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In Vitro Studies on Chelating Agents as Potential Iron Absorption Promoters

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ABSTRACT

Ligands which can bind iron to keep it in solution with good stability at the neutral or alkaline pH of the small intestine can serve as useful absorption promoters of dietary iron. A number of biologically compatible compounds were screened in vitro *for this ability at pH 7·5, employing three different systems.*

Studies carried out with iron–amino acid complexes have shown that amino acids and their derivatives were stable only in the acid pH range with stability constants of 10·9 or lower. Compounds which could solubilize insoluble ferric orthophosphate at pH 7·5 exhibited efficiencies in the order: hydroxamate of lysine > nicotinic acid > EDTA > ascorbic acid > picolinic acid > cysteine. A large number of compounds were tested for their ability to keep iron in solution at pH 7·5 at different iron: ligand ratios. Of these, ascorbic acid, inorganic polyphosphates, hydroxamates of nicotinic acid and lysine, EDTA, caffeic acid, citric acid, xanthurenic acid and phytic acid showed high chelating ability. Some of these compounds can be further exploited as iron absorption-promoting ligands.

INTRODUCTION

Iron deficiency anaemia is one of the major nutritional problems affecting a large proportion of the world's population (FAO/WHO, 1970). Chemical analyses suggest that an appreciable quantity of iron is present in many cereal-based diets (Ramalingaswami & Patwardhan, 1949). The

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content availability of iron from diets mainly based on staple vegetables has been found to be very poor (Martinez-Torres & Layrisse, 1973; Narasinga Rao & Prabhavathi, 1978).

Iron balance may be improved by (1) providing additional iron by fortifying a commonly consumed food with inorganic iron salts or (2) improving iron availability from habitual diets that have adequate iron levels but are poorly absorbed.

There have been several attempts to improve the iron nutrition of populations at risk by fortifying appropriate 'vehicles' such as table salt (Narasinga Rao, 1978), sugar (Layrisse *et al.*, 1976) and cereals (Anderson *et al.*, 1976) with inorganic iron salts. An alternative would be to increase the availability of the intrinsic iron of the food itself, since many diets contain adequate levels of iron. It has been shown that amino acids (Kroe *et al.*, 1963; Martinez-Torres & Layrisse, 1970) and ascorbic acid (Conrad & Schade, 1968; Sayers *et al.*, 1973), when added at appropriate levels, can enhance the absorption of iron from foods. The enhancing effect of these ligands is believed to be due to the formation of soluble chelates with non-heme iron at the neutral or alkaline pH of the small intestine which facilitates iron uptake by the intestinal mucosa. On the other hand, compounds such as carbonates, oxalates, phosphates and tannates form sparingly soluble iron complexes of high stability and inhibit iron absorption (Forth & Rummel, 1975; Bothwell *et al.*, 1979). The formation of such sparingly soluble and poorly absorbed iron complexes in the gut can be prevented by the addition of some promoter ligands (Lee & Clydesdale, 1979). The effectiveness of the absorption promoters will depend upon the level of the inhibitors in the diet, the stability constants of bound ligands and the level of promoter added.

The ability of several ligands to chelate iron was studied *in vitro* by measuring: (1) the stability constants of complexes of amino acids and some of their metabolites with ferric iron, (ii) the capacity of various ligands to solubilize iron from insoluble ferric orthophosphate at pH 7·5 and (iii) the ability of ligands to chelate iron and keep it in soluble form at pH 7·5.

MATERIALS AND METHODS

Materials

All chemicals used as ligands were obtained from the Sigma Chemical Company, USA. Other chemicals were of AnalaR grade and were

obtained from S.M. Chemicals, India. $^{59}FeCl_3$ (in HCl solution) was purchased from the Bhaba Atomic Research Centre, Bombay, India.

Methods

Potentiometric determination of stability constants
Stability constants were determined potentiometrically using a potentiometer (Baird and Tatlock Ltd, London, Great Britain) with platinum and calomel electrodes (Elico Co. Pvt. Ltd, India) with a readability of 1 mV. The potentiometer was standardized using a Weston Standard Cell of Type 402-A. pH was measured by means of a combination glass electrode (Elico Co. Pvt. Ltd, India). Potentiometric titration of a mixture of equimolar Fe^{++} and Fe^{+++} (0·003M) perchlorates and ligands (at different concentrations, as given in Table 1) was carried out in the pH range 0·65–3·50. Stability constants were calculated according to the method of Perrin (1958*a*).

Solubilization of iron from ferric orthophosphate at pH 7·5
Labelled ferric orthophosphate (^{59}Fe) was prepared according to the method of Steinkamp *et al.* (1955). Insoluble ferric orthophosphate (25 mg) was agitated in a stoppered conical flask with ligand at different molar ratios to iron in a volume of 25 ml of water, adjusted to pH 1·5. Incubation was carried out at 37 °C in a shaker water bath for 1·5 h at a speed of a hundred to a hundred-and-twenty oscillations a minute. The pH was then raised to 7·5 with 0·5N NaOH and incubation was continued for another 1·5 h. At the end of incubation, the contents of the flask were centrifuged at 20 000 *g* for 30 min and an aliquot of the supernatant was counted for ^{59}Fe radioactivity in a well-type gamma scintillation counter (Tracerlab, USA).

Iron-chelating ability of ligands at pH 7·5
Labelled ferric chloride ($^{59}FeCl_3 . 6H_2O$) and ligand at various molar ratios were incubated in stoppered conical flasks in a volume of 25 ml water, adjusted to pH 1·5, and treated as described in the previous section.

RESULTS AND DISCUSSION

The stability constants (log K_s) of the amino acids and their metabolites studied are given in Table 1. Although the stability constants of some of

TABLE 1
Stability Constants of Amino Acids and their Metabolites with Fe^{+++}

Ligand	*Ligand concentration (molarity)*	*pH range of titration*	*log K_s*
Glycine	1·00	0·65–2·45	10·4
L-Alanine	1·00	0·75–2·60	10·7
β-Alanine	1·00	1·10–3·50	10·8
DL-Serine	0·30	1·30–3·10	8·2
L-Valine	0·30	1·20–3·35	8·8
L-Leucine	0·20	1·30–2·75	9·0
L-Isoleucine	0·20	1·00–2·50	10·5
L-Proline	0·05	1·60–2·35	10·1
DL-Phenylalanine	0·15	1·15–2·65	9·9
L-β-Phenylalanine	0·15	1·60–2·40	9·9
L-Tyrosine	0·10	0·50–1·20	10·5
L-Tryptophan	0·05	1·35–2·25	9·1
L-Methionine	0·20	1·30–2·75	8·7
L-Aspartic acid	0·05	1·40–2·65	10·7
L-Asparagine	0·15	1·20–2·25	8·5
L-Glutamic acid	0·05	1·60–2·30	10·8
L-Glutamine	0·05	1·20–2·50	8·9
L-Lysine	0·10	1·60–3·20	10·9
L-Arginine	0·11	1·65–2·75	8·3
L-Histidine	0·10	1·90–2·75	8·1
Picolinic acid	0·05	0·85–1·40	6·8
Quinolinic acid	0·05	0·85–1·20	5·6
Nicotinic acid	0·05	1·35–2·70	11·9
Anthranilic acid	0·05	0·85–1·40	4·0

the amino acids included in the present study have been reported by Perrin (1958*b*), they are reported again here for the purpose of comparison. The K_s values of the compounds studied ranged from 4·0 to 11·9. Of the amino acids, glycine, L-alanine, β-alanine, L-isoleucine, L-proline, L-tyrosine, L-aspartate, L-glutamate and L-lysine have log K_s values slightly above 10. Picolinic, quinolinic and anthranilic acids have low stability constants. The highest log K_s value of 11·9 was observed for nicotinic acid. Except in the case of histidine, the log K_s values of other amino acids are comparable with those reported by Perrin (1958*b*).

The stability constants (log K_s) for complexes of amino acids and their derivatives which have been suggested as possible iron absorption

promoters (Kroe *et al.*, 1963, 1966; Van Campen & Gross, 1969; Martinez-Torres & Layrisse, 1970) ranged between 8·1 and 10·9. It was observed that these iron–amino acid complexes were stable only in the acid pH range (0·5–3·5). It was also observed that, by increasing the pH of the solution containing the iron–amino acid complex above 3, iron was precipitated as ferric hydroxide. On the basis of both these observations—i.e. the actual values of the stability constants of the amino acid–iron complexes and their dependence on pH—it would appear that amino acids and their derivatives may not be very effective iron absorption promoters. Some, if used at fairly high concentrations, may enhance food iron absorption, but their practical utility will be limited.

Solubilization of iron from ferric orthophosphate at pH 7·5

Ethylene diamine tetra-acetic acid (EDTA), citric acid, phytic acid, quinolinic acid, nicotinic acid, picolinic acid, fructose, glycine, L-cysteine, L-aspartic acid and L-glutamic acid were tested up to a ligand:iron ratio of 40:1—and ascorbic acid up to a ratio of 220:1—for their capacity to solubilize iron from insoluble ^{59}Fe-labelled ferric orthophosphate at pH 7·5. As shown in Table 2, only a few of the compounds tested showed any significant iron solubilizing capacity. EDTA was observed to have the highest capacity of all the ligands tested. Ascorbic acid, picolinic acid and cysteine showed low solubilizing capacity. Others showed no iron solubilizing capacity.

TABLE 2
Per cent Iron Solubilized from Ferric Orthophosphate at pH 7·5

EDTA		*Ascorbic acid*		*Picolinic acid*	*Cysteine*	*L/I*	*LH*	*NAH*	*G-γ-H*
L/I	*Per cent iron*	*L/I*	*Per cent iron*	*Per cent iron*	*Per cent iron*		*(per cent iron)*		
2	6·3	20	6·2	9·6	4·6	1	8·5	7·7	0·0
4	8·5	40	13·4	14·1	7·1	2	12·8	20·1	4·4
20	33·4	100	18·2	—	—	—	—	—	—
40	56·5	200	20·5	—	—	—	—	—	—

L/I = Molar ratio of ligand/iron.
LH = Lysine hydroxamate.
NAH = Nicotinic acid hydroxamate.
G-γ-H = Glutamate-γ-hydroxamate.

Other biologically relevant compounds such as hydroxamates of glycine, leucine, histidine, lysine, γ-glutamate, nicotinic acid, 1-methyl and 3-methyl histidine, OH-proline and dipeptides such as anserine and carnosine, were tested up to a ligand:iron ratio of 2. Higher molar ratios could not be tested due to constraints on the availability of these expensive compounds. Of these, only hydroxamates of lysine, nicotinic acid and γ-glutamates could solubilize appreciable amounts of iron, as shown in Table 2. Lysine and nicotinic acid hydroxamates appear to have even better solubilizing effects than EDTA.

Phosphates in foods are known to form insoluble ferric phosphates and inhibit iron absorption. Compounds which can solubilize insoluble ferric orthophosphate at pH 7·5 can be considered as fairly powerful iron chelating agents and can serve as very useful promoters of food iron absorption. Because of its stability and non-reactivity, ferric phosphate is often used as an iron fortificant but iron availability from such compounds is unacceptably low. Ligands which can solubilize iron from ferric phosphate may serve as useful additives to promote iron absorption from ferric phosphate used as a fortificant.

Although a large number of compounds were screened at different iron:ligand ratios, only a few were found to solubilize iron from $FePO_4$ at pH 7·5. These compounds had to be used at fairly high levels, i.e. an iron:ligand ratio of 1:40, for any significant effect to be observed. These compounds, in decreasing order of efficiency, were: hydroxamates of lysine and nicotinic acid, EDTA, ascorbic acid, picolinic acid and cysteine. None of the amino acids, except cysteine, had any solubilizing effect (even cysteine could extract only 7% of iron when used at an iron:cysteine molar ratio of 1:40). Hydroxamates of lysine and nicotinic acid were found to have a high capacity for solubilizing iron from $FePO_4$—even better than EDTA.

Ability of ligands to keep iron in solution at pH 7·5

All the amino acids, EDTA, ascorbate, citrate, pyruvate, picolinate, phytate and inorganic polyphosphates such as sodium hexametaphosphate, sodium tripolyphosphate, sodium trimetaphosphate, phosphate glass and tetrasodium pyrophosphate were tested for their iron-chelating ability. Amino acids were tested up to a ligand:iron ratio of 40. Picolinic acid was tested up to a ratio of 100. Other ligands were tested up

to a ratio of 3 because of their high binding capacity. Of the amino acids only glycine and histidine were able to retain 8% and 20% iron, respectively in solution at a ligand:iron ratio of 40.

The compounds found to chelate iron at a 1:1 molar ratio of iron:chelate are listed in Table 3. At a 1:1 molar ratio, compounds such as ascorbic acid, hexametaphosphate, tripolyphosphate and trimetaphosphate could retain all their iron in soluble form at pH 7·5.

TABLE 3
Per cent Iron Solubility by Ligands at a Ligand:Iron Ratio of 1:1 at pH 7·5

Ligand	*Per cent soluble iron*
Ascorbic acid	100
Sodium hexametaphosphate	100
Tripolyphosphate	100
Trimetaphosphate	100
Dihydroxyplenylalanine	88
Tetrasodium pyrophosphate	87
Nicotinic acid hydroxamate	84
Caffeic acid	81
EDTA[a]	78
Phytic acid	65
Xanthurenic acid	65
Citric acid	61
Phosphate glass	59
Lysine hydroxamate	30
Salycylic acid	23
L-Carnosine	13
L-3-Methyl histidine	12
Quinolinic acid	10
Glutamate-γ-hydroxamate	9
L-1-Methyl histidine	6
Nicotinic acid	6
Taurine	6
Histidine hydroxamate	6
GABA[b]	5
L-Anserine	4
6-OH-Nicotinic acid	4

[a] Ethylene diamine tetra acetic acid.
[b] Aminobutyric acid.

Dihydroxyphenylalanine (DOPA), tetrasodiumpyrophosphate, nicotinic acid hydroxamate, caffeic acid, xanthurenic acid and citric acid were able to retain more than 60% iron in soluble form. Lysine hydroxamate, salicylic acid, L-carnosine, L-3-methyl histidine and quinolinic acid were able to retain between 10 and 30% iron in the solution. Other compounds showed low iron-chelating ability and retained less than 10% iron in solution.

Several physiological compounds with an amino, hydroxyl or carboxyl group were also tested for their iron-chelating capacity at equimolar ligand–iron concentrations. Those compounds which showed no chelating ability at equimolar ratios were anthranilic acid, kynurenic acid, hydroxamates of glycine and leucine, dicarboxylates such as succinate, fumarate, α-ketoglutarate, oxaloacetate, malate and picolinic acids and hydroxy carboxylic acids such as *p*-coumaric acid and vanillic acid, D-glucuronolactone and D-galacturonic acids.

By simulating the physiological process in the gastro-intestinal tract, the ability of compounds to chelate iron and prevent its precipitation, when the pH is changed from acid (pH 1·5) to neutral (pH 7·5), was tested. During the normal passage of food in the gastro-intestinal tract, iron is released into solution in the acid pH of the stomach and is later precipitated when food enters the duodenum where the pH is neutral or alkaline, unless such precipitation is prevented by the chelating agents present in the food.

A large number of compounds were tested for their ability to maintain iron in solution at pH 7·5 at various iron:ligand ratios. Of these, ascorbic acid, inorganic polyphosphates, nicotinic acid, hydroxamate, EDTA, caffeic acid, citric acid, xanthurenic acid and phytic acid showed high chelating ability, retaining 60–100% iron in solution when used at iron:ligand molar ratios of 1:1. Lysine hydroxamate, salicylic acid, L-carnosine, 3-methyl histidine and quinolinic acid had moderate chelating abilities and could retain 10–30% of iron in solution at 1:1 molar ratios. It was interesting to observe that phytate—generally considered an inhibitor of iron absorption—showed a very high chelating ability. It is reported that monoferric phytate is soluble (Morris & Ellis, 1976) and that, when phytate is present in excess relative to iron, it may form soluble complexes. Inorganic polyphosphates—used in the food industry as leavening agents, rancidity inhibitors and acidulants (Ellinger, 1972)—showed very high iron-binding capacities. They were able to retain nearly 100% iron in solution at molar ratios of 1:1 or lower.

TABLE 4
Iron-Chelating Ability of Various Ligands at pH 7·5

Ligand	*Ligand/Iron molar ratio to retain 50% iron in soluble form*
Ascorbic acid	0·09
Sodium hexametaphosphate	0·12
Tripolyphosphate	0·22
Trimetaphosphate	0·23
EDTA[a]	0·32
Tetrasodium pyrophosphate	0·52
Phytic acid	0·61
Dihydroxyphenylalanine	0·68
Citric acid	0·80
Caffeic acid	0·82
Phosphate glass	0·85
Nicotinic acid hydroxamate	0·87
Xanthurenic acid	0·90

[a] Ethylene diamine tetra acetic acid.

Table 4 gives the ligand:iron ratios necessary to retain 50% iron in soluble form at pH 7·5 for several compounds which showed a significant chelating effect. At ligand:iron molar ratios of less than 1·0, ascorbic acid, EDTA, citric acid and polyphosphates were able to retain 50% iron in solution, thus exhibiting a very high iron-chelating ability.

Based on all three approaches to measuring iron-chelating ability, the following compounds were found to possess high chelating ability and can serve as potential ligands to enhance iron absorption: EDTA, ascorbic acid, inorganic polyphosphates such as sodium hexametaphosphate, tripolyphosphate, phosphate glass (sodium salt), phytic acid, hydroxamates of nicotinic acid and lysine, citric acid, DOPA, caffeic acid, L-carnosine, L-3-methyl histidine and quinolinic acid. Of these, ascorbic acid and EDTA are known to be powerful iron-chelating agents and the former is recommended as a promoter of iron absorption. Although some of the amino acids are suggested as ligands useful for promoting iron absorption, none was found to be useful at the concentrations tested.

A number of compounds have been identified in this study as having iron-chelating capacity. However, before they can be used as potential iron absorption promoters, the following points need to be considered.

(1) Although these compounds chelate iron and keep it in solution, it has to be ascertained whether the resulting complex can surrender its iron to acceptor sites in the intestinal mucosa. It is quite possible that complexes with very high stability constants may not surrender iron.

(2) In the present study the chelating ability of these compounds has been determined in pure solutions. In foods there are many agents which may combine either with the metal or the ligand and thus modify the ability of the ligand to chelate iron and keep it in solution. These potential compounds have to be tested in the presence of food for their iron-chelating ability and promotion of iron absorption.

(3) The acceptability and safety of these compounds for humans should be tested before they can be recommended as iron absorption promoters. Some of the compounds, such as polyphosphates used as food additives, may not pose any problem in this respect. However, the acceptability and safety of other compounds, such as hydroxamates, need to be tested.

All these aspects are being studied in respect of compounds which have shown high chelating ability, particularly the polyphosphates.

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