Iron Chelators of the Pyridoxal 2-Pyridyl Hydrazone Class. Part 4.[1] pK_a Values of the Chelators and their Relevance to Biological Properties

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The proton binding constants (pK_a) and species distribution over pH range 1.5–12.0 of two types of biologically active iron chelators (*a*) pyridoxal type (L_x)—pyridoxal 2-pyridyl hydrazone (PPH) and pyridoxal isonicotinoyl hydrazone (PIH); (*b*) pyridoxal-betaine type (L_y)—1-[*N*-methyl-pyridoxylidenium]-2-[2′-pyridyl]hydrazine iodide (MPH) and 1-[*N*-ethoxy-carbonylmethylpyridoxyl-idenium]-2-[2′-pyridyl]hydrazine bromide (EPH] have been determined by glass electrode potentiometry. The lowest pK value in type (*a*), in the range 2.62 (PPH)–2.45 (PIH) was assigned to pyridinium protonation; the following ionization constants, pK_{a_2} = 4.63 (PPH)–4.54 (PIH), to pyridoxylidenium protonation; pK_{a_3} = 7.96 (PPH)–7.44 (PIH), to phenolate protonation, and pK_{a_4} = 9.96 (PIH)–9.84 (PPH) to amine–hydrazone protonation. At pH < 2, all ligands exist in the respective protonated forms ($H_4L_x^{2+}$, H_3Ly^{2+} and $H_3L_x^{+}$) and at pH > 11, in the fully deprotonated forms (L_x^{2+}, and Ly^{-}). At pH *ca.* 5.0, the pyridoxal-betaines, MPH and EPH, exist predominantly as zwitterions, whereas PPH and PIH are present at that pH predominantly in the neutral, non-zwitterionic, H_2L_x form. At higher pH (7.2), PPH and PIH, are present as mixtures of the neutral and the negatively charged monodeprotonated forms (**IV**).

Acyl-hydrazones of the class of pyridoxal isonicotinoyl hydrazone (PIH)[2] and pyridoxal benzoyl hydrazone (PBH)[3] have been identified as promising candidates for replacing desferrioxamine (desferal, DF), the only therapeutically safe drug in clinical use, for treatment of iron overload in thalassemic patients.[4] Studies have demonstrated that arylhydrazones of the pyridoxal pyridyl hydrazone (PPH)‡ class and its betaines, 1-[*N*-methylpyridoxylidenium]-2-[2′-pyridyl]hydrazine iodid (MPH) and 1-[*N*-ethoxycarbonylmethyl-pyridoxyl-idenium]-2-[2′-pyridyl]hydrazine bromide (EPH) are considerably more effective[2a,c] as iron mobilizers than either PIH or PBH when administered orally.

Of particular chemotherapeutic interest is the mechanism-based discovery of antimalarial activity (IC_{50} = 1.9 μmol dm^{-3})[6] shown by EPH against the chloroquine-resistant species of *Plasmodium falciparum*, the main causative agent of human malaria. The search for new chelators has been driven in part by the urgent need to enhance immunity to malaria in man by inducing a transient state of iron deficiency at sites of parasite proliferation (red blood cells). The cell compartment targeted for the chemotherapeutic action is the iron-dependent enzyme ribonucleotide diphosphate reductase, which controls the DNA synthesis.§ Studies have shown[9] that the antimalarial activity of EPH is, in fact, iron-dependent and that the mode of action involves carbon-centred Fe^{II}-chelate free radicals,[10] which cause damage to parasite DNA.

In analogy to PIH and its analogues,[11,12] the pyridoxal type ligands (L_x), PIH and PPH, are presumed to exist in aqueous media as five distinctly different species: two protonated (positively charged), **I–II**, **XII–XVI**, one neutral (**III** and **XIV**) and two deprotonated species, **IV–V** and **XV–XVI**. The four corresponding species in the cases of MPH and EPH comprise **VI–IX** (Schemes 1 and 2). Each of these can react differently with iron ions to yield iron complexes with different composition, stability, passage through membranes and redox potentials. Metal coordination and biological properties must therefore depend on the acidity of the cell compartments in which they occur. It is, accordingly, important to determine the

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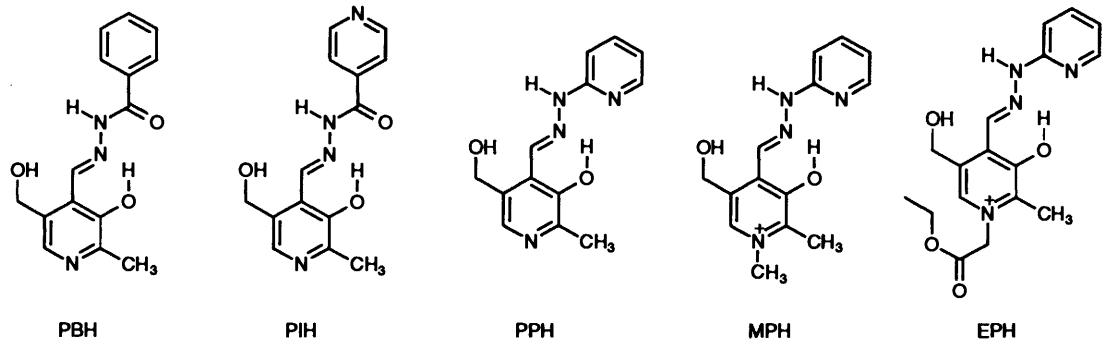
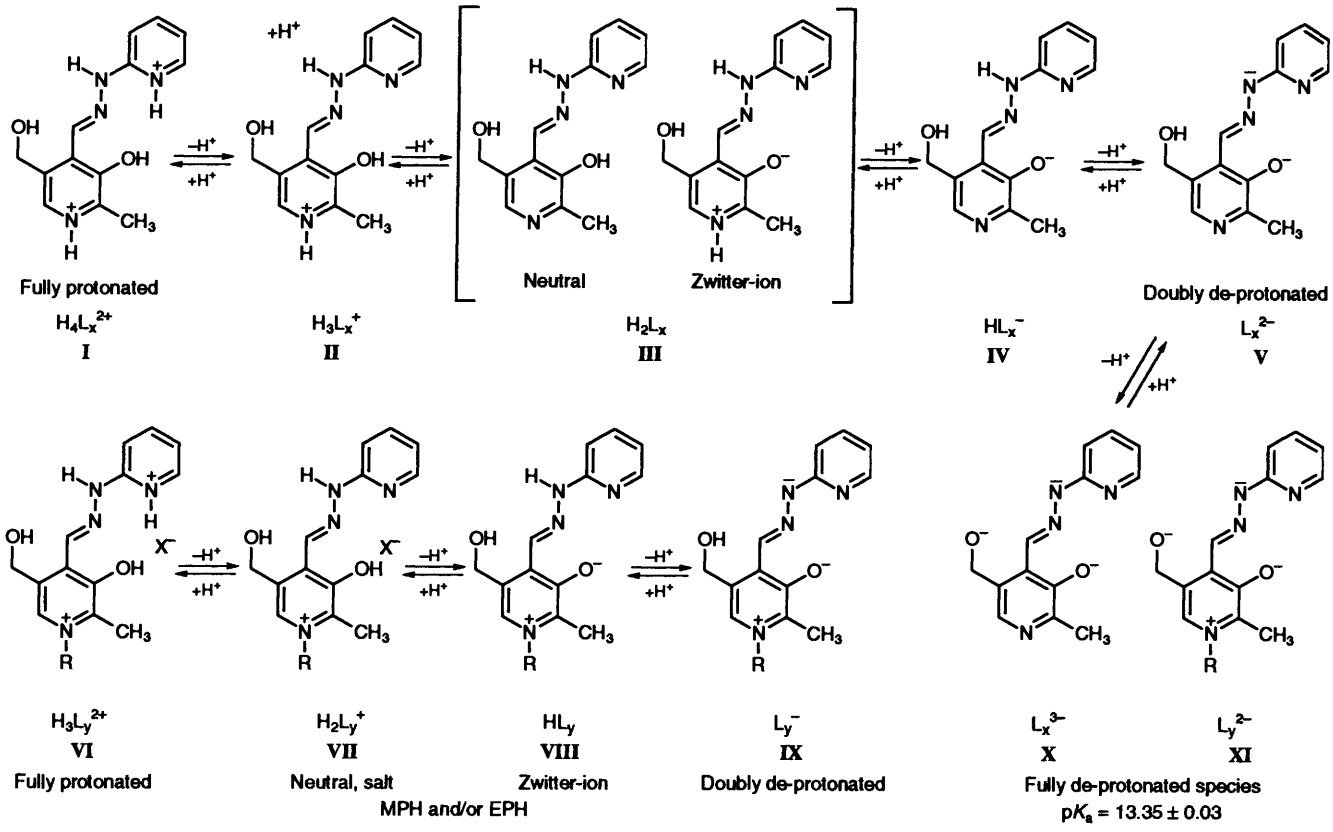
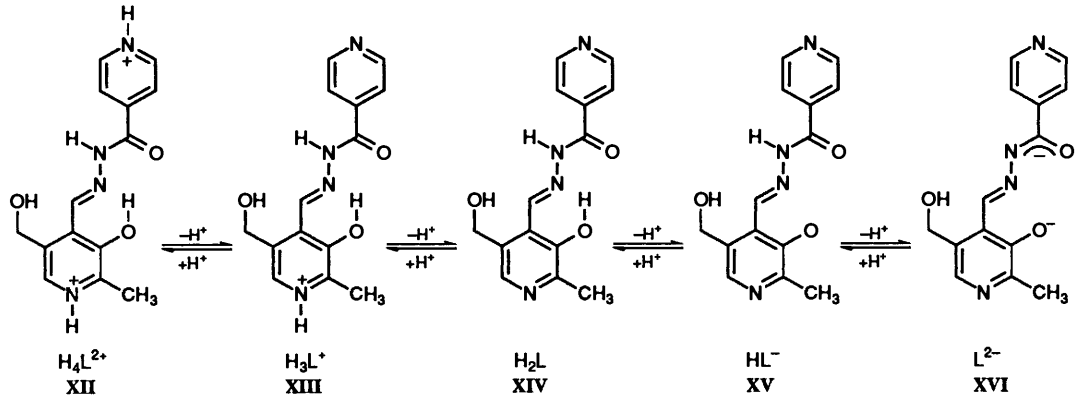
‡ Studies[2a,c,d] have shown that on PIH ⟶ PPH ⟶ EPH conversions, the %-radioiron (^{59}Fe) excretion in rats increase from 37 to 66 to 96%, respectively.

§ Earlier,[7] we reported that EPH inhibits the activity of ribonucleotide diphosphate reductase[8] by 87% at 10 μmol dm^{-3} concentration, relative to 100% hydroxyurea under similar conditions.





Scheme 1 pH-Dependent species of PPH (I–V), MPH and EPH (VI–XI)



Scheme 2 pH-Dependent species of PIH

pK_a values of these biologically-active chelators. Towards this end, we have adopted two distinctly different experimental methods. One, the technique of pH-dependence of carbon-13 nuclear magnetic resonance spectroscopy (^{13}C NMR) is to be published elsewhere,[13] and the second, glass electrode potentiometry[14] is the subject of the present paper. The protonation constants, together with speciation of protonation constants as a function of pH, included here, are required for estimation of complex-formation between the ligands and Fe^{2+} and Fe^{3+} ions.[15]

Experimental

Chelating Agents.—PPH, m.p. 288 °C (free base); MPH, m.p. 235–236 °C (free base); and PIH, m.p. 245 °C (monohydrochloride) were prepared according to literature and recrystallized from methanol.

1-[N-*Ethoxycarbonylmethylpyridoxylidenium*]-2-[2′-*pyridyl*]*hydrazine bromide* (*EPH*). This was prepared by allowing 1-[pyridoxylidene]-2-[2′-pyridyl]hydrazine (PPH) (0.93 g, 3.5 mmol) in dry ethanol (15 cm^3) to react with ethyl bromoacetate (1.2 g, 7 mmol) under reflux for 24 h. The yellow crystalline product (81%) deposited after removal of solvent and washings with diethyl ether, was recrystallized once from methanol and then from ethanol afforded crystals melting at 204 °C; λ_{max}/nm(MeOH, *c* 5.10^{-6} mol dm^{-3}) 651 (log ε 1.55), 407.7 (4.41), 242.5 (4.13), 205.5 (4.20); *m*/*z* (EI): 258 [M^+ – ($BrCH_2CO_2Et$)], 108 [$C_6H_8N_2^+$, base-peak]; δ_H(300 MHz, [$^2H_6$]Me_2SO) 8.579 (s, 1 H), 8.467 (s, 2 H), 8.31 (t, 1 H, *J* 1.6 Hz), 7.83–7.77 (sextet, 1 H), 7.06–6.94 (m, 2 H), 5.71 (s, 2 H), 4.79 (d, 2 H, *J* 2 Hz), 4.26 (q, 2 H, *J* 2.3 Hz), 2.60 (s, 3 H) and 1.26 (t, 3 H, *J* 2.4 Hz) (Found: C, 48.8; H, 5.3; Br, 18.2; N, 12.2. Calc. for $C_{18}H_{24}BrN_4O_4$: C, 49.09; H, 5.45; Br, 18.18; N, 12.73).

Protonation Constants.—Preparation of ligand solutions. Nitric acid and sodium hydroxide solutions were obtained from Merck (Titrisol). The concentrations of these solutions were checked regularly by acid-base titrations in the course of

Table 1 pK_a Values of PPH, MPH, EPH, PIH and PBH[a]

pK	PIH	PPH	MPH	EPH	PBH[a]	Assignment
pK_a 1	2.45 ± 0.10	2.62 ± 0.09	2.39 ± 0.31	2.31 ± 0.15	—	PYR ring N
pK_a 2	4.54 ± 0.07	4.63 ± 0.05	—	—	4.59 ± 0.04	PDX ring N
pK_a 3	7.44 ± 0.14	7.96 ± 0.07	7.30 ± 0.42	7.43 ± 0.35	8.47 ± 0.04	PDX OH
pK_a 4	9.94 ± 0.50	9.84 ± 0.07	9.96 ± 0.76	9.70 ± 0.51	11.40 ± 0.04	NH–N=

[a] Data from ref. 18.

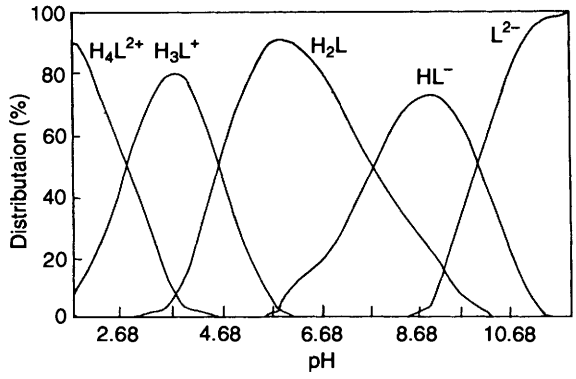


Fig. 1 Species distribution of PPH over pH range 1.5–12.0

electrode calibration.[16] Stock solutions of 0.2 mmol dm^{-3} of PPH, MPH, EPH and PIH were freshly prepared before each titration by dissolving the respective ligand in 0.15 mol dm^{-3} KNO_3 (Merck Analytical grade). Doubly-distilled water was used throughout the experiments.

Potentiometric titrations. Potentiometric (pH) titrations were used to determine the protonation constants for PPH, MPH, EPH and PIH. Potentiometric titration data were obtained using a purpose-built automatic titrator of PHM62 Radiometer pH meter, with a 5 cm^3 piston burette, a digital electrometer and stirrer, which were all under the control of a programable calculator. Potentiometric measurements were carried out in a capped titration vessel, fitted with a GK 2322C combine electrode and a fibre-tipped saturated calomel electrode. The titrations were performed under an inert atmosphere maintaining a flow of pure N_2 through the titration vessel. The entire apparatus was kept in a thermostat at 37 ± 0.5 °C. The electrode calibrations were made in a 0.15 mol dm^{-3} KNO_3 at 37 °C by acid-base titration[16] under identical conditions. Analysis of the titration data for PPH, MPH, EPH and PIH was generally performed using the SUPERQUARD computer program[17] under the IBM RISC/6000 computer machine of The Hebrew University of Jerusalem. The data were between 300 and 500 for the calculations. The assignment of the pK_a values assembled in Table 1, was based on *ab initio* calculations,[1] validated by pH-dependent (^{13}C NMR) measurements.[1] Species distribution of PIH, PPH, MPH and EPH, over the pH range 2–12, are displayed in Figs. 1–4.

Results and Discussion

The ligands described above comprise of pyridoxylidene and pyridyl rings, each of which exist in the aqueous media as several prototropic species.[14,18] Pyridoxal prototropic behaviour has been thoroughly analysed, and this species is known to be present in aqueous solutions as at least four pH-dependent structures.[11,12] Furthermore, both the pyridyl and the isonicotinoyl moieties can protonate at the heterocyclic ring nitrogens (N^4). All of them can deprotonate at the phenolic hydroxyl (HO^1), and the amine of the hydrazone bridge (N^3) can be either deprotonated or protonated. These pyridoxal-based series of synthetic ligands exist hence in aqueous media as several pH-dependent structures (**I–XVI**), as portrayed by the structurally related ligands: PPH, and MPH–EPH (see Schemes 1 and 2). Each of the other synthetic ligands would, therefore, correspond to a case described in Scheme 3.

$$H_4L_x^{2+} \longrightarrow H_3L_x^{+} + H^+ K_1 = [H_3L_x^{+}][H^+]/[H_4L_x^{2+}] \quad (1)$$

$$H_3L_x^{+} \longrightarrow H_2L_x + H^+ K_2 = [H_2L_x][H^+]/[H_3L_x^{+}] \quad (2)$$

$$H_2L_x \longrightarrow HL_x^{-} + H^+ K_3 = [HL_x][H^+]/[H_2L_x] \quad (3)$$

$$HL_x^{-} \longrightarrow L_x^{2-} + H^+ K_4 = [L_x^{2-}][H^+]/[HL_x] \quad (4)$$

$$H_3Ly^{2+} \longrightarrow H_2Ly^{+} + H^+ K_{1'} = [H_2Ly^{+}][H^+]/[H_3Ly^{2+}] \quad (5)$$

$$H_2Ly^{+} \longrightarrow HLy + H^+ K_{3'} = [HLy][H^+]/[H_2Ly^{+}] \quad (6)$$

$$HLy \longrightarrow Ly^{-} + H^+ K_{4'} = [Ly^{-}][H^+]/[HLy] \quad (7)$$

Scheme 3

The positively charged species: $H_4L_x^{2+}$ (**I**) and H_3Ly^{2+} (**VI**) represent the respective fully protonated forms of PPH, PIH, MPH and EPH. The H_2L_x (**III**) and H_2Ly^{+} (**VII**) forms are electrically neutral, and the HL_x^{-} (**IV**) and HLy (**VIII**) species represent the corresponding monodeprotonated forms. The negatively charged species, L_x^{2-} (**V**) and Ly^{-} (**IX**), are the respective doubly deprotonated forms. In the cases of MPH and EPH, the pyridoxylidenium protonation stage (eqn. 2) could not take place and so this type of ligand could have only three pK_a values: pK_{a_1}, pK_{a_3} and pK_{a_4}.

Analysis of the titration curves yielded, indeed, four pK_a values for PIH and PPH, and three each for MPH and EPH. From examination of the pK_a values of PIH and PPH (Table 1), it is apparent that the extra carbonyl in PIH enhances the electron withdrawing power of the pyridyl group. The pK_a of 7.96 in PPH is associated with the ionization of phenolic ^1O–H proton which is one half unit higher than that of PIH (7.44), but is one half unit lower than that of pyridoxal pyridyl hydrazone (PBH, 8.47).[18] However, the pK_a of 9.84 in PPH, associated with the ionization of the proximal NH–N proton, is essentially identical to that of PIH (9.94), but is 1.6 units lower than that of PBH (11.40).[18] Typically, quaternization of the pyridoxal ring nitrogen (N^1) in PPH acts similarly to a nitro group by reducing the electron density on the aromatic ring. Thus, the phenolic pK_a of 7.30 in MPH is 0.66 units lower than that of PPH, but the ionization constant of the distant NH–N proton in MPH (9.76) compares with that of PPH (9.84). On the other hand, removal of the carbonyl group from the acyl hydrazone, PIH, does not have a substantial effect on the pK_a values of the two distant ring nitrogens, N^1 and N^4 (compare PIH, PPH, MPH, EPH and PBH in Table 1).

Species Distribution Plot as a Function of pH.—Plotting of species distribution in solution of PIH, PPH, MPH, EPH, against pH, in the range pH 1.5–12.5 (Figs. 1–4), clearly indicate

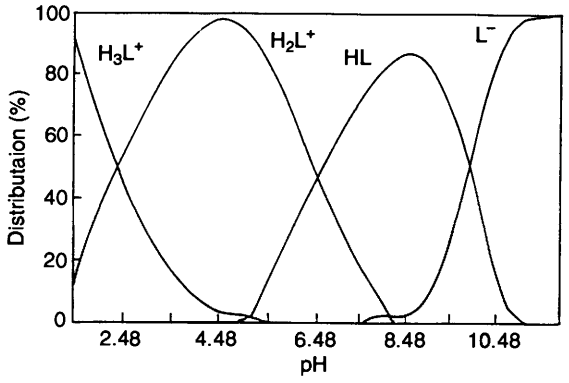


Fig. 2 Species distribution of MPH over pH range 1.5–12.0

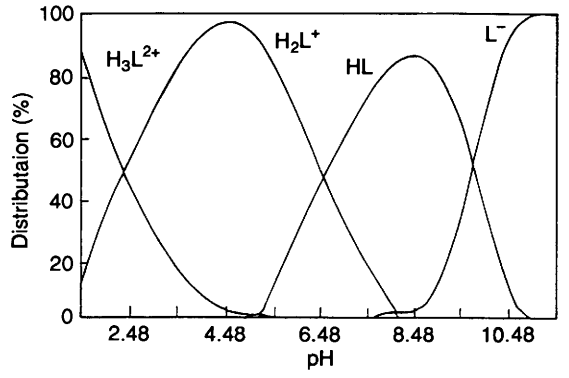


Fig. 3 Species distribution of EPH over pH range 1.5–12.0

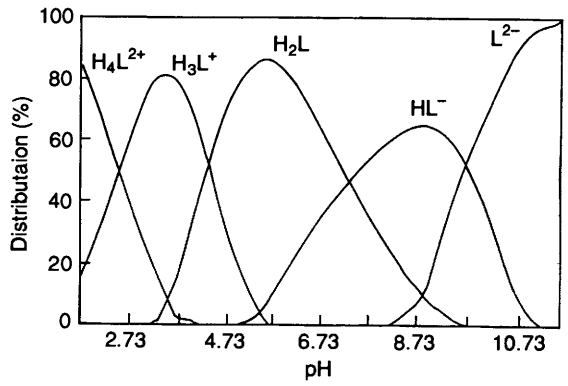


Fig. 4 Species distribution of PIH ocver pH range 1.5–12.0

Table 2 Species distribution (%) of protonated, neutral and deprotonated forms of PPH, MPH, EPH and PIH at pH 5.2 and 7.2

	pH	
Ligand	5.2	7.2
	(II–XIII):(III–XIV)	**(III–XIV):(IV–XV)**
PPH	20:80	65:35
PIH	17:83	55:45
	(VII):(VIII)	**(VII):(VIII)**
MPH	87:13	33:67
EPH	93:7	37:63

that at pH < 3, three to four sites on the ligand molecules become fully protonated. From plots in Figs. 1–4, it can be seen that at pH 5.2 (the biological significance of which is underlined in the sequence), the molecules of PIH and PPH exist in solution as 1:4–**[II]**:**[III]** mixtures, whereas MPH and EPH at same pH (5.2), exist as 9:1–**[VIII]**:**[VII]** mixtures. The monodeprotonated species, **(IV)**, appears to be totally absent in the PPH–PIH type. However, at physiological pH (7.2), PPH and PIH exist to a large extent (55–65%) in the neutral **(III)** form, whereas MPH and EPH, at that pH (7.2) exist predominantly (65%) in the monodeprotonated **(VIII)** form.

Biological relevance. The pK_a values of the phenol functional groups in the less potent antimalarials, PIH and PPH (Table 1), are in the physiological range, such that at blood pH (7.2–7.4) they populate either the neutral or the protonated states. The presence of a positively-charged pyridoxylidenium group in the potent antimalarials MPH and EPH, render the latter at blood pH to be populated both by neutral and deprotonated states (see Table 2). Following the passage of **VII** (neutral forms of the most potent antimalarials) from the blood media to the parasitized red blood cell,¶ and then to the trophozoite (a well defined stage in the intraerythrocytic life-cycle of *Plasmodium falciparum*,[24] the pH of its food vacuole is estimated to be approximately 5.2),[25–27] the percentage of this positively charged species **(VII)** increases three-fold, at the expense of its respective neutral monodeprotonated species **(VIII)**. In the corresponding biochemical event, however, the percentage of the neutral forms of PPH **(III)**, and of PIH **(XIV)**, both weaker iron mobilizers and antimalarials than their respective parent compounds (PIH and PPH), increase by only 0.3-fold, as they reach the parasite food vacuole. This associates metal coordination and biological properties with the acidity of the cell compartment[28] in which the metal-complexation occurs.[6] Hence the pyridoxal-based antimalarials are not assumed to concentrate in a definite organelle of the trophozoite, as the quinoline-based antimalarials, chloroquine, does.[23] Indeed, they were shown to operate by a distinctly different mechanism.[7]

The results suggest that the lipophilic ligands could diffuse through the cell membrane, allowing absorption from the stomach and access to intracellular ion pools. In PIH and PPH, the presence of the neutral species would be maximal at pH 6 and hence maximal absorption would probably occur in the small intestine where the pH is in this range.[29]

All ligands are virtually fully deprotonated at pH > 11. The species distribution of PPH, MPH, EPH and PIH as a function of pH are shown in Figs. 1–4. They agree well with the data of related structures in the literature.[14,18–22]

Conclusions

The pK_a values of PPH, MPH, EPH and PIH, produced here by glass electrode potentiometry are in harmony with the values produced by the ^{13}C NMR spectrometric method, and in excellent agreement with the *ab initio* calculations of proton binding energies to relevant sites of the ligands. This allows us to assign the pK_a values of the ligands in the following: pyridinium protonation < pyridoxylidenium protonation < phenolate protonation < amine-hydrazone protonation < alkoxide protonation.

It is apparent that the extra carbonyl in PIH (PPH ⟶ PIH) enhances the electron withdrawing power of the pyridyl group. Moreover, quaternization of the pyridoxal ring-N in PPH acts similarly to a nitro group by reducing the electron density on the aromatic ring, and by promoting the acidity of the phenolic group. This renders the neutral species of MPH–EPH predominancy at pH 5.2, which promotes biological iron-

¶ The diffusion of pyridoxal isonicotinoyl hydrazone (PIH) into the red blood-cell has been shown to be energy-independent.[23] PPH is believed to behave similarly to PIH in this sense.

complexation and hence iron-mobilization at relevant cell compartments. The significance of this in antimalarial action is still unknown.

Acknowledgements

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