*Biochimica et Biophysica Acta*, 649 (1981) 297–304
Elsevier/North-Holland Biomedical Press



BBA 79488

# IRON UPTAKE BY RABBIT INTESTINAL MUCOSAL MEMBRANE VESICLES

JOANNES J M. MARX * and PHILIP AISEN

*Departments of Biophysics and Medicine, Albert Einstein College of Medicine, Bronx, NY 10461 (U.S.A.)*

(Received May 27th, 1981)

*Key words Iron uptake, Brush border membrane, (Rabbit mucosal membrane)*

**Iron uptake by brush border membrane vesicles isolated from rabbit small intestine was studied using a rapid filtration technique. Special attention was directed at avoiding hydrolysis of iron at Fe(III) and oxidation of iron at Fe(II). Uptake of Fe(III) was studied from media containing ferric citrate with a 1000-fold molar excess of citrate to iron, calculated to be sufficient to prevent simple hydrolysis and polynuclear complex formation. Fe(III) bound to the mucosal membranes in a temperature-dependent and saturable fashion. Transport into the intravesicular space, however, could not be demonstrated. Fe(III) uptake was almost identical after heat denaturation of membrane proteins, which probably rules out the involvement of a specific protein receptor. Membrane binding of Fe(III) was higher when media with a lower pH were used and when there was only a 20-fold molar excess of citrate to iron. The major part of the $^{55}$Fe(III) is bound to the membranes very strongly, and cannot be removed by washing with excess unlabeled Fe(III). Under similar experimental conditions uptake of Fe(II), from a medium with a 20-fold molar excess of ascorbate to iron, was several hundred times higher than that of Fe(III). Again, there was no difference between the uptake of Fe(II) by normal or boiled vesicles. In contrast to results with Fe(III), however, uptake of Fe(II) into an osmotic space could be demonstrated. The large amount of Fe(II) transported may be explained by assuming an intravesicular iron trap, possibly provided by oxidation and hydrolysis in the vesicular lumen. Our findings indicate that the uptake of iron by microvillous membrane vesicles probably entails both binding and simple diffusion.**

## Introduction

It has been recognized for almost half a century that fluctuations in the body's need for iron are met by varying iron uptake from the intestine [1]. There is good evidence that iron absorption consists of two steps. mucosal uptake, followed by mucosal transfer of iron, both possibly subject to regulation [2,3]. The mechanism of mucosal uptake has been studied in vitro by incubation of intestinal brush border segments with iron [4,5]. In this system the iron has access, however, to the luminal and the cytoplasmal site of the mucosal membrane. Moreover it is not possible to distinguish binding from transport. These problems can be overcome if purified mucosal membrane vesicles are used. The technique for production of those vesicles has been improved considerably in the last few years, and vesicles have been used to investigate epithelial transport of a number of nutrients [6]. Few studies have been published of iron transport using mucosal membrane vesicles [7,8], with conflicting results. In the present work we investigated some biochemical aspects of the interaction of Fe(III) and Fe(II) with intestinal mucosal membrane vesicles. Much effort was given to using well-defined ferric citrate and ferrous ascorbate test media to minimize artifactual hydrolysis of Fe(III) and oxidation of Fe(II) which might influence the results considerably.

* Permanent address Dr J.J M. Marx, Department of Hematology, University Hospital, Catharijnesingel 101, Utrecht, The Netherlands.

Some of the uptake studies were also repeated with vesicles in which proteins were denaturated by heating

## Methods

*Preparation of brush border membrane vesicles*

Pooled material of two normal New Zealand White rabbits was used for every preparation. The animals were killed by a blow in the neck. The proximal one-third part of the small intestine was removed and rinsed immediately with ice-cold 0.15 M NaCl. All subsequent steps were performed at temperatures between 0°C and 4°C. Membrane vesicles were isolated according to a method described by Kessler et al [9] with only minor alterations. Intestinal loops of about 12 cm were opened, rinsed again with saline and blotted carefully with soft tissue paper to remove as much mucus as possible. The mucosa was scraped off gently with a glass slide, suspended in a hypotonic buffer solution (50 mM mannitol, 2 mM Tris-HCl, pH 7.1), 25 ml for 1 g wet weight mucosa, and homogenized in a Waring blender for 2 min at maximum speed. Solid $CaCl_2$ was then added to a final concentration of 10 mM to cross-link and precipitate intracellular organelles and basolateral plasma membranes [9]. This suspension was centrifuged for 15 min at 3000 × *g* in a refrigerated Sorvall RC-5B (rotor SS-34). The pellet was discarded and the supernatant spun for 30 min at 27 000 × *g*. The resulting pellet was resuspended in 100 mM mannitol/20 mM Tris-Hepes, pH 7.4, and homogenized with a Thomas glass/Teflon tissue grinder with serrated pestles, 8 strokes with 1500 rev./min. The homogenate was centrifuged for 15 min at 6000 × *g* to remove remaining particles, followed by a final centrifugation of the supernatant for 30 min at 23 000 × *g* to yield pelleted vesicles These were resuspended by aspiration in a syringe through a 25-gauge needle The vesicles were frozen immediately in 200 µl samples at −70°C until use.

*Preparation of the incubation medium*

The incubation medium consisted of two equal parts A and B. Part A was identical in most experiments and is called the 'standard medium'. It was made to have, after reconstitution with part B, the following composition: 50 mM manitol, 50 mM NaCl, 20 mM Tris-Hepes, pH 7.4 and 1 mM Dglucose [9]. In the uptake studies with different osmolarities a variable amount of mannitol was included in medium A Part A was freshly prepared or filtered through a 22 µm Millipore filter before use.

Part B was variable in the different experiments In the studies of D-glucose uptake, 5–10 µCi of D-[1-$^3$H]glucose (Amersham), specific activity 6.7 Ci/mmol, was included in 50 µl of medium B, which was preincubated with an equal part of A before use.

In the iron uptake studies, Part B included radioactive and carrier-iron The final iron concentration is given for every single experiment in the results section. Iron test doses were freshly prepared for every set of experiments. For studies of Fe(III) uptake a chelating agent had to be used because of the hydrolytic tendencies of Fe(III) and the low solubility of ferric hydroxide complexes [10]. For this purpose citrate was chosen. We calculated that a 1000-fold molar excess of citrate was necessary at pH 7.4 in order to obtain a maximum [Fe] less than $10^{-19}$ M, using Eqn. 7 from Aisen et al [11]. This is sufficiently low to prevent hydrolysis. Ferric dicitrate complex formation was taken into account [12]. In all experiments $^{55}FeCl_3$ (Amersham) in 0.1 M HCl, specific activity 4 86 Ci/mmol, was used. To obtain the desired total concentration of Fe, an appropriate amount of 0 5 mM $FeCl_3$ in 0 1 M HCl was added to the tracer. Enough citric acid (J.T Baker) was added to obtain a 1000-fold molar excess After 10 min at low pH a calculated amount of NaOH solution was added in dropwise fashion with active stirring, to increase the pH to 7.0–7 4 After addition of buffer (Tris-Hepes, pH 7.4 in medium A) the measured pH in the final reaction mixture was 7.4.

During preparation of the reaction medium for Fe(II) uptake studies several precautions were taken to avoid oxidation. First, $^{55}FeCl_3$ was added to at least a 5-fold molar excess of ferrous sulphate (Sigma) in 0.1 M HCl. A 20-fold molar excess of L-ascorbic acid (J T. Baker) was then added to the iron. After 20 min the pH was brought to pH 7.4 with $NaHCO_3$. Medium B was saturated with $N_2$ as were all the other solutions for preparation of the Fe(II) incubation medium, and reconstituted with medium A immediately before every test.

All reagents were analytical grade and doubly distilled, deionized water was used in all experiments

*Uptake method*

The uptake of labeled iron and glucose was studied with a rapid Millipore filtration technique. The vesicles were freshly prepared the same day or taken from stock, frozen at −70°C and thawed at 37°C before a set of experiments. Vesicles were kept on ice until directly before the uptake test. Usually 20 µl vortexed vesicle suspension was added to 100 µl incubation medium in a glass culture tube in a shaking waterbath or on melting ice. The reaction was terminated at desired times by removal, after brief vortexing, of a 20 µl aliquot from the medium, which was immediately applied to a wet Millipore filter (PHWP 025 00, pore size 0.3 µm), mounted on a glass fritted disc, connected to a vacuum pump. Use of a battery of four filters allowed filtration of samples within short time intervals. The vesicles were washed on the filters immediately after application with at least 4 ml of an ice-cold NaCl stop-solution, which had always a higher osmolarity than the incubation medium. In the D-glucose uptake studies 0.7 mM phloridzin was added to the stop-solution, as a specific inhibitor of D-glucose uptake. In a number of iron uptake studies the 20 µl samples were first diluted in an ice-cold 1 ml stop-solution, containing 0.1 mM $FeCl_3$ and 100 mM citrate, neutralized to pH 7.0 with NaOH. After 1 min this solution was Millipore filtered and washed as described. The filters were solubilized in Aquasol (New England Nuclear) and radioactivity was measured, after an interval of at least 6 h in the dark, with a liquid scintillation counter (Beckmann LS 7500), equipped with automatic quench compensation. In every experiment results were corrected for nonspecific binding of radioactivity to the filters. This binding was always very low in the experiments with D-glucose and Fe(III) citrate, the total counts being less than twice the amount derived from a wet Millipore filter blank. Nonspecific binding of $^{55}Fe(II)$ was about five times as high, and could not be abolished by a number of variations of the washing method. The amount of nonspecifically bound $^{55}Fe$ in these experiments, however, was usually less than 5% of the counts associated with the vesicles.

All uptake studies, if not done in triplicate, were repeated several times with similar results using vesicles from different preparations. When uptake values were calculated as nmol Fe/mg membrane protein, variation of results was usually less than 20%, for identical concentrations of iron even when vesicles from different batches were used, provided Fe concentrations in the incubation media were expressed as nmol/mg protein.

*Other methods*

Protein was estimated according to Peterson [13], using bovine serum-albumin (Schwarz-Mann) as a standard. Sucrase activity, which is highly specific for mucosal membranes, was measured by the method of Dahlquist [14]. The glucose liberated in this reaction was assayed by a glucose oxidase method using Statzyme Glucose 500 (Worthington Diagnostics).

## Results

*Characterization of vesicles*

The purification method used yields a highly purified suspension of brush border membrane vesicles [9]. Vesicles from the different preparations used in this study were characterized routinely by assessment of the increase of specific sucrase activity. The final vesicle suspension ($n = 4$) contained 2.75 ± 0.26% of the protein ($m \pm$ S.E.) and 25.2 ± 2.2% of the sucrase activity of the first homogenate, representing a mean increase of the specific sucrase activity of 9 2.

Functional integrity of the vesicles was tested by the measurement of carrier mediated D-glucose transport. The electrochemical gradient, which was provided by NaCl included in part A of the medium, resulted in an initial facilitated diffusion of the D-glucose, which is very specific for microvillous membranes [15]. The overshoot was usually more than 10-times the equilibrium value after back diffusion of D-glucose into the medium, which compares very well with other studies using the same system [9,16,17]. Moreover, this established that the vesicles were sealed and oriented right-side out. The fast initial uptake of D-glucose could be inhibited with 0.7 mM phloridzin. When the vesicles were preincubated with 50 mM NaCl there was only a slow diffusion of D-glucose into the vesicles until equilibrium occurred. The same pattern was observed when the D-glucose carrier was inactivated by heating of the vesicle suspension for 5 min at 100°C. The intravesicular space, calcu-

lated from the amount of included D-[$^3$H]glucose, was 0.038% of the final volume of the reaction mixture before and after heating indicating that the vesicles remained intact. When increasing amounts of mannitol were added to the media to increase their osmolarity, a linear reduction of D-glucose uptake was seen, which could be extrapolated to zero at infinite osmolarity, showing that there is true membrane transport of D-glucose rather than simple binding. No difference was found with respect to D-[$^3$H]glucose uptake between freshly prepared and 4-month-old vesicles stored at −70°C.

*Uptake of ferric citrate*

*Temperature dependence.* Uptake of Fe(III) at 37°C was consistently 5–6-times higher than at 0°C (Fig. 1). The uptake velocity gradually decreased after 1 min, but total uptake of $^{55}$Fe-labeled ferric citrate was still increasing after 60 min incubation. There was a good reproducibility of Fe(III) uptake at 37°C and 0°C, measured during different experiments using vesicles from the same preparation. Vesicles stored for 2 months at −70°C showed the same characteristics of Fe(III) uptake as fresh vesicles.

*Binding or transport.* To distinguish between binding of ferric citrate to the membrane and transport into an intravesicular space, vesicles were incubated

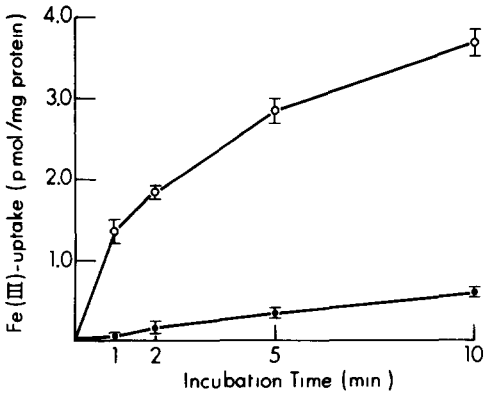


Fig 1. Uptake of $^{55}$Fe(III) citrate by rabbit intestinal microvillous membrane vesicles. The reaction mixture contained 1.72 μM Fe(III) and 1 72 mM citrate at pH 7 4. Final concentration of the vesicles was 0.77 mg protein/ml. Indicated are mean ± S.E of independent experiments at 37°C (○——○, $n$ = 4) and 0°C (●——●, $n$ = 3) Before Millipore filtration every sample was diluted in an ice-cold stop-solution with 100 μM Fe(III) and 100 mM citrate.

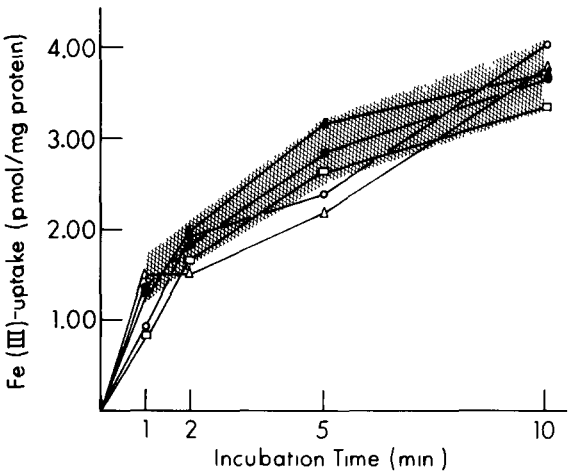


Fig. 2. Influence of osmolarity on uptake of $^{55}$Fe(III) citrate by intestinal mucosal membranes at 37°C and pH 7 4. Concentration of Fe(III), citrate and vesicles as in Fig 1. The reaction mixture contained in addition 20 mM Tris-Hepes, 42 mM NaCl, 0.9 mM D-glucose and sufficient mannitol to reach the following osmolarities. 0.200 osM (●), 0 275 osM (○), 0.286 osM (△), 0.322 osM (▲), and 0 390 osM (□). The shaded area indicates the range of values of four experiments using a 0.200 osM reaction mixture.

in hypertonic media with increasing osmolarity to promote shrinkage of the vesicles [18]. To eliminate the contribution of weakly bound, exchangeable $^{55}$Fe, every sample was washed in a 1 ml ice-cold stop-solution with excess ferric citrate as described in the methods. No difference was observed in the amount of $^{55}$Fe associated with the vesicles during incubation in media with increasing osmolarity (Fig. 2), indicating that the Fe(III) was merely bound to the membrane and not transported into an osmotically active space.

*Binding of Fe(III) or of citrate.* To investigate whether uptake of $^{55}$Fe by the vesicles results from binding of the ferric citrate complex or only the iron, doubly labeled media with $^{55}$Fe(III) and [$^{14}$C]citrate (New England Nuclear) were studied with results shown in Fig. 3. Binding of $^{55}$Fe continued to increase for at least 60 min while binding of $^{14}$C exhibited saturation within 2 min. It should be noted that, in our experiments, the molar ratio of citrate to iron on the vesicles is always much greater than unity. We cannot tell, therefore, whether vesicle associated $^{55}$Fe represents a ferric citrate complex,

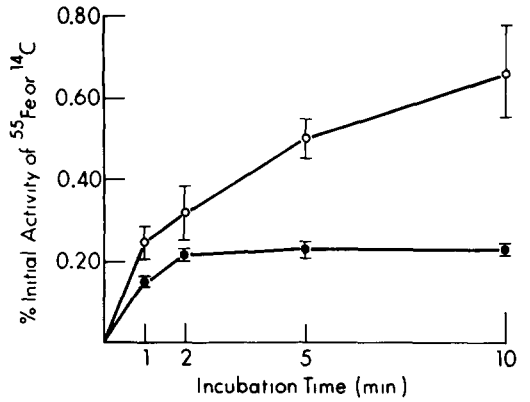


Fig 3 Uptake of $^{55}$Fe(III) (○———○) and [$^{14}$C]citrate (●———●) by mucosal membrane vesicles from a reaction medium containing 1.77 μM Fe(III) and 1.77 mM citrate, at 37°C and pH 7.4 Final concentration of vesicles was 0.66 mg protein/ml. Indicated are mean ± S.E. of three different experiments All samples were washed directly on the filter with 4 ml ice-cold 0.154 M NaCl.

or Fe(III) dissociated from citrate.

*Involvement of protein as an iron receptor.* In order to investigate whether or not the Fe(III) was bound to protein, serving as an iron receptor in the mucosal membrane, vesicle proteins were denaturated by heating during 5 min at 100°C in a waterbath. Iron uptake was compared with that in non-heated vesicles. A typical set of experiments is shown in Fig. 4. It appeared that the vesicles retained their capacity to bind Fe(III) after boiling. Fe(III) uptake by the boiled vesicles was still temperature-dependent and five times as high at 37°C than at 0°C. In both normal and boiled vesicles the binding of most of the Fe(III) to the membranes was very tight, because only a limited amount of the $^{55}$Fe could be removed by dilution of the vesicles in a large excess of ice-cold nonradioactive ferric citrate. The amount of reversibly bound $^{55}$Fe, which could be chased by cold iron, was even larger in the boiled vesicles. Substantially identical results were obtained with vesicles from different preparations and different iron and vesicle concentrations.

*Concentration dependence of iron binding.* The concentration dependence of iron binding to the mucosal membranes of both normal and boiled vesicles was studied using incubation media with Fe(III) concentrations between 1 and 10 μM, always with a 1000-fold molar excess of citrate. Reliable experiments with higher iron concentrations are not feasible with this system because both osmolarity and ionic strength of the reaction medium would increase prohibitively. In the concentration range studied Fe(III) uptake exhibited saturation kinetics for normal as well as for boiled vesicles (Fig. 5). As in the previous experiment different washing procedures were used. Uptake kinetics were the same for heated and non-heated vesicles when the samples were diluted in excess ferric citrate before filtration. Boiled vesicles, however, exhibited a higher affinity for Fe(III) when washed only with saline. In a Lineweaver-Burk

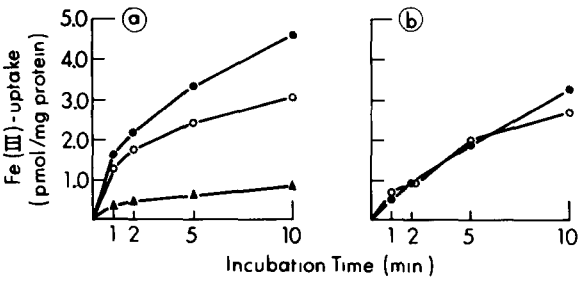


Fig. 4 Influence of membrane-protein denaturation on $^{55}$Fe-(III)citrate uptake by mucosal membrane vesicles. Normal vesicles were tested at 37°C (○) and vesicles, heated for 5 min at 100°C, at 37°C (●) and 0°C (▲), all at pH 7.4. The reaction mixtures contained 0.86 μM Fe(III) and 0.86 mM citrate Vesicle concentration was 1 67 mg protein/ml. The vesicles were either washed directly on the filter with 6 ml ice-cold 0.15 M NaCl (a) or first diluted in 1 ml ice-cold stop-solution with 100 μM Fe(III) and 100 mM citrate (b).

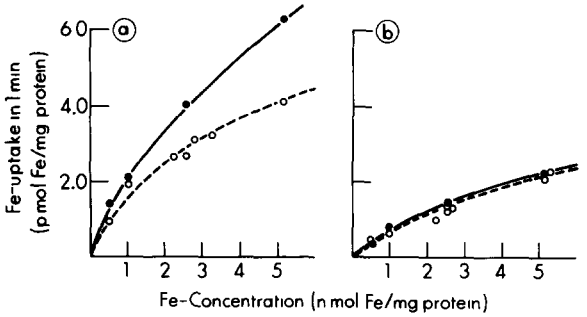


Fig. 5. Concentration dependence of initial Fe(III) uptake by normal mucosal membrane vesicles (○) and vesicles heated for 5 min at 100°C (●). Vesicle concentration was always 1.67 mg protein/ml Fe(III) concentration ranged from 0.86 to 8.58 μM, always with a 1000-fold molar excess of citrate. Temperature was 37°C and pH 7.4. Vesicles were washed directly on the filter (a) or were first diluted in a stop-solution with excess Fe(III) and citrate (b).

plot all four curves could be transformed into straight lines, suggesting Michaelis-Menten saturation kinetics. This is not meant to imply, however, that specific enzymic reactions are entailed in iron uptake. The curves can as well be analyzed on the basis of adsorption isotherm analysis with the same results. Furthermore, because of the similarity between the normal and the heated vesicles, it is unlikely that a specific protein is responsible for the binding of Fe(III) to the membranes. The difference in affinity for Fe(III) by saline-washed vesicles before and after heat treatment may be the result of changes in the lipid bilayer, which can be irreverible after heating above

*Influence of lower pH and lower citrate concentration.* When vesicles were incubated in a reaction medium with the same Fe(III) concentration but with a lower pH, an increase of iron uptake by the membranes was observed (Table I). This may be a result of dissociation of the ferric citrate complex at the lower pH Reduction of the liberated Fe(III) may also have occurred because of the presence of reducing agents like D-glucose Even a small amount of $^{55}Fe(II)$ would have resulted in increased binding of $^{55}Fe$. When uptake tests were performed in a medium with only a 20-fold molar excess of citrate, uptake of Fe(III) was higher than when a 1000-fold molar excess of citrate was used (Table II) This difference may be an effect of competition by the citrate and the membrane for the iron. With a 20-fold molar excess of citrate, however, at pH 7.4, hydrolysis of iron and formation of polynuclear complexes are possible. Interpretation of iron uptake from such media is difficult, therefore.

TABLE I

INFLUENCE OF pH ON Fe-UPTAKE BY MUCOSAL MEMBRANE VESICLES FROM A STANDARD REACTION MIXTURE, CONTAINING 4.3 $\mu$M Fe(III) AND 4.3 mM CITRATE, AT 37°C

Final concentration of vesicles was 1.67 mg protein/ml.

| | Fe uptake (pmol/mg protein) after | | | |
|---|---|---|---|---|
| | 1 min | 2 min | 5 min | 10 min |
| pH 7.4 | 2.5 | 3.2 | 5.5 | 5 9 |
| pH 3 7 | 151 | 216 | 211 | 233 |

TABLE II

INFLUENCE OF THE AMOUNT OF CITRATE IN THE REACTION MEDIUM ON Fe(III) UPTAKE BY MUCOSAL MEMBRANE VESICLES, AT 37°C, IN THE STANDARD REACTION MIXTURE

Final concentration of vesicles was 1.67 mg protein/ml.

| Medium | Fe uptake (pmol/mg protein) after | | |
|---|---|---|---|
| | 2 min | 5 min | 10 min |
| 1 $\mu$M $Fe^{3+}$ | | | |
| 1 000 $\mu$M citrate | 1.2 | 2 2 | 2.7 |
| 1 $\mu$M $Fe^{3+}$ + | | | |
| 20 $\mu$M citrate | 13 3 | 19 3 | 26.4 |

*Uptake of Fe(II)*

Precautions were taken to retain the iron in the divalent form during these experiments. It could be calculated from the equilibrium studies of Ulmgren and Wahlberg [20] that practically all the Fe(II) under the conditions used was complexed as ferrous ascorbate. The uptake of Fe(II) was, using the same vesicles and the same wash procedure, several hundred times higher than that of Fe(III) from a ferric

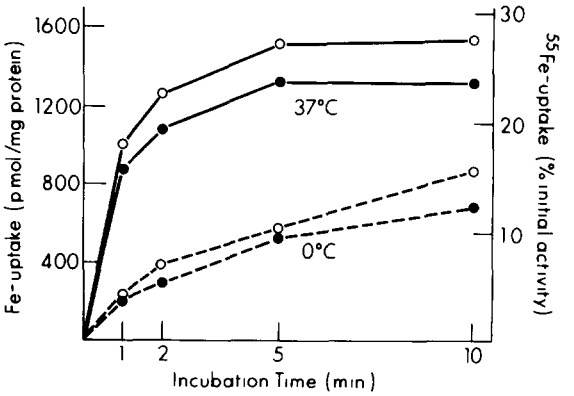


Fig 6 Uptake of $^{55}Fe(II)$ by intestinal mucosal vesicles at 37°C (———) and 0°C (- - - - -) using normal (○) and heat-treated (●) vesicles The incubation media contained 4.29 $\mu$M Fe(II) with a 20-fold molar excess of L-ascorbic acid, pH 7.4. Concentration of vesicles was 0.77 mg protein/ml The osmotic space within the vesicles was 0.045% of the total reaction medium

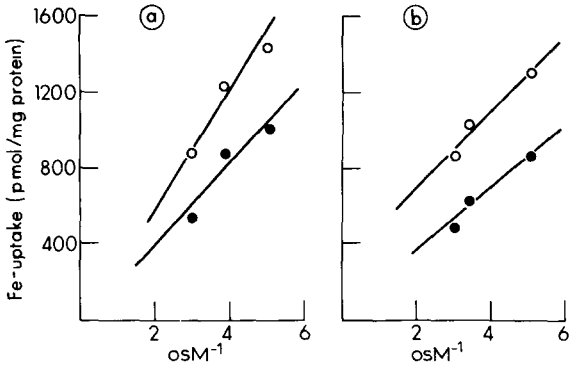


Fig. 7 Influence of osmolarity on uptake of $^{55}$Fe(II) by normal vesicles (a) and vesicles heated for 5 min at 100°C (b) Uptake values after 1 min (•) and 10 min (○) are shown. The media contained 4 3 μM Fe(II) with a 20-fold molar excess of L-ascorbic acid, 20 mM Tris-Hepes, 42 mM NaCl, 0 9 M D-glucose and sufficient mannitol to reach the indicated osmolarity Temperature was 37°C and pH 7 4

citrate medium (Fig. 6). Identical results were found for normal and heat treated vesicles. The influence of temperature on uptake of Fe(II) was much less than on Fe(III) uptake. The high uptake of Fe(II), even after short incubation times, reduced the iron concentration of the medium during the experiment considerably. This became even more evident when the samples were not diluted in a ferric citrate stop-solution, but washed with 4 ml ice-cold saline directly on the filter. Using the same non-heated vesicle as in Fig. 6, the amount of $^{55}$Fe associated with the vesicles after 1 min incubation was 35% and after 10 min was 49% of the initial activity in the medium.

To differentiate between membrane binding and transport into an intravesicular space also $^{55}$Fe(II) uptake was studied from reaction media with increasing osmolarity. Samples were washed in excess ferric citrate to remove reversibly bound $^{55}$Fe from the membranes. The results, as shown in Fig. 7, indicate that Fe(II) is transported into the lumen of the vesicles. The effect of increasing osmolarity of the reaction media was obscured when the vesicles were washed in saline, presumably because large amounts of bound $^{55}$Fe remained on the membrane surface.

## Discussion

The absorption of iron depends not only on factors related to microvillous and basolateral membranes but also on intraluminal and intracellular factors, on the rate and extent of submucosal perfusion and the concentration and turnover of iron and iron-binding molecules in cells and extracellular fluids. Purified microvillous membrane vesicles provided a system for investigating solely the interaction of iron with intestinal mucosal membranes.

The aqueous chemistry of iron necessitated special precautions in the preparation of the Fe(III) and Fe(II) test doses. We calculated that, using ferric citrate, a 1000-fold molar excess of citrate compared to iron is necessary at pH 7.4 to prevent formation of insoluble ferric hydroxide. Although hydrolysis of Fe(III) already occurs at pH 1, at neutral pH precipitation of ferric hydroxides is slow and may require several years to attain equilibrium [21]. At insufficient concentration of citrate or other iron complexing agents, hydrolysis of iron and formation of polynuclear clusters are inevitable, frustrating investigation of the physiology of membrane binding of Fe(III). Unless care is taken to prevent hydrolysis, binding of Fe(III) to membranes may merely represent the chemical process of hydroxide formation localized on the membranes. The amount of $^{55}$Fe bound to mucosal membrane vesicles from a ferric citrate solution, in which hydrolysis should not occur, as demonstrated in our experiments, is very low. Higher uptake values were observed at lower citrate concentrations and lower pH, allowing hydrolysis and dissociation of ferric citrate respectively. This may explain the higher uptake of Fe(III) by mucosal membrane vesicles observed by Cox and O'Donnell [8]. Other authors, using intact mucosal cells instead of mucosal vesicles [22], found uptakes of iron 100-times greater than ours. In these experiments, however, ascorbic acid was added in 8-fold molar excess to the $FeCl_3$ at pH 2, so that in fact Fe(II) uptake may have been studied.

We found no transmembrane transport of Fe(III). Binding, however, was temperature dependent, followed saturation kinetics, and was almost identical in heat-treated vesicles. Apparently the iron-binding sites on mucosal membranes are heat resistant, which makes involvement of specific iron-binding proteins as receptors very unlikely One possibility is that Fe(III) binds to glycolipids or to sugars on glycoproteins by metabolically passive processes which may be spared by heat treatment. A cation gradient

from superficial towards deeper layers of the membrane can develop with a slow diffusion rate Even if binding of $^{55}$Fe(III) to deeper sites in the membrane is reversible, exchange with nonradioactive iron in a wash solution can be insignificant over a brief time, especially when diffusion-rate is slowed down and membrane properties change at low temperature. Superficially bound $^{55}$Fe, however, should readily exchange with excess iron in the medium. In our experiments with Fe(III) no difference was seen in the binding of 'nonexchangeable' iron between normal and heat-treated vesicles. The amount of Fe(III) bound to the membranes after only washing with saline is even larger in heat-treated vesicles 'Exchangeable' iron, the difference between membrane related $^{55}$Fe after wash with saline and with excess ferric citrate, might then represent the superficially bound iron.

In the uptake experiments with Fe(II) a large proportion of $^{55}$Fe in the medium was bound to the vesicles even after short incubation times. Approximately 50% of this $^{55}$Fe was exchangeable with cold iron. In contrast to results with ferric iron, it appeared that the nonexchangeable Fe(II) was not membrane-bound, but internalized into an intravesicular space. This finding is identical with the results of Eastham et al. [7] with Fe(II) in rat enterocyte membrane vesicles. The accumulation of $^{55}$Fe inside the vesicles against an enormous concentration gradient is remarkable Only mannitol and Tris-Hepes, pH 7.4 were included in the vesicles, and were also present in the incubation medium An active uptake mechanism for Fe(II) was not established and is unlikely in the face of the high uptake of Fe(II) by heat-treated vesicles. Uptake against a concentration gradient can only be explained if there exists a 'trap' inside the vesicles which decreases the intravesicular aqueous iron concentration. Such a trap could be provided by rapid oxidation of Fe(II) and hydrolysis of the resulting Fe(III) on nucleation centers located on the inner cell surface.

We interpret our findings as indicating that uptake of Fe(III) by microvillous membrane vesicles entails binding to strong sites, perhaps on glycolipids or glycoproteins. Transport of Fe(II) is a passive process which entails both binding and diffusion, and is probably limited by the intravesicular volume and the available intravesicular, or intracellular, iron-binding sites. Our results are consistent with the view that the intestinal mucosal membrane plays only a passive role in the regulation of iron absorption

## Acknowledgements

This study was supported by the Netherlands Organisation for the Advancement of Pure Research (Z.W.O ), Grant No. S91-104, and the National Institutes of Health, Grant No. AM 15056. For valuable advice we are greatly indebted to Dr. Jill Eveloff, Dr. Eva Kinne-Saffran, and Dr. Rolf Kinne.

## References

1 MacCance, R A. and Widdowson, E.M. (1937) Lancet 1, 680–684
2 Manis, J.G. and Schachter, D (1962) Am. J Physiol. 203, 73–80
3 Marx, J J.M (1979) Scand. J. Haematol. 23, 293–302
4 Greenberger, N J , Balcerzak, S P. and Ackerman, G A. (1969) J Lab Clin. Med. 73, 711–721
5 Kimber, C L., Mukherjee, T and Deller, D J. (1973) Dig Des 18, 781–791
6 Murer, H and Kinne, R (1980) J Membrane Biol 55, 81–95
7 Eastham, E.J , Bell, J.I and Douglas, A P (1977) Biochem J 164, 289–294
8 Cox, T M. and O'Donnell, M.W (1981) Biochem. J 194, 753–759
9 Kessler, M , Acuto, O , Storelli, C , Murer, H., Muller, M. and Semenza, G (1978) Biochim. Biophys. Acta 506, 136–154
10 Sylva, R.N (1972) Rev Pure Appl. Chem. 22, 115–131
11 Aisen, P , Leibman, A. and Zweier, J (1978) J Biol Chem 253, 1930–1937
12 Spiro, T C , Bates, G. and Saltman, P (1967) J Am. Chem. Soc 89, 5559–5562
13 Peterson, G.L (1977) Anal. Biochem. 83, 346–356
14 Dahlquist, A. (1964) Anal Biochem. 7, 18–25
15 Murer, H , Hopfer, U , Kinne-Saffran, E and Kinne, R. (1974) Biochim Biophys Acta 345, 170–179
16 Lucke, H , Berner, W , Menge, H. and Murer, H (1978) Pflugers Arch. 373, 243–248
17 Klip, A , Grinstein, S and Semenza, G (1979) FEBS Lett 99, 91–96
18 Hopfer, U , Nelson, K , Perrotto, J and Isselbacher, K.J (1973) J Biol Chem 248, 25–32
19 Brasitus, T A , Tall, A.R. and Schachter, D. (1980) Biochemistry 19, 1256–1261
20 Ulmgren, P and Wahlberg, O (1974) Acta Chem. Scand. A28, 631–637
21 Baes, C F , Jr. and Mesmer, R.E (1976) The Hydrolysis of Cations, pp 226–237, John Wiley and Sons, New York
22 Savin, M.S. and Cook, J.D. (1978) Gastroenterology 75, 688–694