pH greater than 2; (2) even in methanol most of the iron remains as ferric ion to as low as "pH" 1. These conclusions are now further supported by the infrared data reported here, which show no significant semiquinone C–O bands in the IR spectra of the blue, protonated iron complexes of enterobactin or the synthetic tricatechoylamide ligands.

Hider's apparent misinterpretation of the Mössbauer data may have stemmed from their failure to *quantitate* the relative amounts of ferrous and ferric ion in their very concentrated samples and the fact that ferric ion can be difficult to observe if the relaxation times are slow. These problems can be exacerbated by the difficulties in preparing pure samples of ferric enterobactin. Because

(43) Wicklund, P. A.; Beckman, L. S.; Brown, D. G. *Inorg. Chem.* **1976**, *15*, 1996.

enterobactin is hydrolytically and oxidatively unstable in basic solution, it is common to add iron to the ligand in acidic solution and then adjust the pH to the desired value. However, while the mixture is still acidic, a small amount of ligand oxidation usually occurs. Thus in our own work, the ferric enterobactin is passed through an alumina column, as described in Materials and Methods, to separate oxidized ligand and ferrous ion from the ferric enterobactin. Since Hider et al.[12] do not report their method for preparing and purifying ferric enterobactin, it is not possible to determine whether this explanation could account for the ferrous ion observed in the Mössbauer spectra. In any event, this consideration emphasizes the need to quantitate the relative concentrations of the species observed in the Mössbauer experiments.

On the Protonation Scheme of Ferric Enterobactin. Hider et al.[25] have also challenged our previous reports that the ferric complexes of enterobactin as well as most of the synthetic ligands are protonated in discrete one-proton steps. Instead, they propose that there is only a single, three-proton reaction, so that only two species are involved. We have discussed the existence of the one-proton stoichiometry in these systems in three previous papers and feel that the evidence in support of such a scheme is overwhelming. The strongest evidence is based on a very simple consideration of the absorbance data and a ferric ion mass balance. Any system which contains only two species that are related to one another by a simple protonation of unknown stoichiometry is described by the set of equations

$$ML + nH^+ \rightleftharpoons MH_nL \quad K_{MHL} = [MH_nL]/[ML][H]^n \quad (1)$$

$$[M]_{tot} = [MH_nL] + [ML] \quad (2)$$

$$\text{absorbance}_i = \epsilon_{ML} + \epsilon_{MH_nL}[MH_nL] \quad (3)$$

These equations can easily be rearranged to give eq 4

$$\epsilon_i = (\epsilon_{ML} - \epsilon_i)/K_{MH_nL}[H]^n \quad (4)$$

where $\epsilon_i = \text{abs}_i/[M]_{tot}$. As long as only two species are present, as indicated by an isosbestic point, a plot of ϵ_i vs. $1/[H]^n$ should be linear for the appropriate value of n, the stoichiometry of the hydrogen ion term in eq 1, with a slope equal to $1/K_{MH_nL}$. Our published results for enterobactin, MECAM, MECAMS, CYCAM, CYCAMS, and LICAMS all show that plots of eq 4 are linear only for $n = 1$. Only for ferric TRIMCAMS, which lacks any α-carbonyl for salicylate bonding, did we obtain a linear plot of eq 4 for $n = 2$.

All our titration data are on aqueous solutions. Hider et al.[25] reported a spectrophotometric titration of ferric enterobactin in 50% aqueous methanol and claimed that three protons were added simultaneously to the ferric enterobactin complex. However, instead of reporting a series of spectra with an isosbestic point to support their claim that there are no intermediates between $[Fe(ent)]^{3-}$ and $[Fe(H_4ent)]^0$, they show a plot of the absorbance at 450 nm as a function of pH and erroneously claim that such data constitute proof of their reaction scheme. To clarify this point, we have been taken absorbance vs. pH data from their Figure 10 and analyzed it as described above. If eq 4 is multiplied by the total iron concentration, one obtains eq 5

$$A_i = \frac{(A_0 - A_i)}{K_{MH_nL}[H]^n} + A_\infty \quad (5)$$

where A_0 is the absorbance of the initial $[Fe(ent)]^{3-}$ red complex, A_i is the absorbance at any pH_i, and A_∞ would correspond to the absorbance if all the iron were converted cleanly to the protonated species. A plot of eq 5, using Hider's data,[25] is shown in Figure 12. Not only is the plot clearly linear when $n = 1$, not $n = 3$, but also the calculated value of the first chelate protonation constant is very similar to the value determined by us for an aqueous solution. Thus it appears that the addition of 50% methanol does not greatly change the solution chemistry of ferric enterobactin.

Hider et al. also reported a gradual spectral change over the pH range 7–10.[25] To the best of our knowledge, such a change has never been reported elsewhere, since the $[Fe(ent)]^{3-}$ complex is very stable over this entire pH range.[8] The explanation offered for this observation was that it represented the reaction of ferric ion with free enterobactin.[25] This cannot possibly be correct, since both the stability constant of ferric enterobactin (which, for excess enterobactin, maintains an $[Fe^{3+}]$ activity equivalent to 10^{-35} M) and the solubility product ferric hydroxide[46] (which in any case maintains $[Fe^{3+}]$ below 10^{-18} M) both rule out the existence of any free ferric ion over this pH range. One possible explanation is that the absorbance was measured at 450 nm, rather than at the λ_{max} of the $[Fe(ent)]^{3-}$ complex (495 nm).

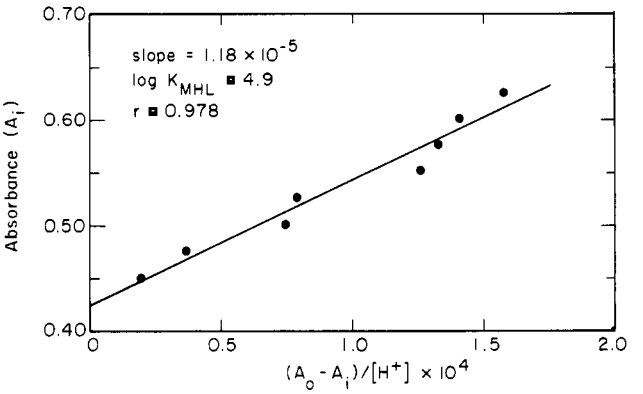


Figure 12. A plot of the absorbance at 450 nm of ferric enterobactin solutions in 50% aqueous methanol as the pH is changed. The data are from Figure 10 of ref 25. The vertical axis is absorbance (A_i). The horizonatal axis is the function $(A_0 - A_i)/[H^+]$ and has been multiplied by 10^4. A linear relationship implies a single-proton stoichiometry in the reaction. A least-squares fit (the line shown) gives a correlation coefficient of 0.978, a slope of 1.18×10^{-5} (the inverse of the protonation constant), and a intercept of 0.425 (A_∞).

On the Coordination of Simple Catechols to Ferric Ion. Hider et al.[25] have also disagreed with our earlier report that catechol and simple catechol derivatives react with ferric ion above pH 3 to form simple mono-, bis-, and tris(catecholato)iron(III) complexes.[8,40] On the basis of spectrophotometric, potentiometric, and Mössbauer data, Hider et al. proposed the formation of four complexes in the iron–catechol system. They suggested that the green species observed at low pH is a 1:1 ferrous semiquinone complex and that by pH 6.5 the iron has been converted to a 3:1 ferrous semiquinone species which they formulate as $Fe(cat)_2(cat\cdot)$. They then proceed to make two contradictory statements. They first claim that the blue complex is converted to a purple hydroxy-bridged dimer of bis(catecholato)iron(III) over the pH range 6.5–9.0. Later in the same paper they claim that the isosbestic point which is maintained from pH 10 to 7.85 corresponds to a transition of the purple dimer to the tris(catecholato)iron(III) complex. Clearly both these claims cannot be true.

There also appear to be some serious discrepancies with the potentiometric titrations reported by Hider et al. on the ferric–catechol system.[25] Their Figure 3 shows titration curves for 1:1, 3:1, and 6:1 ratios of ligand:metal. Since the stability constants of mono(catechol) complexes of Fe(III) are not sufficiently great to avoid precipitation of ferric hydroxide at the pH values reported, there is no point in discussing the 1:1 ratio data. In the 3:1 titration, Hider et al. report sharp breaks at 1.4 equiv of base (pH 4), at 3.6 equiv of base (pH 7), and at 5.5 equiv of base (pH 9.5). These authors do not appear to appreciate the significance of such data, since a break in this type of titration curve almost always indicates a pH range over which only a *single* species exists in solution. The position of the break, in terms of equivalents of base, gives the proton stoichiometry of that complex. Thus a noninteger break can be explained only by polymeric species. The data of Hider et al. are quite different from those previously reported,[8,40]

(44) Schwarzenbach, G.; Willi, A. *Helv. Chim. Acta* **1951**, *34*, 528.
(45) Murakami, Y.; Nakamura, K. *Bull. Chem. Soc. Jpn.* **1963**, *36*, 1408.
(46) Martell, A. E.; Smith, R. M. "Critical Stability Constants"; Plenum Press: New York, 1977; Vol. III.

which showed breaks at 2, 4, and 6 equiv that are readily explained by the formation of mono-, bis-, and tris(catecholato)iron(III) complexes. Similar results have also been reported by a variety of other workers for catechol complexes of iron[43-45] and other metal ions.[46]

Furthermore, the species proposed by Hider et al. for the ferric–catecholate system[25] are inconsistent with their own titration data, as well as ours. The formation of a 1:1 ferrous semiquinone complex at pH 4 would produce a break in the titration curve at 2 equiv of base, while the break in their titration curve actually appears at about 1.4 equiv. The second break could be due either to their proposed bis(catecholato)(semiquinone)iron(II) complex or the hydroxy-bridged iron(III) dimer. The semiquinone would produce a break at 3.0 equiv, while the dimer would produce a break at 4.5 equiv; but the break actually appears at 3.6 equiv. The final break could be due to either their proposed dimer or tris(catecholato)iron(III), which should produce a break at 4.5 or 6.0 equiv, respectively. The actual break appears at 5.5 equiv. Thus the titration data of Hider et al.[25] are inconsistent with every species which they postulated for the ferric–catecholate system. As in the enterobactin study,[12] the hypothesis of Hider et al.[25] that the blue species formed in the iron–catechol system is a ferrous semiquinone complex is based on the detection of an iron(II) signal in the Mössbauer spectrum of this system at pH 6. However, these workers mention the precipitation of a blue precipitate which we strongly suspect of being oxidized ligand. This virtually assures the presence of a small amount of ferrous ion in solution, and since Hider et al. failed to quantitate the relative amounts of iron(III) and iron(II) in their sample,[25] we suspect that they have based their reaction scheme on the detection of a material that is essentially an impurity. Such an explanation could also account for the fact that the iron(II) signal closely resembles that of ferrous chloride, because based on the stabilities of other divalent first-row transition-metal ions,[46] one would not expect catechol to bind ferrous ion at pH less than 8.

On the Reduction of Ferric Enterobactin at Low pH. While the reduction potential of ferric enterobactin at pH 10 is −1.0 V (all potentials here are versus the normal hydrogen electrode), this potential increases as the pH is lowered and at pH 7 has been estimated as −750 mV.[8,10] That remains an estimate, since the individual formation constants for the protonated *ferrous* enterobactin complexes are unknown. However Fe(II) will not be significantly complexed by catechols at pH 4[26] and so the calculation of the redox potential at this pH is straightforward. The individual equilibrium constants for the following reactions are products of the conditional formation constant and individual stepwise protonation constants of ferric enterobactin (the equilibrium constants described by eq 20 through 22 of ref 8, respectively)

$$Fe^{3+} + H_6ent = Fe(H_2ent)^- + 4H^+ \quad K = 10^{-1.75} \qquad (6)$$

$$Fe^{3+} + H_6ent = Fe(Hent)^{2-}, + 5H^+ \quad K = 10^{-4.9} \qquad (7)$$

For a total Fe^{3+} concentration of 10^{-6} M and a total enterobactin concentration of 10^{-5} M, the concentrations of individual species at pH 4 are $[Fe^{3+}] = 6.8 \times 10^{-17}$, $[Fe(ent)]^{3-} = 1.2 \times 10^{-7}$, $[Fe(Hent)]^{2-} = 7.7 \times 10^{-7}$, $[Fe(H_2ent)]^- = 1.1 \times 10^{-7}$, and $[H_6ent] = 9 \times 10^{-6}$. For a standard state chosen such that the total concentration of Fe(II) and Fe(III) species are equal, the $[Fe^{2+}]$ concentration is 10^{-6} M. Thus, since the molar free energies of all ferric ion containing species are equal at equilibrium, the reduction potential for ferric enterobactin at pH 4 is $E = 0.77 - 0.059 \log ([Fe^{2+}]/[Fe^{3+}]) = +170$ mV.

Summary

By monitoring the carbonyl stretching frequency of enterobactin and synthetic analogues, we are able to understand better the mode of bonding of the ferric complexes in acidic media. Previous work has demonstrated that the proton stoichiometry, visible spectra, and oxidation state of iron catechoylamides is consistent with a salicylate mode of bonding in weakly acidic solution. The data are presented herein give the first direct evidence for participation of the carbonyl moiety in metal ion coordination.

The IR spectra in the region of 1400–1500 cm^{-1} were examined to detect the formation of ferrous semiquinone complexes. The results are in agreement with the Mössbauer data in the previous paper[-1] and indicate that the initial protonation of the ferric complexes does not produce any significant quantity of ferrous semiquinones. Such species are observed at very low pH (1–2), where partial dissociation of the ferric complexes also occurs, as noted earlier for acid solutions of catechols and ferric ion.

An early mechanism for iron release from enterobactin involves cleavage of the central ring by an esterase, which would raise the physiological reductants. However, it has been shown recently that catechoylamides which do not have the central ester ring of enterobactin, but still have very negative redox couples, will act as growth factors for bacteria. An alternative mechanism for iron release by enterobactin which would also apply to the synthetic analogues is to postulate slightly more acid conditions at a point in the cellular uptake of the iron enterobactin complex, with a concomitant shift in the bonding about the iron. This change in the ferric ion coordination to the salicylate type raises dramatically the potential of the ferric catechoylamide complex, which in turn would make it available, via reduction, to microorganisms. At pH 4 this potential is +170 mV for the conditions described in the previous section. This mechanism differs from that of Hider et al.,[12,25] in that we conclude that the iron is *not* reduced internally by one of the catechol groups of enterobactin to form an insoluble semiquinone complex. Rather we conclude that the sequential protonation of the ferric complex merely lowers the redox potential of the complex so that enzymatic reduction becomes possible. The presence of an enterobactin esterase enzyme may then be due to the necessity to eliminate free enterobactin, following iron removal, in order to prevent the indiscriminant chelation of intracellular iron.

Acknowledgment. We thank Dr. Jan Offenbach and Heinz Frei for experimental assistance. We thank Dr. Mel Klein for access to his instrumentation and his assistance. The American Cancer Society is acknowledged for a postdoctoral fellowship (G.B.W.). The Berkeley FTIR factility was funded by the National Science Foundation (Grant CHE77-08923). This research was supported by NIH Grants AI 11744 and, in part, HL 24775.

Registry No. $[Fe(ent)]^{3-}$, 61481-53-6; $[Fe(MECAM)]^{3-}$, 69058-77-1; $[Fe(MECAMS)]^{6-}$, 85710-04-9; $[Fe(TRIMCAMS)]^{6-}$, 85710-05-0; $[Fe(3,4\text{-}LICAMS)]^{6-}$, 85710-06-1; $[Fe(3,4,3\text{-}CYCAMS)]^{6-}$, 85710-07-2; $[Fe(MESAM)]^0$, 85719-49-9; $[Cu(H\text{-}MECAMS)]^{6-}$, 85719-50-2; $[Cu(MECAMS)]^{7-}$, 85710-08-3; $[Cu(H_2\text{-}MECAMS)]^{5-}$, 85710-09-4; $[Fe(Hent)]^{2-}$, 85719-51-3; $[Fe(H_2ent)]^-$, 85710-10-7; $[Ga(MECAMS)]^{3-}$, 85710-11-8; $[Fe(H_3MECAM)]^0$, 85710-12-9; $[Fe(H_3ent)]^0$, 85710-13-0; $Fe(H_3CYCAMS)^{3-}$, 85710-14-1; $Fe(H\text{-}MECAMS)^{5-}$, 85710-15-2; $Fe(H_2\text{-}MECAMS)^{4-}$, 85710-16-3; $Fe(H_3\text{-}MECAMS)^{3-}$, 85710-17-4; Me_3MESAM, 85710-18-5; MESAM, 85710-19-6; 1,3,5-tris(aminomethyl)benzene trihydrochloride, 69146-57-2; 2-methoxybenzoyl chloride, 21615-34-9.