# Iron Chelators of the Pyridoxal Isonicotinoyl Hydrazone Class Part II. Formation Constants with Iron(III) and Iron(II)

LUCIA M. WIS VITOLO, GLENN T. HEFTER, BRIAN W. CLARE and JOHN WEBB*

*School of Mathematical and Physical Sciences, Murdoch University, Murdoch, WA 6150 (Australia)*

(Received August 18, 1988; revised May 4, 1989)

## Abstract

Formation constants for the iron(III) complexes of the orally effective iron chelator pyridoxal isonicotinoyl hydrazone (PIH) and three analogues: pyridoxal benzoyl hydrazone (PBH), 3-hydroxyisonicotinaldehyde isonicotinoyl hydrazone (IIH) and salicylaldehyde isonicotinoyl hydrazone (SIH), have been determined by a combination of spectrophotometry and potentiometry. All four ligands bind iron(III) strongly giving, at physiological pH 7.4, values of pM (−log[uncomplexed metal]) between 27.7 and 50, comparable to or greater than those for transferrin (25.6) and desferrioxamine B (28.6). The complexation of Fe(II) by PIH has also been studied and has been found to be appreciable but very much weaker than that for Fe(III).

## Introduction

The chelating agent pyridoxal isonicotinoyl hydrazone (PIH, Fig. 1(a)) has attracted considerable attention in the search for high affinity iron chelators [1, 2] due to its high activity in mobilising cellular iron in a variety of cellular and animal-based bioassays [3–14]. The affinities of PIH for iron(II) and iron(III) are significant aspects of this activity.

In this paper we report the results of solution studies of the formation constants with iron(III) of PIH and three structural analogues PBH (pyridoxal benzoyl hydrazone), IIH (3-hydroxyisonicotinaldehyde isonicotinoyl hydrazone) and SIH (salicylaldehyde isonicotinoyl hydrazone) (Fig. 1(b)–(d)). In addition, the interaction between PIH and iron(II) is characterised, following an earlier report of this formation constant [10]. Brief reports have appeared elsewhere [15, 16].

X-ray structural data indicate that coordination to iron(III) in the solid state occurs via the deprotonated phenolic –OH, the C=O and NH=N– of the hydrazone bridge [15, 17, 18].

## Experimental

The ligands were prepared as described in Part I [19]. Stock solutions of 0.1 M $Fe(NO_3)_3$ in 0.05 M

*Author to whom correspondence should be addressed.

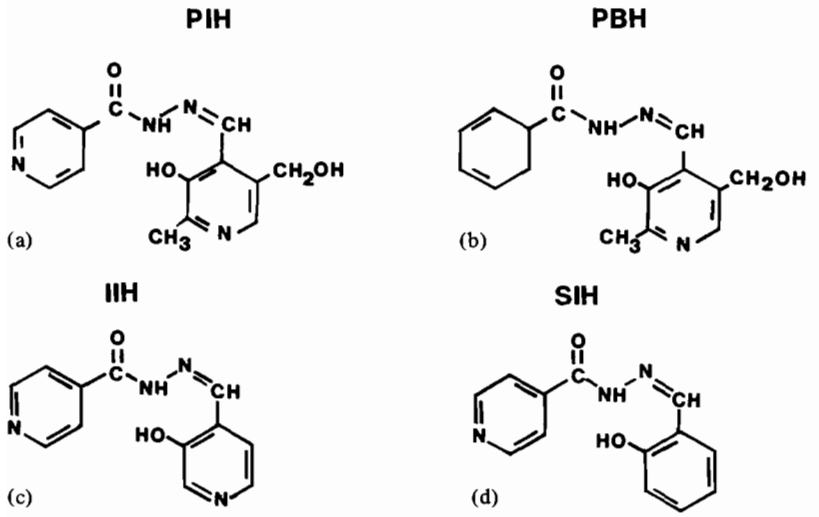


Fig. 1. Structures of the chelating agents studied: (a) pyridoxal isonicotinoyl hydrazone (PIH); (b) pyridoxal benzoyl hydrazone (PBH); (c) 3-hydroxyisonicotinaldehyde isonicotinoyl hydrazone (IIH); (d) salicylaldehyde isonicotinoyl hydrazone (SIH).

0020-1693/90/$3.50 

$HNO_3$ were standardised by reduction with $SnCl_2$ followed by titration with standard $KMnO_4$. Solutions of $Fe(NO_3)_2$ were prepared by mixing equimolar solutions of $FeSO_4$ and $Ba(NO_3)_2$ in freshly distilled water, under a positive pressure of argon [20]. After standing overnight at 0 °C, the supernatant was decanted and filtered under Ar before being added to $HNO_3$, giving a final acid concentration of 0.05 M and a final [Fe(II)] of approx. 0.1 M. The exact [Fe(II)] was determined by titration with standard $KMnO_4$. Such solutions could be stored at 0 °C for several days without significant oxidation to Fe(III).

Formation constants of the Fe(III) complexes were determined by a combination of spectrophotometry and potentiometry. Spectrophotometry was employed to measure initial complexation at pH 1.5–3.2. Potentiometric titrations ($H^+$–titrant) were then used to determine the acid dissociation constants of the Fe(III) complexes at higher pH. Very dilute solutions, with [Fe(III)] = $1–5 \times 10^{-5}$ M were used to avoid precipitation. The Fe(II) complexes are more soluble and were studied at [Fe(II)] = $2.5 \times 10^{-4}$ M over the full pH range by potentiometry although great care had to be taken to exclude oxygen.

For spectrophotometric measurements, solutions of $[L]_T:[Fe]_T = 1$, 2 and 4 were prepared for each ligand in succinic acid–succinate buffer solutions. The subscript T refers to the total or analytical concentration. The final concentrations were adjusted to give absorbance readings between 0.1 and 1.0 at 440–470 nm (typically, $2.5–5 \times 10^{-5}$ M). Spectra in the UV–Vis regions were recorded with a Hewlett-Packard HP8450 spectrophotometer equipped with thermostatted (±0.1 °C) cell holders. The titration apparatus and procedures for pH measurements have been described previously [19].

All measurements were made at 25.0 °C at an ionic strength of 0.1 M ($KNO_3$).

## Calculation Procedures

For the analysis of the spectrophotometric data, each experimental point was considered to represent four variables: $[L]_T$, $[M]_T$, pH and absorbance. It was assumed that $H_2L$ was the most protonated form of the ligand that can bind significantly to the metal ions. Hence data fitting began with 8 parameters; the formation constants and molar absorptivities ($\epsilon$) of the four mono- and bis-complexes of $H_2L$, i.e. $M(H_2L)$, $M(H_2L)_2$, and of HL, i.e. M(HL), $M(HL)_2$, with the residuals (on the absorbance) and their standard deviation providing a measure of goodness-of-fit using the non-linear algorithm of Marquardt [21]. Various combinations of metal complexes were evaluated in this manner to give the best standard deviation. Speciation plots were calculated from the mass balance for M and L at each pH value. Analysis of the potentiometric data was performed in a manner analogous to the spectrophotometric data. Variables were the measured pH, $[L]_T$ and $[M]_T$ derived from the initial totals and the titration volumes, whilst the parameters were the acid dissociation constants of the complexes (see eqn. (3) below).

## Nomenclature

Because of the large number of ionisable protons on the ligands, iron–PIH and related systems are potentially complicated and it is necessary to specify the equilibria and the stoichiometry of the complexes carefully. This can be done in a number of ways. The following approach has the advantage of relating the experimental data to the major equilibria likely to be occurring in solution.

The association of the ligand with the metal can be represented generally as follows (omitting charges for simplicity)

$$Fe + aH_xL + bH_yL \longrightarrow Fe(H_xL)_a(H_yL)_b$$

where $a$ and $b$ are 0 or 1. The formation constants for these equilibria are then

$$\beta_{xy} = \frac{[Fe(H_xL)_a(H_yL)_b]}{[Fe][H_xL]^a[H_yL]^b}$$

Complex formation can also be represented as a proton-dependent equilibrium

$$Fe + aH_{x'}L + bH_{y'}L \longrightarrow Fe(H_xL)_a(H_yL)_b + nH^+$$

where $n = (x' - x) + (y' - y)$ and $a$ and $b = 0$ or 1. Formation constants for these equilibria are thus

$$\beta^*_{xy} = \frac{[Fe(H_xL)_a(H_yL)_b][H^+]^n}{[Fe][H_{x'}L]^a[H_{y'}L]^b}$$

The acid dissociation constants for the complexes are represented as $K_{ci}$ ($i \leqslant 2$), where $K_{ci}$ is the equilibrium constant for the following reactions

$$Fe(H_xL)_a(H_yL)_b \longrightarrow Fe(H_{x-1}L)_a(H_yL)_b + H^+$$

or

$$Fe(H_xL)_a(H_yL)_b \longrightarrow Fe(H_xL)_a(H_{y-1}L)_b + H^+$$

where $x$ and $y$ are 0 or 1.

The overall formation constants $\beta_{xy}$ for each species can then be calculated from $\beta^*_{xy}$ when the acid dissociation constants for the ligands ($K_i$) and the complexes ($K_{ci}$) are known.

## Results and Discussion

*Complex Formation with Iron(III)*

Study of the interaction of Fe(III) with PIH and its analogues is of considerable interest but presents particular experimental difficulties. Not only are the formation constants large but the quite limited solubility of the ligands and especially the neutral complexes in aqueous solution necessitates the use of metal and ligand concentrations that are 2 or even 3 orders of magnitude below those usually employed in such studies. Moreover, the low solubility restricts the pH range available for study. The situation is complicated further by the large number of possible complexes. Inevitably the errors associated with the formation constants reported in this work are somewhat larger than the authors would prefer. However, the importance of these data in helping to understand the interaction of Fe(III) with this potentially important class of ligands far outweighs these limitations.

In the pH range 1.5–3.2, both PIH and IIH associate with iron(III) with the loss of two or three protons from their fully protonated form $H_4L^{2+}$, giving $Fe(H_2L)^{3+}$, $Fe(H_2L)_2^{3+}$ and $Fe(HL)_2^{+}$. The species $Fe(HL)^{2+}$ forms with IIH but not PIH. In the case of the other two ligands PBH and SIH, only the two species $Fe(HL)^{2+}$ and $Fe(HL)_2^{+}$ are detected. The formation constants for all these species are shown in Table 1.

The affinities of PIH and IIH for Fe(III) are comparable, indicating, not surprisingly, that the $-CH_3$ and $-CH_2OH$ side chains on the pyridoxal ring have little influence on metal-binding. However, the substitution of a benzene for a pyridine ring results in a dramatic increase in iron-binding capability. Thus $\log \beta_{11}^*$ increases from 8.80 for PIH to 21.8 for PBH and 32.2 for SIH (see structures in Fig. 1). This effect is much more pronounced for SIH than for PBH. Clearly the pyridine nitrogen has a more pronounced electron-withdrawing effect on the phenolate group of the pyridine residue than on the carbonyl side chain of the isoniazid residue. In this context, the complex formation with salicyl benzoyl hydrazone (SBH), the PIH analogue with two benzene rings, would be of particular interest. Unfortunately, attempts to study the Fe(III)/SBH equilibria were thwarted by precipitation problems.

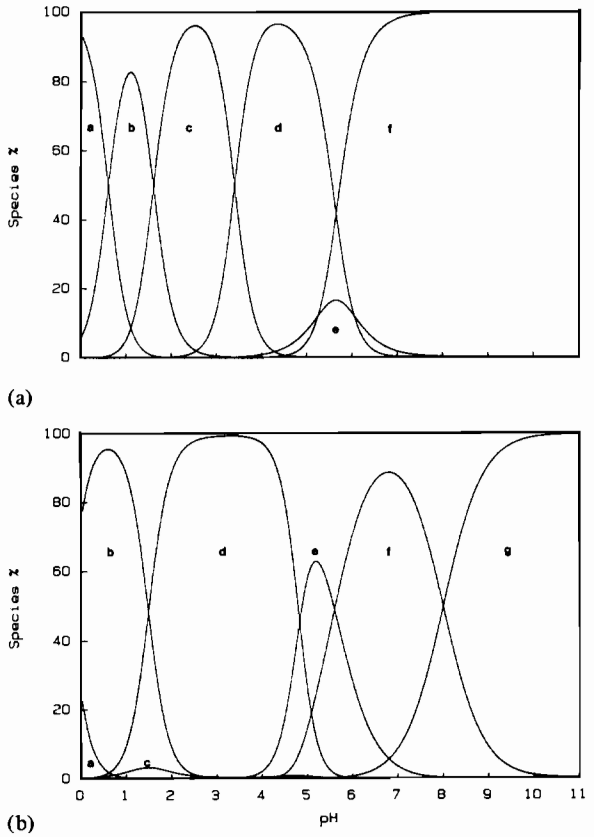


Fig. 2. Distribution of iron(III) complex species over pH 0–11 for [Fe(III)] = $10^{-6}$ M and [ligand] = $10^{-3}$ M, when ligand is (a) PIH; (b) IIH. a = Uncomplexed Fe(III), b = $Fe(H_2L)^{3+}$, c = $Fe(HL)^{2+}$, d = $Fe(H_2L)_2^{3+}$, e = $Fe(HL)_2^{+}$, f = $Fe(HL)L^0$, g = $FeL_2^-$.

At pH > 3.2, the Fe(III) is present almost exclusively as the bis-complexes for all of the ligands studied (Figs. 2 and 3). Subsequent spectral changes were attributed to deprotonation of the complexes. As the pH was raised a sparingly soluble product, presumably the neutral species $Fe(HL)L^0$, precipitated. This redissolved as further base was added, presumably as the result of the formation of $FeL_2^-$.

TABLE 1. Formation constants for iron(III) complexes of PIH and its analogues determined by spectrophotometry[a]

| Ligand | $\log \beta_2$ $Fe(H_2L)^{3+}$ | $\log \beta_{22}$ $Fe(H_2L)_2^{3+}$ | $\log \beta_1^*$ $Fe(HL)^{2+}$ | $\log \beta_{11}^*$ $Fe(HL)_2^{+}$ |
|---|---|---|---|---|
| PIH | 8.93 ± 0.11 | 15.89 ± 0.11 | n.d. | 8.80 ± 0.8 |
| PBH | n.d. | n.d. | 18.8 ± 1.6 | 21.8 ± 1.9 |
| IIH | 10.43 ± 0.07 | 17.4 ± 1.5 | 7.73 ± 0.15 | 10.1 ± 0.9 |
| SIH | n.d. | n.d. | 30.0 ± 2.6 | 32.2 ± 2.8 |

[a]n.d. = not detected.

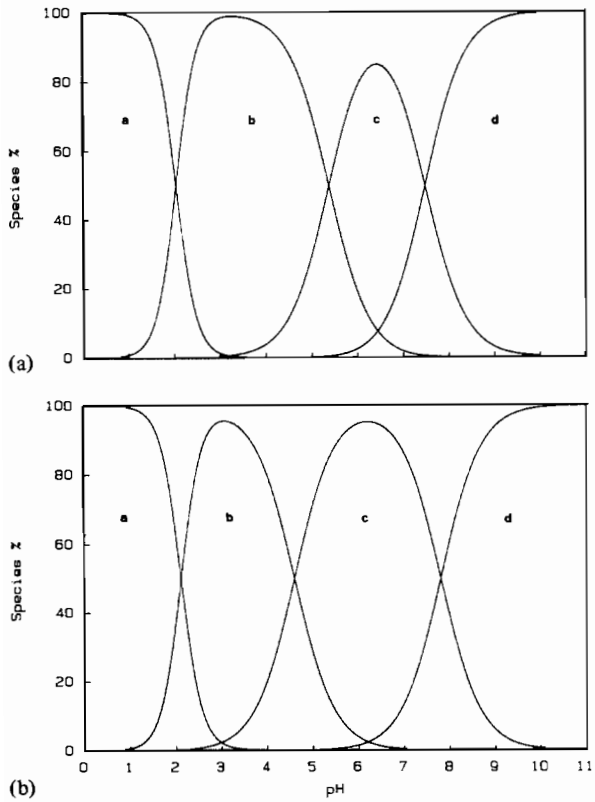


Fig. 3. Distribution of iron(III) complex species over pH 0–11 for [Fe(III)] = $10^{-6}$ M, [ligand] = $10^{-3}$ M when ligand is (a) PBH; (b) SIH. a = $Fe(HL)^{2+}$; b = $Fe(HL)_2^{+}$; c = $Fe(HL)L^0$; d = $FeL_2^{-}$.

The last two deprotonations were quantified by potentiometric pH titrations, adding acid to a basified metal–ligand mixture to minimise precipitation problems. The $pK_a$ values were approximately 5 and 7 for all four ligands studied (Table 2). The consistency of these values suggests that they refer to closely related reactions in all the ligands. This common structural feature is the –NH–N group in the hydrazone bridge (Fig. 1) which has been shown elsewhere [19] to be characterised by a $pK_a$ of 10–11 in the uncomplexed ligands.

Using these $pK_a$ values (i.e. $K_{ci}$, see above), the overall formation constants of the species $Fe(HL)L^0$ and $Fe(L)_2^{-}$ were calculated (Table 3). These data confirm the observation noted earlier that PIH and IIH have comparable affinities for iron(III) while the affinities of PBH and SIH are considerably greater.

TABLE 2. Acid dissociation constants for the complex species $Fe(HL)_2^{+}$ determined by potentiometry

| Ligand | $pK_{c1}$ | $pK_{c2}$ |
|---|---|---|
| PIH | 5.38 ± 0.09 | 7.45 ± 0.09 |
| PBH | 5.2 ± 0.3 | 6.8 ± 0.5 |
| IIH | 5.4 ± 0.7 | 7.4 ± 0.7 |
| SIH | 4.6 ± 2.7 | 7.8 ± 0.7 |

The distributions of the complex species as a function of pH and in the presence of a 1000-fold molar excess of ligand over iron(III) are shown in Figs. 2 and 3. At physiological pH (7.4), the complex with PIH exists as the singly charged anion $FeL_2^{-}$ but the other three ligands occur as a mixture of this anionic species and the neutral species $Fe(HL)L^0$. This is consistent with the fact that the excretion of the Fe–PIH complex occurs primarily in bile [5].

Comparison of the metal-binding affinities of ligands that form complexes of different stoichiometries can best be achieved by calculation of their pM (= $-\log[M]$) values, where M is the uncomplexed or 'free' metal ion [2]. Such values for PIH and its analogues are shown in Table 4 together with those for the serum iron-transport protein transferrin and three high affinity siderophores: desferrioxamine B (currently in clinical use), rhodotorulic acid and enterobactin [22]. The pM values for the PIH ligands are greater than that for transferrin, indicating that these ligands are thermodynamically able to mobilise transferrin-bound iron. A kinetic barrier, however, inhibits this exchange process, as in the case of desferrioxamine [23]. The rates of mobilisation of iron from transferrin by desferrioxamine and PIH are comparable [4].

TABLE 3. Overall formation constants for iron(III) complexes with PIH and its analogues

| Ligand | $\log \beta_1$ $Fe(HL)^{2+}$ | $\log \beta_{11}$ $Fe(HL)_2^{+}$ | $\log \beta_{10}$ $Fe(HL)L^0$ | $\log \beta_{00}$ $FeL_2^{-}$ |
|---|---|---|---|---|
| PIH | n.d.[a] | 24.8 ± 0.8 | 29.0 ± 2.0 | 34.0 ± 3.0 |
| PBH | 27.2 ± 1.7 | 38.5 ± 1.9 | 44.3 ± 2.2 | 48.0 ± 2.4 |
| IIH | 15.7 ± 0.3 | 23.6 ± 0.9 | 28.0 ± 1.4 | 30.0 ± 1.8 |
| SIH | 38.3 ± 2.7 | 48.8 ± 2.8 | 54 ± 4 | 56 ± 4 |

[a]n.d. = not detected.

TABLE 4. Unbound iron concentrations at pH 7.4, [Fe(III)] = $10^{-6}$ M, [L] = $10^{-3}$ M

| Compound | pM[a] |
|---|---|
| PIH | 27.7 |
| PBH | 39.7 |
| IIH | 24.5 |
| SIH | 50 |
| Transferrin | 25.6[b] |
| Desferrioxamine B | 28.6[b] |
| Rhodotorulic acid | 25.0[b] |
| Enterobactin | 37.6[b] |

[a]pM = − log[uncomplexed metal]. [b]Taken from ref. 19.

*Complex Formation with Iron(II)*

Potentiometric pH titration of PIH with iron(II) (2:1 mole ratio) led to the detection of only two species, $Fe(H_2L)_2^{2+}$ and $Fe(HL)_2^0$ with $\log \beta_{22}$ and $\log \beta_{11}$ values that were considerably lower than those for the corresponding Fe(III) species (Table 5), i.e. PIH shows a strong preference for Fe(III). This investigation was limited by the sensitivity of the complex to oxidation and to its instability to hydrolysis especially at high pH. This sensitivity to oxidation has also been reported [24] for pyridoxal–amino acid Schiff base complexes with iron(II).

TABLE 5. Comparison of PIH formation constants with Fe(II) and Fe(III)

| | $\log \beta_{22}$ $Fe(H_2L)_2$ | $\log \beta_{11}$ $Fe(HL)_2$ |
|---|---|---|
| Fe(II) | 6.98 ± 0.05 | 12.47 ± 0.32 |
| Fe(III) | 15.89 ± 0.11 | 24.8 ± 0.8 |

A previous report [10] of $\log K = 8.67$ for a bis-PIH complex with iron(II) gave no details on the protonation of the ligand but the reported value falls between the $\beta_{11}$ and $\beta_{22}$ values determined in the present study. However, failure to take the necessary precautions to limit oxidation of iron(II) suggests that this undefined $\log K$ value refers to the Fe(III)–PIH system.

It has been suggested [25] that PIH has a biological role as an iron(II) chelator and it is interesting to note that the formation constants are appreciable and significantly greater than for the metal ions calcium(II) and magnesium(II) [26]. If Fe is released as iron(II) from transferrin that has been carried inside cells then this affinity of PIH for iron(II) may be a significant factor in its ability to mobilise iron from cells.

## Acknowledgements

The award of a Commonwealth Postgraduate Scholarship to M.L.V. is gratefully acknowledged as is the financial assistance of the Telethon Foundation, Perth, for the purchase of the HP8450 spectrophotometer.

## References

1 B. Modell and V. Berdoukas, *The Clinical Approach to Thalassemia*, Grune and Stratton, New York, 1984.
2 A. E. Martell, W. French-Anderson and D. G. Badman (eds.), *Development of Iron Chelators for Clinical Use*, Elsevier-North Holland, New York, 1981.
3 P. Ponka, J. Borova, J. Neuwirt and O. Fuchs, *FEBS Lett., 97* (1979) 317.
4 P. Ponka, J. Borova, J. Neuwirt, O. Fuchs and E. Necas, *Biochim. Biophys. Acta, 586* (1979) 278.
5 T. Hoy, J. Humphrys, A. Jacobs, A. Williams and P. Ponka, *Br. J. Haematol., 43* (1979) 443.
6 M. Cikrt, P. Ponka, E. Necas and J. Neuwirt, *Br. J. Haematol., 45* (1980) 275.
7 C. Hershko, S. Avramovici-Grisaru, G. Link, L. Gelfand and S. Sarel, *J. Lab. Clin. Med., 98* (1981) 99.
8 D. K. Johnson, M. J. Pippard, T. B. Murphy and N. J. Rose, *J. Pharm. Exp. Ther., 221* (1982) 399.
9 A. Williams, T. Hoy, A. Pugh and A. Jacobs, *J. Pharm. Pharmacol., 34* (1982) 730.
10 S. Avramovici-Grisaru, S. Sarel, G. Link and C. Hershko, *J. Med. Chem., 26* (1983) 298.
11 M. L. Vitolo, J. Webb and P. Saltman, *J. Inorg. Biochem., 20* (1984) 255.
12 E. Baker, M. L. Vitolo and J. Webb, *Biochem. Pharmacol., 34* (1985) 3011.
13 B.-K. Kim, H. A. Huebers and C. A. Finch, *Am. J. Hemat., 24* (1987) 277.
14 E. Baker, in S. Fucharoen, P. T. Rowley and N. W. Paul (eds.), *Thalassemia: Pathophysiology and Management, Part B*, Vol. 23, Birth Defects Foundation, Original Article Series, 1988, p. 49.
15 J. Webb and M. L. Vitolo, in S. Fucharoen, P. T. Rowley and N. W. Paul (eds.), *Thalassemia: Pathophysiology and Management, Part B*, Vol. 23, Birth Defects Foundation, Original Article Series, 1988, p. 63.
16 M. L. Vitolo, B. W. Clare, G. T. Hefter and J. Webb, in S. Fucharoen, P. T. Rowley and N. W. Paul (eds.), *Thalassemia: Pathophysiology and Management, Part B*, Vol. 23, Birth Defects Foundation, Original Article Series, 1988, p. 71.
17 T. B. Murphy, D. K. Johnson, N. J. Rose, A. Arufo and V. Schomaker, *Inorg. Chim. Acta, 66* (1982) L69; *67* (1982) L25.
18 S. Avramovici-Grisaru, S. Sarel, S. Cohen and R. E. Bauminger, *Israel J. Chem., 25* (1985) 288.
19 D. R. Richardson, M. L. Vitolo, G. T. Hefter, P. M. May, B. W. Clare, J. M. Webb and P. Wilairat, *Inorg. Chim. Acta, 170* (1990) 165.
20 A. M. Bond and G. T. Hefter, *J. Inorg. Nucl. Chem., 34* (1972) 603.
21 D. R. Sadler, *Numerical Methods for Nonlinear Regression*, University of Queensland Press, St. Lucia, Qld., 1975.

22 W. R. Harris, C. J. Carrano and K. N. Raymond, *J. Am. Chem. Soc., 101* (1979) 2722.
23 S. Pollack, P. Aisen, F. D. Lasky and G. Vanderhoff, *Br. J. Hematol., 34* (1976) 235.
24 H. N. Christensen, *J. Am. Chem Soc., 79* (1957) 4073.
25 R. W. Grady, in A. E. Martell, W. French-Anderson and D. G. Badman (eds.), *Development of Iron Chelators for Clinical Use,* Elsevier-North Holland, New York, 1981, p. 156.
26 D. R. Richardson, G. T. Hefter, P. M. May, J. Webb and E. Baker, *Biol. Met., 2* (1989) 161.