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Analytical Methods Section

Speciation of trace metals, with special reference to intestinal iron absorption

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Mechanisms of intestinal iron absorption are reviewed: there are three principal pathways of mucosal inorganic iron uptake. These include the electrogenic-carrier and fatty-acid-mediated transcellular pathways and a nonspecific paracellular route. A novel method is described for the speciation of dietary and luminal iron during its transit along the gastro-intestinal tract. Extrinsic labelling of the diet with $^{59}FeCl_3$ revealed a differential distribution of chemical and radio-iron in the various fractions, indicating that absorption and bioavailability data obtained with extrinsically labelled diets must be interpreted with caution.

In standard laboratory chow, two-thirds of the iron is in a residual (acid-soluble) fraction, with only 1% in an exchangeable (aqueous $MgCl_2$-soluble) fraction. However, analysis of gastric contents after feeding the diet indicated that most of the iron had become redistributed from the residual to the exchangeable fraction. This process could only be partially mimicked by acid incubation of the diet.

In the duodenal lumen there was a significant decrease in the exchangeable fraction consistent with its availability for absorption. In the jejunum and ileum most of the remaining exchangeable iron had become redistributed to carbonate and oxide-bound fractions: this redistribution could be mimicked *in vitro* by neutralisation of gastric contents with sodium bicarbonate.

Use of Fe(II)-specific indicators shows that the gastric lumen is a major site of reduction of dietary Fe(III). Recent studies have demonstrated, additionally duodenal mucosal ferri-reductase activity, highlighting the key role of Fe(II) as an intermediate in iron absorption.

INTRODUCTION

Knowledge of the speciation of micro-nutrient elements is essential for our understanding of the physiological processes in their intestinal absorption. In addition to analyses of the foodstuffs themselves, studies of gastro-intestinal lumen contents are key to dissecting out the many possible absorptive steps. Speciation analyses are also important in the understanding of the bioavailability of these elements in human and animal foodstuffs, estimation of nutritional requirements, dietary interactions and possible toxicological effects.

This paper describes a novel method for investigating the speciation of trace metals in foodstuffs and gastro-intestinal contents which can assess both endogenously and exogenously-labelled experimental diets. The method has been applied to the study of intestinal iron absorption, but is clearly applicable to other elements. It will also be necessary to give a brief review of the physiological mechanisms and regulation of intestinal iron absorption.

Characteristic of iron chemistry is the key role of the redox couple Fe(II)/Fe(III) in the absorptive process and in the intracellular functions of iron complexes. Data on the valency state of iron during the digestive-absorptive process will be reviewed and linked to recent data on the importance of Fe(III) reduction during the mucosal uptake step for this element.

Physiological mechanisms of intestinal iron absorption

Luminal iron is present, as both heme iron complexes and as low molecular weight iron-ligand complexes. Absorption of heme iron, which occurs via a distinct mechanism, is clearly important, but is not the subject of this review (Conrad *et al.*, 1966; Forth & Rummel, 1973; Charlton & Bothwell, 1983). Absorption of

inorganic iron, whether ingested as such or released from dietary complexes, occurs mainly in the duodenum (Raja *et al.*, 1987*a*) under basal conditions and particularly in situations of enhanced iron absorption (Raja *et al.*, 1986, 1987*b*; Simpson *et al.*, 1986*a*). Much, however, still remains to be learned of the transport processes involved. A small amount of dietary iron, particularly in the form of low molecular weight complexes, can be absorbed by the paracellular route. This normally represents a minor pathway (~10%), but, as it is not subject to adaptive regulation, significant amounts may be absorbed by this route when the dietary iron content is very high (Peters *et al.*, 1988). The paracellular route of absorption has recently become of interest because of the striking increases in permeability which occur in certain small-intestinal diseases, the effect of commonly administered drugs on this pathway and the marked variation in small-bowel permeability between individual animal species (Peters & Bjarnason, 1988). The permeability pathway is believed largely to reflect the paracellular route and is readily monitored by assessing absorption of various probes, particularly ^{51}CrEDTA. Thus, chronic ingestion of high levels of iron, particularly if accompanied by alcohol abuse, a known disruptive agent of the permeability barrier, will lead to generalised tissue iron overload, so-called Bantu siderosis.

There are two transcellular routes for iron uptake. The major pathway is believed to be an electrogenic energy-requiring carrier-mediated pathway (Raja *et al.*, 1989). The transporter, by analogy with known carrier mechanisms, is believed to be a transmembrane protein and may correspond to one of the iron-binding (glyco)proteins already identified in small-intestinal cell membranes (Cox & O'Donnell, 1981; Stremmel *et al.*, 1987). It is not known whether Fe(II) and Fe(III) share the same transporter, but this is unlikely in view of the marked physico-chemical differences between these two ions (Simpson *et al.*, 1986*b*). The most striking feature of this mechanism is that it undergoes an adaptive change in response to enhanced body iron requirements, notably iron deficiency, chronic hypoxia and late pregnancy (Raja *et al.*, 1987*a*). Iron uptake by this pathway may increase two- to four-fold, but the molecular basis of this response and the physiological mechanisms involved remain to be determined.

Recent studies have demonstrated a duodenal mucosal ferri-reductase activity (Raja *et al.*, 1991). This activity shows adaptive increase in chronic hypoxia and nutritional iron deficiency, and displays many of the properties formerly attributed to the Fe(III) transporter (Cox & Peters, 1980). The reduction of iron may, therefore, represent a rate-limiting step in Fe(III) absorption.

The second transcellular pathway has only recently been identified. It involves the formation of a Fe(II)-fatty acid complex with subsequent lipophilic transfer across the membrane (Simpson *et al.*, 1988, 1989). The pathway undergoes only a limited adaptive response to increased iron demands and is probably responsible for approximately 10% of duodenal iron absorption. Transfer of the iron across the cytoplasm of the enterocyte probably involves low molecular weight ligands: ferritin plays a passive permissive role.

At the baso-lateral aspect of the enterocyte, a key site at which absorption of iron is regulated (Raja *et al.*, 1988), an iron-binding domain has recently been identified (Snape *et al.*, 1990). This presumed carrier probably translocates iron to the external aspect of the plasma membrane, where the iron is then transferred to the portal plasma as a low molecular weight ligand complex. In spite of claims to the contrary (Huebers *et al.*, 1983), transferrin is clearly not implicated in the absorption mechanism for iron (Simpson *et al.*, 1986*c*) and this has been recently confirmed in studies with a strain of mice lacking transferrin: these animals show greatly enhanced intestinal iron absorption (Craven *et al.*, 1987). Non-transferrin-bound iron is, thus, the principal mediator of iron transfer from gut to liver. Transferrin is implicated in the transfer of iron to many other tissues most notably the bone marrow. It is clear that many questions remain unanswered with respect to the mechanisms of iron absorption and their regulation, the basis of the molecular deficits in genetic haemochromatosis and the mechanisms for modulation of iron absorption.

In studies of iron absorption, various chemically defined chelators, e.g. citrate, ascorbate and nitrilotriacetate, are used and their physiological relevance is uncertain. Redox clearly plays an essential role in determining the physico-chemical properties of iron and, thus, in its bioavailability and absorption (Wollenberg & Rummel, 1987). For these reasons, we have investigated the speciation and redox state of iron in animal chow and in the various segments of the gastro-intestinal tract during the digestion-absorption of this chow.

METHODS

Chemical speciation of dietary and luminal iron

The principal extraction steps in the speciation of the chow and gut contents are described in Table 1. The procedure is adapted from a method of Tessier *et al.* (1979) for the speciation of metals in soil samples. The samples are successively extracted with a series of buffers with a wash step between each extraction. The combined supernatants are counted for radioactivity and analysed for iron by atomic absorption spectrophotometry. Further details are published elsewhere (Simpson *et al.*, 1991).

Table 1. Speciation of dietary iron

Fraction	Extraction procedure
Exchangeable	1 M $MgCl_2$, 1 h
Carbonate	1 M Na Acetate (pH 5·0), 4 h
Oxide	0·04 M NH_2OH.HCl in 25% acetic acid, 16 h at 95°C
Organic	0·02 M HNO_3 + 30% H_2O_2, 5 h at 85°C
Residue	Final pellet

Samples extracted with appropriate reagent, centrifuged (10000 *g* × 30 min) and washed pellet extracted with following reagent. Extracts assayed for iron by AA.

Characterisation of the fractions was performed using model compounds, and is shown in Table 2. Ferric phytate forms a soluble iron complex and was largely recovered in the exchangeable fraction. Ferric bicarbonate was largely recovered in the carbonate fraction and ferric hydroxide in the carbonate (minor) and oxide (major) fractions. Ferric phosphate was largely recovered in the oxide fraction. The nomenclature used for the fractions is that of Tessier *et al.*, and does not reflect the sole constituent of the individual fraction. None of the model compounds contributes significantly to the organic or residual fractions and ferric bicarbonate, hydroxide and phosphate do not contain a significant exchangeable fraction. Assays of Fe(II) in gastro-intestinal contents were based on reaction with the specific chelator ferrozine (Simpson & Peters, 1990).

RESULTS

Speciation of diet

Table 3 shows the speciation of intrinsic iron assayed by atomic absorption spectroscopy in laboratory chow and compares this with the distribution of the same diet labelled extrinsically with $^{59}FeCl_3$. Approximately two-thirds of the total iron is found in the residual fraction, with one-fifth in the oxide fraction, but only 1% in the exchangeable fraction. In contrast, the radioactivity is more evenly distributed between the various fractions. Note that over 10% of the label is associated with exchangeable fractions.

Table 2. Iron speciation of model compounds

Compounds	Fraction (%)			
	Exchangeable	Carbonate	Oxide	Organic/residue
Fe Phytate	94·5	5·5	<1	<1
$FeHCO_3$	4·0	88·2	7·8	<1
$Fe(OH)_3$	2·3	27·0	65·4	5·0
Fe Phosphate	2·6	6·3	90·2	1·0

$^{59}FeCl_3$ in 10 mM HCl mixed with appropriate Na salt for 48 h at 20°C prior to speciation.

Table 3. Speciation in laboratory diet

	Fraction (%)				
	Exchangeable	Carbonate	Oxide	Organic	Residue
Total iron	1·4	5·9	21·2	8·3	63·3
Radio-labelled iron	13·4	18·0	19·3	11·0	38·6

Mean of two or three experiments. Labsure Diet ERD (rodent breeding diet with iron and vitamin supplements) hydrated, mixed with $^{59}FeCl_3$ and dried prior to speciation.

Speciation of diet in gastro-intestinal tract

Table 4 compares the forms of iron in the diet and in the various segments of the gastro-intestinal tract of rats after consuming the diet. The most striking change in the stomach is the large increase in the amount of iron in the exchangeable fraction reflecting mobilisation of the iron, particularly from the residual fraction. In the duodenum, consistent with it being the principal site of iron absorption, the amount in the exchangeable fraction falls. In the jejunum (middle third of small intestine), following entry of pancreato-biliary secretion, the exchangeable fraction falls further with carbonate and oxide fractions, showing significant increases in iron content. Note there is no significant difference between jejunal and ileal contents.

Table 4. Iron speciation of laboratory diet and gastro-intestinal contents

	Fraction (%)				
	Exchangeable	Carbonate	Oxide	Organic	Residue
Diet	1	6	21	8	63
Stomach	58	2	20	2	18
Duodenum	27	10	33	5	25
Jejunum	2	24	46	3	25
Ileum	3	25	44	3	26

Mean of two to four experiments.

Table 5 shows the concentration of ferrozine-available iron (identified at low pH as Fe(II)) in the diet and in gastric and duodenal contents. As expected, there was no detectable ferrozine-available iron in the diet. However, the stomach contents had a high concentration of Fe(II) and in duodenal contents lower, but

Table 5. Ferrozine-available iron

	Fe(II) (μmol)
Diet	<1
Stomach contents	113 ± 28 (11)
Duodenal contents	19 ± 5 (14)

Mean ± SD for (*n*) experiments.

Table 6. Iron speciation: effect of acidification of diet

	Fraction (%)				
	Exchangeable	Carbonate	Oxide	Organic	Residue
Diet	1	6	21	8	63
pH 4·8	11	3	23	3	59
pH 2·5	27	2	20	2	48
pH 0·9	39	2	10	2	47

Diet hydrated and maintained at appropriate pH (HCl) for 15 min prior to speciation. Mean of two to four experiments.

definitely detectable, levels of ferrozine-available iron were present.

In-vitro simulation of dietary iron digestion

In an attempt to investigate the mechanisms underlying the changes in iron speciation during passage along the gastro-intestinal tract, in-vitro studies have been performed in an attempt to simulate the in-vivo changes. Table 6 shows the composition of the experimental diet before and after acidification. Although acidification leads to a decrease in the residual and other insoluble fractions with a marked and progressive increase in the exchangeable fraction, it, however, does not achieve a similar degree of solubilisation of iron as that found *in vivo*, even under the most acidic conditions. However, neutralisation of gastric contents sampled *in vivo* with bicarbonate led to a redistribution of iron species very similar to that seen in jejunal contents (Table 7).

Redox state of dietary iron

Table 8 shows the effect of ferricyanide and ferrocyanide on ^{59}Fe(III) uptake. The ferricyanide oxidises any Fe(II) formed, reducing iron uptake by the mucosa. Ferrozine is a potent chelator of Fe(II) and almost completely inhibits the uptake of ^{59}Fe(III) by mouse duodenum. Table 9 shows the in-vivo effect of ferrozine in inhibiting iron absorption, both as an acute and a chronic effect.

Table 7. Effect of in-vitro and in-vivo neutralisation of gastric contents

	Fraction (%)				
	Exchangeable	Carbonate	Oxide	Organic	Residue
Gastric contents	58	2	20	1	18
Neutralised gastric contents	2	17	45	7	29
Jejunal contents	2	23	45	4	26

Gastric contents neutralised *in vitro* with 0·5 M $NaHCO_3$. Mean of two experiments prior to speciation. Comparison with gastric and jejunal contents collected *in vivo*.

Table 8. ^{59}Fe(III) uptake by mouse duodenal mucosa *in vitro*

	Uptake (pmol/min/mg tissue)
Control	5·4 (±0·5) (5)
Ferricyanide	3·8 (±0·5) (5) $p < 0·04$
Ferrocyanide	5·0 (±0·6) (6) $p > 0·5$
Ferrozine	0·5 (±0·2) (4) $p < 0·01$

Uptake, mean (±SE) determined over 5 min without or with (1 mM) inhibitor for (*n*) tissue samples.

DISCUSSION

The principal findings reported in the present study are the development and application of a method for the speciation of both dietary and gastro-intestinal contents for iron. The method is based on a sequential extraction procedure, which has been widely applied in geological and food sciences, but this is the first systematic study using dietary components. The method is clearly applicable to studies of other minerals and heavy metals and has recently been used to study lead speciation within the gut (J. A. Blair, personal communication).

The initial evaluation of the method investigated the distribution of model ferric salts between the various fractions. The exchangeable (soluble in aqueous $MgCl_2$) fraction is presumed to include the iron species that are available for intestinal uptake. The finding that ferric phytate distributed to the exchangeable fraction was surprising, as dietary fibre renders minerals, including iron, non-bioavailable (Simpson *et al.*, 1981). However, more detailed studies indicate that the inhibition of iron absorption by dietary fibre is not attributable to its phytate content (Akhtar *et al.*, 1987). Further work is needed to correlate iron speciation and bioavailability, both *in vivo* and *in vitro*. It is, however, noteworthy that ferric phosphate is almost entirely recovered

Table 9. Effect of oral ferrozine on intestinal iron absorption and hepatic stores

	Controls	Ferrozine
Absorption (pmol/min/mg intestine)[a]	5·9 ± (1·4) (21)	3·2 (±0·9) (25)[c]
Liver non-haem iron (μg/g tissue)[b]	97·1 ± (30·2) (5)	56·4 (±4·9) (5)[d]

[a] Mice fed ^{59}Fe extrinsically labelled diet ±0·5 mM Ferrozine for 1 h and absorption assessed by carcass/mucosal counting.
[b] Mice fed normal diet ±2 mM Ferrozine for 3 weeks. Values mean (±SE) for (*n*) animals.
[c] $p < 0·04$.
[d] $p < 0·02$.

with the insoluble oxide fraction, a result consistent with the observation that phospho-proteins (Sato *et al.*, 1987) and inorganic phosphate (Peters *et al.*, 1971) are markedly inhibitory for iron absorption.

The comparison of the speciation of intrinsic iron and of extrinsic (radio-labelled) iron added to the diet reveals significant differences. It is claimed that the behaviour of extrinsic label closely follows that of intrinsic iron during the digestive-absorptive process and mirrors its bioavailability (Cook *et al.*, 1972; Monsen, 1974). The present study suggests that, at least for the laboratory chow used here, this conclusion may not necessarily be valid. Further studies comparing bioavailability of extrinsic and intrinsic iron are clearly indicated: it may be that following gastric digestion, the iron pools become identical. Sequential speciation of chemical and radio-labelled iron throughout the gastrointestinal tract could be of particular interest.

The striking effect of the stomach in mobilising insoluble iron salts and organic and residual iron has been highlighted by the present study. It is clear that acidification alone is not responsible for this effect (Lock & Bender, 1980). Equally interesting is the marked reduction of Fe(III) to Fe(II) within the stomach. It is likely that a combination of gastric proteases and reducing agents, e.g. ascorbate (Raffin *et al.*, 1974; Nojeim & Clydesdale, 1981; Rathbone *et al.*, 1989) and cysteine (Martinez-Torres & Layrisse, 1970; Taylor *et al.*, 1986), are responsible for these effects. Extrapolating these data to the clinical situation, the relevance of the gastric disease, e.g. achlorhydria, gastritis and gastric surgery, to the iron status of a patient is well-recognised and the present findings offer an explanation for these observations (Celada *et al.*, 1978).

The key role of Fe(II) as an intermediate in iron absorption is emphasised by the recent demonstration of ecto-ferri-reductase activity in mouse duodenum (Raja *et al.*, 1991), by the demonstration that Fe(II)/Fe(III) redox pairs inhibit iron uptake by the gut, by the striking inhibition of in-vitro iron absorption by the specific Fe(II) chelator bathophenanthroline (Barrand *et al.*, 1990), and by the inhibition of iron absorption, both *in vitro* and *in vivo*, by ferrozine, demonstrated in the present publication.

These studies show that considerable progress has been made in our understanding of the relationship between luminal iron species, redox state and the absorptive process. Much, however, remains to be determined of the detailed cellular events of the absorptive process and of the regulatory process.

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