The Structure, Size and Solution Chemistry of a Polysaccharide Iron Complex (Niferex)

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(Received April 20, 1988)

Abstract

A polysaccharide–iron complex (PIC), synthesized by the neutralization of an $FeCl_3$ carbohydrate solution, is marketed as an oral hematinic ('Niferex'). The chemistry, structure and solution chemistry of this complex have been examined by a variety of techniques. Elemental analyses are consistent with the report that solid PIC contains ferrihydrite in the core. Treatment of this material with ammonium oxalate yields a small molecular weight material which rapidly becomes a second, larger material, a result also consistent with the idenfication of ferrihydrite in PIC. The complex itself appears to be spherical with diameters ranging from 3 to 10 nm as determined by gel permeation chromatography and electron microscopy. The relationship between the charge and pH on the complex was studied by electrophoresis and acid–base titration. The material is negatively charged and soluble above pH 4.6. Between pH 4.0 and 4.6, PIC is neutral and insoluble, and below 4.0 it is positively charged and soluble. The carbohydrate component apparently contributes to its solubility at near neutral pHs and may be important in the reported bioavailability of the iron in this supplement.

Introduction

There is a clear need for oral iron supplements in the treatment and prevention of iron deficiency in humans. Iron loss, although low, is continuous and uncontrolled [1]. Pregnant women cannot meet their daily iron requirement from nutritional sources alone [2, 3]. In fact, the diet of most of the world's population is so deficient in iron that iron deficiency is a worldwide problem [4]. A new oral supplement is needed. Presently available oral supplements are ferrous salts [5–7] whose side effects can include constipation, diarrhea and epigastric pain. These side effects can often be severe enough to cause the termination of treatment. Dosage levels for the reversal of anemia are therefore set to the highest level that a patient will tolerate [5–7].

Polysaccharide iron complex (PIC) is a synthetic complex of ferric iron and carbohydrate marketed under the name 'Niferex' as an oral hematinic by Central Pharmaceuticals, Inc. (Seymour, IN, U.S.A.). It is reported to be effective in the treatment of iron deficiency anemia [8–10], safe (unpublished) and free of side effects. These studies, however, need to be supplemented by additional carefully controlled clinical evaluations. The structure and chemistry of PIC have not been completely examined. The iron component has been shown to be ferrihydrite [11], but nothing is known about particle size, structure or solution chemistry. These characteristics could help explain its claimed effectiveness and lack of side effects.

This study reports additional evidence for the presence of ferrihydrite as well as a number of characteristics which support the claim that PIC may be a useful oral iron supplement.

Experimental

Polysaccharide iron complex, the active ingredient in Niferex preparations, was supplied as a dry powder by Central Pharmaceuticals, Inc., Seymour, IN, U.S.A. Proteins were all from Sigma. Solutions were made from doubly deionized water.

Iron Analysis

Iron was measured by atomic absorption at 248.3 nm on a Perkin-Elmer 370 spectrometer using an iron cathode lamp and air–acetylene flame. Standard additions of $Fe(NO_3)_3$ or $Fe(NH_4)SO_4$ (1 mg/ml Fe) in 0.41 M HNO_3 were made to four unknown samples. The data were analyzed by a least-squares fit.

Sodium Analysis

Sodium was measured at 589.6 nm by either atomic emission or atomic absorption from a sodium lamp. Samples were assayed in the presence of

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2 mg/ml KCl to increase efficiency. Standard additions of 0.8 μg/ml NaCl were made to unknown samples, and the data were analyzed as described for iron.

Chloride Analysis

Chloride was analyzed by potentiometric titration with $AgNO_3$. An Ag/AgCl electrode served as the working electrode while a combination pH electrode with the reference lead detached served as a reference. The sample analyzed contained 0.4 g PIC, 25 mmol sodium acetate and 25 mmol acetic acid in 100 ml.

Results

Elemental Analysis

The results of elemental analyses for Fe, C, H, Na, Cl and O (by difference) appear in Table 1. If one assumes all of the Cl present to be in the form of NaCl and the remaining Na to be in the form of NaOH, then PIC contains 1.8 ± 0.2% NaCl and 3.8 ± 0.2% NaOH. Furthermore, if all of the C is 6-carbon aldose, then PIC is 20.3% carbohydrate, with 0.113 ± 0.001 mol monomer per 100 g PIC. After normalizing remaining H and O to five iron atoms (empirical formula for ferrihydrite), the resulting stoichiometry is $Fe_5O_{9.3}H_{4.7}\cdot(C_6H_{12}O_6)_{0.66}\cdot(NaOH)_{0.55}\cdot(NaCl)_{0.17}$. The literature stoichiometry for ferrihydrite is $Fe_5O_{12}H_9$ [12, 13]. This stoichiometry of PIC can be reconciled with the stoichiometry for ferrihydrite if $\frac{1}{2}H_2O$ per iron is added to the observed stoichiometry. This would be expected if half of the irons are on the surface of the ferrihydrite core and coordinate a ligand other than water, such as a sugar or a hydroxyl ion. If NaOH is considered equal to one water and a carbohydrate monomer equal to 3 waters, the Fe:O:H stoichiometry becomes $Fe_5O_{11.8\pm0.7}H_{9.8\pm0.2}$ which is consistent with the reported stoichiometries for ferrihydrite.

TABLE 1. Elemental Analysis of PIC

Element	Analysis[a] (%)
Iron	47.93 ± 0.38, *47.63
Carbon	*8.13
Hydrogen	*2.28
Sodium	2.85 ± 0.10, *2.98
Chloride	1.06 ± 0.10, *1.09
Oxygen	37.75 ± 1.92

[a]Determinations marked with an asterisk made by Galbraith Laboratories, Knoxville, TN, and errors assumed to be 1%. Oxygen was calculated by difference.

Oxalate Solubility

The solubility of PIC in ammonium oxalate buffer was tested by a method similar to that of Schwertmann [14]. Figure 1 shows the Sephadex G50 chromatography of PIC after different lengths of exposure to ammonium oxalate. It appears that a small intermediate is produced rapidly from the PIC complex, containing at maximum about 63% of all the iron. Almost as quickly this small material disappears, forming a larger material. With longer incubations, a portion of the smaller material remains, with the majority of the iron chromatographing with the peak of large molecules. These results suggest that PIC forms a soluble ammonium oxalate–iron polymer exceeding 5.0 nm in diameter. The solubility of PIC in ammonium oxalate is con-

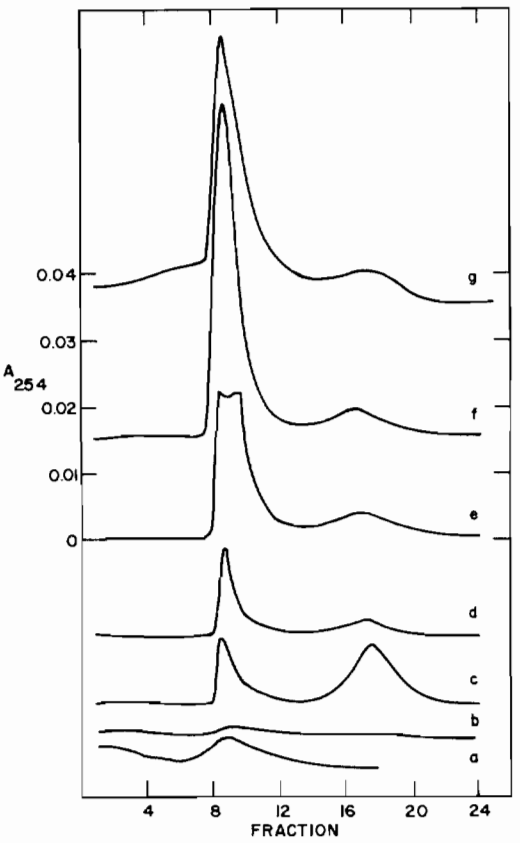


Fig. 1. Sephadex G50 chromatography of PIC after various lengths of exposure to oxalate. PIC (5 mg/ml) was incubated in 100 mM ammonium oxalate buffer (pH 3.0) at 37 °C. Aliquots (50 μl) were removed at various times, diluted into 200 μl 100 mM NaPi buffer (pH 7.4), and filtered through a Millipore HA filter (0.45 μm pore size). Samples (10 μl) were injected onto a Sephadex G50 column (1.2 × 13.5 cm) and eluted with NaPi buffer. Iron was monitored at 254 nm as 1 ml fractions were collected. Each separation took 25 min to complete. a, 0 time; b, 0.5 h incubation; c, 1 h; d, 2 h; e, 4 h; f, 5 h; g, 225 h. Baselines are shifted relative to each other.

sistent with previous identifications of ferrihydrite in PIC [11]. The large size of the complex can explain the *in vivo* inhibition of oxalate on iron bioavailability [15].

Acid–Base Titration

Solutions of PIC at different concentrations were titrated with HCl and backtitrated with NaOH (Fig. 2a and b). The first derivative curves appear in Fig. 2b, with the regions where precipitate was observed enclosed within parentheses. Two inflection points were observed in the base titration (at 450 μeq H^+/g PIC and 900 μeq H^+/g PIC) but only one inflection point in the reverse direction (900 μeq H^+/g PIC). These are marked by dotted lines. Precipitate was observed to dissolve in the base titration and form in the acid titration at about 1350 μeq H^+/g PIC, marked by a third dotted line.

$Fe(NO_3)_3$ was titrated with NaOH and interpreted in terms of known pK_as [16] in order to identify differences between PIC and inorganic FeOOH (Fig. 3). The structures of this material during the titration are unknown as is the dependence of the pK_as on the structure. Since equilibria are expected to be slow [17–20], the titration is somewhat dependent on the speed of the experiment. To aid in the interpretation of the observed transitions (eqn. (1)), the titration was also monitored for conductivity and UV absorption of the supernatant.

$$Fe^{3+} \underset{pK_a = 2.50}{\overset{1}{\rightleftharpoons}} Fe(OH)^{2+} \underset{3.06}{\overset{2}{\rightleftharpoons}} Fe(OH)_2^{+} \underset{6.34}{\overset{3}{\rightleftharpoons}}$$

$$Fe(OH)_3(S) \underset{10.35}{\overset{4}{\rightleftharpoons}} Fe(OH)_4^{-} \qquad (1)$$

High-voltage Electrophoresis on Paper

High-voltage electrophoresis on paper shows PIC to have positively charged components at pHs 2.06

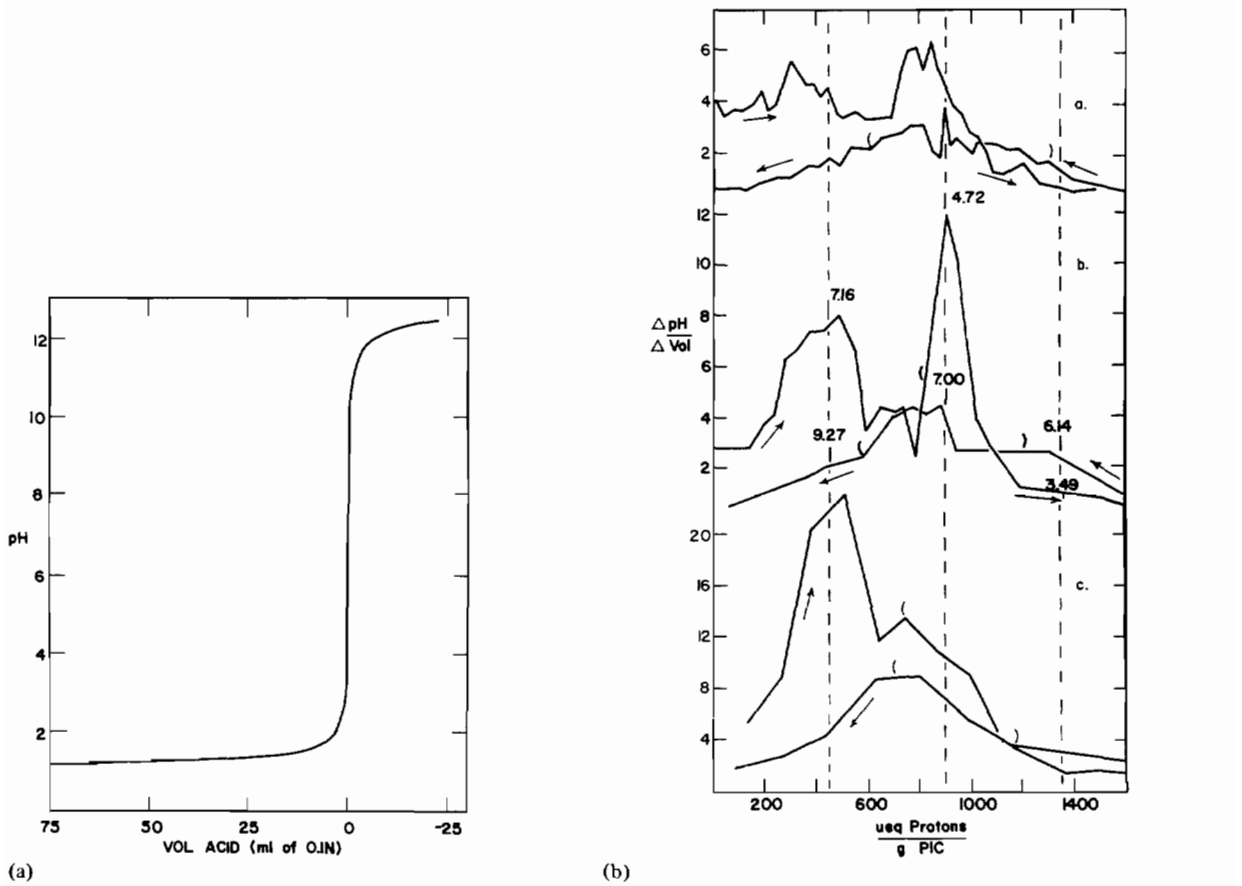


Fig. 2. Acid–Base titration of PIC. HCl (0.10436 M) and NaOH (0.05939 M) were used to titrate (a) 13 ml of 11.17 mg PIC/ml, (b) 89 ml of 1.088 mg PIC/ml, and (c) 297 ml of 110.1 μg PIC/ml. Panel '2a' shows the titration of sample (b). Panel '2b' shows the first derivative curves of the titrations of all three samples. Back titrations are marked with a reverse arrow. Titrations were performed at room temperature with approximately 1 min allowed between additions for equilibrium to be reached. pH was monitored with a combination electrode and a Fisher model 610 pH meter. Theoretical equivalence points are marked by vertical dotted lines. Regions of titrations where precipitate was observed are enclosed by parentheses.

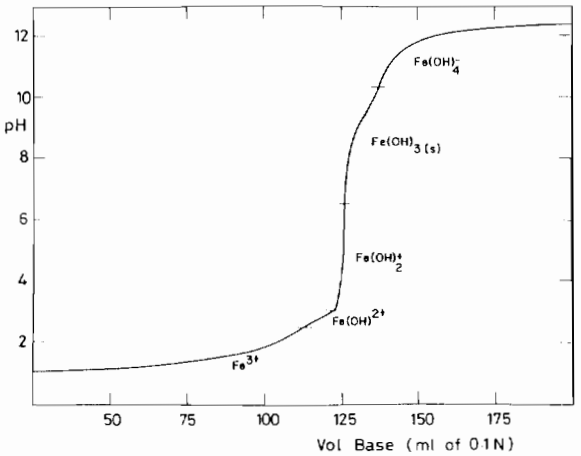


Fig. 3. Acid–Base titration of $Fe(NO_3)_3$. 30 ml of $Fe(NO_3)_3$ solution (1 mg/ml) was titrated with NaOH as described in Fig. 2. Theoretical pK_as are marked by horizontal tick marks.

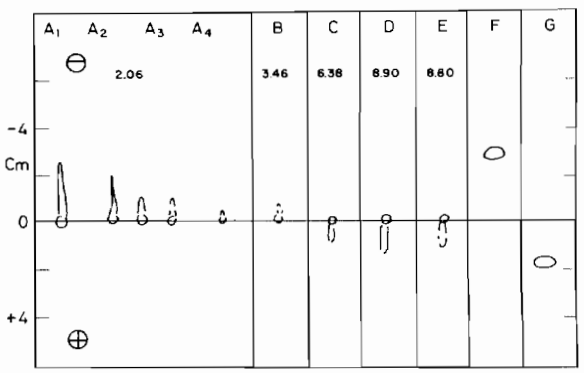


Fig. 4. High-voltage paper electrophoresis of PIC at different pHs. Aqueous samples of PIC were spotted onto Fisher filter paper grade P8 and air-dried. The paper was saturated with buffer and developed for 60 min at 450 V at room temperature in a Savant apparatus. Standards were run in adjacent lanes. Buffers were: A: pH 2.06 (23 ml 89% formic acid, 80 ml glac. acetic acid, 897 ml H_2O); A_1–A_4: increasing amounts of PIC spotted; B: pH 3.46 (4 ml pyridine, 2.4 ml glac. acetic acid, 720 ml H_2O); C: non-buffered; D, E: pH 8.90 (1% $(NH_4)_2CO_3$ in H_2O); F: Malachite green standard run in pH 2.06 buffer; G: Bromophenol blue standard run in pH 2.06 buffer. PIC spots became blue when the dried chromatogram was sprayed with 2% $K_4Fe(CN)_6 \cdot 3H_2O$. The color was shown to closely follow the iron present by eluting sections of unsprayed strips with water and measuring the iron of each section by flame atomic absorption.

and 3.46, and negatively charged components at pHs 6.38 and 8.90 (Fig. 4). This is in accord with the titration evidence above. The patterns reveal several components differing in either their charge or adsorption onto the support. In all patterns the majority of the iron remained at the origin. PIC could not be eluted cleanly from the paper by the chosen buffers, revealing the material at $R_f = 0$ to be adsorbed rather than lacking charge.

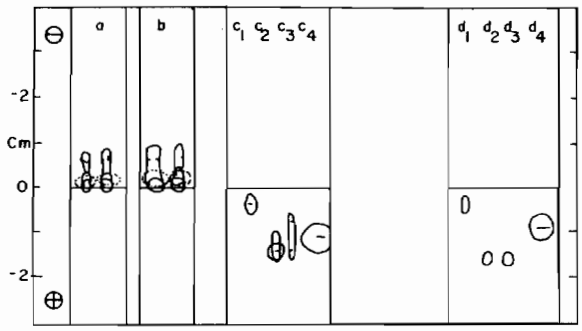


Fig. 5. Cellulose acetate electrophoresis. Cellulose acetate strips (Gelman Instruments), 1 × 6 in, or disks (Millipore) were soaked in buffer and preelectrophoresed 30 min at 0.4 mA/cm width in close contact with a heat sink consisting of a plastic bag filled with room temperature water. Samples were spotted onto the damp, preelectrophoresed strip and electrophoresed immediately for 10 min. Bromophenol blue (mobile standard) and dextrose (solvent front) were standards run in adjacent lanes. Carbohydrate was visualized by 'printing' the electrophoretogram onto a picce of filter paper, which was then air-dried, sprayed with $AgNO_3$ solution (100 μl $AgNO_3$–saturated H_2O and 20 ml n-butanol) and sprayed with 0.5 M NaOH in EtOH. Dextrose appeared dark brown. Iron was visualized by dipping the electrophoretogram in 60 mM $K_4Fe(CN)_6 \cdot 3H_2O$ in 1 M HCl. Buffers were: a, b: glycine–NaCl buffer, pH 2.19 [21]; C: pH 6.06 sodium phosphate (NaPi) (100 mM); d: pH 7.4, NaPi (100 mM). Samples were: a, c_2, d_2: PIC; b, c_3, d_3: PIC in higher conc. than in a, c_2, or d_2; c_1, d_1: bromophenol blue c_4, d_4: dextrose.

High-voltage Electrophoresis on Cellulose Acetate

High-voltage electrophoresis on cellulose acetate shows that sodium phosphate buffer (100 mM) completely solubilized PIC at pH 7.4, while this was incomplete at pH 6.0 (Fig. 5). At pH 7.4, PIC moved toward the anode 1.51 cm in 60 min as a single spot. Dextrose (solvent front) moved 0.96 cm, and bromophenol blue moved 0.32 cm in the same direction. This result further shows that PIC is negatively charged at pH 7.4. At pH 6.0 PIC also moved (1.29 cm) ahead of the solvent (1.09 cm) toward the anode. No heterogeneity was observed.

In buffers with pHs below 6.0 the electrophoretic behavior of PIC on cellulose acetate was similar to that observed on paper: the majority of the material remained at the origin with multiple spots present and some streaking toward the cathode being observed. PIC is soluble in glycine–NaCl buffer (pH 2.19). Electrophoresis in this buffer on cellulose acetate showed freshly dissolved PIC to have neutral and positively charged components at this pH. The neutral component may have been incompletely solubilized PIC. Otherwise this result is consistent with the model represented by eqn. (2).

Dialysis

To establish a lower limit on the size of PIC at several pH points, 5 ml of an aqueous PIC solution (2.17 mg/ml) was dialyzed against 2 l of 50 mM sodium phosphate buffer (pH 7.35) overnight at room temperature. The membrane (Spectrapore) had a molecular weight cut off of 3500 corresponding to a pore size of 2.5 nm. The solutions inside and outside the membrane were analyzed for iron. Only 0.47% was dialyzable (96% yield overall). PIC appears to be larger than 2.5 nm at pH 7.4.

High-pressure Liquid Chromatography

Aliquots (25 μl) of freshly dissolved PIC (1 mg/ml) were injected onto a Water I-125 gel permeation column thermostatted at 37 °C and run at 2.0 ml/min. The buffer was 100 nM sodium phosphate (pH 7.4), purged of gasses with He. Calibration standards were blue dextran, bovine serum albumin, lysozyme, bovine superoxide dismutase, cytochrome *c*, and tryptophan. The effluent was monitored for iron by UV absorption at 254 nm and/or by atomic absorption. All of the iron eluted at the void volume, showing the PIC to exceed 3.5 nm in effective size.

Gel Permeation Chromatography

Gel permeation chromatography on a calibrated column of Sephacryl S-300 (Fig. 6) of PIC freshly dissolved in 100 mM NaPi (pH 7.4) showed the main component to be 6.3 ± 0.6 nm in diameter with minor components of 5.0 ± 0.3 nm, and 3.9 ± 0.3 nm and 12.0 ± 0.7 nm. A representative chromatogram appears in Fig. 7. Samples incubated in buffer for 1 h (room temperature) showed peaks corresponding to

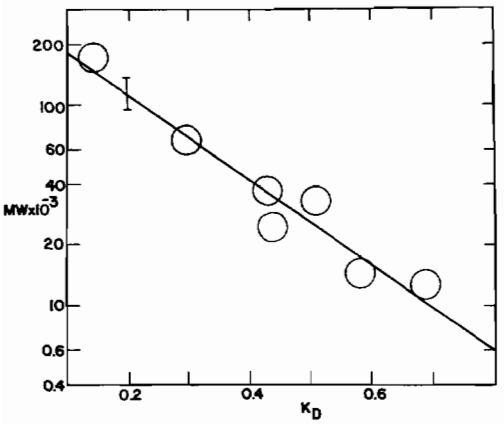


Fig. 6. Calibration of Sephacryl S-300 SF column. Calibration samples (0.75 ml) were injected onto a column (2.6 × 80 cm) of Sephacryl S-300 SF (Pharmacia) equilibrated with buffer at room temperature. The standards were blue dextran, porcine kidney diamine oxidase, bovine serum albumin, β-lactoglobulin, bovine pancreas trysinogen, bovine superoxide dismutase, egg white lysozyme, horse heart cytochrome *c*, and tryptophan.

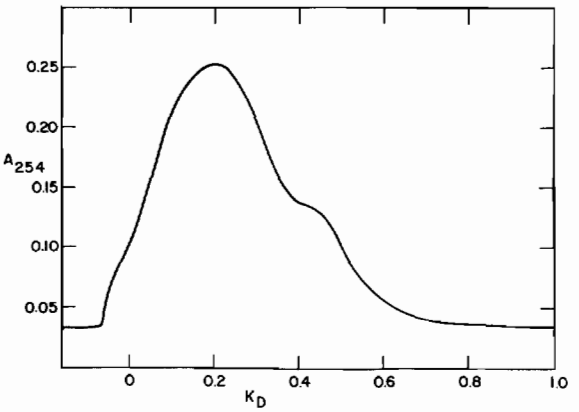


Fig. 7. Sephacryl S-300 gel permeation chromatography of PIC. A 0.75 ml sample of PIC (5 mg/ml) freshly dissolved in 100 mM NaPi (pH 7.4) was injected onto the column described in Fig. 6. UV absorption (254 nm) was monitored as 10 ml fractions were collected, which were later assayed for iron by flame atomic absorption. Errors in the sample measurements were calculated from the least-squares fit of the standards (Fig. 6). Protein molecular weight was converted to diameter of an equivalent sphere by assuming the average protein to be spherical with a density of 0.75 g/ml.

essentially the same sizes but with a larger fraction of 5.0 nm material. Fractions centered on each peak were concentrated 20-fold by rotary evaporation and rechromatographed. The retention volume of each peak corresponded to sizes within experimental error of the original peaks. An additional sample was rechromatographed without concentration with no difference in retention volume. The concentration of solute or buffer must, therefore, not be important. PIC does not appear to contain any free iron resembling, in this respect, Imferon [22].

Electron Microscopy

When PIC is suspended in acetone and deposited on a grid, only very large fractured fragments (0.2–0.4 μm) are observed. This is most likely the form of the air-dried powder, since PIC is not soluble in acetone. A collodion film was also prepared [23], coated with carbon by glow discharge, and the sample applied to the grid [24].

When PIC is first dissociated in water for 1 h, a distribution of particle sizes is seen ranging from 3.0 to 43.3 nm in diameter. The particles exceeding 10 nm are irregularly shaped. The majority of the particles, however, are between 3.0 and 6.0 nm in diameter.

Discussion

Previous studies using Mössbauer spectroscopy and X-ray diffraction have shown the component of PIC to be ferrihydrite. The stoichiometry between

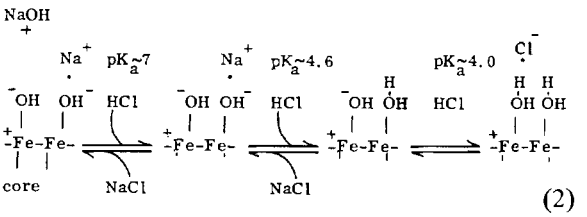
iron, oxygen, and hydrogen reported here (Table 1) are consistent with ferrihydrite. The stoichiometry must be considered approximate, since the elemental analyses are based on the dry weight of the undessicated powder, and may be subject to weight changes due to humidity. Changes in the total degree of hydration may also be effected by particle size due to changes in relative surface area. The presence of ferrihydrite makes PIC similar to the core of ferritin [25] and may, therefore, serve as a useful model. Ferritin cores clearly differ from PIC in one respect, however, in that the former contain phosphate absorbed onto mineral [26].

The titration curve of $Fe(NO_3)_3$ (Fig. 3) does not match what one would expect from the published K_{eq}s [16]. The transition expected at $pK_a = 3.06$ (reaction 2, eqn. (1)) does not appear as a clear inflection point. This is probably due to the high favorability of precipitate formation (reaction 3, eqn. (1)) which shifts the reaction in the forward direction. A second anomaly is that the precipitate does not redissolve easily at pHs above 10.35 as would be expected, which can be attributed, in part, to the slowness of reactions 3 and 4 [17–20]. Solutions of PIC were titrated with acid and back-titrated with base (Fig. 2a and b). Although the attainment of equilibria in these reactions is very slow [17–20], an estimation of the pK_as and the number of charges is possible.

The first inflection point marked by the dotted line on Fig. 2b at 450 μeq H^+ per g PIC is interpreted to be the titration of unbound NaOH. This NaOH is not part of the structure and does not appear in the backtitrations (Fig. 2b). It composes 1.80% of the solid and was not included in the calculation of the stoichiometry in the previous section, even though the changes in the Fe:O:H ratios are not significant.

The second inflection point ($pK_a \cong 4.6$), marked by a dotted line at 900 μeq H^+ PIC on Fig. 2b, is most likely the titration of NaOH coordinated to the iron. The bound hydroxyl ions give the complex a net negative charge which probably contributes to the solubility of PIC at pHs > 4.6. The total of bound and unbound NaOH equiovalents (900 μeq H^+/g) means PIC is 3.6% NaOH, a value which is within experimental error of the determination by elemental analysis.

By analogy to the titration with base of Fe(III) (Fig. 3) a third inflection point is expected in the titration of PIC for the protonation of iron-bound hydroxyls which are counterions to the positive charges on the iron. No inflection in the titration curve is in fact observed, but precipitated PIC does reversibly but slowly redissolve as the pH falls below 4.0 (1350 μeq H^+/g PIC). This point is marked as another dotted line on Fig. 2b at 1350 μeq H^+/g PIC. The inflection point may not be visible due to the slowness of this reaction. This is also consistent with PIC carrying a positive charge at low pHs contributing to its solubility. The number of positive charges at low pHs seems to be the same as the number of negative charges observed at high pHs. At pHs between 4.6 and 4.0 the complex is an insoluble precipitate due to the lack of charge. Equation (2) outlines the interpretation of all the titration points.



(2)

It is notable that PIC is soluble and charged at *both* the neutral pHs encountered in the mouth and intestine and at the low pH of the stomach. Although a similar reversal of charge is seen with $Fe(NO_3)_3$ between low and high pHs, the pK_as observed with PIC are different enough from those of $Fe(NO_3)_3$ to implicate the bound carbohydrates in PIC's solubility. The carbohydrate component of PIC may also be involved in the reversibility of precipitation at pHs 4.6 and 4.0. This characteristic, of being soluble even after neutralization of an acidic solution, may also contribute to the bioavailability of PIC [27].

The charges observed for PIC at different pHs are similar to those observed with similar materials. Imferon is negative at pHs 6.8, 8.6 [27] and pH 7.6 [28]. Iron sorbitol at 7.6 (28) and β_2FeOOH at alkaline pHs [29] also are negatively charged. This contrasts with α-, β-, and δ-FeOOH which do not form soluble carbohydrate complexes at alkaline pHs [29]. All FeOOH forms are positively charged at low pH [29] except iron sorbitol [28].

Within the limits of resolution of the Sephacryl S-300 column used for gel permeation chromatography, a minimum of three sizes for PIC can be detected (Fig. 7), the major fraction containing species which are 6.3 ± 0.6 nm in diameter. A second fraction which decreases in proportion with increasing freshness of the PIC sample contains species which are 5.0 ± 0.3 nm in diameter, while a third minor fraction appears to represent a diameter of 3.9 ± 0.3 nm. All three fractions rechromatograph at the same retention volumes, and thus appear to be stable sizes within the time course of the experiment.

The column does not resolve the peaks well, suggesting that there may be a continuous distribution of sizes, with the three sizes mentioned having a higher probability. In addition, the patterns reported may be specific for the time course of the experiment: one size may actually be more stable than the others.

A greater upper limit on the size of PIC is observed in the electron microscope than has been reported previously for similar material [18, 30]. The larger particles appear to decrease in size and number as PIC remains as an aqueous solution. The size distribution observed is more in agreement with the size range reported for similar materials [18, 30] and with the range determined by Sephacryl S-300 chromatography. We conclude that PIC slowly disintegrates in water from the 1 to 0.1 μm particles of the air-dried powder to a 3.0 to 10.0 nm distribution.

The size range reported here is consistent with Mössbauer results [11] reported previously (3.5–10 nm) and other studies on ferrihydrite (2–5 nm) [31]. Other FeOOH polymers form complexes of similar size. Goethite (α-FeOOH) forms spheres 1.5–3.0 nm in diameter, which can then polymerize and coalesce into cigar-shaped crystals [32]. Akagenéite (β-FeOOH) also forms spheres of similar size (3.0 nm) [30] as well as spindles [33]. Iron citrate forms 7.2 ± 0.9 nm spherical polymers [19]. Imferon, and FeOOH–dextran complex reportedly containing akageneite [33, 34], is also a 3–4.0 nm sphere [33, 35]. PIC is also similar to ferritin cores in size and shape. The maximum core size in ferritin is 6.0–7.0 nm [25, 36, 37].

Acknowledgements

Gratitude is extended to the Burroughs-Wellcome Co. and C. G. Schreiner for the use of high-voltage electrophoresis equipment, and to Professor L. A. Jones for the use of his HPLC facilities. Cellulose acetate strips were a gift from Burroughs-Wellcome Co. The complex, PIC and support for this work were generously supplied by Central Pharmaceuticals, Inc.

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