λ_0^- in water as $\lambda_0^- = 80.0$ has been taken. From $\Lambda_0 = 133.51$ one gets for the transport number at infinite dilution, $n_+^0 = 0.401$. Assuming n_+^0 to remain constant with glycol composition in the glycol mixtures, R_+ and R_- could be calculated.

The semiempirical equations

$$R_+D = A + (R_+)_\infty D$$
$$R_-D = A^1 + (R_-)_\infty D \qquad \text{(IV)}$$

of Fuoss[17] (expressing the relaxation of the solvent dipoles orientation upon the passage of the ions) have been applied in Figure 3 for $MnSO_4$ with H_2O–glycol and H_2O–dioxane[3] where the products R_+D and R_-D are reported *vs.* D. The slopes give $(R_+)_\infty = 3.35 \times 10^{-8}$ cm and $(R_-)_\infty = 2.35 \times 10^{-8}$ cm, the hydrodynamic radii in a medium of infinite dielectric constant. In this medium no ion–solvent interaction could exist; therefore

$$(R_+)_\infty + (R_-)_\infty = a_A = 5.7 \times 10^{-8} \text{ cm}$$

in fair accord with $a_K = 4.6 \times 10^{-8}$ cm and $a(L_2) = (4.4 \pm 0.2) \times 10^{-8}$ cm.

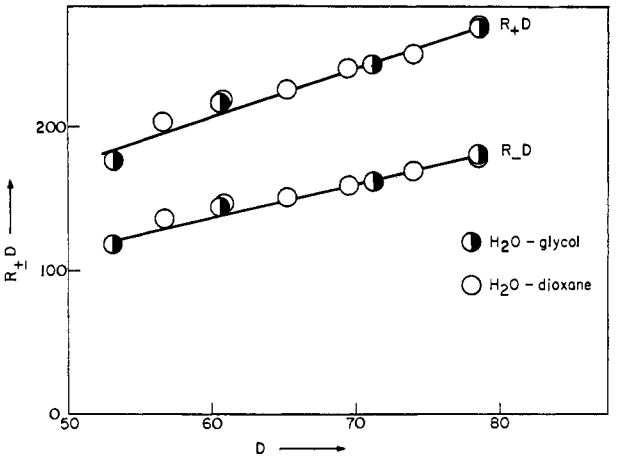

Figure 3. Plot of $R_\pm D$ *vs.* D for $MnSO_4$ in H_2O–glycol and in H_2O–dioxane at 25°.

Conclusion

The parameter a has been calculated through the interaction terms J[14] and L_2,[15] through the association constant K_A and the hydrodynamic radii, giving a consistent value (a_J having the most scattering as predicted) for two different solvent systems like H_2O–dioxane and H_2O–glycol. The viscosity and the dielectric constant of the solvent seem to suffice to describe the hydrodynamic and electrostatic properties of the above systems.

The validity of formulas II and IV and the satisfactory fitting of the conductance equations to the conductance data lead to the conclusion that the sphere in a continuum model and the Fuoss–Onsager theory[14] hold physically and mathematically for these systems.

Acknowledgment. The authors wish to express their thanks to Dr. D. Ebdon of the University of Maryland for having run the conductance data through an IBM program. This work has been sponsored by the National Science Foundation through the Science Development Program of the Polytechnic Institute of Brooklyn.

(17) R. M. Fuoss, *Proc. Natl. Acad. Sci. U. S.*, **45**, 807 (1959).

The Hydrolytic Polymerization of Ferric Citrate. I. The Chemistry of the Polymer

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Abstract: The chemistry of ferric citrate at equal molar concentrations of iron and citrate has been examined by a variety of techniques including ultraviolet and visible spectrophotometry, glass-electrode measurements, and equilibrium dialysis. On titration with base, an anionic chelate, $FeCit^-$, is formed at low pH and then polymerizes in a buffer region at pH 8–9, which terminates at 3 base equiv per mole of iron. About 85% of the citrate dissociates from the polymer in the process. The polymer was isolated using the techniques of gel and membrane filtration. Electron microscopy shows the polymer particles to be spherical, with a diameter of 72 ± 9 A. Its molecular weight determined by its volume and density measurements is $2.1 \pm 0.1 \times 10^5$. The spheres appear to consist of an iron hydroxide core with citrate ions bound to the surface. The polymer may be a good model for the iron storage protein, ferritin.

Recently we have shown that the hydrolysis of ferric ion can lead to the formation of a high molecular weight soluble polymer.[2,3] This polymer has a molecular weight of approximately 1.5×10^5 and contains about 1200 iron atoms linked by hydroxy and oxy

(1) (a) Princeton University. (b) Research Career Development Awardee, U. S. Public Health Service.

(2) T. G. Spiro, S. E. Allerton, J. Renner, A. Terzis, R. Bils, and P. Saltman, *J. Am. Chem. Soc.*, **88**, 2721 (1966).

(3) S. E. Allerton, J. Renner, S. Colt, and P. Saltman, *ibid.*, **88**, 3147 (1966).

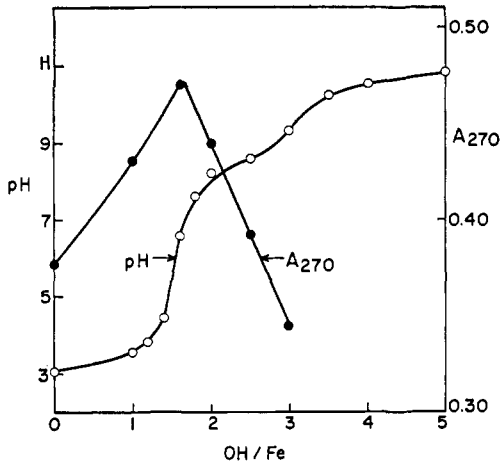

Figure 1. Titration curve (○) of solutions containing 10^{-3} M ferric nitrate, 10^{-3} M sodium citrate, and 10^{-1} M sodium nitrate as a function of increasing base equivalents per mole of iron, added as 10^{-2} M NaOH. The absorbancy, 1-mm light path, at 270 mμ (●) for these same solutions is also presented.

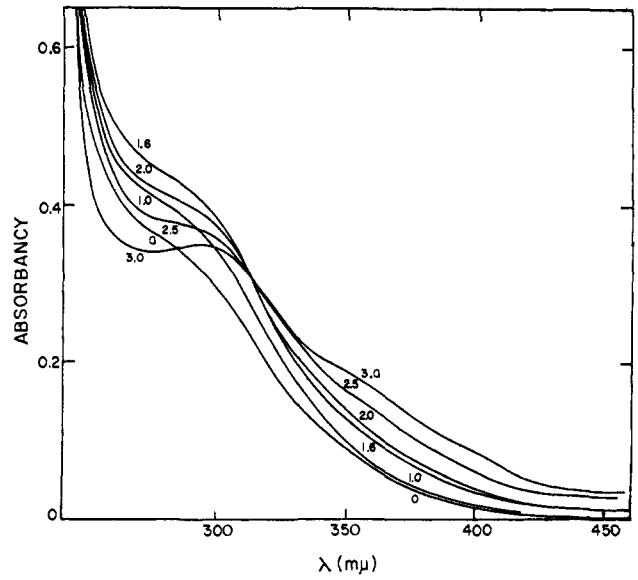

Figure 2. Visible and ultraviolet spectra for the same series of solutions used in Figure 1 (path length, 1 mm). Base equivalents per mole of iron are indicated by number on curve.

bridges. It is a polycation and carries a charge of 0.5 per iron atom. Preliminary studies in our laboratory indicated that chelation does not necessarily prevent iron polymerization and that high polymers might be present in various iron chelate solutions. Previous studies of iron chelates have been limited to low molecular weight species, principally monomers and dimers. Evidence of polymer formation has usually been taken as grounds for discarding the solution in question. We felt that the characterization of chelate polymers would be intrinsically interesting, and also that the variation in the properties of the iron polymer caused by chelation might elucidate the nature of the polymerization process. We chose citrate as the chelate for this investigation because the stoichiometry of the polymerization reaction is well defined, as detailed below. In addition, citrate has been widely used in biochemical investigations on transmembrane movement[4] and specific metal binding to proteins.[5]

Iron complexes with citric acid have been studied by several investigators.[6-9] All of these researches were carried out at acid pH. It was noted on occasion that at pH values higher than about 4.0 a characteristic red-brown color appeared and that redox and glass electrode potentials became sluggish. The pH region above 4.0 has occupied our primary interest. We find that under alkaline conditions a high polymer forms, which appears to be similar to that found in chelate-free solutions,[2] except that a coating of citrate prevents its precipitation at high pH.

Experimental Section

Titration Studies. All solutions were prepared using analytical reagent grade ferric nitrate, sodium citrate, sodium nitrate, and CO_2-free standardized NaOH. A series of solutions was prepared containing a final concentration of 10^{-3} M ferric nitrate, 10^{-3} M sodium citrate, and 0.1 M sodium nitrate. Increasing amounts of 0.01 M sodium hydroxide were added last with vigorous stirring. The pH of each solution was measured with a Beckman Research Model pH meter standardized with 10^{-3} M HNO_3 in 0.1 M sodium nitrate. The resulting titration curve is shown in Figure 1. At pH values higher than 4.0 the glass electrode potentials tended to drift. The uncertainty introduced into the titration curve, no greater than 0.1 pH unit, was insufficient to obscure the stoichiometry of the buffer regions. The initial, low pH buffer region ends upon the addition of 1.5 base equiv per mole of iron. Beyond this point there is a second buffer region at pH 8–9 which terminates upon the addition of 3 base equiv per mole of iron. Addition of further base leads to precipitation.

Visible and ultraviolet spectra were obtained for each of the solutions using a Cary Model 15 recording spectrophotometer with a 1-mm path length quartz cell. These spectra are shown in Figure 2. As base is added to ferric citrate, the initial yellow-orange solution turns lime green as the end of the first buffer region is approached. This is reflected in the spectra labeled 0 and 1.0 in Figure 2. As the second buffer region is entered, the solutions become red-brown, a color characteristic of many hydrolyzed ferric species. These changes are reflected in spectra 1.6 to 3.0 where an isosbestic point is noted at approximately 315 mμ. The isosbestic point suggests the presence of only two absorbing chromophores in this buffer region. The changes in absorbancy at 270 mμ of these solutions are superimposed upon the pH titration curve of Figure 1. The end point at 1.5 base equiv per iron is confirmed in this plot.

Dialysis Experiments. The solutions that were prepared above for the titrimetric and spectrophotometric determinations were subjected to dialysis using the techniques of Lindskog and Malmström.[10] In these experiments duplicate solutions were prepared using tracer amounts of ^{59}Fe (approximately 60,000 cpm/0.1 ml) added prior to addition of base. The solutions were introduced on opposite sides of a Visking dialysis membrane. Periodically, samples were taken from both compartments and their radioactivity determined. The cells were continuously agitated to ensure complete mixing. Since no concentration gradients are established across the membrane, dialysis is a measure of self-diffusion of radioactive iron from one side to another. The results are shown in Figure 3, where the log of the fraction of undialyzed material is plotted against time. Solutions containing 0 or 1.0 added base equiv rapidly reached equilibrium with a $t_{1/2}$ = 3.5 to 4.0 hr. Solutions containing 1.5 or more base equiv showed a slowly dialyzing fraction, as well as a rapid one. The fraction of iron in the slow component can be estimated by extrapolation of the later portion of the dialysis curve to zero time. These estimates are shown in Table I. The rate of dialysis of the slowly

(4) J. M. Hopping and W. S. Ruliffson, *Am. J. Physiol.*, 210, 1316 (1966).
(5) G. W. Bates, C. Billups, and P. Saltman, *J. Biol. Chem.*, 242, 2810 (1967).
(6) M. Bobtelsky and J. Jordan, *J. Am. Chem. Soc.*, 69, 2886 (1947).
(7) O. E. Lanford and J. R. Quinan, *ibid.*, 70, 2900 (1948).
(8) R. C. Warner and I. Weber, *ibid.*, 75, 5086 (1953).
(9) C. F. Timberlake, *J. Chem. Soc.*, 5075 (1964).
(10) S. Lindskog and B. Malmström, *J. Biol. Chem.*, 237, 1129 (1962).

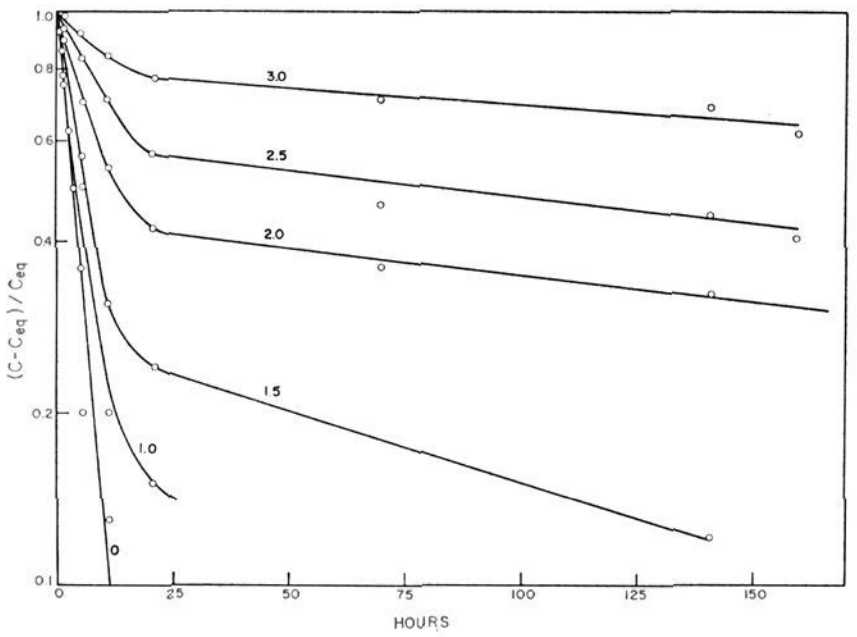

Figure 3. Fraction of ^{59}Fe undialyzed as a function of time for solutions containing 10^{-3} *M* ferric nitrate, 10^{-3} *M* sodium citrate, and 10^{-1} *M* sodium nitrate at increasing added base equivalents indicated by number on curve. The fraction is given as $(C - C)_{eq}/C_{eq}$, where C is the concentration of ^{59}Fe in the radioactive compartment at any time, and C_{eq} is the equilibrium concentration, *i.e.*, half of the initial concentration.

dialyzing fraction was, however, essentially the same in all solutions.

Dialyses were also carried out in the high pH buffer region on solutions containing varying concentrations of ferric citrate, from 10^{-2} to 10^{-4} *M*. Some decrease in polymer fraction was observed on decreasing the concentration. However, the effect is not large, and it is certain that solutions as dilute as 10^{-4} *M* still contain a sizable fraction of polymer.

Table I. Fraction of Polymer as a Function of Base Equivalents per Mole of Iron[a]

OH/Fe	% polymer
1.5	27
2.0	44
2.5	59
3.0	80

[a] All solutions were 10^{-3} *M* ferric citrate and were prepared as described in Figure 1.

Polymer Isolation. In order to provide reasonable amounts of polymer for isolation, more concentrated solutions, 0.03 *M*, of ferric citrate were prepared and hydrolyzed. Throughout the initial buffer region hydrolysis was carried out with sodium bicarbonate, as hydroxide addition to the more concentrated solutions produced local precipitates which were slow to redissolve at low pH. Near the end of the initial buffer region the solutions were gassed with nitrogen to remove dissolved CO_2. Sodium hydroxide, 0.05 *M*, could then be added with vigorous stirring up to a total of 3 base equiv per mole of iron, before precipitation occurred.

The polymer formed in this way could be isolated by gel filtration as long as the eluent contained at least 10^{-4} *M* sodium citrate to prevent precipitation. On a column of Bio-Gel P-30 (Bio-Rad Chemical Co., Berkeley, Calif.) the polymer fraction was eluted with the void volume. The polymer could also be isolated by ultrafiltration of the hydrolyzed solutions using collodion membranes (Schleicher & Schuell Co., Keene, N. H.) and the appropriate vacuum apparatus. Either isolation procedure led upon lyophilization to a dark brown powder which was readily soluble in water.

Electron Microscopy. A solution of 10^{-3} *M* ferric citrate was adjusted to 3 base equiv per mole of iron, with 0.01 *M* NaOH as described above. The solution was mixed with polystyrene latex balls, diameter 1880 A, and sprayed on 200-mesh electroplate grids previously coated with Formvar and stabilized with carbon. The grids were shadow cast with platinum at 5:1 angle and observed in a RCA-3MU-f electron microscope using a 50-kev electron beam.

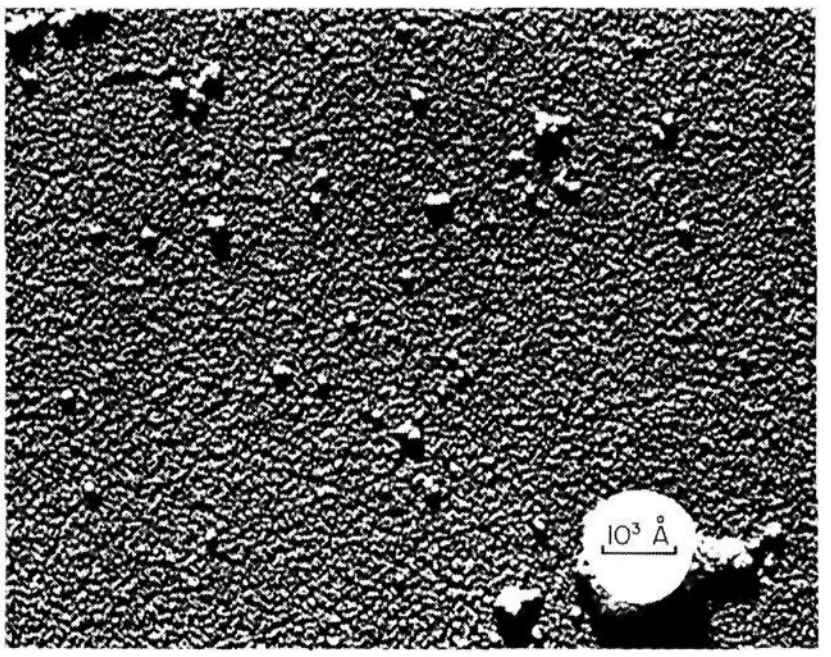

Figure 4. Electron micrograph of typical field of ferric citrate polymers shadow cast with Pt.

The field was characterized by large numbers of spherical particles much the same as those in similar preparations of ferric hydroxy nitrate polymers.[2] Multiple fields each containing at least 50 particles were scanned and the average diameter of the ferric citrate spheroids, as determined by comparison with the latex spheres, was 72 ± 9 A. No discrete particles of diameter less than 50 A or more than 95 A could be seen. Also visible in the field are aggregates of unit particles which seem to have a tendency to be aligned in linear arrays (Figure 4). It is not clear whether this tendency reflects a fundamental particle interaction or results from the electron microscope preparative techniques.

Determination of Molecular Weight. The specific volume of isolated ferric citrate polymer was measured using standard techniques of pycnometry, $\bar{v} = 0.535$ cm³/g. The density of the dry polymer was calculated from this value, $\rho = 1.87$ g/cm³. Using the measured average diameter of the polymer particle, the molecular weight of the polymer was calculated to be $2.1 \pm 0.1 \times 10^5$. Preliminary studies using sedimentation velocity and membrane osmometry confirm this value. A detailed study of the physical chemical properties of the polymer is in progress and will be reported subsequently.

Chemical Analysis of the Polymer. Polymer fractions were isolated by membrane filtration from ferric citrate solutions to which 2.0, 2.5, and 2.8 base equiv per mole of iron had been added and dried *in vacuo* over P_2O_5. Samples were analyzed for iron, carbon, hydrogen, nitrogen, and ash (Fe_2O_3 and Na_2SO_4) by Galbraith Laboratories, Knoxville, Tenn. The results (%) for the sample isolated at 2.5OH/Fe were as follows: Fe, 41.37; C, 7.94; H, 1.24; N, 2.17; ash, 83.54. The nitrogen comes from $NaNO_3$ present in the solid. The samples were not washed exhaustively with water in the filtration apparatus for fear of altering them chemically. The % N result indicates that 13.15% of the solid was $NaNO_3$. Taking this into account the empirical formula of the polymer can be calculated from the analytical results to be $(FeO)_{0.95}(OH)_{0.75}Cit_{0.15}Na_{0.24}$, where Cit represents citrate ion from which the hydroxyl proton has been removed (see Discussion). The base content of the polymer is 2.8OH/Fe, somewhat higher than that of the solution from which it was isolated. The results for the samples at 2.0- and 2.8OH/Fe were quite similar. The ratio of citrate to iron decreases slightly with increasing OH/Fe.

The most striking result of the analyses is that the citrate/iron ratio is much less than unity. The excess of iron over citrate in the polymer was confirmed in an experiment in which a hydrolyzed 10^{-3} *M* 1:1 ferric citrate solution, to which ^{14}C-labeled citrate (New England Nuclear Corp.) had been added before hydrolysis, was allowed to dialyze against distilled water. The concentration of iron remaining in the polymer solution was determined spectrophotometrically using bathophenanthrolene[11] and that of citrate with liquid scintillation counting. After 24 hr some 17% of the original iron and 77% of the original citrate had moved across the membrane, so that the ratio of citrate to iron in the polymer solu-

(11) T. H. Bothewell and C. A. Finch, "Iron Metabolism," Little, Brown and Co., Boston, Mass., 1962, p 18.

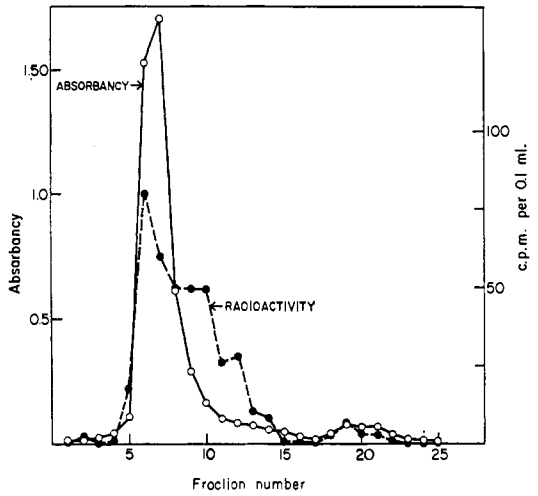

Figure 5. Elution pattern of dialyzed ferric citrate polymer from Bio-Gel P-30 using 10^{-4} M sodium citrate, pH 8.0. Iron was measured by absorbancy at 350 mμ; ^{14}C-citrate was determined using liquid scintillation techniques.

tion had decreased from 1.0 to 0.28. With continued dialysis the ratio continued to decrease slowly, but after a few days a precipitate formed in the solution.

A 0.5-ml aliquot of the above solution, which had been dialyzed for 24 hr, was placed on a 1 × 15 cm Bio-Gel P-30 column and eluted with 10^{-4} M sodium citrate (nonradioactive). Fractions were collected and monitored for iron by light absorbance at 350 mμ and for ^{14}C-citrate by scintillation counting. The elution curve is shown in Figure 5. Eluted under the same conditions, but in the absence of iron, ^{14}C-citrate begins to emerge from the column in tube 9. It can be seen that roughly half of the ^{14}C-citrate in the polymer solution emerged as free citrate. Thus the ratio of bound ^{14}C-citrate to iron in the gel-filtered polymer was about 0.14, very close to the citrate/iron ratio in the isolated polymer as determined by carbon analysis. Apparently little exchange of citrate between polymer and eluent occurred during gel filtration, indicating rather firm binding of the polymer citrate.

Discussion

Equilibria in ferric citrate solutions at low pH have been studied fairly thoroughly, especially by Warner and Weber.[8] These authors concluded from glass electrode and spectrophotometric data that the formation of a 1:1 chelate is accompanied by the loss of three protons from citric acid in a single step, and then a fourth, presumably from the citrate hydroxyl group, to form $FeCit^-$, where Cit^{4-} represents citric acid with four protons removed. Timberlake[9] confirmed the proton stoichiometry but suggested that $FeCit^-$ was dimeric on the basis of Fe(III)–Fe(II) redox potentials. Warner and Weber[8] noted that their titration curve did not break at the expected four protons per citric acid, but rather at about 4.2, and they suggested the involvement of more highly hydrolyzed species.

Our titration curve (Figure 1), covering a wider pH range, shows the midpoint between the low and high pH buffer regions to occur at 1.5 protons ionized per citrate (4.5 protons per citric acid). This could be taken to imply that the end of the first buffer region corresponds to the formation of a species $[(FeCit)_2OH]_n^{3n-}$, with n a small number, perhaps one, since the species is readily dialyzable. However, it seems more likely that the half-integral end point is the result of a kinetic phenomenon. The dialysis experiments showed that the polymer, formed in the high pH buffer region, dissociates extremely slowly. Since it is a more highly hydrolyzed species (2.8OH/Fe), its formation corresponds to the effective removal of base from the solution. To the extent that some polymer can form irreversibly, through local concentration gradients in the low pH buffer region, the end point of this region is delayed beyond what it would be at equilibrium. The fraction of iron polymerized at the end point is about 27% (Table I). If the iron and hydroxide contained in this amount of polymer is subtracted from the composition of the solution, then the OH/Fe ratio of the remainder is very close to 1.0. In other words irreversible polymer formation can account quantitatively for a shift in the end point from 1.0 to 1.5. There is therefore no need to postulate a low pH species more highly hydrolyzed than $FeCit^-$.

The high pH buffer region is characterized by conversion of an increasing fraction of the iron to a high polymer. However, a significant fraction of the iron is readily dialyzable even at the end of the buffer region, indicating that polymerization is not quantitative. The situation here is quite analogous to the polymerization of chelate-free iron.[2] Since the high polymer dissociates very slowly, the polymerization of the low molecular weight species must also be slow. The dialyzable complex cannot therefore be simply the starting material, $FeCit^-$. It is probably a hydroxy–iron citrate complex, possibly an oligomer. Its nature is considered more fully in the succeeding article.[12]

The polymer itself is remarkably similar in size and shape to that formed at low pH in ferric nitrate solutions.[2] It contains some 1.5×10^3 iron atoms in a sphere of diameter ~72 A. The analytical data show the polymer to consist largely of iron and oxide or hydroxide groups, with relatively few citrate ions, and sodium counterions. If bound citrate is assumed to have lost its hydroxyl proton, as presumably it has in $FeCit^-$, then the charge per iron of the hydroxy iron portion of the polymer is +0.35, not far from the +0.5 found in the chelate-free polymer. The hydrolytic polymerization of $FeCit^-$ is evidently accompanied by extensive dissociation of citrate, and it is an attractive hypothesis that the remaining citrates are simply attached to the surface of a hydroxy–iron polymer, which may be similar in structure to the polymer formed in chelate-free solutions. If 1.5×10^3 iron atoms are arranged in a sphere, some 32% of them would be expected to be at the surface. There would therefore be about two surface irons per bound citrate. A variety of structures in which a citrate could bridge two irons can be envisaged. Surface coverage by the citrate would serve to protect the polymer particles from condensing through hydroxyl bridges at high pH.

This model of a citrate-covered hydroxy–iron polymer is similar in structure to the protein ferritin, one of the major storage forms of iron in biological systems. Ferritin consists of an iron hydroxide micelle approximately 70 A in diameter surrounded by 20 protein subunits each of molecular weight about 2.4×10^4, which are essential for maintaining solubility.[13] The mechanism which controls the formation of the iron

(12) T. G. Spiro, G. Bates, and P. Saltman, *J. Am. Chem. Soc.*, **89**, 5559 (1967).

(13) P. M. Harrison in "Iron Metabolism," F. Gross, Ed., Springer-Verlag, Berlin, 1964, p 301.

core and the subsequent symmetrical arrangement of these protein units is currently under investigation in our laboratory.

Acknowledgments. It is a pleasure to thank Mrs. Carolyn Billups and Mr. Clyde Stitt for their valuable technical assistance in various phases of this work. The electron microscope facilities were provided by Dr. Robert Bils. The financial support of the John A. Hartford Foundation, Inc., to the laboratories at the University of Southern California and a U. S. Public Health Service Research Grant (GM 13498) to T. G. S. are gratefully acknowledged.

The Hydrolytic Polymerization of Ferric Citrate. II. The Influence of Excess Citrate

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Abstract: Addition of base to iron(III) solutions containing excess citrate ion leads to the formation at high pH of an anionic chelate which probably contains two citrates, each with its hydroxyl proton removed. This reaction competes kinetically with polymerization of the 1:1 chelate, stable at low pH. For 10^{-3} *M* iron solutions the presence of 0.02 *M* citrate is sufficient to prevent detectable polymerization. Once formed, the polymer is attacked slowly by excess citrate and by EDTA.

In the previous publication,[2] we demonstrated that the addition of base to a solution containing equimolar iron(III) and citrate leads to the formation at low pH of an anionic chelate, probably $FeCit^-$, which then polymerizes at high pH with continued addition of base. The product has a molecular weight of 2×10^5 and is spherical, with a diameter of 72 A. Most of the citrate is dissociated during polymerization and the remainder, ~15%, is probably bound to the surface of a hydroxy-iron polymer, similar to that formed at low pH in chelate-free solutions. We now wish to report on the effect of added citrate on the polymerization process. The finding that excess citrate accelerates the rate of incorporation of iron into transferrin[3] stimulated our interest in this problem. Our conclusion is that there must be a low molecular weight species containing more than one citrate per iron, probably $[Fe(Cit)_2]^{5-}$, whose formation competes with the polymerization reaction.

Experimental Section

Titration Studies at High Citrate Concentration. A series of solutions was prepared containing reagent grade 10^{-3} *M* ferric nitrate, 0.031 *M* sodium citrate, and 0.1 *M* sodium nitrate, with varying amounts of 0.01 *M* CO_2-free sodium hydroxide added last with vigorous stirring. Determination of pH was made with a Heathkit recording pH meter equipped with a Corning combination electrode, which was standardized with 10^{-3} *M* CO_2-free NaOH in 0.1 *M* $NaNO_3$. Visible and ultraviolet spectra of the solutions were recorded with a Cary Model 15 spectrophotometer, using 1-mm light-path cells. The spectra as a function of added base are shown in Figure 1. The pH titration curve for this same series of solutions is presented in Figure 2. The initial pH (no added base) is 7 and is established by citrate buffering. It has previously been shown that protons are released when iron complexes citrate ion. The protons are simply taken up by the excess citrate present. The titration curve then shows a fairly well-defined buffer region ending at 2.0 base equiv per mole of iron. Throughout this region, the spectra show a monotonic variation which ends abruptly at 2.0OH/Fe. An isosbestic point at 315 mμ is preserved throughout the titration, implying that there are only two absorbing species present. The absorbance at 280 mμ is not, however, linear with added base, Figure 2, presumably because of the participation of the excess citrate in the buffering system.

Dialysis as a Function of Excess Citrate. A series of solutions was prepared with 0.001 *M* ferric nitrate, 0.1 *M* sodium nitrate, varying concentrations of sodium citrate, and 0.003 *M* NaOH, added last as 0.01 *M* CO_2-free NaOH, with vigorous stirring. This amount of base corresponds to the end of the polymer buffer region in the 1:1 ferric citrate system, and therefore should yield the maximum polymer fraction possible. Spectra and pH values were determined as above. Duplicate solutions were prepared containing ^{59}Fe tracer, added prior to the addition of base. Dialysis was carried out using the techniques of Lindskog and Malmström[4] as in the preceding study.[2] The dialysis results are shown in Figure 3 as the log of the fraction of iron undialyzed as a function of time. As in the case of 1:1 iron to citrate, the solutions contained both a rapidly and a slowly dialyzing component. The fraction of the latter can be estimated by extrapolation to zero time of the slow phase of the dialysis curves. This fraction decreases with increasing citrate concentration, as shown in Table I, and reaches zero at about 0.02 *M* excess citrate. The rate of dialysis of the slow fraction, however, is about the same for all solutions. At the same time the spectra (Figure 4) show a monotonic variation, with an isosbestic point at 315 mμ, ending in a limiting spectrum at about 0.02 *M* excess citrate. This spectrum is identical with that found at the end of the excess citrate buffer region. The pH rises with increasing citrate concentration (Table I) to a limiting value of 10.8, at about 0.02 *M* excess citrate. This pH corresponds fairly closely to 10^{-3} *M* NaOH, *i.e.*, to the release of about one hydroxide per iron.

Proton Relaxation as a Function of Excess Citrate. The bulk solvent proton magnetic relaxation times were measured for a series of solutions containing 10^{-3} *M* ferric nitrate in the presence of

(1) (a) Princeton University. (b) Predoctoral fellow supported by Training Grant 5TO1-GM-197-08. (c) Research Career Development Awardee, U. S. Public Health Service.

(2) T. Spiro, L. Pape, and P. Saltman, *J. Am. Chem. Soc.*, **89**, 5555 (1967).

(3) G. W. Bates, C. Billups, and P. Saltman, *J. Biol. Chem.*, **242**, 2810 (1967).

(4) S. Lindskog and B. Malmström, *J. Biol. Chem.*, **237**, 1129 (1962).