grams written by J. Blount; UCLA versions of ORFLS (Busing, Martin, and Levy), structure factor calculations and full-matrix, least-squares refinement; ORTEP (Johnson), figure plotting; ABSN (Coppens), absorption correction; and ORFFE (Busing, Martin, and Levy), distance, angle, and error computations. The equations used in data reduction are the same as given in A. K. Wilkerson, J. B. Chodak, and C. E. Strouse, *J. Am. Chem. Soc.*, **97,** 3000–3004 (1975). All least-squares refinements computed the agreement factors R and R_w according to $R = \Sigma||F_o| - |F_c||/\Sigma|F_o|$ and $R_w = [\Sigma w_i||F_o| - |F_c||^2/\Sigma w_i|F_o|^2]^{1/2}$, where F_o and F_c are the observed and calculated structure factors, respectively, and $w_i^{1/2} = 1/\sigma(F_o)$. The parameter minimized in all least-squares refinements was $\Sigma w_i||F_o| - |F_c||^2$. All calculations were performed on the IBM 360-91KK computer operated by the UCLA Campus Computing Network.

(16) The scattering factors for the nonhydrogen neutral atoms were obtained from H. P. Hanson, F. Herman, J. D. Lea, and S. Skillman, *Acta Crystallogr.*, **17,** 1040–1044 (1964). The hydrogen scattering factors were from R. F. Stewart, E. R. Davidson, and W. T. Simpson, *J. Chem. Phys.*, **42,** 3175–3187 (1965). The anomalous dispersion corrections for iron were obtained from D. T. Cromer and D. Liberman, *J. Chem. Phys.*, **53,** 1891–1898 (1970).

(17) See paragraph at the end of the paper concerning supplementary material.

(18) C. E. Strouse, *Rev. Sci. Instrum.*, **47,** 871–876 (1976).

(19) The scattering factors for the nonhydrogen neutral atoms were obtained from "International Tables for X-Ray Crystallography", Vol. IV, Kynoch Press, Birmingham, England, 1974, p 72 ff. The hydrogen scattering factors and anomalous dispersion corrections were obtained from the corresponding sources for these parameters given in ref 16.

(20) J. P. Glusker and H. L. Carrell, *J. Mol. Struct.*, **15,** 151–159 (1973).

(21) C. F. Timberlake, *J. Chem. Soc.*, 5078–5085 (1964).

(22) K. Nakamoto, M. Margoshes, and R. E. Rundle, *J. Am. Chem. Soc.*, **77,** 6480–6486 (1955).

^{13}C NMR Studies of Ferrous Citrates in Acidic and Alkaline Solutions. Implications Concerning the Active Site of Aconitase[1]

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Abstract: ^{13}C NMR studies of the paramagnetic 1:1 Fe(II):citrate system in solution were carried out as a function of pH, temperature, and concentration, with the use of natural abundance and [1,2,4,5-^{13}C]citric acid prepared from ethyl bromo[1,2-^{13}C]acetate and ethyl acetoacetate. The temperature and concentration dependence of the large shifts in the positions of the ^{13}C resonances indicated that in acidic samples, chemical exchange is fast on the NMR time scale. The temperature dependence of the spectra of alkaline samples revealed an equilibrium among several forms of the complex which are not exchanging on the NMR time scale. At temperatures near the freezing point of the solution, a single form predominates in which the two halves of the citrate ion are inequivalent. Molecular weight determinations in solutions of high ionic strength indicated that both Fe(II) and Ni(II) complexes of tetraionized citrate are tetrameric, Fe(II)-triionized citrate is monomeric, and Ni(II)-triionized citrate is primarily monomeric with evidence of a small degree of association. The Ni(II) complex of tetraionized citrate is dimeric in solutions of very low ionic strength. The magnetic susceptibilities of Ni(II) complexes of triionized and tetraionized citrate and Fe(II) complexes of tetraionized citrate in solution were measured by an NMR method. No significant magnetic coupling was observed for any of these complexes. A complex of S_4 symmetry based on a tetrahedral cluster of nonbonded Fe(II) ions is proposed as the predominant form of Fe(II)-citrate in cold alkaline solution. In this complex, each face of the tetrahedron is capped with a triply-bridging citrate hydroxyl oxygen atom. The four citrate ions of the cluster have configurations and conformations similar to those observed in a previous crystallographic characterization of a Ni(II) complex of tetraionized citrate. The observation of a metal bridged configuration of the ionized citrate hydroxyl group in the complexes of tetraionized citrate ions with Ni(II) ions and Fe(II) ions characterized by x-ray crystallography and ^{13}C NMR, respectively, suggests that this configuration may be present in most stable transition metal complexes of tetraionized citrate. The implications of these results concerning the nature of the iron citrate complex at the active site of aconitase are discussed.

The Krebs cycle enzyme aconitase catalyzes the interconversions of citrate, *cis*-aconitate, and 2*R*,3*S*-(+)-isocitrate. The presence of Fe(II) and a reducing agent is required for activation of the catalytic function of the enzyme. Although many other metal ions have been tested, only Fe(II) (in the presence of a reducing agent) produces significant activation of the enzyme.[2] A ternary enzyme–Fe(II)-citrate complex at the active site of aconitase has been detected by ^1H NMR T_1 measurements.[3,4]

The p*K* of the citrate hydroxyl group is substantially lowered when the citrate ion is coordinated to a transition metal ion.[5–8] When citrate ions are coordinated to Fe(II) ions, the hydroxyl groups are predominantly ionized in neutral or alkaline aqueous solution. X-ray structural studies of an Fe(II)-citrate complex crystallized from acidic solution and a Ni(II)-citrate complex crystallized from alkaline solution revealed that the mode of coordination of the citrate ion to transition metal ions is dependent upon the state of ionization of the citrate hydroxyl group.[9] To examine this phenomenon in solution, a ^{13}C NMR investigation of Fe(II)-citrate complexes in acidic and alkaline solution was undertaken. Since the pH optimum for the enzymatic activity of aconitase from a variety of sources in several different buffers lies in the alkaline range,[2] data obtained from solutions of alkaline ferrous citrate complexes are of special interest as models for the coordination of citrate to the active site of aconitase. ^{13}C NMR of ferrous citrate complexes was utilized because the contact shifts of the citrate resonances are extremely sensitive to the configurations and conformations of the citrate ions and because of the greater number and variety of carbon atoms than protons in citrate. Additionally, the paramagnetic NMR technique is potentially very useful for similar investigations of the configuration and conformation of the citrate ion in the ternary aconitase–Fe(II)-citrate complex. Paramagnetic NMR is particularly valuable in enzyme studies because the resonances of atoms which interact with the metal site are shifted away from the complicated envelope of resonances which arises from the rest of the macromolecule. A synthesis of ^{13}C enriched citric acid was undertaken to enhance the sensitivity of the ^{13}C paramagnetic NMR measurements and to aid in the assignment of the observed resonances. A similar study of the interaction of ferrous ion activated aconitase with its substrates would undoubtedly require the use of highly ^{13}C enriched substrates for observation of the spectra.

Experimental Section

Titrations. Solutions of equimolar quantities of citric acid and Fe(II) or Ni(II) ions were titrated with standardized sodium hydroxide solution, and the pH was measured with a Sargent-Welch Micro Combination pH electrode connected to a Leeds and Northrup pH meter. Solutions which contained Fe(II) ions were continuously purged with nitrogen gas and were electrolyzed for several hours prior to the titration to reduce residual Fe(III) to Fe(II) in an apparatus similar to that used for preparation of samples for NMR measurements (vide infra).

Synthesis. A three-step synthesis[10] has been developed for the incorporation of ^{13}C into four of the six carbon positions of citric acid. Ethyl bromoacetate with 90% ^{13}C at both acetate carbon positions (prepared by Dr. D. G. Ott, Los Alamos Scientific Laboratory) was the starting material used for the incorporation of the isotope. The sequence of reactions involved the condensation of 2 mol of ethyl bromoacetate with 1 mol of ethyl acetoacetate, followed by the conversion of the methyl ketone to an acetate group by means of a Baeyer-Villiger oxidation reaction. The esters thus produced were hydrolyzed essentially quantitatively to citric acid (see Figure 1).

Triethyl β-Acetyl[1,2,4,5-^{13}C]tricarballylate (I). The disubstitution of ethyl acetoacetate to produce triethyl β-acetyltricarballylate (I) (step 1) was accomplished by the use of sodium hydride as the base according to the method of Sandberg,[11] with the notable exception that a stoichiometric quantity of sodium hydride (50% in oil; Research Organic/Inorganic Chemical Corp.) was used. The presence of excess sodium hydride in the disubstitution reaction of ethyl acetoacetate was found to favor the deacylation and to significantly lower the yield of I. The yields of several reactions of natural abundance starting materials according to this procedure ranged from 61-64% of the theoretical yield of I [bp 105 °C (0.15 mm)]. ^{1}H NMR spectra were consistent with the assignment of the structure of the product to I.

Triethyl [1,2,4,5-^{13}C]Citrate Acetate (II) and Triethyl [1,2,4,5-^{13}C]Citrate (III). The reaction conditions for Baeyer-Villiger oxidation of ketones to esters vary widely from one reaction to another.[12] Disubstituted ethyl acetoacetate was previously reported to be resistant to oxidation with peracetic acid.[13] However, it was found in the course of this investigation that I was oxidized by trifluoroperacetic acid to produce triethyl citrate acetate (II) with some hydrolysis to triethyl citrate (III) (step 2) by an adaptation of the method used by Emmons and Lucas[14] to oxidize ketones with single carbonyl groups to esters.

A clean, unetched 250-ml three-neck flask was pretreated according to the following procedure. A solution of approximately 0.1 M hydrochloric acid was boiled in the flask for 1 h. The flask was rinsed with water, then rinsed with approximately 0.1 M sodium hydroxide solution, and then rinsed again with water. The dried flask was washed with a 0.01% solution of dithizone in chloroform. Finally, the flask was heated in a flame to further condition the surface.

A 25-ml portion of dichloromethane was placed in the pretreated flask fitted with a reflux condenser and an addition funnel and cooled in an ice bath. Hydrogen peroxide, 90%[15] (1.42 ml, 0.0520 mol), was carefully added to the cooled solvent. Through the addition funnel, 10.5 ml of trifluoroacetic anhydride (0.0745 mol) was added dropwise while the mixture was stirred. After addition was complete, the ice bath was removed and the reaction mixture was allowed to warm to room temperature. A solution of 5.25 g of 90% ^{13}C enriched I (0.0172 mol) in 25 ml of dichloromethane was added dropwise through the addition funnel. The reaction mixture was then refluxed for 24 h. After the mixture had cooled, a 60-ml portion of a 10% solution of sodium carbonate was added slowly. After separation of the two layers, the dichloromethane layer was washed three times with water, and the combined water layers and carbonate wash were extracted three times with dichloromethane. The combined dichloromethane solutions were dried over magnesium sulfate. The dichloromethane was removed on a rotary evaporator, and a proton NMR spectrum was obtained to determine the relative quantities of I, II, and III. The reaction products from a sample of natural abundance starting material were identified by comparison of their ^{1}H NMR spectra with those of triethyl citrate acetate (II) obtained from K & K Laboratories, Inc., and triethyl citrate (III) obtained from Matheson Coleman and Bell.

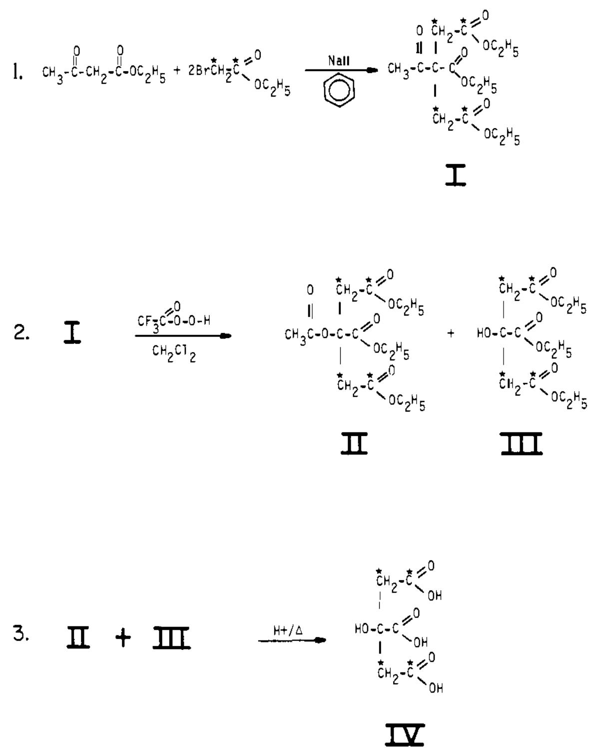

Figure 1. Reaction sequence for the synthesis of [1,2,4,5-^{13}C]citric acid.

Upon completion of the above experimental procedure, the reaction mixture obtained was frequently discolored. The yellow color was removed by addition of silica gel to a dichloromethane solution of the mixture. The silica gel was filtered and washed with three aliquots of dichloromethane. The filtrate was treated with charcoal for 3-4 h. The charcoal was removed by filtration, and the dichloromethane was then removed on a rotary evaporator. This mixture was used for another Baeyer-Villiger reaction. After four cycles, the overall yield of the mixture of II and III obtained from a natural abundance sample of I was 73% of the theoretical yield.

The yield of the reaction was strongly dependent upon the surface of the reaction vessel. The presence of the strong, fluorinated acid visibly etched a Pyrex flask, and it was observed that the fraction of the ketone that was oxidized to the ester was significantly reduced when the reaction was carried out in a flask that had been used two to three times for the same reaction. The same phenomenon was observed with the use of a Teflon reaction vessel. Likewise, the presence of impurities in the reagents affected the yield of the reaction. Although the conditions required for the oxidation of I to II and III were well defined prior to the use of isotopically labeled materials, the use of ^{13}C enriched I as the starting material resulted in almost no reaction. Pretreatment of this material with silica gel and then charcoal allowed the reaction to proceed with a yield similar to that obtained with natural abundance starting material. The phenomenon was presumed to result from the presence (or absence) of trace impurities in the ^{13}C labeled ethyl bromoacetate.

[1,2,4,5-^{13}C]Citric Acid. A 2.9-g sample of a mixture which contained approximately 67% III, 25% II, and 8% I, as determined by NMR, was hydrolyzed according to the method of Rothchild and Fields.[16] Recrystallization of the solid residue was carried out by addition of 20 ml of acetone and 15 ml of benzene. The solution was gently heated until about half of the acetone had evaporated and an oil had separated. The mixture was allowed to stand overnight, and 0.4631 g of white, crystalline [1,2,4,5-^{13}C]citric acid was obtained, which accounts for less than 25% of the starting material of the hydrolysis. Separation of the remaining citric acid from the residual oil will undoubtedly significantly improve the overall yield.

A proton decoupled ^{13}C NMR spectrum of the ^{13}C labeled citric acid is given in Figure 2a. A ^{13}C NMR spectrum of natural abundance citric acid is given in Figure 2b for comparison. The presence of a small amount of β-acetyl[1,2,4,5-^{13}C]tricarballylic acid (the hydrolysis

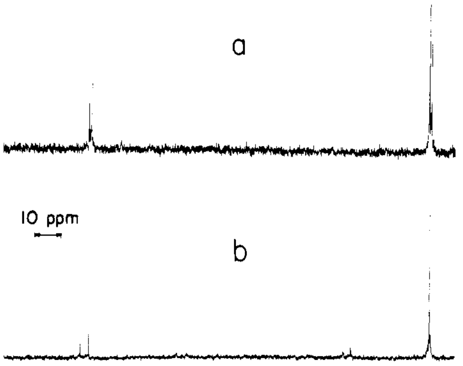

Figure 2. Proton decoupled Fourier transform ^{13}C NMR spectra of citric acid. These and all subsequent ^{13}C NMR spectra were obtained with the use of a superconducting solenoid NMR spectrometer operated at 63.1 MHz. (a) A solution of crystalline [1,2,4,5-^{13}C]citric acid in H_2O. The spectrum was obtained from a single transient. (b) Natural abundance citric acid in H_2O. The spectrum was obtained from 400 transients (1.0-s pulse delay).

product of I) as an impurity in the crystalline sample of citric acid was evident from the small peaks located upfield from the methylene multiplet of [1,2,4,5-^{13}C]citric acid in Figure 2a. This impurity was used as an internal reference in the ^{13}C NMR spectra of ferrous citrate complexes (vide infra). The ^{13}C–^{13}C coupling constant of the adjacent methylene and carboxyl carbon atoms of citric acid was 52 Hz. A long range ^{13}C–^{13}C coupling of about 3-4 Hz was also resolved.

^{13}C NMR Measurements. Sample Preparation. Natural abundance samples were prepared in a glovebag under an atmosphere of nitrogen by the addition of equimolar quantities of solid ferrous chloride and citric acid to water which had been purged with nitrogen gas. The concentration of all natural abundance samples was 1.0 M. The pH of each solution, measured with a Leeds and Northrup pH meter, was adjusted by the addition of concentrated hydrochloric acid or concentrated sodium hydroxide solution which had been purged with nitrogen gas. A small quantity of sodium dithionite, added to some of the samples immediately prior to the NMR measurements, was found not to affect the positions of the resonances. During the development of sample handling techniques, an apparatus was constructed for recrystallization of the ferrous chloride, preparation and titration of the ferrous citrate solution, and measurement of the pH in an inert atmosphere. The sample was drawn off into an NMR tube while argon gas was passed over the top of the tube. A sample of pH 9.5 was obtained by this method, and the NMR tube was capped with an O-ring sealed cap.

A sample of ^{13}C labeled ferrous citrate was prepared by the addition of 1.8 mmol of crystalline [1,2,4,5-^{13}C]citric acid (90% enrichment) and 1.8 mmol of solid ferrous chloride to 4.0 ml of water which had been thoroughly purged with nitrogen gas in an especially constructed apparatus (see Figure 3). The solution was electrolyzed overnight to assure complete reduction of residual Fe(III) to Fe(II). Nitrogen gas was bubbled first through a 10 M sodium hydroxide solution, then through a 9.778 M sodium hydroxide solution which was transferred to the calibrated addition funnel for the titration. Wet nitrogen gas was bubbled into the apparatus through inlets a and b. The outlets, c, d, and e, were connected with Tygon tubing to each other to equalize the pressure in both sides of the electrolysis cell and inside the pH probe, and the junction of the three was connected to a bubbler to prevent diffusion of oxygen back into the apparatus. A side arm with an O-ring sealed Teflon coupling to a 10-mm NMR tube was constructed at an angle such that rotation of the entire apparatus would result in the transfer of the sample into the NMR tube. The apparatus was used inside a glovebag filled with argon gas. The NMR tube was removed from the apparatus and capped immediately with an O-ring sealed Nylon cap, while argon gas was blown directly over the top of the tube. After the spectra of the sample at a particular pH were obtained, the sample was returned to the electrolysis apparatus which had been capped and kept under nitrogen while empty.

^{13}C NMR Spectra. Spectra were obtained on a superconducting solenoid NMR spectrometer which operates at a ^{13}C frequency of 63.1 MHz and a ^{1}H frequency of 251 MHz.[17] All spectra of natural abundance ferrous citrate samples were obtained in the Fourier transform mode with a spectral width of 14 kHz and with 512 data points. Each spectrum was obtained from the sum of 10 000-32 000 transients. The transients were each obtained from a single pulse with no time delay following data acquisition. All but the spectra of the pH 9.50 sample were obtained at the ambient temperature of the probe with the proton decoupler in operation. The spectra of the alkaline natural abundance sample were obtained with no decoupling power at a temperature of 2 °C.

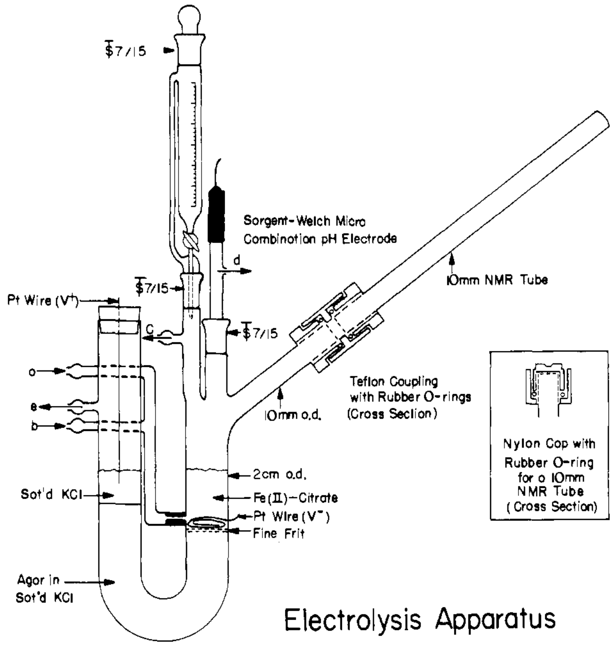

Figure 3. Electrolysis apparatus for the preparation of ^{13}C labeled ferrous citrate for NMR measurements.

The spectra of the ^{13}C labeled ferrous citrate complexes in solutions of pH less than 7 were obtained in the Fourier transform mode with the same spectral parameters as used for the natural abundance samples, except that each spectrum was obtained from 1500-2000 transients. Proton decoupling power was used but the temperature was maintained at 2 or 30 °C.

Spectra of ^{13}C labeled ferrous citrate at pH values of 7.60, 8.15, and 9.25 were obtained in the frequency sweep mode. A spectral width of 82 kHz was used with a receiver–transmitter time sharing frequency of 60 kHz. Each spectrum was signal averaged for 10-15 h, during which 2000-3000 scans were summed at a rate of 15 s per scan. No decoupling power was applied to the nonspinning sample.

Because of the large spectral width and relatively high radio frequency power employed, substantial baseline corrections were required. Functions of the form $A \cos n\Theta$ or $A \sin n\Theta$, where A is an arbitrary constant, $n = 0$, 1, and 2, $\Theta = 0$ at the beginning of the spectrum, and $\Theta = \pi$ at the end of the spectrum, were added to the uncorrected spectra until the baselines appeared reasonably flat. In order to adequately filter the data, the Fourier transform of the averaged data was obtained, a broadening function of 200 Hz was applied to the data in the time domain, the imaginary part of the transform was discarded, and the Fourier transform of the real part was carried out to give the final spectrum.

Molecular Weight Determinations. The degree of association of Fe(II) and Ni(II) citrate complexes in aqueous solution was determined from the depression of the transition temperature of anhydrous sodium sulfate to sodium sulfate decahydrate[18] which arises from the presence of ions other than sodium or sulfate. The technique of Lefebvre[19] was modified such that oxygen-free nitrogen gas was bubbled first through a solution of composition and temperature identical with the test solution, then through the test solution, to prevent oxidation of the ferrous citrate complexes while the concentration of the test solution was kept constant. The test solution (75 ml) was heated to 34 °C and anhydrous sodium sulfate (50 g) was added. The mixture was cooled slowly while it was stirred with a magnetic stirring apparatus. The maintenance of sufficient stirring was found to be essential to the achievement of thermal equilibrium and thus a constant temperature at the transition point.

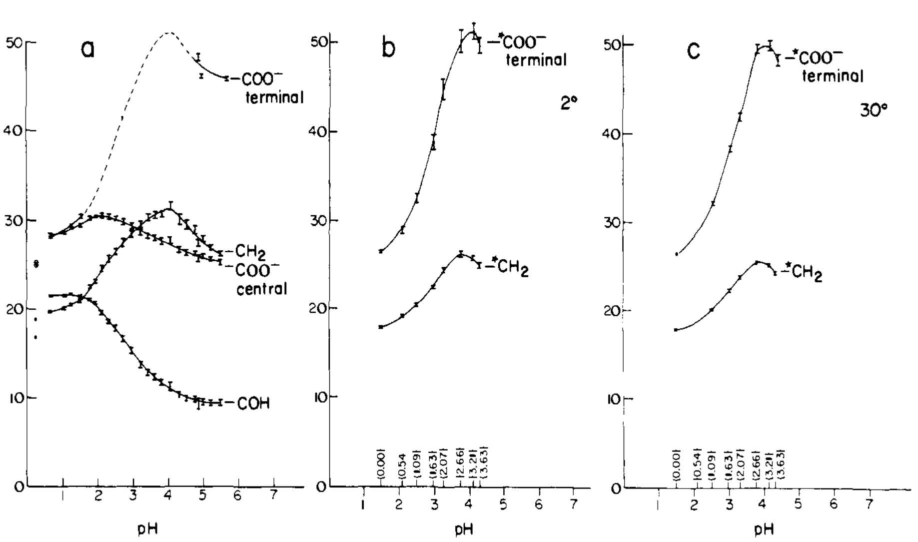

Figure 4. ^{13}C NMR resonance frequencies of solutions of ferrous citrate. The error bars represent the line widths at half-height. (a) 1 M solutions of natural abundance ferrous citrate. The circled data points are the resonance positions of diamagnetic citric acid. (b) 0.45 M solutions of ferrous complexes of [1,2,4,5-^{13}C]citrate at 2 °C. The numbers in parentheses are the equivalents of base added. (c) Same as b except the data were obtained at 30 °C.

The freezing point depression of water which contained Ni(II)-citrate prepared from equimolar quantities of $Ni(OH)_2$ and disodium citrate was measured as a function of solute concentration. No attempt was made to exclude oxygen from these samples. Theoretical curves were calculated from the following equation based on Debye-Huckel theory:[20]

$$\Delta T = -0.059|z^+z^-|\left(\sum_i \nu_i z_i^2\right)^{1/2}\nu K_f m^{3/2} + \nu K_f m$$

where ΔT is the freezing point depression, z^+ and z^- are the charges on the positive and negative ions, respectively, ν is the number of moles of ions per formula weight of the electrolyte ($\nu = \Sigma_i \nu_i$), K_f is the molal freezing point depression of water, and m is the molal concentration of the solute.

The freezing point depression of water to which equimolar quantities of Ni(II) sulfate, trisodium citrate, and sodium hydroxide had been added was measured as a function of the concentration of added electrolyte. The technique was the same as used for the measurement of the transition temperature of sodium sulfate except anhydrous sodium sulfate was not added, and the entire apparatus was placed inside a plastic container which was immersed in an ice-salt bath. The solution was not stirred since, in the absence of solid sodium sulfate, the nitrogen bubbling provided sufficient mixing. Theoretical curves were calculated as for the samples of low ionic strength, with the inclusion of an additive term for the Na_2SO_4 present in these samples.

Magnetic Susceptibility Measurements. The magnetic susceptibilities of Fe(II) and Ni(II) complexes of triionized and tetraionized citrate in solution were measured by an NMR method[21] over the temperature range of 3–71 °C. Equimolar quantities of solid trisodium citrate and Ni(II) sulfate were dissolved in a solution of 2% (v/v) *tert*-butyl alcohol in water to produce a solution 0.100 M in Ni(II) ions. A portion of this solution was placed in an NMR tube with a coaxial capillary which contained a solution of identical composition except for the substitution of magnesium sulfate for Ni(II) sulfate. A sample of Ni(II)-tetraionized citrate was prepared as above except 1 equiv of sodium hydroxide was added. The concentration of the *tert*-butyl alcohol solution used to prepare the sample was such that the final concentration of *tert*-butyl alcohol after the addition of sodium hydroxide solution was 2%.

Solutions of Fe(II)-tetraionized citrate were prepared as above except that they were electrolyzed in a closed apparatus under nitrogen to convert any residual Fe(III) to Fe(II). The electrolysis apparatus was placed inside a glovebag which contained a nitrogen atmosphere, and the solutions were transferred to NMR tubes. Caps were sealed to the NMR tubes with epoxy glue. Three identically prepared samples of this air-sensitive complex were made. Samples of $NiCl_2$ and $FeSO_4$ were also prepared by similar methods.

Proton NMR spectra of these metal citrate solutions were obtained with a Varian A-60 NMR spectrometer equipped with a variable temperature accessory. Sample temperatures were determined by measurement of the temperature of ethylene glycol or methanol calibration samples before and after the susceptibility determinations.

Results

Titrations. The titration data revealed a fourth acid dissociation of Fe(II) and Ni(II) citrates as had been reported previously.[6,7] In solutions of Fe(II) citrate, which contain very little residual Fe(III), a distinct, reproducible color change occurs after the addition of 3 equiv of base. The very pale blue solutions take on a light straw color as the fourth equivalent is titrated.

^{13}C NMR Measurements of Ferrous Citrate Complexes in Acidic Solution. A titration curve of the ^{13}C NMR resonance positions of 1 M ferrous citrate (natural abundance) over an acidic pH range is presented in Figure 4a. The error bars in Figures 4 and 5 represent the line widths at half-height. The circled data points are the resonance positions of diamagnetic citric acid. The difference between the resonance positions of the diamagnetic sample vs. the paramagnetic sample at the lowest pH results primarily from the difference in the bulk magnetic susceptibility of the two samples. As the pH of the solution is increased, the formation of ferrous citrate complexes is favored and the citrate resonances experience large shifts. The smooth curves of resonance positions vs. pH permit the tentative assignment of the resonances throughout the pH range employed. At pH 0.65, the resonances, in order of increasing frequency, are assigned to the methylene carbon atoms, the central carbon atom to which the hydroxyl group is bonded, the terminal carboxyl carbon atoms, and the central carboxyl carbon atom. Corresponding curves for a 0.45 M solution of ferrous complexes of [1,2,4,5-^{13}C]citrate at 2 and 30 °C are given in Figure 4b,c. The absence of the resonances previously assigned to the central carbon atom and the central carboxyl carbon atom confirms these assignments. The substantially greater line widths of the resonances of the ^{13}C labeled ferrous citrate spectra obtained at 2 °C, with respect to those obtained from this sample at 30 °C, are indicative of rapid chemical exchange. Thus, the presence of only two resonances for the tetra-labeled citrate ion probably results from chemical exchange rather than from the formation of a symmetric complex. The large line widths of the terminal carboxyl resonances in the pH range of 1.5 to 4.5 obscured this signal in the natural abundance spectra. The dotted line in Figure 4a

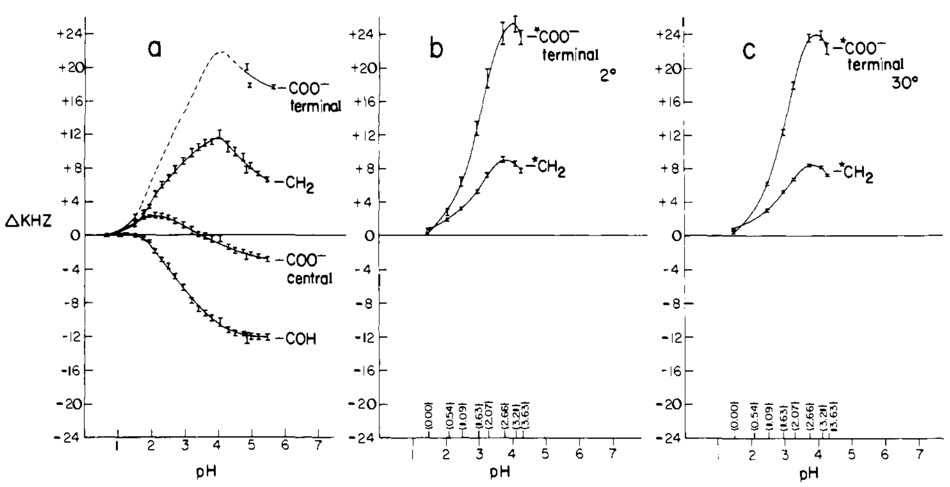

Figure 5. ^{13}C NMR shifts of the carbon atoms of ferrous citrate. The error bars represent the line widths at half-height. (a) These data were obtained from 1 M natural abundance samples, and the references used to determine the shifts were the corresponding resonances at pH 0.65. (b) These data were obtained from 0.45 M solutions of the ^{13}C labeled samples at 2 °C, and the references used to determine the shifts were the corresponding resonances at pH 1.5 of the impurity that was present in the sample. The numbers in parentheses are the equivalents of base added. (c) Same as b except the data were obtained at 30 °C.

was included as an estimate of the resonance positions of these carbon atoms based on a comparison of the corresponding curves obtained with the ^{13}C labeled sample and interpolation of the few resonance frequencies observed for these carbon atoms.

Because of the difference in the concentrations of the natural abundance samples and the ^{13}C labeled sample, the bulk magnetic susceptibilities of the two were significantly different. Thus, an internal reference for each must be used to compare the observed shifts. The difference between the resonance frequency of a given carbon atom at a particular pH value and its resonance frequency at the lowest pH measured was determined for the natural abundance samples, and the resultant shifts are plotted for each carbon atom as a function of pH in Figure 5a. The most acidic solution which was used to obtain spectra of the ^{13}C labeled sample was pH 1.5. Figure 5a indicates that the methylene and terminal carboxyl carbon atoms have been measurably shifted at pH 1.5 and thus would not function as good reference resonance positions. The sample of ^{13}C labeled citric acid contained a small quantity of β-acetyl[1,2,4,5-^{13}C]tricarballylic acid as an impurity. The resonance positions of this compound in the ferrous citrate solutions were virtually invariant with pH and temperature. Since the impurity appears to participate very little in complex formation, the positions of its resonances in the pH 1.5 sample were used as the references. The differences between the methylene and terminal carboxyl carbon resonance positions of ferrous citrate and the corresponding reference resonance positions are plotted as a function of pH for the spectra of the ^{13}C labeled sample at 2 and 30 °C in Figure 5b,c.

The titration curves for the ^{13}C labeled sample were obtained with a quantitative addition of a standardized NaOH solution. The number of equivalents of base which had been added during the preparation of the samples for NMR measurements is given in parentheses above the abscissa of the graphs in Figures 4b,c and 5b,c. The maxima in the curves occurred approximately at the point at which 3 equiv of hydroxide had been added. The decrease in the shift with the addition of more base is attributed to the decrease in the concentration of the Fe(II)-citrate(−3) complex which accompanies the formation of the more stable Fe(II)-citrate(−4) complex. Similarly, the difference in the height of the maximum of the curve for the methylene carbon atoms of the ^{13}C labeled sample vs. the natural abundance samples is attributed to the difference in the concentrations of these samples. The lower concentration of the ^{13}C labeled sample (0.45 M as compared with 1.0 M natural abundance samples) favors dissociation of the complex, and thus smaller shifts were observed for the rapidly exchanging citrate.

^{13}C NMR Measurements of Ferrous Citrate Complexes in Alkaline Solution. Spectra of the ^{13}C labeled sample of ferrous citrate in alkaline solution were obtained over a range of temperatures and are given in Figure 6 for the sample at pH 7.60. It is clear from these spectra that several forms of the ferrous complex with citrate are present in alkaline solution. However, the spectrum at −8 °C indicates that a single form predominates near the freezing point of the solution. Spectra of the sample at pH 8.15 and 9.25 were superimposable with those of the pH 7.60 sample at the same temperatures.

A graph of the resonance frequencies with respect to an arbitrary reference as a function of the inverse temperature for the pH 7.60 sample is given in Figure 7. Although the complexity of the spectra at the higher temperatures precludes a definite assignment of the resonances, straight lines through the data (Curie dependence) were drawn such that two groups of resonances were separated with the same number of resonances in each group. The resonances assigned to the predominant form at low temperature are indicated with arrows to the right of the graph. Extrapolation of these four lines to infinite temperature yielded values of 7 ± 2, 10 ± 2, −2 ± 2, −1 ± 2 kHz in order from top to bottom in Figure 7. If a Curie dependence is valid, these would be the positions of the corresponding resonances in a diamagnetic complex. The resonance frequencies of the methylene and terminal carboxyl carbon atoms of diamagnetic citric acid are 2 and 10 kHz, respectively, referred to the same frequency. On the basis of the difference in the extrapolated frequencies, the two higher frequency resonances were assigned to the terminal carboxyl carbon atoms, and the two lower frequency resonances were assigned to the methylene carbon atoms of this ferrous citrate complex.

The difference between the frequencies of diamagnetic citric acid and the extrapolated frequencies of the paramagnetic sample probably results from the error associated with such a large extrapolation. The resonances due to ^{13}C labeled β-acetyltricarballylic acid present in the ^{13}C labeled citric acid used for the preparation of this sample were essentially independent of temperature and are included in Figure 7.

Spectra of a natural abundance sample of ferrous citrate in a solution of pH 9.5 were obtained at 2 °C. Table I contains

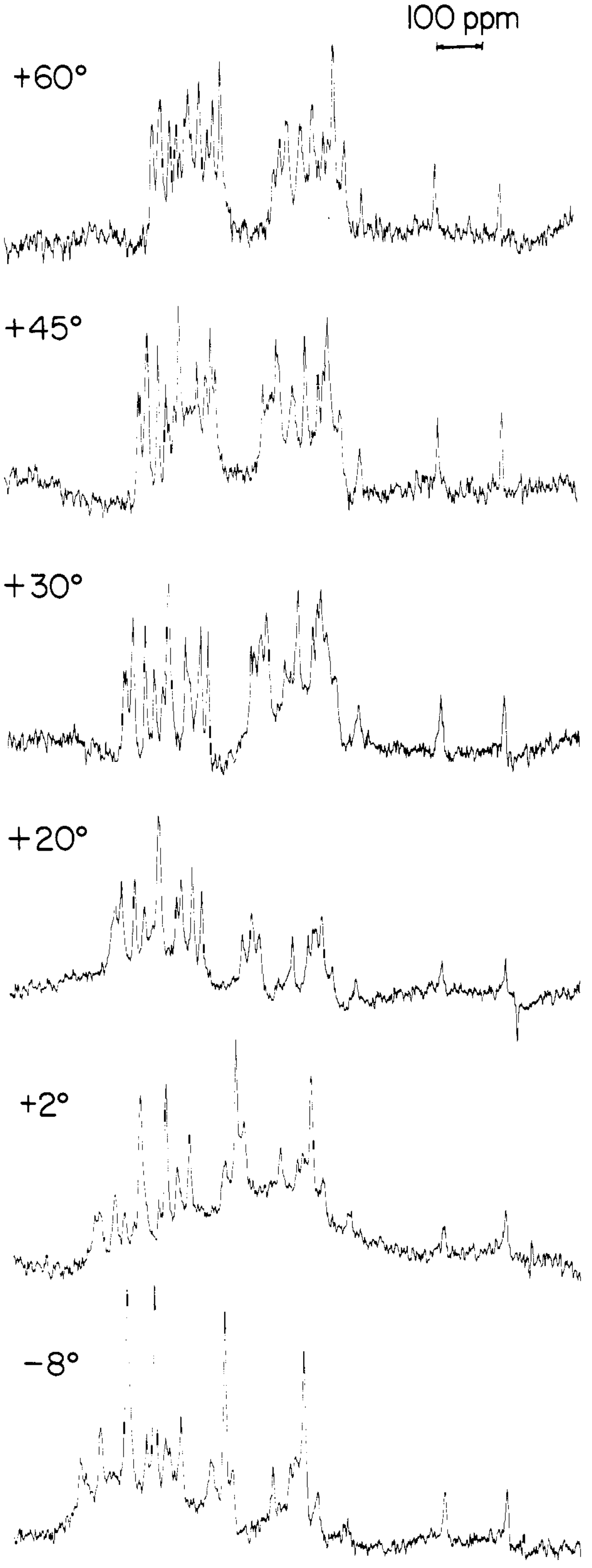

Figure 6. ^{13}C NMR spectra of the complexes of Fe(II) with [1,2,4,5-^{13}C]citrate at pH 7.60 as a function of temperature (°C). The frequency decreases from left to right and the two lowest frequency resonances in each spectrum are due to the presence of an impurity in the sample.

a list of the observed resonances of this sample and the positions of the corresponding resonances observed in the 2 °C spectrum of the ^{13}C labeled sample. The line widths at half-height (in kHz), as determined from the spectra of the natural abundance sample, are given in parentheses. The resonance positions in Table I are given with respect to the position of the citrate terminal carboxyl resonance at pH 0.65 for the natural abundance sample and with respect to the carboxyl resonance of the impurity at pH 1.50 for the ^{13}C labeled sample. The resonances at −39.2 and 21.2 kHz in the natural abundance spectra are due to the central carbon atom and the central carboxyl carbon atom. However, on the basis of the data available, it is not possible to make a definite assignment of these two resonances.

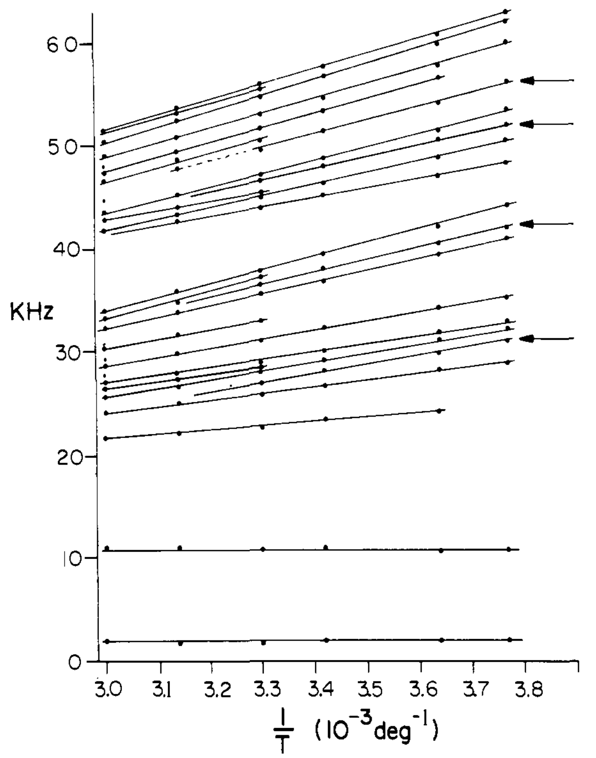

Figure 7. ^{13}C NMR resonance frequencies of the complexes of Fe(II) with [1,2,4,5-^{13}C]citrate at pH 7.60 as a function of the inverse temperature (K).

Table I. ^{13}C NMR Resonance Positions of Alkaline Ferrous Citrate at 2 °C

Natural abundance[a]		^{13}C labeled[b]	
Frequency (kHz)	ppm	Frequency (kHz)	ppm
−39.2 (0.5)	−621		
18.6 (0.4)	+295	18.7	+296
21.2 (0.4)	+336		
29.7 (0.4)	+471	29.4	+466
40.3 (0.4)	+639	39.4	+624
43.1 (0.4)	+683	42.9	+680

[a] The resonance positions are referred to the position of the citrate terminal carboxyl resonance in a solution of ferrous citrate of the same concentration at pH 0.65. The line widths at half-height (kHz) are given in parentheses. [b] The resonance positions are referred to the position of the carboxyl resonance of the impurity present in the ^{13}C labeled sample of ferrous citrate at pH 1.50.

The complex nature of the spectra of the ^{13}C labeled sample at higher temperatures, the fact that the line widths are not temperature dependent, and the observation that the shifts are independent of pH and concentration are all indicative of a system in which most chemical exchange mechanisms are slow on the NMR time scale.

Further ^{13}C NMR studies of the alkaline ferrous citrate complexes could undoubtedly yield valuable information about

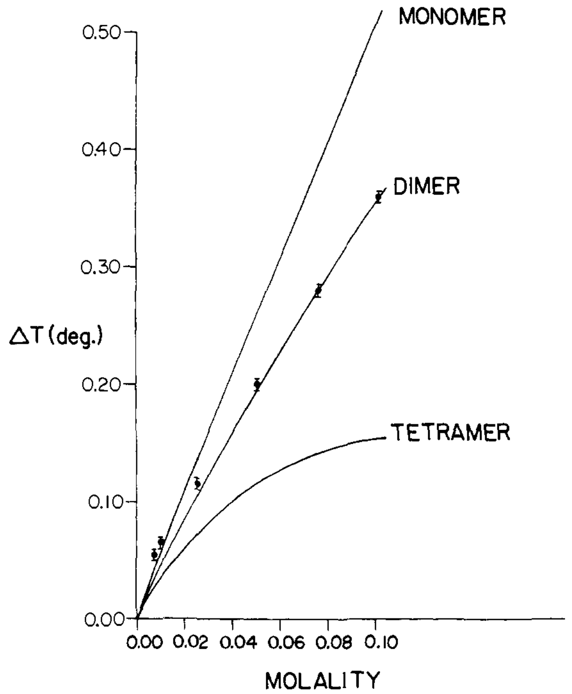

Figure 8. Freezing point depressions of low ionic strength solutions of Ni(II)-citrate(−4). The concentrations are based on the molecular weight of the monomer. The solid curves are theoretical curves based on the degrees of association indicated to the right.

the different forms of the complexes which are in equilibrium. For example, the concentration dependence of the spectral features at a constant temperature which is high enough to yield a significant concentration of the various forms of the complex should reveal whether some of these forms are dissociation products of tetrameric ferrous citrate (vide infra). The sampling rates of the spectrometer at present limit the spectral width in the Fourier transform mode to a maximum of 14 kHz which is small compared with the observed ^{13}C NMR shifts of the alkaline ferrous citrate complexes. Therefore, these complex spectra must be obtained in the continuous wave mode which is an inefficient and time consuming process. Further ^{13}C NMR studies of this system have been postponed pending the completion of modifications of the ^{13}C NMR instrumentation in order that much larger spectral widths can be obtained in the Fourier transform mode.

Molecular Weight Determinations. The data presented in Table II indicate that in solutions of high ionic strength, complexes of Fe(II) and Ni(II) with triionized citrate are monomeric although, in the case of Ni(II), a small degree of association is indicated by the slightly smaller depression of the sodium sulfate transition temperature produced by trisodium citrate in the presence of Ni(II) ions than in the absence of the metal ions. On the other hand, complexes of Fe(II) and Ni(II) with tetraionized citrate produce a depression of the transition temperature which is only one-fourth the depression produced by trisodium citrate. Thus, both of these transition metal ions form complexes that are tetrameric in alkaline solution.

In view of the stabilization of highly charged complexes by the environment of very high ionic strength under the conditions of these experiments, molecular weight studies of the Ni(II)-tetraionized citrate complexes were also carried out at lower ionic strengths. Nickel(II)-tetraionized citrate complexes in solutions which contain only the anionic complexes and their counterions are predominantly dimeric. Figure 8 demonstrates the excellent fit of the experimental freezing point depression data to the calculated curve for dimer formation. However, when samples are prepared by simple mixing of salts of the ions required, 2 equiv of sodium ions and one of sulfate ions are present for every equivalent of Ni(II) and citrate ions in the solution. Comparison of experimental data points with calculated curves indicates that samples of the Ni(II)-tetraionized citrate complexes prepared by this method result in the formation of tetramers.

Table II. Depression of the Transition Temperature of Anhydrous Sodium Sulfate to Sodium Sulfate Decahydrate by the Presence of Fe(II) and Ni(II) Citrate Complexes

Sample[a]	$\Delta T \pm 0.005$, °C	pH
Trisodium citrate	0.190	
Fe(II)-citrate(−3)	0.190	
Fe(II)-citrate(−4)	0.045	7.30
Ni(II)-citrate(−3)	0.170	5.85
Ni(II)-citrate(−4)	0.045	8.78

[a] All samples were prepared such that the concentration of each was 0.100 M based on the molecular weight of the monomer. pH measurements of the last three samples were made immediately following the transition temperature determinations.

Magnetic Susceptibility Measurements. The values for the magnetic moments and Weiss constants of Ni(II) complexes of triionized and tetraionized citrates were identical within experimental error, and the magnetic moment was not significantly different from that of a sample of $NiCl_2$. Likewise, the magnetic moment and Weiss constant of samples of Fe(II)-tetraionized citrate were not significantly different from those of $FeSO_4$.

Discussion

The results of solution studies of transition metal citrate complexes are consistent with the substantially different configurations of transition metal citrate complexes with triionized and tetraionized citrate ions observed in the crystallographic investigations of Fe(II)-citrate(−3) and Ni(II)-citrate(−4).[9] The molecular weight determinations indicate that studies of these complexes in solution should be interpreted in terms of *monomeric* transition metal complexes of *triionized* citrate and *tetrameric* transition metal complexes of *tetraionized* citrate.

Comparison of the molecular weight data with the results of the x-ray crystallographic studies suggests that the coordination of both oxygen atoms of a citrate carboxyl group to two different metal ions is easily disrupted in solution. The 1:1 Fe(II):citrate(−3) tridentate chelate forms an infinite chain in the solid state by the coordination of both oxygen atoms of the terminal carboxyl group not involved in the tridentate chelation to two other symmetry-related Fe(II) ions. The replacement of these carboxyl oxygen atoms by water molecules in the coordination sphere of the chelated Fe(II) ions would result in a monomeric complex of Fe(II) with triionized citrate.

Ni(II) forms a discrete complex with citrate(−4) in the solid state that is composed of eight Ni(II) ions and six tetraionized citrate ions. The centrosymmetric complex contains two clusters of four nonbonded Ni(II) ions which form highly distorted tetrahedra. Three of the faces of each tetrahedron are capped by the coordination of citrate ions with doubly or triply bridging hydroxyl oxygen atoms and the fourth face is capped by a triply bridging hydroxide ion. The coordination of both oxygen atoms of one of the terminal carboxyl groups of the doubly bridging citrate ion to two different metals of the symmetry-related tetrahedron provides the only linkage between the two clusters.[22] Separation of the two clusters, by disruption of this type of coordination, followed by the replacement of the triply bridging hydroxide ion with a triply bridging hydroxyl oxygen atom of a tetraionized citrate ion would result in the formation of a tetrameric structure of 1:1 Ni(II):citrate stoichiometry.

Proposed Structure of Ferrous Citrate in Alkaline Solution. The x-ray crystallographic investigation of a nickel–citrate complex crystallized from a solution of pH 9.20[9] provides an excellent model for the configurations and conformations of transition metal–citrate complexes in basic solution, because discrete Ni(II)–citrate complexes are surrounded by water molecules and positive counterions. On the basis of this model and the molecular weight data, it is proposed that the predominant form of the ferrous citrate complex in cold alkaline solution is a tetrameric cluster in which four Fe(II) ions form a nonbonded tetrahedron in which each face of the tetrahedron is capped by a citrate tetraanion. A structure in which the two terminal carboxyl groups and the two methylene groups of each citrate ion are inequivalent, but all four citrate ions of the tetramer are symmetry related is the simplest model that is consistent with the NMR data. A complex with S_4 symmetry has these properties. A nonsymmetric structure in which the individual citrate ions are averaged by a rapid dynamic NMR effect is also possible but a more complex model of this type is not required by the data.

Photographs of a ball-and-stick model of an S_4 tetrameric Fe(II)–tetraionized citrate complex are shown in Figure 9. The ionized citrate hydroxyl oxygen atom is coordinated to the three Fe(II) ions of the face in a triply-bridged configuration, and the three carboxyl groups each have one oxygen atom coordinated to the three metal ions of that face in a manner similar to that observed for two of the three independent citrate ions in the crystallographic study. The coordination sites of each Fe(II) ion are occupied by the hydroxyl oxygen atoms of three of the four citrate ions of the complex and three carboxyl oxygen atoms in an approximately octahedral arrangement. The carboxyl oxygen atoms located within the coordination sphere of each metal ion are contributed by the central carboxyl group of one citrate ion of the complex and by the terminal carboxyl groups of two other citrate ions. The conformations of the individual citrate ions in this model are similar to those observed in the crystallographic investigation.

This tetrameric ferrous citrate complex is an example of a "cubane" structure comprised of two interpenetrating tetrahedra of Fe(II) ions and alkoxide oxygen atoms. The formation of transition metal cluster compounds with metal-bridging ligands suggests the possibility of magnetic interactions among the bridged metals. Herskovitz et al.[23] have reported the synthesis of an analogue of the active site of non-heme iron–sulfur proteins in which substantial antiferromagnetic coupling is observed. This tetrameric cluster contains a distorted tetrahedron of nonbonded, tetrahedrally coordinated iron atoms with triply-bridging sulfur ligands on each tetrahedral face. In contrast, the octahedrally coordinated metals of the tetrameric Fe(II)–citrate complex exhibit no measurable magnetic interactions over the temperature range investigated.

^{13}C Contact Shifts. Strouse and Matwiyoff[24] have reported patterns of upfield and downfield contact shifts for the carbon resonances of Ni(II) complexes of propylamine and substituted ethylenediamines and α-amino acids. They observed large upfield shifts for the carboxyl carbon atoms, even larger upfield shifts of the carbon atoms located α to the nitrogen donor atom, and downfield shifts for carbon atoms located β and γ to the nitrogen atom which are attenuated with distance from the donor atom. These patterns were attributed to a combination of direct delocalization and spin polarization mechanisms.

In acidic solutions of equimolar Fe(II) and citrate, a large upfield shift of the central carbon atom to which the hydroxyl group is attached and a smaller upfield shift of the central carboxyl carbon atom were observed in the ^{13}C NMR spectra at pH values high enough for significant complex formation. These results are consistent with those reported for the diamine and amino acid complexes of Ni(II) with the simple substitution of the nitrogen donor atom with the hydroxyl oxygen atom in the Fe(II)–citrate complexes. Likewise, the large downfield shifts of the methylene carbon atoms of Fe(II)–citrate are consistent with the previously reported shifts of carbon atoms located β to the nitrogen donor. However, a model for the expected shift of a carboxyl carbon atom which is not located β to another donor atom is unavailable. The very large downfield contact shifts of these carbon atoms probably result from a large contribution from the direct delocalization mechanism, which dominates any spin polarization mechanism for the transmission of the unpaired spin to a carboxyl carbon atom of this configuration.

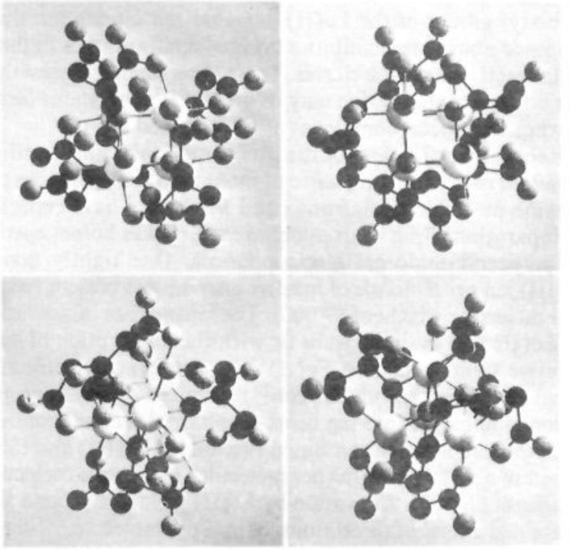

Figure 9. Ball-and-stick model of a tetrameric complex of Fe(II) citrate(−4) with S_4 symmetry. Upper left: View through the face of the cube of Fe(II) and citrate hydroxyl oxygen atoms which contains the C_2 axis of the complex. Upper right: View through a face of the cube of Fe(II) and citrate hydroxyl oxygen atoms which lacks a symmetry axis. Lower left: View from above one of the vertices of the nonbonded tetrahedron of Fe(II) ions. Lower right: View from above one of the faces of the nonbonded tetrahedron of Fe(II) ions.

In alkaline solution, only one resonance was shifted upfield and all the other resonances experienced large downfield shifts. Unfortunately, a model for the prediction of the ^{13}C contact shifts of complexes with a bridging donor atom is not currently available.

The large shifts observed for the ferrous complexes could contain significant dipolar contributions although they are not required to do so.[25] Wicholas[26] has shown that contact-shifted proton resonances of the octahedral complex of Fe(II) with diphenyl sulfoxide do not contain significant contributions from dipolar interactions. Even in the absence of a detailed understanding of the origin of the shifts and the mechanisms of delocalization of unpaired spin to the ligands, the ^{13}C NMR technique is a sensitive probe of configurations and conformations of the ligands and is therefore a powerful tool for the elucidation of the structures and dynamics of a wide variety of coordination complexes in solution.

The Active Site of Aconitase. Since the pH optimum for the catalytic activity of aconitase from a variety of sources lies in the alkaline range,[2] the structures of transition metal citrate complexes in basic media are of particular interest to studies of the mechanism of aconitase activity. All three crystallographically independent citrate ions of the Ni(II)–tetraionized citrate structure reported previously exhibit a bridged configuration of the ionized citrate hydroxyl groups to two or three Ni(II) ions of the cluster.[9] Likewise, each of the ionized citrate hydroxyl groups of the Fe(II)-tetraionized citrate tetramer proposed above also exhibits a bridged configuration to three of the Fe(II) ions of the cluster. These observations suggest that this bridged configuration may be present in most stable forms of transition metal complexes of tetraionized citrate.

Recent investigations of the iron content of highly purified aconitase indicate the presence of more than one iron atom per enzyme molecule. Villafranca and Mildvan[27] have reported a preparation of pig heart aconitase which was homogeneous by polyacrylamide gel electrophoresis. One tightly bound Fe(III) ion per molecule of inactive enzyme was present (based on a molecular weight of 89 000). The kinetic data of activation by Fe(II) and cysteine were fit with the assumption of consecutive binding of two Fe(II) ions per enzyme molecule. Kennedy, Rauner, and Gawron[28] reported a different preparation of homogeneous pig heart aconitase. Inactive aconitase from this preparation contained two ions of Fe(III) and three atoms of acid labile sulfur per molecule (based on a molecular weight of 66 000). Activation by Fe(II) yielded a protein that contained a total of three atoms of iron per molecule.[29] Suzuki et al.[30,31] obtained crystalline samples of yeast aconitase that contained two iron atoms and one acid labile sulfur atom per enzyme molecule (based on a molecular weight of 68 500). The presence of more than a single atom of iron per enzyme molecule introduces the possibility that the ternary aconitase-Fe(II)-citrate complex may also involve a bridged coordination of tetraionized citrate to at least two iron atoms at the active site of aconitase.

Villafranca and Mildvan[4] have measured the paramagnetic contributions to both the longitudinal ($1/T_1$) and transverse ($1/T_2$) relaxation rates of the methylene protons of citrate in binary complexes with Mn(II), a competitive inhibitor of aconitase, and with Fe(II), and in ternary complexes with aconitase-Mn(II) and aconitase-Fe(II) in D_2O at pD 7.5. From these data, root-mean-sixth distances and lower limits to the hyperfine interactions between a single Fe(II) ion and the protons observed were calculated. By the detection of similar distances and hyperfine interactions for solutions of citrate and Mn(II) or Fe(II) compared with solutions of citrate and aconitase-Mn(II) or aconitase-Fe(II), Villafranca and Mildvan[4] provided the first direct evidence that a ternary Fe(II)-citrate complex is present at the active site of aconitase.

These calculated metal ion to proton distances were based on the assumption of a single Mn(II) or Fe(II) ion per citrate ion in the complexes. However, since the calculated distances are proportional to the sixth root of the paramagnetic contributions to the longitudinal relaxation times, the assumption of more than a single paramagnetic ion per citrate ion in each complex would not substantially alter the results of the calculations. Thus, the insensitivity of the technique precludes a definite conclusion on the basis of T_1 data regarding the participation of more than one metal ion per citrate ion in the ternary complex.

Examination of the parameters used to obtain the ^{13}C NMR spectra of the alkaline ferrous citrate complexes demonstrates that, with the use of ^{13}C labeled citrate, direct observation of the ternary aconitase-Fe(II)-citrate complex by ^{13}C Fourier transform NMR may be feasible. Experiments of this kind which could further elucidate the nature of the interaction of the citrate ion with the iron atom(s) at the active site of aconitase will be undertaken following completion of the modifications of the Fourier transform capability of the present ^{13}C NMR spectrometer.

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References and Notes

(1) This paper is based in part on a dissertation submitted by Jane Strouse to the Graduate School of the University of Wisconsin-Madison in partial fulfillment of the Ph.D. degree, December 1975.
(2) J. P. Glusker, "The Enzymes", Vol. 5, P. D. Boyer, Ed., 3d ed, Academic Press, New York, N.Y., 1971, pp 413–439, and references cited therein.
(3) J. J. Villafranca and A. S. Mildvan, *J. Biol. Chem.*, **246**, 5791–5798 (1971).
(4) J. J. Villafranca and A. S. Mildvan, *J. Biol. Chem.*, **247**, 3454–3463 (1972).
(5) A. K. Grzybowski, S. S. Tate, and S. P. Datta, *J. Chem. Soc. A*, 241–245 (1970).
(6) R. E. Hamm, C. M. Shull, Jr., and D. M. Grant, *J. Am. Chem. Soc.*, **76**, 2111–2114 (1954).
(7) R. K. Patnaik and S. Pani, *J. Indian Chem. Soc.*, **34**, 619–628 (1957).
(8) A. I. Shnarevich, *Russ. J. Inorg. Chem.* (*Engl. Transl.*), **8**, 1083–1086 (1963).
(9) J. Strouse, S. W. Layten, and C. E. Strouse, preceding paper in this issue.
(10) The synthetic procedure was suggested by Dr. D. F. Evans, California Institute of Technology.
(11) R. Sandberg, *Acta Chem. Scand.*, **11**, 748 (1957).
(12) H. O. House, "Modern Synthetic Reactions", 2d ed, W. A. Benjamin, Menlo Park, California, 1972, p 321 ff.
(13) C. H. Hassall, "Organic Reactions", Vol. 9, R. Adams, A. H. Blatt, A. C. Cope, D. Y. Curtin, F. C. McGrew, and C. Niemann, Ed., Wiley, New York, N.Y., 1957, pp 73–106.
(14) W. D. Emmons and G. B. Lucas, *J. Am. Chem. Soc.*, **77**, 2287–2288 (1955).
(15) 90% hydrogen peroxide is highly explosive and should be handled with extreme caution.
(16) S. Rothchild and M. Fields, *J. Am. Chem. Soc.*, **74**, 2401 (1952).
(17) See F. A. L. Anet and A. K. Cheng, *J. Am. Chem. Soc.*, **97**, 2420–2424 (1975), and references cited therein.
(18) F. E. W. Wetmore and D. J. LeRoy, "Principles of Phase Equilibria", 1st ed, McGraw-Hill, New York, N.Y., 1951, pp 38–41.
(19) J. Lefebvre, *J. Chim. Phys. Phys. Chim. Biol.*, **54**, 581–600 (1957).
(20) W. J. Moore, "Physical Chemistry", 3d ed, Prentice-Hall, Englewood Cliffs, N.J., 1962, pp 348, 356.
(21) D. F. Evans, *J. Chem. Soc.*, 2003–2005 (1959).
(22) See especially Figures 5 and 6 of J. Strouse, S. W. Layten, and C. E. Strouse, preceding paper in this issue.
(23) T. Herskovitz, B. A. Averill, R. H. Holm, J. A. Ibers, W. D. Phillips, and J. F. Weiher, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 2437–2441 (1972).
(24) C. E. Strouse and N. A. Matwiyoff, *Chem. Comm.*, 439–440 (1970).
(25) W. DeW. Horrocks, Jr., "NMR of Paramagnetic Molecules", G. N. LaMar, W. DeW. Horrocks, Jr., and R. H. Holm, Ed., Academic Press, New York, N.Y., 1973, pp 127–177.
(26) M. Wicholas, *J. Inorg. Nucl. Chem.*, **32**, 873–878 (1970).
(27) J. J. Villafranca and A. S. Mildvan, *J. Biol. Chem.*, **246**, 772–779 (1971).
(28) C. Kennedy, R. Rauner, and O. Gawron, *Biochem. Biophys. Res. Commun.*, **47**, 740–745 (1972).
(29) O. Gawron, A. Waheed, A. J. Glaid, III, and A. Jaklitsch, *Biochem. J.*, **139**, 709–714 (1974).
(30) T. Suzuki, O. Yamazaki, K. Nara, S. Akiyama, Y. Nakao, and H. Fukuda, *Agric. Biol. Chem.*, **37**, 2211–2212 (1973).
(31) T. Suzuki, O. Yamazaki, K. Nara, S. Akiyama, Y. Nakao, and H. Fukuda, *J. Biochem.* (*Tokyo*), **77**, 367–372 (1975).