The Molecular Formula and Proposed Structure of the Iron–Dextran Complex, IMFERON

E. LONDON

School of Pharmacy and Biomedical Sciences, St. Michael's Building, White Swan Road, University of Portsmouth, Portsmouth, PO1 2DT, UK

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ABSTRACT: The first iron–dextran complex was discovered in 1953, when we attempted to synthesize an analog of ferritin, by substituting polysaccharide for its protein shell. This new complex soon became the most widely used parental therapy for hypochromic anemia in humans. No molecular formula has been proposed, but Cox has attributed an outline structure to it. The present article proposes a structure greatly different from the Cox model, by having a polynuclear β-ferric oxyhydroxide core, closely similar or identical to Akaganeite, chelated firmly by an encircling framework of dextran gluconic acid chains and surrounded by a removable outer sheath of colloidal dextran gluconic acid. The molecular weight of the iron–dextran core molecule, including its chelated framework, has been determined by gel filtration and analysis and its molecular formula (1.3) calculated. Also, these new data and existing electron photomicrographic, X-ray diffraction and crystallographic studies, have enabled a molecular weight, formula, and model structure to be proposed for its complex (2), which includes the outer sheath. The 480 iron atoms in both the core molecule and its sheathed complex are close to the number calculated from the core's unit cell dimensions and volume. © 2004 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 93:1838–1846, 2004

Keywords: iron–dextran complex; IMFERON; proposed structure

INTRODUCTION

The first synthesis of an iron–dextran complex was announced in a letter[1] to the British Medical Journal by Fletcher & London (1954), wherein the medical need for such a product was described, together with an outline of its properties. In the same year a patent[2] was assigned to London & Twigg, giving details of the preparation and properties of the complex. Samples are still stable nearly 50 years after manufacture. This iron–dextran complex called IMFERON (Fison^{TM}), is specified in the British Pharmacopoeia (2000) and referred to forthwith as the complex or "Product A."

It regenerates hemoglobin quickly and efficiently in humans and piglets, and is well tolerated by both. It can be a life-saving treatment for mothers close to confinement with a low hemoglobin level.

Essentially, all piglets in the UK and many elsewhere, receive their life store of iron from it within about 2 weeks of birth.

A resurgence of interest in iron–dextran occurred in the 1990s following its widening use in hemodialysis and in imaging techniques. It has the ability during dialysis to effectively reduce blood makeup, which conserves both supply and expenditure.

About 40 years after the discovery of IMFERON, Fison withdrew from the market, following an alleged difference on quality control with the FDA. in their American plant.

The new prospective manufacturers such as Luitpold Pharmaceuticals, Inc., Shirley, NY, etc.,

Correspondence to: Eric London (Telephone: 02 392 551366; E-mail: londonye@aol.com)

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have concentrated their efforts on securing control of molecular size, stability, reproducibility, and sterility of their iron–dextran complexes, as indicated by their publications, for example, Lawrence RJ, 1998.[22]

The elucidation of factors controlling absorption at the intramuscular site and those influencing the antigenicity of the complexes should also be important objectives.

IMFERON may no longer appear under this trademark, as new legitimate synthesizers may well use their own for essentially the same product, or indeed market different iron–dextran complexes under various trademarks.

We had found initially that alkaline treatment of the dextran was essential for good stability of the iron–dextran complex but only realized later that the reducing end groups of the dextran had largely been converted to carboxyl.[3] This led to a study of the behavior of the complex in distilled water on a mixed-bed resin, showing that the main fraction passed readily through the column, while any uncomplexed carboxylated dextran was retained.[4] These important findings appeared as internal company reports by E. London, Head of Organic Research, 1951/1956, and were held unpublished until 1968, when a Fison patent[5] revealed the carboxylation.

Numerous clinical,[6–8] veterinary,[9,10] pharmacological[11]/physiological,[12] and several structural publications[13–18] appeared pertaining to this complex during 1959–1972. Later references primarily concern the iron core,[19–21] with a publication in 1998[22] describing new developments of iron dextran products.

However, none of these publications has given a molecular or structural formula for the complex or any of its components.

This article has gone some way towards rectifying the situation by isolating a core molecule from the complex, determining its molar mass and molecular formula (1.3) and then proposing a chemical structure (Fig. 2) for the core molecule; as well as a molecular formula (2) and model (Fig. 6) for the whole iron–dextran complex ("Product A"), comprising the core molecule wrapped in its colloidal sheath.

MATERIALS AND METHODS

Methodology for Gel Filtration[23]

A column 1.2×50 cm of Sephacryl S-300 HR (allyldextran-N, N1-methylene bisacrylamide) was used, together with 0.05 M potassium phosphate buffer (pH7.4) as eluant, containing 6.8 g KH_2PO_4 and 8 g NaOH per liter.This was fed from a header tank connected to the top of the column, a constant pressure (5–6 psi.) being supplied to it by an air pump with a controlled leak valve. The pressure line was transferred directly to the top of the column when the iron–dextran solution was being absorbed prior to development. Iron–dextran (1 mL) containing at least 2 mg/mL was usually applied, giving a clean separation of the core molecule from its sheath. One milliliter fractions per minute were collected automatically for analysis. The concentrations of other compounds applied to the column such as dextrans and their fluorescent derivatives, varied from 2–5 mg/mL, depending on the analytical procedure, molar absorptivity, or fluorescence.

Iron Assay

Gel filtration fractions were assayed spectroscopically at 430 nm. Standard curves prepared from commercial samples of "Product A," followed the Beer-Lambert law at least over the range of 0.01–0.2 mg/mL Fe, even though 430 nm is not a true λ max. The iron potency of "Product A" was based on an ammonium cerium (IV) sulphate/ferroin assay of the reduced test solution. (BritishPharmacopoeia, 2000).[24]

Dextran/Dextran Gluconic Acid Assays

The British Pharmacopoeia assay[24] for dextran was found to work satisfactorily for dextran and dextran gluconic acid, after some important modifications in detail but not in principle. The assay involves reaction of an aqueous solution of the dextran or its derivative with a 2% Anthrone solution in concentrated sulphuric acid containing 5% water. On heating a deep blue colour develops with a λ max. of 625 nm.

As the extent of the absorbance at 625 nm was sensitive to both time and temperature, we optimized these factors (to 16 min at 90°C), following literature references[25,26] and our own studies. The precision was further improved by keeping the Anthrone reagent at 0–5°C or cooler and using it within 2 or 3 days. Also, dextran gluconic acid was used as its own internal standard, a fresh calibration curve being prepared for each series of assays. These were carried out in triplicate, giving a precision of $\pm 5\%$ of the mean triple absorbance. The precision was improved to

±3% if the reagent and test solution were separated by 1 mL of water to prevent premature interaction. This was achieved by dispensing 4 mL of Anthrone reagent at 0–5°C into each test tube, followed carefully by 1 mL water and then 1 mL of test solution without mixing. Triplicate tubes and their contents were then shaken together and heated at 90°C for 16 min in a waterbath, cooled in crushed ice and read at 625 nm.

A blank correction should be made to the final absorbance by substituting 2 mL of water for the 1 mL of water and 1 mL of test solution in the triplicate assay.

Knowing the absorptivity of the test solution, its potency could be derived by reference to the current standard curve.

Determination of the Molar Mass of the Core Molecule

Gel filtration on a column of Sephacryl S-300 HR was used (see methodology above), taking advantage of the relationship between elution volume (V_e), void volume (V_0), packed bed volume (V_t) and molecular weight/size established by Laurent and Killander[27]/Ogston[28]/Granath[33] and summarized in the equations $\text{Kav} = V_e - V_0 / V_t - V_0$ and $\text{Kav} = \text{m log MW}$. Consequently, determination of the V_e values of a series of polysaccharides of known molar masses, allows their Kav values to be calculated and a plot to be drawn of Kav versus log MW. Then a related compound of unknown molar mass can be allocated one, simply by determining its V_e value on the same column, calculating its Kav, and using the log plot of the series.

In practice, the elution volumes of a series of dextran fractions or their fluorescein isothiocyanate (FITC) derivatives of known molecular weight (ex Sigma Chemical Company, Poole, Dorset, BH12 4QH, England) were determined, using the same Sephacryl S-300 HR column and packing at approximately 14°C. The FITC derivatives, where available, enabled the elution to be followed visually and measured quantitatively using their λ max at 490 nm. For colourless dextran fractions, such as dextran MW.74,000 (ex Sigma), the Anthrone assay was used on multiple fractions collected at timed intervals (usually 1 min), as neither a flow-through refractometer or pulsed amperometric detector were available at the time. Plotting the cumulative elution volume versus absorbance for each fraction, gave a series of curves, the peak value of each representing the elution volume (V_e) of the particular solute. The use of blue dextran (MW $\sim 2 \times 10^6$) gave the void volume (V_0) and the pack dimensions gave (V_t).

Added precision could sometimes be given to a peak value by differentiating the equation for the curve in question (of absorptivity versus cumulative eluant volume) and obtaining the x value where $dy/dx = 0$. This is the (V_e) value at peak absorptivity used to calculate Kav above.

Progressive Release of Iron from the Iron–Dextran Complex ("Product A"): Expt.544/1

The reduction of the iron–complex with thioglycollic acid (or hydroxylamine) resulted in the release of ferric ions as ferrous, giving a deep red color with α,α′-dipyridyl [Fe^{2+}(dipyridyl)$_3$]. The extent and rate of this loss of ferric ions was revealed as follows: 1 mL of "Product A" containing 50 mg Fe was diluted with deionised water to 100 mL. To 50 mL of this 0.5 mg/mL Fe solution was added 46 mg of thioglycollic acid (equivalent to ~25 mg Fe), retaining the remaining 50 mL for Expt. 544/2. The solution of complex + thioglycologlycollic acid was diluted with water to 0.1 mg/mL Fe and 5 mL of this solution was added to1 mL of 20 mg/mL α,α′-dipyridyl in 0.5 N HCl. After further dilution with water to 0.01 mg/mL Fe, the absorptivity at 522 nm [λ max for Fe^{2+}(α,α′-dipyridyl)$_3$] and the time were recorded. These readings were repeated at hourly intervals until the absorptivity plateaued.

Expt.544/2

The above experiment was repeated using only half the thioglycollic acid.

RESULTS AND DISCUSSION

Initially (1953), our search for the structure of the iron–dextran complex ("Product A") began with the use of mixed bed ion exchange incorporating both strong anionic and cationic resins (formerly "Biodeminrolit") (see Introduction). In the current work, Amberlite 400 and 402 (Rohm & Haas Co., Philadelphia, PA) were at first used to purify the commercial iron–dextran complex ("Product A") from any uncomplexed dextran gluconic acid, possibly remaining from the biosynthetic process. However, gel filtration on Sephadex or Sephacryl columns made it possible to visualize the devel-

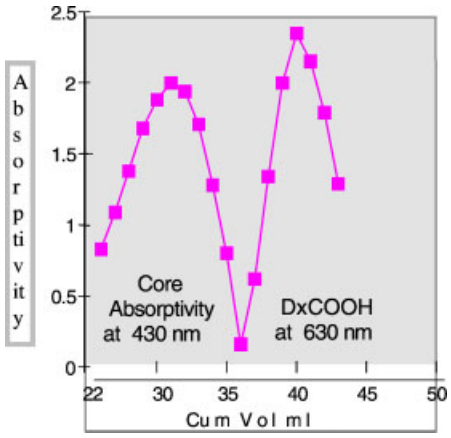

Figure 1. Separation of iron–dextran core molecule from its sheath (DxCOOH) in "Product A." [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

opment and elution. Sephacryl S-300 HR also showed good stability, flow rate, high resolution, and covered the dextran molar mass range of 2000–400,000 Da, and so was used in subsequent work.

The complex was resolved into two fractions, one containing iron and dextran gluconic acid, the other dextran gluconic acid (abbreviated to dextran acid or DxCOOH; this consists of 31 essentially 1:6 linked anhydroglucose units, the reducing end group of the final unit having been oxidized to carboxyl, MW 5056) in a clean separation. A typical graph of this separation is shown in Figure 1.

Attempts to repeat the gel filtration of the iron–dextran acid fraction to see if more could be removed, always resulted in polymodal separations. This led to the conclusion that before its removal from the column, the uncomplexed DxCOOH had probably been acting as a protective sheath around what it is proposed to call the "core molecule," the sheath preventing interaction between the core molecule and the dextran derivative column (see Materials and Methods). This was partially confirmed by comparing the stabilities of the eluted core molecule and the original complex, after each had been diluted (1:900) with water and allowed to stand. The core precipitated ferric oxyhydroxide after 35 days, while the complex survived 63 days. The use of a nondextran derivative column in place of Sephacryl, for example, "Toya Pearl," may well allow repeat gel filtration without degradation and support our hypothesis of column interaction.

The molar mass estimate (MW) of this isolated core molecule was determined as described under Materials and Methods, yielding the data summarized in Table 1.

The key relationship, $\mathrm{Kav} = V_e - V_0/V_t - V_0$ allowed the conversion of elution volumes (V_e) to Kav values, representing the fractions of the gel volume available to the solutes used.

Plotting Kav versus log MW from Table 1 gave a sigmoidal curve linear over a wide range, the linear section having an equation $y = 3.6749x + 5.6101$, where $R^2 = 0.9855$ ($y = \log$ MW and $x =$ Kav).

This technique enabled a molar mass estimate to be made for any related gel filtration fraction, given its elution volume and gave a molar mass estimate for the iron–dextran core molecule of ~105,000 Da, replacing the 73,000 assigned by Cox et al.[18]

Having now determined the molar mass estimate of the core molecule and the molecular formulae of both the core molecule and the original complex, the molar mass estimate of the complex could be calculated, providing we knew the ratios of iron to dextran gluconic acid in each. So using the same assays for iron and dextran gluconic acid as had been used to obtain the elution profiles in Figure 1 (see Materials and Methods), these ratios were found to be 1:1.7 and 1:4 for the core and complex, respectively. Knowing the ratios, the molar mass of the core and the fact that the iron is present as β-FeO.OH,[17,21] the molecular formulae of both the core (1.3), the original complex (2), and the molar mass of the complex were calculated as shown below.

Calc. of Mol. Formula of Core Molecule

Let the molecular formula of the core molecule be

$$(\mathrm{FeO.OH.2H_2O^{*}})_a\ (\mathrm{DxCOOH})_b \qquad (1)$$

where a and b are uknown integers.

This formula must also account for the molar mass estimate of 105,000 Da determined above. This gives the equation:

$$(55.84 + 69)\mathbf{a} + (5056^{**})\mathbf{b} = \mathrm{MW} = 105{,}000 \qquad (1.1)$$

Having already found the ratio of iron to dextran gluconic acid in the core molecule to be 1:1.7, we obtain $55.84\mathbf{a}{:}5056\mathbf{b} = 1{:}1.7$. giving the equation:

$$55.84\mathbf{a} \times 1.7 = 5056\mathbf{b} \qquad (1.2)$$

Table 1. Data Required for MW Determination

Sample Ref.	MW	V_0	V_e[b]	Kav	Log MW
Blue Dx IX/63	$\sim 2 \times 10^6$	22	—	—	—
FITC[a] Dx IX/70	50,700		28.15	0.24	4.7050
Dx IX/35	74,000		27.25	0.21	4.8692
FTIC Dx IX/41	145,000		25.05	0.12	5.1614
Iron–Dx IX/31	(**105,200**)		26.2	0.16	(5.013)

[a]Fluorescein derivative label (ex Sigma plc.)
[b]Each Ve value is an average of a number of expts.

where **a** and **b** are the same unknown integers as in eq 2.

Solving eqs 1.1 and 1.2 for their common roots **a** and **b**, we find

$$a = 477.8 \quad \text{and} \quad b = 8.87^{***}$$

Therefore, the molecular formula of the core molecule is:

$$(FeO.OH.2H_2O)_{478}\,(Dx.COOH)_9 \qquad (1.3)$$

$$MW = 105,000 \text{ Da (by expt.)}$$

*This agrees with the dihydrate of FeOOH being present, as required by the proposed structural formula, Figure 2. **MW 5056, based on an intrinsic viscosity of (0.05) for the original dextran, with the addition of a carboxyl end group to the terminal anhydroglucose unit. ***Further details can be obtained from the author.

Calc. of the Mol. Formula of the Complex ("Product A")

Fe:(DxCOOH) in Core Mol. = 1:1.7 by wt

Fe:(DxCOOH) in the Complex = 1:4.0 by wt. No. of (DxCOOH) in Core is 9.

No. of (DxCOOH) in the Complex is 9x (4.0/1.7) = 21.2

Therefore Molecular Formula of Complex is:

$$\{(FeOOH.2H_2O)_{478}(DxCOOH)_9\}(DxCOOH)_{12} \qquad (2)$$

covalent core + colloidal sheath

$$MW = 166,000 \text{ Da (by calc.)}$$

As the molar mass estimate of the Core Molecule (1.3) was 105,000 Da, the MW calculated for the Complex (2) from its molecular formula = 166,000 Da.

This is supported by the zone centrifugation data (>156,000 Da.) of Ricketts et al.[14]

Our earlier work using iron exchange (see Introduction) had established that this main fraction eluted by gel filtration had a smaller molar mass than the original complex and is named here the "core molecule."

To accommodate the new molecular features of the iron–dextran complex ("Product A"), that is, two new molecular formulae and their corresponding molar mass estimates, it is proposed that a ligand structure is adopted for the polynuclear β-ferric oxyhydroxide core molecule. This was inspired by Muller's[30] 1967 article on iron hydroxide complexes.

This proposal seals the core between two DxCOOH ligands, the remaining seven ligands being wrapped probably in random helices (an expandable coil formation for the ligands would be typical of dextran chains of MW ≅ 2000–10,000[32]) around the core and attached to it by covalent links at their carboxyl ends and hydrogen bonds at the other.

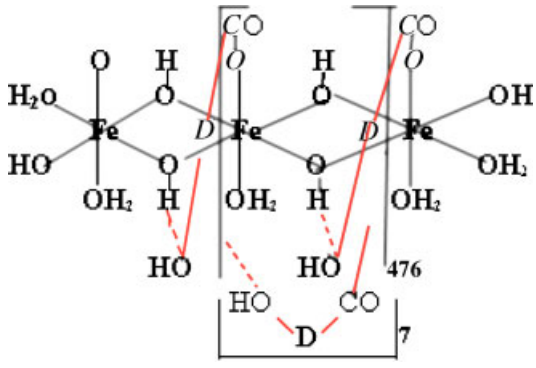

Figure 2. Proposed iron dextran core molecule. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

Figure 2 shows this proposal, the initial and terminal dextran gluconic acid ligands appearing in italics.

The dimensions of the metal shadowed electron photomicrographs of either the core molecule or complex do not support the extended radial dextran acid chains proposed by Cox,[18] although at that time the separate existence of a core molecule was not recognized. It was thought to be the iron dextran complex ("Product A"). The complex is now considered to combine both the core molecule and its associated dextran acid sheath.

However, Figure 2, while quantitatively accounting for the iron–dextran hydrated polynuclear ferric oxyhydroxide core with its dextran gluconic acid framework and newly determined molar mass, nevertheless does not indicate the intricate 3D nature of the core, which is shown essentially as a ferric oxyhydroxide polymer for purposes of clarity.

The core detail was revealed first by the work of Towe[19] and extended by Kilcoyne and Lawrence,[21] the latter using Mossbauer spectroscopy and Rietveld refinement of their X-ray powder diffraction data. These techniques were used earlier by Buckwald and Post,[31] on powdered crystallites of Akaganeite obtained from an iron–nickel meteorite. The iron–dextran cores and the natural Akaganeite were shown to be essentially identical by Kilcoyne and Lawrernce, although Towe had indicated their great similarity.

Kilcoyne and Lawrence concluded that the core consisted of β-FeOOH with a monoclinic unit cell, space group I2/m, containing two nonequivalent Fe^{3+} sites in a distorted octahedral environment. Their data gave a projected crystal structure for an iron–dextran core down the b-axis (Fig. 4), which could be regarded as a ball and stick equivalent of the space-filled unit cell of natural Akaganeite (Fig. 5), based on Buchwald and Post's article.

This article gave unit cell dimensions for Akaganeite of $10.6 \times 3.0339 \times 10.513$ Å containing 8 Fe, 16 O, and 2 Cl atoms (Fig. 5), giving a core density of 0.0422 Fe atoms/nm^3.

These data are close to those of Kilcoyne and Lawrence.[21]

A 3.5-nm diameter spherical core with this structure would contain 532 Fe atoms and so approximates to our formula (1.3), which contains 478. Rutherford[17,18] had proposed a 3–4 nm core from electron photomicrographs.

The chlorine atoms shown in the unit cell (Fig. 5), are usually considered essential for the formation of this polynuclear ferric oxyhydroxide crystal cell structure, and their complete or partial removal after synthesis would leave channels in the structure that could facilitate the ready transport of ferrous ions. In fact, Kilcoyne and Lawrence only claimed an occupancy of 0.91 atoms of Cl per unit cell, so the average degree of substitution at any one Cl site must be less than 50%, and some channels must already exist in the structure.

Such a possibility for an ion transport mechanism would again be reminiscent of that considered to operate in Ferritin.

As each unit cell of the core contains 8 Fe atoms, then the 478 Fe atoms found in the molecular formula calculation of the Core Molecule (1.3), should probably be 480, equivalent to 60 unit cells in the core.

Further study has shown that, as with Ferritin, the complex loses its iron atoms progressively by reduction with thioglycollic acid, and the $(DxCOOH)_{12}$ sheath seemed to remain intact

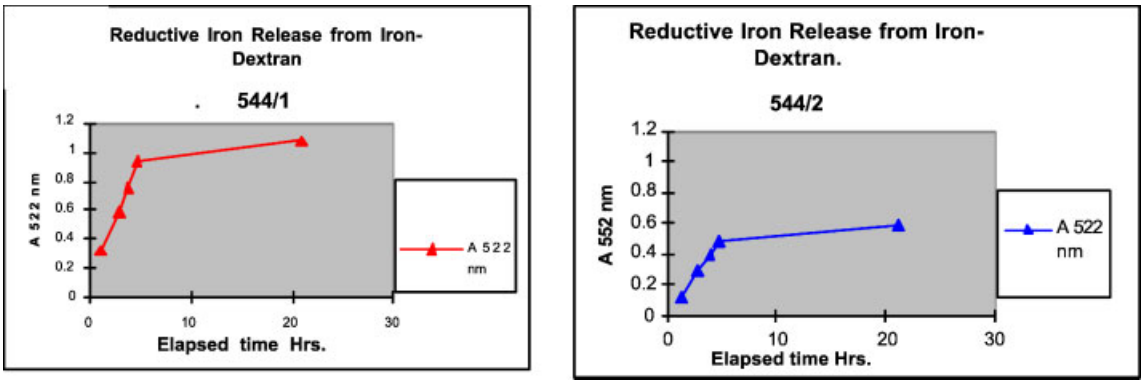

Figure 3. Iron release by reduction. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

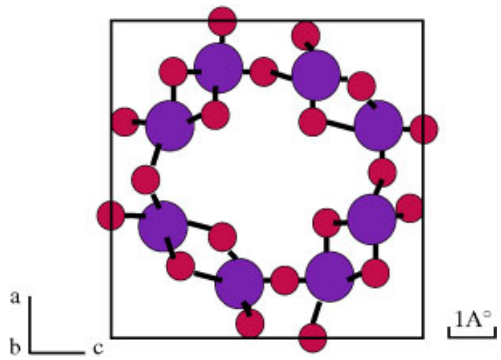

Figure 4. Iron–dextran core. A projection of the crystal structure of the iron–dextran core down the b-axis as determined by Rietveld refinement. [© 1999. Reprinted with permission. S.H. Kilcoyne, J.L. Lawrence, Z. Kristallogr 214: 668.[21]] [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

during their loss, as there was little change in the viscosity of the reaction medium, but this work needs extending.

The progressive loss of ferric ions after reduction to ferrous is illustrated in Figure 3.

These curves show that reduction, as expected, proceeds more rapidly at the higher thioglycollic acid to iron concentration (544/1) of 1 mol equivalent of each and its extent is roughly proportional to the amount of reductant used, that is, 0.5 mol equivalent of thioglycollic acid to iron (544/2), liberates 53% of the ferrous ions freed by 1 mol equivalent (544/1).

About 42 and 80% of the theoretical amount of iron available to be freed were accounted for in Figure 3, 544/2 and 544/1, respectively.

Reduction Data
Chart 544/1

Elapsed Time Hrs	A 522 nm
1.08	0.32
2.83	0.59
3.75	0.76
4.67	0.93
21	1.08

Chart 554/2

Elapsed Time Hrs	A 522 nm
1.25	0.12
2.75	0.29
3.83	0.4
4.75	0.49
21.08	0.58

Further study is needed to explore the possibility of replacing the lost iron atoms or adding more

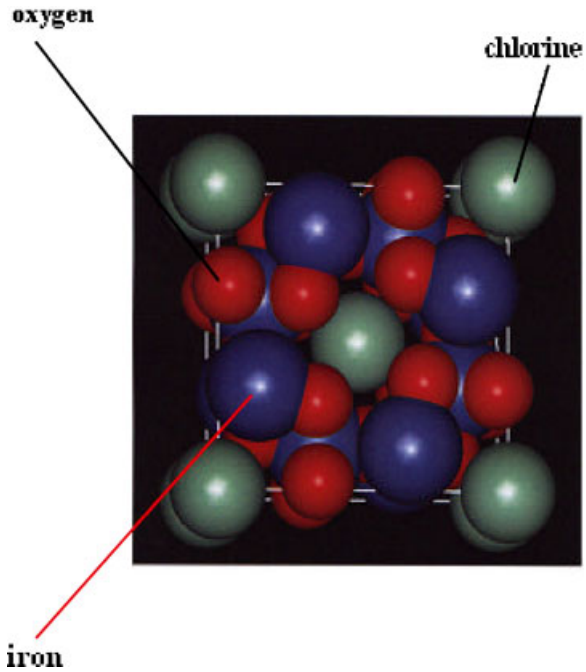

Figure 5. Akaganeite unit cell. Chlorine is usually considered essential for the buildup of this structure during the formation of polynuclear β-FeOOH. Its removal leaves space for core access. [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

than were originally present, as can be done with Ferritin. If it is confirmed that the iron–dextran complex is essentially intact after removal of some or all of the iron in a reversible process, then its modus operandi as a synthetic iron store in man would seem to be similar to that of Ferritin.

However, metal shadowing of the complex revealed an electron translucent sheath around the core (but not around the core freed from uncomplexed dextran acid), giving an overall size for the complete complex of $\sim 11.5 \times 7.5$ nm. If one assumes a symmetrical even thickness sheath around the core, the complex could be represented by Figure 6, agreeing with the lozenge shape of some electron photomicrographs[17] and Schneider's[29] β-Fe.O.OH models. This suggests a partially filled cavity of ca 9.5×5.5 nm may exist in the "Product A" complex. If correct, this would allow iron–dextran complexes like Dexferrum[22] with its higher MW to exist within a similar structure, by enlarging the core and if necessary the sheath.

Ferritin is known[32] to have a cavity that is normally incompletely filled.

While it is recognized that electron photomicrographs may not allow precise measurement, they

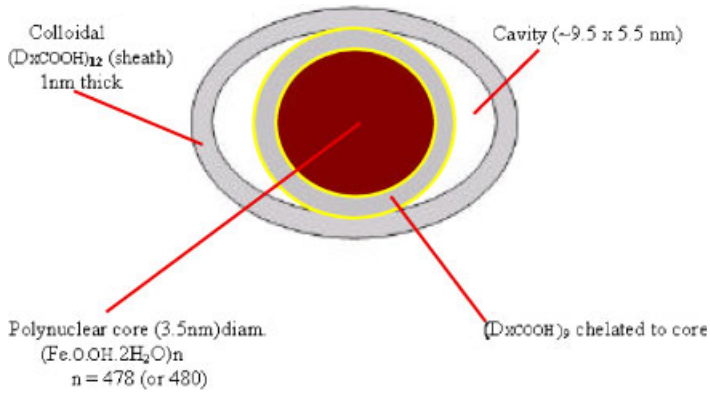

Figure 6. Proposed cross section of iron–dextran complex, Imferon. (~11.5 × 7.5 nm). [Color figure can be seen in the online version of this article, available on the website, www.interscience.wiley.com.]

will usually give a sufficiently correct order of magnitude to justify a model of the type proposed in Figure 6, which considerably aids the visualization of this somewhat unusual structure.

Finally, it is possible that the use of the core molecule shown as the central feature of Figure 6, in place of the whole iron–dextran complex normally used, would sharpen the data obtained using such techniques as X-ray diffraction and Mossbauer spectroscopy and make them easier to interpret.

In particular, the broad overlapping Bragg peaks referred to in the Kilcoyne and Lawrence[21] article might be further resolved, and could simplify the determination of the space group using X-ray diffraction, and possibly remove any residual uncertainty from the very close similarity already established by Kilcoyne and Lawrence between the iron–dextran core structure and that of natural Akaganeite.

By so excluding the sheath around the core, less than 43% of the total complex polysaccharide would be available to interfere with these sensitive investigations, and none of it would be present as a purely polysaccharide barrier at the outer surface of the complex, as it has been in previous investigations on iron–dextran cores. (The author could supply such material given reasonable notice.)

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