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Pecoraro, Vincent L.

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ANALOGUES OF ENTEROBACTIN

Vincent L. Pecoraro, Frederick L. Weigl, and
Kenneth N. Raymond

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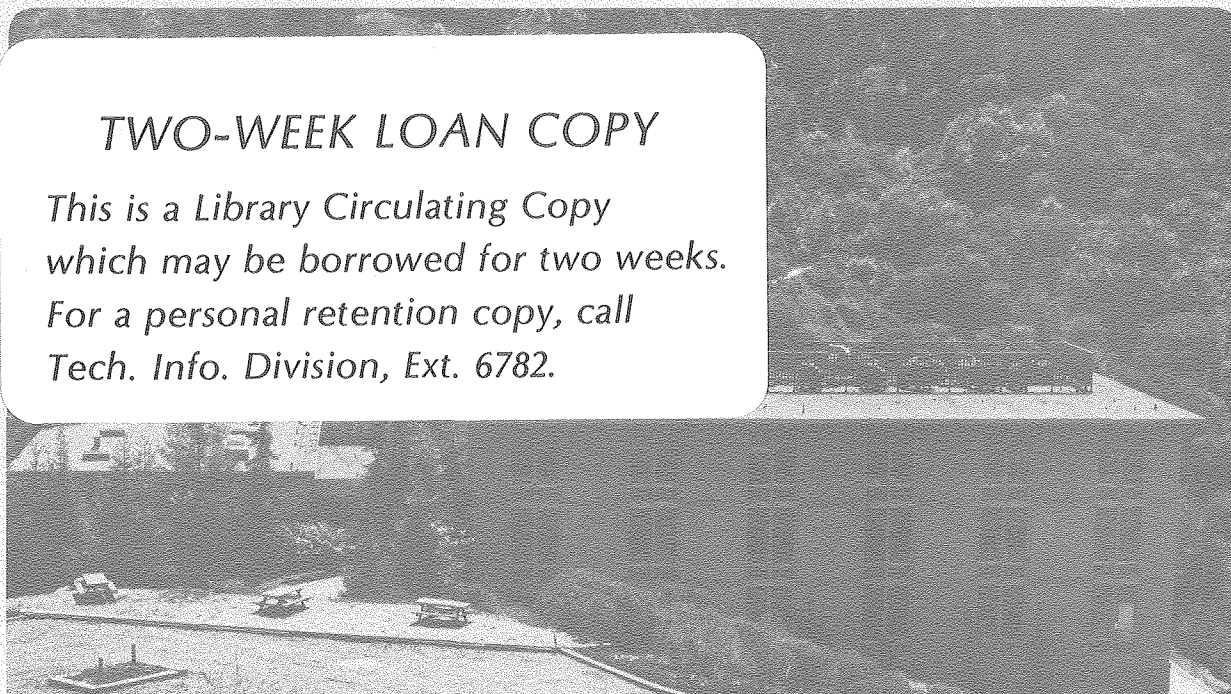
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Ferric Ion-Specific Sequestering Agents. 7. Synthesis, Iron Exchange
Kinetics, and Stability Constants of N-Substituted, Sulfonated
Catechoylamide Analogues of Enterobactin¹

By

Vincent L. Pecoraro, Frederick L. Weigl² and Kenneth N. Raymond*³

Contribution from the Department of Chemistry and Materials and Molecular
Research Division, Lawrence Berkeley Laboratory, University of
California, Berkeley, California 94720

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Abstract

For treatment of chronic iron overload (as occurs in Cooley's anemia), ferric ion sequestering agents with specific properties are necessary. Two analogues of enterobactin [a microbial chelating agent with the greatest stability constant known for an Fe(III) complex] are reported which exhibit: i) hydrolytic stability; ii) water solubility; iii) N-substitution to block peptidase hydrolysis. The first compound, N,N',N''-trimethyl-N,N',N''-tris(2,3-dihydroxysulfo benzoyl)1,3,5-triaminomethylbenzene, [Me₃MECAMS, 6] was prepared from the amide of trimesoyl chloride (1) and MeNH₂. The resulting amide was reduced to the triamine (3) and converted in three steps to the final product 6 in 6% overall yield. The proton-dependent formation constant (log K*) for the reaction:



is 4.87, which gives an equilibrium concentration of [Fe³⁺] at pH 7.4 of 2×10^{-27} M for 10^{-5} M L (6) and 10^{-6} M total Fe³⁺. The estimated formation constant (log β₁₁₀) is 40. At low pH the FeL⁶⁻ complex undergoes a series of three, one-proton reactions which probably gives a tris-salicylate complex formed by the carbonyl and ortho-catechol oxygen of the 2,3-dihydroxybenzoyl units (the same reaction that occurs with ferric enterobactin). After six hours in the presence of 6 mM ascorbate, Me₃MECAMS (6.0 mM) removed 3.7% of the ferric ion initially sequestered by the iron storage protein, ferritin. The human iron transport protein transferrin gives up iron to Me₃MECAMS with a pseudo first-order rate constant of $1.9 \times 10^{-3} \text{ min}^{-1}$ (ligand concentration 2×10^{-4} M). This rate is comparable to that of enterobactin and other catechoyl amide

sequestering agents, and greatly exceeds that of desferrioxamine B (Desferal[®]), the current drug of choice in treating iron overload. Two related compounds have been prepared in which the catechol ring is attached to the amide nitrogen through a methylene group, with amide formation with an acetyl group. In N,N',N''-triacetyl-N,N',N''-tris(2,3-dihydroxysulfobenzoyl)-N,N',N''-triaminomethylbenzene [NacMECAMS, 11] and its unsulfonated precursor, the amide linkage of the catechoyl amides such as Me₃MECAMS (6) has been shifted from an endo position relative to the benzene and catechol rings to an exo position in which the amide carbonyl is not conjugated with the catechol ring and cannot form a stable chelate ring in conjunction with a catechol oxygen. The preparation of 11 and 10 proceeded from the previously described precursor of TRIMCAM, 7, borane reduction to the triamine 8, and amide formation with acetyl chloride to 9, followed by deprotection of the catechol oxygens with BBr₃/CH₂Cl₂ to give 10. Sulfonation of 10 to NacMECAMS, 11, is carried out in fuming sulfuric acid. In comparison with Me₃MECAMS, the protonation of NacMECAMS (11) proceeds by an initial two-proton step in contrast to the one-proton reactions typical of the catechoyl amides, which can form a salicylate mode of coordination involving the amide carbonyl group. Also as a result of the removal of the carbonyl group from conjugation with the catechol ring, the acidity of NacMECAMS (11) is less than Me₃MECAMS (6). While the estimated log β₁₁₀ is approximately the same as for Me₃MECAMS (40), the effective formation constant (log K*) and pM. (- log [Fe³⁺_{aq}]) values are lower (4.0 and 25.0, respectively).

Introduction

It is established that virtually all organisms need iron.⁴ Human beings maintain a total inventory of ca. 5 g in the adult through a complex process of iron transport and storage.⁵ In this light, it is a well documented fact that, in excess, Fe^{3+} is very toxic. Indeed, acute iron overload (primarily from ingestion of iron supplement preparations by infants) is one of the most common forms of poisoning in the U.S.A.⁶ In addition, long-term (i.e. chronic) iron overload can result from the transfusion therapy of Cooley's anemia (β -thalassemia major),⁷ a blood disorder affecting approximately three million people worldwide, and is the major cause of death associated with this disease.

The present treatment for transfusional iron overload in the β -thalassemic patient is either subcutaneous or intravenous slow infusion of Desferal[®], the mesylate salt of desferrioxamine B. This therapy has exhibited efficacies as low as 10%, presumably due to the instability of the drug in the body and its rapid excretion.⁸ The pool of labile, low-molecular-weight iron, the iron storage protein ferritin and the iron transport protein transferrin are three possible sources for removable iron. Desferal[®] presumably sequesters ferric ion from the labile iron pool and, with the co-administration of ascorbate, from ferritin. Although Desferal[®] is thermodynamically capable of removing iron from transferrin, the process has been shown to be kinetically slow. We are actively involved in the synthesis and characterization of new iron sequestering compounds which are intended to be potential therapeutic agents in the treatment of iron overload.

These ligands are analogues of the catechol-containing siderophore, enterobactin, which has been shown to be both thermodynamically and kinetically able to remove iron from transferrin.⁹ Unfortunately, enterobactin is unsuitable for clinical use due to its low aqueous solubility, extreme oxidative and hydrolytic lability, and its capacity to cause toxic bacteremia in iron-overloaded mice.¹⁰ Preliminary animal experiments on hypertransfused rats have shown that sulfonated catechoyl-amide sequestering agents are capable of mobilizing iron in vivo. No acute toxic effects were reported; however, after ten days the animals died due to toxic bacteremia.¹¹ Bacterial proliferation is presumably due to mobilization of iron from biological stores which are normally unavailable for bacterial growth. It seems possible that the bacteria are in some way removing iron from the metal complex. A reduction mechanism for iron release at pH 7.4 in aqueous solution seems unlikely since $\text{Fe}(\text{Me}_3\text{MECAMS})^{6-}$, $\text{Fe}(\text{NACMECAMS})^{6-}$ and other tricatecholate compounds have estimated reduction potentials of approximately -750 mV vs nhe at this pH. As with ferric enterobactin, this redox potential is too negative to be affected by physiological reductants. Another possible scenario for extraction of iron would be peptidase cleavage of the amide bond, which would raise the redox potential within the physiological range.¹² Attaching an alkyl group to the amide nitrogen should inhibit this degradation, so that we can test the validity of this mechanism for iron acquisition by microbes.

The title compounds have been designed to avoid the drawbacks of enterobactin. Sulfonation enhances the water solubility and phenolic acidity of the ligand. The inherent chelate effect of a macrocyclic ligand is retained by use of the benzenoid platform. Alkylation of the amide nitrogen still allows the formation of the FeL^{6-} complex and,

moreover, may make the free ligand and iron complex resistant to microbial peptidase activity. We report here the synthesis and stability constants of N,N',N''-trimethyl-N,N',N''-tris(2,3-dihydroxysulfobenzoyl)-1,3,5-triaminomethylbenzene [Me₃MECAMS, 6] and N,N',N''-triacetyl-N,N',N''-tris(2,3-dihydroxysulfobenzoyl)-1,3,4-triaminomethylbenzene [NACMECAMS, 11]. In addition, the kinetics of iron removal from the mammalian transport and storage proteins by Me₃MECAMS have been determined.

Materials and Methods

Potentiometric Measurements. We have previously given a detailed account of the apparatus used and the procedure followed for potentiometric titrations.¹³ Briefly, measurements were made with a Corning 130 digital pH meter equipped with Corning Glass and saturated calomel electrodes. The meter was calibrated with standard acetate and nitric acid solutions to read hydrogen ion concentration, not activity. Solutions (40 mL) were kept under inert atmosphere (argon) and were maintained at $25 \pm 0.05^\circ\text{C}$ by a circulating water bath. The ionic strength was maintained at 0.1 M with KNO₃. Carbonate-free 0.1 M KOH was prepared from Baker Dilut-It ampoules using freshly boiled, doubly distilled H₂O. Potentiometric data were refined using a nonlinear least-squares analysis described previously.¹³

Spectrophotometric Measurements. Spectra were recorded on a Cary 118 spectrophotometer. The visible spectra of ferric Me₃MECAMS as a function of pH were obtained from a single solution. After each adjustment of pH, an aliquot was removed, the spectrum recorded, and the sample returned to the original solution. Spectrophotometric competitions using

$\text{Na}_2\text{H}_2\text{EDTA}$ ¹⁴ were carried out as previously described.¹³ Spectra were recorded 24 and 72 hours after mixing to assure equilibrium had been achieved.

Kinetics. Apotransferrin (Sigma chemicals) was saturated by the procedure of Bates et al.¹⁵ using 0.02 M $[\text{Fe}(\text{NTA})_2]^{3-}$ at pH 7.4. The resulting complex was gel filtered on Sephadex G-25 resin equilibrated with 0.1 M tris pH 7.4 buffer. The ratio $A_{280}/A_{466} = 11$ demonstrated greater than 95% saturation of transferrin. Kinetic studies were monitored at 520 nm. The time of mixing was taken as time zero. The concentration of ligand used was 0.2 mM in order to make comparisons with previously published results.⁹ Linear plots of $\ln[A_t - A_\infty]/(A_0 - A_\infty)$ vs time were obtained for 1.5 half lives. The values of k_{obs} were obtained by linear least-squares analysis.

Horse spleen ferritin was purchased from United States Biochemical. The ferritin was purified by centrifugation for a period of two hours followed by gel permeation chromatography on Sephadex G-25 using 0.05 M tris HCl¹⁴ buffer at pH 7.4 as an eluant. Ferric ion concentration was determined by the absorption at 420 nm ($E_{1\text{cm}}^{1\%} - 100$).¹⁶ The procedure of Lowry et al. was followed for the protein assay.¹⁷ The $\ell(+)$ -ascorbic acid (Matheson, Coleman and Bell) was used as received. Freshly prepared stock solutions of the ligand and ascorbic acid were used for all kinetic runs.

Kinetic studies were performed in a Cary 118 uv/vis spectrophotometer equipped with a thermostated quartz cell (1 cm path length) maintained at $37.0 \pm 0.05^\circ\text{C}$. Reaction mixtures were stored in a water bath at 37°C in between the time of each absorbance measurement. Time zero was taken to be the time of addition of the stock ferritin solution to the

remainder of the reaction mixture. Absorbance measurements at 487 nm were taken once every hour for a period of at least 12 hours for all samples. Reaction mixtures were buffered at pH 7.4 (tris HCl). No attempt was made to exclude oxygen from the sample vials.

The percent iron removed in six hours was calculated by correcting the absorbance change at 487 nm for the baseline absorbance due to ferritin alone. Using a molar extinction coefficient of $5,400 \text{ M}^{-1} \text{ cm}^{-1}$ for $[\text{FeMe}_3\text{MECAMS}]^{6-}$, the number of millimoles of iron removed was calculated. This value was compared to the initial concentration of ferritin-bound iron.

Syntheses

Experimental Methods. Melting points were taken on a Buchi apparatus in open capillaries and are uncorrected. ^1H NMR spectra were recorded on a Varian A-60 instrument using Me_4Si (with organic solvents) or 3- Me_3Si -1-propane sulfonic acid, sodium salt (with D_2O) as internal standard. Infrared spectra were recorded on a Perkin-Elmer 283 instrument. Evaporations were accomplished in vacuo with a Buchi Rotovapor-RE at $\leq 55^\circ\text{C}$. Thin layer chromatography (tlc) was performed on precoated 60F-254 silica gel sheets which were developed in tetrahydrofuran (93 mL)/ C_6H_{12} (7 mL)/ H_2O (5 mL), then visualized with UV, iodine, or $\text{Fe}^{3+}/\text{H}_2\text{O}/\text{EtOH}$ spray. Column chromatography was performed using 60-200 mesh silica in a 35 x 2.5 cm o.d. column and fractions were monitored with tlc. Microanalyses and mass spectra (EI-70 eV) were performed by Analytical Services, Chemistry Department, University of California, Berkeley. Trimesoyl chloride, I , was purchased from Aldrich Chemical

Co., Milwaukee, Wisconsin. Compound 7 was prepared according to the literature method.¹⁸

N,N',N''-Trimethyl-1,3,5-carboxamidobenzene (2). To a vigorously stirred water bath cooled solution of trimesoyl chloride, 1 (20 g, 75 mmol) in tetrahydrofuran (THF, 300 mL) was added excess monomethylamine via gas diffusion tube. The reaction mixture was stirred overnight under a Drierite tube then was filtered. The resulting cake was washed well with H₂O to remove amine·HCl. The cake was then recrystallized from boiling aqueous ethanol to obtain 2 (16.0 g, 86%): mp 312-14°. Anal. Calcd. for C₁₂H₁₅N₃O₃: C, 57.82; H, 6.07; N, 16.86. Found: C, 57.89; H, 6.02; N, 16.91.

N,N',N''-Trimethyl-1,3,5-triaminomethylbenzene trihydrochloride (3). Under argon atmosphere, a slurry of triamide 2 (11.0 g, 44 mmol) in BH₃/THF (1 N, 30 mL) was slowly brought to reflux, maintained for 24 hr. Then aq. HCl (6 N, 50 mL) was added (Caution, H₂↑) dropwise and maintained at reflux for 60 hr. Evaporation in vacuo, followed by coevaporation with methanol to volatilize the borates gave crude, dry solid which, when crystallized from boiling methanol by the addition of ethanol (cooling and scratching), gave hygroscopic 3 (9.9 g, 70%, dried in vacuo at 70°, 6 hr): mp 252-5°. Anal. Calcd. for C₁₂H₂₁N₃·3HCl: C, 45.51; H, 7.64; N, 13.27; Cl, 33.58. Found: C, 45.19; H, 8.26; N, 12.93; Cl, 33.04

N,N',N''-Trimethyl-N,N',N''-tris(2,3-dimethoxybenzoyl)-1,3,5-triaminomethylbenzene (4). To 2,3-dimethoxybenzoyl chloride (45 mmol) dissolved

in THF (75 mL) was added solid 3 (4.75 g, 15 mmol), followed by NEt_3 (12.5 mL, 90 mmol). The reaction mixture was stirred vigorously in a stoppered flask (60 hr). Filtration removed the $\text{NEt}_3 \cdot \text{HCl}$. Evaporation of the THF solution gave crude, oily product which was dissolved in CCl_4 , washed well with aq. NaOH, then entered onto a silica gel column. Elution with (0-5%, v/v) CH_3OH in CHCl_3 mixture provided pure product; tlc, R_f 0.63. Coevaporation with CCl_4 to remove all other solvents, then Abderhalden drying at 60° , 48 hrs, gave glassy 4 (8.3 g, 75%): mp $70-5^\circ$; ir (neat, NaCl) 2935, 2835 ($-\text{CH}-$), 1635 ($-\text{CONR}_2$), 1580, 1480, 1428, 1400, 1308, 1278, 1230, 1050, 1000, 795, 750 cm^{-1} ; $^1\text{H NMR}$ (CCl_4) δ 2.72, 2.92 (two s, 9 H, CH_3N), 3.83 (s, 18 H, $-\text{OCH}_3$), 4.32, 4.70 (two s, 6 H, ArCH_2), 6.7-7.4 (complex m, 12 H, ArH); mass spectrum, m/e (rel. intensity), 699 (M, 11), 668 (M- OCH_3 , 12), 577 (2), 549 (9), 534 [M- $\text{C}_6\text{H}_3(\text{OCH}_3)_2\text{CO}$, 10], 165 [$\text{C}_6\text{H}_3(\text{OCH}_3)_2\text{CO}$, 100].

Anal. Calcd. for $\text{C}_{39}\text{H}_{45}\text{N}_3\text{O}_9 \cdot 1/4\text{CCl}_4$: C, 63.84; H, 6.14; N, 5.69.

Found: C, 63.50; H, 6.22; N, 5.59.

$\text{N,N}',\text{N}''$ -Trimethyl- $\text{N,N}',\text{N}''$ -tris(2,3-dihydroxybenzoyl)-1,3,5-triamino-methylbenzene (5). A solution of 4 (8 g, 10.8 mmol) under argon atmosphere in CH_2Cl_2 (75 mL) was added dropwise via addition funnel to a vigorously stirred solution of BBr_3 (8 mL, 80 mmol) in CH_2Cl_2 (200 mL), cooled by a room-temperature water bath. An immediate precipitate formed and the slurry was stirred overnight. Dropwise addition (Caution, $\text{HBr}\uparrow$) of water (50 mL) followed by aq. NaOH (40 mL, 6 M) gave a (pH $\sim$ 6) mixture which was filtered and the cake washed well with water. This crude light tan product was dissolved in ethanol and precipitated by the addition of large volumes of ethylacetate. Filtration, ethylacetate wash,

then drying in vacuo, at 90°, overnight, provided amorphous powder 5 (4.6 g, 70%): mp 130-40° (glass); ir (KBr) 1610 ($-\text{CONR}_2$), 1585, 1470, 1408, 1280, 1100, 1070, 790, 750, 735 cm^{-1} ; $^1\text{H NMR}$ (TFA) δ 3.38 (broad s, 9 H, N-CH_3), 5.22 (v. broad s, 6 H, ArCH_2N), 7.0-7.5 (complex m, 12 H, ArH); mass spectrum m/e (rel. intensity), 615 (M, 2), 479 [$\text{M-C}_6\text{H}_3(\text{OH})_2\text{CO}$, 6], 343 [$\text{M}-(\text{C}_6\text{H}_3(\text{OH})_2\text{CO})_2$, 5], 136 [$\text{C}_6\text{H}_3(\text{OH})_2\text{CO}$, 26].

Anal. Calcd. for $\text{C}_{33}\text{H}_{33}\text{N}_3\text{O}_9 \cdot 1-1/2\text{H}_2\text{O}$: C, 61.67; H, 5.65; N, 6.54.

Found: C, 61.38; H, 5.35; N, 6.46.

N,N',N'' -trimethyl- N,N'-N'' -tris(2,3-dihydroxysulfo benzoyl)-1,3,5-triaminomethylbenzene, trisodium salt (6). Solid 5 (4.0 g, 6.0 mmol) was added in portions to vigorously stirred 30% $\text{SO}_3\text{-H}_2\text{SO}_4$ (36 mL) at room temperature, and stirring was continued in a stoppered flask for 20 hr before quenching on excess ice. This solution was brought to pH 4 by slow addition of conc. aq. NaOH with external ice-bath cooling. The addition of an equal volume of CH_3OH , filtration of the boiling mixture, and washing of the Na_2SO_4 cake with hot, 1:1 $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ gave product-free cake (colorless Fe^{+3} test). The product solution and wash was concentrated in vacuo to a brown water solution. Initially CH_3OH , then EtOH, finally acetone were sequentially added in small amounts to precipitate less soluble, colored impurities each time removed by filtration. Finally, several volumes of acetone were added to give white product 6· $6\text{H}_2\text{O}$ (4.3 g, 69%) mp 290°; ir(KBr) 3700-2500 ($-\text{OH}$, $-\text{CH}-$), 1615 ($-\text{CONR}_2$), 1492, 1421, 1290, 1230-1170 (SO_3^-), 1108, 1046, 620 (SO_3^-) cm^{-1} ; $^1\text{H NMR}$ (D_2O) δ 2.8-3.3 (broad m, 9 H, $\text{CH}_3\text{N}<$), 4.4-4.8 [broad m, 6 H, $-\text{CH}_2\text{N}(\text{CH}_3)-$], 7.2-7.7 (m, 9 H, ArH).

Anal. Calcd. for $C_{33}H_{30}N_3O_3SNa_3 \cdot 6H_2O$: C, 38.49; H, 4.11; N, 4.08; S, 9.34.

Found: C, 38.66; H, 3.76; N, 4.01; S, 8.66.

N,N',N''-Tris(2,3-dimethoxybenzyl)-1,3,5-triaminomethylbenzene (8).

To an argon flushed system containing 7 (10.6 g, 16.1 mmol) was added at once, via syringe and septum, BH_3 /THF solution (1 N, 150 mL), then was heated in a 70° oil bath (60 hr). Next, aq. HCl (6 N, 25 mL) was added dropwise (Caution, $H_2\uparrow$) and reflux continued overnight. Evaporation of the THF followed by $CHCl_3$ -extraction gave crude aqueous $8 \cdot 3HCl$ solution. Excess 6 N NaOH was added and the strongly basic solution was extracted well with Et_2O , then the organic layer was dried with $MgSO_4$ to give a solution of 8: mass spectrum m/e (rel. intensity), 615 (M, 3), 478 [$M-C_6H_3(OCH_3)_2$, 34], 464 [$M-CH_2C_6H_3(OCH_3)_2$, 49], 450 [$M-NHCHC_6H_3(OCH_3)_2$, 100]; ir (neat, KBr plate) 3310 (-NH-), 2940, 2815 (-CH-), 1585, 1475, 1270, 1220, 1075, 1005, 775, 750 cm^{-1} ; 1H NMR (TFA) δ 4.04, 4.12 (two s, 18 H, $-OCH_3$), 4.6 (broad, 12 H, $-CH_2NH_2CH_2-$), 7-7.5 (complex m, 9 H, ArH) 7.8-8.0 (complex m, ArH). The addition of HCl, via gas diffusion tube, caused the separation of a white semi-solid. The Et_2O was decanted and the compound dissolved in CH_2Cl_2 which was evaporated to leave a white powder and further dried over $P_2O_5/NaOH$ overnight to yield $8 \cdot 3HCl$ (10.6 g, 91%).

Anal. Calcd. for $C_{36}H_{45}N_3O_3 \cdot 3HCl$: Cl, 14.67.

Found: Cl, 15.20.

N,N',N''-Triacetyl-N,N',N''-tris(2,3-dimethoxybenzyl)-1,3,5-triamino-
methylbenzene (9). Acetylation of 8 was achieved as follows: $8 \cdot 3HCl$ (10.6 g, 15 mmol) and NEt_3 (6.2 mL, 45 mmol) were slurried in THF (100 mL)

for 30 min before the sequential, dropwise addition of CH_3COCl (3.6 mL, 50 mmol), and NEt_3 (6.9 mL, 50 mmol) dissolved in THF (50 mL). The resulting mixture was stirred overnight in a closed flask at 20–5°. Filtration removed $\text{NEt}_3\cdot\text{HCl}$ and the THF solution was evaporated to residual oil. The latter was dissolved in CCl_4 and eluted from an aluminum oxide column (neutral, grade 1) sequentially with CCl_4 , CH_2Cl_2 and CHCl_3 to obtain, in the first fractions, the pure oil 9 (5.0 g, 45%): tlc, R_f 0.63; EI-mass spectrum m/e (rel. intensity), 741 (M, 10), 698 (M-COCH₃, 1), 590 [M-CH₂C₆H₃(OCH₃)₂, 5], 548 (23), 534 [M-CH₃CONHCHC₆H₃(OCH₃)₂, 10], 208 [CH₃CONHCHC₆H₃(OCH₃)₂, 54], 151 [CH₂C₆H₃(OCH₃)₂, 100]; ir (neat, KBr plate) 3100–2800 (–CH–), 1650 (CH₃CON<), 1480, 1420, 1270, 1220, 1080, 1062, 1005, 770, 750 cm^{-1} ; $^1\text{H NMR}$ (CCl_4) δ 2.10 (broad s, 9 H, CH₃CO), 3.73, 3.80 (two s, 18 H, CH₃O–), 4.40 [broad s, 12 H, –CH₂N(COCH₃)CH₂–], 6.6–7.2 (m, 12 H, ArH).
 Anal. Calcd. for $\text{C}_{42}\text{H}_{51}\text{N}_3\text{O}_9$: C, 68.00; H, 6.93; N, 5.66.
 Found: C, 67.79; H, 6.73; N, 5.45.

N,N',N''-Triacetyl-N,N',N''-tris(2,3-dihydroxybenzyl)-1,3,5-triamino-methylbenzene (10). In an argon atmosphere, 9 (5.0 g, 6.7 mmol) dissolved in CH_2Cl_2 (50 mL) was added dropwise via addition funnel to a vigorously stirred solution of BBr_3 (5 mL, 50 mmol) in CH_2Cl_2 (200 mL) immersed in a 20–5° water bath. After stirring overnight, H_2O (50 mL) was added dropwise (Caution, $\text{HBr}\uparrow$) followed by sufficient 6 N aq. NaOH to maintain pH 4 in the aqueous layer. Solid was collected by filtration, washed well with H_2O , then crystallized from hot aqueous EtOH upon overnight cooling. Filtration, water wash and vacuum desiccator drying over $\text{P}_2\text{O}_5/\text{NaOH}$ gave white powder 10·1–1/2 H_2O (3.0 g, 65%): mp 186–9°;

ir (KBr) 3600-2700 (-OH, -CH-), 1615 ($\text{CH}_3\text{CON}<$), 1590, 1485, 1440, 1360, 1272, 743 cm^{-1} ; ^1H NMR (TFA) δ 2.57, 2.97 (two s, 9 H, CH_3CO), 5.07 [broad s, $-\text{CH}_2\text{N}(\text{COCH}_3)\text{CH}_2-$], 7.0-7.7 (broad m, 12 H, ArH); mass spectrum m/e (rel. intensity), 658 (M, 19).

Anal. Calcd. for $\text{C}_{36}\text{H}_{39}\text{N}_3\text{O}_9 \cdot 1\frac{1}{2}\text{H}_2\text{O}$: C, 63.15; H, 6.18; N, 6.14.

Found: C, 63.27; H, 6.06; N, 6.14.

N,N',N''-Triacetyl-N,N',N''-tris(2,3-dihydroxysulfobenzyl)-1,3,5-triaminomethylbenzene, trisodium salt (11). Compound 10 (2.6 g, 3.8 mmol) was dissolved in $\text{SO}_3\text{-H}_2\text{SO}_4$ (30%, 25 mL) vigorously stirred, at ambient temperature, and allowed to react overnight in a stoppered flask. It was then poured onto ice and neutralized to pH 4 by the slow addition of 6 N NaOH with vigorous stirring and ice bath cooling. Slow addition of an equal volume of MeOH to the boiling solution, filtration, and evaporation of the filtrate gave crude product. The latter was dissolved in a small amount of H_2O and adjusted to turbidity by successive addition of CH_3OH , EtOH, Et_2O ; each time filtering out the inorganic and colored impurities. Finally, several volumes of Et_2O precipitated white product from colorless filtrate. The product was redissolved in H_2O and evaporated to dryness to remove organic solvents, then dried over P_2O_5 , in vacuo, at 20-5° to obtain hygroscopic 11 ($\cdot 6\text{H}_2\text{O}$) (3.3 g, 80%): mp 260-2°d; ir (KBr) 3700-2500 (-OH, -CH-), 1615 ($\text{CH}_3\text{CON}<$), 1490, 1435, 1360, 1300-1150 ($-\text{SO}_3^-$), 1108, 1048, 630 cm^{-1} ; ^1H NMR (D_2O) δ 2.20, 2.37 (two s, 9 H, $\text{CH}_3\text{CO}-$), 4.3-5.0 [broad, 12 H, $-\text{CH}_2\text{N}(\text{COCH}_3)\text{CH}_2-$], 6.6-7.5 (m, 6 H, ArH).

Anal. Calcd. for $\text{C}_{36}\text{H}_{36}\text{N}_3\text{O}_{18}\text{S}_3\text{Na}_3 \cdot 6\text{H}_2\text{O}$: C, 40.34; H, 4.51; N, 3.92, S, 8.97.

Found: C, 40.17; H, 4.02; N, 3.81; S, 9.45.

Results and Discussion

Thermodynamics — Me₃MECAMS. The potentiometric equilibrium curves (pH versus OH⁻ added) for deprotonation of Me₃MECAMS (6) and complexation of Fe(III) are shown in Figure 3. Since the individual catechol groups of Me₃MECAMS are essentially the same as the mono-catechol ligand N,N-dimethyl-2,3-dihydroxy-5-sulfobenzamide (7), the protonation constants of 7 ($\log K_1 = 11.5$, $\log K_2 = 7.26$)¹ should be very similar to the average values for Me₃MECAMS. Indeed, the six protonation reactions of Me₃MECAMS fall into two groups of three protons each. The first (more basic) protonations occur at pH > 11 with no evidence of appreciable dissociation below this pH. No attempt was made to determine directly $\log K_n^H$ for n = 1 to 3. An estimate for these constants, based on the protonation of 7, is $\log K_n^{H \text{ avg}} = 11.8$ for n = 1 to 3 for Me₃MECAMS. The three more acidic protons have a $\log K_{4-6}^{H \text{ avg}} = 7.6$ (see Table II). The break at a = 3 is consistent with these results. These protons are much more acidic than those of enterobactin and previously synthesized unsulfonated catechoylamide sequestering agents.

The potentiometric titration of the ferric complex (curve b in Figure 3) shows that initial complexation is quite strong, as is typical for iron(III) catecholate complexes. There is a three-proton buffer region between pH 5.5 and 7.3. Least-squares refinement of these data gives the protonation constants listed in Table II. The first metal protonation constant was also determined spectrophotometrically by the method of Schwarzenbach in which ϵ vs $[(\epsilon - \epsilon_0)/[H^+]^n]$ is graphed.¹⁹ A linear plot is obtained by choosing the appropriate value for n, which is simply the proton stoichiometry for the reaction. The slope of the

line directly gives K_{ML}^H . The multiple, overlapping equilibria made it impossible to use this method to refine the lower constants. The visible spectra for the first protonation equilibrium are shown in Figure 4. The λ_{max} for the tris complex is 487 nm ($\epsilon = 5390 \text{ M}^{-1} \text{ cm}^{-1}$). Upon protonation, the peak shifts to lower energy with a maximum near 512 nm. The proton-dependent formation constant (K^* , Table II) was calculated from competition experiments with NaH_2EDTA at $\text{pH} \sim 6.8$. This value was corrected for metal ligand protonation and hydrolysis equilibria observed in this pH regime.

NACMECAMS. The phenolic protons of NACMECAMS are much more basic than for Me_3MECAMS as can be seen in Table I. The decreased acidity is a direct result of the relocation of the amide carbonyl to a position where it can no longer be an electron withdrawing substituent of the catechol ring. As with Me_3MECAMS the higher protonation constants have been estimated using DMBS as a model; however, due to the slightly higher pK' s of the acidic protons of NACMECAMS, a value of 12.1 for the basic protons has been used.

At high pH, the ferric complex of NACMECAMS is similar to other catecholate ligands investigated with $\epsilon = 5400 \text{ M}^{-1} \text{ cm}^{-1}$ at 487 nm for the tris complex. Figure 5 illustrates the change in the visible absorption spectra as the pH is lowered. Unlike Me_3MECAMS and other tricatecholate ligands with carbonyl oxygens α to the ring, NACMECAMS undergoes a single, two-proton step to yield a ferric bis-catecholate complex. This protonation scheme is similar to that observed for TRIMCAMS and DMBS.¹ Competition experiments (as above) with NaH_2EDTA were again used to determine K^* .

Kinetics. The visible spectra for the reaction of Me_3MECAMS with diferric transferrin is shown in Figure 6. The absorbance maximum shifts from 466 nm, indicative of an iron transferrin complex, to the 487 nm peak of the tris catecholate species. A plot of $\ln[(A_t - A_\infty)/(A_0 - A_\infty)]$ vs time, gave a first order rate constant, k_{obs} , for the reaction (Figure 6). Under the conditions previously used,⁹ $k_{\text{obs}} = 1.9 \times 10^{-3} \text{ min}^{-1}$ with a half life of 6 hr. After 30 min four percent of the iron had been removed from transferrin.

Stability Constants. Table I lists all relevant stability constants. A direct effect of sulfonating the catecholate ring is the increased acidity of the phenolate oxygens. The final three protonations of Me_3MECAMS occur at pH values much lower than observed for enterobactin or other unsulfonated catechoylamides (9.2, 8.4, and 7.6 for $\log K_n^{\text{ent}}$ where $n = 4-6$, respectively). Although NACMECAMS is sulfonated, the $\log K_{1-3}^{\text{avg}}$ for this ligand is comparable to that of enterobactin. Since the carbonyl group is no longer conjugated to the catechol ring, its electron withdrawing capacity has been replaced by a slightly electron donating methylene group. In essence, the increased electron withdrawing ability of the sulfonyl group is canceled by the loss of the alpha carbonyl and is reflected in the ligand protonation constants. In general, however, sulfonated ligands, as discussed below, become more effective chelating agents at physiological pH.

Since the corresponding trialkyl-substituted ligand $\text{N},\text{N}',\text{N}''$ -tri(isopropyl)- $\text{N},\text{N}',\text{N}''$ -tris(5-sulfo-2,3-dihydroxybenzoyl)-1,3,5-triaminobenzene $[(i\text{Pr})_3\text{MECAMS}]^{21}$ does not form a tris complex with Fe(III) below pH 10, there was some concern that the methyl groups on

the amide nitrogen of Me_3MECAMS might hinder the formation of tris-catecholato coordination about the metal ion. However, Me_3MECAMS and NacMECAMS apparently form tris complexes with Fe(III) . The visible spectra ($\lambda_{\text{max}} = 487$, $\epsilon = 5390$), potentiometric titration curve (break at $a = 6$) and magnitude of the formation constant (vide infra) are all consistent with a tris species.

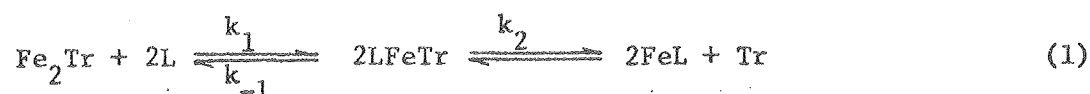
The metal chelate protonation equilibria for Me_3MECAMS are similar to those of enterobactin, MECAM, and MECAMS. Upon protonation, a shift from a catecholate to a salicylate mode of bonding (via the amide carbonyl and ortho phenolate oxygens) has been proposed for these three ligands.¹³ The visible spectra and proton stoichiometry determined from a Schwarzenbach plot for $\text{Fe}(\text{Me}_3\text{MECAMS})^{6-}$ are consistent with those of the previously studied ligands, and would suggest that this complex also undergoes a shift to salicylate coordination.

In contrast to ligands with a carbonyl group alpha to the catechol ring, the low pH mode of bonding in NacMECAMS is via bis-catecholate coordination. This arrangement of iron binding groups is preferable to a salicylate bonding mode for two reasons. First, the carbonyl oxygen has less anionic character when it is not conjugated to the catecholate ring. This decrease in charge on the oxygen lowers its affinity for the hard Fe^{3+} ion. Second, the formation of a complex via the *o*-phenol and carbonyl oxygens would require an eight-membered chelate ring, whereas salicylate coordination is achieved by a stable five-membered ring.

The usual proton-independent formation constant, $\log \beta_{110} (\text{Fe}^{3+} + \text{L}^{6-} \rightarrow \text{FeL}^{3-})$, is estimated to be 40.3 for both Me_3MECAMS and NacMECAMS .

This value is nearly 13 orders of magnitude lower than the value reported for enterobactin and 6 orders of magnitude less than values for the unsulfonated derivatives MECAM or LICAM.¹³ However, these constants are strictly valid only at pH = 14, where the reacting species is a fully deprotonated L^{9-} ligand. Below this pH, ligand and metal chelate protonation equilibria have a profound effect. For this reason we have used pM, defined as $-\log [M^{n+}]$, as a basis for comparison of relative ligand complexing strength at pH 7.4 and a defined metal and ligand concentration, thus affording a direct evaluation of sequestering ability under physiologically relevant conditions. The pM value for $Me_3MECAMS$ is within 2.5 log units of MECAM under the conditions listed in Table III, even though MECAM has a much greater formation constant. Of more importance is that $Me_3MECAMS$ has a pM which is equal to ferri-oxamine B and a thousand times greater than transferrin. Thus, $Me_3MECAMS$ is thermodynamically capable of removing iron from transferrin under physiological conditions.

Kinetics. Carrano and Raymond⁹ have investigated the ability of catecholate chelating agents to remove iron from transferrin. The mechanism of iron removal is believed to involve a ternary complex as is shown in Eq. 1:



The results indicated that both natural and synthetic catechoylamides were capable of kinetically affecting exchange of transferrin-bound iron. The rate constant reported for $Me_3MECAMS$ (Table IV) is consistent

with these results. Although iron exchange with Me_3MECAMS is slightly slower than with other catechoylamides, it is still able to sequester 4% of the transferrin bound iron in 30 min at a 1:1 ligand to transferrin ratio. In contrast, Desferal[®], at a 100:1 ligand to transferrin ratio, can remove only 5% of the transferrin bound iron. These results suggest that Me_3MECAMS is both thermodynamically and kinetically able to remove iron from transferrin.

Mobilization of storage type ferric ion from ferritin, through mediation of ascorbate, is one mechanism by which Desferal[®] is believed to sequester in vivo iron.⁸ For this reason, we investigated the ability of Me_3MECAMS to remove iron from ferritin. The ascorbate dependence and mechanism of iron exchange are discussed more fully in a separate paper.¹⁹ Under the conditions of 6 mM Me_3MECAMS and 6 mM ascorbate, 3.7% of total ferritin-bound iron was removed in six hr at pH 7.4. This value is equal to or better than that reported for ferrioxamine B,^{22,23} suggesting that the sulfonated catechol derivative would as effectively sequester storage iron forms.

Experiments performed by Dr. Rowe Byers' group at the University of Mississippi have shown that $\text{FeMe}_3\text{MECAMS}$ acts as a growth factor for B. subtilis.²⁴ Thus, we can rule out the possibility of amide bond cleavage as a mechanism for iron release in synthetic catechoylamide sequestering agents.

Summary

There are many considerations involved in the rational design of ferric ion chelating agents for the treatment of acute and chronic iron overload. A high efficacy, low toxicity, ability to remove iron

from most available stores and rapid excretion from the body after effecting mobilization of the metal are major requirements. Desferal[®], the present drug of choice, suffers from a short biological half life, kinetic inability to remove iron from transferrin, and a less than adequate ability to maintain negative iron balance in transfused patients. We have described here a new ligand, Me₃MECAMS, which has a high affinity for ferric ion at physiological pH and can remove iron from transferrin at a significant rate. Moreover, Me₃MECAMS can mobilize iron from ferritin when ascorbate is present. Sulfonation of the ligand affords a high water solubility, as well as lowering the ligand protonation constants. The solution chemistry of Me₃MECAMS is analogous to enterobactin, in that it forms a tris catechol complex at high pH and most likely shifts to a salicylate mode of bonding upon protonation. A second compound, NACMECAMS, forms a tris catecholate iron(III) complex at high pH, but undergoes a 2 H⁺ step which causes dissociation of an arm to form a bis catecholate coordination geometry. Unlike previously prepared catecholamides, Me₃MECAMS and NACMECAMS contain only tertiary amide nitrogens. Although N-substitution presumably does not affect microbial acquisition of iron from FeMe₃MECAMS, this modification may be important for future design of orally active drugs which could encounter nonspecific peptidase.

Acknowledgment

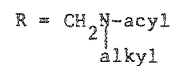
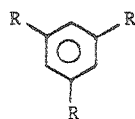
We thank Mr. Thomas P. Tufano for experimental aid and discussion of kinetic data, Dr. Geoffrey B. Wong for assistance with the electrochemical apparatus, and Dr. Wesley R. Harris for thermodynamic data on (iPr)₃MECAMS. This research is supported by the National Institutes of Health through Grant HL 24775 and the Division of Nuclear Sciences, U.S. Department of Energy under Contract No. W-7405-Eng-48.

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14. Abbreviations used: Disodium ethylenediaminetetraacetic acid ($\text{Na}_2\text{H}_2\text{EDTA}$); Nitrilotriacetic acid (NTA); Tris(hydroxymethyl)-aminomethane hydrochloride (Tris HCl); Human serum transferrin (Tr); N,N',N''-Tris(2,3-dihydroxy-5-sulfobenzyl)-1,3,5-tricarboxamido-benzene (TRIMCAMS); N,N',N''-Tris(2,3-dihydroxybenzoyl)-1,3,5-tri-aminomethylbenzene (MECAM); N,N',N''-Tris(2,3-dihydroxybenzoyl)-1,3,9-triazadecane (LICAM).
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Table I. Symmetrical 1,3,5-trisubstituted benzene catecholamides (3°).



No.	Alkyl	Acyl	Yd, %	mp, °C	M ⁺ (rel. int)	ν_{CO} (KBr) cm ⁻¹	Spectral Data	
							¹ HNMR δ^a	
							$\text{CH}_3\text{N}<$	$\text{CH}_3\text{CON}<$
4 ~	CH ₃	2,3-dimethoxybenzoyl	75	- 70-5	699(11)	1635	2.72, 2.92 (CCl ₄)	-
9 ~	2,3-dimethoxybenzyl	acetyl	45	oil	741(10)	1650	-	2.10 (CCl ₄)
5 ~	CH ₃	2,3-dihydroxybenzoyl	70	130-40	615(2)	1610	3.38 (TFA)	-
10 ~	2,3-dihydroxybenzyl	acetyl	65	186-9	658(19)	1615	-	2.57, 2.97 (TFA)
6 ~	CH ₃	2,3-dihydroxybenzoyl-sodiumsulfonate	69	290 d	-	1615	2.8, 3.3 (D ₂ O)	-
11 ~	2,3-dihydroxybenzyl-sodiumsulfonate	acetyl	80	260-2 d	-	1615	-	2.20, 2.37 (D ₂ O)

^aBroad/overlapping double resonance due to hindered rotation about the amide bond.

Table II. Physical data for Me₃MECAMS and NAcMECAMS with Fe(III).Me₃MECAMS:

$\log K_{1-3}^{Avg} = 11.8(2)^a$	$\log \beta_{013}^f = 34.5(3)^a$	$\log K_{ML}^H = 6.80(3)^b$
$\log K_4 = 8.52(2)$	$\log \beta_{014} = 43.9(8)^a$	$\log K_{ML}^H = 6.91(3)^c$
$\log K_5 = 7.57(2)$	$\log \beta_{015} = 51.5(8)^a$	$\log K_{MLH}^H = 6.18(3)^c$
$\log K_6 = 6.72(2)$	$\log \beta_{016} = 58.2(8)^a$	$\log K_{MLH_2}^H = 5.50(4)^c$
$\log K^* = 4.87(3)^g$	$\log \beta_{110} = 40.3(8)^a$	$pM = 26.6^d$

Iron removed from horse spleen ferritin after six hours: 3.7%^e

NAcMECAMS:

$\log K_{1-3}^{Avg} = 12.1(2)^a$	$\log \beta_{013} = 36.3(3)^a$	$\log K^* = 4.00(5)^g$
$\log K_4 = 9.3(1)$	$\log \beta_{014} = 45.6(8)^a$	$\log \beta_{110} = 40.3(8)^f$
$\log K_5 = 8.4(1)$	$\log \beta_{015} = 54.0(8)^a$	$\log K_{ML}^{2H} = 14.48(3)^b$
$\log K_6 = 7.7(1)$	$\log \beta_{016} = 61.7(8)^a$	$pM = 25.0^d$

^aBased on estimate for high protonation constant using N,N-dimethyl-2,3-dihydroxy-5-sulfobenzamide.^bDetermined from spectrophotometric titration.^cDetermined from potentiometric titration.^dConditions are: pH 7.4, 10 μ M total ligand, 1 μ M total Fe³⁺.^e[Me₃MECAMS] = 6.0 mM; [ascorbate] = 6.0 mM.^fThe formation constant, β_{mlh} , for the reaction $mM + lL + hH^+ \rightarrow M_lL_h$.

$$^g K^* = \frac{[H^+]^3 [FeL^{6-}]}{[H_3L^{6-}] [Fe^{3+}]}$$

Table III. pM values of selected Fe(III) sequestering agents.

Ligand	pM ^a (-log [Fe ³⁺] _{aq})
Enterobactin	35.5
HBED ^b	31.0
MECAM	29.4
MECAMS	29.1
3,4-LICAMS	28.5
Me ₃ MECAMS	26.6
Ferrioxamine B	26.6
EHPG ^c	26.4
TRIMCAMS	25.1
NACMECAMS	25.0
NTPA ^d	24.7
Transferrin	23.6
EDTA ^e	22.2
Tiron ^f	19.5

^aCalculated for 10 μM ligand, 1 μM Fe³⁺, pH 7.4.

^bN,N-bis(2-hydroxybenzyl)-ethylenediamine-N,N'-diacetic acid.

^cEthylene-1,2-bis(2-hydroxyphenylglycine).

^dDiethylenetriaminepentaacetic acid.

^eEthylenediaminediacetic acid.

^f1,2-Dihydroxy-3,5-disulfobenzene.

Table IV. Kinetic ability of ligands to remove transferrin-bound iron.

Ligand	k_{obs} ($\times 10^3 \text{ min}^{-1}$) ^a	% Fe Removed (30 min)	
		40:1 ^b	1:1
Enterobactin	2.2	-	6
MECAM	3.4	-	13
3,4-LICAMS	2.2	50	6
Me ₃ MECAMS	1.9	-	4
3,4-LICAM-CO ₂ H ^c	2.1	-	6
Desferal ^R	-	5 ^d	-
EDTA	-	37 ^e	-
DMBS	-	36	0

^aLigand concentration 0.2 mM.

^bRatio of ligand to transferrin.

^cSynthesis and characterization to be reported separately, Ref. 20.

^d100:1 ratio.

^e2500:1 ratio.

Figure Captions

Figure 1. a) Enterobactin; b) N,N',N''-trimethyl-tris(2,3-dihydroxy-sulfobenzoyl)-N,N',N''-triaminomethylbenzene [Me₃MECAMS];
c) N,N',N''-triacetyl-tris(2,3-dihydroxysulfobenzoyl)-N,N',N''-triaminomethylbenzene.

Figure 2. Synthesis of symmetrical 1,3,5-trisubstituted benzene tertiary catecholamides.

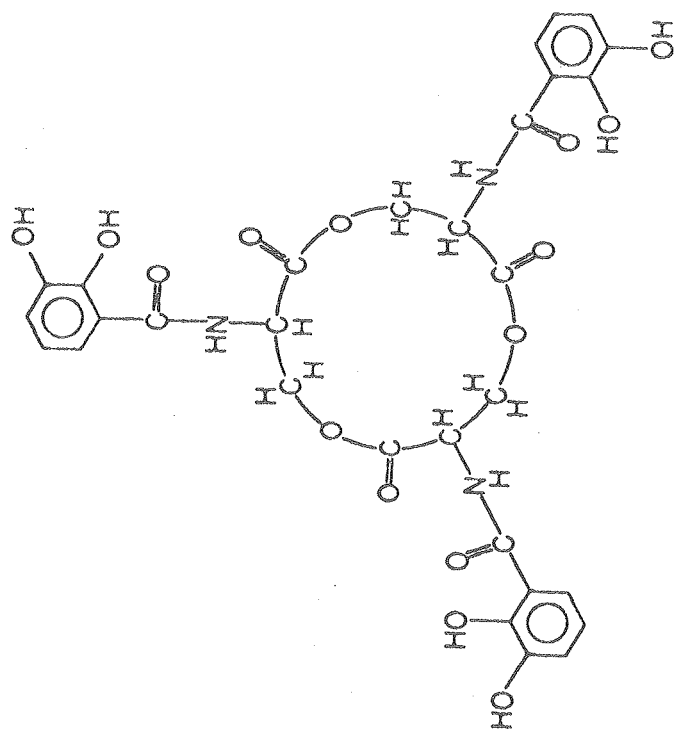
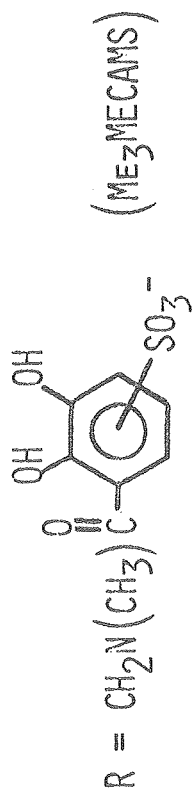
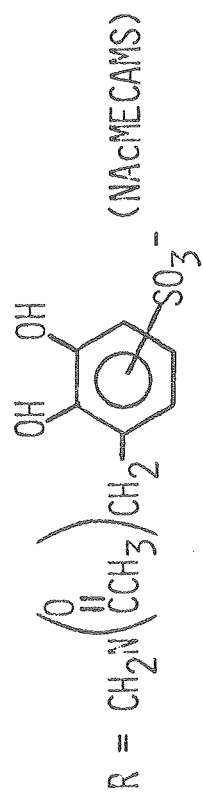
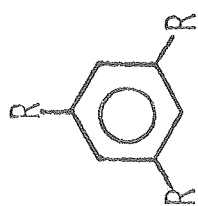
Figure 3. Potentiometric equilibrium curves for Me₃MECAMS. a) 1.5×10^{-3} M Me₃MECAMS; b) Me₃MECAMS + Fe³⁺, 1:1, 1.3×10^{-3} M.

Figure 4. Visible spectrophotometric titration of [FeMe₃MECAMS]⁶⁻ + H⁺ → [HFeMe₃MECAMS]⁵⁻ from pH 6 to 9.5. [FeMe₃MECAMS] = 2×10^{-4} M; $\mu = 0.10$ (KNO₃); t = 25°C.

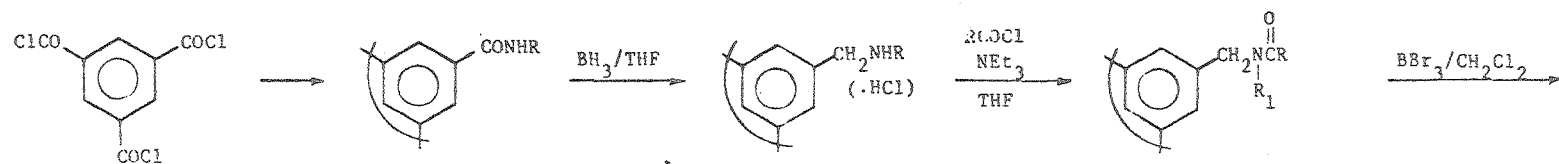
Figure 5. Visible spectrophotometric titration of [FeNacMECAMS]⁶⁻ + 2H⁺ → [H₂FeNacMECAMS]⁴⁻ from pH 7 to 8. [FeNacMECAMS] = 1.9×10^{-4} M; $\mu = 0.10$ (KNO₃); t = 25°C.

Figure 6. Spectral changes associated with exchange of transferrin bound iron with Me₃MECAMS [Me₃MECAMS] = 2.0×10^{-4} M; [Fe₂Tr] = 2.9×10^{-5} M; pH = 7.4; t = 25°C.

Figure 7: Plot in $\ln[(A_t - A_\infty)/(A_0 - A_\infty)]$ vs time for the removal of iron from transferrin. The slope of this line gives the observed rate constant, k_{obs} .



ENTEROBACTIN



1 Trimesoyl
chloride

2 $\text{R} = \text{CH}_3$

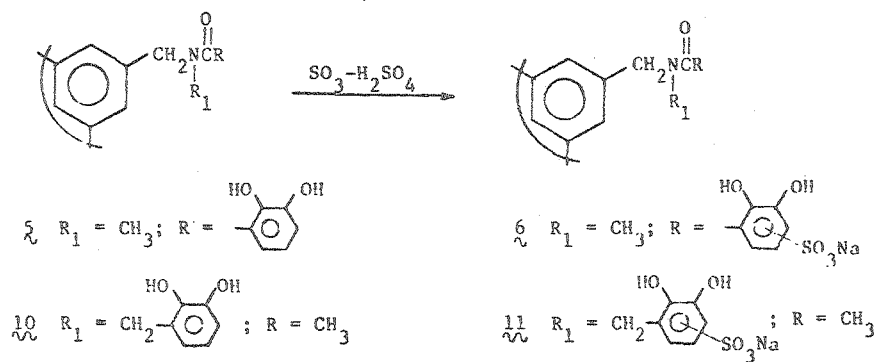
3 $\text{R} = \text{CH}_3$

4 $\text{R}_1 = \text{CH}_3$; $\text{R} =$

7 $\text{R} = \text{CH}_2$ -

8 $\text{R} = \text{CH}_2$ -

9 $\text{R}_1 = \text{CH}_2$ -; $\text{R} = \text{CH}_3$

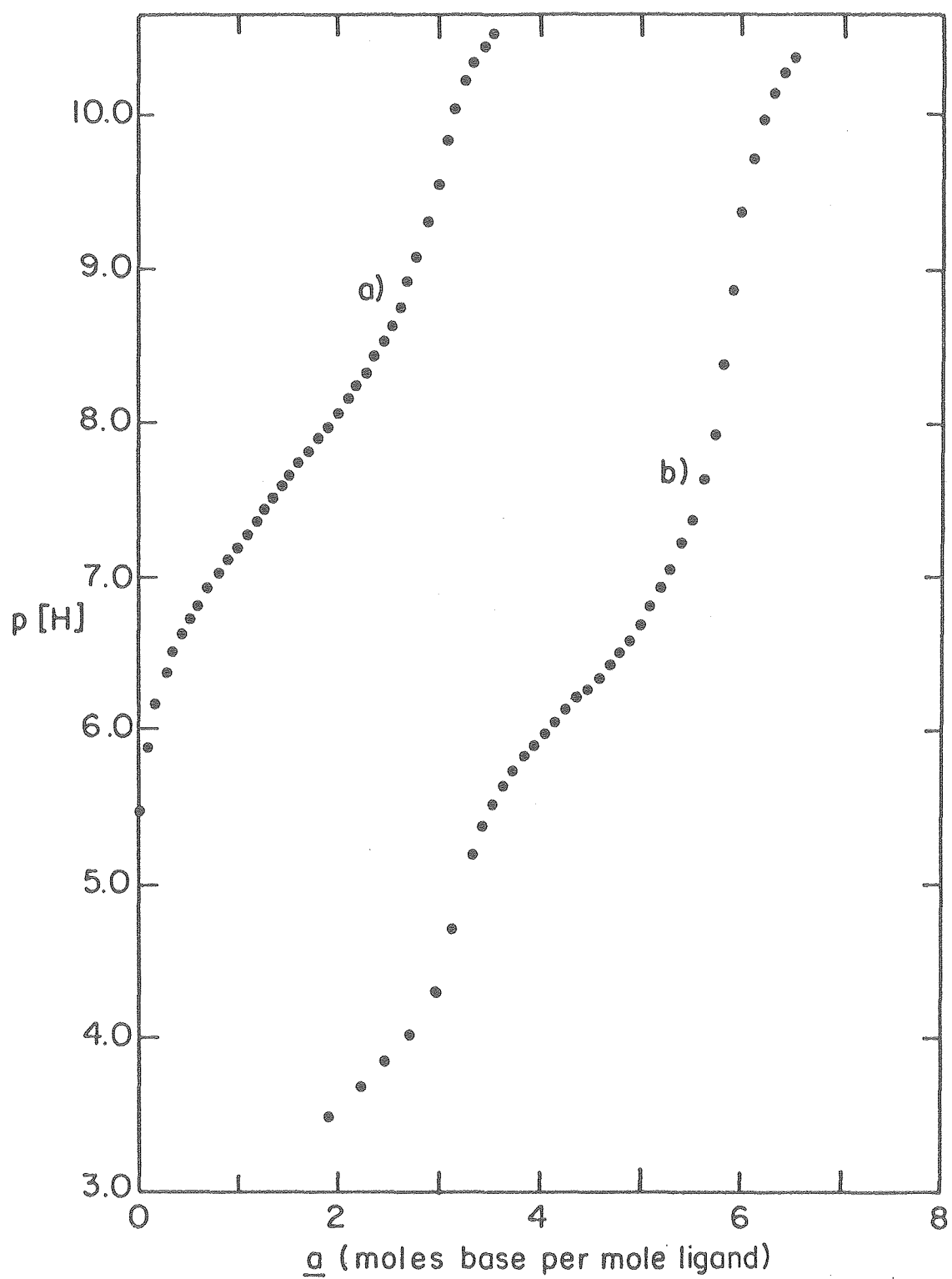


5 $\text{R}_1 = \text{CH}_3$; $\text{R} =$

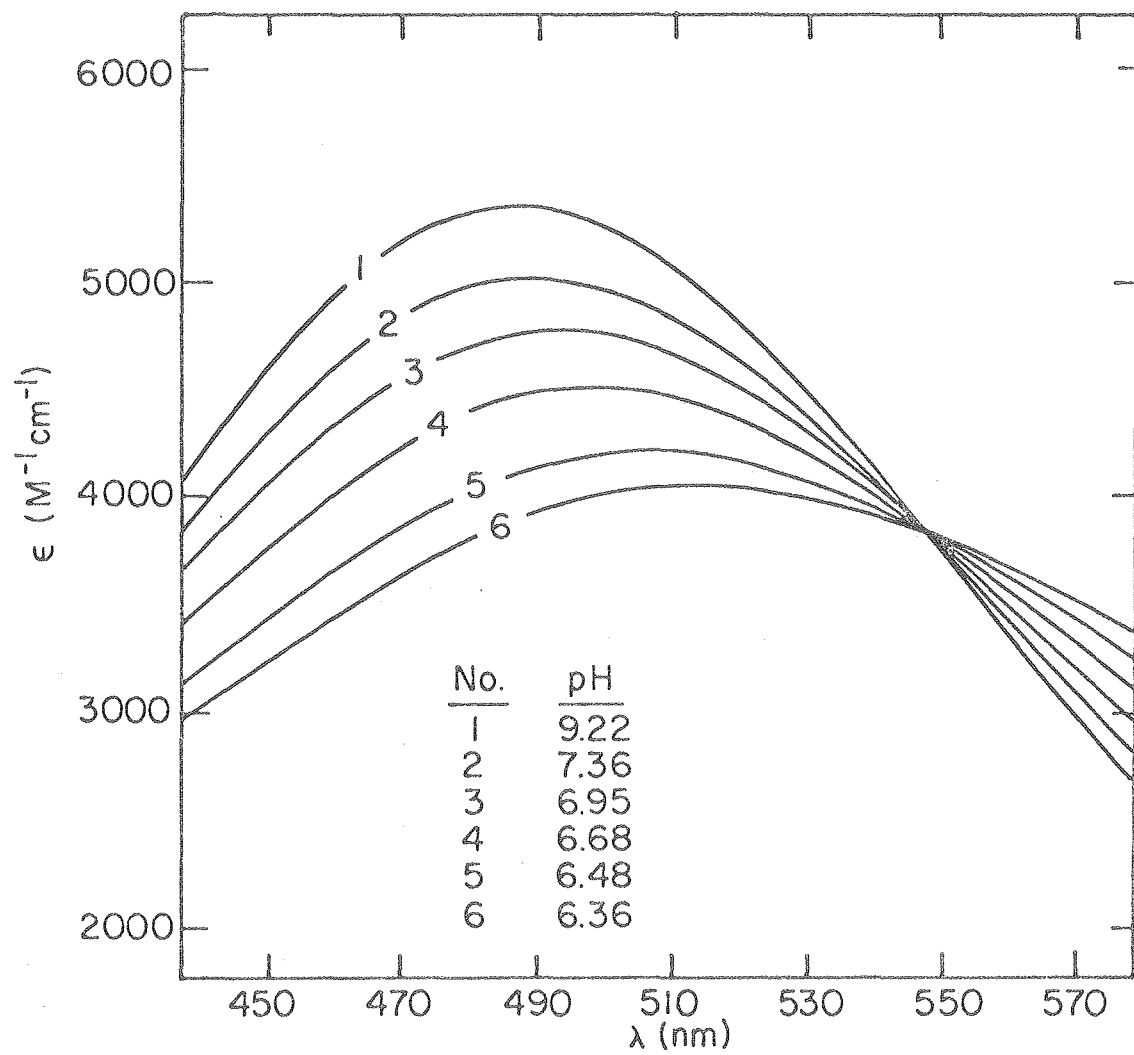
6 $\text{R}_1 = \text{CH}_3$; $\text{R} =$

10 $\text{R}_1 = \text{CH}_2$ -; $\text{R} = \text{CH}_3$

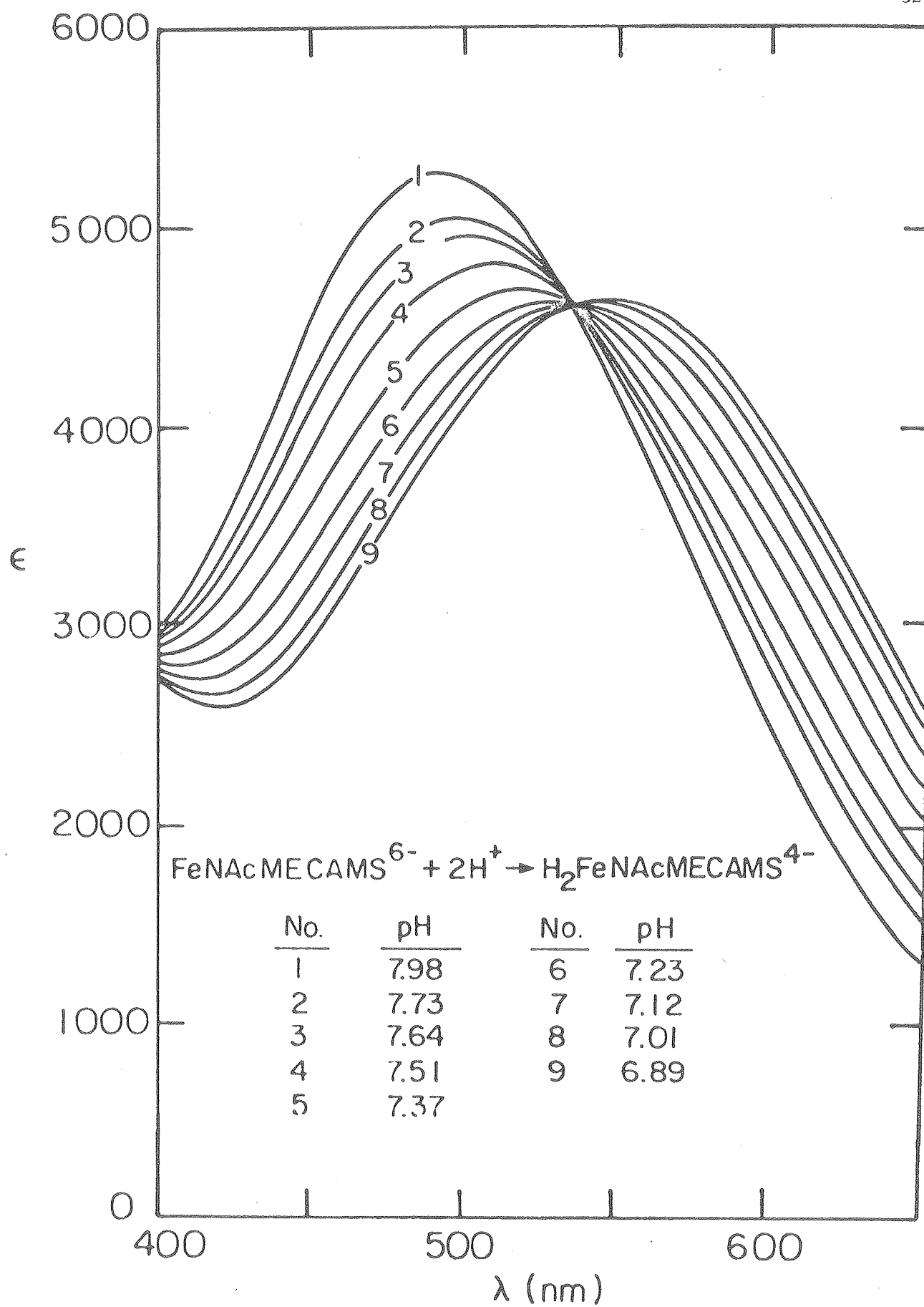
11 $\text{R}_1 = \text{CH}_2$ -; $\text{R} = \text{CH}_3$

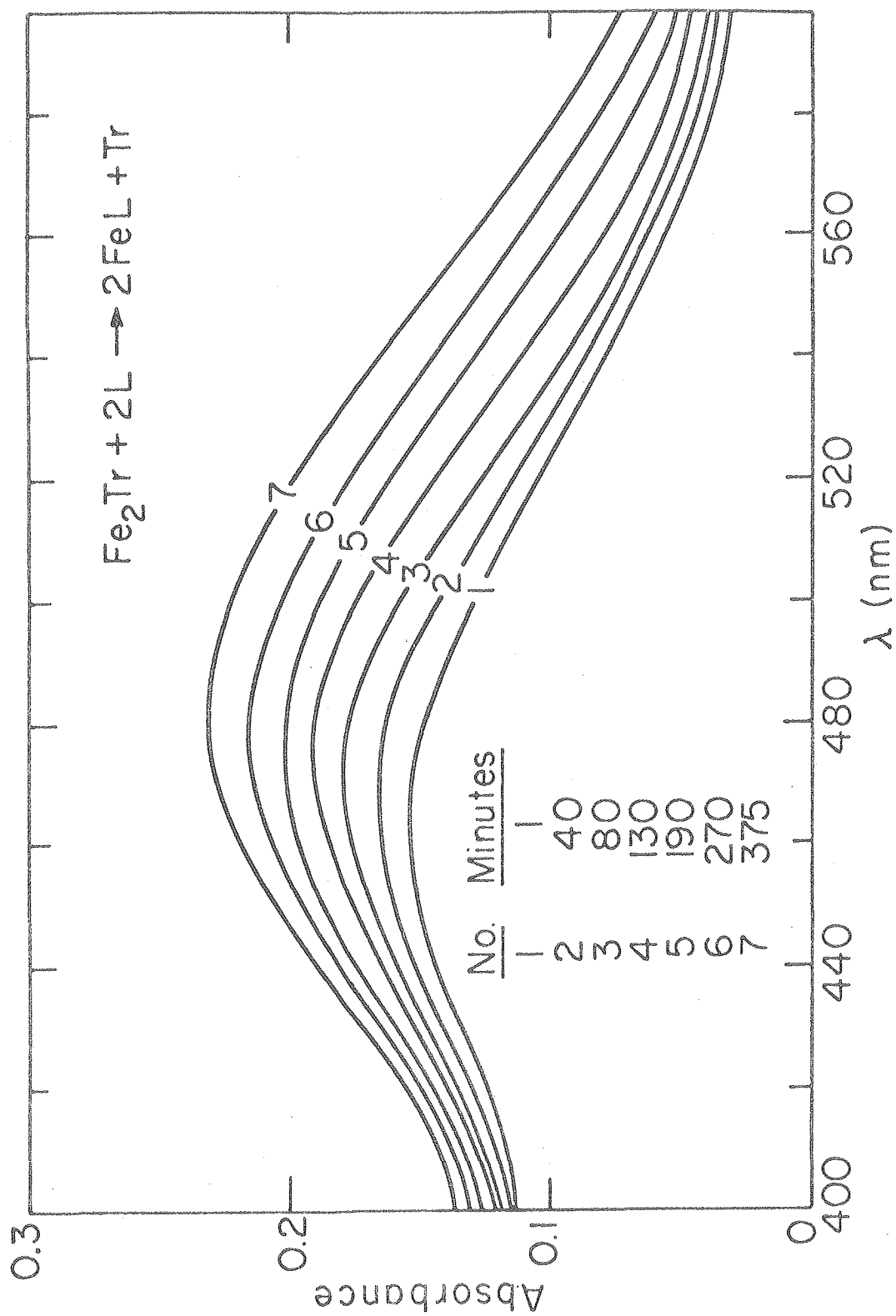


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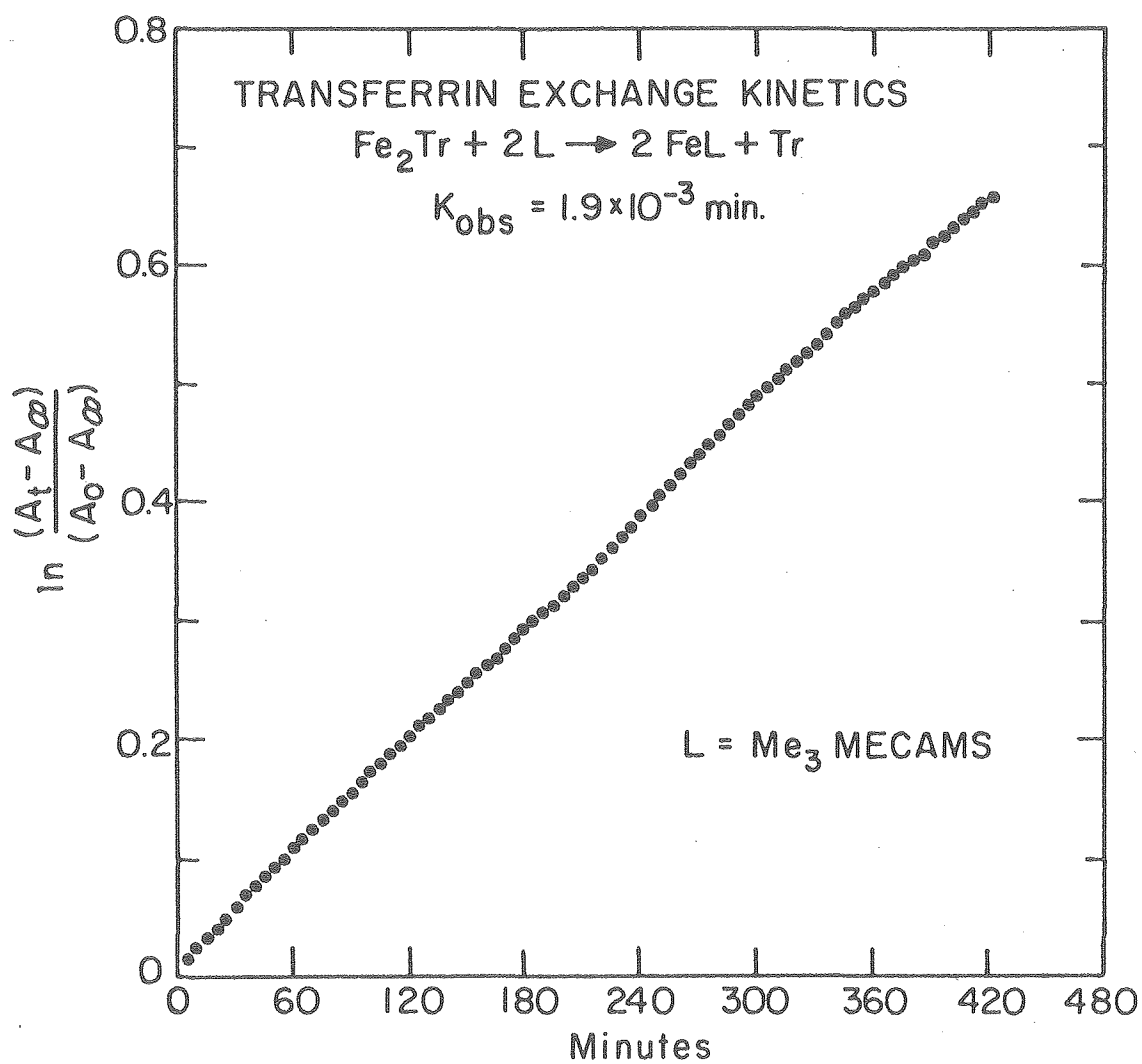


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