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Ferric Ion Sequestering Agents. 15. Synthesis, Solution Chemistry, and Electrochemistry of a New Cationic Analogue of Enterobactin[1]

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A new ferric ion sequestering tricatecholate, TRENCAM, has been synthesized from β,β′,β″-triaminotriethylamine (TREN) in high yield. The solution coordination chemistry of the ligand and its iron(3+,2+) complexes have been studied by means of pH potentiometric, spectrophotometric, and voltammetric methods. The last five stepwise protonation constants (log *K*) to $(H_7TRENCAM)Br$ are 11.26, 8.75, 8.61, 6.71, and 5.88 (from least-squares refinement of the potentiometric data from pH 3.5 to 10.7). The potentiometric data of the ferric complex have shown that the last three protonation constants are positioned close to one another between pH 5 and 5.6. These are resolved by spectrophotometric data from pH 8 to 5.6, which show that the first protonation constant (log *K*) of ferric(TRENCAM) is 5.59. From the reduction potential vs. pH curve of the iron(3+,2+) complexes the corresponding protonation constant (log *K*) of the ferrous complex has been determined to be 11.2. The specific affinity of TRENCAM for ferric ion relative to ferrous ion results in a highly negative reduction potential of the iron(3+,2+) center: −1.04 V vs. (NHE). From competition experiments with EDTA, the formation constant (log *K*) of ferric(TRENCAM) has been estimated to be 43.6. At pH 7.4, $[ligand]_{tot}$ = 10 μM, and $[Fe^{3+}]_{tot}$ = 1 μM, $p[Fe^{3+}]$ = 27.8. Implications of the results for in vivo iron removal are presented.

Introduction

Since 1978 we have been actively involved in the synthesis and characterization of ferric ion sequestering agents toward the ultimate development of iron removal drugs for therapeutic use.[1-4] With the natural ferric ion sequestering agent, enterobactin,[5,6] of unrivaled stability constant (K_{ML} = 10^{52})[7] as a model, we have prepared and characterized the tricatecholates MECAM,[4,8-10] MECAMS,[11-13] (Me_3)MECAMS,[14] (NAc)MECAMS,[14] *N,N′*-dialkyl LICAM derivatives, TiPMECAMS,[16] TRIMCAMS,[11,12] 3,3,4-CYCAM,[9,10] 3,3,4-CYCAMS,[11,12] 3,4-LICAMS,[11-13] DB-3,4-LICAMS,[14,16] DC-3,4-LICAMS[14,16] and DiP-3,4-LICAMS.[14,16] Recently a series of macrocyclic catecholates has been prepared.[17] While previous studies of these ligands have shown them to possess many thermodynamic and kinetic properties useful to an iron removal agent, it is clear that the future direction in the design of such an agent must directly address the in vivo biological distribution properties of the ligand. In the present work, we wish to report the preparation and evaluation of a new ferric ion sequestering agent based on the backbone of β,β′,β″-triaminotriethylamine (TREN). A salient feature of this new ferric ion sequestering agent is the introduction of tertiary amine into the backbone, which is expected to increase water solubility while conferring a net positive charge on the ligand. All previous catecholate ligands have been highly anionic, and it was anticipated that cationic ligands, such as the one presented here, might possess desirable in vivo distribution properties.

Experimental Section

Reagents. Stock solutions of ferric ion were prepared by dissolution of $Fe(NO_3)_3 \cdot 9H_2O$ (Mallinckrodt) into standardized 0.1 M nitric acid. The actual ferric ion concentration (0.1 M) was determined by standard methods.[18-20] Carbonate-free 0.1 M KOH solutions were prepared from J. T. Baker DILUT-IT ampules by using freshly boiled, doubly distilled water and were standardized with potassium hydrogen phthalate. The absence of carbonate was confirmed by Gran's plots.[21,22] Titrant and sample solutions were kept under N_2.

pH Measurements. The pH measurements were made with a Fisher Accumet digital pH meter with a Sigma E-4753 combination electrode. All standardizations and titrations were carried out at 25 °C and 0.1 M KNO_3. The apparatus was calibrated with NBS buffers and standard acid solutions to give pH meter readings of hydrogen ion *concentration*. All equilibrium constants given are concentration quotients valid at 25 °C and μ = 0.1 M. Potentiometric titrations employed an automatic titrator as before.[10] The thermodynamic reversibility was checked by cycling the titrations from low to high and back to low pH (or the reverse). The data were refined with use of a weighted, nonlinear least-squares analysis in which log β's were varied to minimize the sum of the squared differences between the observed and calculated pH at each point in the titration equilibrium curve as previously described.[16] Solutions for ligand titrations contained 2% CH_3OH because of the low ligand solubility in water. Control acid/base titration experiments confirmed that 2% CH_3OH did not have any observable effects on the response of the pH meter or the conversion of pH to $[H^+]$ over the measured pH range 3.5 to 10.7.

Spectral Measurements. All spectra were recorded on a Hewlett-Packard 8450A UV/vis spectrophotometer. All solutions contained 10 μM phosphate (to conform with the electrochemical studies) and were adjusted to 0.1 M ionic strength by the addition of KNO_3. The visible spectra of 0.01–0.03 mM ferric complexes were monitored as a function of pH, which was adjusted by adding nitric acid or potassium hydroxide. The 10-cm cell was designed for measurements to be done at 25 °C.[22] Absorbance measurements for the spectrophotometric competition reactions with Na_2H_2EDTA were done after samples were allowed to equilibrate for at least 1 week at 25 °C. The experiments were done at pH 6.5–7.0. Equilibrium was approached from both directions with TRENCAM and Fe^{III}EDTA or Fe^{III}TRENCAM and EDTA as starting reagents. The appropriate protonation and ferric ion formation constants

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† Deceased.

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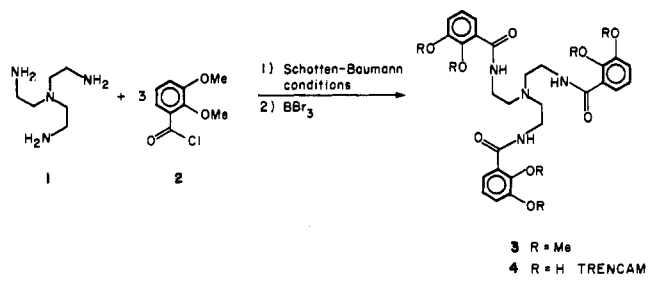

Figure 1. Synthesis of the tricatechol ligand TRENCAM from the tetraamine TREN.

for EDTA were taken from the critical compilation of Martell and Smith.[23]

Electrochemistry. Conventional electrochemical instrumentation, cells, and electrodes were employed for the cyclic voltammetric, polarographic, and controlled-potential coulometric measurements.[24] Buffers used were 40 mM phosphate (above pH 11) and 50 mM bicarbonate (below pH 11). Experiments were conducted in 0.4 M $NaClO_4$ solution at the laboratory temperature, 22 ± 2 °C. While a saturated calomel electrode was the reference, quoted potentials are with respect to the NHE.

Synthesis. Melting points were taken on a Mel-Temp apparatus and are uncorrected. The ^1H NMR spectra were recorded on a 200-MHz spectrometer by using Me_4Si as internal standard. Solvent was removed under vacuum (rotovap). Thin-layer chromatography was performed on precoated Analtech GHLF silica gel sheets and visualized by UV, I_2, or Fe(III)/MeOH spray. Microanalyses and positive FAB mass spectrum (glycerol or thioglycerol matrix) were performed by the Analytical Services Laboratory at the Chemistry Department, University of California, Berkeley, CA. Reagents were distilled before use: benzene from sodium benzophenone ketyl; thionyl chloride from triphenyl phosphite; dichloromethane from calcium hydride. Pure TREN was isolated from technical grade triethylenetetramine (TRIEN).[25]

Me_6TRENCAM (3). The procedure of Weitl[26] was used for the preparation of the methyl-protected acid chloride; 2,3-dimethoxybenzoic acid (10.0 g, 54.9 mmol) was heated to reflux in 30 mL of $SOCl_2$ and 50 mL of benzene for 12 h under a Drierite tube. After coevaporation with benzene (three times, 40 mL) the acid chloride (**2**) was dissolved in 110 mL of CH_2Cl_2. This was added dropwise over 45 min simultaneously with 110 mL of 0.5 M NaOH to a vigorously stirred solution of TREN (2.29 g, 15.7 mmol) in 50 mL of H_2O and 50 mL of CH_2Cl_2 at 0 °C. The pH was maintained above 10 during the course of the reaction, and after the mixture was stirred for 3 h, the layers were separated. The aqueous layer was extracted with CH_2Cl_2 (four times, 100 mL) and the combined CH_2Cl_2 layers were dried (Na_2SO_4) and evaporated to dryness. Recrystallization from EtOAc/cyclohexane afforded 9.12 g (91%) of white crystalline flakes of **3**: mp 120–122 °C; R_f (10% MeOH/CH_2Cl_2) 0.63; ^1H NMR (200 MHz, $CDCl_3$) δ 2.88 (t, 6 H, J = 6.6 Hz), 3.6 (m, 6 H), 3.85 (s, 9 H), 3.86 (s, 9 H), 6.99 (dd, 3 H), 7.08 (dd, 3 H), 7.61 (dd, 3 H), 8.20 (t, 3 H, J = 5 Hz); ^{13}C NMR (55.48 MHz, $CDCl_3$) δ 165.437, 152.510, 147.503, 126.780, 124.211, 122.588, 115.206, 61.292, 55.997, 53.449, 37.860.

Anal. Calcd for $C_{33}H_{42}N_4O_9$: C, 62.05; H, 6.63; N, 8.77. Found: C, 62.10; H, 6.67; N, 8.68.

TRENCAM·HBr (4·HBr). A solution of BBr_3 (10 mL, 106 mmol) in 50 mL of CH_2Cl_2 was added dropwise to **3** (500 g, 7.8 mmol) dissolved in 250 mL of CH_2Cl_2 under N_2 at 0 °C. The resulting suspension was allowed to stir overnight at room temperature. To the reaction mixture at 0 °C under N_2 was added 50 mL of MeOH dropwise, and the reaction mixture was stirred at room temperature for 2 h. After repeated evaporation with MeOH (10 times, 100 mL) to remove the borates, the product was dissolved in hot MeOH and precipitated with Et_2O, yielding 4.45 g (87%) of an off-white hygroscopic powder: ^1H NMR (200 MHz, Me_2SO-d_6) δ 3.52 (br m, 6 H), 3.75 (br m, 6 H), 6.67 (m, 3 H), 6.93 (m, 3 H), 7.34 (m, 3 H), 9.13 (br m, 3 H), 10.6 (br s, 1 H), 12.3 (br s, 2 H); ^{13}C NMR (55.48 MHz, Me_2SO-d_6) δ 170.00, 149.321, 146.196, 119.103, 118.163, 117.735, 115.080, 51.382, 33.790.

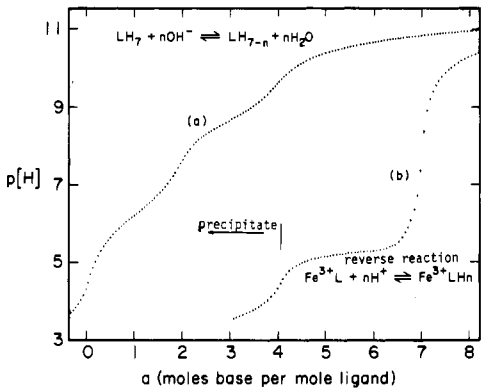

Figure 2. Potentiometric titration curves: (a) 3.9×10^{-4} M TRENCAM; (b) TRENCAM + Fe^{3+}, 1:1, 3.1×10^{-4} M. All solutions were at 25 °C and μ = 0.10 M (KNO_3). For the region noted as "precipitate" in the iron compex titration curve, the data could not be used in refinements because some precipitate of the neutral $Fe(H_3L)^0$ complex was present.

Anal. Calcd for $C_{27}H_{30}N_4O_9$·HBr·H_2O: C, 49.62; H, 5.09; N, 8.57; Br, 12.23. Found: C, 49.51; H, 5.24; N, 8.19; Br, 12.33. FAB-MS: m/e (M + H) 655.

Results and Discussion

Preparative Scheme. A scheme for the synthesis of TRENCAM is shown in Figure 1. Acylation of TREN (**1**) with 3 equiv of 2,3-dimethoxybenzoyl chloride (**2**) under modified Schotten–Baumann conditions afforded Me_6TRENCAM (**3**) as a highly crystalline solid in 91% yield. Removal of the methyl protecting group with excess BBr_3 yielded TRENCAM (**4**) as the HBr salt in 87% yield.

Solution Chemistry. The potentiometric equilibrium curves for TRENCAM[27] and Fe^{III}TRENCAM are shown in Figure 2, in which TRENCAM has been treated as H_7L^+ (L ≡ totally deprotonated form of TRENCAM) and a is the number of moles of base added per mole of ligand. A noteworthy feature of the ligand curve is two well-developed, two-proton buffer regions at pH 6–7 and 8–9, respectively. Typical tricatecholates previously characterized[10,12–14,16] display only one three-proton buffer region in the pH range 4–10. As will be developed, the acid/base activity of the tertiary amine proton causes this deprotonation behavior of the tricatecholate. The last four ligand protonation constants have been determined to be log K^H_7 = 5.88 (1), log K^H_6 = 6.71 (1), log K^H_5 = 8.61 (1), and log K^H_4 = 8.75 (1), where K^H_n is defined by eq 1. We assign three of these constants to the more

$$K^H_n = \frac{[LH_n]}{[LH_{n-1}][H]} \qquad (1)$$

acidic catechol OH groups. The fourth is apparently due to the tertiary ammonium, which is made substantially more acidic by strong interactions with the three amide NH protons. The average of these protonation constants is 7.49, which is significantly lower than the lower protonation constant, log K^H_2 = 8.42, of *N*,*N*-dimethyl-2,3-dihydroxybenzamide.[7] This is also lower than the average value of the three more acidic protonation constants of previously prepared unsulfonated tricatecholates,[10] which we ascribe to the presence of the protonated tertiary amine in the ligand backbone. These low protonation constants contribute to a relatively high water solubility, 0.8 mM, of the ligand. In addition it is noteworthy that the similar values of log K^H_5 and log K^H_4 result in a relatively high correlation coefficient, −0.86, between them.

The potentiometric equilibrium curve of Fe^{III}TRENCAM[28]

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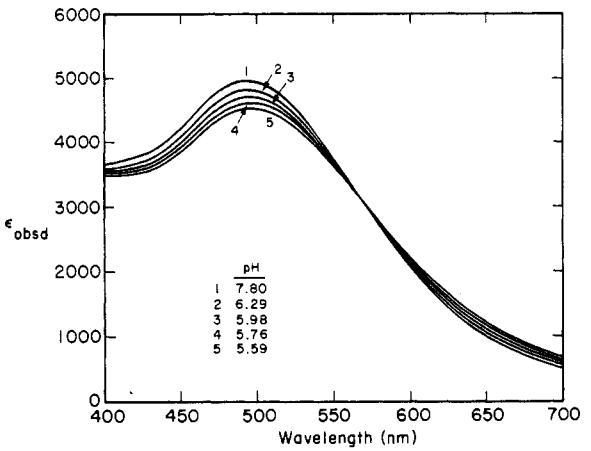

Figure 3. Visible spectra of Fe^{III}TRENCAM as a function of pH ([Fe(TRENCAM)] = 2.6×10^{-5} M; 10-cm cell; 0.1 M KNO_3; 25 °C).

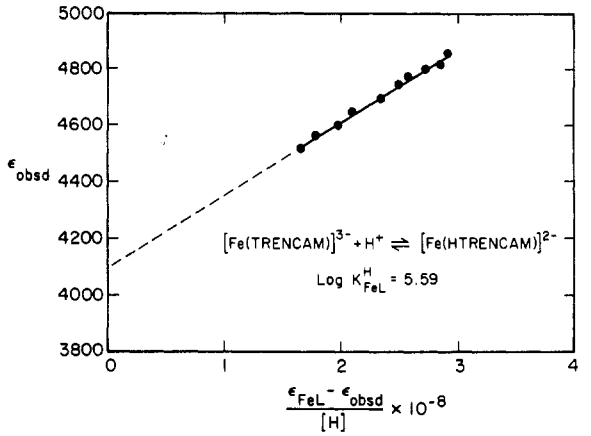

Figure 4. Schwarzenbach plot for the first chelate protonation reaction of $[Fe(TRENCAM)]^{3-}$. Conditions are given in Figure 3.

shows a large pH jump at $a = 7$, which indicates that the seven protons of the six catechol groups plus the protonated tertiary amine are released when the ligand binds ferric ion above pH 7.5. At this point of the titration, the solution was deep red with $\epsilon = 4900$ M^{-1} cm^{-1} at $\lambda_{max} = 496$ nm. The visible spectrum of this complex is very similar to those of the tris(bidentate) model compounds in which the iron is coordinated to six catecholate oxygens[29] and the previously characterized ferric tricatecholates, including enterobactin.[7,9,12-16] The intense band of $\epsilon = 4900$ M^{-1} cm^{-1} at $\lambda_{max} = 496$ nm is assigned to be ligand to metal charge transfer (LMCT).[30,31] Thus, the red complex in the solution can be identified as the $[Fe(TRENCAM)]^{3-}$ species, in which the iron is coordinated through the six catecholate oxygens of the three TREN side arms.

The titration curve of Fe^{III}TRENCAM has a three-proton buffer region from $a = 4$–7, which spans pH 5–5.6. Because of the three consecutively overlapping protonations in the short pH range, the precipitation of a neutral complex was expected at a relatively high pH during the titration. Careful visual observation confirmed that a significant amount of complex began to precipitate out as purple solid below pH 5 under the present experimental conditions. The observed physical change does not appear to originate from oxidation or chemical changes other than protonation, since increasing the solution pH recovered the spectral and electrochemical properties of original solution. The existence of the precipitate during the titration precluded the use of these data in refinement procedures. However, it is clear from the potentiometric data that the first three protonation constants of Fe^{III}TRENCAM are positioned close to one another between pH 5 and 5.6.

Although the protonation constants of the ferric complex could not be determined from potentiometric data, the intense LMCT band of the metal complex allowed the determination of the first protonation constant. Between pH 8 and 5.6, the visible spectra display an isosbestic point at 572 nm, as shown in Figure 3, indicating that a simple equilibrium exists in this pH range between two metal complexes, $Fe^{3+}L$ and $Fe^{3+}HL$, absorbing in the visible region. The data can be analyzed by eq 2[7,23] where ϵ_{obsd}

$$\epsilon_{obsd} = \epsilon_{FeH_nL} + \frac{\epsilon_{FeL} - \epsilon_{obsd}}{K_{FeH_nL}[H]^n} \quad (2)$$

= (absorbance)/$[Fe]_{tot}$ and $K_{FeH_nL} = [FeH_nL]/[FeL][H]^n$. Plots of ϵ_{obsd} vs. $(\epsilon_{FeL} - \epsilon_{obsd})/[H]^n$ are linear only for $n = 1$ (Figure 4), indicating that Fe^{III}TRENCAM reacts with a single hydrogen ion to form a $[Fe(HTRENCAM)]^{2-}$ complex. Values of log K_{FeHL} = 5.59 and $\epsilon_{FeHL} = 4100$ were determined from the slopes and intercepts, respectively. The first protonation constant determined is consistent with the potentiometric data; it corresponds to the pH range of the $a = 7$–6 buffer region.

Because the complex is fully formed over the entire pH range accessible to the titration and there is essentially no free (unchelated) ferric ion present in solutions, the formation constant of Fe^{III}TRENCAM cannot be determined from titration data. Instead, from the competition experiments with EDTA[10,16] the proton dependent formation constant is determined as log $K^* = -22.7$ from eq 3. Assuming that the average of the three largest

$$K^* = \frac{[Fe(TRENCAM)][H]^7}{[Fe][H_7TRENCAM]} \quad (3)$$

log K^H values are 12.1[7,10] the formation constant (log K_{FeL}) is estimated to be 43.6. From this formation constant and the protonation constants K^H_n ($n = 4$–7) and K_{FeHL} (see Table I) we calculated p[Fe^{3+}] (defined as −log [Fe^{3+}]) to be 27.8 at pH 7.4, $[Fe^{3+}]_{tot} = 1$ μM, and $[ligand]_{tot} = 10$ μM.

Table I. Comparison of Thermodynamic and Spectroscopic Data for TRENCAM and Enterobactin[a]

	TRENCAM	enterobactin
hydrolytic stability	stable	unstable
solubility,[b] mM	0.8	0.1[c]
$E°_{Fe^{3+/2+}L}$,[d] V vs. NHE	−1.04	−0.99[e]
log K_{H_nL}[f]		
$n = 1$	12.9[g]	12.9[g]
$n = 2$	12.1[g]	12.1[g]
$n = 3$	11.26 (2)[h]	11.3[g]
$n = 4$	8.75 (1)[h]	9.2[g]
$n = 5$	8.61 (1)[h]	8.4[g]
$n = 6$	6.71 (1)[h]	7.6[g]
$n = 7$	5.88 (1)[h]	
p[Fe^{3+}][i]	27.8	35.5[j]
$\epsilon_{Fe^{3+}L}$, M^{-1} cm^{-1}	4900 (496 nm)	5600 (495 nm)[j]
$\epsilon_{Fe^{3+}HL}$,[k] M^{-1} cm^{-1}	4100	4400[j]

	L = TRENCAM		L = enterobactin	
	Fe^{3+}	Fe^{2+}	Fe^{3+}	Fe^{2+}
log K_{FeL}	43.6[l]	12.6[m]	52[j,l]	22[j,m]
log K_{FeHL}[n]	5.59[k]	11.2[o]	4.80[j,k]	10.4[e]

[a] At 25 °C, aqueous potassium nitrate (0.1 M) media unless specified. The esd's of the last digit are in parentheses. [b] In pure water. [c] From ref 4. [d] Formal potential measured in 0.4 M $NaClO_4$. [e] From ref 24. [f] $K_{H_nL} = [LH_n]/[LH_{n-1}][H]$. [g] Estimated on the basis of *N,N*-dimethyl-2,3-dihydroxybenzamide.[6] [h] Determined from potentiometric titration data in 2% methanol solution. [i] p[Fe^{3+}] = −log [$Fe(OH_2)_6^{3+}$] at pH 7.4, 1 μM $[Fe^{3+}]_T$, 10 μM $[L]_T$. [j] From ref 6. [k] Determined from spectrophotometric titration data. [l] Determined from competition experiments with EDTA. [m] Calculated by using $E_{complex} - E_{aquo} = 59$ log $(K_{Fe^{3+}L}/K_{Fe^{2+}L})$. [n] For the reaction FeL + H^+ = FeHL. [o] Determined from electrochemical data.

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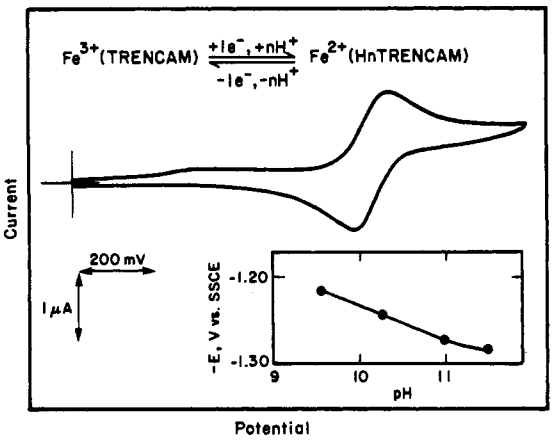

Figure 5. Cyclic voltammogram of $[Fe(TRENCAM)]^{3-}$ in 0.4 M $NaClO_4$ at pH 11.5 (40 mM phosphate). Negative potentials (V vs. SSCE) are plotted to the right and reduction currents are plotted upward. Conditions: area of working electrode (HMDE) 0.032 cm^2; intial potential −0.06 V; scan rate 0.1 V s^{-1}. The inset shows the pH dependence of the formal potentials for the iron(3+,2+) complexes of TRENCAM.

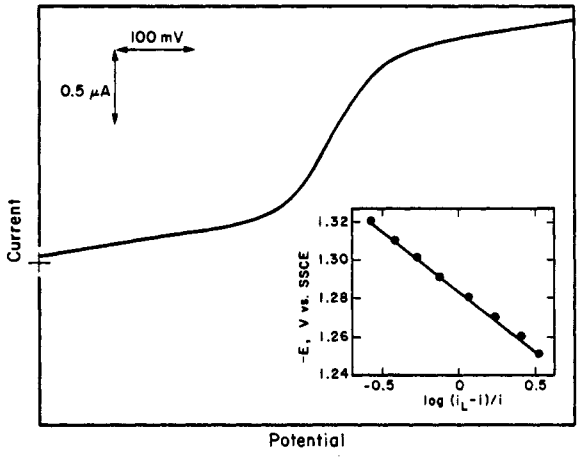

Figure 6. Normal pulse polarogram at a dropping mercury electrode: flow rate 1.2 (± 0.1) mg s^{-1}; initial potential −0.90 V; scan rate 5 mV s^{-1}. Other conditions are the same as in Figure 5. The inset shows −E vs. log $[(i_L - i)/i]$ for the normal pulse polarogram.

Electrochemistry. Figure 5 shows the cyclic voltammogram recorded with 0.4 mM $[Fe(TRENCAM)]^{3-}$ in an aqueous sodium perchlorate electrolyte at pH 11.5, with use of a hanging mercury drop working electrode. The ratio of cathodic and anodic peak currents is 1.0, and the peak separation is 60 mV. The log plot of a normal pulse polarogram, shown in Figure 6, is linear with a sloe of 60 mV (Figure 6 inset), which is consistent with a one-electron, Nernstian reaction. In addition, preparative reduction of Fe^{III}TRENCAM yielded colorless solutions where the LMCT band was absent, and the reoxidation of the resulting solution recovered the LMCT band of the original solution. Both oxidation and reduction data confirmed that the number of electrons involved was 1. Thus, the formal potential of the iron(3+,2+) complexes of TRENCAM was determined from cyclic voltammetry and normal pulse polarography.[33]

The inset of Figure 5 shows a variation of the measured formal potential for the iron(3+,2+) center from pH 11.5 to 9.6. At lower pH, the electron transfer was sluggish. Since the first protonation constant of $[Fe(TRENCAM)]^{3-}$ is $10^{5.59}$ (vide supra), the electrode reaction is assigned to be

$$[Fe^{3+}(TRENCAM)]^{3-} \underset{-1e^-,\,-nH^+}{\overset{+1e^-,\,+nH^+}{\rightleftharpoons}} [Fe^{2+}(H_nTRENCAM)]^{n-4} \quad (4)$$

There is a clear break at pH 11.2, where protonation occurs. Below pH 11.2, the slope is −60 mV/pH unit, indicating that a one-proton transfer ($n = 1$) follows the electron transfer. The first protonation constant, log K^H_1 = 11.2, of Fe^{II}TRENCAM is larger than that of ferrous enterobactin (10.4), which is consistent with the fact that the first protonation constant of Fe^{III}TRENCAM is larger than that of ferric enterobactin. Above pH 11.2, the reduction potential does not depend on pH (i.e. $n = 0$). This limiting high pH potential (−1.04 V) is as negative as that for any tricatecholate complexes of iron(3+, 2+) we have examined to date, including enterobactin. This is directly related to the stability constants of ferric and ferrous complexes by eq 5,[34] where K_{II} and K_{III} represent the FeL stability constants for

$$E^\circ_{complex} - E_{aquo} = 59 \log \left(\frac{K_{III}}{K_{II}} \right) \quad (5)$$

the ferrous and ferric oxidation states, respectively. Given log K_{III} as 43.6 (vide supra), log K_{II} is calculated to be 12.6. The results are summarized in Table I.

It is noteworthy that the specificity of TRENCAM for ferric ion vs. ferrous ion is comparable to that of enterobactin, although TRENCAM forms a weaker ferric complex than does enterobactin. We are preparing bicapped macrocyclic tricatechol ligands that may more closely mimic the stability of the enterobactin complex due to preformation of the coordination cavity.[35,36]

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