Biochemical Pharmacology, Vol. 34, No. 17, pp. 3011–3017, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

IRON CHELATION BY PYRIDOXAL ISONICOTINOYL HYDRAZONE AND ANALOGUES IN HEPATOCYTES IN CULTURE

E. BAKER, M. L. VITOLO* and J. WEBB*

Department of Physiology, University of Western Australia, Perth, Western Australia 6009; and
*School of Mathematical and Physical Sciences, Murdoch University, Perth, Western Australia 6150

(*Received* 3 *January* 1984; *accepted* 20 *December* 1984)

Abstract—Pyridoxal isonicotinoyl hydrazone (PIH) and several analogues were synthesized and assessed in the rat hepatocyte culture for their potential in iron chelation therapy. Pyridoxal isonicotinoyl hydrazone and pyridoxal benzoyl hydrazone were as effective as desferrioxamine (DFO) in reducing both net uptake of rat transferrin-^{59}Fe and incorporation into ferritin by hepatocytes. Dialysis studies showed that this was due to a cellular action and not to the extracellular chelation of transferrin-bound ^{59}Fe. The analogues of PIH were more effective in mobilization studies than PIH and DFO, releasing more ^{59}Fe from ferritin as well as from the stroma-mitochondrial membranes in hepatocytes prelabelled using transferrin-^{59}Fe. Chelator action was dependent on incubation time, concentration, temperature and lipophilicity. Pyridoxal benzoyl hydrazone, the most effective iron chelator, was also the most lipophilic, suggesting that access to cellular iron compartments as well as iron-binding affinity is important in effective iron chelation.

Treatment with an iron chelator is essential to reduce toxic iron overload in patients with chronic anaemia (e.g. thalassemia) who require regular blood transfusions for survival [1]. Negative iron balance can be achieved using desferrioxamine (DFO), the only chelator in clinical use at present. However, DFO is expensive and is only effective when given over long periods of time by subcutaneous infusion. A wide variety of alternative iron chelators with clinical potential is being evaluated *in vivo* and *in vitro* [2–5]. Clinically, the ideal iron chelator would have a high affinity and specificity for iron and be membrane-permeable, non-toxic, cheap and effective given orally rather than parentally.

Pyridoxal isonicotinoyl hydrazone (PIH) has been shown to mobilize iron from reticulocytes [6] and Chang cells [7] and increase biliary iron excretion in rats when given orally and intraperitoneally [7–9]. Recent studies *in vivo* [10, 11] and in Chang Cells [11] have indicated that some other inexpensive analogues of pyridoxal may be more effective in iron mobilization then PIH. However, their exact locus and mechanism of action in the hepatocyte, the principal iron storage cell in which toxicity is observed in diseases of iron overload, is not known. In this study several analogues of PIH (Fig. 1) have been synthesized and their efficacy in iron chelation and mechanism of action investigated using hepatocytes in culture. Physiological studies on iron metabolism in hepatocytes in suspension [12] and culture [13–16] indicate that the cultured hepatocyte is a convenient as well as appropriate model for assessing the clinical potential of new iron chelators.

The effect of the chelators on iron uptake by hepatocytes from plasma transferrin was studied, as well as iron mobilization from intracellular sites on prelabelled hepatocytes. Effective iron chelation during the uptake phase of liver-iron exchange and/or increased liver-iron release would reduce liver iron and hence hepatotoxicity. Desferrioxamine was included as a reference chelator in both uptake and efflux experiments.

MATERIALS AND METHODS

Reagents

Iron-59 as ferric chloride in 0.1 M HCl (sp. act., 10–30 μCi/μg) and iodine-125 as sodium iodide (sp. act., 4–12 mCi/μg) were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. Collagenase (Grade II) was supplied by Boehringer Mannheim, Mt. Waverley, Victoria, Australia. Eagle's Minimum Essential Medium (MEM) was supplied in powder form by Flow Laboratories, Annandale, New South Wales, Australia. Foetal calf serum (FCS) and insulin were both supplied by Commonwealth Serum Laboratories, Melbourne, Australia. Fungizone (amphotericin B), penicillin/streptomycin and glutamine were obtained from Gibco, Grand Island, NY. Pyridoxal hydrochloride and isonicotinic acid hydrazide were obtained from Sigma Chemical Co., St. Louis, MO., benzhydrazide and salicylaldehyde from Fluka. All other chemicals were of analytical reagent quality.

Chelators

Compounds I–V (Fig. 1) were synthesized by Schiff-base condensation between the respective aldehydes and acid hydrazide. All were commercially available except 3-hydroxyisonicotinaldehyde, which was synthesized as described in the literature [17–19]. Desferrioxamine and compounds I–V were screened as 1 mM or saturated solutions in the incubation medium after roller preincubation for 2 hr at 37° and centrifugation. The concentrations of the saturated supernatants, measured using an Hp-8450

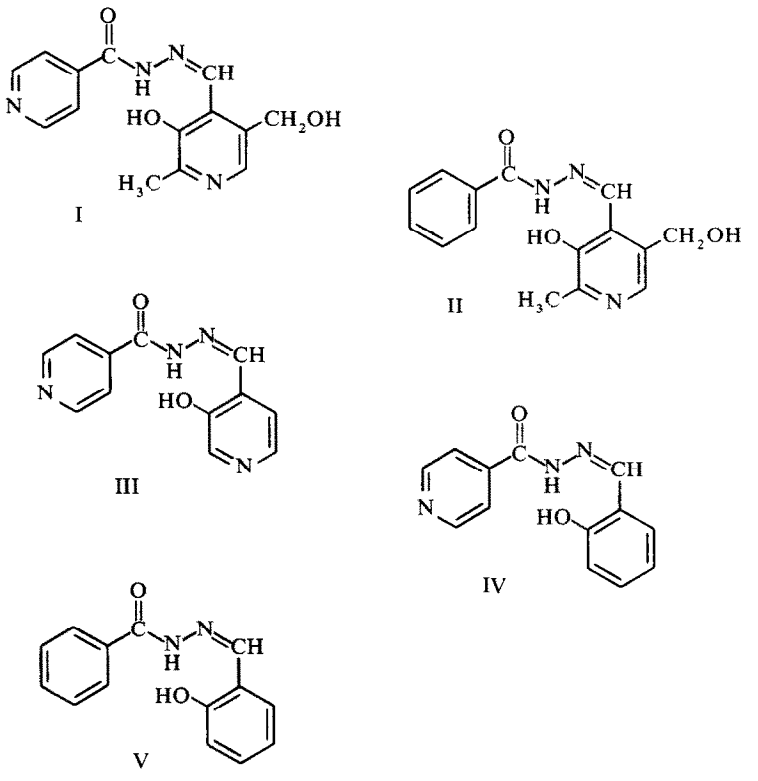

Fig. 1. Structures of pyridoxal isonicotinoyl hydrazone and analogues: I, pyridoxal isonicotinoyl hydrazone (PIH); II, pyridoxal benzoyl hydrazone (PBH); III, 3-hydroxyisonicotinaldehyde isonicotinoyl hydrazone (IIH); IV, salicylaldehyde isonicotinoyl hydrazone (SIH); and V, salicylaldehyde benzoyl hydrazone (SBH).

u.v. visible spectrophotometer, were 0.5 mM for pyridoxal benzoylhydrazone (PBH), 0.3 mM for salicylaldehyde isonicotinoyl hydrazone (SIH), 0.1 mM for salicylaldehyde benzoyl hydrazone (SBH) and 0.1 mM for 3-hydroxy isonicotinaldehyde isonicotinoyl hydrazone (IIH).

Protein purification and labelling

Rat transferrin was isolated and labelled with iodine-125 and iron-59 by previously described methods [15] to give a sp. act. of approximately 1 μCi ^{125}I and 0.2 μCi ^{59}Fe/nmole transferrin. Antiserum to rat liver ferritin, isolated by the method of Huebers *et al.* [20], was prepared from rabbits using standard procedures.

Hepatocyte isolation and culture

Fetal hepatocytes were used in these studies in addition to adult hepatocytes as they are similar in iron metabolism to adult cells (ref. [21]; Torrance, Baker and Morgan, to be published) and their preparation is much less sensitive to variation in collagenase quality. Their responses to PIH and its analogues and to DFO were similar and the results have been combined in this paper.

Adult hepatocytes were isolated and set up in culture as described previously [15]. Livers from 250 g male Wistar rats were perfused by the method of Crane and Miller [22], using a collagenase concentration of 0.05%. The hepatocytes were suspended in a packed cell volume of 4% in MEM supplemented with insulin (2×10^{-6} M), glutamine (2.4×10^{-3} M), FCS (10%), Hepes (20×10^{-3} M), Fungizone (28 μg/ml), and penicillin/streptomycin (57 units/ml and 570 μg/ml, respectively). Aliquots of the suspension were plated onto collagen-coated plastic culture dishes (Disposable Products, Melbourne, Australia) and incubated at 37° in an atmosphere of 5% CO_2/95% air.

Fetal hepatocytes were isolated from 19-day pregnant rats by the procedure of Yeoh *et al.* [23]. Livers were finely chopped using a Mickle chopper and incubated for 15 min at 37° with collagenase (0.05%). The washed cells were then set up for culture as described for adult cells. Adult cells were used on the 2nd–3rd days of culture and fetal cells on the 3rd–4th days. At this time the cells were almost confluent and had regained the morphological and biochemical characteristics observed *in vivo* [15, 23].

Experimental procedure

Uptake. To measure the effect of the chelators on iron uptake by hepatocytes, the medium was replaced with MEM containing the chelators and diferric transferrin (0.05–0.10 mg/ml) doubly labelled with ^{59}Fe and ^{125}I, or freshly labelled plasma-^{59}Fe diluted in MEM to the same final transferrin concentration. The labelled proteins were added to the medium immediately before the start of the experiment and incubated with the cells for approx.

20 hr (inhibition of iron uptake was linear with uptake time). The cell monolayer was then washed four times, detached from the plate with a Teflon spatula, sonicated, diluted to 5 ml and fractionated after the method of White *et al.* [24]. An aliquot (2.0 ml) was used to estimate total cell radioactivity and a further aliquot for the DNA assay. The remainder was separated into membrane (stroma-mitochondrial) and post mitochondrial supernatant fractions by centrifugation (20,000 *g*, 40 min, 5°). For brevity these fractions will be referred to subsequently as membrane and cytosol. Ferritin was isolated from the cytosol using rabbit anti-rat ferritin serum [24].

Radioactivity was determined in cell, membrane and ferritin fractions in a well-type gamma scintillation counter (Packard Tricarb model 5360) and corrected for cross counting. Correction was also made, using blank plates, for the binding of radioactive label to the plastic culture plates. This was generally 5% of the cellular uptake after 24 hr incubation at 37°. The sp. act. of the radioactively labelled transferrin was approx. 250,000 cpm/μg ^{59}Fe and 10,000 cpm/μg transferrin. Uptake per plate varied between about 5,000 and 20,000 cpm for both ^{59}Fe and ^{125}I (depending on cell density, cell type and concentration and specific activity of transferrin).

As the cells were grown on collagen-coated plates, protein levels could not be estimated accurately. Instead, DNA was measured by the method of Hinegardner [25], using highly polymerized calf thymus DNA as a standard [23]. The usual range was between 20 and 40 μg DNA per plate in different experiments. All data were calculated as cpm/μg DNA to correct for variation in cell density and then expressed as a percentage of the control value in each experiment.

Efflux. The effect of the chelators on iron and transferrin release from the hepatocytes was measured using cells that had been preincubated for up to 24 hr at 37° with MEM containing transferrin-^{125}I-^{59}Fe or plasma-^{59}Fe (in a final concentration of 0.05–0.10 mg transferrin/ml). The cells were then washed four times before reincubation at 37° either in MEM (the control) or in MEM containing the test chelator. At the end of the reincubation period (overnight, approx. 20 hr, unless stated otherwise) radioactivity was determined in the efflux medium overlaying the cells and in cell, membrane and ferritin fractions obtained after cell fractionation, as described for the uptake procedure. The action of the chelators was assessed from ^{59}Fe efflux and from the change in cpm ^{59}Fe/μg hepatocyte DNA. Data were expressed as a percentage of the control value obtained in each experiment.

Toxicity evaluation. Toxicity of the chelators in hepatocytes in culture was assessed from morphological changes, trypan blue staining and the release of aspartate aminotransferase, EC 2.6.1.1 (glutamic oxaloacetic transaminase, GOT) into the overlaying medium; GOT was measured spectrophotometrically (Roche GOT Opt. Test. No. 07 14569).

Dialysis studies. Dialysis experiments were performed to determine the extent of direct extracellular chelation of transferrin-bound ^{59}Fe. Incubation media containing transferrin-^{125}I-^{59}Fe (0.1 mg/ml) with or without the chelator to be tested were incubated at 37° under the same conditions of time, concentration and pH as used in cell uptake studies. Aliquots (1 ml) were then dialysed against balanced salt solution for 48 hr at 4°. Aliquots of the dialysate and the retentate were counted to determine the percentage of ^{59}Fe chelated in the absence of cells.

RESULTS

Effect of chelators on iron uptake and intracellular distribution in hepatocytes

As established previously [13–16], iron uptake was linear with time of incubation while transferrin uptake plateaued after about 5 hr, due to the recycling of apotransferrin back to the incubation medium. Fractionation studies showed that 76 ± 2% (means ± S.E.M.; N = 22) of the cellular ^{59}Fe was in the post mitochondrial supernatant, of which 72% (i.e. 55 ± 3% of the cellular ^{59}Fe) was in ferritin. The membrane fraction contained 24 ± 1% of cellular ^{59}Fe and 67 ± 5% of cellular transferrin-^{125}I.

Uptake of ^{59}Fe from transferrin-^{125}I and transferrin ^{59}Fe was reduced to a similar level by DFO, PIH and PBH (Table 1). Iron-59 uptake decreased to 48% of control in the presence of DFO and to 45 and 41% of the control in the presence of PIH and PBH, respectively. The other three analogues of PIH had less effect, reducing ^{59}Fe uptake to 55–71% of the control (Table 1). Desferrioxamine also reduced transferrin uptake slightly, but PIH and its analogues had no apparent effect.

Cell fractionation experiments showed that all the chelators except SBH reduced ^{59}Fe incorporation into ferritin to a greater degree than total ^{59}Fe uptake by the hepatocytes (Table 2). Pyridoxal benzoyl hydrazone, DFO and IIH had the greatest effect, decreasing ^{59}Fe incorporation into ferritin to 20% or less of the control uptake. The amount of ^{59}Fe in the ferritin-free cytosol decreased slightly in the presence

Table 1. Effect of iron chelators on the uptake of ^{59}Fe and transferrin-^{125}I by hepatocytes

Chelator	^{59}Fe uptake (% control)	Transferrin-^{125}I uptake (% control)
DFO	48 ± 10 (8)	72 ± 10 (4)
PIH	45 ± 9 (8)	103 ± 8 (4)
PBH	41 ± 9 (5)	101 ± 13 (5)
IIH	62 ± 17 (4)	112 ± 22 (4)
SIH	55 ± 7 (3)	102 ± 7 (3)
SBH	71 ± 10 (5)	103 ± 9 (5)

Results are expressed as the percentage of the uptake obtained when the cells were incubated without any chelator present. Results are mean ± S.E.M. (number of experiments). Triplicate determinations were made in each experiment.

Table 2. Effect of iron chelators on the cellular distribution of ^{59}Fe taken up by hepatocytes during incubation for 20 hr

Chelator	^{59}Fe uptake (% control) Total	Ferritin	Ferritin-free cytosol	Membranes
DFO (4)	41 ± 12	20 ± 5	74 ± 16	39 ± 8
PIH (4)	46 ± 11	31 ± 7	68 ± 13	44 ± 7
PBH (4)	42 ± 10	14 ± 6	96 ± 16	45 ± 8
SIH (2)	60 (71,50)	37 (35,39)	71 (63,79)	69 (60,79)
IIH (2)	49 (68,30)	20 (28,14)	75 (87,63)	79 (97,60)
SBH (2)	64 (58,70)	73 (71,76)	45 (27,63)	53 (49,58)

Results are expressed as the percentage of the uptake obtained when the cells were incubated without any chelator present. Results are mean values from 2–4 experiments, with triplicate determinations in each experiment. S.E.M. are given where appropriate.

of the chelators (Table 2). All chelators except IIH produced a marked decrease in incorporation of ^{59}Fe into the membrane fraction, which paralleled the reduction in total cell uptake. The release of GOT to the medium increased 10–20% above the control in the presence of IIH, suggesting slight toxicity and membrane damage.

It should be noted that the PIH analogues as saturated solutions were at different concentrations (see Materials and Methods). A comparison of the effect of concentration on ^{59}Fe uptake by hepatocytes indicated that PIH, IIH, SIH and DFO were markedly concentration dependent. When ^{59}Fe uptake was compared on an equimolar basis at 0.10 mM, PBH (64% control), IIH (73% control) and DFO (70% control) were much more active than PIH. Salicylaldehyde benzoyl hydrazone had little effect up to 0.1 mM, its saturation concentration.

Dialysis experiments. Dialysis experiments using aliquots of the uptake media showed that, in the absence of cells and chelators, only 1–3% of transferrin-bound ^{59}Fe was released from the protein. In the presence of IIH, PIH, SBH, PBH and SIH this release increased slightly to 2–8%. In contrast, in the presence of DFO, transferrin lost a significant proportion of bound ^{59}Fe, namely 20–25%.

Effect of chelators on iron and transferrin release from hepatocytes

The amount of ^{59}Fe in hepatocytes labelled by preincubation (as described in Materials and Methods) was only slightly reduced by reincubation in the presence of PIH or DFO, decreasing to 87% of the control (Table 3). Similarly there was little change in ferritin-^{59}Fe or in transferrin-^{125}I binding (Table 3). In contrast to PIH and DFO, the chelators PBH, IIH and SIH markedly decreased hepatocyte-^{59}Fe content and ferritin-^{59}Fe levels (Tables 3 and 4). Transferrin binding was little affected. Of these chelators PBH was the most effective and SBH the least effective at promoting iron efflux. As in the uptake experiments, the action of the chelators was dependent on concentration. When compared on an equimolar basis at a concentration of 0.1 mM, PBH and IIH released more ^{59}Fe (232 and 276% of the control value, respectively) than SIH (130%), SBH (120%), PIH (140%) and DFO (150%).

Each of the four analogs of PIH promoted the release of ^{59}Fe and markedly reduced ferritin-^{59}Fe levels, to between 60 and 80% of the control (Table 3). Table 4 compares the decrease in cellular ^{59}Fe (expressed as a percentage of the value obtained in the control) with changes in the intracellular dis-

Table 3. The effect of iron chelators on iron and transferrin levels in hepatocytes. The cells were radioactively labelled by preincubation with transferrin-^{125}I-^{59}Fe and reincubated in the presence of the chelators

Chelator	Hepatocyte-^{59}Fe (% control)	Ferritin-^{59}Fe (% control)	Hepatocyte-^{125}I transferrin (% control)
DFO	87 ± 10	95 ± 9	80 ± 9
PIH	87 ± 4	95 ± 7	91 ± 12
PBH	67 ± 5	59 ± 7	93 ± 4
IIH	76 ± 4	61 ± 8	98 ± 5
SIH	75 ± 5	61 ± 5	109 ± 7
SBH	85 ± 7	79 ± 5	91 ± 3

Results are expressed as a percentage of the values obtained when the hepatocytes were reincubated without any chelator present. Results are shown as mean ± S.E.M. for 4 experiments in which hepatocytes were preincubated with transferrin-^{125}I-^{59}Fe for 24 hr, washed and reincubated in the presence of the chelators. Triplicate determinations were made in each experiment.

Table 4. Effect of analogues of PIH on the cellular levels and intracellular distribution of ^{59}Fe in hepatocytes. The cells were radioactively labelled by preincubation with transferrin-^{125}I-^{59}Fe and reincubated in the presence of the chelators

Chelator	Hepatocyte-^{59}Fe† (% control)		Intracellular distribution of Iron-^{59}Fe* (% cell total)		
	Total	Ferritin	Membranes	Ferritin	Non-ferritin cytosol
Control	100	100	30 ± 4	61 ± 8	10 ± 8
PBH	70 ± 11	63 ± 12	26 ± 2	55 ± 12	23 ± 13
IIH	83 ± 7	68 ± 10	28 ± 2	50 ± 9	22 ± 8
SIH	82 ± 6	68 ± 3	32 ± 3	51 ± 7	17 ± 7
SBH	87 ± 10	80 ± 11	32 ± 3	56 ± 9	13 ± 7

* Radioactivity in each fraction is expressed as a percentage of the total radioactivity in the cells at the end of the reincubation.

† Percentage of the value obtained in cells reincubated without chelators.

Mean ± S.E.M. for 4 experiments. Hepatocytes were preincubated with transferrin-^{59}Fe-^{125}I for 24 hr and reincubated for 20 hr in the presence of the chelators. Triplicate determinations were made in each experiment.

tribution of iron (expressed as a percentage of the total cell ^{59}Fe). The chelators had less effect on the intracellular distribution of ^{59}Fe than on the total hepatocyte-^{59}Fe content (Table 4). At the end of the reincubation, 30% of hepatocyte-^{59}Fe was in the membrane fraction in the control cells. This value was not reduced significantly in the presence of the chelators. The proportion of cellular ^{59}Fe in the ferritin fraction was slightly reduced and the proportion in the ferritin-free cytosol increased in the presence of the chelators. These data suggest that the analogues of PIH may act indirectly on ferritin, by chelating membrane and cytoplasmic ^{59}Fe from pools in equilibrium with hepatocyte ferritin-^{59}Fe.

The mechanism of action of the PIH analogues was studied further using PBH, the most active chelator. The action of PBH was dependent on temperature and concentration. The release of ^{59}Fe from prelabelled hepatocytes was 145% of the control at 4° and 485% of the control at 37° during incubation for 24 hr in the presence of PBH (0.5 mM). Similarly, with increasing concentrations of PBH the ^{59}Fe released increased from 170% of the control (0.05 mM) through 230% of the control (0.1 mM) to 320% (0.5 mM) during incubation for 16 hr at 37°. The changes in the intracellular distribution of hepatocyte ^{59}Fe in the presence of PBH are shown in Fig. 2. Iron-59 in ferritin in PBH-treated cells rapidly decreased to 80% of the control within 2 hr and decreased further over 20 hr incubation (see Table 4). There was a corresponding increase in the proportion of ^{59}Fe present in the ferritin-free cytosol. The effect of IIH (0.1 mM) and SIH (0.3 mM) were qualitatively similar to PBH, but less marked. However, both compounds produced a slight increase in GOT release (10–20% greater than the control) after 20 hr reincubation.

The lipophilicity of PIH and its analogues was assessed by extraction in ethyl acetate and diethyl ether. As shown in Table 5, PBH showed a much greater partitioning into the organic solvents than PIH, both as the apochelator and the iron complex.

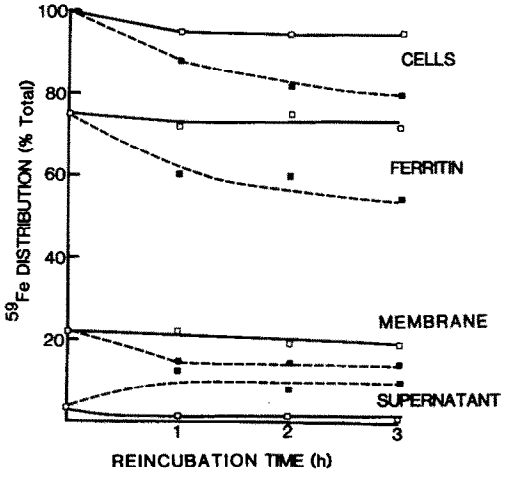

Fig. 2. Cellular distribution of ^{59}Fe in hepatocytes preincubated with transferrin-^{59}Fe and reincubated in the presence (■) or absence (□) of PBH (0.5 mM).

DISCUSSION

Comparison of action of DFO and PIH

Desferrioxamine, the only chelator in clinical use [3], and PIH, shown to have high potential in animal experiments [6, 9], have a similar action on hepatocytes in culture, affecting iron uptake to a much greater degree than iron mobilization. Both chelators markedly reduced both net iron uptake from plasma transferrin (Table 1) and the incorporation of hepatocyte-^{59}Fe into ferritin (Table 2). DFO and PIH may act entirely intracellularly. However, the degrees of inhibition of uptake into the whole cell and membrane fractions were similar and not as marked as the inhibition of incorporation of ^{59}Fe into ferritin (Table 2). This suggests either that the diffusion of the iron complex from the cell is restricted or that the chelators may act at two sites, binding iron at the site of iron transfer from transferrin into the cell and also chelating intracellular iron prior to its incorporation into ferritin. *In vivo* studies on the action of DFO on iron uptake by the liver have shown urinary as well as fecal excretion [26] when the donor molecule is transferrin-^{59}Fe. In addition, when PIH

Table 5. Relative lipophilicity of chelators assessed by extraction in organic solvents

Solvent	Complex	% Extraction* PBH	SIH	IIH	PIH
Ethyl acetate	Chelator	72	82	28	26
	Iron complex	48	17	8	8
Diethyl ether	Chelator	33	44	22	5
	Iron complex	24	11	19	<10

* Mean of duplicates; chelator concentration, 0.1 mM.

is given simultaneously with transferrin-^{59}Fe *in vivo* (as in this study, *in vitro*), PIH-induced excretion of ^{59}Fe is much greater than when given to the prelabelled cells [8]. These observations also suggest that the chelators may act at the cell membrane, as well as intracellularly.

Dialysis studies showed that the mechanism of inhibition by DFO of iron uptake by hepatocytes may also involve the direct extracellular chelation of transferrin-bound ^{59}Fe. In contrast, PIH and its analogues did not chelate transferrin-bound ^{59}Fe in the absence of cells.

The decrease in total hepatocyte-^{59}Fe during reincubation with PIH and DFO (Tables 3 and 4) largely reflected mobilization of membrane-^{59}Fe, as there was little change in ferritin-^{59}Fe. The slight decrease in transferrin binding (Table 3) in the presence of DFO suggests that one site of iron chelation was the small fraction of ^{59}Fe attached to transferrin bound to the outer cell membrane, followed by the dissociation of the apotransferrin. However, taking into consideration the high net accumulation of iron over transferrin at the start of reincubation [15, 16], dissociation of 20% of the transferrin-^{125}I could not account for release of 20% of hepatocyte-^{59}Fe.

The limited mobilization of hepatocyte-^{59}Fe by DFO contrasts with the rapid mobilization seen in Chang cells [27] and fibroblasts [28]. Pyridoxal isonicotinoyl hydrazone also mobilized cellular iron from Chang cells [7] while having less effect on hepatocytes (Table 3) or fibroblasts [28]. This suggests there may be important differences in iron or ferritin metabolism between these cell types and hence differences in the locus of action of chelators.

Action of analogues of PIH

The effects of the analogues of PIH on both iron uptake and mobilization of hepatocyte iron indicate their definite potential in iron chelation therapy relative to PIH and DFO. All analogues decreased net ^{59}Fe uptake by hepatocytes (Table 1) and PBH, SIH and IIH markedly reduced the incorporation of hepatocyte-^{59}Fe into ferritin (Table 2). Hence, like PIH and DFO, the PIH analogues reduced net iron transfer from transferrin to the cell and/or acted intracellularly, inhibiting the incorporation of cytoplasmic iron into ferritin. When compared on a molar basis, several of the PIH analogues were more effective than DFO in reducing iron uptake and incorporation into ferritin, without affecting transferrin binding.

In addition all the analogues of PIH mobilized ^{59}Fe from hepatocytes prelabelled by incubation with transferrin-^{59}Fe (Tables 3 and 4). The reduction in ferritin-^{59}Fe was greater than in total hepatocyte-^{59}Fe indicating some restriction in the release of the iron complexes from the cell. This was reflected in the increase in the fraction of hepatocyte-^{59}Fe present at the end of the reincubation which was in the non-ferritin cytosol (Table 4). This increase was apparent within 1 hr of exposure to PBH (Fig. 2) and sustained for at least 20 hr (Table 4).

Overall, PBH showed the most potential in this system as an alternative iron chelator for clinical use. In view of its ease of synthesis and its relative cheapness, PBH should certainly be investigated further. It was as effective as DFO and PIH in reducing ^{59}Fe uptake and incorporation into ferritin in hepatocytes. In addition it mobilized hepatocyte-^{59}Fe without evidence of toxicity and cell damage, as indicated by cell morphological changes or GOT release. 3-Hydroxy isonicotinaldehyde isonicotinoyl hydrazone and SIH also decreased iron uptake and promoted iron mobilization but showed evidence of slight toxicity.

The greater efficacy of PBH in this system may be partly related to its greater lipophilicity. A higher proportion of both PBH and its iron complex were extracted into the organic solvents, ethyl acetate and diethyl ether than observed with PIH and other analogues (Table 5). In contrast, biochemical studies have shown that PBH and PIH mobilize similar amounts of iron from ferritin in a cell-free system [29] but are less effective than DFO. This suggests that chelator lipophilicity and access to cellular iron compartments, as well as iron-binding affinity, are important in effective iron chelation.

Acknowledgements—The authors gratefully acknowledge the technical assistance of Miss Clare Cooper. This work was supported by the National Health and Medical Research Council (Grant No. 81/3285) and the Telethon Foundation.

REFERENCES

1. D. J. Weatherall, in *Development of Iron Chelators for Clinical Use* (Eds. A. E. Martell, W. French-Anderson and D. G. Badman), p. 3. Elsevier-North Holland, New York (1981).
2. A. Jacobs, *Br. J. Haematol.* **43**, 1 (1979).
3. A. E. Martell, W. F. Anderson and D. G. Badman, in *Development of Iron Chelators for Clinical Use*. Elsevier-North Holland, New York (1981).
4. M. J. Pippard and S. T. Callender, *Br. J. Haematol.* **54**, 503 (1983).
5. E. Baker, E. N. Maslen, K. J. Watson and A. H. White, *J. Am. chem. Soc.* **106**, 2860 (1984).
6. P. Ponka, J. Borova, J. Neuwirt, O. Fuchs and E. Necas, *Biochim. biophys. Acta* **586**, 278 (1979).

7. T. Hoy, J. Humphreys, A. Jacobs, A. Williams and P. Ponka, *Br. J. Haematol.* **43**, 443 (1979).
8. M. Cikrt, P. Ponka, E. Necas and J. Neuwirt, *Br. J. Haematol.* **45**, 275 (1980).
9. C. Hershko, S. Avramovici-Grisaru, G. Link, L. Gefand and S. Sarel, *J. Lab. clin. Med.* **98**, 99 (1981).
10. D. R. Johnson, M. J. Pippard, T. B. Murphy and N. J. Rose, *J. Pharmac. exp. Ther.* **221**, 399 (1982).
11. A. Williams, T. Hoy, A. Pugh and A. Jacobs, *Pharmac. Pharmacol.* **36**, 730 (1982).
12. E. Baker, F. Vicary and E. Huehns, *Br. J. Haematol.* **47**, 493 (1981).
13. J.-C. Sibille, J.-N. Octave, Y.-J. Schneider, A. Trouet and R. R. Crichton, *FEBS Lett.* **150**, 365 (1982).
14. E. S. Cole and J. Glass, *Biochim. biophys. Acta* **762**, 102 (1983).
15. M. Page, E. Baker and E. H. Morgan, *Am. J. Physiol.* **246**, G26 (1984).
16. E. Baker, M. Page and E. H. Morgan, *Am. J. Physiol.* **248**, G93 (1985).
17. J. U. Dejardin and C. L. Lapiere, *Bull. Soc. chim. Fr.* **3, 4**, 530 (1976).
18. D. Heinert and A. E. Martell, *Tetrahedron* **3**, 49 (1958).
19. D. Heinert and A. E. Martell, *J. Am. chem. Soc.* **81**, 3933 (1959).
20. H. Huebers, E. Huebers, W. Rummel and R. R. Crichton, *Eur. J. Biochem.* **66**, 447 (1976).
21. H. Munro and M. Linder, *Physiol. Rev.* **58**, 317 (1979).
22. L. J. Crane and D. L. Miller, *Cell Biol.* **72**, 11 (1977).
23. G. C. T. Yeoh, F. A. Bennett and I. T. Oliver, *Biochem. J.* **180**, 545 (1979).
24. G. P. White, R. Bailey-Wood and A. Jacobs, *Clin. Sci. molec. Med.* **50**, 145 (1976).
25. R. T. Hindgardner, *Analyt. Biochem.* **39**, 197 (1971).
26. M. J. Pippard, D. K. Johnson and C. A. Finch, *Br. J. Haematol.* **52**, 211 (1982).
27. G. P. White, A. Jacobs, R. W. Grady and A. Cerami, *Blood* **48**, 923 (1976).
28. R. Rama, J.-N. Octave, Y.-J. Schneider, J.-C. Sibille, J. N. Limet, A. Trollet and R. R. Crichton, *FEBS Lett.* **127**, 204 (1981).
29. M. L. Vitolo, J. Webb and P. Saltman, *J. inorg. Biochem.* **20**, 255 (1984).