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# Iron chelators of the pyridoxal isonicotinoyl hydrazone class

## III. Formation constants with calcium(II), magnesium(II) and zinc(II)

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**Summary.** Formation constants for the calcium(II), magnesium(II) and zinc(II) complexes of the orally effective iron chelator, pyridoxal isonicotinoyl hydrazone (PIH) and three analogues, pyridoxal benzoyl hydrazone (PBH), pyridoxal *p*-methoxybenzoyl hydrazone (P*p*MBH) and pyridoxal *m*-fluorobenzoyl hydrazone (P*m*FBH) have been determined by potentiometry at 25° C and $I = 0.1$ M [$KNO_3$]. The four ligands bind calcium(II) weakly and magnesium(II) only slightly more strongly, as a 1:1 complex which is formed at pH > 8. The chelation of zinc(II) for all the ligands studied was greater than that for calcium(II) and magnesium(II), with complexation generally becoming significant at about pH 5. Thus, chelation of zinc(II) but not calcium(II) or magnesium(II) at physiological pH, 7.4 may be expected. Calculated values of the concentration of uncomplexed metal ion indicate that the selectivity of these ligands towards Fe(III) is comparable to that of the clinically used chelator desferrioxamine.

**Key words:** Fe chelation — Thalassemia — Fe overload — Calcium — Magnesium — Zinc

### Introduction

A clinically useful iron chelator must have high affinity for iron(III) in vivo compared to haemosiderin, ferritin, transferrin and the intracellular iron pool, but in addition it should also have a low affinity for all physiologically important cations other than iron (Martell et al. 1981). Strong affinities of chelators for essential metal ions present in high concentrations in vivo, especially calcium(II) and magnesium(II), would result in gross metabolic disturbances. Affinity towards zinc(II) is also of interest since the metabolism of this ion is known to be greatly perturbed during treatment of iron overload with the chelating agent diethylenetriaminepentaacetic acid (Modell and Berdoukas 1984). Clearly, determination of the formation constants of the calcium(II), magnesium(II) and zinc(II) complexes is vital in the context of the evaluation of potential iron chelators for clinical use.

In this paper we report the results of such studies for pyridoxal isonicotinoyl hydrazone (PIH) which has been shown to be effective in treating iron overload in vivo and in vitro (Ponka et al. 1979a, 1979b; Hoy et al. 1979; Cikrt et al. 1980; Herschko et al. 1981; Johnson et al. 1982; Williams et al. 1982; Avramovici-Grisaru et al. 1983; Wis-Vitolo et al. 1984; Baker et al. 1985; Kim et al. 1987). We consider here also analogues of PIH that have considerable activity in various in vitro bioassays (Richardson et al. 1988; Ponka et al. 1988): pyridoxal benzoyl hydrazone (PBH), pyridoxal *p*-methoxybenzoyl hydrazone (P*p*MBH) and pyridoxal *m*-fluorobenzoyl hydrazone (P*m*FBH) (Fig. 1). Previous papers in this series have dealt with the determination of the protonation constants of PIH and several analogues (Richardson et al. 1989b) and their formation constants (Wis Vitolo et al. 1989) with iron(II) and iron(III).

### Experimental procedures

*Materials.* Commerical reagents of the highest quality available were used without further purification: calcium(II) carbonate (BDH), magnesium(II) nitrate hexahydrate (Mallinck-

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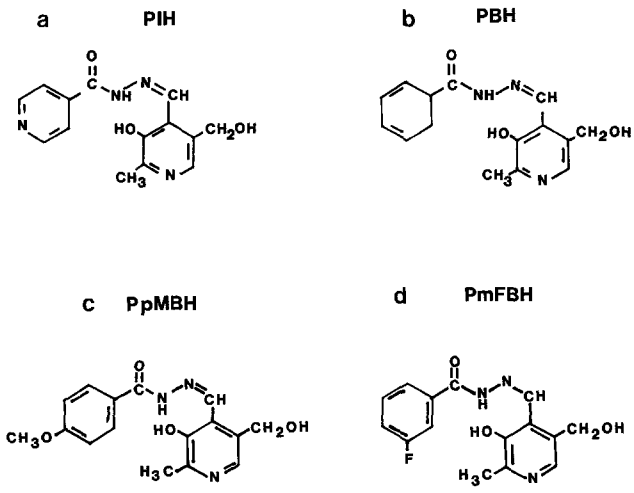


**Fig. 1.** Structures of the chelating agents studied: **(a)** pyridoxal isonicotinoyl hydrazone (PIH); **(b)** pyridoxal benzoyl hydrazone (PBH); **(c)** pyridoxal *p*-methoxybenzoyl hydrazone (P*p*MBH); **(d)** pyridoxal *m*-fluorobenzoyl hydrazone (P*m*FBH)

rodt) and zinc(II) oxide (BDH). Chelators were synthesised as described previously (Richardson et al. 1989b). Formation constants of PIH, PBH, P*p*MBH and P*m*FBH with calcium(II) ion, magnesium(II) ion and zinc(II) ion were determined with the potentiometric titration apparatus described earlier (Richardson et al. 1989b). Stock solutions of the metal salts (0.167 M) were prepared in a 3-fold excess of nitric acid (0.5 M). Calcium(II) carbonate was weighed directly into the acid. The reaction was accelerated to completion by sonication and gentle warming. The last traces of carbon dioxide were removed from the solution by sonication and heating followed by careful degassing with a stream of high-purity nitrogen through the solution for 20 min. In all cases metal ion concentrations were cross-checked by complexometric titrations with EDTA (0.02000 M, volumetric standard ampules, May and Baker). Due to the low solubility of the ligands, metal:ligand ratios could only be varied over a limited range. Metal concentrations of 0.2–1.7 mM were used, but for the weaker interactions, concentrations less than 0.5 mM usually resulted in failure of the optimisation sequence of the computer program.

*Calculation procedures.* Analysis of the titration data was performed using the computer program library, ESTA, Equilibrium Simulation for Titration Analysis (Murray and May 1984; May et al. 1985). The estimates of the errors used by ESTA were 0.05 mV for emf readings and 0.001 mL for titre volumes.

## Results and discussion

Concentrations of the acid added as $HNO_3$ ($c_H$), total ligand ($c_L$) and total metal ($c_M$) used in the pH ranges investigated in the potentiometric titrations are shown in Table 1. The low $c_L$ values used were necessitated by the poor solubility of the neutral ligands. The total metal and ligand concentrations are considerably lower than optimal and this limited the precision achieved in the determinations of the formation constants, as discussed previously (Richardson et al. 1989b). Furthermore, conditions of extreme pH (<2 or >12), were avoided due to incipient hydrolysis of the ligands (Richardson et al. 1989a).

**Table 1.** Experimental conditions used in determination of formation constants: initial total concentrations of metal ($c_M$); ligand ($c_L$); added nitric acid ($c_H$) and pH range investigated

| Ligand | Ion | $c_M$ (mM) | $c_L$ (mM) | $c_H$ (mM) | pH range |
|---|---|---|---|---|---|
| PIH | $H^+$ | | 1.0 | 0.6 | 3.9–10.9 |
| | | | 1.0 | 5.0 | 2.5–11.4 |
| | Ca(II) | 1.7 | 1.0 | 1.7 | 3.4–11.4 |
| | | 0.5 | 1.0 | 5.5 | 2.4–11.7 |
| | Mg(II) | 1.7 | 1.0 | 5.0 | 2.5–10.7 |
| | | 0.5 | 1.0 | 4.5 | 2.6–11.1 |
| | Zn(II) | 0.2 | 0.1 | 1.2 | 3.0–11.0 |
| PBH | $H^+$ | | 0.5 | 0.8 | 3.4–11.7 |
| | Ca(II) | 0.5 | 0.5 | 1.5 | 3.0–11.6 |
| | | 0.2 | 0.5 | 1.2 | 3.1–11.5 |
| | Mg(II) | 0.5 | 0.5 | 1.5 | 3.0–11.5 |
| | Zn(II) | 0.5 | 0.5 | 1.5 | 3.0–11.6 |
| P*p*MBH | $H^+$ | | 0.25 | 2.0 | 2.7–11.4 |
| | Mg(II) | 0.3 | 0.25 | 1.0 | 3.1–11.2 |
| | Zn(II) | 0.3 | 0.25 | 1.3 | 3.0–11.6 |
| P*m*FBH | $H^+$ | | 0.5 | 0.8 | 3.5–11.5 |
| | Ca(II) | 0.5 | 0.5 | 1.5 | 3.0–11.5 |
| | Mg(II) | 0.5 | 0.5 | 1.5 | 3.0–11.5 |
| | Zn(II) | 0.5 | 0.5 | 1.5 | 3.0–11.6 |

The interactions of the ligands with the metal ions are expressed as follows, omitting charges for simplicity:

$$pM + qL + rH \rightleftharpoons M_pL_qH_r$$

for which the overall equilibrium constant $\beta$ is:

$$\beta_{pqr} = \frac{[M_pL_qH_r]}{[M]^p[L]^q[H]^r}.$$

Values for these constants are listed in Table 2, together with the number of data points and the objective function, a measure of the agreement between the observed and calculated data, derived from the ESTA program (Murray and May 1984).

Formation constants of all four ligands with calcium(II) were less than those for magnesium(II) and both were much less than those for zinc(II) ion (Table 2). This is in accordance with

**Table 2.** Formation constants of PIH and analogues with Ca(II), Mg(II), Zn(II)

| Ligand | Ion | $p$ | $q$ | $r$ | $\log \beta_{pqr}$[a] | Objective[b] function | $n$[b] |
|---|---|---|---|---|---|---|---|
| PIH | Ca(II) | 1 | 1 | 0 | $3.06 \pm 0.02$ | $4.8 \times 10^2$ | 334 |
| | Mg(II) | 1 | 1 | 0 | $4.46 \pm 0.04$ | $2.1 \times 10^3$ | 169 |
| | Zn(II) | 1 | 1 | 0 | $10.6 \pm 0.4$ | $6.7 \times 10^2$ | 36 |
| | | 1 | 1 | −1 | $0.0 \pm 0.4$ | | |
| PBH | Ca(II) | 1 | 1 | 0 | $2.86 \pm 0.05$ | $8.7 \times 10$ | 159 |
| | Mg(II) | 1 | 1 | 0 | $4.61 \pm 0.04$ | $1.9 \times 10^2$ | 86 |
| | | 1 | 1 | −1 | $-7.31 \pm 0.07$ | | |
| | Zn(II) | 1 | 1 | 0 | $9.26 \pm 0.03$ | $1.5 \times 10^2$ | 135 |
| | | 1 | 1 | −1 | $-0.21 \pm 0.04$ | | |
| | | 1 | 1 | 1 | $16.59 \pm 0.04$ | | |
| P*p*MBH | Ca(II) | 1 | 1 | 0 | $2.7 \pm 0.5$[c] | | |
| | Mg(II) | 1 | 1 | 0 | $4.40 \pm 0.03$ | $9.3 \times 10$ | 117 |
| | Zn(II) | 1 | 1 | 0 | $9.55 \pm 0.09$ | $2.7 \times 10^3$ | 120 |
| P*m*FBH | Ca(II) | 1 | 1 | 0 | $2.56 \pm 0.05$ | $3.3 \times 10$ | 152 |
| | Mg(II) | 1 | 1 | 0 | $4.41 \pm 0.02$ | $6.7 \times 10$ | 80 |
| | | 1 | 1 | −1 | $-7.50 \pm 0.03$ | | |
| | Zn(II) | 1 | 1 | 0 | $9.45 \pm 0.10$ | $3.9 \times 10^3$ | 89 |
| | | 1 | 1 | −1 | $-0.10 \pm 0.17$ | | |

[a] $\beta_{pqr} = [M_p L_q H_r]/[M]^p[L]^q[H]^r$
[b] See text and Murray and May (1984)
[c] Estimated, see text

the Irving-Williams series (Irving and Williams 1948). The interaction of all four ligands with each metal ion is similar. This is probably due to the involvement of the same ligating groups in each case and the relative remoteness of the substituents (*p*-$OCH_3$, *m*-F) from the metal-binding sites.

The main species present in all the metal-ligand titrations was shown to be the neutral 1:1 ligand-metal ion complex, (ML), where the ligands are in their fully deprotonated forms ($L^{2-}$). There was no evidence for the formation of the bis-complex, but some (minor) protonated species were detected. Given that PIH and its analogues are tridentate ligands, at least for iron(III) (Webb and Vitolo 1988), it can be presumed that the remaining sites in the co-ordination sphere of the metal ion are occupied by water molecules.

### *Interaction of ligands with Ca(II) and Mg(II)*

The formation constant for the chelation of calcium(II) ion by P*p*MBH could not be measured. This is probably a reflection of the weakness of the interaction and the difficulties of titrating such dilute solutions, as discussed above. On the basis of the similarities of the formation constants of these ligands with the other metal ions (Table 2), a formation constant of $2.7 \pm 0.5$ can be estimated for the binding of calcium(II) by P*p*MBH.

Diagrams showing the distribution of the metal ion–ligand complex species as a function of pH for PIH, PBH, P*p*MBH and P*m*FBH with a 1000-fold molar excess of L over M are shown in Figs 2–5. For calcium(II) and magnesium(II), the pres-

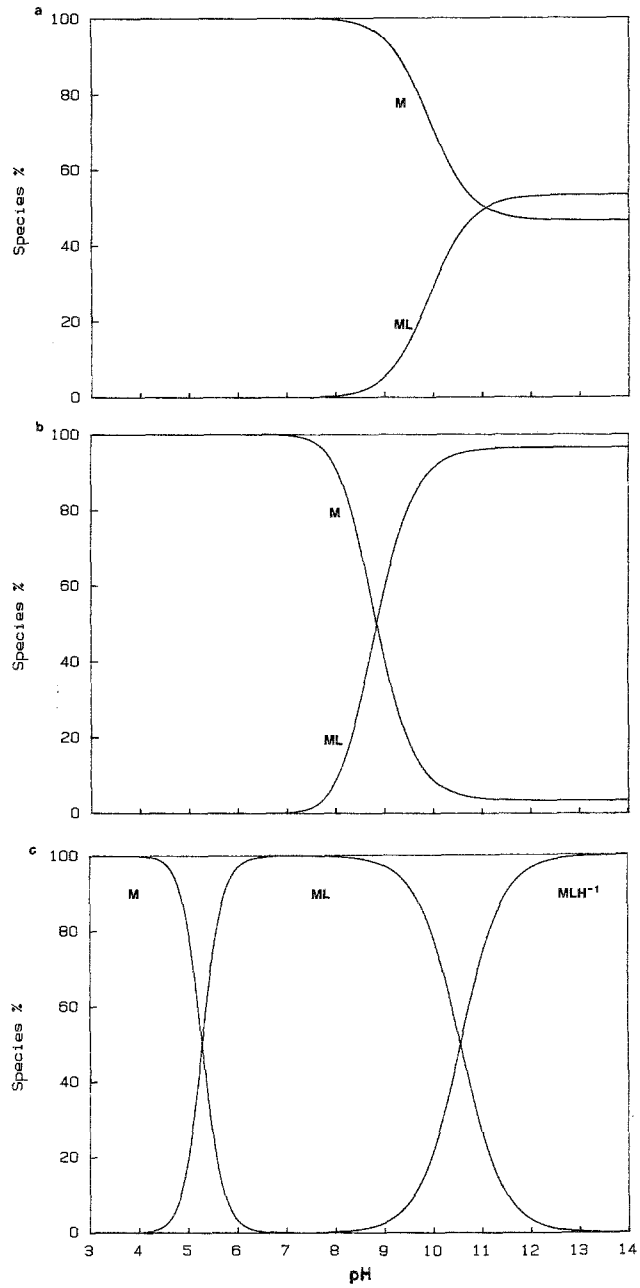


**Fig 2.** Distribution of metal-ligand complex species over pH 3–14 for PIH and **(a)** Ca(II), **(b)** Mg(II), **(c)** Zn(II) for $[M] = 10^{-6}$ M, $[L] = 10^{-3}$ M

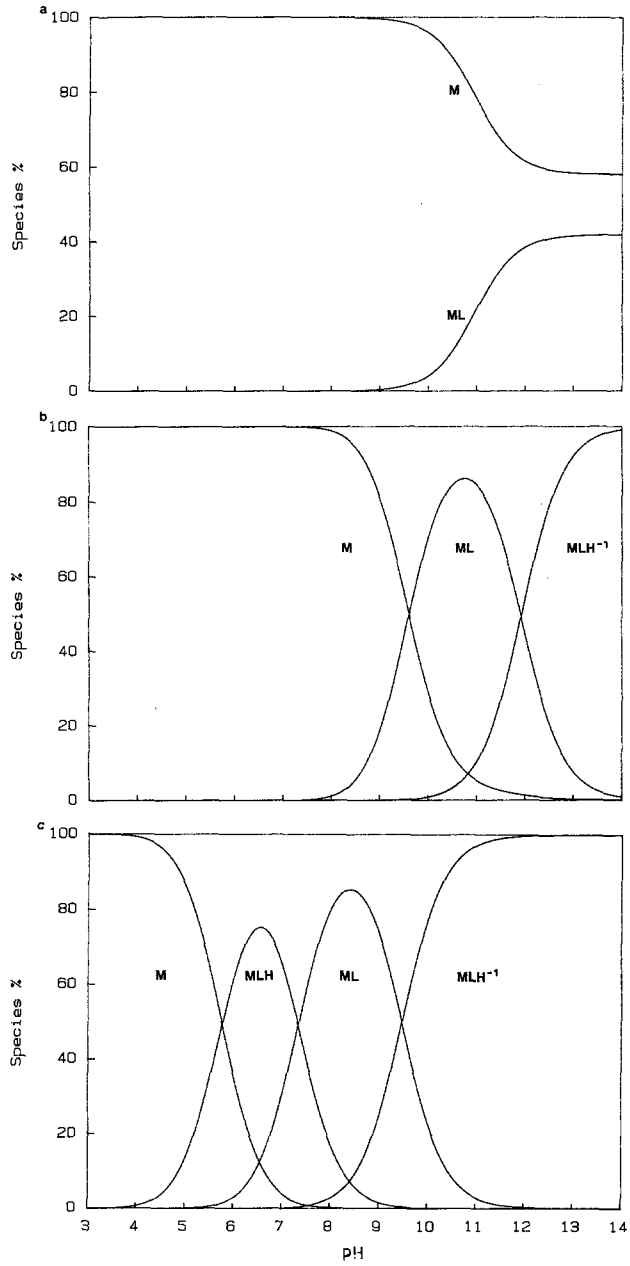


**Fig. 3.** Distribution of metal-ligand complex species over pH 3–14 for PBH and **(a)** Ca(II), **(b)** Mg(II), **(c)** Zn(II) for $[M] = 10^{-6}$ M, $[L] = 10^{-3}$ M

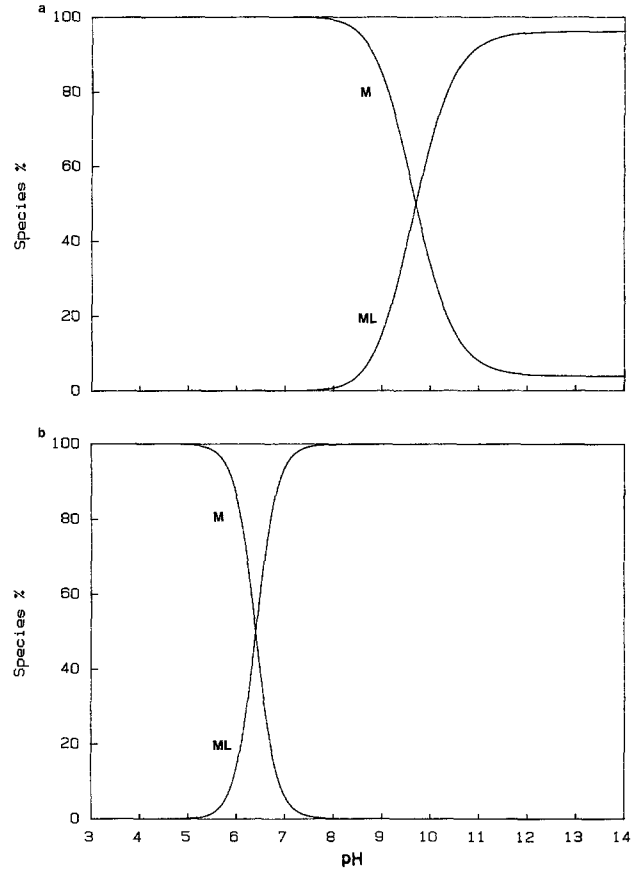


**Fig. 4.** Distribution of metal-ligand complex species over pH 3–14 for P*p*MBH and **(a)** Mg(II), **(b)** Zn(II) for $[M] = 10^{-6}$ M, $[L] = 10^{-3}$ M

ent results indicate that significant complexation only occurs at pH > 8 (Figures 2a, 3a, 5a). Thus, it can be concluded that chelation of calcium(II) and magnesium(II) ion by these ligands is insignificant at physiological pH (pH 7.4). This is important because it overcomes the disadvantage of a ligand such as DTPA which has a strong affinity for calcium(II) and magnesium(II) at physiological pH and has been shown to interfere with the metabolism of essential trace elements (Modell and Berdoukas 1984; May and Bulman 1983). Furthermore, chelators having strong interactions with metal ions which occur at high concentrations in vivo, such as calcium(II) and magnesium(II), would result in the suppression of the avidity of such ligands for target toxic metals. Hence, the lack of interaction of PIH and its analogues with calcium(II) and magnesium(II) may partly explain their effectiveness at removing iron in vivo and in vitro.

An additional species, $MLH^{-1}$, i.e. $ML(OH)^{-}$, was noted for titrations involving magnesium(II) and the ligands PBH and P*m*FBH. This species generally became important only at pH > 10 (Figs 3b, 5b) and thus is unlikely to be of biological significance. The existence of the species $MLH^{-1}$ may be explained by the presence of an ionisable proton which is present in the complex but not in the ligand alone. Since only three sites on the metal ion are likely to be co-ordinated to the ligand, the remaining sites will be occupied by water molecules, and a proton may dissociate

from one of these co-ordinated water molecules at high pH. This is consistent with the hydrolysis constants for the deprotonation of the simple hydrated metal ions calcium(II) and magnesium(II), which have been reported as 12.7 and 11.42 respectively (Yatsimirskii and Vasilév 1984).

*Interaction of ligands with Zn(II)*

The interaction of the ligands with zinc(II) was far greater than that obtained for either calcium(II) or magnesium(II) (Table 2). The major species formed with zinc(II) was the complex ML which became significant at a lower pH (4–5.5) than for calcium(II) and magnesium(II) (Figs 2c, 3c, 4b, 5c). In addition to the ML species, the $MLH^{-1}$ species (for PIH, PBH, P*m*FBH) and MLH species (PBH) were also evident. The $MLH^{-1}$ species generally became significant at a pH $>7$ (Figs 2c, 3c, 5c). Again this is consistent with the hydrolysis of a co-ordinated water molecule, since the hydrolysis constant of the aquated zinc(II) is 9.6 (Yatsimirskii and Vasilév 1984) i.e. $10^{2-3}$ less than for calcium(II) and magnesium(II).

It is important to note that, by pH 7.5, zinc(II) is fully bound by all four ligands, i.e. the concentration of unchelated M has dropped to zero (Figs 2c, 3c, 4b, 5c). Considering this and the strong interaction of the ligands with zinc(II), a significant physiological effect may be expected. However, the extent of binding in vivo would depend on competition with other naturally occurring ligands. The importance of this binding can be estimated by computer simulation (May and Bulman 1983). However, experimental studies that monitored the concentration of zinc(II) in the bile of rats after administration of PIH detected no increase in zinc(II) excretion (Cikrt et al. 1980). However, zinc(II) excretion in the urine was not measured and hence excretion of zinc(II) by this route needs to be determined before a conclusion is made that loss of zinc(II) is not associated with PIH administration. Even if PIH and its analogues do chelate appreciable quantities of zinc(II), in vivo oral supplements of zinc(II) could be given as has been suggested regarding DTPA iron chelation therapy (Modell and Berdoukas 1984).

Calculation of the pM values (the uncomplexed or 'free' metal ion concentration) in the presence of different ligands provides a simple means of comparing their relative chelation efficacy (Martell et al. 1981). These have been calculated for the four chelating agents of the present study, for desferrioxamine (DFO), the only chelating agent in clinical use for treating iron overload, and for two aminocarboxylic acid chelating agents, EDTA and DTPA (Martell and Smith 1977). The larger the value of pM, the more effective is the ligand as a chelating agent for the particular metal ion. A pM of 6 in the present calculations, i.e. $[M]=10^{-6}$ M, shows that there is neg-

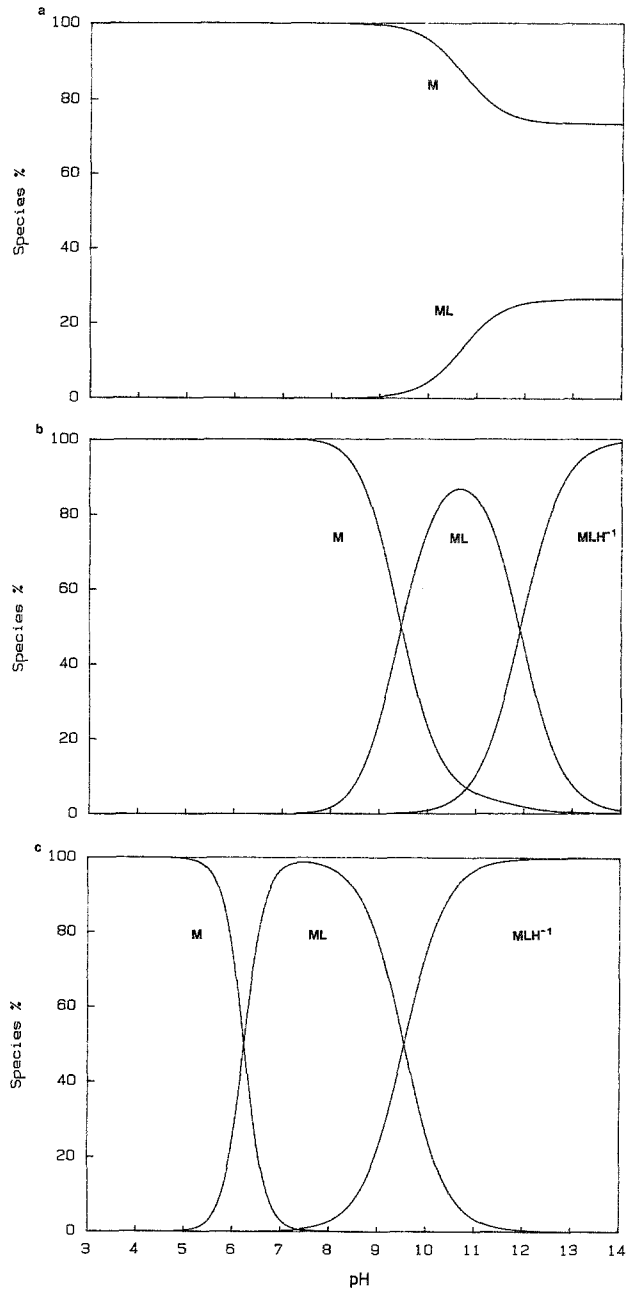


**Fig. 5.** Distribution of metal ligand complex species over pH 3–14 for P*m*FBH and **(a)** Ca(II), **(b)** Mg(II), for $[M]=10^{-6}$ M, $[L]=10^{-3}$ M

ligible binding by the chelating agent, since the calculations assume $[M]_{total} = 10^{-6}$ M.

From Table 3 it is evident that the selectivity of PIH and PBH for metal ions is comparable to that of DFO and much greater than that for EDTA and DTPA. Although the formation constants of P*p*MBH and P*m*FBH with iron(II) and iron(III) have not yet been determined, it is reasonable to assume that they will be similar to that of the parent compound PBH (see Fig. 1), which has been reported (Wis Vitolo et al. 1989). All three have similar pM values for Ca(II), Mg(II) and Zn(II). In addition, the high value of pFe(III) and the low values of pM seen for calcium(II), magnesium(II) and zinc(II) for PBH suggest that this chelator may possess greater selectivity than that of DFO and hence may explain its high efficacy and low toxicity seen in vitro and in vivo (Richardson et al. 1988; Ponka et al. 1989; Richardson et al. 1989b; Wis Vitolo et al. 1989).

Considering all the available evidence, it must be concluded that PIH and especially PBH, P*p*MBH and P*m*FBH remain outstanding candidates as drugs suitable to treat iron overload and deserve further investigation.

**Table 3.** Unbound metal ion concentration expressed as pM values, for PIH, PBH, P*p*MBH, P*m*FBH, DFO, DTPA and EDTA at pH 7.4. Calculated for $[metal] = 10^{-6}$ M and $[ligand] = 10^{-3}$ M

| Ligand | Metal ion | pM[a] |
|---|---|---|
| PIH | Ca(II) | 6.0 |
| | Fe(II) | 6.0[b] |
| | Fe(III) | 27.7[b] |
| | Mg(II) | 6.0 |
| | Zn(II) | 10.1 |
| PBH | Ca(II) | 6.0 |
| | Fe(III) | 39.7[b] |
| | Mg(II) | 6.0 |
| | Zn(II) | 8.1 |
| P*p*MBH | Ca(II) | 6.0[c] |
| | Mg(II) | 6.0 |
| | Zn(II) | 8.1 |
| P*m*FBH | Ca(II) | 6.0 |
| | Mg(II) | 6.0 |
| | Zn(II) | 8.4 |
| DFO[d] | Ca(II) | 6.0 |
| | Fe(II) | 7.6 |
| | Fe(III) | 28.6 |
| | Mg(II) | 6.0 |
| | Zn(II) | 9.2 |
| EDTA[d] | Ca(II) | 6.6 |
| | Fe(II) | 14.3 |
| | Fe(III) | 25.2 |
| | Mg(II) | 8.8 |
| | Zn(II) | 16.4 |
| DTPA[d] | Ca(II) | 9.2 |
| | Fe(III) | 25.9 |
| | Mg(II) | 8.0 |
| | Zn(II) | 17.4 |

a Note that pM = 6 represents no complexation
[b] From Wis Vitolo et al. (1989)
[c] Calculated using estimated value of $\log \beta = 2.7$
[d] Calculated from data in Martell and Smith (1977)

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