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STUDIES OF Fe^{3+} TRANSPORT ACROSS ISOLATED INTESTINAL BRUSH-BORDER MEMBRANE OF THE MOUSE

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Mouse intestinal brush-border membrane vesicles take up iron from media containing $^{59}Fe^{3+}$-nitrilotriacetic acid. The iron uptake by the vesicles represents accumulation of iron which relates to an osmotically active space. Uptake is linearly related to vesicle protein concentration and is inhibited by low incubation temperature and low medium free Fe^{3+} concentrations. Experiments with the lipid soluble iron ligand 8-hydroxyquinoline and with Triton X-100 imply that the uptake is rate limited by membrane transport.

Introduction

The mechanism of iron translocation from the intestinal lumen to the portal plasma is ill understood, consequently the regulation of this process is obscure. Research so far has delineated two steps, termed uptake and transfer, in this process. Both of these steps appear to be regulated in adaptive increases in iron absorption [1].

We have been investigating iron absorption both in man and the experimental animal using in vitro measurements of Fe^{3+} uptake by segments of intestinal tissue from media containing Fe^{3+} and nitrilotriacetic acid [2–4]. Recent studies of Fe^{3+} uptake by isolated rabbit brush-border membrane vesicles failed to demonstrate transport of Fe^{3+} across the brush-border membrane [5]. Fe^{2+} transport has been demonstrated in such systems [5,6] although similar studies by other workers reached contradictory conclusions [7,8]. We therefore set out to investigate the role of brush-border membrane in the mechanism and regulation of iron uptake by the intestine relating the studies with vesicles to experiments with intact duodenal segments and to in vivo results. In this paper the uptake of Fe^{3+} by brush-border membrane vesicles is studied to reveal the nature of the uptake process and the rate-limiting step for the process.

Materials and Methods

Materials. Biochemicals were from Sigma Chemical Co. Ltd., Poole, Dorset; other chemicals and solvents were analar grade, radiochemicals and PCS scintillation fluid were from Amersham International, Amersham, U.K.

General methods. Enzyme assays were performed as in Refs. 9, 10 on freshly prepared or frozen vesicle preparations. Protein was determined by a modified Lowry method [11] with bovine serum albumin as standard. The specific activity of $^{59}FeCl_3$ was determined by assaying Fe^{3+} with ferrozine and by gamma spectrometry in a Beckman Instruments Inc. (Fullerton, CA, U.S.A.) Gamma 7000 Counter.

Brush-border membrane vesicles. Brush-border membrane vesicles were isolated from mouse (6–8-week old, male, To strain) duodenal or duodenal plus jejunal scrapings according to a

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

method based on that of Kessler et al. [12]. $MgCl_2$ was used in place of $CaCl_2$ to reduce phospholipid cleavage by Ca^{2+}-activated phospholipases [13]. Mucosal scrapings were suspended in 10 ml (minimum) or 28 ml/g of tissue of 50 mM mannitol/2 mM Hepes (pH 7.1). This was homogenised at top speed for 2 min in a cooled Waring Blender. Solid $MgCl_2$ was added to 10 mM and the mixture maintained for 20 min on ice. After centrifuging for 10 min at $3000 \times g$, the pellet was discarded and the supernatant recentrifuged at $27000 \times g$ for 30 min. The vesicles were resuspended by repeated passage through a 21-gauge needle in 5 ml (minimum) or 20 ml/g of original tissue of resuspension buffer (0.1 M mannitol/0.1 M NaCl/0.1 mM $MgSO_4$/20 mM Hepes-NaOH (pH 7.4), filtered through a Millipore filter (GSWP) before use). After centrifuging for 15 min at $6000 \times g$, the supernatant was recentrifuged at $27000 \times g$ for 30 min. The resulting pellet of brush-border membrane vesicles was resuspended as above in resuspension buffer to a final protein concentration of approx. 5 mg/ml. All the above procedures were performed at 4°C.

Uptake studies. Studies with Fe^{3+} in physiological media require the presence of a chelating agent, e.g. citrate, nitrilotriacetic acid or EDTA, in order to prevent precipitation of iron hydroxide [14]. Nitrilotriacetic acid is an especially suitable agent as it preserves Fe^{3+} in a soluble monomeric form at neutral pH [15] while the iron in the complex is biologically available.

Fe^{3+} uptake studies were performed on duodenal vesicles freshly prepared in resuspension buffer as above and preincubated for 30 min at 37°C. The incubation medium was prepared by mixing $^{59}FeCl_3$ (3–20 mCi/mg Fe) in 10 mM HCl with 10 mM $FeCl_3$ in 10 mM HCl then adding the appropriate volume of 10 mM trisodium nitrilotriacetate. After 10 min at room temperature, H_2O and Millipore-filtered mannitol/NaCl/Hepes buffer were added to give the appropriate Fe^{3+} and nitrilotriacetic acid concentration in 0.1 M mannitol/0.1 M NaCl/20 mM Hepes (pH 7.4). Where additional compounds were used, these were added as a solution in place of H_2O. The specific radioactivity of ^{59}Fe was adjusted to be 20000–40000 cpm per nmol. Uptake was initiated by adding 5 μl of membrane preparation to 50 μl of incubation medium and vortexing. After incubation, 50 μl of mixture was added to a 0.22 μm Millipore filter (previously moistened with 0.15 M NaCl) in a Millipore sampling manifold (Cat. No. XX2702550) and immediately washed with two 5-ml portions of ice cold 0.15 M NaCl/0.1 mM Fe^{3+}/0.22 mM nitrilotriacetic acid (pH at room temperature approx. 4.5). Filters were dried and counted at constant geometry in a γ-counter (Beckman Gamma 7000).

Vesicle uptake was corrected with blanks in which incubation mixtures were incubated and filtered as above except that appropriate quantities of final resuspension buffer were added instead of the vesicle suspension. These blanks only exceeded 2-times the background count from a filter at very low (less than 1.5 : 1) nitrilotriacetic acid : Fe^{3+} ratios. Assay of Zn^{2+}-resistant α-glucosidase (a brush-border marker enzyme [9]) in filtrates from uptake experiments revealed that greater than 99.5% of vesicles were retained on the 0.22 μm filter after washing, even in experiments where the vesicles were shrunk in hypertonic medium. Preliminary studies were performed with 0.45 μm filters as used by previous workers [8]. Assays of the brush-border enzyme Zn^{2+}-resistant α-glucosidase in the filter effluent indicated that up to 16% (mean value $6.3 \pm 1.5\%$ (S.E., $n = 11$)) of the vesicles were not retained by the filters.

This uptake assay system was found to give most reproducible results. Uptake was found to be essentially the same if the volume of wash was increased to 15 ml. Omission of unlabelled Fe^{3+} and nitrilotriacetic acid from the wash solution gave uptake values generally about 10% higher. Nitrilotri[1-^{14}C]acetic acid uptake was performed as above except that ^{59}Fe was omitted. Appropriate adjustments to the unlabelled Fe^{3+} and nitrilotriacetic acid concentrations were made. Filters were incubated overnight in PCS before counting in a scintillation counter.

Na^+-dependent glucose transport was performed on vesicles freshly prepared in 0.3 M mannitol/0.1 mM $MgSO_4$/20 mM Hepes (pH 7.4) as resuspension buffer. Vesicles were mixed with 1 volume of 0.9 mM [U-^{14}C]glucose (60000 cpm per nmol)/0.15 M NaCl/20 mM Hepes (pH 7.4) to start uptake and incubated at 20°C. Aliquots of 50 μl were removed, filtered as above and washed two

times with 5 ml of ice cold 0.7 mM phloridzin/0.15 M NaCl. Filters were counted as above.

Electron spin resonance spectroscopy. Vesicles were prepared from duodenal-jejunal mucosal scrapings of up to 10 mice as above for iron uptake experiments to yield a final protein concentration of 10–20 mg/ml. 20 μl of 1 mM Fe^{3+}/2 mM nitrilotriacetic acid in 0.1 M NaCl/0.1 M mannitol/20 mM Hepes (pH 7.4) was added to 180 μl of vesicle suspension in the ESR tube. Incubations were terminated by freezing the sample in liquid N_2. Tubes were stored at -70°C and ESR spectra (full field scan) obtained on a Varian E-9 spectrometer at 100 K.

Electron microscopy. Vesicle suspensions were fixed for 1 h at room temperature by adding more than 10 volumes of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) containing 5% sucrose. The vesicles were then washed in cacodylate buffer and post-fixed for 1 h with 1% osmium tetroxide in cacodylate buffer for 1 h. The samples were then dehydrated through acetone and embedded in Spur resin. Thin sections stained with uranyl acetate and lead citrate were viewed under a Phillips 300 electron microscope operating at 60 kV.

Results and Discussion

Brush-border membrane vesicles

The duodenal brush-border membrane vesicle preparations showed a mean final specific activity of Zn^{2+}-resistant α-glucosidase of 106 ± 6 (mean $\pm$ S.E., $n = 10$) munits per mg of protein and a enrichment relative to the homogenate of 13 ± 2 (mean $\pm$ S.E., $n = 4$) fold. Yields were 30 ± 4% (mean $\pm$ S.E., $n = 4$). The vesicles were essentially free from contaminating organelles as judged by electron microscopy and similar preparations have been shown to be free of marker enzymes for contaminating organelles by many laboratories [16]. The purification ratio tended to vary more from preparation to preparation than did the final specific activity of marker enzyme in the vesicles, perhaps reflecting variation in the depth of the initial scraping. Marker enzymes for mitochondria and cytoplasm were essentially absent (enrichment factors for malate dehydrogenase and lactate dehydrogenase less than 0.02-fold). This is important as mitochondria demonstrate a large capacity for iron uptake [17,18].

Electron microscopy revealed exclusively right-side-out vesicles similar to those shown in [12] and predominantly filled with core proteins. The vesicles appear essentially unchanged in morphology after incubation for several hours at 37°C in the Fe^{3+} containing incubation mixture although a decrease in the proportion of vesicles in which core proteins were visible was noted.

Studies of Na^+-dependent glucose transport revealed the characteristic overshoot shown by other workers [12] with an equilibrium endpoint for glucose uptake corresponding to roughly 1 μl of solvent space per mg of vesicle protein. This value has been found by other workers in studies of amino acid transport with mouse intestinal brush-border membrane vesicles [19], confirming that this apparent volume is a true solvent space. It is noteworthy that this is a much smaller solvent space (per mg of protein) than is found with cells, even red cells (approx. 2 μl solvent per mg protein [20]), reflecting the extremely high protein content of the vesicles and their small size (mean apparent diameter approx. 0.2 μm). These studies also validate the preparation of mouse duodenal brush-border vesicles and their use in transport studies.

Fe^{3+} uptake by brush-border vesicles

Fig. 1 shows the time-course for uptake of

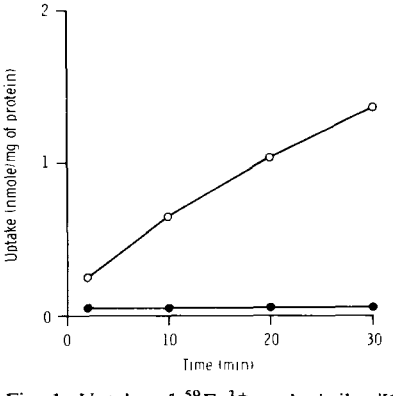

Fig. 1. Uptake of $^{59}Fe^{3+}$ and nitrilotri[1-^{14}C]acetic acid by mouse duodenal brush-border membrane vesicles. The uptake of Fe^{3+} (○) and nitrilotriacetic acid (●) were measured as described in Materials and Methods. Time-courses were run in parallel on the same vesicle preparation at 37°C. Medium concentration of Fe^{3+} was 91 μM and nitrilotriacetic acid was 182 μM.

$^{59}Fe^{3+}$ and nitrilotri[1-^{14}C]acetic acid by the vesicles in parallel incubations. Using the above estimate of the solvent space, it can be seen that even at the shortest incubation time, iron uptake represent accumulation with respect to the medium iron concentration ($t = 2$ min uptake of 0.26 nmol/mg protein is equivalent to 260 μM Fe^{3+} within the vesicles). The uptake of nitrilotriacetic acid is low relative to the ^{59}Fe. The Fe^{3+} uptake proceeds for several hours at this temperature, reaching a plateau value of 10.5 ± 0.4 nmol/mg protein (mean $\pm$ S.E., $n = 6$). The uptake rate was found to vary by a factor of up to 2–3-fold from one membrane preparation to another, even when prepared and assayed in parallel on the same day. Data shown are generally from representative experiments but qualitative results were highly reproducible.

The concentration of iron within the vesicles at the uptake end point may be calculated on the basis of the solvent space, giving a value of 10 mM, or 100-times the medium iron concentration. Clearly, since Fe^{3+} cannot achieve such concentrations in the hydrated state at neutral pH [14], the Fe^{3+} uptake must represent some binding or precipitation event on or within the vesicles. Fe^{3+}-nitrilotriacetic acid solutions are themselves stable with respect to precipitation for many days in the incubation media used in these experiments. Since all experiments were performed with a wash containing unlabelled Fe^{3+} plus excess nitrilotriacetic acid, uptake should not contain significant components of weak or nonspecific binding of Fe^{3+}. Uptake was measured at 5 min and was found to be linearly related to vesicle protein in the assay system (Fig. 2). In other experiments, the linear range was found to extend up to at least 2 mg of vesicle protein per ml of assay mixture.

Does Fe^{3+} uptake represent transport across the brush-border membrane?

This critical question in transport studies is usually answered by shrinking the vesicles in hypertonic media while studying uptake [12]. Fe^{3+} uptake was found to be strongly dependent on an osmotically active space (Fig. 3). Similar results are found if the vesicles are shrunk with NaCl or mannitol. As noted in Materials and Methods, shrinking does not cause the vesicles to pass through the filters. Greater than 90% of Fe^{3+} uptake at low nitrilotriacetic acid : Fe^{3+} ratios (approx. 2 : 1) is observed to relate to the inside of the vesicles. At higher nitriloacetic acid : Fe^{3+} ratios, the proportion of the uptake which represents transport is reduced. Thus at an nitrilotriacetic acid : Fe^{3+} ratio of 10 : 1, 40% of uptake at 10 min was found to be unrelated to the osmotically active space, presumably representing binding to the outside of the vesicles.

Previous studies of Fe^{3+} uptake by rabbit intestinal brush-border membrane vesicles [5], which failed to find any significant transport of Fe^{3+}, were performed at very high chelate : Fe^{3+} ratios such that the free Fe^{3+} concentration was calcu-

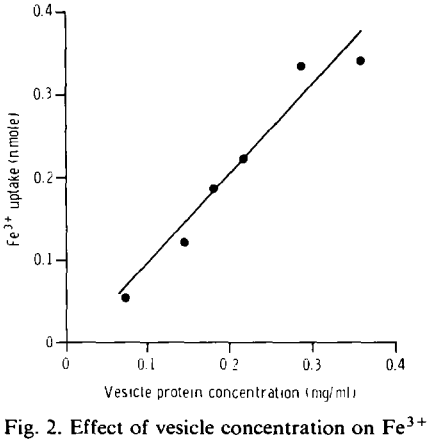

Fig. 2. Effect of vesicle concentration on Fe^{3+} uptake. Uptake was performed as in Fig. 1 for 5 min at 37°C. The medium Fe^{3+} was 370 μM and nitrilotriacetic acid was 730 μM.

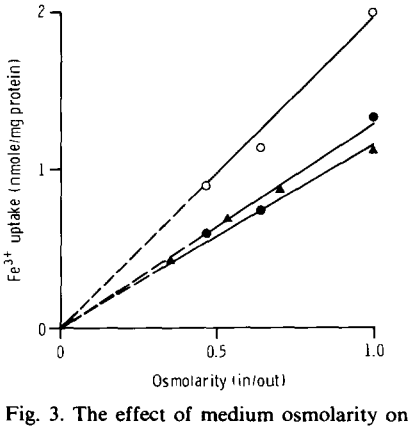

Fig. 3. The effect of medium osmolarity on $^{59}Fe^{3+}$ uptake at two incubation times. Uptake was performed as described in Fig. 1 for 5 min (●, ▲) or 10 min (○). Medium osmolarity was increased by adding mannitol (●, ○) or NaCl (▲).

lated to be several orders of magnitude lower than that employed in our study [5]. Extrapolating our data to such low free Fe^{3+} levels would also reveal relatively little transport compared to binding of Fe^{3+} to the outside of the vesicles.

The iron inside the vesicles is unlikely to be free in solution (see below) yet uptake is strongly inhibited by shrinking the vesicles. This is difficult to explain, although a similar finding has been made with Fe^{2+} uptake by rabbit brush-border membrane vesicles [5]. The uptake in that case also represented accumulation (presumably binding [21]) of the Fe^{2+} within the vesicle.

Characteristics of Fe^{3+} uptake by brush-border vesicles

Fig. 4 shows the effect of varying the medium nitrilotriacetic acid concentration at constant Fe^{3+} concentration on Fe^{3+} uptake. Uptake is potently inhibited by increasing the nitrilotriacetic acid concentration. Studies of the time course of Fe^{3+} uptake at high nitrilotriacetic acid : Fe ratio (10 : 1) revealed rapid and slower components, presumably reflecting binding (to the outside of the vesicles) and transport components, respectively, of the overall uptake. Bearing in mind the decrease in the proportion of uptake which is transport into the vesicles noted above, it can be seen that the rate of transport across the brush-border membrane may be driven to very low values at very low free Fe^{3+} concentrations. This explains the quantitative difference between the Fe^{3+} uptake reported here and that in Ref. 5 which was performed at the very low free Fe^{3+} concentration of 10^{-19} M. Similarly, the lower Fe^{3+} uptake noted by Cox and O'Donnell [8] was at an nitrilotriacetic acid : Fe^{3+} ratio of 110 : 1. These results are consistent with the rate of uptake of Fe^{3+} by the vesicles being determined by the free Fe^{3+} concentration in the medium. Further evidence for this was found in experiments where the total Fe^{3+} and nitrilotriacetic acid concentrations were varied by a factor of 72 at a constant nitrilotriacetic acid : Fe^{3+} ratio of 2 : 1. The initial uptake rate was found to vary little over this range of nearly two orders of magnitude of total nitrilotriacetic acid : Fe^{3+} concentration. If the Fe^{3+}-nitrilotriacetic acid complex is assumed to be predominantly the 1 : 1 complex [20] then the free Fe^{3+} concentration will remain constant in such an experiment while the Fe^{3+}-nitrilotriacetic acid concentration will vary markedly. Thus Fe^{3+} uptake again relates to the free medium Fe^{3+} concentration.

Studies of the effect of divalent metal ions on the uptake are again dominated by the free Fe^{3+} concentration, which increases when metals which compete with Fe^{3+} for nitrilotriacetic acid are added. The results presented in Table I show increasing uptake due to the cations Mg^{2+}, Ca^{2+}, Mn^{2+} and Cu^{2+}, respectively. This order reflects the reported increasing affinity of this series of

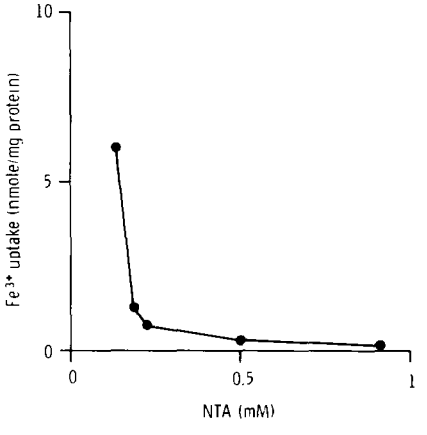

Fig. 4. The effect of medium nitrilotriacetic acid (NTA) concentration at constant Fe^{3+} on the uptake of $^{59}Fe^{3+}$. The Fe^{3+} concentration was 91 μM. Incubations were performed as in Fig. 1 for 5 min.

TABLE I

THE EFFECT OF DIVALENT CATIONS ON Fe^{3+} UPTAKE BY DUODENAL BRUSH-BORDER MEMBRANE VESICLES

Incubations were performed as in Fig. 1 with a medium Fe^{3+} concentration of 91 μM and a nitrilotriacetic acid concentration of 182 μM. Incubation time was 5 min. All samples contained 18 μM $MgSO_4$ in addition to the salts shown.

Addition	Final concn. (mM)	Relative Fe^{3+} uptake
None	-	1
$MgSO_4$	0.1	1.16
$MgCl_2$	1	1.9
$CaCl_2$	1	7.3
$MnCl_2$	1	36
$CuCl_2$	1	49

cations for nitrilotriacetic acid [22]. It is noteworthy that at the concentrations of Mg^{2+} present in the assay from the resuspension buffer (18 μM) there is negligible effect on the uptake (Table I).

Fig. 5 shows the effect of temperature on Fe^{3+} uptake. Overall uptake is potently inhibited by lowering the incubation temperature. Experiments in hypertonic mannitol reveal that uptake at 0°C for 10 min reflects approx. 30% binding to the outside of vesicles compared to less than 10% at 37°C. Thus the transport is reduced 9-fold between 37°C and 0°C.

The rate determinating step in the uptake process

The overall rate of uptake of Fe^{3+} could be determined by dissociation of Fe^{3+} from nitrilotriacetic acid, transport across the membrane, or some process, for example binding or precipitation, occurring inside the vesicles. In order to establish which step determined the observed rate of uptake, studies were undertaken with the lipid soluble Fe^{3+} ligand 8-hydroxyquinoline. This molecule has been used as a membrane carrier for trivalent cations [23] to load liposomes and has also been found to stimulate Fe^{3+} absorption in jejunal loops in situ in rat [14]. Fig. 6 shows that this compound can, at low concentrations, greatly accelerate the Fe^{3+} uptake of brush-border membrane vesicles. The final equilibrium uptake is not greatly affected and ESR spectra show no difference from those loaded by simple incubation

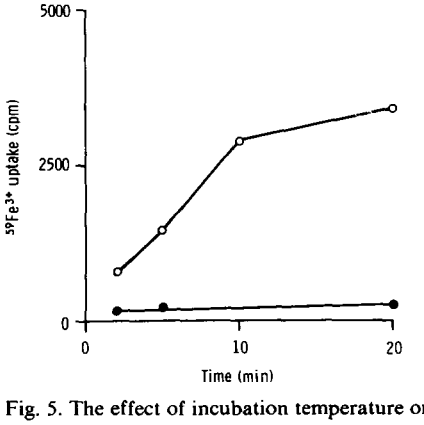

Fig. 5. The effect of incubation temperature on $^{59}Fe^{3+}$ uptake. Incubations were performed as in Fig. 1, either at 37°C (○) or on melting ice (●). Medium Fe^{3+} was 400 μM and nitrilotriacetic acid was 790 μM.

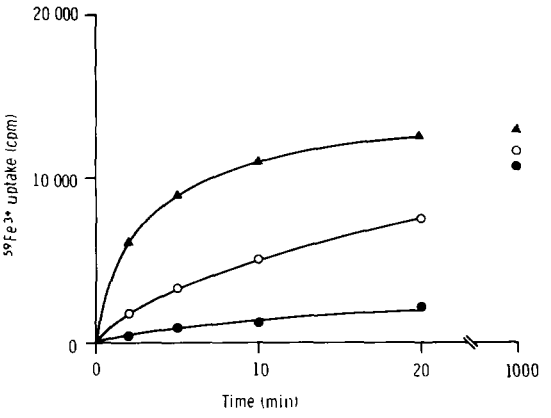

Fig. 6. The effect of 8-hydroxyquinoline on the $^{59}Fe^{3+}$ uptake time-course. Incubations were performed as in Fig. 1, only 8-hydroxyquinoline in ethanol was added to give final concentrations of 4.5 μM (▲), 0.9 μM (○) and 0.0 μM (●). In each case the final ethanol concentration was 1% (v/v), however, controls containing neither ethanol nor 8-hydroxyquinoline superimpose over controls containing ethanol.

with Fe^{3+}-nitrilotriacetic acid. Previously reported ESR and electron microscopic studies [24] indicated that the uptake represented binding of iron by components of the vesicles. Further ESR studies with Triton X-100-solubilised vesicles also reveal accelerated uptake, while the end point is unaffected. Boiling the vesicles for 15 min prior to uptake experiments also accelerated the uptake time-course, presumably due to damaging the membrane and allowing entry of Fe^{3+}-nitrilotriacetic acid complex.

These results imply that measurements of Fe^{3+} uptake by brush-border membrane vesicles do indeed represent Fe^{3+} transport rates across the brush -border membrane.

Conclusions

This work demonstrates, for the first time, transport of Fe^{3+} across the membrane of isolated brush-border vesicles. Transport can be detected only when a significant free Fe^{3+} concentration is present. The uptake of Fe^{3+} by brush-border membrane vesicles from Fe^{3+}-nitrilotriacetic acid solutions gives a measure of the rate of transport of Fe^{3+} across the brush-border membrane. This transport rate depends on the free Fe^{3+} concentration and incubation temperature. Damage to

the integrity of the brush-border membrane and the lipid soluble Fe^{3+} carrier 8-hydroxyquinoline accelerate the binding of Fe^{3+} by internal components of the vesicles.

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