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Lipid peroxidation effects of a novel iron compound, ferric maltol. A comparison with ferrous sulphate

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Abstract—Lipid peroxidation effects of ferric maltol have been compared with those of ferrous sulphate both in lecithin liposomes and in brush border and mitochondrial membranes prepared from rat small intestine. Ferrous sulphate, but not ferric maltol, initiated peroxidation in liposomes as measured by conjugated diene production, but, with 500 μM ascorbic acid present, both caused intense peroxidation which was inhibitable by N_2, tocopherol, maltol and ferrous chelators, but not by OH or H_2O_2 scavengers. The rate of peroxidation increased with ferrous sulphate concentration up to 100 μM but was independent of ferric maltol concentration between 5–500 μM. Material eluted from rat small intestine contained a reducing factor, similar in size to ascorbic acid, capable of generating ferrous ions from ferric maltol and initiating peroxidation. Peroxidation in mitochondrial membranes appeared unaffected by addition of iron whilst that in brush border membranes was detectable only in the presence of iron. At iron concentrations of 100 μM and above ferric maltol produced less liposomal peroxidation than ferrous sulphate. Maltol itself may delay recycling of Fe^{3+} to Fe^{2+}. Thus ferric maltol could provide a less toxic alternative to ferrous salts in the oral treatment of iron-deficiency.

It has generally been observed that the most effective preparations for oral administration of iron in the treatment of iron deficiency contain iron in the ferrous form (Dietzfelbinger 1987), due in large part to the lower bioavailability of ferric iron. Unfortunately, the presence of high concentrations of ferrous iron in the gastrointestinal tract can cause irritation and damage to the mucosa. Fatalities resulting from iron overdose appear to be associated with intestinal injury, although death itself is usually caused by cardiovascular damage rather than by the local intestinal injury (Nayfield et al 1976; Robotham & Lietman 1980). One of the factors thought to underlie such injury is the capacity of ferrous ions to generate free radicals (Slivka et al 1986) and to initiate lipid peroxidation.

An iron pyrone complex (ferric maltol) has recently been developed which holds its iron in the ferric form yet appears to be effective, both in-vivo (Barrand et al 1987) and in-vitro (Levey et al 1988), in allowing intestinal iron uptake. Since the pyrone binds the ferric ions with high affinity (log $\beta_3 = 28$) and the resultant complex is stable in aqueous media over a wide range of pH, it was suggested that ferric maltol might prove to be less toxic than ferrous preparations (Levey et al 1988). Recently it has been shown that the iron in ferric maltol undergoes reduction in the presence of elutable factors from the rat intestinal lumen (Barrand et al 1990). Ferric maltol is thus capable of generating ferrous ions within the intestinal lumen and, indeed, this appears to be vital for normal iron uptake across the intestine (Barrand et al 1990).

The present work was undertaken to determine the potential capacity of ferric maltol to produce lipid peroxidative damage.

Materials and methods

Materials. The pyrone, maltol, was purchased as Veltol from Pfizer Ltd (Sandwich, Kent, UK). All other chemicals were either obtained from Sigma Chemical Co. Ltd (Poole, Dorset, UK) or were standard laboratory reagents of analytical grade. Ferric maltol solutions were prepared by mixing $FeCl_3$ with maltol in Hepes buffer to a metal:ligand ratio of 1:4.

Liposome preparation. This was done by dissolving L-α-phosphatidylcholine derived from soya bean in chloroform and evaporating under N_2 in a flask to form a thin film of lipid. This was resuspended in buffer (composition mM Hepes, 16; NaCl, 125; KCl, 3·5; $CaCl_2$, 1; $MgSO_4$, 10 and pH 7·4) and soaked for 1 h under N_2; it was then sonicated for 20 s immediately before use.

Mitochondrial and brush border membrane preparation. Male Wistar rats (200–400 g), previously fasted for 18 h, were killed by stunning and cervical dislocation. Mucosal scrapings were taken from duodenum and jejunum after rinsing in saline and homogenized either in (1) 0·25 M sucrose/2 mM Hepes containing 0·001 mM EDTA at pH 7·4 for mitochondria or in (2) 50 mM mannitol/2 mM Hepes at pH 7·4 for brush border membranes. Both suspensions were then spun at 4°C for 10 min at 600 *g* to remove unbroken cells and debris. The resultant supernatants were treated as follows:

either (1) for mitochondria by centrifugation at 9000 *g* for 15 min. The pellet was resuspended in distilled water and recentrifuged at 27 000 *g* for 30 min. The final pellet was taken up in Hepes buffer as above.

or (2) for brush border membranes, solid $MgCl_2$ was added to a final concentration of 10 mM (Stremmel et al 1987) and left 20 min at 4°C before centrifugation at 6000 *g* for 10 min. The resultant supernatant was centrifuged at 27 000 *g* for 30 min to obtain a pellet which was resuspended in 100 mM mannitol/2 mM Hepes/0·1 mM $MgCl_2$ and recentrifuged at 6000 *g* for 10 min. The final supernatant was again centrifuged at 27 000 *g* for 30 min and the pellet from this taken up in Hepes buffer as above. Both membrane preparations were stored at −20°C under N_2 until use.

Preparation and gel filtration of gut washings. Lengths of duodenum and jejunum were filled with Hepes buffer containing 10 mM glucose and incubated at 37°C for 45 min. Buffer within the gut lumen was then collected, centrifuged at 600 *g* for 10 min at 4°C to remove cell debris and used immediately. Washings were also obtained in a similar manner from the small intestine in-situ in terminally anaesthetized rats. One mL samples of the washings 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.

Lipid peroxidation assay. Although the thiobarbituric acid assay is the most frequently used method for detecting lipid peroxidation in-vitro, it is not so suitable for in-vivo studies due to metabolism of the end product, malondialdehyde. Initial events in peroxidation involve rearrangement of double bonds leading to formation of conjugated dienes measurement of which provides a sensitive and more versatile assay of peroxidation (Buege & Aust 1978). Liposomes (at a final lipid concentration of 10 mg mL^{-1}) and membrane preparations (at a final protein concentration of 1–5 mg mL^{-1}) were incubated at 37°C with the

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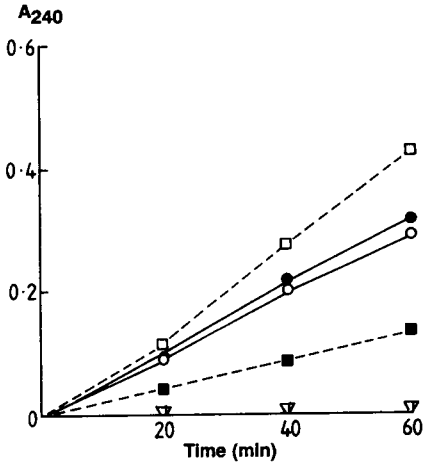

FIG. 1. Time course of peroxidation in liposomes induced by $FeSO_4$ at 0·005 mM (■), 0·05 mM (□) or ferric maltol at 0·005 mM (●), 0·05 mM (○) in the presence of 500 μM ascorbic acid or with buffer alone (▽). Each point is the mean of data taken from duplicate measurements in four separate experiments.

iron solutions and other test reagents in an atmosphere of air. 50–100 μL samples were taken at various times up to 60 min into 2 mL of chloroform–methanol (2:1) and absorbance measured at 240 nm with reference to chloroform–methanol alone. When protein was present in the mixture, the tubes were centrifuged for 5 min at 6000 *g* before assay. The change in absorbance noted from the start of incubation was taken as a measure of the peroxidation rate.

Results and discussion

In the presence of 100 μM ferrous sulphate, lipid peroxidation in liposomes, as measured by a change in absorbance at 240 nm, was linear up to 60 min. The presence of 500 μM ascorbic acid greatly facilitated the process, presumably by recycling the ferric ions formed by oxidation back to the ferrous form. No peroxidation was evident with liposomes alone, with 500 μM ascorbic acid or with 100 μM ferric maltol alone (Fig. 1). However, when 500 μM ascorbic acid was added to the ferric maltol, peroxidation was detectable.

Table 1. Effects of various treatments on lipid peroxidation in liposomes induced by 100 μM $FeSO_4$ or 100 μM ferric maltol in the presence of 500 μM ascorbic acid.

Treatment	with $FeSO_4$	with Ferric maltol
Control	7·10±1·12	5·32±0·99
Without ascorbic acid	0·88±0·09	0·16±0·10
Under nitrogen	1·00±0·14	0·75±0·14
+Tocopherol (1 mM)	3·81±0·41	2·28±0·16
+Retinol (1 mM)	6·65±0·27	4·59±0·25
+Mannitol (10 mM)	6·72±0·43	5·00±0·44
+Maltol (1 mM)	0·35±0·14	0·84±0·05

Values are means±s.e.m. (n=4–6) of changes in absorbance at 240 nm min^{-1} g^{-1} lipid.

In an atmosphere of N_2, peroxidation was significantly reduced. Inhibition was also seen in the presence both of α-tocopherol and retinol (Table 1). These agents interfere with propagation of the peroxidation chain reaction by trapping lipid peroxide radicals (Burton & Ingold 1984). Antioxidants that reduce chain initiation by scavenging OH radicals (mannitol) or H_2O_2 (catalase) had no effect on the iron-induced liposomal peroxidation. Both the ferrous chelator, bathophenanthroline sulphonate and maltol itself which has a high affinity for ferric iron only inhibited peroxidation. This suggests that initiation of the iron-induced lipid peroxidation seen in the liposomes, results directly from shuttling between the ferrous and ferric forms of iron rather than from generation of free radicals through the Fenton or Haber Weiss reactions. Although both OH^- and O^- radicals have been implicated in iron-induced peroxidation, the evidence is conflicting and many now favour the idea that it is ferrous iron itself which is the active initiating species (Kornbrust & Mavis 1980), the ratio of ferrous to ferric ion being an important determinant (Braughler et al 1986; Minotti & Aust 1987).

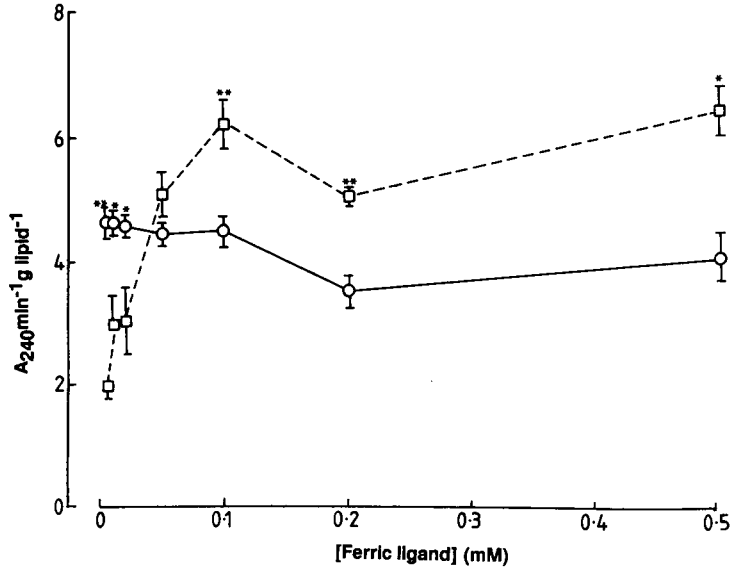

FIG. 2. Effect of concentration of ferric maltol (○) and of $FeSO_4$ (□) on peroxidation in liposomes in the presence of 500 μM ascorbic acid. Samples were removed from the incubation medium at times shown during the linear phase of peroxidation (see Fig. 1). Each point is the mean±s.e.m. of quadruplicate determinations from four separate experiments. Statistical analysis by Student's *t*-test shows significant differences between the two iron compounds at ** $P<0·001$ and at * $P<0·01$.

The peroxidation rate in the presence of 500 μM ascorbic acid increased with increasing concentrations of ferrous sulphate (from 5–100 μM) but showed a slight decrease with increasing ferric maltol concentrations (Fig. 2). This observation agrees with data (Barrand et al 1990) showing decreased rates of ferrous ion generation with increasing amounts of ferric maltol in the presence of high levels of ascorbic acid. The high affinity of maltol for ferric iron may delay reduction of iron to the ferrous form and this would be particularly noticeable at the higher ferric maltol concentrations. However, it has been suggested that the ability of ferric chelates to promote peroxidation may not only be correlated with rates of ferric iron reduction, and that rates of reoxidation and the maintenance of appropriate ferric:ferrous ion ratios may also be important (Vile & Winterbourn 1988).

The effects of ascorbic acid over a range of concentrations (from 0·01–5 mM) on peroxidation induced by ferrous sulphate and by ferric maltol at 100 μM were studied. Previous results (Heikkila & Manzino 1987) have suggested that low concentrations of ascorbate will be insufficient to reduce ferric ions back to the ferrous form whilst high concentrations will again inhibit because of reductive maintenance of iron in the reduced form thus delaying recycling. With both iron compounds, bell-shaped curves were obtained, peak values for peroxidation occurring between 0·5 and 1 mM ascorbic acid, these values being tenfold higher than those seen at 0·01 or 5 mM.

In the presence of 0·5 mM ascorbic acid, ferrous sulphate at 100 μM, or higher, induced significantly greater peroxidation than equivalent concentrations of ferric maltol (Fig. 2). This may even be an underestimate of the capacity of ferrous sulphate to initiate peroxidation since decreases in ferrous ion concentration can occur rapidly in ferrous/ascorbate solutions at pH above 7 (Dorey et al 1987). With the doses required during iron therapy (in the region of 2–3 mmol per dose in man), concentrations greatly in excess of 100 μM could well be present for some time in the duodenal lumen.

Washings from the rat small intestinal lumen which have been shown to contain reducing factors (Barrand et al 1990), were found to initiate peroxidation in the presence of ferric maltol. After subjecting these gut washings to gel filtration, the subsequent fractions were analysed for their ability to cause liposomal peroxidation in the presence of 100 μM ferric maltol (Fig. 3). As with reducing activity (Barrand et al 1990), peroxidation was evident mainly associated with fractions 6, 7 and 8 corresponding to material of low molecular mass (< 1000).

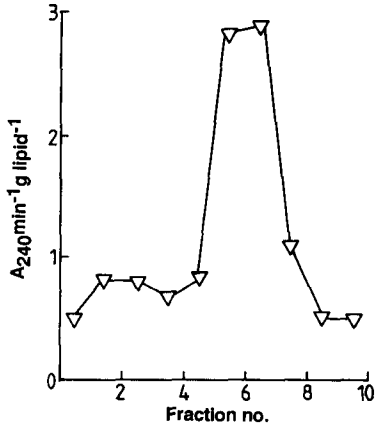

FIG. 3. Gel filtration profile of gut washings showing peroxidative activity eluting at fractions 6, 7 and 8. The capacity of each fraction to initiate iron induced peroxidation in liposomes was tested using 0·05 mM ferric maltol. Data shown are from one of four separate experiments.

Table 2. Effects of NADH and of ferric maltol on rates of peroxidation in mitochondrial and brush border membranes prepared from rat small intestine.

	Mitochondrial membranes	Brush border membranes
No additions	14·76 ± 1·23	0·99 ± 0·84
+ NADH (1 mM)	29·48 ± 1·90*	67·18 ± 6·30*
+ Ferric maltol (0·1 mM)/ ascorbic acid (0·5 mM)	19·24 ± 3·00	9·67 ± 1·70*

Values are taken from duplicate determinations in four separate experiments and are given as changes in absorbance at 240 min^{-1} g^{-1} protein. Statistical analysis by Student's *t*-test shows significance from control values as * $P > 0·001$.

No significant absorbance changes were observed in the presence of bathophenanthroline sulphonate (1 mM). However, a high background reading due to the formation of colour during ferrous iron chelation with this agent prevented the detection of any very small changes in absorbance that may have occurred.

Apart from reducing factors that can be eluted from the gut lumen, there may also be cell membrane associated reducing activity. Reductase enzymes fuelled by NADH have been identified in intestinal brush border membranes (Goldenberg 1982). It has been suggested that some cell surface reductase may, in fact, be involved in conversion of external ferric iron to ferrous iron before uptake (Sun et al 1984). Brush border and mitochondrial membranes were tested against 100 μM ferric maltol to determine whether reduction of ferric maltol leading to peroxidation could occur within these membranes. In the absence of added iron, peroxidation was apparent with mitochondrial but not brush border membranes (Table 2). A significant increase in peroxidation was seen in both preparations on addition of ferric maltol and NADH. However, a similar effect occurred with NADH alone. Presumably this was unrelated to iron reduction since neither 1 mM maltol acting as a ferric chelator nor 1 mM bathophenanthroline sulphonate acting as a ferrous chelator prevented the effect. In fact addition of 100 μM ferric maltol or 100 μM ferrous sulphate in association with 500 μM ascorbic acid increased peroxidation only with brush border and not with mitochondrial membranes.

It appears, therefore, that lipid peroxidation can take place in the presence of ferric maltol as a consequence of metal reduction by factors present in the intestinal lumen. However, peroxidation is decreased by the ligand maltol itself and at high concentrations the ferric maltol complex initiates significantly less peroxidation than equimolar concentrations of ferrous sulphate. Thus, in doses needed for the effective oral treatment of anaemia or where there is accidental overdose, ferric maltol may prove to be less toxic than ferrous sulphate.

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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