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Dissociation of a ferric maltol complex and its subsequent metabolism during absorption across the small intestine of the rat

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1 The fate and disposition of [^{59}Fe]-ferric [^{3}H]-maltol after intravenous administration were investigated in anaesthetized rats. Immediate dissociation of ferric iron from maltol took place in the circulation even with high doses of ferric maltol (containing 1 mg elemental iron). In plasma samples withdrawn within 1 min of injection and subjected to gel filtration, ^{59}Fe eluted with the high molecular weight proteins whilst the tritium was associated with low molecular weight material.

2 The rates of elimination of ^{59}Fe and of tritium from the plasma and their ultimate fate were very different. The half life for ^{59}Fe in the plasma was around 70 min and ^{59}Fe appeared mainly in the bone marrow and liver. There was an initial rapid exit of tritium from the plasma with a half life of around 12 min. This was followed either by a plateau or by a rise in tritium levels, involving entry of maltol metabolites into the circulation. These metabolites could be recovered in the urine.

3 Entry of ^{59}Fe and of tritium into the blood plasma after intraduodenal administration of [^{59}Fe]-ferric [^{3}H]-maltol was also very different. At low doses of ferric maltol (containing 100 μg elemental iron), the tritium appeared in the plasma in highest amounts within seconds and then decreased whilst there was a slow rise in ^{59}Fe levels. At higher doses of ferric maltol (containing 7 mg elemental iron), levels of ^{59}Fe in the plasma were highest at 5 min and then fell whereas tritium levels rose steadily. Mucosal processing of ^{59}Fe prevented further entry of iron at high dose into the circulation.

4 Initial rates of uptake of [^{3}H]-maltol into isolated intestinal fragments were measured over a range of concentrations and revealed that maltol alone could diffuse freely into the tissues whereas maltol complexed to iron showed saturable uptake kinetics similar to those seen with the iron itself.

5 After intestinal uptake, ^{59}Fe and tritium were associated with different subcellular fractions, maltol itself being metabolized to the glucuronide conjugate within the intestinal mucosa.

6 It is concluded that dissociation of metal and ligand takes place before entry into the intestinal mucosa. Iron is then taken up on the endogenous carrier and processed in the normal way whilst maltol enters by diffusion, its rate of entry being limited by the degree of dissociation. It is subsequently metabolized by conjugation and eliminated rapidly from the body in the urine.

Introduction

It is generally supposed that ferrous preparations are more effective in the oral treatment of iron deficiency than ferric compounds partly at least because of the low bioavailability of ferric iron (Dietzfelbinger, 1987). Ferrous preparations may cause irritation and damage to the mucosal lining, particularly in overdose (Nayfield *et al.*, 1976). Thus compounds that can hold ferric iron in absorbable form might be a therapeutic advantage. The hydroxypyrone, maltol, has a very high affinity for ferric iron (K_{aff} value of ferric iron for maltol of log $\beta 3 = 28$) and appears able to hold the metal in soluble form within the pH range which would be encountered in the duodenal lumen i.e., up to 8 (Hungerford & Linder, 1983; Hider *et al.*, 1984). Yet the ferric maltol complex is kinetically labile and will readily donate its iron to transferrin (half life of transfer to apotransferrin of 2 min (M.E. Bakaj, personal communication). It has already been shown in rats that substantial absorption of iron can occur after oral treatment with ferric maltol (Barrand *et al.*, 1987).

Since the intestinal mucosa provides the only means of controlling body iron status, it is important to ensure that the mechanism by which iron from ferric maltol crosses the intestinal wall does not involve by-passing the normal regulatory system, thereby leading to iron overload. It was originally supposed that the uncharged ferric maltol complex (structure given in Barrand *et al.*, 1987) on account of its relatively high oil/water partition coefficient (0.5; Bakaj, 1984), could diffuse across the intestinal mucosa and donate the ferric ions directly to transferrin in the blood. Recent studies have indicated however, that the iron taken up from ferric maltol probably passes to the normal control pathways within the intestinal wall (Barrand & Callingham, 1991). Furthermore, it appears that reduction of ferric iron to the ferrous form can occur within the intestinal lumen and may well precede any uptake (Barrand *et al.*, 1990). If this is so, then metal and ligand must dissociate before iron absorption, since maltol has a very low affinity for the ferrous form of iron.

The present work extends these previous studies by following concomitantly the passage of metal and ligand across the intestinal wall and by investigating the fate and metabolism of the ligand itself. Further evidence that dissociation takes place before entry is provided indicating that the ferric maltol complex itself never exists beyond the lumen of the gut. Preliminary results of this work have been reported to the British Pharmacological Society (Gee *et al.*, 1987).

Methods

Absorption and pharmacokinetic studies in whole animals

Male Wistar rats, 150–400 g body weight, were fasted for 18 h before use. For pharmacokinetic studies, rats were anaesthetized with sodium pentobarbitone (Sagatal, Rhône Poulenc, Dagenham, Essex; 60 mg kg^{-1}, i.p.) and cannulae

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inserted into the jugular vein and carotid artery. Heparin (500 i.u.), further anaesthetic as required and the iron solutions were introduced via the jugular venous cannula. Blood was sampled from the carotid arterial cannula at suitable time intervals, care being taken to ensure that total blood loss was kept to a minimum, i.e., no more than 10% of the blood volume. The volume of blood removed in each sample was replaced by an equivalent volume of physiological saline. In studies where ferric maltol solutions were given into the duodenum the jugular vein was not cannulated and heparin and further anaesthetic were administered i.p. The animals remained under anaesthesia throughout the experiments and were killed by overdose at the end. Samples of liver together with the kidneys, femurs and urine were taken for analysis of their ^{59}Fe content in a Packard Auto Gamma 500 counter. The blood samples taken during the experiments were centrifuged at 6000 g for 5 min to remove the cells and the plasma analysed for ^{59}Fe and tritium content by liquid scintillation β-particle spectrometry with automatic external standardization to correct for quenching and to allow conversion to d.p.m. for each radioisotope. The efficiency of counting for tritium was around 30% whilst that for ^{59}Fe almost 100%.

Gel filtration

Plasma samples, up to 1 ml in volume, were passed down 10 ml columns (PD-10) of Sephadex G-25 (Pharmacia, Uppsala, Sweden) previously equilibrated with 20 mM HEPES buffer at pH 7.4 and eluted in 1 ml fractions. The supernatants obtained after centrifugation of intestinal homogenates (see below) were subjected to gel filtration in a similar manner. Recovery of tritium from the columns was around 96% to 98% of that applied whilst that of ^{59}Fe was 80% to 90%. Some dissociation of iron from maltol occurred on mixing the ferric maltol complex *in vitro* with either plasma or intestinal homogenates due to the presence of transferrin in these samples. Ferric iron has a higher affinity for transferrin than for maltol and rapidly transfers from the ligand to the protein (Barrand *et al.*, 1987).

Thin layer chromatography

Samples of the gel fractions were concentrated by freeze-drying and spotted in 100 μl volumes on to cellulose thin layer plates together with 2 μl samples of unlabelled maltol (10 mg ml^{-1}) and run to a distance of 12 cm (1 to 2 h duration) at room temperature in the following solvent system: methyl ethyl ketone:acetone:water:formic acid (80:6:12:2 by volume). The unlabelled maltol spots could be visualized under u.v. light at 254 nm. The plates were divided into 1 cm lengths and the tritium content of the cellulose analysed after dissolution in scintillation fluid. Recovery of tritium was generally over 95% of that applied to the plates.

Uptake into isolated intestinal fragments

Uptake of ^{59}Fe and of tritium into isolated fragments of small intestine was carried out as described previously (Levey *et al.*, 1988). Intestinal fragments, 30 to 50 mg in weight, were removed immediately from rats, which had been killed by cervical dislocation, and immersed in buffer containing (mM): HEPES 16, NaCl 125, KCl 3.5, $CaCl_2$ 1, $MgSO_4$ 10 and D-glucose 10 at pH 7.4. After incubation for 10 min at 37°C with the appropriate ^{59}Fe and tritium solutions (see above), fragments were removed, digested in Soluene-350 (Packard, Reading) and analysed for their ^{59}Fe and/or tritium content. Where uptake of tritium alone was being monitored, [^{14}C]-mannitol (0.001 μCi ml^{-1}) was included in the incubation mixture as an extracellular fluid marker. Calculations of ^{59}Fe, ^{3}H or their simultaneous accumulation were corrected for wet weight of each tissue fragment and for extracellular fluid volume. Over the course of 10 min, both ^{59}Fe and tritium accumulation increased in a linear fashion so it was possible to calculate initial rates of uptake from the data.

Maltol metabolite studies

Isolated intestinal fragments, 2 to 3 cm in length, were incubated for 10 to 20 min with appropriate ^{59}Fe and tritium solutions, the mucosa then scraped off with a glass slide and homogenized in 20 mM HEPES buffer at pH 7.4. After centrifugation for 5 min at 600 g to remove unbroken cells, the homogenate was centrifuged at 100,000 g for 1 h. Samples of pellet and supernatant were analysed for ^{59}Fe and tritium content by liquid scintillation counting. Supernatant samples were also subjected to gel filtration (see above). In studies to investigate the nature of metabolites formed, mucosal scrapings from isolated intestinal fragments, previously incubated for 30 min in 0.1 mM [^{3}H]-maltol, were homogenized in 80% ethanol. The precipitated proteins were centrifuged into a pellet and excess ethanol was removed from the supernatant under a jet of N_2. Supernatant samples were subjected to gel filtration and the tritium-rich fractions concentrated by freeze-drying before incubation for 1 h with 10–20 units of β-glucuronidase in Na acetate buffer at pH 5. It was found from preliminary experiments in which the incubation time was varied that incubation for at least 1 h was necessary for conversion of 75%–100% of the tritiated metabolite. After incubation, the pH was readjusted to 7 and 100 μl samples spotted on to thin layer plates to undergo separation as described above. In studies to determine the effect of maltol concentration on conjugation, uncentrifuged homogenates were adjusted to pH 6 by addition of 0.05 M HCl and were washed 3 times with equal volumes of chloroform to remove unchanged [^{3}H]-maltol before being analysed for tritiated metabolite content. Initial recovery experiments showed that, with this extraction technique, 96–100% maltol could be removed leaving 95–100% of the metabolite for radiochemical assay. Conjugation rates can be much modified by isolation conditions and it is probable that tissue systems more closely mimic the *in vivo* situation than subcellular fractions (Koster & Noordhoek, 1983; Koster, 1985).

Preparation of [^{3}H]-maltol

Preparation of [^{3}H]-methylfurfurylalcohol Methyl iodide (3.63 g) was dissolved in 75 ml sodium-dried ether, chilled in ice and mixed with [^{3}H]-methyl iodide (Amersham, 25 mCi; 101 mCi mg^{-1}). The specific radioactivity of the methyl iodide used in the first stage of the synthesis was therefore 1 Ci mol^{-1}. The ethereal methyl iodide solution was poured into an addition funnel attached to a 250 ml 3-necked round-bottomed flask containing 0.77 g Mg turnings. After running approximately half of the methyl iodide solution on to the magnesium, a crystal of iodine was placed in the flask, to initiate formation of the Grignard reagent. Over a period of a few min the yellow iodine colour disappeared, being replaced by the white 'cloudiness' of the Grignard reagent. At this stage, stirring was begun and the mixture heated until the ether refluxed. Stirring was continued until the reaction subsided. The remaining methyl iodide solution was added dropwise over a 5 min period with continued stirring. The mixture was refluxed for a further 15 min in order to ensure maximum Grignard preparation. On cooling a cloudy white solution resulted with little magnesium being visible.

Furfural (2.49 g) was dissolved in 25 ml sodium-dried ether, poured into a second addition funnel and added dropwise to the Grignard reagent over a period of 10 min with constant stirring. On completion of this addition the mixture was refluxed for 15 min to ensure completion of the reaction.

After cooling, 75 ml of ice/water was added to the reaction flask and the mixture shaken until all the resulting solid dissolved. The ethereal/aqueous mixture was decanted into a separating funnel and thoroughly mixed with 100 ml saturated NaCl together with 100 ml 1 M sodium metabisulphite solu-

tion. The aqueous layer was washed with dichloromethane (2 × 200 ml), and the organic fractions were combined for final washing with 1 M sodium metabisulphite (2 × 200 ml), dried over anhydrous sodium sulphate, and evaporated *in vacuo* to give [^{3}H]-methylfurfuryl alcohol as an oil, 1.9 g, 66% yield.

Preparation of [^{3}H]-maltol A mixture of methanol (4 ml) and water (6 ml) contained in a 250 ml 3-necked round-bottomed flask was chilled to −10°C by external application of dry ice/acetone. Two additional funnels attached to the flask contained [^{3}H]-methylfurfuryl alcohol (1.9 g) dissolved in methanol (8 ml)/water (4 ml) and bromine (5.7 g) respectively. The alcohol and bromine were added simultaneously in a rapid dropwise fashion, maintaining the temperature of about −10°C, such that all the alcohol was added when 1/3 of the bromine remained. Stirring was maintained throughout the additions, the final portion of bromine being added slowly at −10°C.

After replacing one of the additional funnels with a sidearm and condenser, the flask was heated to 80°C and an 8 ml portion of methanol was distilled from the reaction mixture. The sidearm was then rapidly replaced by a double condenser and the mixture was refluxed at 90–95°C for 3.5 h.

The brown mixture was cooled, decanted into a beaker and adjusted to pH 8 by the addition of 2 M NaOH. After reducing the volume of the resulting solution to approximately 5 ml by rotary evaporation, it was extracted with dichloromethane (4 × 30 ml). The combined organic layers were dried over anhydrous Na_2SO_4 filtered and evaporated *in vacuo* to give [^{3}H]-maltol, 0.75 g, 35% yield. The crude product was recrystallized from absolute ethanol. The purity of each batch of [^{3}H]-maltol was tested alongside unlabelled maltol on thin layer silica gel plates with methanol:chloroform (10:1 by volume) as the solvent system.

Materials

$^{59}FeCl_3$ and [^{14}C]-mannitol were purchased from Amersham International (Bucks). Unlabelled maltol (Veltol) was obtained from Pfizer Ltd (Sandwich, Kent). All other chemicals either were bought from Sigma Chemical Co Ltd (Poole, Dorset) or were standard laboratory reagents of analytical grade. For the *in vitro* uptake experiments, non-radioactive stock solutions of ferric maltol were prepared by mixing $FeCl_3$ with maltol in HEPES buffer to a metal:ligand ratio of 1:4. It is known from *in vitro* studies that on mixing ferric iron with maltol at neutral pH, there is immediate formation of an uncharged 1:3 iron-maltol complex (Stefanovic *et al.*, 1968). Radioactive stocks were prepared in a similar way, $^{59}FeCl_3$ and [^{3}H]-maltol being added so as to achieve final concentrations in the incubation media of 0.05–0.25 μCi ml^{-1} for ^{59}Fe and 0.1–0.5 μCi ml^{-1} for tritium. Where appropriate, [^{14}C]-mannitol was used at a final concentration of 0.01 μCi ml^{-1}. For the whole animal uptake studies, ferric maltol solutions were prepared just before use by mixing solid $FeCl_3$, $^{59}FeCl_3$[^{3}H]-maltol and maltol powder at a molar ratio of 1:4 in sufficient saline to provide 100 μg, 1 mg or 7 mg elemental iron, 5, 15 or 25 μCi of tritium and 1, 3 or 5 μCi of ^{59}Fe in each 500 μl dose.

Results

Pharmacokinetic studies after intravenous injection of [^{59}Fe]-ferric [^{3}H]-maltol

The elimination of ^{59}Fe and of tritium from the blood plasma after intravenous injection of [^{59}Fe]-ferric [^{3}H]-maltol at doses of 100 μg and of 1 mg elemental iron was followed by analysing samples of blood plasma taken at 10 to 20 min intervals for ^{59}Fe and tritium content. Elimination of ^{59}Fe appeared to obey single compartment first order kinetics with a half life of around 70 min (Figure 1); 60 min after injection of 100 μg of [^{59}Fe]-ferric maltol, the tissues with the highest

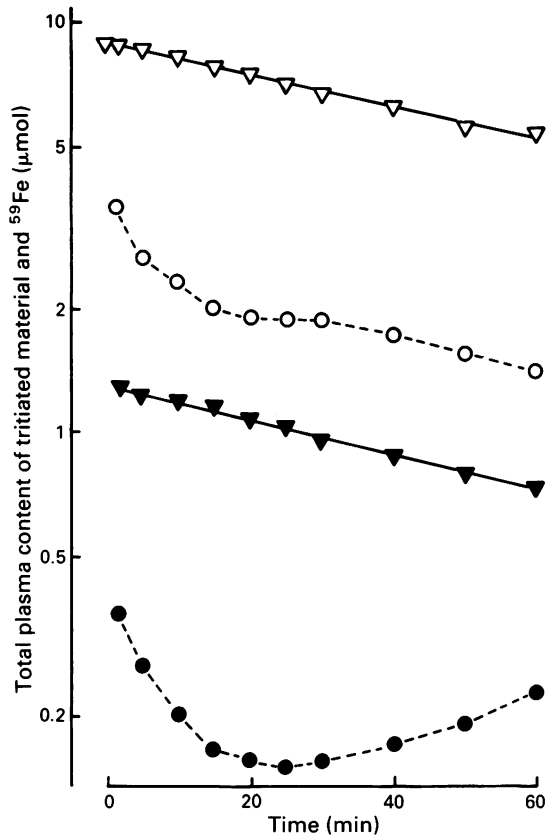

Figure 1 Changes in total plasma content of ^{59}Fe (▼, ▽) and tritium (●, ○) during 1 h following intravenous injections of [^{59}Fe]-ferric [^{3}H]-maltol at doses containing 100 μg (closed symbols) and 1 mg (open symbols) of elemental iron. Each value is the mean of data from 3 animals and is given as μmol of radioactive material in the total plasma.

content of ^{59}Fe were the bone marrow (11 ± 2% of administered dose, $n = 4$ rats) and liver (18 ± 1%). A small amount of ^{59}Fe was detected in the urine (2.6 ± 1%). The shapes of the elimination curves for tritium were much more complex. There seemed to be an initial phase with a half life of about 12 min which was followed in some cases by a pleateau and in some cases by a rise in tritium content. Tissue content of tritium was not measured but a substantial proportion of the dose injected was found in the urine after 60 min (30–40% at 100 μg, 5–10% at 1 mg).

Disposition of ^{59}Fe and tritium in the plasma after intravenous injection

Gel filtration of plasma samples taken 2 and 60 min after injection of [^{59}Fe]-ferric [^{3}H]-maltol at a dose containing 1 mg elemental iron showed that even within 2 min, metal and ligand no longer were present as the complex (Figure 2). ^{59}Fe eluted from the gel at the void volume which corresponded to high molecular weight proteins whereas tritium was retained within the gel. Partial dissociation of ferric maltol can also occur in plasma samples *in vitro*, presumably due to the ready donation of ferric iron to apotransferrin. In the plasma samples taken after 2 min, the tritium eluted with material corresponding to a molecular weight of maltol itself but in the 60 min sample, the tritium peak was shifted slightly to a position corresponding to material slightly larger than maltol. The tritium in the urine showed a similar elution profile. Identical results were obtained after injection of ferric maltol at the 100 μg dose.

The tritium containing fractions were analysed further by a thin layer chromatography system that separated the relatively lipophilic maltol from more hydrophilic compounds.

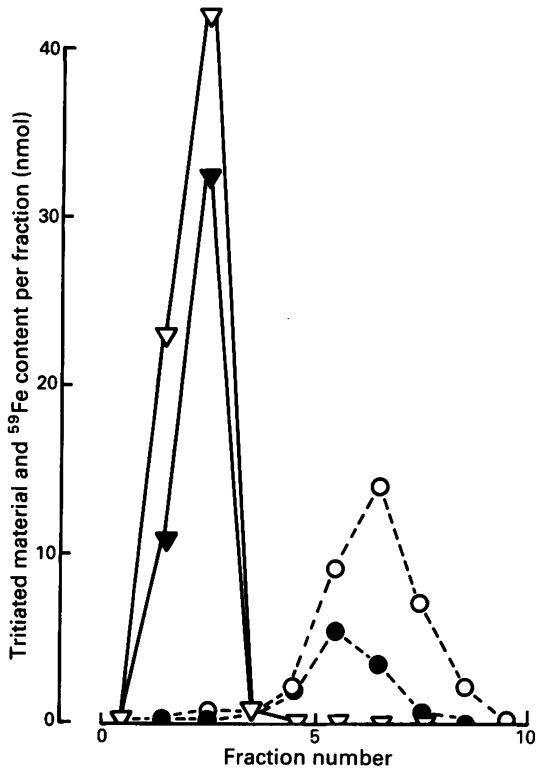

Figure 2 Gel filtration profiles of ^{59}Fe (▼, ▽) and tritium (●, ○) in plasma samples taken 2 min (open symbols) and 60 min (closed symbols) after intravenous injection of [^{59}Fe]-ferric [^{3}H]-maltol at a dose of 1 mg elemental iron; 100 μl volumes were applied to 10 ml columns of Sephadex G-25 and 1 ml fractions eluted. Values are shown as nmol of radioactive material in each fraction.

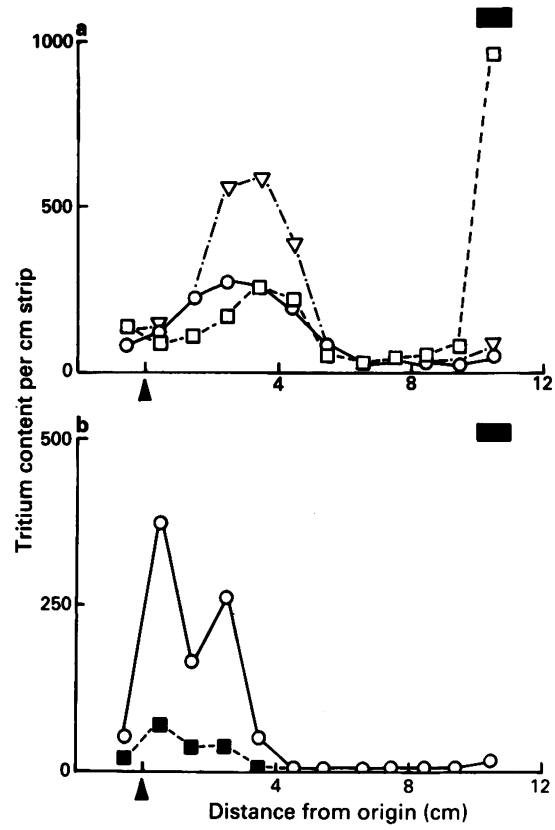

Figure 3 Thin layer chromatographic separation of tritiated material in plasma samples taken 2 min (□), 5 min (■), 20 min (▽) and 60 min (○) following intravenous (a) or intraduodenal (b) administration of [^{59}Fe]-ferric [^{3}H]-maltol at a dose of (a) 1 mg or (b) 7 mg elemental iron. Radioactive fractions obtained from plasma samples after gel filtration were concentrated, spotted on to thin layer plates and developed 1–2 h in methyl ethyl ketone:acetone:water:formic acid (80:6:12:2 by volume). Values are given as d.p.m. of tritiated material eluted from each 1 cm strip. The position of non-radioactive maltol is indicated by the black band and the origin is shown by the arrow head.

Most of the tritium in the gel fractions obtained from the 2 min plasma samples ran with unlabelled maltol just behind the solvent front (Figure 3a). But the tritium contained in the gel fractions from the 60 min plasma sample appeared in a peak near the origin, corresponding to a substance of more hydrophilic nature, most probably a conjugate. Although the nature of this conjugate was not analysed, the identity of the maltol conjugate formed by intestinal tissue was investigated in more detail as described below.

Pharmacokinetics after intraduodenal administration of [^{59}Fe]-ferric [^{3}H]-maltol

The entry of ^{59}Fe and of tritium into the plasma after intraduodenal administration of [^{59}Fe]-ferric [^{3}H]-maltol was followed by measuring the ^{59}Fe and tritium content of plasma samples taken at 5 to 10 min intervals. After the lower dose of ferric maltol (100 μg elemental iron), tritium entered the circulation rapidly, peak values being attained within 10 min whilst ^{59}Fe levels rose slowly reaching a plateau after about 60 min (Figure 4a). After the higher doses of ferric maltol (7 mg elemental iron), tritium levels rose gradually over the course of 60 min whilst the ^{59}Fe content was highest initially and then declined (Figure 4b). At both doses therefore ^{59}Fe and tritium must cross the intestinal wall at different rates. Thin layer analysis of plasma samples taken 5 and 60 min after intraduodenal administration (Figure 3b) indicated that maltol must enter the circulation entirely in the conjugated form, no unchanged maltol being detected. The presence of two radioactive peaks near the origin suggests that two separate conjugates may have been formed.

[^{3}H]-maltol uptake into isolated intestinal fragments

The way in which maltol itself can cross the brush border membranes and enter the intestinal cells was investigated by studying tritium accumulation in isolated fragments of rat small intestine. In the absence of iron, [^{3}H]-maltol appeared to diffuse rapidly into the fragments (Figure 5a) but when presented to the tissues as the ferric maltol complex, [^{3}H]-maltol entry was very much slower and showed saturable kinetics (Figure 5b) similar to those seen previously for iron entry from ferric maltol (Levey *et al.*, 1988). Indeed when entry of both ^{59}Fe and tritium were followed simultaneously, the uptake curves obtained for metal and for ligand were superimposable.

Disposition of ^{59}Fe and tritium in intestinal tissue

The disposition of ^{59}Fe and of tritium within the intestinal fragments after uptake was investigated. This was done by analysing the distribution of radioactivity in pellet and supernatant following centrifugation of homogenates of mucosal scrapings at 100,000 *g* for 1 h. After incubation of isolated fragments for 20 min with 0.05 mM [^{59}Fe]-ferric [^{3}H]-maltol, 99% of the absorbed tritium was localized in the supernatant whilst 60–70% of the ^{59}Fe appeared bound to the membrane pellet.

Metabolism of maltol

The nature of the tritiated material recovered from the supernatant was identified following gel filtration and thin layer chromatography. After incubation of intestinal fragments with 0.1 mM [^{3}H]-maltol, tritium was found in the gel fractions containing material slightly larger than maltol itself. After thin

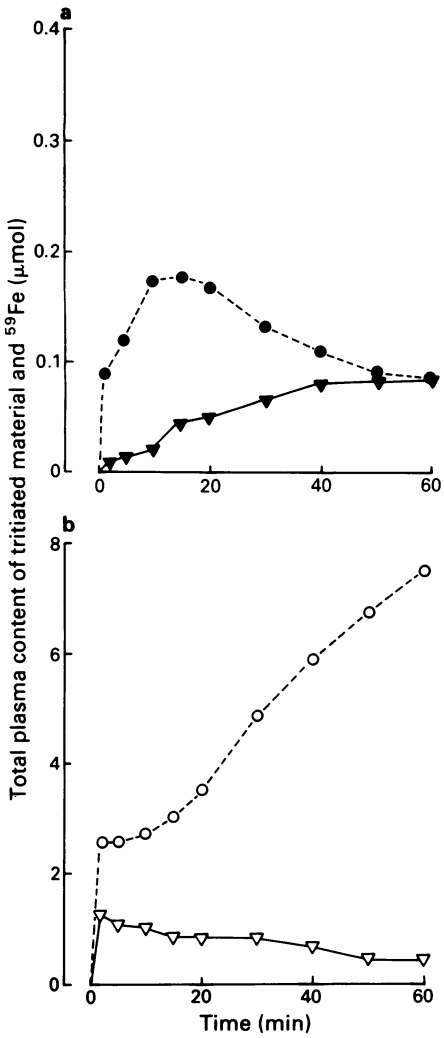

Figure 4 Changes in total plasma content of ^{59}Fe (▼, ▽) and tritium (●, ○) during 1 h following intraduodenal administration of [^{59}Fe]-ferric [^{3}H]-maltol at doses containing (a) 100 μg (closed symbols) and (b) 7 mg (open symbols) of elemental iron. Each value is the mean of data from 3 animals and is shown as μmol of radioactive material in the total plasma.

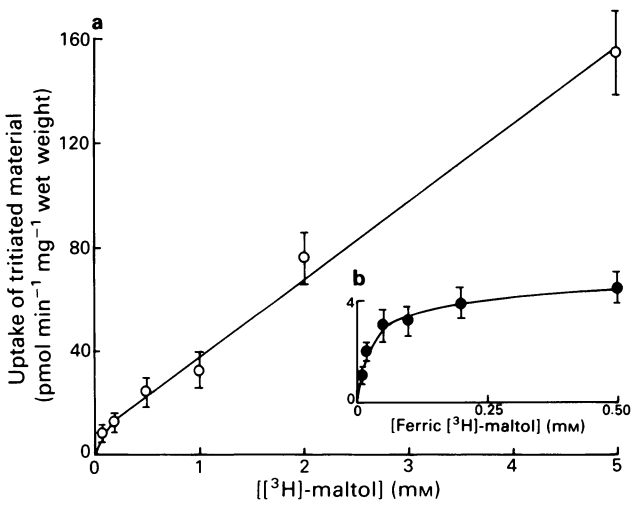

Figure 5 Effect of concentration on initial rates of uptake of tritium into isolated intestinal fragments after incubation with (a) [^{3}H]-maltol (○) or (b) ferric [^{3}H]-maltol (●). Each value represents the mean of data from 3 separate experiments, quadruplicate determinations being obtained from each experiment; s.e.mean shown by vertical bars. Values are given as pmol of tritiated material min^{-1} mg^{-1} wet weight of tissue.

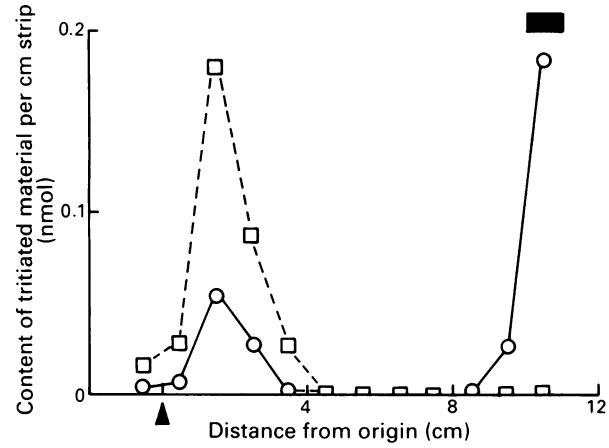

Figure 6 Thin layer chromatographic separation of tritiated material obtained from intestinal mucosa after incubation for 30 min with 0.1 mM [^{3}H]-maltol. Following homogenization and gel filtration, tritiated fractions were concentrated and incubated with (○) or without (□) β-glucuronidase before thin layer separation. Values are shown as nmol of tritiated material eluted from each 1 cm strip. The position of non-radioactive maltol is indicated by the black band and the origin is shown by the arrowhead.

layer separation it was detectable as a single peak of material near the origin, thus more hydrophilic than maltol (Figure 6). After incubation of the gel fractions with β-glucuronidase, the tritiated material then ran on the thin layer plates just behind the solvent front in a position corresponding to the unchanged maltol (Figure 6). The amount of tritiated metabolite converted to maltol varied from experiment to experiment, ranging from 75% to 100%. It is possible that in some cases the incubation time or amount of enzyme available was insufficient for complete conversion.

The proportion of maltol transformed to the conjugate varied with maltol concentration. After incubation of intestinal fragments with 1 mM [^{3}H]-maltol, two tritium peaks could be discerned on the thin layer plates, one corresponding to the hydrophilic metabolite and the other to the unchanged maltol. Uptake and metabolism of maltol over a range of maltol con-

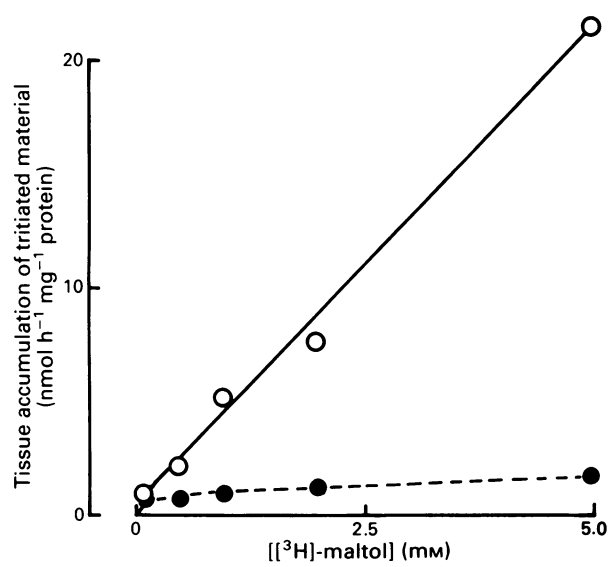

Figure 7 Effect of concentration on the proportion of maltol metabolized during uptake into isolated intestinal fragments. Following incubation at 37°C for 30 min with [^{3}H]-maltol, mucosal scrapings from the fragments were homogenized and the content of total tritium (○) and of tritiated metabolite (●) remaining after chloroform extraction of unchanged maltol analysed. Each value is the mean of triplicate determinations and is given as nmol of tritiated material h^{-1} mg^{-1} protein in the homogenate.

centrations were investigated together in the isolated intestinal fragment, unchanged maltol being separated from its metabolite by chloroform extraction. Conjugation was clearly saturable (Figure 7) so that at the higher maltol concentrations, most of the tritium accumulated by the isolated fragments was still in the form of unchanged maltol. Attempts to study maltol metabolism in intestinal homogenates proved unsuccessful. Although the homogenates retained the ability to conjugate other phenols, metabolism of maltol was not detectable despite the addition of the requisite cofactors (Bock et al., 1983) in the absence of normal subcellular compartmentation.

Discussion

It is apparent from these results that ferric maltol dissociates rapidly within the circulation. The complex is known to be kinetically labile and able to donate the iron rapidly to apotransferrin (Barrand et al., 1987). This dissociation also occurred with doses of iron that must have far exceeded the iron carrying capacity of transferrin in the plasma. It is possible that under these conditions iron then binds to albumin. Generally in the iron overload situation, non-transferrin-bound iron in the plasma is complexed to low molecular weight compounds such as amino acids or to citrate (Grootveld et al., 1989) but to a lesser extent attachment to albumin may also occur (Brissot et al., 1985; Wright et al., 1988). Gel filtration revealed the ^{59}Fe still to be associated with high molecular weight proteins although the identity of these proteins was not resolved. Separation of albumin and transferrin cannot be achieved on the basis of molecular size but requires exploitation of differences in charge and such methodology might well alter the capacity of these two proteins to bind the iron. Although non-transferrin bound iron may enter body tissues by routes different from those involved in transferrin-bound iron uptake (Brissot et al., 1985; Wright et al., 1988), no obvious effects on elimination of ^{59}Fe from the plasma were seen after the intravenous ferric maltol dose containing 1 mg elemental iron.

After dissociation, [^{3}H]-maltol itself seems to remain in the plasma as low molecular weight material, unbound by proteins. Its complicated elimination pattern together with the slight change in gel filtration profiles for tritium seen between 2 min and 60 min after injection into the circulation strongly suggest the presence of more than one species of tritiated material. This was confirmed by thin layer chromatography, which revealed the presence of a metabolite, larger and more hydrophilic in nature than the original maltol and therefore probably a conjugate. Conjugation is a common pathway for metabolism and elimination of phenols (Smith & Williams, 1966). Certainly the tritiated material collected in the urine after 1 h appeared to be entirely in the form of the conjugate. The liver is probably the main site for conjugation of maltol injected directly into the circulation but conjugation can also take place within the small intestines. This was demonstrated by incubating isolated intestinal fragments with [^{3}H]-maltol. Both glucuronide and sulphate conjugation can take place in the rat small intestine, the relative proportions of each depending on the nature of the substrate (Koster, 1985). The conjugate formed from maltol in the intestines appeared to be a glucuronide since enzymic digestion with β-glucuronidase led to recovery of the original [^{3}H]-maltol. Previous studies in dogs identified both glucuronide and sulphate conjugates in the urine after oral dosing with ethyl maltol (Rennhard, 1971). Preliminary data (Hider, unpublished observations) suggests that both forms of conjugate may be produced from maltol and eliminated in the urine in human subjects.

During absorption of ferric maltol from the duodenal lumen, ^{59}Fe and tritium enter the circulation at different rates, the pattern of entry depending on the dose of ferric maltol given. This strongly suggests that dissociation of metal and ligand takes place before reaching the blood. Subcellular fractionation studies indicated that once within the intestinal tissues, iron and maltol become localized to different compartments. The initial uptake of maltol across the intestinal brush border in the presence of iron is a saturable process and similar to the uptake of the iron itself. However, in the absence of iron, maltol can diffuse rapidly into the tissues. This would seem to suggest that iron and maltol pass into the intestinal cells as the complex before dissociation. However, this seems unlikely since previous studies have shown that reduction of the ferric iron to the ferrous state occurs in the intestinal lumen and appears to be a vital step in the uptake of iron from ferric maltol (Barrand et al., 1990). Dissociation of iron and maltol takes place on reduction of the iron in the gut lumen. Thus iron and ligand will be able to enter the tissues by separate pathways, the ferrous iron binding to the endogenous carrier at the cell surface and maltol simply diffusing across the cell membrane. The entry of maltol will still appear to be saturable since the rate of its diffusional entry will be governed by the rate of release of maltol from its complex with iron.

The different rates of entry of ^{59}Fe and of tritium into the circulation from the duodenum with the different doses of ferric maltol may be explained in the following way. At low dose, rapid and extensive dissociation may occur in the gut lumen. Free maltol would then be able to diffuse rapidly into the blood whilst the iron would be passed into the intestinal cells for normal mucosal processing. It has already been established that at low doses of ferric maltol much of the iron absorbed into the intestinal mucosa of iron replete animals becomes associated with ferritin (Callingham & Barrand, 1987; Levey et al., 1988; Barrand & Callingham, 1991). Transfer of iron from the serosal surface to transferrin in the circulation will then be regulated by the demands for iron in the peripheral tissues (Peters et al., 1988) and may well take place as a slow release. At high doses, dissociation of metal and ligand may be very slow, hence the slow entry of maltol into the circulation. Indeed after oral administration with high doses of ferric maltol i.e. those containing 1 or more mg elemental iron, it has been possible to visualize the deep red coloured undissociated ferric maltol complex not only in the stomach but also throughout the length of the small intestine (Barrand & Callingham, 1991). Certainly the rate of ferrous iron formation from ferric maltol decreases at the higher concentrations (Barrand et al., 1990). Iron passing initially into the blood will quickly saturate all binding sites being in much higher absolute amounts, so no further transfer of iron from serosal surface to blood will take place even though dissociation of iron and maltol continue. Presumably the released iron is either precipitated in the gut lumen or sequestered by ferritin in the mucosal cells and eventually shed back into the gut lumen. Either way, it does not gain access to the circulation and so lead to iron overload in peripheral tissues.

By following the fates of metal and ligand independently during absorption of ferric maltol, it has been possible to demonstrate that dissociation of iron and maltol probably due to reductive mechanisms occurs in the gut lumen before uptake of either component. The ligand, maltol, is then free to diffuse across the intestinal wall. Conjugation both within the intestines and in other peripheral tissues renders the hydroxypyrone more hydrophilic and it is rapidly eliminated from the body in the urine. The reduced iron, no longer complexed to maltol, passes into the intestinal mucosa on the endogenous carrier and is processed via normal regulatory mechanisms. Ferric maltol thus provides a way of administering ferric iron to the gut lumen in bioavailable form. Since maltol limits the lipid peroxidation brought about by ferrous iron generation (Singh & Barrand, 1990) ferric maltol acts as a reservoir of soluble iron in a form less likely to produce tissue damage (Slivka et al., 1989). Once reduced, however, the iron is readily available for uptake at the intestinal cell surface.

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