

Studies on the control of iron uptake by rabbit small intestine

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1. Whole-body retention in vivo and uptake of ^{59}Fe -labelled ascorbate and nitrilotriacetate chelates by intestinal slices in vitro were determined in groups of normal control rabbits and rabbits with experimentally-induced Fe deficiency.
2. Over-all absorption as measured by retention of doses of either chelate was greatly increased in conditions of Fe deficiency.
3. Intestinal Fe uptake in vitro was inhibited up to 77% in the presence of 2,4-dinitrophenol and sodium fluoride. Initial rates showed saturation within the concentration range 18–450 $\mu\text{mol/l}$, suggesting that uptake was brought about by an active transport process.
4. When studied at chelate concentrations of 450 $\mu\text{mol/l}$, significant regional differences in uptake rates were observed. Uptake in duodenal slices was increased when compared with slices from jejunum and ileum.
5. Fe uptake from ferric and ferrous chelates was greatly enhanced in Fe deficiency. This was chiefly due to increases in uptake by slices from the duodenum, but uptake into slices of distal intestine was also stimulated.
6. Kinetic analysis of Fe uptake by duodenal slices from animals rendered Fe deficient by diet or repeated bleeding indicated in both groups an increased apparent maximum velocity (V_{max}) for influx of Fe without significant changes in apparent affinity for Fe.
7. The experiments provide further insight into the nature and regional distribution of transport of Fe into the intestine and suggest, in the rabbit, that important control of Fe absorption may be exerted by an active process operating at this initial entry step.

Although it is generally accepted that the small intestine maintains iron homeostasis by regulating absorption of dietary Fe to meet body needs (McCance & Widdowson, 1937), the rate-limiting step in the transfer of Fe from lumen to the portal blood has yet to be defined. *A priori*, control of Fe absorption could be exerted at several loci within the luminal epithelium of the intestinal mucosa: at the brush border membrane; at intracellular sites of Fe transfer or at the level of efflux across the baso-lateral membrane.

Recent experiments employing biopsy specimens of human duodenal mucosa incubated in vitro with radio-labelled ferric chelates indicate that entry of Fe into the absorptive epithelium is a saturable process with features of active transport (Cox & Peters, 1979). Moreover, in conditions of Fe deficiency, initial rates of Fe entry were reversibly stimulated, suggesting, in man, that the uptake step may be important in over-all control of Fe absorption (Cox & Peters, 1980).

In this paper we report further observations in experimental preparations of animal intestine with special reference to modulations of mucosal Fe uptake occurring as adaptive responses to biological stress. The study provides additional evidence, in the rabbit, that control of over-all Fe absorption is exerted at the initial uptake step across the brush border, which may thus be a rate-limiting transport process.

EXPERIMENTAL

Animals

Healthy male New Zealand White Rabbits were obtained from Froxfield Laboratory Animal Breeders, Hants. Animals weighing approximately 2.5 kg were pair-matched for age and weight and caged individually. Weanling animals were used in nutritional studies at 8 weeks of age when they weighed approximately 1 kg.

Induction of Fe deficiency

Haemorrhagic anaemia was induced by twice-weekly venesection of blood (10 ml/kg body-weight) for 4–6 weeks in one partner of eight matched pairs of animals. The animals were maintained on Labsure RAF diet, containing 230 mg Fe/kg.

Nutritional anaemia was induced in the group of weanling animals maintained for 3–4 weeks on a casein-based low-Fe diet containing less than 10 mg/kg Fe (Labsure Animal Foods, PO Box 6, Agrarian House, Castle Street, Poole, Dorset). No further control animals for this sub-group of Fe-deficient rabbits were used.

Tissue criteria of Fe status

Blood (10 ml) was removed immediately before death and analysed for haemoglobin concentration and serum Fe by the methods of Drabkin & Austin (1932) and Peters *et al.* (1956) respectively. Hepatic non-haem Fe was measured in tissue obtained *post mortem* by the method of Weinfeld (1964): it was related to hepatic protein as assayed by the fluorescamine method in the presence of 5 g sodium dodecyl sulphate/l (Van Frank, 1975).

Measurement of whole body iron retention

Whole body retention of ^{59}Fe was determined simultaneously in matched animals given 2 μmol ferrous ascorbate or ferric nitrilotriacetate in 3 ml neutral saline (9 g sodium chloride/l) by gavage after an overnight fast. The value for Fe:chelate concentration was 1:20 and 1:2 for ferrous ascorbate and ferric nitrilotriacetate respectively. Radioactivity was measured in a twin-crystal small-animal body gamma counter and the emission was determined in four positions to correct for geometrical variation. The radioactivity from the animals was compared with a known standard at 1 h and periodically for 14 d after administration of the dose. The counting error was set to less than 1%; the coefficient of variation due to geometrical factors was generally less than 5%.

Determination of intestinal Fe uptake

Pairs of matched animals were killed after an overnight fast by injecting 30 ml air rapidly into the circulation. The abdomen was opened immediately and the small intestine removed, flushed with ice-cold neutral saline and placed in incubation buffer. Appropriate segments of intestine were gently everted and full-thickness crescentic slices, weighing 5–15 mg, were cut from the duodenum (50 mm distal to the pylorus); jejunum (600 mm distal to the pylorus); or ileum (200 mm proximal to ileo-caecal valve).

Uptake of ^{59}Fe (II) ascorbate or ^{59}Fe (III) nitrilotriacetate into duplicate slices was determined essentially as described previously (Cox & Peters, 1979). Fe chelates were prepared fresh by adding tracer $^{59}\text{FeCl}_3$ (IFSI; Radiochemical Centre, Bucks.) to 50 μl 1 M-sodium ascorbate or a similar volume of 0.1 M-sodium nitrilotriacetate at pH 3. Maximum impurity of contaminating non-radioactive ferrous iron was 0.5 $\mu\text{mol/l}$; it was not determined in the ferric chelates. Sufficient carrier-Fe as FeCl_3 in 0.1 M-hydrochloric acid (5–15 μmol) was added to give a specific radioactivity of 5–100 $\mu\text{Ci}/\mu\text{g}$ atom Fe. The chelate was diluted with 5 ml warm buffer (20 mM-Hepes, 130 mM-NaCl, 3.5 mM-potassium chloride, 10 mM-D-glucose, 1 mM-calcium chloride, pH 7.35) immediately before incubations were started. The chelate solutions were stable for at least 1 h: more than 98.5% passed through 0.22 μ millipore filters; the radioactivity remained dialysable and, in the instance of the ferrous chelates, assay with o-phenanthroline reagent indicated no appreciable oxidation to the ferric form. ^{57}Co cyanocobalamin (CT_{12} ; Radiochemical Centre) was also present as a non-absorbable marker of extra-cellular fluid at a final concentration of 10 $\mu\text{g/ml}$ and specific radioactivity 30 $\mu\text{Ci}/\mu\text{mol}$. The incubations, at 37°, were vigorously

Table 1. *Body-weights and iron status in experimental groups of rabbits*
(Mean values with their standard errors)

| Group† | n | Wt (kg) | | Haemoglobin concentration (g/l) | | Serum Fe $\mu\text{mol/l}$ | | Liver non-haem Fe (ng atom/mg protein) | |
|---------------------|---|---------|------|---------------------------------|----|----------------------------|-----|--|----|
| | | Mean | SE | Mean | SE | Mean | SE | Mean | SE |
| Normal controls | 8 | 3.22 | 0.15 | 141 | 3 | 58.4 | 6.6 | 156 | 59 |
| Chronic haemorrhage | 8 | 3.13 | 0.13 | 89** | 4 | 25.9** | 4.1 | 43** | 7 |
| Low-Fe diet | 3 | 1.82* | 0.14 | 94* | 7 | 10.0* | 3.7 | 11* | 3 |

Mean values were statistically significantly different from control values: * $P < 0.05$, ** $P < 0.01$.

† For details, see p. 252.

bubbled with pure oxygen and stopped as previously described: uptake was expressed as pg atoms Fe/unit weight of intestine and in kinetic experiments as pg atoms/min per mg after 5 min incubation.

Statistical analysis

Differences between groups were assessed by *t* tests for paired or unpaired observations. The significance of differences between groups where several stratified or matched sets of measurements were available was assessed by analysis of variance.

RESULTS

Fe status of experimental groups

The values are shown in Table 1. Controlled haemorrhage induced sideropenic anaemia which was maintained in spite of weight gains not significantly different from control animals. As shown, both haemorrhagic and nutritional sideropenic anaemia in the rabbits were associated with significantly reduced Fe stores in liver, when compared with control animals. As expected, the animals with Fe deficiency induced by diet gained little weight during the experimental period; the matched animals remained within 200 g of the mean weight during the period of controlled haemorrhage, and were not significantly different from matched controls.

Whole body Fe retention

Over-all absorption of the doses of ferrous and ferric chelates was judged in vivo by the measurement of Fe retention in control and bled rabbits (Fig. 1).

In normal control animals mean retention after 14 d of the dose of ferrous Fe was 1.5% of the dose and of the ferric dose 3.9% of the dose. Whole body retentions in the two matched pairs of bled animals were 28.2 and 17.8% of the dose of ferrous and ferric chelates. The results confirmed that absorption of chelated Fe in vivo was stimulated several fold in conditions of haemorrhagic Fe deficiency ($P < 0.01$).

Time course of intestinal Fe uptake and effects of metabolic poisons

As shown in Fig. 2, uptake of radio Fe by full-thickness slices continues incrementally over a prolonged incubation period in vitro, confirming previous observations with mucosal biopsy specimens of human duodenum (Cox & Peters, 1979). Also displayed are the effects of metabolic poisons on Fe uptake: addition of fluoride and 2,4-dinitrophenol to this incubation mix progressively inhibited radio Fe influx. The uptake rate was reduced by 77% over the 30 min incubation period ($P < 0.05$). Comparable effects were observed on reducing the incubation temperature to 10°.

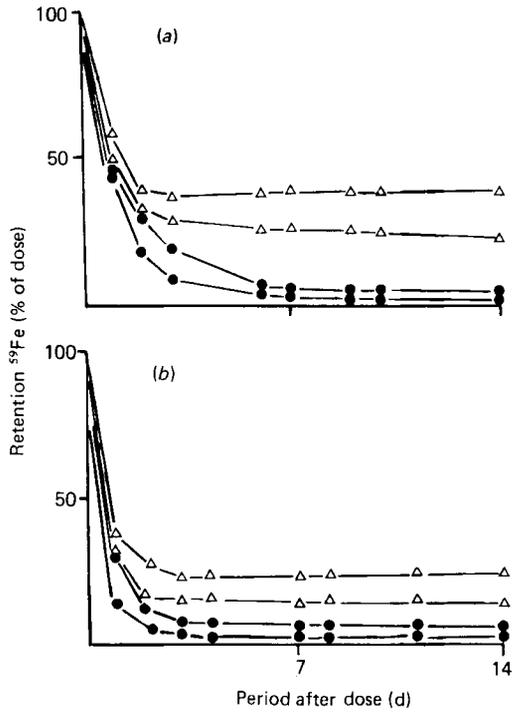


Fig. 1. Whole-body iron retention (% of dose) in normal control (●) and Fe-deficient (△) rabbits given intragastric doses of $2 \mu\text{mol}$ ferrous ascorbate (a) or ferric nitrilotriacetate (b). Each point represents a single determination on one animal; the difference between values for the control and chronically-bled animals was statistically significant ($P < 0.01$).

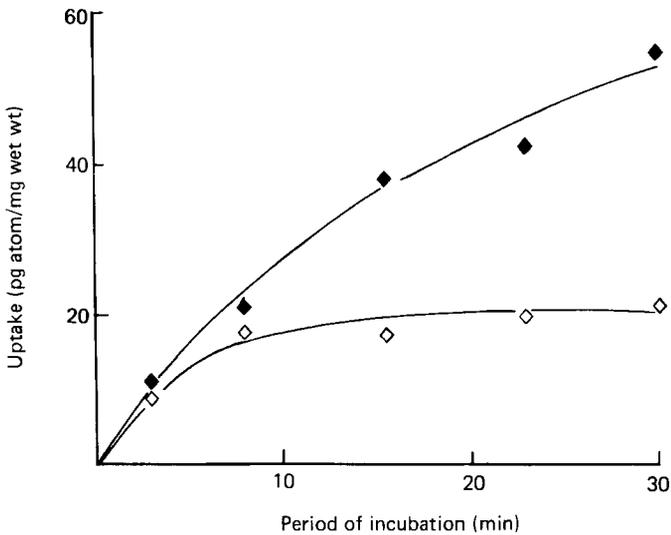


Fig. 2. Time-course of ^{59}Fe uptake (pg atom/mg wet weight) by proximal intestinal slices from a normal control animal incubated in the presence of ferrous ascorbate at a concentration of $25 \mu\text{mol/l}$ (●). Uptake in the presence of 0.1 mM -2,4-dinitrophenol and 10 mM -sodium fluoride is shown (◇). Each point represents the mean of duplicate determinations and the difference between values for the two treatments was statistically significant ($P < 0.05$).

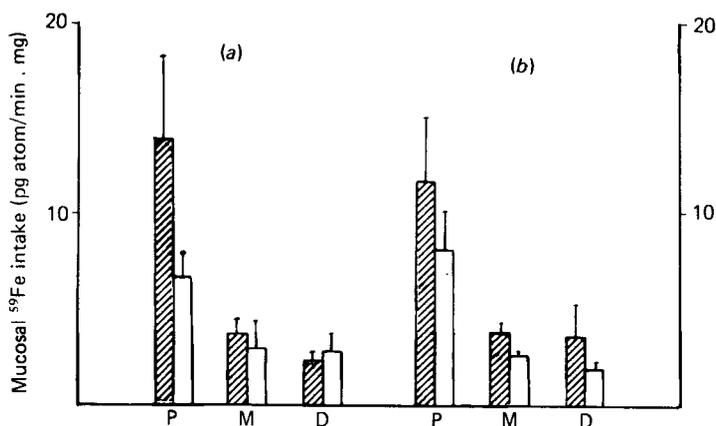


Fig. 3. Effect of location on mucosal ^{59}Fe uptake from $450\ \mu\text{mol/l}$ ferrous ascorbate (a) and $450\ \mu\text{mol/l}$ ferric nitrilotriacetate (b). Intestinal slices from proximal duodenum (P), mid-jejunum (M) and distal ileum (D) were incubated for 5 min (for details, see p. 252). (▨), Slices obtained from Fe-deficient animals; (□), obtained identically from normal control animals. Values are means with their standard errors for six paired determinations in duodenum and three paired determinations in distal intestine represented by vertical bars.

Effects on location on intestinal Fe uptake

Influx of Fe into intestinal slices obtained from distal intestine was substantially reduced when compared with slices from the duodenum (Fig. 3). This regional gradation was observed in the paired experiments employing both the ferrous and ferric chelates and was significant at $P < 0.01$. In matched experiments employing intestinal slices from rabbits rendered chronically Fe deficient by controlled bleeding, influx of Fe was significantly enhanced but this effect was greatest in the proximal segments ($P < 0.02$). Influx of ferric Fe into slices of ileum was also stimulated in Fe deficiency; this effect was only evident in experiments employing ferric nitrilotriacetate.

Kinetic studies of Fe uptake in conditions of Fe deficiency

In order to examine further the nature of the modulations by Fe transport induced by conditions of body Fe deficiency, kinetic studies of uptake velocity over a range of medium concentrations of ferrous ascorbate were undertaken. Kinetic studies in normal control rabbits, chronically-bled animals, and a group rendered Fe-deficient by feeding a low-Fe diet are shown in Fig. 4. Duodenal uptake velocities in both groups of Fe-deficient animals were significantly enhanced when compared with duodenal slices from normal control animals. As previously shown in studies with mucosal biopsy specimens of human duodenum, initial Fe uptake velocities showed evidence of saturation within the range of concentrations used ($18\text{--}450\ \mu\text{mol/l}$). Kinetic constants, calculated by linear regression of individual experiments from Eadie-Hofstee plots (Hofstee, 1957) are set out in Table 2. Both haemorrhagic and nutritional Fe deficiency induced comparable increases in the apparent maximum transport capacity for Fe influx with insignificant alterations in the apparent affinity constant (K_t) for transport.

DISCUSSION

Fe absorption in mammals is regulated by the intestine and has long been known to increase adaptively in response to biological stresses including haemorrhage, nutritional Fe deficiency and pregnancy. However, the intestinal control mechanisms are incompletely understood

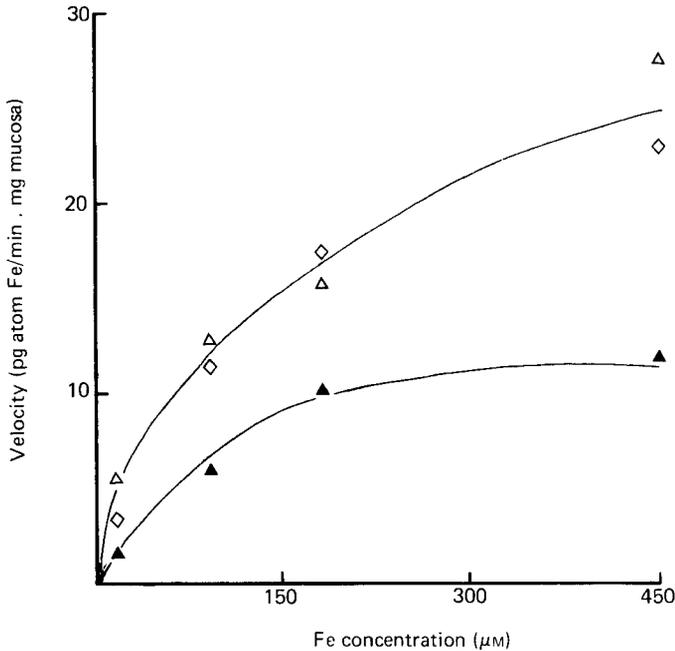


Fig. 4. Intestinal iron uptake kinetics. Uptake velocity (pg atom Fe/min per mg mucosa) over a range of concentrations of ^{59}Fe ascorbate was determined in slices of proximal intestine from normal control animals (▲) and pair-matched animals rendered Fe-deficient by controlled haemorrhage (△). Uptake kinetics in a group of nutritionally-Fe deficient rabbits are also shown (◇). In normal and chronically-bled animals, each point represents the mean of duplicate determinations for five animals ($P < 0.01$); in the nutritionally-Fe-deficient group, each point represents the mean of duplicate determinations in three animals. Mean values were statistically significantly different from control values: for chronically-bled animals $P < 0.01$; for nutritionally-Fe-deficient animals $P < 0.05$.

Table 2. Kinetic constants of duodenal Fe uptake in iron-deficient rabbits
(Mean values with their standard errors)

| Group† | n | V_{\max} (pg atom/min per mg) | | K_t ($\mu\text{mol/l}$) | |
|---------------------------------|---|------------------------------------|-----|--------------------------------|----|
| | | Mean | SE | Mean | SE |
| Normal controls | 5 | 16.1 | 3.4 | 138 | 16 |
| Chronic haemorrhagic anaemia | 5 | 27.2* | 4.8 | 98 | 17 |
| Low-Fe diet nutritional anaemia | 3 | 25.3 | 7.1 | 110 | 8 |

V_{\max} , maximum transport capacity; K_t , apparent affinity constant for iron transport.

* Mean values were statistically significantly different from control values, $P < 0.025$.

† For details, see experimental section (p. 252).

(Forth & Rummel, 1973). Moreover, variations in Fe handling by different species of experimental animal as well as wide differences in methodology, especially in regard to measurement of absorption, have added confusion to the available literature (Cox, 1979).

In the present study attention has been focused on the regulatory significance of the initial step in the over-all absorption of Fe, namely influx across the luminal surface of the mucosa. The use of a freely-diffusible non-absorbable marker and incubation periods short enough to avoid intra-cellular accumulation with reflux of radio Fe into the medium, allows valid

measurements of initial uptake velocities of this probe to be made (Sallee *et al.* 1972; Cox & Peters, 1979). The use of divalent instead of trivalent Fe chelates additionally avoids binding of Fe to plasma transferrin entrapped within interstices at the cut surfaces of the intestinal slices: it is remotely possible that such binding could contribute to apparent uptake values in the presence of nitrilotriacetate chelates.

In the rabbit duodenum Fe uptake was inhibited powerfully by the addition of metabolic poisons to the incubation medium and, as in human duodenal mucosa, initial velocities showed evidence of saturation within a physiological range of Fe concentration. These properties are features shared by active transport processes where carrier-mediated fluxes are dependent on metabolically-derived electrochemical gradients. By analogy with regulation of biochemical pathways, such a transport process would be expected to be amenable to cellular control mechanisms, especially in view of its primary location in the absorptive sequence.

Over-all absorption and intestinal uptake of Fe presented as stable ferrous or ferric chelates were comparable, suggesting, in agreement with the experiments of Forth *et al.* (1965), that the biological availability of these redox states may be related to aqueous solubility and kinetic lability of individual complexes, rather than intrinsic atomic differences. Furthermore, the gradation of Fe uptake in the more distal segments of intestine was observed with both chelates. Absorption and uptake of both chelates were also stimulated significantly in conditions of haemorrhagic Fe deficiency.

In experimental Fe deficiency initial uptake velocities for Fe were markedly stimulated and the kinetic analyses indicated an enhanced V_{\max} for transport with insignificant alterations in apparent affinity, or K_t , for Fe. These kinetic perturbations suggest that the intestinal Fe transport is stimulated by increased carrier activity rather than changes in carrier affinity.

Several carrier moieties for Fe have been localized to the mucosal epithelium, and the mucosal transferrin purified from epithelial cytosol in rodent intestine is an example of a transport protein whose synthesis is stimulated in conditions of Fe deficiency (see Hübers *et al.* 1976). Notwithstanding the existence of cytosolic Fe transport proteins, we have recently provided evidence that enhanced transfer of Fe across the intestinal brush border is mediated in the rabbit by transport moieties within the microvillus membrane. We have shown that Fe binding sites in membranes purified from Fe-deficient animals were reversibly increased in number but that affinity for Fe was not altered when compared with membranes from normal control rabbits (Cox & O'Donnell, 1980). The kinetic changes in membrane Fe binding were comparable to those observed in Fe transport into intestinal slices, and suggest that the two processes are linked functionally.

Many previous studies of intestinal Fe uptake neither examined authentic initial rates nor corrected for adherent, unabsorbed fluid. Acheson & Schultz (1972) were the first to measure instantaneous rates of Fe influx into segments of rabbit duodenum: they demonstrated an enhanced influx in rabbits made Fe deficient by bleeding and used 1 mmol ferrous ascorbate/l. The increased Fe influx was restored to normal on repleting the animals with Fe parenterally. The present experiments confirm and extend these findings. Both nutritional and haemorrhagic anaemia induced comparable increases in influx of ferrous and ferric chelates and kinetic analysis revealed that this was related to stimulation of V_{\max} for uptake, as had been previously observed *in vitro* in specimens of human duodenal mucosa (Cox & Peters, 1980). In contrast, similar experiments employing everted gut segments and isolated epithelial cells from rat intestine failed to show any evidence for increased Fe influx rates in conditions of Fe deficiency, or any evidence of active transport (Sheehan, 1976; Savin & Cook, 1978). Furthermore, Fe uptake by brush borders isolated from proximal rat intestine was not enhanced in preparations from Fe-deficient animals

(Greenberger *et al.* 1969). The results strongly suggest that species differences account for these inconsistencies. Our observations in the rabbit support the view that Fe absorption may be controlled by regulation of the initial uptake of Fe by the intestine and is related to the activity of Fe-binding sites in the brush-border membrane. Fe deficiency would appear to be associated with an extension of the uptake capacity for Fe along the intestine and this, combined with enhanced transport per unit of intestine, would greatly increase over-all uptake and absorption.

Fe deficiency induced by haemorrhage or low-Fe diet represents a profound biological stress and rapidly stimulates compensatory responses in the intestine. In spite of this, the mechanism of Fe deficiency in the two circumstances is quite different: bleeding removes body Fe without affecting the availability of dietary Fe in the lumen; nutritional deficiency merely reduces the availability of lumen Fe below the concentrations necessary for absorption to match requirements. Irrespective of the stimulus, the changes in intestinal Fe uptake are the same: the capacity of the uptake process is increased without material changes in affinity. It is remotely possible that, owing to differences in formulation, other dietary factors in the nutritionally Fe-deficient group may have modified Fe uptake. Although these effects cannot be ruled out, the similarities of uptake response in the two groups of anaemic animals make such a possibility unlikely.

As has been pointed out in the instance of human nutrition (Cox, 1980) enhanced carrier capacity under conditions of reduced bioavailability may only result in modest enhancement of over-all Fe absorption since at low luminal concentrations full exploitation of the increased capacity of the intestine could not occur. The behaviour of the rabbit intestine *in vitro* appears to match that of human duodenal mucosa closely, and, if parallel behaviour can be assumed *in vivo*, these considerations would partially account for the prevalence of Fe deficiency in human populations occurring in spite of intestinal compensatory mechanisms.

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