Modulation of duodenal iron uptake by hypoxia and fasting in the rat

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(*Received 6 November 1995 – Revised 11 June 1996 – Accepted 14 June 1996*)

The effect of hypoxic exposure on *in vitro* duodenal Fe uptake kinetics was studied in tissue fragments from rats that were fed or fasted overnight before study. Hypoxic exposure was for 3 d at 0·5 atm and fasting was for the last 18–24 h before Fe uptake determinations. The non-permeable Fe^{2+} chelator 3-(2-pyridyl)-5,6-bis-(4-phenyl-sulphonic acid)-1,2,4-triazine (ferrozine), and medium deoxygenation inhibited uptake in all experimental groups. Ferrozine sensitivity and mucosal Fe^{3+} reductase activity were greatest in hypoxic animals. Fe uptake was inhibited by membrane depolarization only after fasting or hypoxic exposure of the rats. The data demonstrated that Fe uptake by rat duodenal fragments involves at least two mechanisms: a membrane-potential-independent mechanism which is not responsive to hypoxia and a second mechanism, induced by fasting or hypoxia, which is inhibited by membrane depolarization. Uptake is partially dependent on reduction of Fe^{3+} to Fe^{2+} and this is primarily associated with the second mechanism for uptake. These properties have been reported also in mouse and human Fe uptake, suggesting that the rat is a useful model for the study of basic mechanisms of Fe absorption.

Iron: Intestine: Membrane potential

Transport studies with *in vitro* model systems have been of key importance in the elucidation of the mechanisms of intestinal absorption of nutrients, for example, glucose (Bihler & Crane, 1962). We have developed and validated for Fe-uptake studies the *in vitro*-incubated intestinal fragment system (Cox & Peters, 1979; Raja *et al.* 1987*b*). This system is of particular value in the study of the initial uptake step for Fe absorption, which probably represents the key site for the interaction of mucosal and dietary factors for the determination of Fe absorption. Determination of mucosal Fe uptake kinetics by incubating intestinal fragments *in vitro* has been applied extensively to mice (Raja *et al.* 1987*a*) and human subjects (Cox & Peters, 1979, 1980). Such studies have led to hypotheses concerning the defect in human haemochromatosis (Cox & Peters, 1980), a membrane-potential dependence of mouse duodenal Fe uptake (Raja *et al.* 1989) and a requirement for reduction of Fe^{3+} to Fe^{2+} before uptake by mouse (Raja *et al.* 1992) and human duodenum (Raja *et al.* 1996). Recently, attempts have been made to extend the membrane potential and reduction hypotheses to the rat (O'Riordan *et al.* 1994; Wein & Van Campen, 1994), the most-frequently-used small-animal model for Fe nutrition studies. It is of key interest to test whether the duodenal uptake of Fe by this species shows similar properties to those observed in the mouse and human subjects. In the present study we have investigated the interaction between modulators of Fe absorption, i.e. chronic hypoxia and fasting, in

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determining Fe uptake. This was achieved by exposing rats to normoxic or hypoxic conditions for 3 d with or without feeding on the last day. The role of reduction and membrane potential in the uptake process was studied in rats subjected to these various treatments.

MATERIALS AND METHODS

Animals

Male Wistar rats (*n* 180) were obtained from an out-bred colony maintained in the Comparative Biology Unit at King's College School of Medicine and Dentistry. They were maintained on a common chow (Diet CRM; supplied by Special Diet Services, Witham, Essex) from weaning. This chow contained 106 mg Fe/kg diet (manufacturer's data) and was provided, together with tap water *ad libitum* throughout the study, except as indicated below. The procedure was approved by the UK Home Office. At the start of each procedure animals were 6–8 weeks old and weighed 250 (SE 10) g. Rats were housed in plastic and stainless-steel grid-bottomed cages (two to three per cage). Rats were weighed at the time of receipt and the time of killing. Rats were exposed to a normal cycle of 12 h light and 12 h darkness in a temperature-controlled room held at 21°. All animals were killed within 1 week of receipt. Two types of experiment were performed. In one experiment, the effect of hypoxia and fasting on a number of variables, i.e. Fe uptake with or without membrane depolarization, Fe-uptake kinetics, mucosal free fatty acid levels, mucosal reductase activity or Fe uptake in the presence or absence in the medium of ferrozine was determined. This required several studies as only one or two variables could be determined in a single study. This was achieved by randomly assigning rats to four groups of four to eight animals, i.e. normoxic fed (control), normoxic fasted, hypoxic fed and hypoxic fasted. The latter two groups were exposed to chronic hypoxia by placing animals in a hypobaric chamber set at 0·5 atm (53·3 kPa) for 3 d. Such treatment simulates an altitude of approximately 5000 m above sea level and has been shown to stimulate Fe absorption in rats (Osterloh *et al.* 1987). The two fasted groups were allowed water but denied food for 18–24 h before killing. At the end of the treatments animals were killed in a randomized order and used for studying *in vitro* Fe uptake or mucosal free fatty acid levels as described on pp. 461–463. The number of animals used for each treatment in a given study is detailed with the results. The second type of experiment involved varying the time of hypoxic exposure. Batches of up to eight rats were placed in the hypobaric chamber and groups of two to three rats were removed after various times of exposure. Normoxic controls were studied in parallel. Up to four cages (eight rats) were housed in the hypobaric chamber at any one time, including both fed and fasted animals. The hypobaric chamber was not large enough to house metabolic cages, thus food spillage is included in values for food consumption. Food was generally absent from the duodenum of all animals at the time of death. Fasted animals had little or no food in the stomach or small intestine. Fed animals generally did not eat after daylight on the day of study; however they still had food in their stomach at the time of killing.

Materials

Reagents were purchased at the highest appropriate grade from Merck Ltd (Lutterworth, Leics.) except L-ascorbate, EDTA, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), nitrilotriacetic acid (NTA), 2,2′,2″-nitrilotriethanol (triethanolamine), palmitic acid, phloridzin and 3-(2-pyridyl)-5,6-bis-(4-phenyl-sulphonic acid)-1,2,4-triazine (ferro-

zine) which were obtained from Sigma Chemical Company Ltd (Poole, Dorset); Metophane (methoxyfluorane) from C-Vet (Bury St Edmunds, Suffolk); Ecolume scintillation fluid from ICN Biomedicals Inc., (High Wycombe, Bucks.); Lumatom tissue solubilizer from Hans Kurner Analysen Technik (Brude-Grimm-Strasse, 6 Postfach, Germany); cyanocobalamin (Cytamen injection BP) from Duncan Flockhart and Co. Ltd (Uxbridge, Middlesex) and 1,5-diphenyl carbazide which was obtained from Aldrich Chemical Company Ltd (Gillingham, Kent).

^{59}Fe as $FeCl_3$ was supplied by NEN-Dupont (Stevenage, Herts.) or Amersham International Plc (Aylesbury, Bucks.); 0·5 mCi of NEN-Dupont ^{59}Fe (5–75 Ci/g) was supplied in 100 μl 0·5 M-HCl and was diluted 1 : 4 (v/v) with 10 mM-HCl immediately on delivery while ^{59}Fe (3–50 Ci/g from Amersham International Plc) was supplied in 1 ml 0·1 M-HCl. Before use 100 μCi of either stock was diluted to a volume of 0·5 ml with 10 mM-HCl. [^{57}Co]cyanocobalamin was supplied by Amerlite Diagnostics Services Ltd (Amersham, Bucks.) in 10 μCi portions (0·014–0·027 mCi/nmol; 0·5–1·0 MBq/nmol) in benzyl alcohol (9 ml/l). ^{51}Cr EDTA was supplied by Amersham International Plc (Health Care Division) as a 1 : 1 complex of EDTA with chromic ion in an aqueous solution containing benzyl alcohol (10 ml/l; 1–2 mCi/mg). Methyl (α-D-[U-^{14}C] gluco)-pyranoside (50–200 mCi/mmol) was supplied by Amersham International Plc as a solution in aqueous ethanol (30 ml/l).

^{59}Fe uptake by duodenal fragments

The *in vitro* method for measuring ^{59}Fe uptake by intestinal fragments was based on that described by Cox & Peters (1979) and Raja *et al.* (1987*b*). Rats were anaesthetized with Metophane and bled by cardiac puncture. A portion (5 μl) of non-heparinized blood was immediately taken into 1 ml Drabkin's reagent for measurement of haemoglobin levels (see p. 462).

Rats were then killed by cervical dislocation and approximately 20 mm of the duodenum, immediately distal to the bile duct, was removed. The piece of intestine was opened longitudinally and then cut into small pieces of 3–15 mg wet weight. The fragments (up to twenty per animal) were washed by gentle vortex-mixing in HEPES medium, at room temperature (to remove any adhering food particles and mucus) and then pre-incubated for approximately 1 min at 37° in HEPES medium containing 5 nM-cyanocobalamin. The fragments were then transferred to a second tube containing a medium with ^{59}Fe and ^{57}Co at 37°. Fe uptake was terminated after 5 min by lightly blotting the pieces of gut and vortexing them for 3s in ice-cold HEPES medium. This washing time has been shown by Raja *et al.* (1987*a*) to remove non-specifically-bound radioactivity. The wet weight of the fragments was recorded before counting individual fragments for ^{59}Fe and ^{57}Co, simultaneously, for 1 min in a gamma-counter (LKB-Wallac 1282 Compugamma; LKB-Wallac, Helsinki, Finland). Within-animal CV values were found to be in the range $< 25\%$, while between-animal CV values were generally $> 25\%$. Uptake values for fragments from a given animal (four to six fragments for given treatment and incubation conditions) were, therefore, averaged and the average taken as the uptake value; *n* values refer to the number of these averaged values.

In experiments where the ferri-reductase activity of the gut fragments was investigated, the fragments were transferred from the pre-incubation medium into the radioactive medium together with 100 mM-ferrozine (100 : 1, v/v). A 100 μl portion of the medium was immediately taken (*t* 0 min) and added to 400 μl deionized water. Further portions were taken at 1, 2, 3, and 5 min. After 5 min incubation the fragments were blotted, rinsed,

weighed and counted. As ferrozine forms a purple complex with Fe^{2+}, the rate of Fe^{3+} reduction can be determined by spectrophotometric measurement (562 nm) of colour formation in the incubation media. After measuring the absorbance of the diluted 100 μl portions of incubation medium, the Fe in these portions was fully reduced by addition of excess ascorbic acid and the absorbance measured. This measurement provided a standard with which to relate the absorbance in the portions of medium to the amount of Fe reduced.

^{59}Fe uptake by gut sacs

To determine the effective contributions to overall fragment uptake by the mucosal and serosal surfaces, the uptake of Fe^{3+} by 'right-side out' and everted gut sacs was investigated. After anaesthetization of animals with Metophane and subsequent cervical dislocation, approximately 100 mm of the duodenum (immediately distal to the bile duct) was removed, rinsed through with 100 ml HEPES medium and divided into two pieces. One of these was everted over a plastic rod. The location of the portion of gut taken for evertion was alternated between sequential replicates within experimental groups. Both pieces were then filled with HEPES medium (to prevent the sacs floating) and each tied-off with surgical thread into two closed sacs, which were then separated from each other using a scalpel. The everted sacs were well rinsed and blotted to remove mucus. The four sacs were then pre-incubated in oxygenated HEPES medium for approximately 1 min at 37°. After this time interval they were incubated for 5 min in 5 ml oxygenated radioactive medium at 37°. Sacs were removed from the incubation media, blotted and thoroughly rinsed with ice-cold HEPES medium. The sacs were blotted again and the ends cut off, inside the ligatures, in order to prevent counting of radioactivity adsorbed to the surgical thread. 'Right-side out' sacs were cut open and the inside thoroughly blotted to remove mucus, ensuring that wet weights were comparable with those of the everted sacs. Sacs were weighed and ^{59}Fe and ^{57}Co counted for 1 min using a LKB-Wallac 1282 Compugamma gamma-counter.

Na^+-dependent glucose uptake by fragments

Duodenal fragments were prepared as described previously and then up to twenty were pre-incubated in oxygenated HEPES medium at 37° for approximately 1 min before placing them (four to six fragments from a single animal in a given incubation medium) in HEPES medium containing 50 mM-methyl-α-glucopyranoside; the pH was adjusted with 1 M-Tris to 7·3. [^{14}C]Methyl glucopyranoside (1·8–2·2 mCi/ml) was added in trace amounts and ^{51}Cr EDTA (0·8–1·2 mCi/ml) was used as an extracellular-fluid marker. For the Na^+-free medium, NaCl was replaced with choline chloride, and for the phloridzin-containing medium the inhibitor was included at 1 mM. After incubation for 5 min the fragments were removed, blotted and rinsed. After weighing and ^{51}Cr counting, the fragments were placed in β-counting vials with 200 μl Lumatom tissue solubilizer. The vials were left overnight to allow complete digestion of the tissue before addition of 5 ml Ecolume scintillant, then counted on a LKB-Wallac 1214 Rackbeta Spectral liquid scintillation counter.

Biochemical assays

Haemoglobin was determined by adding a 5 μl portion of freshly collected, non-heparinized, blood to 1 ml Drabkin's solution (Drabkin & Austin, 1932), mixing and recording absorption at 540 nm.

For the purpose of performing precise kinetic determinations, the Fe concentration of radioactive stocks was determined spectrophotometrically using ferrozine. The procedure used was adapted from the colour-reaction step in the non-haem-Fe assay of Foy *et al.* (1967). Standards contained 1 and 2 nmol $FeCl_3$.

Mucosal free fatty acid levels

Animals were anaesthetized and then killed by cervical dislocation. Approximately 100 mm of the duodenum distal to the bile duct was removed and immediately rinsed through with 50 ml ice-cold 0·15 M-NaCl. This has been shown to stop effectively the formation of free fatty acids in the isolated tissue (Simpson *et al.* 1989). All instruments and equipment that came in contact with tissue thereafter were ice-cold. The gut was cut open and the mucosa scraped off with a microscope slide. The serosa was discarded and the mucosa homogenized in 5 ml ice-cold HEPES medium in a Dounce homogenizer. A 50 μl portion of the homogenate was transferred to a stoppered glass tube containing 6 ml chloroform–heptane (1 : 1, v/v) with methanol (20 ml/l) and 1 ml KH_2PO_4 (33 mM)–Na_2HPO_4 (33 mM; 2 : 1, v/v) and shaken for 90 s. The remaining homogenate was transferred to a 30 ml vial and incubated at 37° for up to 120 min. At intervals, further portions were taken into organic mix. Free fatty acid concentrations were determined in the extracts as described by Falholt *et al.* (1973). A standard curve was constructed from palmitic acid over the range 0–2 mM.

Data analysis and statistical methods

All data from investigations of the concentration dependence of Fe^{3+} uptake were fitted to the Michaelis–Menten equation for a single-binding site using the direct linear plot method of Eisenthal & Cornish-Bowden (1974). Two types of statistical analysis were performed on the data. Where only one comparison was involved (i.e. between one control and one test group) the Student's *t* test was used (paired or unpaired as appropriate). In cases where multiple comparisons were to be made, two-way or three-way ANOVA was performed using the general linear model (Minitab Inc., 1992). Where appropriate, multiple comparison of means was performed, following ANOVA, by the Student–Newman–Keuls test for all pairwise comparisons (Glantz & Slinker, 1990). The threshold for significance was set at $P < 0.05$.

RESULTS

Tissue viability

Viability of duodenal fragments was demonstrated by uptake of the glucose analogue [^{14}C]methyl-α-glucopyranoside. This uptake was significantly inhibited by replacement of NaCl with choline chloride (44% inhibition; $P < 0.001$) and by the inclusion of 1 mM-phloridzin in the incubation medium (98% inhibition; $P < 0.001$). These results demonstrate the uptake of methyl-glucopyranoside by the Na^+-dependent glucose transporter, thus indicating the functional viability of fragments. In addition, examination of gut morphology after 5 min incubation in 450 μM-Fe^{3+}–NTA (1 : 2, v/v) at 37° showed that rat duodenal tissue remains intact under these conditions. These results agree with those of Raja *et al.* (1987*b*, 1989) and, hence, there is good evidence to suggest that duodenal fragments from the rat are functionally viable for the duration of the experiments presented here.

^{59}Fe uptake by duodenal fragments

There was a good correlation between fragment weight and $^{59}Fe^{3+}$ uptake (r 0·95) and between weight and [^{57}Co]cyanocobalamin (extracellular-fluid marker) uptake (r 0·93). However, ^{59}Fe was associated with fragments in excess of [^{57}Co]cyanocobalamin and this indicates that the association is not entirely due to the extracellular-fluid space. The uptake of ^{59}Fe, after correction for the extracellular fluid and extrapolation to zero fragment size, was not significantly different from zero. This indicates that the cut surfaces of the fragments did not contribute significantly to the uptake of ^{59}Fe.

Everted and 'right-side out' gut sacs were used to investigate the contribution, to total fragment uptake, of uptake through the serosal surface. The combined uptake via the serosal and mucosal surfaces in these sac experiments approximated to the uptake values (pmol/mg wet weight per min) seen in fragments from fed (n 13; sacs 5·04 (SE 0·24) v. fragments 7·5 (SE 3·3)) and for fasted animals (n 5; sacs 6·21 (SE 0·61) v. fragments 9.3 (SE 4·2)). The data show that the total uptake increased significantly ($P < 0·05$) in fasted animals. This increase was accompanied by a marginally significant increase ($P = 0·0553$) in mucosal uptake (uptake by everted sacs), without any change in the serosal uptake in fasted animals. In normoxic animals, serosal uptake comprised approximately 45 and 41% of the uptake in fed and fasted animals respectively.

Effect of hypoxia and fasting on animal weight and haemoglobin levels

The effect of fasting and/or hypoxia on haemoglobin levels is shown in Table 1. There were significant main effects of both fasting and hypoxia on haemoglobin and body weight ($P < 0·001$). Fasting, exposure to hypoxia, and the combination of hypoxia and fasting caused an increase in haemoglobin levels.

It was found that during the first day of exposure to hypoxia the food consumption of the rats used here was reduced by two-thirds; animals ate 4·4 (SE 0·8) g (n 7) compared with the normal rate of 15 g/d. Feeding was back to normal by the second day (14·4 (SE 0·4) g; n 5) and remained so on the third day (16·9 (SE 1·5) g; n 3). The decrease in food consumption seen on day 1 of hypoxic exposure was reflected in a decrease in the body weight of hypoxic animals as compared with normoxic fed animals (Table 1). As expected a decrease in body weight was also seen in animals which had been fasted for 18–24 h before the experiments and in animals which had been exposed to 3 d of hypoxia with fasting over the last 24 h.

Effect of fasting, hypoxia and membrane depolarization on ^{59}Fe uptake

Preliminary *in vitro* studies in fed rats showed that Fe uptake was not affected by membrane depolarization and 3 d hypoxia caused only a small increase in Fe uptake. This is in contrast to the effect of hypoxia on intestinal Fe uptake in mice. It was thought that the differing responses in mice and rats to hypoxia may be due to differences in food intake; it has been shown that the rates of feeding in mice are decreased over the entire 3 d of hypoxic exposure (R. J. Simpson, unpublished results). To test this hypothesis the effect of fasting and hypoxia on Fe uptake was investigated (Table 1). Fasting of normoxic animals (i.e. normoxic fasted) caused a small increase in Fe uptake and, as seen in the hypoxic fed animals, uptake was inhibited (35%) in high-K^+ solutions (Fig. 1). Fasting of hypoxic animals, over the last 18–24 h of exposure to hypoxia, caused a 2–4-fold increase in Fe^{3+} uptake and this uptake was decreased by approximately 42% in a high-K^+ medium. Three-way ANOVA indicated that there was a significant effect of hypoxia, fasting and of high-

Table 1. *Effect of hypoxia and fasting on body weight, haemoglobin levels and kinetic variables of Fe^{3+} uptake by duodenal fragments from rats*†

(Mean values with their standard errors for nos. of rats shown)

Physiological status	Body wt‡ (g)			Haemoglobin levels (g/dl)			K_m (mM)			V_{max} (pmol/mg wet wt per min)		
	n	Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*	Mean	SE
Normal: Fed	91	234[a]	2·7	83	15·8[a]	0·2	4	326	92	4	9·2	1·2
Fasted	36	204[b]	3·0	53	16·4[b]	0·2	8	132	9	8	12·5	1·6
Hypoxic: Fed	19	220[a]	6·6	36	16·3[a,b]	0·4	5	162	29	5	12·0	1·5
Fasted	33	190[c]	4·7	39	18·0[c]	0·3	6	129	15	6	20·9[a]	2·4
Statistical significance of effect of §:												
Fasting		***			***			***			***	
Hypoxia		***			***			***			***	

[a,b,c]Mean values in columns with unlike superscript letters were significantly different ($P < 0·05$).
***$P < 0·001$.
†For details of animals and procedures, see pp. 460–463.
‡At the time of killing.
§Two-way ANOVA.

K^+ buffer ($P < 0·001$ in all cases) on Fe^{3+} uptake. Subsidiary tests suggested that there was no significant increase in uptake by normoxic fasted and hypoxic fed animals as compared with normoxic fed. However, there was a significant increase in uptake in hypoxic fasted animals as compared with the other three animal groups. In the presence of high-K^+ medium, there were no significant differences between groups. The decrease in uptake seen when fragments from hypoxic fed or hypoxic fasted animals were incubated in high K^+ is significant. These data suggest that both fasting and hypoxia induces a K^+-sensitive (i.e. membrane-potential-dependent) uptake mechanism.

Time-course of hypoxic response

To further investigate the hypoxic response of Fe^{3+} uptake, the time dependence of the response in fed animals was investigated. Fig. 2 shows the results of the present study. As previously shown, there was no significant increase in Fe uptake by duodenal fragments from fed animals after 3 d of hypoxia; there was, however, a significant inhibition of uptake when fragments were incubated in high-K^+ solution. Fig. 2 shows that the effect is evident after 1 d of hypoxia. Two-way ANOVA showed that there were significant effects of both duration of hypoxic exposure ($P < 0·001$) and of high-K^+ buffer ($P < 0·001$) on Fe uptake. In addition there was a significant interaction between duration of hypoxic exposure and the buffer ($P < 0·001$). Subsidiary tests suggested that uptake was not significantly increased after 1, 2 or 3 d of hypoxia compared with normoxic fed animals. Uptake by tissue from normoxic fed animals was unaffected by high K^+, but uptake was significantly inhibited by membrane depolarization in tissue from animals exposed to hypoxia for 1 (41%), 2 (48%) or 3 (45%) d. The data show that the sensitivity to membrane depolarization developed on the first day of hypoxic exposure.

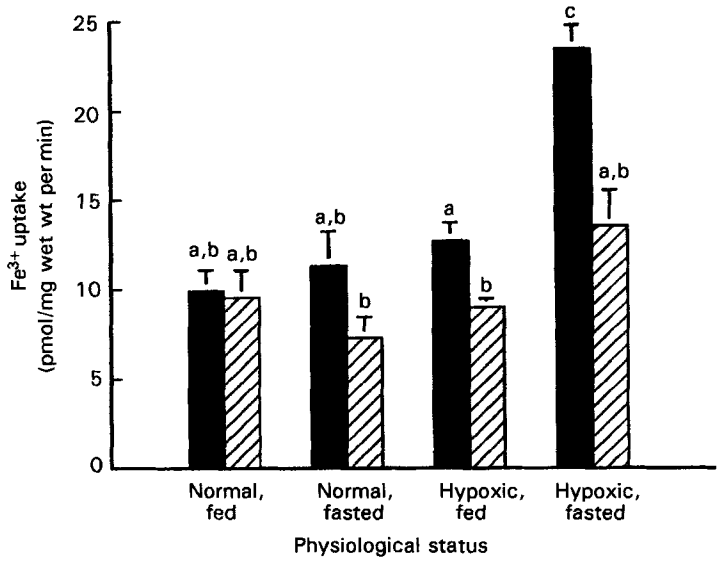

Fig. 1. Effect of hypoxia and fasting on Fe^{3+} uptake in duodenal fragments from rats. Duodenal fragments were prepared from normal fed, normal fasted, hypoxic fed or hypoxic fasted animals and incubated in normal 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid medium (■) or in medium containing a high K^+ concentration (▨). Each solution contained 450 mM-$^{59}Fe^{3+}$ in 900 mM-nitrilotriacetic acid and included [^{57}Co]cyanocobalamin as an extracellular-fluid marker. Values are means with their standard errors represented by vertical bars for seven to eight rats. ^{a,b,c}Mean values with unlike superscript letters were significantly different ($P < 0.05$). For details of animals and procedures, see pp. 460–463.

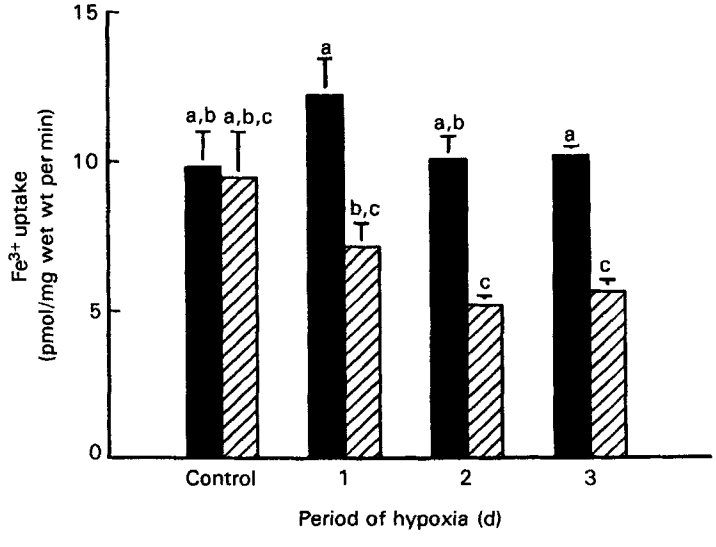

Fig. 2. Time-course of Fe^{3+} uptake response to hypoxia in duodenal fragments from fed rats. Duodenal fragments were prepared from normal fed animals and from animals exposed to hypoxia for 1, 2 or 3 d. Uptake of Fe^{3+} from 450 mM-$^{59}Fe^{3+}$ in 900 mM-nitrilotriacetic acid was measured after 5 min incubation at 37° in normal 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid medium (■) or medium containing a high K^+ concentration (▨); [^{57}Co]cyanocobalamin was the extracellular-fluid marker. Values are means with their standard errors represented by vertical bars for six rats. Mean values with different superscript letters were significantly different ($P < 0.05$). For details of animals and procedures, see pp. 460–463.

Kinetics of ^{59}Fe uptake by rat duodenum

The uptake of Fe^{3+} by duodenal fragments from all four groups of animals was linear over 10 min at both 450 and 45 μM. Fig. 3 shows the concentration dependence of Fe^{3+} uptake in normoxic fed, normoxic fasted, hypoxic fed and hypoxic fasted animals. The data from each group could be fitted by the Michaelis–Menten equation for a single binding site and the V_{max} and K_m were calculated for each group (Table 1). There was a significant effect of both hypoxia and fasting on the K_m for Fe^{3+} uptake. The K_m was decreased in normoxic fasted, hypoxic fed and hypoxic fasted animals compared with that for normoxic fed animals. The V_{max} for Fe^{3+} uptake by fragments was increased in normoxic fasted, hypoxic fed and hypoxic fasted animals compared with normoxic fed rats. Two-way ANOVA indicated that there was a significant effect of hypoxia ($P < 0{\cdot}001$) and of fasting ($P < 0{\cdot}001$) on the V_{max} for Fe^{3+} uptake. These data suggest that a low K_m mechanism of uptake is induced by hypoxia and fasting.

Contribution of media components to uptake characteristics

There was no significant change in the rate of uptake in any of the animal groups when glucose was omitted from the medium. This suggests that the inhibition in Fe uptake by replacement of medium Na^+ with K^+ is not due to effects on glucose uptake. When fragments from any of the animal groups were incubated without oxygenation, uptake was significantly decreased ($P < 0{\cdot}001$; 31 % for both normoxic fed and normoxic fasted and 21 % and 22 % for hypoxic fed and hypoxic fasted respectively; not shown). It has been shown in the mouse that removal of Ca^{2+} from the media causes an increase in uptake (Raja *et al.* 1987*c*). It has been suggested that this is due to an increase in the stability of the

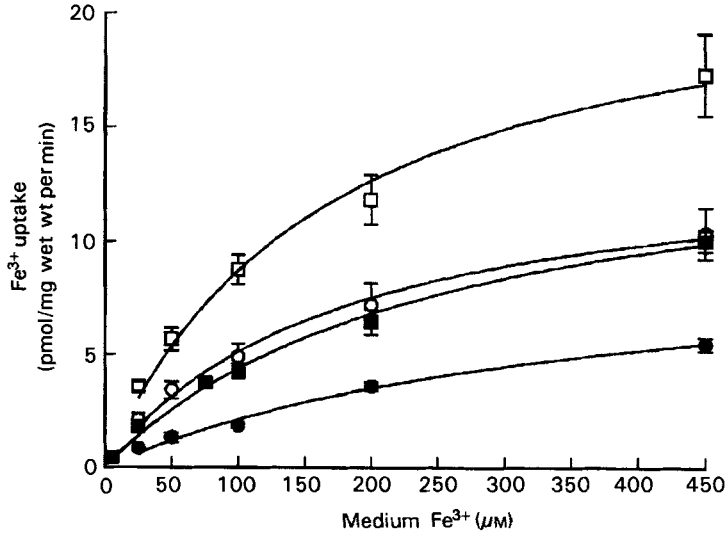

Fig. 3. Kinetics of Fe^{3+} uptake by duodenal fragments from rats. Concentration dependence of Fe^{3+} uptake from 450 mM-$^{59}Fe^{3+}$–900 mM-nitrilotriacetic acid was studied in fragments prepared from normal fed (●), normal fasted (○), hypoxic fed (■) and hypoxic fasted animals (□). Fragments were incubated for 5 min at 37° in 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid medium containing 5–450 mM-$^{59}Fe^{3+}$; [^{57}Co]cyanocobalamin was added to the medium as an extracellular-fluid marker. The Michaelis–Menten equation was fitted to each set of data; values for K_m and V_{max} are shown in Table 1. Values are means with their standard errors represented for vertical bars for four to eight rats.

Fe^{3+}–NTA complex. We found that Ca^{2+} caused no significant change in the levels of uptake in rat fragments in any animal group (values not shown).

Reductase activity and effect of Fe^{2+} chelation on Fe^{3+} uptake by fragments

It has been suggested recently that a mucosal ferri-reductase plays a central role in the specific uptake of Fe^{3+} in the mouse duodenum (Raja *et al.* 1992). It was, therefore, of interest to investigate the role of reduction in the uptake of Fe from Fe^{3+}–NTA by rat duodenum. Table 2 shows the effect, on Fe^{3+} uptake, of including 1 mM-ferrozine in the incubation medium; ferrozine is a specific Fe^{2+} chelator which would rapidly bind any Fe^{2+} produced. In all four animal groups the presence of the Fe^{2+} chelator decreased Fe^{3+} uptake significantly ($P < 0.001$; three-way ANOVA). There was also a significant interaction between the effect of hypoxia and ferrozine in the medium. Subsidiary tests suggest that there was a significant increase in uptake in normoxic fasted, hypoxic fed and hypoxic fasted animals as compared with normoxic fed animals. There was a further significant increase in uptake in hypoxic fasted animals as compared with all other animal groups. There were no significant differences in uptake between groups in the presence of ferrozine. Table 2 shows the interrelations between uptake, the decrease in uptake caused by ferrozine and the level of ferri-reductase activity in each of the groups. In all groups the quantity of medium Fe chelated by ferrozine was less than 10% of the total. Both uptake and reductase activity was enhanced in hypoxic fasted animals. Two-way ANOVA showed

Table 2. *Effect of Fe^{2+} chelation on Fe^{3+} uptake and ferri-reductase activity in duodenal fragments from rats*†

(Mean values with their standard errors for nos. of rats shown)

		^{59}Fe uptake (pmol/mg per min)								
	Ferrozine in medium	Uptake with or without ferrozine			Ferrozine-dependent uptake			Reductase activity (pmol/mg per min)		
Physiological status		Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*
Normal: Fed	−	3·54[a]	0·41	5	1·42[a]	0·52	5	74·2[a,b]	9·6	5
	+	2·12[b]	0·15	5						
Fasted	−	5·19[c]	0·28	9	2·67[a]	0·32	9	62·7[a]	5·9	6
	+	2·52[b]	0·20	9						
Hypoxic: Fed	−	5·36[c]	0·51	8	3·76[a,b]	0·47	8	96·2[a,b]	20·0	6
	+	1·72[b]	0·22	8						
Fasted	−	7·42[d]	0·93	8	4·84[b]	0·96	8	113·8[b]	12·4	6
	+	2·57[b]	0·28	8						
Statistical significance‡ of effect of:										
Fasting			*			NS			NS	
Hypoxia			*			*			*	
Ferrozine			*							

[a,b,c]Mean values in columns with unlike superscript letters were significantly different ($P < 0.05$).

−, Absent; +, present.

* $P < 0.05$.

† For details of animals and procedures, see pp. 460–463.

‡The effect of fasting, hypoxia and ferrozine on Fe uptake was analysed by three-way ANOVA and the effect of fasting and hypoxia on ferrozine-dependent uptake and reductase activity by two-way ANOVA.

that fasting had no significant effect on reductase activity, while hypoxia significantly increased reductase activity ($P < 0.05$).

Mucosal free fatty acid levels

There is evidence to suggest that free fatty acids could play a role in the facilitated transport of Fe across the mucosal brush border (Simpson *et al.* 1989). The effect of fasting on the response of Fe^{3+} uptake may be due to some change in the levels of free fatty acids in the rat brush-border membrane; therefore, the effect of fasting on free fatty acid levels in mucosa was investigated. There was rapid, linear production of free fatty acids in mucosal scrapings between 0 and 30 min after which production proceeded slowly, if at all. Two-way ANOVA showed that there was no difference in the rate of production (fed 26 (SE 4); n 4 *v.* fasted 30 (SE 3); n 4 pmol/min per mg protein) of free fatty acids by mucosa from fed or fasted animals, in the magnitude of production nor in initial levels (fed 0·17 (SE 0·05); n 4 *v.* fasted 0·15 (SE 0·03); n 4 μmol/mg protein). This suggests that mucosal fatty acids may not be important in the adaptive effect of fasting on Fe uptake mechanisms. Note, however, that local changes in fatty acid levels in the brush-border membrane could not be investigated by this technique.

DISCUSSION

In mice, the predominant mechanism involved in duodenal Fe^{3+} uptake is a saturable process which is dependent on metabolic energy (Raja *et al.* 1987*b*). The active uptake of Fe^{3+} in mice is also dependent on the membrane potential across the brush border and is enhanced in hypoxia (Raja *et al.* 1989). The latter two characteristics are useful markers for the carrier-mediated uptake of Fe^{3+} in mice and, since the rat is the most common animal model used in Fe nutrition studies, it was of interest to investigate the response of Fe^{3+} uptake by rat duodenal fragments to hypoxia and to membrane depolarization by incubation in high-K^+ solutions.

The data presented here demonstrated several differences in duodenal fragment Fe uptake between mice and rats, i.e. higher serosal uptake in rats (cf. Raja *et al.* 1987*a*), no effect of Ca^{2+} (cf. Raja *et al.* 1987*c*) or glucose omission (cf. Raja *et al.* 1992), a significant change in K_m for uptake in hypoxia (cf. Raja *et al.* 1987*a*), in normoxic fed animals Fe^{3+} uptake was not inhibited by membrane depolarization and hypoxia had little effect in fed animals, in contrast to mice (Raja *et al.* 1987*a*, 1989, 1992). It has been reported that some mouse strains fail to exhibit increased *in vivo* Fe absorption after 3 d hypoxia (Simpson, 1992). This finding was related to rapidly increased haemoglobin levels. The present data indicate that this was not a factor in the rat (Table 1).

On the other hand, uptake by rat duodenum does show a dependence on medium oxygenation, as occurs in the mouse (Raja *et al.* 1992). When fed rats were exposed to 3 d of hypoxia the uptake was inhibited by membrane depolarization (30%). When animals were fasted, uptake inhibition by membrane depolarization and induction by hypoxia were evident. It is possible, therefore, that the differences between the mice and rats may be partly explained by differences in food consumption over the 3 d of hypoxic exposure.

Previous studies of the effect of fasting on Fe absorption have given contradictory results. Osterloh *et al.* (1987) demonstrated reduced *in vivo* absorption of Fe in semi-starved rats, while O'Riordan *et al.* (1994) observed increased absorption in overnight fasted rats. Murray & Stein (1967) observed a decrease in *in vitro* Fe uptake in 18 h fasted rats. It is noteworthy, however, that no effect of semi-starvation or fasting on Fe^{3+} absorption has been observed by us in mice (Raja *et al.* 1988; R. J. Simpson and K. B. Raja,

unpublished results). Earlier studies of the effect of fasting on Fe absorption are complicated by the presence of diet in the intestinal lumen of fed animals (Forrester *et al.* 1962; Donati *et al.* 1964; Conrad *et al.* 1967). Some of the contradictions noted previously may be explained by variations in the duration of fasting (Forrester *et al.* 1962). In our study, the use of *in vitro* incubated tissue fragments enables the mucosal effects of fasting to be observed, free from the direct effects of diet itself. We find that fasting induces a membrane-potential-sensitive Fe uptake mechanism with a low K_m. This is consistent with the findings of Debnam & Thompson (1984) who reported that fasting induces an increased brush-border-membrane potential in the rat. Hypoxia also increases this mechanism of Fe uptake. The *in vitro* uptake observations are complicated by the presence of other uptake mechanisms, however, such that fasting alone has no significant effect on uptake by tissue from non-hypoxic rats at high medium-Fe concentrations (450 μM).

An important consideration is whether fasting or hypoxia might influence Fe absorption by an effect on mucosal Fe or by an effect similar to low-Fe–diet feeding (Fairweather-Tait & Wright, 1984; Schümann *et al.* 1989). Such studies generally show that low dietary Fe intakes increase Fe absorption, although it takes 2–3 d to produce a significant increase (Pearson *et al.* 1967). We have shown that the increased Fe absorption induced by hypoxia is not associated with a change in mucosal Fe level (Raja *et al.* 1987*a*) and that hypoxia and dietary Fe levels act independently to affect Fe absorption in mice (Simpson, 1996). It is noteworthy that the rats employed in the present study ate normally over the last 2 d of hypoxic exposure. Further investigation of the possible effect of fasting on mucosal Fe levels is necessary.

The importance of the present findings is the demonstration that the overall effect of a single perturbation on Fe uptake can be complicated by other factors which affect Fe absorption, thus the effect of hypoxia or fasting alone on Fe uptake depends on the status of the rats with regard to the other perturbation. Several physiological factors are known to affect Fe absorption, including dietary Fe levels, fasting, body Fe stores, erythropoietic rate, pregnancy, hypoxia and inflammation (Turnbull, 1974). Only limited data are available on interactions between these factors (Bothwell *et al.* 1958; Murray & Stein, 1972; Batey & Gallacher, 1977; Flanagan *et al.* 1980; Southon *et al.* 1989; Schümann *et al.* 1989, 1990; Raja *et al.* 1990), therefore the effect of a single factor on Fe absorption cannot always be predicted.

We further investigated the possible involvement of reported mechanisms of Fe uptake, i.e. free fatty acids (Simpson *et al.* 1989) and reduction-dependent processes. No evidence in support of an effect of fasting on total mucosal free fatty acid levels was observed. It should be noted, however, that previous studies have noted decreased brush-border-membrane fatty acids in fasted mice (Simpson *et al.* 1989) and rats (Alcorn *et al.* 1991). The sensitivity of Fe uptake to the Fe^{2+} chelator ferrozine is greatest in tissue from fasted hypoxic rats, consistent with the induction of an uptake mechanism dependent on reduction of Fe^{3+} to Fe^{2+}, as proposed by Raja *et al.* (1992).

The reduction of Fe^{3+} ions to Fe^{2+} has been implicated as a key step in cellular Fe uptake processes in a variety of systems (Bienfait, 1985; Dancis *et al.* 1992; Raja *et al.* 1992; Hodgson *et al.* 1994; Nunez *et al.* 1994). In the case of intestinal Fe absorption, the uptake of Fe by the duodenum has been suggested to require a rate-determining reduction either in the intestinal lumen (Wollenberg & Rummel, 1987; Barrand *et al.* 1990; Wien & Van Campen, 1991), or at the mucosal surface. We have demonstrated that a mucosal surface reductase is present on mouse duodenum and that this reductase is regulated, in parallel with Fe uptake, by hypoxia and Fe deficiency (Raja *et al.* 1992). Others have recently failed to demonstrate regulation of mucosal reductase in isolated brush-border

membranes from Fe-deficient rat duodenum (Wien & Van Campen, 1994), however, studies of isolated membranes may lack an important regulatory factor.

The present data implicate a mucosal surface reductase in the uptake of Fe by the rat duodenum. This reductase is observed in intact tissue incubated in physiological medium. The findings further demonstrate that Fe uptake by rat duodenum involves a low K_m mechanism which depends on an intact membrane potential. Other mechanisms of uptake are implicated, particularly in normoxic fed animals. These observations show similarities to findings made with human duodenum (Raja *et al.* 1996; K. B. Raja, unpublished results). This provides support for the use of rats as a model for human Fe absorption. It should be noted, however, that the presence of multiple uptake mechanisms which show independent responses to physiological modulations such as fasting, means that extrapolation from one species to another requires caution, particularly at the present stage, where we remain ignorant of the relative importance of these various mechanisms for Fe nutrition in the various species.

We thank Dr D. J. Pountney for assistance with Fe uptake and reduction assays and Dr T. Mayall for communications support. E.M.T. was supported by a Wellcome Trust Prize Studentship. This is a contribution from the King's College Centre for the Study of Metals in Biology and Medicine.

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