Biol Metals (1989) 2:69–76

BIOLOGY OF METALS

Pyridoxal isonicotinoyl hydrazone and analogues

Study of their stability in acidic, neutral and basic aqueous solutions by ultraviolet–visible spectrophotometry

Des Richardson[1], Lucia Wis Vitolo[2], Erica Baker[1], and John Webb[2]

[1] Department of Physiology, University of Western Australia, Perth, Western Australia, 6009
[2] School of Mathematical and Physical Sciences, Murdoch University, Perth, Western Australia, 6150

Summary. The ultraviolet-visible absorption spectra 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 measured in aqueous solution with various concentrations of added acid or alkali. Assignment of absorption bands to various molecular species in equilibrium in aqueous solution is made by reference to their acid ionisation constants. All four hydrazones were stable at physiologial pH, but hydrolysed in strongly acidic and basic solutions, resulting in the liberation of pyridoxal and the acid hydrazide. In acidic solutions this resulted in a dramatic decrease in the intensity of absorption at wavelengths of 225 nm and above 300 nm, allowing a quantitative estimate of the degree of acid-catalysed hydrolysis of the ligands. These results indicate that for oral administration the chelator should be administered with calcium carbonate or provided with an enteric coating to minimise acid-catalysed hydrolysis in the stomach. At high pH, base-catalysed hydrolysis occurred, resulting in a decrease in the absorption at a wavelength of 387 nm.

Key words: Pyridoxal isonicotinoyl hydrazone — Ultraviolet-visible spectrophotometry — Acid/base-catalysed hydrolysis

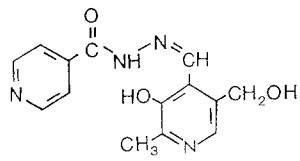
Introduction

Abnormal accumulation of iron in the parenchymal cells of the liver and other organs is a serious complication of thalassemia major and other chronic anaemias requiring long-term blood transfusion (Modell and Berdoukas 1984). These haemoglobinopathies are now recognised as being of wide distribution, particularly in the Mediterranean region and South East Asia (WHO 1983). The only practical method of iron mobilisation in chronic anaemias is with the use of an iron-chelating drug. Desferrioxamine is at present the only iron chelator which is commercially available and of relatively low toxicity (Martell et al. 1981). However, the high cost of desferrioxamine (approximately $10 000 per patient per year in Australia) and its poor intestinal absorption (Kattamis et al. 1981) are serious limitations and underline the need for an alternative iron-chelating compound, especially one wich is effective when given orally (Baker 1988; Webb and Vitolo 1988).

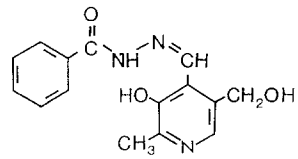
Extensive evaluation of pyridoxal isonicotinoyl hydrazone (PIH) in vivo (Avramovici-Grisaru et al. 1983; Cikrt et al. 1980; Hershko et al. 1981; Hoy et al. 1979; Johnson et al. 1982; Ponka et al. 1979b; Williams et al. 1982) and in vitro (Baker et al. 1985; Hoy et al. 1979; Ponka et al. 1979a, b; Williams et al. 1982) has shown that this orally effective iron-chelating agent (Cikrt et al. 1980; Hershko et al. 1981; Hoy et al. 1979; Williams et al. 1982) holds substantial promise in the treatment of iron-loading diseases such as thalassemia. A recent clinical trial of this compound in a limited number of iron-loaded patients has shown encouraging results (Brittenham, personal communication).

Recent studies have shown that three analogues of PIH, pyridoxal benzoyl hydrazone (PBH), pyridoxal *p*-methoxybenzoyl hydrazone (P*p*MBH) and pyridoxal *m*-fluorobenzoyl hydrazone (P*m*FBH), are more active on a molar basis

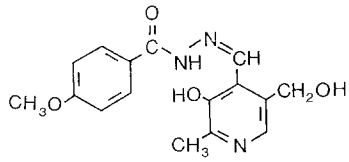
Offprint requests to: J. Webb



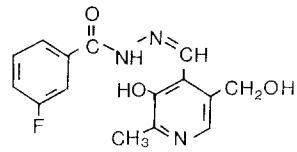
I PIH



II PBH



III PpMBH



IV PmFBH

Fig. 1. Structural formulae of *(I)* pyridoxal isonicotinoyl hydrazone (PIH), *(II)* pyridoxal benzoyl hydrazone (PBH), *(III)* pyridoxal *p*-methoxybenzoyl hydrazone (P*p*MBH) and *(IV)* pyridoxal *m*-fluorobenzoyl hydrazone (P*m*FBH)

than PIH and desferrioxamine in the hepatocyte, macrophage and reticulocyte screens (Ponka et al. 1988; Richardson et al. 1988). Further investigation of these compounds showed that at pH 7.5 the ligands are present predominantly as the neutral molecule, allowing access to intracellular iron pools (Richardson et al., unpublished results). In additon, PIH and some analogues are highly specific for iron(III), showing a selectivity comparable to that seen with desferrioxamine and much greater than that seen with the aminocarboxylic chelating agents, DTPA and EDTA (Richardson et al. and Vitolo et al., unpublished results).

In the light of these encouraging reports, the ligands were investigated further to gain additional understanding of their chemical properties in solution which may be of use in their clinical application. In this paper we report the ultraviolet-visible spectrophotometric investigation of the stability of PIH and its three analogues, PBH, P*p*MBH and P*m*FBH (Fig. 1) in acidic, neutral and basic solutions.

The solution chemisty of these ligands may be important to consider in terms of: (a) the stability of these compounds towards acid-catalysed hydrolysis which could occur in the stomach; (b) the protocol used for potentiometric titrations over a wide pH range (pH 2–12), the results of which will be reported elsewhere (by Richardson et al. and Vitolo et al.), and (c) the methods used to dissolve the ligands prior to experimentation in vitro and in vivo.

Materials and methods

PIH, PBH, P*p*MBH and P*m*FBH were prepared as will be described elsewhere (by Richardson et al.).

The ultraviolet-visible spectra were obtained using a Hewlett-Packard 8450 spectrophotometer with a pair of matched 1-cm quartz cuvettes. All spectral studies were carried out immediately after preparing solutions.

The hydrolysis of PIH and analogues was investigated under pseudo-first-order conditions with a ligand concentration much lower than the acid or base concentration. For kinetic measurements concerning acid-catalysed hydrolysis, the ligands at a concentration of 0.05 mM were prepared in nitric acid (50 mM, pH 1.3) diluted from a 0.1000 M nitric acid stock solution prepared from concentrated volumetric ampules (BDH, UK). All solutions were prepared using distilled water that had been passed through a Millipore Milli-Q System, henceforth referred to as 'millipore water'. At pH 7.5, the buffer used was Hepes (Sigma Chemical Co., St. Louis, MO, USA). For kinetic measurements of base-catalysed hydrolysis, the ligands at a concentration of 0.1 mM were prepared in 0.5 mM KOH (pH 10.7) diluted from a 0.1000 M potassium hydroxide stock solution prepared from concentrated volumetric ampules (BDH, UK) diluted with fresh, degassed millipore water. The pH of the solutions was measured using a DDS pH meter equipped with a Beckman glass electrode which was calibrated with Beckman calibration buffers.

Kinetic studies were performed using the spectrophotometer described above fitted with a cell block thermostatted at $25 \pm 0.1°$ C. First-order rate constants were calculated as described by Chang (1981).

Results and discussion

The absorption spectra of PIH at pH 1, 7.4 and 12 are illustrated in Fig. 2. Similar spectra were also noted for PBH, P*p*MBH, P*m*FBH and 35 other analogues of PIH (Edward et al. 1988). As is evident from Fig. 2, the spectral changes associated with these ligands are complex. The changes which occur in the spectra can be explained by the complex protonation-deprotonation equilibria of the ligands in accordance with their acid dissociation constants (Richardson et al. unpublished results).

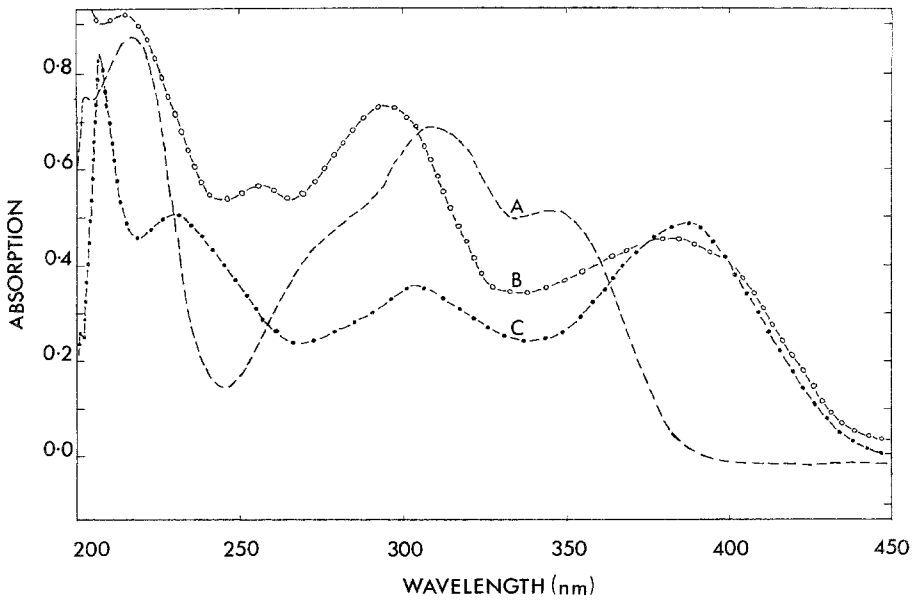


Fig. 2. The ultraviolet-visible absorption spectra of PIH at pH 1.0 **(A)**, pH 7.5 **(B)** and pH 12.0 **(C)**. Similar spectral features were observed with 35 other aroylhydrazones of similar structure (Edward et al. 1988)

Ultraviolet–visible spectra in acidic solutions

For PIH at pH 1 there are three strong absorption bands at 233 nm, 307 nm and 343 nm (Fig. 2A), corresponding to the fully protonated ligand (H_4L^{2+}), where the two ring N atoms (Fig. 1) are protonated (Richardson et al., unpublished). It should be noted that the phenolic hydrogen is probably H-bonded to the imine nitrogen, two tautomeric forms being possible, as suggested for other Schiff bases of similar structure (Dudek and Dudek 1966; Ferguson and Kelly 1951; Matsushima and Martell 1967; Metzler 1957).

If the spectrum of the ligand in acidic solution (pH 1) is observed over a number of hours, there is a dramatic decrease in absorption at the wavelengths of 233 nm and greater than 300 nm, and a blue shift of the maximum absorbance (Fig. 3). The final spectrum obtained is the same as that for equimolar amounts of pyridoxal and isonicotinic acid hydrazide (INH) in acidic solution. Similar observations were made for the remaining three hydrazones. Comparable observations have been reported for the acid-catalysed hydrolysis of pyridoxylidenevaline into pyridoxal and valine (Matsushima and Martell 1967).

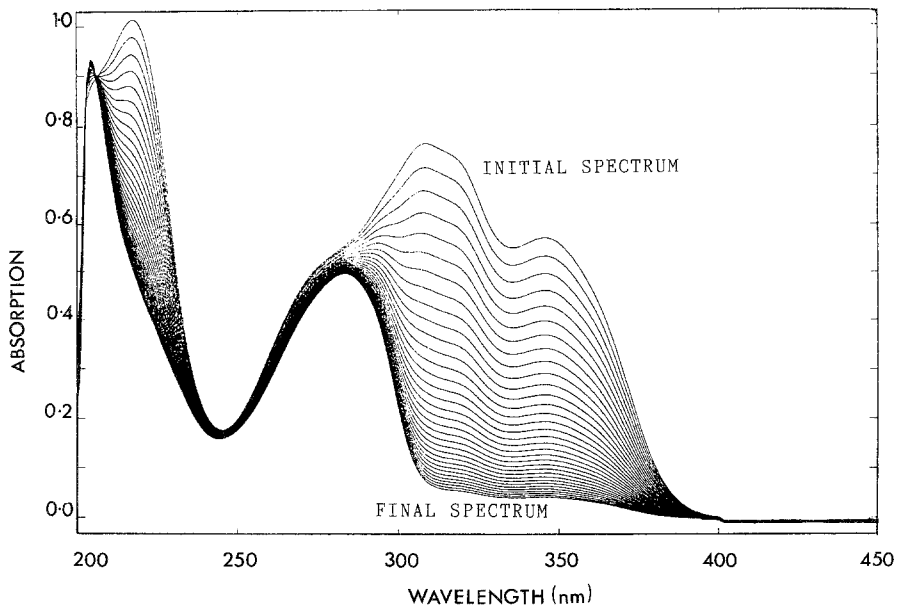


Fig. 3. The acid-catalysed hydrolysis of PIH at pH 1. Spectra were recorded every 100 s. There was a marked decrease in absorbance of 233 nm and at all wavelengths greater than 300 nm

This evidence indicated rapid hydrolysis of PIH and its analogues into pyridoxal and INH in acidic solution (pH 1). This suggestion is also supported by the fact that when either acid-hydrolysed PIH or an acidic solution of equimolar amounts of pyridoxal and INH were adjusted to pH 12, the final spectrum is identical to that of a freshly prepared solution of PIH at pH 12 (Fig. 2C). These changes are associated with isosbestic points at 240, 250, 275 and 310 nm. Hence pyridoxal and INH are condensing to form the hydrazone at high pH. The marked increase in absorbance which occurs at 387 nm may suggest that this peak is characteristic of the hydrazone linkage at basic pH (see below).

In addition to this high-pH peak, the absorption peak above 300 nm, typically at 340 nm in acidic solutions, is also dependent on the existence of the hydrazone linkage. Hence these peaks can provide a quantitative estimate of the degree of hydrolysis of PIH and the analogues into pyridoxal and the acid hydrazide.

Kinetic measurements were performed in order to calculate the rate constants and half-life values of the ligands in acidic solution. From these measurements the hydrolysis of the hydrazones in acidic solution was shown to follow a first-order rate law. At pH 1 and 25° C, the rate constants varied only slightly over $3\text{–}5 \times 10^{-4}\ s^{-1}$, corresponding to half-life values of 1–2 ks. More precise measurements at pH 1.3 yielded the results shown in Table 1. The rate constant for the hydrolysis of the ligands is approximately 10 times less than that seen at pH 1. The rate constants for PIH, PBH and P*m*FBH are similar, varying over $4.04\text{–}4.14 \times 10^{-5}\ s^{-1}$ which correspond to half-life values of 16.7–17.2 ks. The stability of P*p*MBH in 50 mM nitric acid appears to be greater than that of PIH, PBH and P*m*FBH (see Table 1), perhaps due to the electron-donating properties of the methoxy substituent, stabilising the hydrazone linkage. Unsubstituted *o*-hydroxy Schiff bases have been shown to hydrolyse more rapidly than those with methoxy substituents (Chatterjee et al. 1963). Compared with other hydrazones, PIH and its analogues are relatively stable in acidic solutions. For example, *N*-salicylideneaniline has a half-life of 6 min in a 50% (by vol.) water/dioxane solution at a concentration of 0.02 mM (Chatterjee et al. 1963).

Table 1. First-order rate constants (k) and half-life values ($t_{1/2}$) for the acid-catalysed hydrolysis of PIH, PBH, P*p*MBH and P*m*FBH using a mineral acid concentration of 50 mM and a ligand concentration of 0.05 mM

Ligand	Wavelength (nm)	$10^5 \times k$ (s^{-1})	$t_{1/2}$ (ks)
PIH	345	4.14 ± 0.08	16.7 ± 0.3
PBH	345	4.08, 3.99	17.0, 17.4
P*p*MBH	344	3.47 ± 0.09	20.0 ± 0.5
P*m*FBH	345	4.14 ± 0.04	16.8 ± 0.2

Results given are the mean ± SD for three experiments, except for PBH where the results of two separate experiments are given

A possible mechanism for the hydrolysis of the hydrazone linkage involves the protonation of the imino nitrogen as the first step in the acid-catalysed hydrolysis of the carbon-nitrogen double bond (Bruylants and Feytmants-de Medicis 1970; Bruyneel et al. 1966; Cordes and Jencks 1962; Dash and Nanda 1969; Subha and Rao 1982). It should be noted that, in acidic solution, the rate-determining step is the attack of the amine on the carbonyl group (Cordes and Jencks 1962), whereas the rate-determining step in neutral solution is the addition of water to form the carbinolamine intermediate (Willi 1956). Hydrolysis of resacetophenone isoniazid hydrazone proceeds by a mechanism involving the positively charged protonated substrate and the hydronium ion (Subha and Rao 1982). It has also been demonstrated that the rate of hydrolysis is inversely proportional to the concentration of the Schiff base (Chatterjee et al. 1963; Subha and Rao 1982). Preliminary experiments in the present study also demonstrated this effect (data not shown).

Clinical implications. Several authors have reported that suitable solutions of PIH and other aroylhydrazones can be obtained by dissolution in acid followed by adjustment to neutral pH (Cikrt et al. 1980; Ekblom et al. 1986; Ponka et al. 1979b; Williams et al. 1982). This procedure would probably result in hydrolysis of some of the ligand (depending on the concentration of acid and ligand used and the time of exposure of the ligand to the acidic solution) and contamination with the aromatic aldehyde and acid hydrazide; hence it cannot be recommended.

An orally effective iron chelator should be stable towards acid hydroylsis which could occur in the stomach. Parietal cells of the stomach secrete 0.16 M HCl (Junqueira and Carneiro 1980), resulting in gastric juice with a pH of 0.8–1.5 which may act on food for over 4 h (Schmidt and Thews 1983). Hence, whether a compound is stable to these highly acidic conditions will dictate its mode of administration. The results of the present

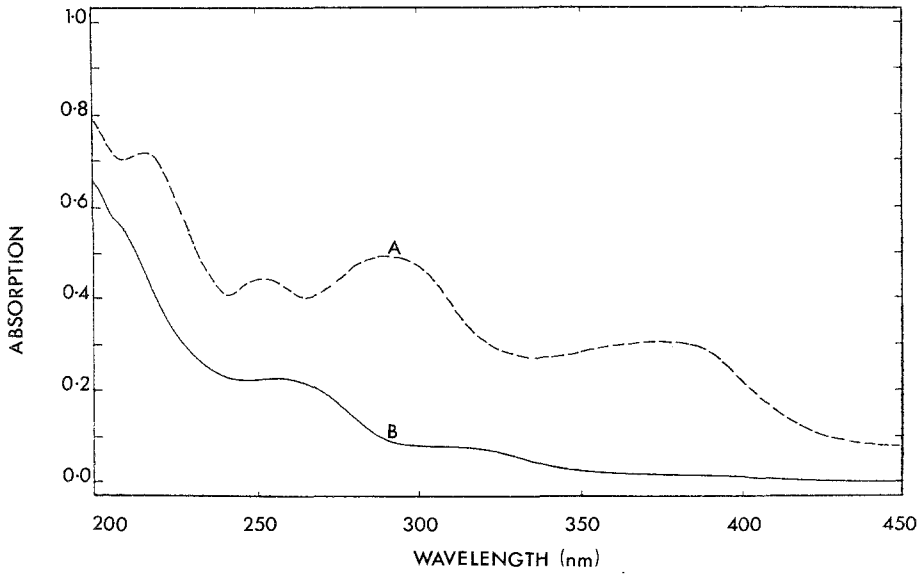


Fig. 4. The ultraviolet-visible spectra of PIH at pH 7.5 at 20° C recorded **(A)** when initially prepared and **(B)** 161 days later

study indicate that, if these ligands reach clinical trials, it would be desirable to administer the compounds to the fasting patient and/or administer them with calcium carbonate to limit hydrolysis in the stomach. In addition, enteric coating of drugs has been developed to prevent hydrolysis in the stomach (Goodman and Gilman 1975, 1980) and should also be employed in these clinical studies.

The data supporting acid-catalysed hydrolysis of the ligands may explain the reported failure of PIH and PBH to increase long-term iron excretion in the rat (Williams et al. 1982). In this study short-term administration of the ligand to fasting rats resulted in a 630% increase in iron excretion in the urine and faeces compared to controls. However, when the compounds were mixed with the normal rat diet at a comparable dose over a period of 10 weeks no increase in iron excretion over the control was noted. When the ligands were administered to fasting rats, acid hydrolysis of the ligand would have been minimal, as the fasting stomach secretes small amounts of gastric juice with a neutral to alkaline pH (Schmidt and Thews 1983). However, when food is eaten, gastric juice with a high concentration of HCl is produced (Schmidt and Thews 1983), which may have resulted in hydrolysis of some of the ligand, decreasing the amount of PIH available for iron chelation.

Ultraviolet-visible spectra in neutral solutions

For PIH in neutral solution (pH 7.5) four bands are observed at 210, 295, 308 and 384 nm (Fig. 2B). At physiological pH (pH 7.5) for PIH, the species H_2L^0 predominates (73%) over HL^- (27%) (Richardson et al., unpublished) and the spectral bands at this pH cannot be assigned to either species. The 384-nm band (absent at pH 1) extends into the blue region of the visible spectrum and the solutions are consequently yellow, the intensity of the yellow colour increasing as the pH is raised. It was important from a practical view-

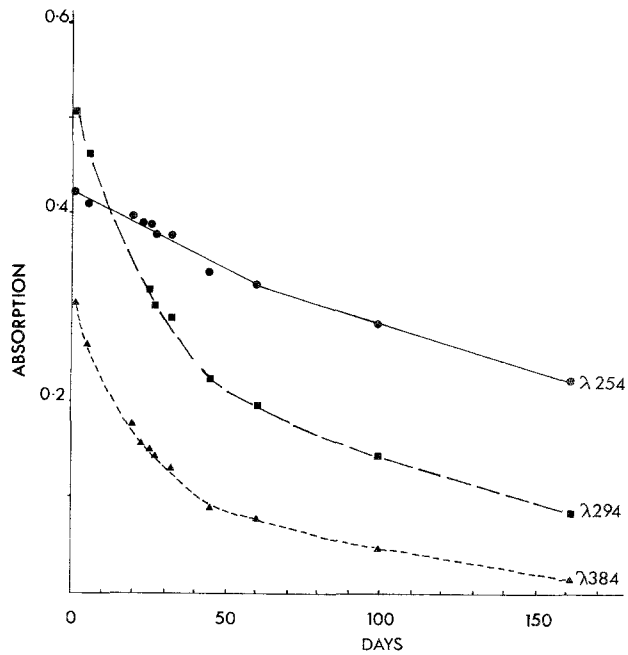


Fig. 5. Changes in absorption intensity at selected wavelengths for PIH, pH 7.5, 20° C, over 161 days. Curves shown are illustrative only

Table 2. First-order rate constants and half-life values for the hydrolysis of PIH, PBH, P*p*MBH and P*m*FBH measured at pH 7.5 at room temperature (≈20° C) over 161 days

Ligand	Conc. (mM)	Wave-length (nm)	$10^7 \times k$ (s^{-1})	$t_{1/2}$ (days)
PIH	0.05	254	0.44	182
		294	1.26	84
		384	2.22	36
PBH	0.05	295	1.20	67
		376	1.76	46
P*p*MBH	0.05	252[a]	—	—
		301	0.99	88
P*m*FBH	0.1	294	1.17	69
		382	1.90	42

[a] For chelator P*p*MBH at a wavelength of 252 nm a first-order rate law was not obeyed

point to investigate the stability of the chelators at physiologial pH. A 0.05 mM solution of PIH, PBH, P*p*MBH and a 0.1 mM solution of P*m*FBH were prepared at pH 7.5 and left standing for 161 days at room temperature (≈20° C) with the absorption spectrum being recorded periodically. The absorption spectrum of a freshly prepared solution of PIH compared to the spectrum of the same solution 161 days later is presented in Fig. 4. Changes in absorption intensity at three wavelengths are shown for PIH in Fig. 5. For PIH, PBH and P*m*FBH the band at the highest wavelength (384 nm, 376 nm and 382 nm, respectively) decreases with a blue shift and shows the greatest decrease in intensity (Table 2). Chelator P*p*MBH showed a different mode of spectral collapse from the other three ligands. In this case a band at 252 nm increased for a period and then, later, slowly decreased in intensity while a band at 301 nm decreased in a similar way to those of the other ligands. The greatest decrease in intensity is evident for the band at the highest wavelength and it is thought that this band corresponds to the hydrazone linkage at neutral pH. Rate constants and half-life values for the hydrolysis reaction of the ligands at room temperature and pH 7.5 are shown in Table 2.

From Table 2 it is apparent that the rate of hydrolysis of the ligands in neutral solution is much slower than that observed for acidic and alkaline solutions (see below).

Ultraviolet-visible spectra in alkaline solutions

Base-catalysed hydrolysis of the ligands was important to investigate in terms of the protocol used for potentiometric titrations of these ligands (Richardson et al. and Vitolo et al. unpublished) and the procedures used to dissolve the ligands prior to biological screening in vitro and in vivo. Due to the low solubility of PIH and its analogues, several authors have reported that concentrations of the ligands suitable for bioassays can be attained by dissolving them at high pH i.e. 10–14 (Fuchs et al. 1988; Johnson et al. 1982; Ponka and Schulman 1985a, b).

For PIH alkaline solution (pH 12) three bands are observed at 210, 304 and 387 nm (Fig. 2C), corresponding to the fully deprotonated ligand (L^{2-}), which is the predominant species at this

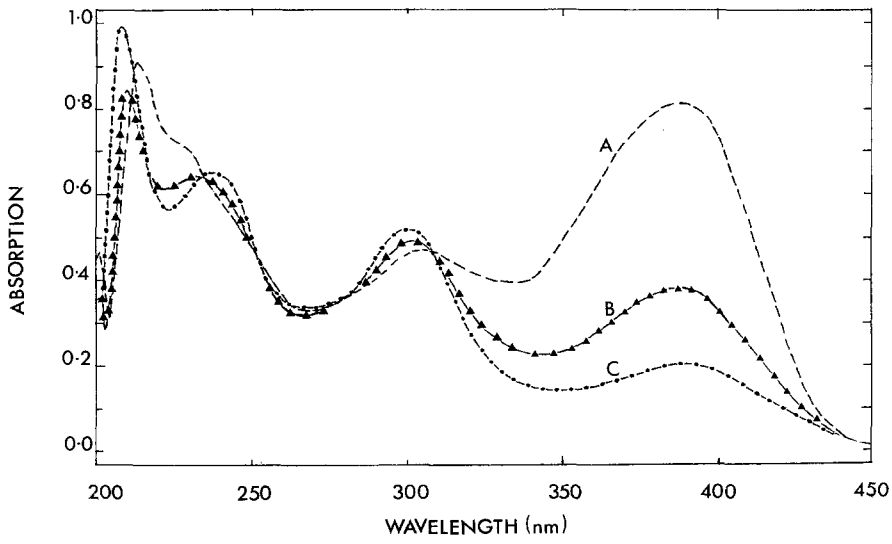


Fig. 6. Changes in absorption intensity of PIH at pH 12; **(A)** freshly prepared PIH (0.05 mM) at pH 12; **(B)** the resulting spectrum after solution **A** was heated on a steam bath for 30 min; **(C)** initial spectrum obtained by mixing equal volumes of pyridoxal (0.1 mM) and isonicotinic acid hydrazide (0.1mM)

pH (Richardson et al., unpublished). This spectrum changes gradually with time, resulting in a significant lowering in intensity of the 387-nm band.

When a freshly prepared solution of PIH (0.05 mM; Fig. 6A) at pH 12 was heated on a steam bath for 30 min and cooled to room temperature the absorbance of the peak at 387 nm had decreased to 46% of its original value. In addition, a new peak at 233 nm appeared (Fig. 6B). This spectrum is similar, with identical peak assignments (210, 233, 300 and 387 nm), to that obtained initially when equimolar solutions of pyridoxal and isonicotinic acid hydrazide are mixed at pH 12 (Fig. 6C). These results suggested that hydrolysis of PIH and its analogues occurred in basic solutions, resulting in cleavage across the hydrazone linkage and liberation of pyridoxal and INH. Cleavage of the carbon-nitrogen double bond has been postulated as the mechanism of Schiff base hydrolysis in alkaline solutions (Bruylants and Feytmants-de Medicis 1970; Kakemi et al. 1970).

As discussed previously, the absorption peak at 387 nm was also shown to increase when acid-hydrolysed PIH or an acidic solution of equimolar amounts of pyridoxal and INH was adjusted to high pH, the final spectrum being identical to a freshly prepared solution of PIH in basic solution. Also, the peak at 233 nm was shown to disappear upon the condensation of pyridoxal and INH to form the hydrazone at basic pH. It should be noted that the isosbestic points were in the same positions (240 nm, 250 nm, 275 nm and 310 nm) as those found when either acid-hydrolysed PIH or an acidic solution of equimolar amounts of pyridoxal and INH are adjusted to pH 12.

From these data it was concluded that the absorption peak at a wavelength of 387 nm is characteristic of the hydrazone linkage at basic pH and provides a quantitative estimate of the base-catalysed hydrolysis of the ligands into pyridoxal and the acid hydrazide.

To examine the decrease in absorbance at 387 nm, PIH, PBH, *Pp*MBH and *Pm*FBH were prepared at a concentration of 0.05 mM at pH 12 and 25° C. For each ligand it was shown that the rate of hydrolysis followed a first-order rate law, with approximately 30%–34% of the ligands hydrolysed at pH 12 after 2.8 h. More precise measurements were performed at a base concentration of 0.5 mM (pH 10.7) and a ligand concentration of 0.1 mM (Table 3). The greater lability of PIH towards base-catalysed hydrolysis may be due to the presence of the extra ring nitrogen in PIH (Fig. 1) which acts in an analogous way to a nitro group by withdrawing electron density (Richardson et al., unpublished). This may result in an enhancement of the positive character of the imine carbon resulting in increased attack of the hydroxyl anion at this site. A comparable, although less dramatic, effect was also observed comparing PBH and *Pm*FBH, the latter chelator having an electron-withdrawing fluoro group (Fig. 1).

Table 3. First-order rate constants and half-life values for the base-catalysed hydrolysis of PIH, PBH and *Pm*FBH at 25° C using a base concentration of 0.5 mM (pH 10.7) and a ligand concentration of 0.1 mM

Ligand	$10^5 \times k$ (s^{-1})	$t_{1/2}$ (ks)
PIH	6.99 ± 0.34 (4)	9.9 ± 4.6 (4)
PBH	1.01 ± 0.15 (3)	70.0 ± 11.6 (3)
*Pm*FBH	1.76 ± 0.29 (3)	40.0 ± 6.4 (3)

*Pp*MBH could not be tested due to its limited solubility at this pH. Results given are the mean ± SD with the number of experiments in parentheses

It can be concluded that dissolution of these ligands in highly alkaline solutions prior to use in a bioassay at pH 7.4 results in decomposition products that would make interpretation of the results difficult.

Acknowledgements. E.B. and D.R. acknowledge the support from the National Health and Medical Research Council of Australia. The Telethon Foundation (W.A.) is thanked for assistance in the purchase of the H.P. 8450 spectrophotometer.

References

Avramovici-Grisaru S, Sarel S, Link G, Hershko C (1983) Synthesis of iron bis(pyridoxal isonicotinoyl hydrazone)s and the in vivo iron-removal properties of some pyridoxal derivatives. J Med Chem 26:298–302

Baker E (1988) Biological screens for iron chelators. Birth Defects Orig Artic Ser 23:49–61

Baker E, Vitolo L, Webb J (1985) Iron chelation by pyridoxal isonicotinoyl hydrazone and analogues in hepatocytes in culture. Biochem Pharmacol 34:3011–3017

Bruylants A, Feytmants-de Medicis E (1970) Cleavage of the carbon-nitrogen double bond. In: Patai S (ed) The chemistry of the carbon-nitrogen double bond. Interscience, New York, pp 465–504

Bruyneel W, Charettee JJ, De Hoffmann E (1966) Kinetics of hydrolysis of hydroxy and methoxy derivatives of *N*-benzylidene-2-aminopropane. J Am Chem Soc 88:3808–3813

Chang R (1981) Physical chemistry with applications to biological systems, 2nd edn, Macmillan, New York

Chatterjee KK, Farrier N, Douglas BE (1963) Copper complexes of *o*-hydroxy Schiff bases and the hydrolysis of the Schiff bases. J Am Chem Soc 85:2919–2922

Cikrt M, Ponka P, Necas E, Neuwirt J (1980) Biliary iron excretion in rats following pyridoxal isonicotinoyl hydrazone. Br J Haematol 45:275–283

Cordes EH, Jencks WP (1962) On the mechanism of Schiff base formation and hydrolysis. J Am Chem Soc 84:832–837

Dash AC, Nanda RK (1969) Reactions of coordinated ligands. I. Kinetics and mechanism of hydrolysis of *N*-salicylideneaniline in the presence of metal ions. J Am Chem Soc 91:6944–6947

Dudek GO, Dudek EP (1966) Spectroscopic studies of the *keto-enol* equilibria. IX. *N*-Substituted anilides. J Am Chem Soc 88:2407–2412

Edward JT, Gauthier M, Chubb FL, Ponka P (1988) Synthesis of new acylhydrazones as iron-chelating compounds. J Chem Engin Data 4:538–540

Ekblom P, Landschulz W, Andersson LC (1986) A lipophilic iron chelator induces an enhanced proliferation of human erythroleukemia (HEL) cells. Scand J Haematol 36:258–262

Ferguson LN, Kelly I (1951) Chelation and association of some ethylenediamine Schiff bases. J Am Chem Soc 73:3707–3709

Fuchs O, Borova J, Hradilek A, Neuwirt J (1988) Non-transferrin donors of iron for heme synthesis in immature erythroid cells. Biochem Biophys Acta 969:158–165

Goodman LS, Gilman A (1975, 1980) The pharmacological basis of therapeutics. Macmillan, London

Hershko C, Avramovici-Grisaru S, Link G, Gelfand L, Sarel S (1981) Mechanism of in vivo iron chelation by pyridoxal isonicotinoyl hydrazone and other imino derivatives of pyridoxal. J Lab Clin Med 98:99–108

Hoy T, Humphrys J, Jacobs A, Williams A, Ponka P (1979) Effective iron chelation following oral administration of an isoniazid-pyridoxal hydrazone. Br J Haematol 43:443–449

Johnson DK, Pippard MJ, Murphy TB, Rose NJ (1982) An in vivo evaluation of iron-chelating drugs derived from pyridoxal and its analogues. J Pharmacol Exp Ther 221:399–403

Junqueira LC, Carneiro J (1980) Basic histology, 3rd edn. Lange Medical Publications, Los Altos, California 94022

Kakemi K, Sezaki H, Iwamoto K, Kobayashi H (1970) General base-catalysed hydrolysis of furylmethylketone isonicotinoyl hydrazone. Chem Pharmacol Bull 18:834–838

Kattamis C, Fitsialos J, Sinopoulou C (1981) Oral desferrioxamine in young patients with thalassemia. Lancet I:51

Martell AE, French-Anderson W, Badman DG (eds) (1981) Development of iron chelators for clinical use, Elsevier/North-Holland, New York

Matsushima Y, Martell AE (1967) Pyridoxal analogues. IX. Electron absorption spectra and molecular species in methanol solution. J Am Chem Soc 89:1322–1330

Metzler DE (1957) Equilibria between pyridoxal and amino acids and their imines. J Am Chem Soc 79:485–490

Modell B, Berdoukas V (1984) The clinical approach to thalassemia. Gruner and Stratton, New York

Ponka P, Schulman HM (1985a) Regulation of heme synthesis in erythroid cells: hemin inhibits transferrin iron utilisation but not protoporphyrin synthesis. Blood 65:850–857

Ponka P, Schulman HM (1985b) Acquisition of iron from transferrin regulates reticulocyte heme synthesis. J Biol Chem 260:14717–14721

Ponka P, Borova J, Neuwirt J, Fuchs O (1979a) Mobilisation of iron from reticulocytes. FEBS Lett 97:317–321

Ponka P, Borova J, Neuwirt J, Fuchs O, Necas E (1979b) A study of intracellular iron metabolism using pyridoxal isonicotinoyl hydrazone and other iron-chelating agents. Biochim Biophys Acta 586:278–297

Ponka P, Richardson D, Baker E, Schulman HM, Edward JT (1988) Effect of pyridoxal isonicotinoyl hydrazone and other hydrazones on iron release from macrophages, reticulocytes and hepatocytes. Biochim Biophys Acta 967:122–129

Richardson DR, Baker E, Ponka P, Wilairat P, Vitolo ML, Webb J (1988) Effect of pyridoxal isonicotinoyl hydrazone and analogs on iron metabolism in hepatocytes and macrophages in culture. Birth Defects Orig Artic Ser 23:81–88

Schmidt RF, Thews G (1983) Human physiology. Springer Berlin Heidelberg New York

Subha MCS, Rao SB (1982) Kinetics of acid hydrolysis of resacetophenone isoniazid hydrazone in the presence and absence of molybdenum(VI) in dimethylformamide. Indian J Chem 21A:125–127

Webb J, Vitolo ML (1988) Pyridoxal isonicotinoyl hydrazone (PIH): a promising new iron chelator. Birth Defects Orig Artic Ser 23:63–70

Willi AV (1956) Helv Chim Acta 39:1193

Williams A, Hoy T, Pugh A, Jacobs A (1982) Pyridoxal complexes as potential chelating agents for oral therapy in transfusional iron overload. J Pharm Pharmacol 34:730–732

World Health Organisation (1983) Community control of hereditary anaemias. Bull WHO 61:63–80

Received March 30, 1989