It is known that catecholamines are photolysed (Takayanagi et al 1976). The principle of this study is based on the fact that a radical generated from dopamine makes a covalent bond with an amino acid residue in the binding site. Photoinactivation was eliminated in the presence of haloperidol, a protector of the dopamine receptor. It is well known that the dose-response curve of the agonist for which there are spare receptors is shifted by an irreversible agent (Nickerson 1956). There is a considerable shift in the dose-response curve for dopamine by photoinactivation of the dopamine receptor in this study. This possibly points to a certain receptor reserve for dopamine in this muscle. Furthermore, the fraction (q) of an amount of the dopamine receptor remaining in active form after photoactivation of the receptor and the dissociation constant (K) of the dopamine-receptor complex were estimated. The data in Fig. 1 were analysed (Furchgott 1966) by plotting reciprocals of doses of dopamine against reciprocals of equiactive doses after photoinactivation of the dopamine receptor. From the straight line best fitting the points on the reciprocal plot, the q- and K-values were calculated. The estimated q-value was 0·34. This indicates that the amount of receptor in active form is reduced after irradiation, though the maximum response to dopamine is still obtained. And the estimated K-value was $1·1 \times 10^{-6}$ M. This value coincided with the K_i-value for dopamine against the specific binding of [^{3}H]haloperidol to calf brain membranes (Burt et al 1976) or of [^{3}H]domperidone to mouse striatal membranes (Baudry et al 1979). This muscle is probably suitable for studies on the dopamine receptor. We thank Miss Y. Nakahara for skilful technical assistance.

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Pyridoxal complexes as potential chelating agents for oral therapy in transfusional iron overload

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Iron chelation therapy for patients maintained on a regular transfusion regime is at present best carried out by means of daily infusions of desferrioxamine (Hussain et al 1977; Pippard et al 1978) but this is onerous for the patient and has social and economic disadvantages. Many recent attempts to provide more effective drugs for iron chelation have been summarized by Jacobs (1979) and increasing attention is now being paid to the possibility of oral iron chelation therapy. Hoy et al (1979) showed that when isonicotinic acid hydrazide (INH) and pyridoxal are mixed in equimolar amounts a hydrazone is formed which chelates iron, and oral administration of this compound to rats results in an eightfold increase in faecal iron excretion. It is effective on repeated administration (Cikrt et al 1980), the main route of iron excretion being through the bile. Long term studies in the rat have not been successful in reducing the iron load of test animals and this appears to be related both to their high dietary iron content and instability of the hydrazone. Its effective shelf life at room temperature is no longer than one month and this is a considerable disadvantage from a therapeutic point of view.

Pyridoxal is known to form a Schiff base with many amino acids and its reactivity has led us to examine complexes of pyridoxal with a number of substances in an attempt to find an alternative iron chelator of greater stability than the INH complex and of comparable effectiveness on oral administration. The screening procedures used were the effects on Chang cell iron metabolism (White et al 1976) and on iron excretion in the rat (Hoy et al 1979).

Materials and methods

All chemicals were obtained from Sigma (S), Koch-Light (KL) or British Drug House Ltd (BDH), as indicated in Table 1. Pyridoxal hydrochloride (Sigma) was allowed to react with an equimolar concentration of each substance in 0·1 M HCl in amounts which resulted in 60 mg of the complex being present in 1 ml of solution. There was usually a rapid spectral change in the solution on mixing and in a few cases a precipitate formed. Solutions of pyridoxal-complex formed in this way were later used for oral administration to rats. In

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Table 1. Iron chelation by complexes of pyridoxal with a variety of compounds, showing ^{59}Fe incorporation into Chang cell ferritin and excretion after a single oral administration of 100 mg complex kg^{-1} rat. All values are shown as a percentage of the control values.

		^{59}Fe incorporation in Chang cell ferritin	^{59}Fe excretion in rats	
			Faecal	Urinary
Benzhydrazide	(KL)	11*	630	630
Cysteine	(BDH)	66*	172	125
Glutamine	(KL)	61*	165	130
Fucose	(S)	42*	139	70
p-Hydroxybenzaldehyde	(KL)	31*	172	80
Inosine	(S)	41*	153	70
Tyrosine hydroxamate	(S)	57*	139	78
Valine hydroxamate	(S)	61	190	101
Tyrosine amide	(S)	41*	145	120
Pyridoxamine	(S)	55	173	123
Salicylamide	(BDH)	44*	146	120
Salicaldehyde oxime	(BDH)	43	160	210
Tyrosyl-valine	(S)	56	153	122
Salicyl hydrazide	(S)	19	199	244
p-Aminobenzoic acid	(KL)	48	151	101
Salicylhydroxamic aicd	(S)	55	150	111
Nicotinamide	(KL)	31	172	113
Overnight fast	—	—	163	69
			Max 139 Mean	75

* Ferritin synthesis or cellular iron uptake also decreased by more than 30%.
(KL) Koch Light. (S) Sigma. (BDH) British Drug Houses Ltd.

Table 2. Comparison of haematological data from rats fed for ten weeks on diets with and without added chelating agents.

(n)	RBC (10^{12} litre^{-1})	Hb (g dl^{-1})	Liver non-haem iron μmol g^{-1} tissue	Whole body ^{59}Fe loss % per day with 95% confidence limits
PINH (8)	8·41 ± 0·11	15·1 ± 0·1	0·238 ± 0·021	0·214 0·171–0·257
Control (8)	8·21 ± 0·13	14·9 ± 0·2	0·292 ± 0·018	0·215 0·154–0·279
PBH (8)	7·59 ± 0·10	14·6 ± 0·3	0·236 ± 0·041	0·260 0·223–0·296
Control (7)	7·69 ± 0·21	14·9 ± 0·3	0·246 ± 0·011	0·230 0·159–0·302

Mean ± s.e.
PBH pyridoxal benzoyl hydrazone.
PINH pyridoxal isonicotinic acid hydrazide.

the case of poorly soluble complexes the suspended precipitate was given. All complexes were also produced at a concentration of 1mM in Minimal Essential Medium with 50% (v/v) ^{59}Fe-labelled serum and [^{3}H]leucine. This mixture was incubated with Chang cells for 22 h and followed by the measurement of total cell iron uptake, iron incorporation into ferritin, total cytosol protein synthesis and apoferritin synthesis using the method of White et al (1976). All results are compared with control cultures in which no potential chelator had been added. ^{3}H incorporation into total protein showing more than 20% inhibition compared with the control has been arbitrarily taken as an index of toxicity. Decreased ferritin iron incorporation appears to be the most sensitive index of intracellular iron chelation and all compounds producing more than 30% reduction were considered as possible candidates and administered orally to rats. Other parameters measured were ferritin synthesis and cellular iron incorporation.

All active complexes were administered by stomach tube to groups of six male Wilstar rats at a dose of 100 mg kg^{-1} following an overnight fast (Hoy et al 1979). These rats' iron stores had previously been labelled with ^{59}Fe and two weeks allowed for uniform distribution amongst the iron pools in the body. Faecal and urinary iron excretion during 24 h following the oral dose was compared with the results on three control days for each animal, using the Mann-Whitney U-test.

Long term effectiveness was determined by adding compounds to normal rat diet. This was first powdered and then reconstituted into pellets by adding a solution of the required compound and drying in an oven at 80 °C. The solution was of such a strength to give a concentration of 1 g kg^{-1} dry food which approximates to an average daily intake of 100 mg kg^{-1} body weight, assuming that all rats in a cage were eating identical amounts of food.

Excretion was monitored weekly by measuring whole body activity and intermittently by collecting faeces and urine while the animals were kept in metabolic cages for 24 h periods. After 10 weeks the animals were killed, blood taken for haematological investigation and liver and spleen samples removed for determination of non haem iron concentrations by the method of Bruckmann & Zondek (1940).

Results

The pyridoxal complex with *p*-dimethyl aminobenzaldehyde was toxic in cell cultures and was not examined further. Complexes with histidine, tyrosine, valine, arginine, hydroxylamine, arginine hydroxamate, glutathione, glycinamide, glycylglycinamide and lactamide, galactosamine, glucosamine had no significant effect on any aspect of Chang cell iron metabolism and were investigated no further.

The most sensitive index of intracellular iron chelation appears to be inhibition of ^{59}Fe incorporation into ferritin by Chang cell cultures. Table 1 shows those complexes which produced more than 30% inhibition. The hydrazone formed from pyridoxal and benzhydrazide and the pyridoxal-salicyl hydrazide complex both reduced iron incorporation into ferritin to less than 20% of control values. The mean faecal and urinary iron excretion resulting from a single dose of each compound to rats is given as a percentage of the mean control values on the preceding three days. The increase in iron excretion can be due in part to the effect of overnight fasting. In three experiments with six rats an overnight fast alone was found to increase faecal iron excretion by between 10–60% on the following day. An increase of less than 60% in faecal iron excretion cannot therefore be assumed to be due entirely to the chelator. Eight of the complexes produced more than a 60% increase in iron excretion. The two which were most effective in reducing iron incorporation into ferritin in the Chang cell cultures gave faecal iron excretion results of 199 and 630% of control values respectively. A comparison between faecal excretion after administration of a

chelator and the maximum excretion produced by fasting showed only the pyridoxal benzoyl hydrazone to be significantly effective ($P < 0{\cdot}001$). This complex appears equally effective to the pyridoxal-INH hydrazone in increasing faecal iron excretion after oral administration and it apears to be stable with no loss of chelating potential after 1 year's storage as a dry powder at room temperature. Absolute values of iron loss based on the specific activity of hepatic and urinary non haem iron are about 35 μg Fe/day for normal rats of which 97% is faecal excretion.

The long term effects of pyridoxal benzoyl hydrazide (PBH) and pyridoxal isonicotinoyl hydrazide (PINH) added to normal rat food for a period of 10 weeks are unimpressive. Faecal and urinary ^{59}Fe loss was measured on four occasions during this period in groups of eight animals and the mean values, expressed as % whole body activity lost per 24 h ± s.e., are 0·132 ± 0·005 (control), 0·123 ± 0·007 (PINH) and 0·160 ± 0·004 (PBH). In view of this a single dose of PINH was given by mouth to a group of animals after eight weeks on diet containing PINH. The increase in faecal excretion was 3·88 × during the 24 h following administration compared with 4·18× for a group of rats on a normal diet. The animals were killed after 10 weeks and no differences were found between either of the treated groups and the control group with respect to haemoglobin concentration, red cell indices, transferrin saturation or the liver and spleen non-haem iron concentration (Table 2).

Discussion

Previous studies of iron metabolism in reticulocytes showed pyridoxal benzoyl hydrazone to be only slightly less effective than pyridoxal-INH in mobilizing intracellular iron (Ponka et al 1979) and the present study shows it to be a stable iron chelator which is effective in mobilizing body iron stores in the rat after oral administration. Screening by the measurement of ^{59}Fe excretion in rats is an effective method for identifying those compounds which are well absorbed, as well as being good in vivo chelators. A number of pyridoxal compounds have been investigated for their iron chelating properties and the two complexes most effective in reducing iron incorporation into Chang cell ferritin, and resulting in the highest faecal iron excretion in rats, are both hydrazones. This appears to be the most favourable configuration for iron chelation in this group of compounds.

The long term administration of the most effective complexes in rat food highlighted some of the problems of oral chelation therapy. The failure to increase long term iron excretion may be due either to a reduced response by the test animals or to interaction with gut contents, making the chelator unavailable. The normal response to an oral dose given after fasting towards the end of the study period suggests that interaction of the complexes with intraluminal food is an inhibitory factor. The iron content of the diet might have been high enough to utilize some 40% of the administered complex but this alone cannot account for its ineffectiveness. Our results suggest that while it may be possible to identify potential iron chelating agents for oral administration in human subjects, animal models may be inadequate for precise prediction of a clinical response, partly because of the possibility of intrinsic species differences and partly because of intraluminal reactions of the drug which are unlikely to be comparable to those in the human gut. While such studies provide valuable pointers, the ultimate determination of therapeutic effectiveness must be carried out in human subjects.

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