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The importance of reductive mechanisms for intestinal uptake of iron from ferric maltol and ferric nitrilotriacetic acid (NTA)

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Abstract—Intestinal iron absorption is thought to proceed with iron mainly in the ferrous form, yet the novel iron complex, ferric maltol is an effective oral preparation. Although possessing a high oil: water partition coefficient, ferric maltol does not diffuse across the intestine but donates its iron to the endogenous uptake system. Reduction of the ferric iron in the gut lumen appears to precede iron uptake both from ferric maltol and from ferric nitrilotriacetic acid (NTA) which is a non-penetrating iron ligand. Uptake of radiolabelled iron (^{59}Fe) into isolated fragments of rat small intestine was inhibited by the ferrous chelator, bathophenanthroline sulphonate (BPS) and enhanced at low concentrations by the reducing agent ascorbic acid. Spectrophotometric evidence was obtained that ferrous ions are generated from these ferric complexes in the presence of ascorbic acid and other reducing agents. The rate of ferrous ion formation was independent of ferric maltol concentration at low ascorbic acid levels and decreased with increasing ferric maltol concentration at higher levels of ascorbate. Maltol has a high affinity for ferric ions and may delay reduction at higher concentrations. By contrast, a higher rate of ferrous ion generation was seen with ferric NTA and this increased with iron ligand concentration. Washings from the intestinal lumen also brought about ferrous ion formation from these ferric ligands. Gel filtration revealed these reducing factors to be of low molecular weight. The washings, however, interfered with ^{59}Fe uptake into the isolated fragments, but when reducing fractions only from the filtered washings were used, enhanced iron uptake was seen. There are thus additional, heat stable factors, unrelated to reduction, present in-vivo in the gut lumen that interfere with iron absorption. Intestinal absorption of iron from ferric maltol appears to occur as with other ferric chelates by initial reduction of the metal. Since maltol has a high affinity for ferric iron it may limit ferrous ion generation but it has a low affinity for the reduced form of the iron and so can donate the ferrous iron once formed immediately to the intestinal cell surface carrier. Ferric maltol may thus act as a relatively non-toxic reservoir for soluble iron in the intestinal lumen.

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It has been repeatedly claimed that only ferrous iron can be used for therapeutic purposes because bioavailability of ferric iron is very poor (Dietzfelbinger 1987) due to hydrolysis at around pH 2 or above to the insoluble ferric hydroxide (Erni et al 1984). Unfortunately, orally administered ferrous iron preparations, particularly ferrous sulphate, may cause irritation and damage to the intestinal mucosa. Attempts have therefore been made to develop iron complexes that hold ferric iron in a more bioavailable form (Sas et al 1984). The hydroxypyrone, maltol, is one such compound that can form stable and uncharged complexes with ferric iron in aqueous media over a wide range of pH and it has been shown in the rat both in-vivo and in-vitro to enhance iron absorption from the small intestine (Barrand et al 1987; Levey et al 1988).

The mechanism by which the ferric iron is absorbed with maltol is not entirely clear. Ferric maltol has a partition coefficient (n-octanol/Tris HCl, 20 mM, pH 7·4, 20°C) sufficiently high enough (0·5) for it to diffuse readily into liposomes and red cell ghosts (Bakaj 1984). However, a diffusional component of iron entry from ferric maltol must be small since saturable uptake kinetics are observed in intact tissues both in-vivo and in-vitro (Barrand et al 1987; Levey et al 1988).

Although maltol has a very high affinity for ferric iron ($\log_{\beta 3} = 28$), it is able to donate its iron readily to transferrin with a half-life for transfer of about 2 min (Bakaj, personal communication). Transferrin, identified both in the duodenal cells and in the intestinal lumen, has been suggested to play a role in the transcellular movement of iron from the gut lumen to plasma transferrin (Huebers et al 1983) possibly via endocytotic mechanisms similar to those described for erythroid cells. However, there is evidence refuting this idea (Simpson et al 1986) and our own studies indicate, not only with ferric maltol but also with ferric nitrilotriacetic acid (NTA) and ferric transferrin, that endocytosis is of little importance (Jones et al 1988). Proteins which bind both ferrous and ferric iron have been isolated from

the rat intestinal brush border membranes. It has been suggested that these proteins function as carriers for iron uptake (Stremmel et al 1987). Others have however concluded that a transport system for ferric iron is lacking and only the ferrous form of iron is taken up by an endogenous intestinal cell surface carrier. Therefore reduction of ferric iron is a prerequisite for uptake (Wollenberg & Rummel 1987). The present experiments investigate the role of reduction in the uptake of iron from ferric maltol and compare this with iron uptake mechanisms from the ferric ligand, nitrilotriacetic acid (NTA), a non-penetrating ligand which must therefore donate its iron to the endogenous uptake system.

Materials and methods

Materials. $^{59}FeCl_3$ and [^{3}H]inulin were purchased from Amersham International (Bucks, UK) and the pyrone, maltol (Veltol) obtained from Pfizer Ltd (Sandwich, Kent, UK). All other chemicals either were bought from Sigma Chemical Co. Ltd (Poole, Dorset, UK) or were standard laboratory reagents of analytical grade. Non-radioactive stock solutions of the metal: ligand complexes were prepared by mixing $FeCl_3$ with each ligand in Hepes buffer to a metal:ligand ratio of 1:4 (with maltol) or of 1:5 (with NTA). Radioactive stocks (1 mM) were prepared in a similar way, $^{59}FeCl_3$ being added to the $FeCl_3$ so as to achieve a final concentration of ^{59}Fe in the incubation media of 0·1–0·25 μCi mL^{-1}.

Iron uptake into isolated fragments of small intestine. This was carried out as described previously (Levey et al 1988). Briefly, intestinal fragments, 30–50 mg in weight were removed from freshly killed male Wistar rats (200–400 g), immersed in buffer (composition in mM: Hepes, 16; NaCl, 125; KCl, 3·5; $CaCl_2$, 1; $MgSO_4$, 10; D-glucose, 10 and pH 7·4) and incubated for 10 min at 37°C with the appropriate iron ligand solutions (see above) containing also [^{3}H]inulin (0·01 μCi mL^{-1}) as extracellular fluid marker. Fragments, removed at intervals and digested in Soluene, were analysed for their ^{59}Fe and tritium content by liquid scintillation β-particle spectrometry. Calculations of ^{59}Fe accumulation were corrected for extracellular fluid volume and wet weight of each tissue fragment. It has already been established that iron uptake over 10 min is linear (Levey et al 1988). Thus it was possible from the data to estimate initial rates of uptake.

Ferrous iron detection. Reduction of ferric iron was detected spectrophotometrically using 0·1 mM bathophenanthroline sulphonate in Hepes buffer at pH 7·4 and was followed in a cuvette as a change in absorbance at 530 nm on addition of appropriate reducing agents.

Preparation and gel filtration of gut washings. 30 cm lengths of proximal small intestine, filled with buffer, were incubated at 37°C for 45 min. The buffer was collected, centrifuged at 600 *g* for 10 min at 4°C to remove any cell debris and used immediately. One mL samples were passed over 10 mL columns (PD-10) of Sephadex G-25 (Pharmacia, Uppsala, Sweden), previously equilibrated with Hepes buffer, and eluted in 1 mL fractions.

Results and discussion

The effects of a ferrous chelator, bathophenanthroline sulphonate (BPS) and of reducing agents, e.g. ascorbic acid, on initial rates of ^{59}Fe uptake into the isolated fragments were investigated. As shown previously (Levey et al 1988), the chelating agent, BPS (1 mM), decreased ^{59}Fe uptake from ferric maltol significantly, both at 0·01 and at 0·1 mM (Table 1). Lower ^{59}Fe uptake from ferric NTA was also observed in the presence of BPS. Ferrous ions appear, therefore, to be vital for normal absorption of iron both from ferric maltol and from ferric NTA.

Table 1. Effects of reducing agents and of a ferrous chelator on ^{59}Fe uptake into isolated fragments of rat small intestine from ferric maltol and from ferric NTA.

	Ferric maltol 0·01 mM	Ferric maltol 0·1 mM	Ferric NTA 0·01 mM
Control	0·66 ± 0·14	5·91 ± 0·79	0·55 ± 0·04
+ BPS (1 mM)	0·14 ± 0·02***	1·88 ± 0·15***	0·14 ± 0·01***
+ Ascorbic acid (1 mM)	1·45 ± 0·24**		0·58 ± 0·03
(100 μM)	1·93 ± 0·31***	5·17 ± 0·94	0·59 ± 0·07
(10 μM)	1·33 ± 0·09**		0·64 ± 0·04
+ GSH (1 mM)	1·44 ± 0·28*		0·55 ± 0·07
+ Cysteine (1 mM)	1·98 ± 0·22***		0·58 ± 0·04

Values represent the means ± s.e.m. of data taken from four to six separate experiments, four measurements being obtained from each and are given in pmol min^{-1} mg^{-1} wet tissue. Statistical analysis by Student's *t*-test shows significance compared with control values as: *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.02$.

By contrast, the potent reducing agent, ascorbic acid (0·01, 0·1 and 1 mM), significantly increased ^{59}Fe uptake from ferric maltol but not from ferric NTA (Table 1). This effect was noticeable only at low concentrations of ferric maltol (0·01 mM) and was maximal at 0·1 mM ascorbic acid. Other reducing agents, i.e. reduced glutathione and cysteine, showed similar enhancing effects on ^{59}Fe uptake from ferric maltol, this activity being maximal at 1 mM rather than 0·1 mM. It is well documented that ascorbic acid can enhance absorption of ingested iron in man (Levine 1986; Hallberg et al 1987) and similar effects with cysteine and reduced glutathione have also been reported (Layrisse et al 1984).

Direct evidence was obtained spectrophotometrically that these reducing agents were generating ferrous ions from the ferric ligands. With 0·1 mM BPS alone, no changes in absorbance were evident with either ferric ligand, but on addition of ascorbic acid, rapid increases in absorbance at 530 nm occurred, indicating the formation of ferrous ions (Fig. 1). The initial rate of ferrous ion production was higher from ferric NTA than from ferric maltol at equimolar concentrations. The affinity of NTA for ferric iron is lower than the affinity of maltol for ferric iron by a factor of 10 (Levey et al 1988) and this difference might well explain the more rapid reduction that was seen with ferric NTA.

Over a range of ascorbic acid concentrations, rates of ferrous ion production were proportional to the concentration of ferric NTA (Fig. 2b). With ferric maltol however (Fig. 2a), ferrous ion production appeared to be independent of the amount of ferric maltol at least at low ascorbic acid concentrations and to decrease with increasing ferric maltol at the higher ascorbic acid concentrations. It is known that more dilute ferric maltol solutions contain a mixture of iron:ligand species, i.e. not only 1:3 but also 1:2 ferric maltol (Gerard & Hugel 1980). The 1:2 complex is more susceptible to reduction than the 1:3 complex (Hider, unpublished observations), a factor which would explain the dependence of reduction rate on concentration of ferric maltol. These observations also offer an explanation as to why the presence of ascorbic acid enhances ^{59}Fe uptake into the isolated fragments only at the low concentrations of ferric maltol (see above). The lack of effect of ascorbic acid on ^{59}Fe uptake from ferric NTA is probably because of the relative ease with which the iron in ferric NTA can be reduced. A supply of iron in the reduced form may not be the rate limiting step with this ligand, there being sufficient reducing activity already present in the incubation media (see below). Other factors such as

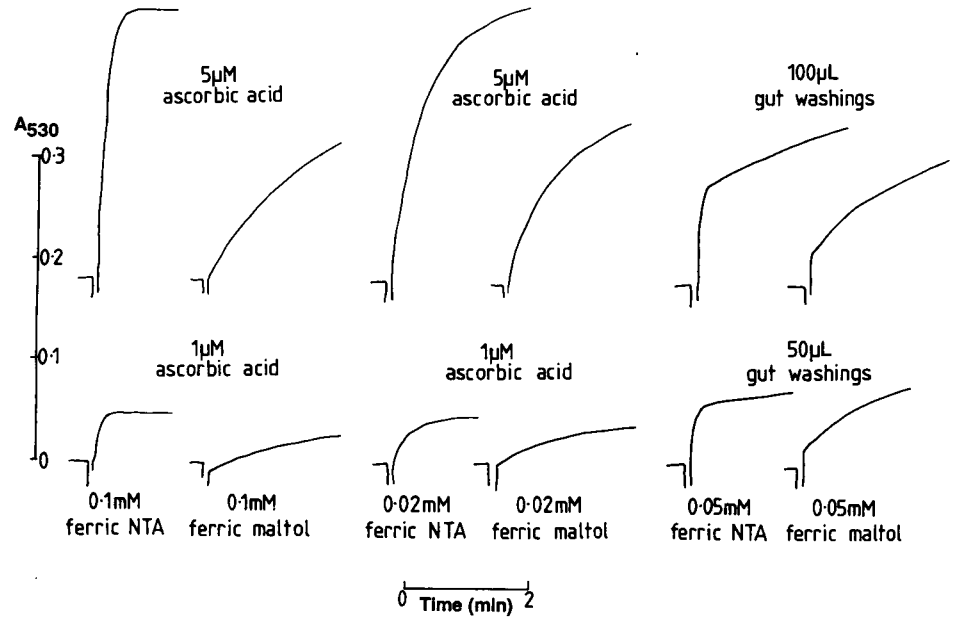

FIG. 1. Spectrophotometric traces showing formation of ferrous ions from ferric maltol and from ferric NTA on addition of ascorbic acid and of gut washings. Ferrous ion formation is indicated by the absorbance changes at 530 nm in the presence of 0·1 mM bathophenanthroline sulphonate in Hepes buffer at pH 7·4.

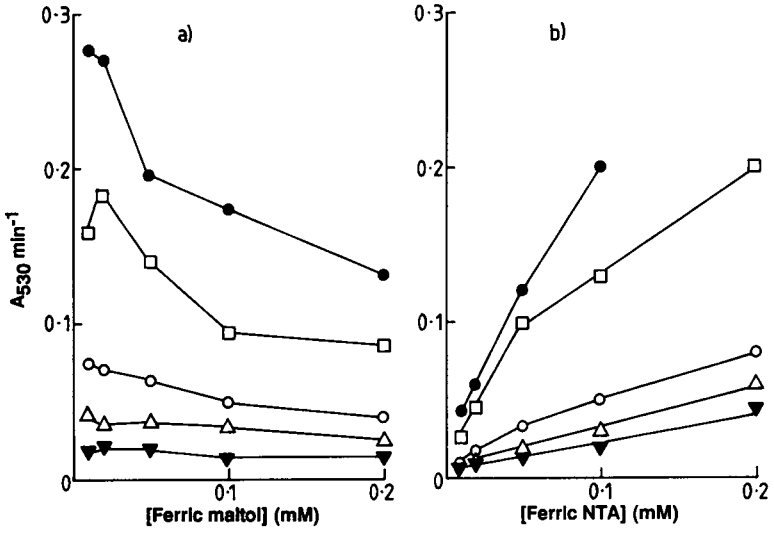

FIG. 2. Effects of iron ligand concentrations on the initial rates of formation of ferrous ions from ferric maltol (a) and from ferric (b) NTA on addition of ascorbic acid at 0·5 (▼), 1 (△), 2 (○), 5 (□) and 10 μM (●). Values represent duplicate determinations from two separate experiments.

relatively high affinity of ferrous iron for NTA, compared with that for maltol, may be responsible for slowing the transfer of iron to the membrane bound carrier. Indeed, Kurimura et al (1968) showed that the ratio of iron III to iron II bound to NTA was 10^7, while with maltol the ratio is 10^{12} (R.C. Hider and A. Hall, unpublished observations).

To determine whether reduction of iron from these ferric ligands could occur in-vivo, samples of buffer washings taken from the intestinal lumen were examined in the spectrophotometer and found to cause absorbance changes with 0·1 mM BPS in the presence of both ferric ligands (Fig. 1). Similar effects were produced by samples of buffer taken from around the isolated intestinal fragments after incubation. When the gut washings were subjected to gel filtration, reducing activity was found mainly in the low molecular weight fractions 6, 7 and 8 (Fig. 3). These same fractions contained reducing activity when ascorbic acid was applied to the column. The reducing factors in the gut washings are thus of similar size to ascorbic acid. In a previous study, measurement of reducing activity in saline washings from 20 cm lengths of rat gut yielded the equivalent of between 40 and 200 μg of ascorbic acid (Wollenberg & Rummel 1987). Certainly, substantial amounts of ascorbic acid can be detected in rat tissues, almost tenfold of those found in human tissues (Levine & Hartzell 1987). However, other agents such as reduced glutathione could also account for this reducing activity.

Despite their capacity to generate ferrous ions, the gut washings did not enhance ^{59}Fe uptake from ferric maltol into the isolated intestinal fragments but actually had an inhibitory effect with both ferric ligands (Table 2). However, if only the reducing fractions taken from the washings after gel filtration were used,

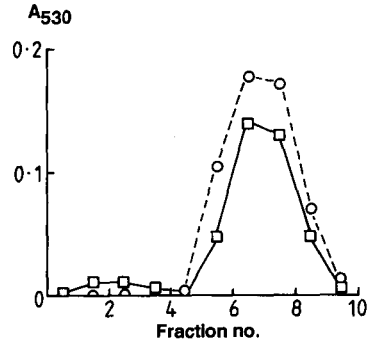

FIG. 3. Gel filtration profiles of gut washings (□) and of ascorbic acid (○) showing reducing activity eluting in the same fractions, i.e. 6, 7 and 8. The reducing capability in each fraction was calculated from the absorbance change obtained after 30 min in the presence of 0·05 mM ferric maltol and 0·1 mM bathophenanthroline sulphonate in Hepes buffer at pH 7·4. Data shown are from one of four separate experiments.

Table 2. The influence of gut washings before and after gel filtration on ^{59}Fe uptake into isolated fragments of rat small intestine from ferric maltol and from ferric NTA.

	Ferric maltol 0·01 mM	Ferric NTA 0·01 mM
Control	0·62 ± 0·04	0·55 ± 0·05
+ Gut washings	0·27 ± 0·03***	0·25 ± 0·01***
+ Heat-treated washings	0·26 ± 0·02***	
+ Protein fractions 2/3	0·59 ± 0·04	0·50 ± 0·06
+ Reducing fractions 6/7/8	0·88 ± 0·06**	0·55 ± 0·08

Values represent the means ± s.e.m. of data taken from four separate experiments, four to six measurements being obtained from each and are given in pmol min^{-1} mg^{-1} wet tissue. Statistical analysis by Student's *t*-test shows significance compared with control values as: *** $P < 0.001$ and ** $P < 0.01$.

iron uptake from ferric maltol was again increased. Clearly there are also factors within the gut lumen, unrelated to reducing activity, that interfere with iron uptake. These appear to be heat-stable but have yet to be identified since they could not be found in any of the fractions eluted from the gel column. Elution of transferrin is known to take place from isolated enterocytes on washing (Osterloh et al 1985) but such mucosal transferrin is thought to be essential and not inhibitory to normal iron uptake, since decreased absorption was observed from tied-off gut segments if the lumen was first washed out (Huebers et al 1974). But others have shown that the decreased absorption seen on washing may be associated with removal of reducing agents since it can be reversed by addition of ascorbic acid (Wollenberg & Rummel 1987). In the present study, elution of necessary reducing agents did indeed occur but was accompanied by inhibitory factors that could not be removed from the gel column after filtration. It is possible that such factors may include phosphate containing lipid micelles which bind avidly to Sephadex and could well render iron unavailable for absorption.

It is concluded that ferric maltol, like other ferric chelates including ferric NTA, will release iron in the ferrous form if exposed to reducing agents. This can be demonstrated in-vitro, but must surely also take place in-vivo since elutable factors in the gut lumen are capable of bringing about this reduction. Whether, in addition, intestinal cell surface reductases are able to reduce tightly bound ferric iron, as seen in liver cells (Low et al 1986), has yet to be determined. Formation of ferrous ions appears to be critical for effective intestinal uptake of iron from ferric maltol.

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