Biochimica et Biophysica Acta 860 (1986) 229–235
Elsevier

BBA 72935

Fe^{2+} uptake by mouse intestinal mucosa in vivo and by isolated intestinal brush-border membrane vesicles

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(Received March 18th, 1986)

Key words: Hypoxia; Fe^{2+} binding; Fe^{2+} transport; Intestinal transport; Kinetics; Brush-border membrane; (Mouse intestine)

In vivo kinetics of mucosal uptake of luminal $^{59}Fe^{2+}$ by tied segments of normal mouse duodenum are characterised by a K_m of approx. 100 μM and a V_{max} of approx. 9 pmol/min per mg wet weight of intestine. These values were determined at pH 7.25 in the presence of excess sodium ascorbate. Studies with luminal Fe^{2+} concentrations of 100 μM reveal: (1) uptake is relatively independent of ascorbate:Fe ratio and luminal pH and (2) uptake is potently inhibited by 1 mM Co^{2+} or Mn^{2+} and large luminal NaCl concentrations but not by Ca^{2+}. 3 days of hypoxia (0.5 atmospheres) yields no significant increase in subsequent total mucosal uptake by in vivo tied segments while uptake is significantly reduced by semi-starvation. Quantitative comparison of in vivo mucosal uptake with subsequent determination of isolated brush-border membrane $^{59}Fe^{2+}$ transport in individual mice reveals a positive correlation ($P < 0.01$) between the two parameters. These results, in conjunction with studies of isolated mouse duodenal brush-border membrane (Simpson, R.J. and Peters, T.J. (1985) Biochim. Biophys. Acta, 814, 381–388 and (1986) Biochim. Biophys. Acta 856, 109–114) suggest that the Fe^{2+} transport properties of isolated brush-border membrane are quantitatively adequate to explain in vivo mucosal uptake in normal and hypoxic mice at Fe^{2+} concentrations up to 100 μM.

Introduction

Recently, comparison of Fe^{3+} transport by isolated brush-border membrane with in vivo mucosal Fe^{3+} uptake by mouse intestine [1] suggested that while ileal mucosal Fe^{3+} uptake is explained adequately by the properties of the isolated membrane vesicles, an extra uptake mechanism, localised to proximal intestine and not observed in preparations of brush-border membrane vesicles, was necessary to explain in vivo duodenal Fe^{3+} uptake. The relative significance of Fe^{2+} and Fe^{3+} in dietary iron absorption remains to be elucidated, consequently we wished to extend our studies to include iron in the divalent form.

Iron absorption in vivo has been extensively studied, particularly in normal and iron-deficient rats [2,3]. In this paper, in vivo Fe^{2+} uptake from tied off segments of normal, hypoxic and control-fed mouse proximal intestine was investigated and the results compared with those obtained in studies of Fe^{2+} uptake by mouse brush-border membrane vesicles [4,5] and in studies of Fe^{3+} uptake [1,6,7].

Materials and Methods

The in vivo determination of ^{59}Fe uptake was performed as in [1] except that the incubation

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

media and wash solutions were prepared as in Ref. 4. Materials were obtained as described previously [1,6,7]. Mice were 6–8 weeks old, male, To strain and control feeding was performed as in Ref. 5. Data were analysed using Student's *t*-test as the distribution of the in vivo data did not differ significantly from the normal distribution [8]. Details of animal diets, induction of hypoxia and mucosal iron determinations are given in Refs. 7 and 9. Reticulocytes were stained with methylene blue. Haemoglobin was determined as the cyanomet derivative using a Coulter Electronics (Luton, U.K.) haemoglobinometer.

A series of experiments were performed with either normal, control-fed or hypoxic mice (see refs. 4 and 5 for details) in which brush-border membrane vesicles were prepared following in vivo tied-off segment uptake experiments. A segment of 6–10 cm (duodenum plus proximal jejunum) was prepared and injected as above with 90 μM Fe^{2+}, 1.8 mM sodium ascorbate, 0.1 M NaCl, 0.1 M mannitol, 20 mM Hepes (pH 7.25). After incubation for 10 min, the segment was removed, weighed and washed as in Ref. 1. Counting of the gut segment for ^{59}Fe was performed within 5 min in a cooled chamber counter (Beckman γ-7000, Beckman Instruments Inc., Fullerton, CA, U.S.A.). The gut segment was then used to prepare brush-border membrane vesicles as described in Ref. 6. The vesicle preparation was counted for ^{59}Fe activity and this was used to correct subsequent $^{59}Fe^{2+}$ uptake experiments performed by millipore filtration as described in [4,5]. Enzyme and protein determinations were performed as in Ref. 6.

Gel filtration analysis of ^{51}CrEDTA and $^{59}Fe^{2+}$/ascorbate media was performed on a Sephadex G-25 column (25 × 1 cm) equilibrated and eluted with 0.15 M NaCl, 10 mM NaHepes (pH 7.0) at 6°C (flow rate 0.3 ml/min). Fractions (1.2 ml) were collected and counted for ^{59}Fe or ^{51}Cr as appropriate.

Results

Haematological parameters for experimental mouse groups

Table I displays haemoglobin values, reticulocyte counts and duodenal mucosal iron levels for normal, hypoxic and control-fed mice. It can be seen that reticulocyte counts and haemoglobin levels are enhanced ($p < 0.001$) in hypoxic (compared with both normal and control-fed) mice, reflecting enhanced erythropoiesis. No significant differences ($p > 0.1$) were observed between control-fed and normal mice in any of the parameters reported in Table I.

TABLE I

HAEMOGLOBIN LEVELS, RETICULOCYTE COUNTS AND MUCOSAL IRON IN NORMAL, HYPOXIC AND CONTROL FED MICE

Blood was obtained from mice by cardiac puncture. Mucosal iron was determined by atomic absorption spectrophotometry as in Refs. 7 and 9. Reticulocyte counts were obtained by counting 1000 cells. Data are mean ± S.E. for (n) mice.

Animal group	Blood haemoglobin (g/dl)	Reticulocyte count (%)	Duodenal mucosal iron (nmol/mg protein)
Normal	16.1 ± 0.4 (7)	1.6 ± 0.4 (7)	5.94 ± 1.8 (6)
Hypoxic	19.6 ± 0.2 (7) [a]	5.8 ± 0.6 (7) [a]	7.56 ± 0.72 (6)
Control-fed	15.1 ± 0.4 (6)	1.3 ± 0.2 (6)	9.02 ± 0.46 (6)

[a] $p < 0.001$ compared with control-fed and normal.

Characteristics of in vivo $^{59}Fe^{2+}$ uptake by mouse duodenum

The initial time-course of total mucosal $^{59}Fe^{2+}$ uptake by mouse duodenum at two medium Fe^{2+} concentrations and a constant ascorbate to Fe ratio of 20 : 1 is shown in Fig. 1. Also shown is the uptake of the marker ^{51}CrEDTA. This extracellular fluid marker [10] was considered to be appropriate for studies with Fe^{2+} as uptake of such markers shows some dependence on molecular size [11] and ^{51}CrEDTA was found to elute in the same position within the included region as Fe^{2+} ascorbate on Sephadex G-25 chromatography. It is noteworthy that, as was previously noted with $^{59}Fe^{3+}$ uptake, the permeation of ^{51}CrEDTA is much lower than that of $^{59}Fe^{2+}$, especially at 10 min incubation. Correction of mucosal $^{59}Fe^{2+}$ uptake with the ^{51}CrEDTA leads to reasonably linear time-courses, similar to those obtained in vivo in studies of mucosal $^{59}Fe^{3+}$ uptake [1], thus allowing an estimate of the initial rate of mucosal uptake.

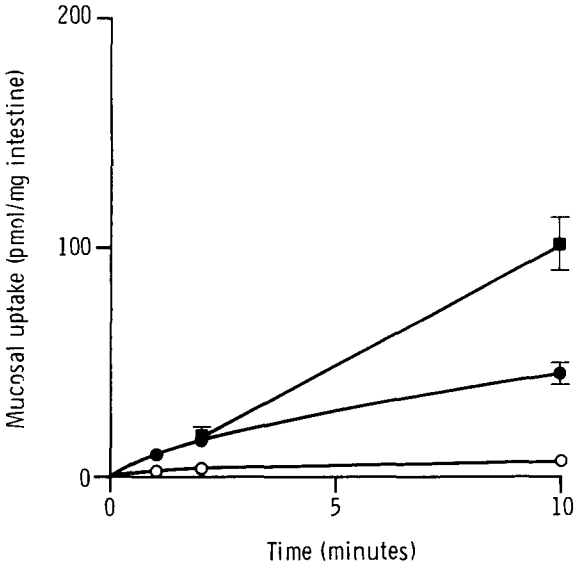

Fig. 1. Time-course for mucosal $^{59}Fe^{2+}$ uptake by mouse duodenum in vivo. Tied-off segments (1–2 cm) of mouse duodenum, immediately distal to the bile duct, were prepared under anaesthesia [1]. Segments were sealed after injection of approx. 50–100 μl of 0.1 M NaCl, 0.1 M mannitol, 20 mM Hepes, $^{59}Fe^{2+}$ and 20-fold molar excess sodium ascorbate (final pH 7.25). After incubation, the segment was removed, opened and washed with 20 ml of ice cold 0.1 mM Fe^{2+}, 2 mM sodium ascorbate, 0.15 M NaCl. After blotting and removal of connective and pancreatic tissue, the segment was weighed and counted. The mouse was killed and counted for ^{59}Fe as in [1]. The counts in the intestinal segment and mouse were used, along with the weight of the segment and its length before and after removal from the mouse, to calculate total mucosal uptake of ^{59}Fe per mg (wet weight) of intestine as in Ref. 1. Medium Fe^{2+} concentrations were 400 μM (■) and 100 μM (●). Also shown are data for uptake of 100 μM ^{51}CrEDTA (○) in the same medium lacking Fe^{2+}/ascorbate.

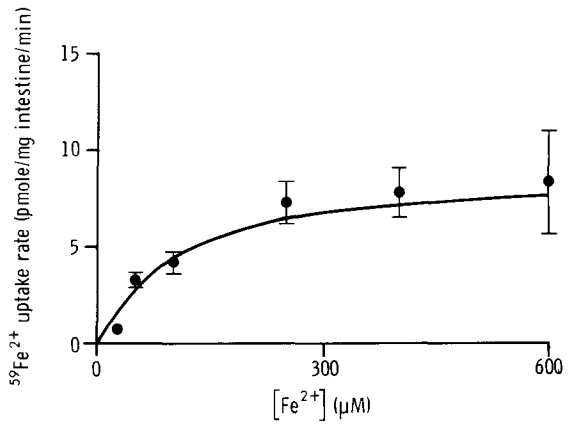

Fig. 2. Concentration dependence of initial mucosal $^{59}Fe^{2+}$ uptake by normal mouse duodenum in vivo. Total mucosal uptake values were obtained from in vivo tied-off segments of duodenum incubated for 10 min with 0.1 M NaCl, 0.1 M mannitol, 20 mM Hepes (pH 7.25), containing the indicated Fe^{2+} concentrations and a constant ascorbate:Fe^{2+} ratio of 20:1. Values were corrected for permeation by non specific mechanisms with ^{51}CrEDTA (see Methods). The curve is a hyperbola characterised by a V_{max} of 9 pmol/mg intestine per min and a K_m of 100 μM.

Fig. 2 demonstrates that varying the Fe^{2+} concentration at constant ascorbate:Fe^{2+} ratio results in a saturable dependence of uptake on medium Fe^{2+} concentration. Note that this curve is obtained after correcting for the uptake of ^{51}CrEDTA, which was found to be linearly dependent on ^{51}CrEDTA upto 1 mM (not shown). This dependence is qualitatively similar to the Fe^{2+} concentration dependence of $^{59}Fe^{2+}$ uptake by mouse duodenal brush-border membrane vesicles as reported previously [4].

Table II shows the results of an investigation of the effect of varying the reducing agent (ascorbate) on initial in vivo uptake with a single concentration of $^{59}Fe^{2+}$. It can be seen that varying the ascorbate:Fe ratio by a factor of 20 or substituting isoascorbate (D-erythrohex-2-enoic acid, sodium salt) have little effect on initial total mucosal uptake of $^{59}Fe^{2+}$. This observation is similar to that made with $^{59}Fe^{2+}$ uptake by isolated brush-border membrane vesicles [4], that is uptake relates to $^{59}Fe^{2+}$ rather than to the Fe^{2+}-ascorbate complexes which may be present.

TABLE II

EFFECT OF VARYING REDUCING AGENTS ON $^{59}Fe^{2+}$

In each experiment, uptake was determined after 10 min incubation in an in vivo tied-off segment as described in Fig. 1. The $^{59}Fe^{2+}$ concentration was 100 μM and the reducing agent was added as the sodium salt.

Reducing agent	Ascorbate: Fe^{2+} ratio	Total mucosal uptake of $^{59}Fe^{2+}$ (pmol/mg intestine, mean ± S.E. for (n) determinations)
0.5 mM ascorbate	5	56.5 ± 6.5 (4)
2 mM ascorbate	20	44.5 ± 4.5 (16)
10 mM ascorbate	100	57.7 ± 6.9 (8)
2 mM isoascorbate	–	35.0 ± 2.5 (5)

Effect of hypoxia on in vivo mucosal $^{59}Fe^{2+}$ uptake by mouse duodenum

Table III shows the effect of exposing mice to 3 days of hypoxia or of control feeding prior to the determination of $^{59}Fe^{2+}$ uptake at 100 μM Fe^{2+}. Total mucosal uptake shows no significant change in hypoxia compared with normal mice. Prewashing the intestinal segment had no significant effect in normal or hypoxic animals and, if anything, obscures any slight increase. There is a striking 2-fold increase in $^{59}Fe^{2+}$ transfer to the carcass in hypoxia, accompanied by a drop in ^{59}Fe retained in the gut segment. This observation provides further strong evidence that the uptake and transfer steps in iron absorption are sequential steps in the overall absorption process (see Ref. 2). When mice are fed the same daily quantity of diet consumed by mice exposed to hypoxia, a significantly lower mucosal uptake is observed ($p < 0.01$; Table III). The reduction appears to be effected at the uptake step as both gut and carcass ^{59}Fe levels are reduced compared with normal animals. These changes in total mucosal Fe^{2+} uptake agree with our studies of $^{59}Fe^{2+}$ uptake by normal, hypoxic and control-fed mouse duodenal brush border membrane vesicles [4,5] conducted with similar media.

Inhibition of Fe^{2+} uptake

Table IV shows that Co^{2+} and Mn^{2+} are potent inhibitors of Fe^{2+} absorption while Ca^{2+} has no significant effect. The effect of Co^{2+} and Mn^{2+} appears to be at the initial uptake step as total mucosal uptake, transferred radioiron and mucosal radioiron all decline roughly in parallel (Table IV). The inhibitory effect of these ions on iron absorption is well documented [2] and inhibition of Fe^{2+} transport by isolated mouse brush-border membrane vesicles by Co^{2+} and Mn^{2+} has been recorded [4] though the effect was less than occurs in vivo. Ca^{2+} has no significant effect on Fe^{2+} transport by isolated brush-border membrane vesicles [4].

Addition of large concentrations of NaCl to the incubation medium had a pronounced inhibitory effect on mucosal Fe^{2+} uptake (Table IV). Lowering the pH of the medium from 7.25 to 6.55 had no significant effect on mucosal uptake (Table IV). Interpretation of these results must be influenced by the complexity of the in vivo system. In particular, alteration of the luminal pH does not alter the pH close to the brush-border membrane significantly, due to the well known capacity of duodenal mucosa to neutralise acid [12]. Reported values for the pH adjacent to

TABLE III

EFFECT OF HYPOXIA ON $^{59}Fe^{2+}$ ABSORPTION FROM TIED-OFF SEGMENTS

Uptakes (pmol/mg intestine, mean ± S.E. for (n) determinations) were determined after 10 min incubation as described in Fig. 1 with 100 μM $^{59}Fe^{2+}$ and 2 mM ascorbate in 0.1 M NaCl, 0.1 M mannitol, 20 mM Hepes (pH 7.25). Prewashed segments were prepared as in Ref. 1 by gently pumping 1 ml of warm (37°C) 0.15 M NaCl through the segment prior to injection of the $^{59}Fe^{2+}$ solution. Hypoxia was induced by placing mice in a hypobaric chamber at 0.5 atmospheres for 3 days. Except for the animals which were control-fed (see Methods) food and water were given freely.

Experiment	^{59}Fe in gut	^{59}Fe in carcass	Total mucosal ^{59}Fe uptake	(n)
Normal mice	22.1 ± 3.0	22.3 ± 3.6	44.5 ± 4.5	16
Normal mice, prewashed segment	18.4 ± 1.7	26.7 ± 6.3	45.4 ± 6.2	8
3 day hypoxic mice	8.5 ± 1.0 [a]	51.3 ± 5.8 [a]	59.2 ± 6.3	18
3 day hypoxic mice, prewashed segment	11.8 ± 1.9 [b]	44.9 ± 4.1 [a,c]	55.7 ± 5.9	11
Control-fed	10.5 ± 1.7 [a]	9.9 ± 1.9 [b,d]	20.5 ± 3.3 [a,d]	10

[a] $p < 0.01$ compared to normal.
[b] $p < 0.02$ compared to normal.
[c] $p < 0.05$ compared to normal prewashed segment.
[d] $p < 0.001$ compared to hypoxic.

TABLE IV

EFFECT OF DIVALENT CATIONS, pH AND NaCl ON $^{59}Fe^{2+}$ ABSORPTION IN VIVO

Incubation media were as described for Table II (sodium ascorbate; 2 mM) with additions as described. Data are expressed as pmol/mg intestine, mean ± S.E. for (n) determinations.

Experiment	^{59}Fe in gut	^{59}Fe in carcass	Total mucosal Fe^{2+} uptake (n)
Control (pH 7.25)			
(no addition)	22.1 ± 3.0	22.3 ± 3.6	44.5 ± 4.5 (16)
+ 1 mM $CaCl_2$	14.4 ± 2.2	22.7 ± 3.5	37.1 ± 3.6 (5)
+ 1 mM $MnCl_2$	3.8 ± 0.9 [b]	8.2 ± 2.1	12.0 ± 2.7 [a] (4)
+ 1 mM $CoCl_2$	6.4 ± 1.3 [b]	4.6 ± 0.9 [b]	11.0 ± 1.8 [a] (8)
+ 1.35 M NaCl	15.2 ± 0.4 [c]	1.33 ± 0.29 [b]	16.5 ± 0.6 [b] (4)
pH 6.55	15.6 ± 3.8	36.1 ± 10.1	51.7 ± 10.9 (6)

[a] $p < 0.001$.
[b] $p < 0.01$.
[c] $p < 0.05$.

brush-border membrane in vivo in rats are not significantly different from 7.25 [12]. The high NaCl concentration used will have marked effects on water flux and the vasculature of the mucosa was visibly affected in these experiments. It is noteworthy that NaCl was inhibitory to brush-border membrane vesicle Fe^{2+} uptake [4,5].

Comparison of $^{59}Fe^{2+}$ uptake by duodenal mucosa in vivo and by isolated brush-border membrane in vitro

As noted above, there are qualitative similarities between total mucosal uptake of $^{59}Fe^{2+}$ in vivo and $^{59}Fe^{2+}$ transport by isolated brush-border vesicles (see Ref. 4). This agreement (little effect of varying ascorbate : Fe^{2+} ratio, of substituting isoascorbate for ascorbate, of Ca^{2+} or hypoxia, inhibition by Co^{2+}, Mn^{2+}, NaCl and control feeding) though not exact, suggests that the kinetic properties of Fe^{2+} transport by isolated brush-border membrane vesicles may be nearly adequate to explain Fe^{2+} uptake kinetics in vivo. It has previously been demonstrated [1] that this is also true for Fe^{3+} uptake by mouse ileum but not mouse duodenum.

The striking difference between Fe^{3+} uptake and Fe^{2+} uptake by isolated duodenal brush-border membrane vesicles is that the latter is much more rapid and extensive (Refs. 13, 14 and 4 compared with Refs. 6 and 7). It would not be surprising, then, that a comparison of Fe^{2+} uptake by mouse duodenum in vitro might show an adequate rate of brush-border membrane vesicle transport.

In order to test the hypothesis that in vivo mucosal Fe^{2+} uptake rates are explained by the properties of isolated brush-border membrane vesicles, the comparison of these two parameters with individual mice was attempted and is shown in Fig. 3.

The initial vesicle transport rate was taken as the difference between uptake values determined at 90 μM and 9 μM Fe^{2+}, in order to correct for a binding component of the uptake process (see Refs. 4 and 5).

The positive, significant correlation ($p < 0.01$) between vesicle and in vivo uptake further confirms that the Fe^{2+} transport properties of mouse brush-border membrane vesicles are related to in vivo mucosal uptake, at least under these conditions. It can be seen from Fig. 3 that all but one of the data points indicates an adequate vesicle uptake rate for the in vivo rate. Both in vivo and vesicle uptake show some variability from animal to animal (see also Refs. 4 and 5). Fig. 3 demonstrates that some of the variability in vesicle uptake data reflects real in vivo variability. It should be noted that this variability was not observed in similar studies with Fe^{3+} or ^{51}CrEDTA uptake in vivo (see Ref. 1) and therefore may not be a reflection of the techniques employed but of real inter-animal variation.

Some determinations of vesicle Fe^{2+} uptake have yielded very large values (see Refs. 4 and 5).

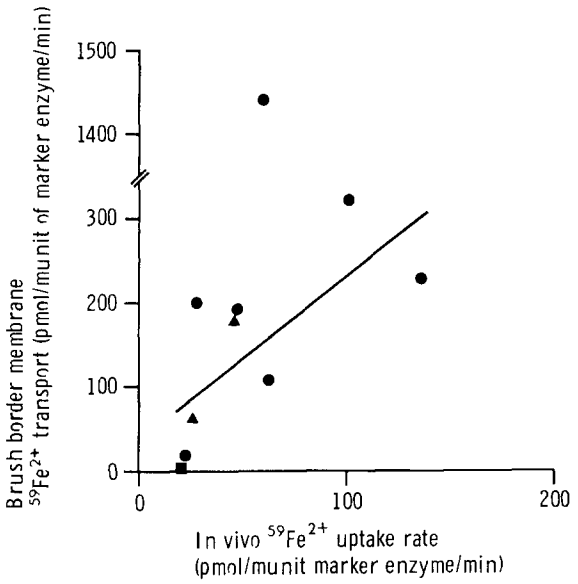

Fig. 3. Correlation of in vivo $^{59}Fe^{2+}$ uptake rates with brush-border membrane vesicle $^{59}Fe^{2+}$ transport. Tied-off segments of duodenum plus proximal jejunum (length 6–10 cm starting distal to the bile duct) were prepared and incubated for 10 min as described in Methods (medium Fe^{2+} concentration 90 μM). After counting for ^{59}Fe, the gut segment was used to prepare brush-border membrane vesicles as described in Ref. 6. The vesicles contained only small quantities of ^{59}Fe from the tied segment experiment and were used to determine $^{59}Fe^{2+}$ uptake (90 μM and 9 μM Fe^{2+}, incubation time 1 min, media as in Fig. 2) by Millipore filtration as described in Ref. 4. The vesicle transport rate was taken as the difference between uptake values at the two medium Fe^{2+} concentrations (see Refs. 4 and 5). The activity of the brush-border marker enzyme Zn^{2+}-resistant α-glucosidase in the two preparations was used to express the vesicle and tissue uptakes in equivalent units. Broadly speaking, this represented multiplication by approx. 20 mg intestine/munit enzyme [1] for in vivo data, and multiplication by approx. 0.1 mg protein/munit enzyme [7] for vesicle data. In vivo total mucosal uptake is expressed as a rate assuming a linear uptake time-course. Mice were either normal (●), control-fed (■), or hypoxic (▲) (see Methods for details). The line was obtained by linear regression ($r = 0.72$, $p < 0.01$). The very large vesicle uptake value was not included in the regression analysis.

In the series of experiments shown in Fig. 3, one vesicle preparation yielded such a very large uptake value. This did not correlate with an especially large in vivo uptake hence this value may represent an occasional artefact of vesicle preparation or, a step subsequent to initial uptake may be rate determining in vivo in this case.

Taken with the small differences between the Fe^{2+} transport properties of brush-border vesicles and in vivo mucosal uptake (slightly greater sensitivity of vesicle transport to high ascorbate concentrations, lower sensitivity of vesicle transport to Co^{2+} and Mn^{2+} inhibition), this suggests that, while quantitatively adequate to explain in vivo uptake, other steps subsequent to the initial brush-border transport may also contribute to determination of the overall rate of uptake.

Discussion

The findings presented above contrast with previous findings relating to Fe^{3+} uptake [1] where brush-border membrane uptake was inadequate to explain in vivo duodenal Fe^{3+} uptake. This suggests that the predominant in vivo mechanism of Fe^{3+} uptake may be different from that for Fe^{2+} under the conditions used for these studies. This proposal is further supported by the finding that in vivo Fe^{2+} mucosal uptake is not enhanced in hypoxic mice compared with normals whereas a significant increase in Fe^{3+} uptake has been observed [1].

Previous studies of enhanced Fe^{2+} absorption in hypoxic mice and rats [15,16] reported 2-fold increases in radioiron retention after oral force feeding of test doses of radio labelled $FeSO_4$ in hypoxic compared with normal animals. This retention parameter is more closely related to ^{59}Fe transfer, in tied segment experiments, than to total mucosal uptake and hence our studies are in reasonable agreement with the earlier work.

It is not yet clear what causes the enhanced ^{59}Fe transfer in hypoxic animals and further studies of subsequent mucosal processing of iron are necessary. The molecular nature of the brush border membrane 'carrier' is not yet known but further studies with isolated brush border membrane are worthwhile, at least for Fe^{2+}.

The finding that distinct, separately regulated mechanisms of mucosal Fe^{2+} and Fe^{3+} uptake may exist means that interpretation of absorption experiments where the luminal Fe redox state is not defined, or poorly controlled, in terms of iron uptake mechanisms, is difficult.

Acknowledgements

We thank Mrs. S. Kingsley for typing this manuscript, Dr. K. Osterloh for useful discussions

and Dr. N. Veall for the use of the large-volume γ-counter.

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