We have studied Fe(III)-citrate and Fe(II)-ascorbate uptake by purified intestinal brush-border membrane vesicles from normal (iron-replete) and iron-deficient mice. In iron-replete mice using a final Fe(III) concentration of 1.43 μM, 25–30 pmol of Fe(III)/mg of protein were bound to the membranes versus 65–70 pmol in iron-deficient mice. Fe(II) uptake in normal mice using a final Fe(II) concentration of 1.79 μM was 1600–1600 pmol/mg of protein versus 3600–4000 pmol in iron-deficient mice. Evidence that Fe(II) was transported into the vesicles by a membrane carrier-mediated process was obtained by observing saturation kinetics under conditions of isotope exchange at equilibrium in mice rendered iron-deficient, but not in iron-replete mice. Eighty per cent of the transported Fe(II) could be removed by strong chelating agents. The remainder was exchangeable with Fe(II) in the medium when measured under equilibrium conditions.

We can explain these results by the following model; iron uptake appears to be a 2-fold process. The first step is the transport of Fe(II) across the membrane by a carrier-mediated process which is biologically regulated. The second step is the subsequent binding of iron on the inside of the membrane. The number of binding sites is also regulated by the iron status of the mouse. The membrane binding affinity for Fe(II) appears to be weaker than that for dithiothreitol but stronger than for ascorbate.

Intestinal absorption of iron is the major pathway for regulating the quantity of body iron stores (1). It seems to consist of two major, functionally separable transport steps: 1) uptake of iron from the lumen into the mucosa, and 2) transfer of iron from the mucosa into the blood (2). An additional factor determining overall intestinal iron absorption may be the intracellular iron pool(s). A recent compartment analysis of iron absorption in beagle dogs actually suggested that the rate-limiting step is uptake of iron from the gut lumen (3). The mechanisms and exact cellular sites of control involved in iron absorption are, however, only poorly understood.

Purified brush-border membrane vesicles constitute a relatively simple experimental system suited to investigate the uptake step without interference from whole cells (4–7), in particular, the intracellular iron pool, cellular metabolism, protein synthesis, and the exit step across the basolateral plasma membrane (8). Only a few studies of iron transport using brush-border membrane vesicles have been published (9–12). The results are conflicting. As discussed by Marx and Aisen (12), the earlier iron uptake studies with brush-border membranes (9–11) are compromised by insufficient attention to the chemistry of Fe(II) and Fe(III) and possibly precipitation of ferric hydroxide during the iron uptake measurements.

The present studies on iron uptake by mouse intestinal brush-border membranes were stimulated by the report of Marx and Aisen (12) that rabbit intestinal brush-border membranes transport Fe(II), but not Fe(III). Since the iron status of mice can be altered relatively easily, and since iron deficiency is associated with increased intestinal iron absorption, the mouse model could provide additional insight into the biochemistry and control of iron absorption. Our studies confirm the observations by Marx and Aisen (12); however, additional experiments (with membranes from control as well as iron-deficient mice) suggest that their major conclusions should be re-evaluated. In particular, the bulk of both Fe(II) and Fe(III) uptake by brush-border membranes results from binding to some component(s) of the isolated brush-border membranes. The amount of binding is biologically regulated and increases with iron deficiency. At least for brush-border membranes from iron-deficient mice, the kinetics of iron uptake suggests a two-step process of transport across the membrane and subsequent binding on the inside of the vesicles. The membrane transport step exhibits saturation, a characteristic of catalytic (carrier-mediated) processes.

EXPERIMENTAL PROCEDURES

Brush-Border Membrane Preparation—For each experiment, the brush-border membrane vesicles were prepared from the upper half of the small intestine of two DBA/2J male mice (Jackson Laboratories, Bar Harbor, ME) and were used within 5 h after preparation. The isolation procedure is based on the divalent cation precipitation method of Schmitz et al. (13). Several major modifications were adopted; however, to optimize the purification and yield and to minimize preparation time with the mouse material. Therefore, a complete description of the method is given. All steps were performed on ice or at 4 °C. Mice were killed by cervical dislocation, and the intestines were immediately removed and rinsed with ice-cold 0.9% NaCl solution. The intestines were everted on a glass rod, and the brush-borders were removed by scraping with glass microscope slides. The scrapings were homogenized with a Tekmar Tissuemizer (Tekmar Co., Cincinnati, OH) at full speed for 12 min in 20 volumes of Solution A consisting of 0.1 M D-mannitol and 1 mM Tris/HEPES.

The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PIPES, pipеразин-N'-N''-бис(2-этансульфонной кислоты); cholamine, [(2-aminoethyl)trimethylammonium chloride hydrochloride]; DFO, 1-amino-6,17-dihydroxy-7,10,18,21-tetraazao-27-(N-acetylhydroxylamino)-6,11,17,22-tetraazahelptasicosane.

*This work was supported by National Institutes of Health Grant AM-25170 and by the American Heart Association, Northeast Ohio Affiliate, and the Genetics Center, Case Western Reserve University, Cleveland, OH 44106. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**To whom correspondence should be addressed at the Department of Medicine, Case Western Reserve University, 2065 Adelbert Road, Cleveland, OH 44106.
pH 7.5. MgCl₂ was added to a final concentration of 20 mM, and the homogenate was stirred in the cold for 30 min to precipitate intracellular Fe, lipid, and basolateral plasma membranes. The homogenate was centrifuged for 10 min at 6,000 × g in a refrigerated Beckman J2-21 centrifuge (rotor JA-20.1). The pellet was discarded, and the supernatant solution was spun for 30 min at 21,000 × g. The resulting pellet from the latter step was resuspended in 10 volumes of Solution A and homogenized with a Teflon-rod in glass homogenizer at 1,000 rpm for six strokes. MgSO₄ was added to a final concentration of 0.1 mM, and the suspension was centrifuged for 10 min at 6,000 × g. The pellet was discarded and the supernatant resuspended in 21,000 × g for 25 min to yield pelleted brush-border vesicles. The membrane vesicles were resuspended in Solution A plus 0.1 mM MgSO₄ by passage through a 27-gauge needle. The suspension was centrifuged for 5 min at 2,000 × g to remove any residual bacteria. The purified membrane vesicles were collected by centrifuging the supernatant at 48,000 × g for 15 min and suspending in Solution A plus 0.1 mM MgSO₄ to the desired concentration prior to the iron uptake experiments.

Vesicles were prepared from iron-deficient mice in an identical fashion. Iron deficiency was produced by feeding mice low iron diets (ICN Nutritional Biochemicals, Cleveland, OH) for 6–8 weeks. Hepatic non-heme iron content of iron-deficient mice was 10 ± 2 μg of Fe/g, wet weight (mean ± S.D.), while normal mouse hepatic non-hemoglobin iron content was 14 ± 2 μg of Fe/g, wet weight. The density determination of the hepatic iron content of liver was determined by the technique of Dahlqvist (14) and alkaline phosphatase by the method of Forstner et al. (15).

Protein Determination—Protein was assayed according to the method of Lowry et al. (16) with crystalline bovine serum albumin as the standard.

D-Glucose Uptake Studies—Uptake of D-glucose served to assess the functional integrity of the vesicles. The membranes at a final concentration of 2–3 mg/ml were incubated at 25 °C in an uptake medium consisting of (final concentration) 0.1 M D-glucose containing D-[³H]glucose (0.2 μCi/μl), D-mannitol (0.1 M), Tris/HEPES (0.1 M), NaSCN (0.1 M), pH 7.4. Uptake was terminated by removal of a sample and dilution with a 50-fold excess of an ice-cold stop solution consisting of 0.9% NaCl, 10 mM Tris/HEPES, and 0.2 mM phenolformalin. The membranes were immediately collected on a wetted Millipore filter (HA025, pore size 0.45 μm) and washed once with 3 ml of the stop solution. Radioactivity was then measured in a Beckman LS-7500 scintillation counter. The fast uptake of D-glucose could be inhibited by thiocyanate, a known inhibitor of Na-glucose cotransport.

Iron Uptake Studies—Unless otherwise indicated, membranes were incubated in an uptake medium which consisted of a 1:1 mixture of Solution B and either of two iron solutions designated as Solution C (Fe(III)) or Solution D (Fe(II)). Solution B had the following composition: 0.1 M mannitol, 0.1 M NaCl, 40 mM Tris/HEPES, pH 7.4, and 250 μM FeCl₃. Where single experiments are presented they represent a typical experiment that was repeated numerous times with similar results.

Solution C—Fe(III)·citrate was prepared just prior to use by adding a 1000-fold molar excess of citrate to 0.1 mM FeCl₃ in 0.1 N HCl. After 10 min, the pH was adjusted to 7.0–7.4 by the dropwise addition of 2 N NaOH. The final volume was increased with water so that the Fe(III) concentration was 3.44 μM, and the citrate concentration was 3.44 mM.

Solution D—Fe(II)·ascorbate used in the Fe(II) uptake studies was prepared by mixing 0.1 mM FeCl₃ in 0.1 N HCl to a 5-fold molar excess of ferrous ions. The solution, 5.4 μM FeCl₃·ascorbate, was diluted to 0.1 N HCI. A 20-fold molar excess of ascorbate (stock solution, 20 mM) was added to the iron, and after 20 min, the pH was adjusted to 4.5 with NaHCO₃. After an additional 30 min at pH 4.5, sufficient NaHCO₃ was added to bring the pH to 7.4 and the solution saturated with N₂. The final iron concentration was 4.3 μM. In studies involving higher iron concentrations, the appropriate amounts of FeSO₄ and unlabeled FeCl₃ were added, always with a 5-fold molar excess of FeSO₄.

In the iron uptake experiments, 20 μl of membranes suspended in Solution A at 4.62 mg/ml in the Fe(III) studies and 2.52 mg/ml in the Fe(II) studies were added to uptake buffer consisting of 1 ml of Solution B and 50 μl of either Solution C or Solution D into a glass culture tube. The reaction mixture was incubated in a 37 °C water bath, and aliquots of 20 μl were removed at set intervals. The aliquots were immediately placed in 1 ml of ice-cold stop solution consisting of 0.1 mM FeCl₃ and 100 mM citrate adjusted to pH 7.0 with NaOH. The stop solution was then filtered through a wetted Millipore filter (HA025, pore size 0.45 μm), and the collected membranes were washed once with 4 ml of cold saline. The filters were γ- counted for determination of the amount of Fe associated with the membrane vesicles. In all experiments, results were corrected for nonspecific binding (incubation without membranes) of labeled iron to the filters. This correction was always less than 1% of the total counts.

Percoll Gradient Centrifugation of Membranes—Membranes were incubated in Solution B + D with 10.75 μM Fe(II) for 15 min at 37 °C for maximal iron uptake and then diluted into a 5-fold volume excess of ice-cold stop solution. Membranes were collected by centrifuging the suspension at 48,000 × g for 20 min. The pellet was then resuspended in Solution A and added to a 17.5% (v/v) Percoll solution containing Tris/HEPES and 0.9% NaCl in a 15-ml polycarbonate centrifuge tube. The tube was spun at 48,000 × g in the Beckman J2-21 centrifuge (JA 20.1 fixed angle rotor) for 20 min. The gradient thus formed was then fractionated into 0.5-ml aliquots from top to bottom by introducing a 65% sucrose solution into the bottom of the tube through a 1 mm steel needle. The fractions were assayed for alkaline phosphatase activity to locate the membranes on the gradient. An identical gradient was run using density marker beads (Pharmacia Fine Chemicals) in place of membrane vesicles for isolation and quantitation of the fraction containing the highest alkaline phosphatase activity. The Percoll gradient as just described. The fractions collected from the second gradient were assayed for alkaline phosphatase activity and then γ-counted to locate the radioactivity within the gradient.

Kinetic Experiments—The kinetics of iron transport in membrane vesicles was investigated by the technique of isotope exchange at equilibrium (18).

Self-exchange was measured as release of labeled Fe(II). Vesicles were first incubated with Fe(II)-ascorbate in Solution B + D at 37 °C for 30–45 min. Isoxophate was then initiated by diluting the vesicles 5-fold with medium of the same chemical composition including Fe(II)-ascorbate, but without labeled isotope. In parallel experiments, increasing concentrations of Fe(II)-ascorbate were used while the amount of radioisotope was held constant.

Velocities were estimated as the time necessary to reach half-maximal isotope release (t₀) on plots of counts/min versus time (t₀ = time at (cpm₀ - cpm)/2, whereby subscript 0 and ∞ refer to zero and equilibrium times, respectively). Velocities are expressed as either the product of Fe(II) concentration times the inverse of t₀ or of Fe(II) amount/mg of protein times the inverse of t₀.

Materials—All solutions used in the preparation of the membranes and in the uptake studies were prepared with acid-washed glassware and double-distilled deionized water. D-[³H]Glucose (specific activity 15 Ci/mmol) was obtained from New England Nuclear, and FeCl₃ (specific activity 0.56 Ci/mmol) was from Amer sham Corp. The Millipore filters used in the uptake studies (HA025) had a pore size of 0.45 μm. Sigma supplied HEPES, PIPES, cholamine, D-mannitol, and FeSO₄. Dithiothreitol was obtained from ICN Nutritional Biochemicals (Cleveland, OH), and Percoll was from Pharmacia (Uppsala, Sweden).

RESULTS

Characterization of the Membrane Preparation

Purity

Yield and purification of the brush-border membranes were routinely assessed by the marker enzyme sucrase (Table I). In normal mice, the specific activity of sucrase in the final membrane was increased 18-fold over the homogenate of intestinal scrapings at a yield of about 30%. The enrichment factor was slightly lower in iron-deficient mice, although the specific sucrase activity of the final membrane was identical (Table I). The complete membrane preparation could be accomplished within 4.5 h so that uptake experiments could routinely be carried out on the same day.

Functional Integrity

To test for sealing of the membranes as vesicles, their transport competence for Na gradient-driven glucose uptake
was measured. It has been shown for several similar small intestinal membrane preparations from different animal species that D-glucose can be taken up into an intravesicular space formed by the membranes. Moreover, electrochemical Na gradients across the membrane will support transport of glucose against a glucose concentration gradient (8, 19).

The mouse membranes prepared without Na (i.e. in Solution A) were incubated with 0.1 M NaCl and 0.1 mM labeled D-glucose, and uptake of labeled glucose was measured as a function of time. A typical overshooting uptake was observed with peak values of 5-7 times the equilibrium. This result is similar to other studies with brush-border vesicles from other animals (8, 19). The glucose uptake pattern was unaltered by 15-min incubations of vesicles with or without the standard Fe(II) or Fe(III) solutions from which NaCl had been omitted. Assuming that the intravesicular glucose concentration at equilibrium is identical to the medium concentration, the intravesicular space can be calculated from the included glucose uptake at equilibrium. This space amounted to about 1.4 μl/mg of protein.

Uptake of Fe(III)

The chemical instability of ferric salts in aqueous solutions, particularly their tendencies to form ferric hydroxide polymers, makes it difficult to study Fe(III) transport by isolated membranes. Marx and Aisen (12) suggested that the problem could be circumvented by the use of Fe(III) complexed to a large excess of citrate (20). When mouse intestinal membranes were incubated with 1.43 μM Fe(III)-citrate in the presence of a 1000-fold molar excess of free citrate, time- and temperature-dependent uptake of Fe(III) could be measured. The Fe(III) uptake represents tightly associated iron since it is not removed by 100 mM citrate or exchanged for unlabeled Fe(III)-citrate in the wash solution. As shown in Fig. 1, there is an initial rapid uptake which levels off at about 10 min at 37°C. The apparent plateau values of Fe(III) uptake increase by about 4-fold between 0 and 37°C (Table II). The plateau values at the lower temperatures probably do not represent equilibrium as shifting the temperature from low to high after 10 min of incubation brought the uptake up to that of the higher temperature, while downshifts of the temperature had no effect on uptake (Fig. 1, dashed lines). To characterize, at least in a limited way, the initial fast uptake, half-times were calculated based on apparent plateau values. Interestingly, the apparent rate constant decreased with increasing temperature (Table II).

To distinguish whether Fe(III) was taken up into the intravesicular space or bound to the membrane, the dependence of Fe(III) uptake on vesicle volume was measured. Vesicles were incubated in media of increasing hypertonic mannitol concentrations to promote shrinkage of vesicles (8). Interestingly, Fe(III) uptake was independent of medium tonicity (or mannitol concentration) during the incubation. This result suggests that Fe(III) uptake was determined by binding of Fe(III) to some component(s) of the membrane preparation.

Fe(III) uptake by membranes from iron-deficient mice appeared qualitatively similar to that of control membranes. More importantly, the membranes from iron-deficient mice took up two to three times as much Fe(III)/mg of protein as controls, suggesting biological regulation of Fe(III)-binding sites (Table II).
Fe(I1) uptake decreased with decreasing temperatures (Fig. 2). As with Fe(III), equilibrium binding of Fe(I1) was temperature-dependent only when raising the incubation temperature from 0 to 37 °C (Fig. 2, dashed lines).

Effect of NaCl or KCl—We investigated the possibility that a Na concentration gradient provided a driving force for Fe(I1) uptake. Fe(I1) uptake experiments were performed with concentrations of NaCl between 0 and 200 mM. As a control, vesicles were preloaded with NaCl by preincubation at 25 °C for 1 h. Fe(I1) uptake was essentially identical under both conditions. When Na was exchanged for K, uptake was unchanged. To control for the effects of ionic strength on membrane uptake of Fe(I1), media containing different combinations of Na and K chloride at a constant total chloride concentration of 200 mM were tested. No difference in overall Fe(I1) uptake was observed.

Effect of Iron Deficiency on Fe(I1) Uptake—When intestinal vesicles prepared from iron-deficient mice were placed in the Fe(I1)-ascorbate test Solution D, plateau values of iron uptake were about twice normal (Fig. 3). This result indicates that the Fe(I1) uptake capacity is regulated in some manner by total body iron deficiency.

Evidence for Binding of Fe(I1) to Brush-Border Membranes

To assess whether the uptake of Fe(I1) by the intestinal membrane preparation was associated with the brush-border membrane rather than a minor contaminant, vesicles preloaded with Fe(I1) were fractionated on a Percoll density gradient (17.5% v/v). All fractions contained some alkaline phosphatase activity. Therefore, the fractions containing most of the enzyme were pooled and recentrifuged on a second Percoll gradient (17.5% v/v) and analyzed for alkaline phosphatase activity and 59Fe. 67% of the total counts/min and 80% of the alkaline phosphatase activity from the first gradient were applied to the second gradient. As demonstrated in Fig. 4, 59Fe(I1) co-migrated with the peak in alkaline phosphatase activity suggesting that the brush-border membranes were taking up the Fe. The density of the alkaline phosphatase peak corresponded to a density of about 1.02 g/cm3 as judged from dextran density markers. Since this value represents the equilibrium density of the membrane particles and is much lower than expected on the basis of protein and lipid composition of the membrane, intravesicular water must contribute substantially to the particle density. Thus, the density behavior of the membrane particles in the Percoll gradient strongly indicates that the isolated membranes form vesicles with the plasma membrane acting as a barrier between the intra- and extravesicular space.

Evidence for Reversibility of Fe(I1) Binding to Membranes

Reversibility of 59Fe(I1)-preloaded Vesicles

To test whether Fe(I1) uptake was reversible, vesicles were incubated in 59Fe(I1) uptake medium (Solution B + D) for 10 min, then pelleted by centrifugation, and resuspended in Tris/HEPES buffer either with or without unlabeled Fe(I1)-ascorbate. Aliquots were sampled over the following 45 min. In neither case was release of labeled Fe(I1) observed. This result contrasts with the situation when strong chelating agents were added, as discussed below.

Effect of Reducing Agents on Fe(I1) Release

Dithiothreitol was first used to study its effect on Fe(I1)-loaded vesicles. The membranes were incubated in Fe(I1) uptake medium for 10 min. At that time, varying concentra-
tions of dithiothreitol were added to the membranes in the Fe(II) medium. Aliquots of vesicles were then sampled over the following 45 min. Concentrations of dithiothreitol less than 1 mM had minimal effect on the amount of iron within the vesicles. On the other hand, in a concentration range of 1–10 mM dithiothreitol, there was a marked reduction in the amount of iron associated with the vesicles (Fig. 5), so that a new equilibrium was reached which amounted to about 20% of the iron originally taken up by the membranes. Experiments performed by first incubating vesicles for 25 min with other reducing agents such as ascorbic acid (0.1–2 mM), 2-mercaptoethanol (0.5–2 mM), and 2-mercaptoethanesulfonic acid (0.5–2 mM), unlike dithiothreitol, did not affect the binding of Fe(II) to vesicles (Table III).

When dithiothreitol in concentrations above 1 mM was added to the incubation medium prior to the addition of Fe(II), there was a marked reduction (75% less) in the amount of iron within Fe(II) associated with the membrane vesicles, and a lower equilibrium was reached in 10–15 min.

Effect of Chelating Agents on Fe(II) Release

EDTA or DFO added to the external media of iron-loaded vesicles at equilibrium also removed about 50% of Fe(II). Detergents such as deoxycholate (0.2 mg/ml) or toluene (2 μl/100 μl of uptake media) either added before or after Fe(II) uptake resulted in no significant change in the amount of Fe(II) associated with the membranes. Loss of membrane integrity was verified by the abolition of D-glucose "overshoot" after deoxycholate and toluene exposure.

EDTA or DFO added to detergent-treated Fe(II)-loaded vesicles resulted in the removal of the same amount of Fe(II) as with the chelating agents alone. Therefore, strong chelating agents appear to be able to reversibly remove Fe(II) taken up by membrane vesicles.

**Binding Versus Membrane Transport of Fe(II)**

One of the major problems in the interpretation of uptake data by membrane vesicles is the question of whether solute uptake represents binding to some components of the membrane or movement into the intravesicular space or a combination of both. If the uptake is a result of solute transport into the intravesicular space, the equilibrium uptake should be proportional to the size of that space. This space, in turn, can be manipulated by exposing vesicles to hypertonic solutions made up of impermeant solutes. The intravesicular spaces, and hence solute uptake, should be inversely proportional to medium osmolarity under these conditions. This approach has been useful for many solutes such as D-glucose, amino acids, and electrolytes such as NaCl with the conclusion that uptake is solely due to transport into the intravesicular space.

However, the observation of an inverse relationship between solute uptake and medium osmolarity by itself is not a sufficient criterion for the conclusion that transport has taken place since uptake will be *apparently* proportional to the inverse medium osmolarity in the case of solute binding to the osmotic agent. To investigate the question of transport versus binding, we performed the following experiments.

**Effect of Increasing Mannitol Concentration on Fe(II) Uptake**

When increasing mannitol concentrations were used to change the tonicity of the medium, Fe(II) uptake was directly proportional to the reciprocal of the mannitol concentration (Fig. 6a). In order to determine whether the decreased uptake with increasing mannitol concentrations was due to an osmotic effect (decrease in the intravesicular volume) or a concentration-related effect, membranes were prepared in high mannitol concentrations (200 mosm, 400 mosm) equal to those used in the subsequent uptake media so that osmotic effects were absent during the subsequent incubation with Fe(II). Under these conditions, the uptake of Fe(II) was also found to be inversely proportional to the mannitol concentration. This finding suggests that Fe(II) has some affinity for mannitol and that the dependence of uptake on hypertonicity due to mannitol cannot be interpreted in terms of intravesicular space effects. Similar results were obtained using the disaccharide cellobiose.

**Effect of Other Osmotic Agents on Fe(II) Uptake**

Agents were chosen which are polar buffers with negligible metal buffer binding constants (21). Zwitterionic aliphatic amines were used such as HEPES, PIPES, and cholamine. In contrast to the results with mannitol and cellobiose, increasing medium osmolarity using HEPES or cholamine had no influence on Fe(II) uptake over a range of 50–400 mosm (Fig. 6b). On the other hand, PIPES appeared to inhibit Fe(II) uptake at all concentrations. The agents shown to be relatively impermeant as increasing medium concentrations (osmolarity) decreased the intravesicular volume, estimated from equilibrium uptake of Na+-dependent glucose (Fig. 6b).

The independence from medium hypertonicity per se suggests that Fe(II) uptake represents binding to the vesicles. This conclusion is further supported by a consideration of the amount of iron uptake and the intravesicular space determined from equilibrium uptake of D-glucose. The observed uptake of about 1800 pmol of Fe(II)/mg of protein would

---

**TABLE III**

| Reducing agent            | Fe(II)/protein pmol/mg |
|---------------------------|------------------------|
| Control                   | 1424                   |
| Dithiothreitol (2 mM)     | 630                    |
| Ascorbic acid (2 mM)      | 1397                   |
| 2-Mercaptoethanol (2 mM)  | 1598                   |
| 2-Mercaptoethanesulfonic acid (2 mM) | 1278 |
Iron Transport across Brush-Border Membranes

Even though iron uptake by the membranes represents binding, membrane transport could be involved if the binding sites are located on the inside of the vesicles. To gain information about the location as well as any membrane transport step, $^{56}\text{Fe}(\text{II}) - ^{59}\text{Fe}(\text{II})$ exchange was measured under equilibrium conditions. These conditions were chosen because the exchange represents purely kinetic events, and independent (Fe(II) concentration) and dependent (velocity measured as $v$) variables are well defined. Since the interpretation of kinetic data is model-dependent, two general models for the exchange of $^{56}\text{Fe}$ for $^{59}\text{Fe}$ and their predictions for isotope exchange were considered. One is a straight stoichiometric exchange of $^{56}\text{Fe}$ for $^{59}\text{Fe}$ attached to macromolecules with forward and backward rate constants, $k_f$ and $k_b$, respectively. In this model, the membrane is considered to be unimportant because of potential extravesicular binding sites or because the membrane does not form an intact barrier.

Model 1

$$^{56}\text{Fe} + ^{59}\text{Fe}-\text{macro} \xrightleftharpoons{k_1}{k_2} ^{56}\text{Fe} + ^{59}\text{Fe}-\text{macro}$$

A second model proposes a saturable membrane catalytic step involved in the exchange of $^{56}\text{Fe} \rightleftharpoons ^{59}\text{Fe}$ from outside to inside (or vice versa) of the vesicles with subsequent binding to macromolecules on the inside.

Model 2

$$^{56}\text{Fe} + ^{59}\text{Fe}-\text{macro} \xrightleftharpoons{k_3}{k_4} ^{56}\text{Fe} + ^{59}\text{Fe}$$

$$^{56}\text{Fe} + ^{59}\text{Fe}-\text{macro} \xrightleftharpoons{k_5}{k_6} ^{56}\text{Fe} + ^{59}\text{Fe}-\text{macro}$$

Subscripts $i$ and $o$ refer to inside and outside for the location of Fe and binding sites as well as inward and outward in the case of the rate constant.

In the latter model, Equation 2 refers to a transmembrane process with $k_i$ and $k_o$ the rate constants of a potential transport reaction.

Model 1 predicts chemical kinetics, e.g. with independence of the half-time of exchange from Fe(II) concentration. In contrast, Model 2, with a (catalyzed) transport step in series with the binding step, predicts potential dependence of the exchange rate on Fe(II) concentration, namely when saturation of the "transporter" occurs.

Isotope exchange experiments were designed to test these models. In these experiments, the $^{56}\text{Fe}(\text{II})$-loaded vesicles were diluted into unlabeled medium of the same composition, and the relative exchange rate was determined from the time curve of radioactive efflux. Freshly prepared membranes were incubated for 30 min at 37 °C in uptake media of varying Fe(II) concentrations (always with a constant amount of $^{59}\text{Fe}$ and a 20 μM excess of ascorbate) in the presence of 2 mM dithiothreitol. At the end of the incubation period, Fe(II) uptake was determined on an aliquot in the usual manner. The vesicles were immediately diluted into a 5-fold volume excess of unlabeled Fe(II)-ascorbate solution at a concentration equal to that of the preincubation medium and containing 2 mM dithiothreitol. Aliquots were sampled at various time intervals. A standard uptake study of Fe(II) with an iron concentration of 1.79 μM was run as a control. In normal mice, the exchange rate of labeled Fe(II) over a 2-4 μM range under equilibrium conditions was found to be directly proportional to the Fe(II) concentration as well as the amount of Fe(II) taken up by the membranes (Fig. 7). Interestingly, all of the Fe(II) bound to the membranes was exchangeable.

When stronger chelating agents (DFO, 200 μM EDTA, 5 mM) were substituted for dithiothreitol, all iron was removed from the vesicles; therefore, no exchange could be measured. At lower DFO and EDTA concentrations (100 μM and 1 mM, respectively), exchange was also absent even though vesicle uptake of iron was still observable at low Fe(II) concentrations (2.15 μM).

Isotope Exchange of Fe(II) in Vesicles Prepared from Iron-deficient Mice

Weaning mice rendered iron-deficient by feeding low iron diets for 6-8 weeks were used in the following experiments.

Fig. 6. Effect of osmotic agents on Fe(II) uptake and intravesicular space in normal mouse brush-border membranes. a, representative uptake of Fe(II) by normal mouse brush-border membrane vesicles using different osmotic agents. Membrane vesicles at a final concentration of 0.42 mg/ml were incubated in uptake media containing 1.79 μM Fe(II) and sufficient HEPES (△), PIPES (♦), ctolamine (◇), or mannitol (●) to reach the indicated osmolarity. Uptake was measured at 10 min in all cases. b, effect of osmotic agents on equilibrium uptake of Na+-dependent glucose. Membrane vesicles at a final concentration of 3 mg/ml were incubated in Tris/HEPES buffer, pH 7.4, and [3H]glucose for 10 min. Sufficient HEPES (◇), PIPES (♦), or mannitol (△) was added to reach the indicated osmolarities. Intravesicular space was calculated from the amount of D-glucose included.
Intestinal membrane vesicles were prepared as in normal mice, and isotope exchange at equilibrium was measured using $^{59}$Fe(II)-ascorbate in the presence of 2 mM dithiothreitol. In contrast to the membranes from control mice, in the iron-deficient mice the rate of Fe(II) exchange demonstrated saturation kinetics typical of a carrier-mediated reaction (Fig. 7). The concentration of Fe(II) that gave half-maximal exchange rate was 8 μM. These data are consistent with Model 1.

To further document that saturation was due to an integral membrane protein. Isotope exchange was then carried out as described above. The iron exchange rate was no longer saturable, consistent with the chemical kinetics predicted by Model 1.

**DISCUSSION**

The cellular mechanisms responsible for the control of iron absorption are poorly understood (2, 4, 22-24). However, experimental observations have provided evidence for two major mechanisms which may be involved in the initiation of iron absorption. One mechanism involves Fe-macromolecular complexes in the gut lumen (25), principally transferrin, to which iron and the recycling of transferrin back to the gut lumen (27). A second mechanism involves Fe-micromolecular (low molecular weight) complexes in the gut lumen (citrate, ascorbate, amino acids, etc.) and the transfer of iron from the complex to the membrane and into the cell by several potential but undefined mechanisms (28). Neither of these mechanisms is necessarily mutually exclusive, and little is known about their relative importance.

In order to circumvent some of the problems in using whole cells or intact animals to study iron absorption at the mucosal level, recent studies have used purified microvillus membrane vesicle preparations to gain mechanistic information about the role of membrane in iron absorption (9-11).

**Complexity of the Aqueous Chemistry of Iron**—The results of the initial vesicle studies were conflicting and may have been confounded by the inability to determine and maintain the desired species of iron in solution. As Marx and Aisen (12) have shown, in order to minimize the formation of ferric hydroxide complexes at increasing pH, as much as a 1000-fold molar excess of citrate to Fe(III) is necessary in order to minimize the free concentration of iron in the solution and prevent hydrolysis (20). Similarly, to prevent oxidation of ferrous iron, at least a 20-fold molar excess of L-ascorbic acid is needed. The presence of ascorbic acid is required at low pH so that complexing the Fe(II) can take place before the pH is adjusted to a more physiological range (17). Using these well defined test media, our results were very similar to that of Marks and Aisen (12) (Fe(III) uptake = 25 pmol/mg of protein versus 4 pmol; Fe(II) uptake = 1700 pmol/mg of protein versus 1500 pmol). The higher values in our studies are probably due to greater purity of the membrane preparation. Several additional parameters of Fe(II) uptake have been investigated and are discussed below.

**Reversibility of Fe(II) Binding to Membrane Vesicles**—Our experiments demonstrate that in the absence of chelating agents, Fe(II) in the concentration range of 2-40 μM is taken up by the vesicles and that this Fe(II) is neither released when the Fe(II) concentration of the buffer is lowered nor exchanged for unlabeled iron at the same concentration. However, reversibility is readily demonstrated by the addition of chelators such as dithiothreitol, EDTA, or DFO to Fe(II)-loaded vesicles as most of the iron is removed. Sulfhydril agents (mercaptoethanol, mercaptoethanesulfonic acid) and ascorbate did not remove bound Fe(II) (Table III). The relative strengths of the chelating agents tested for removing vesicle-bound iron are DFO ≥ EDTA ≥ dithiothreitol >> 2-mercaptoethanesulfonic acid > ascorbic acid ≥ 2-mercaptoethanol. Therefore, the absence of chelating agents appears to prevent Fe(II) exchange because no agent is available to remove iron from presumptive external membrane binding sites.

Interestingly, $^{59}$Fe(II)-$^{59}$Fe(II) exchange between vesicles and medium could only be observed in the presence of the relatively weak chelator dithiothreitol ($K_r \approx 10^5$), but not in the presence of strong chelators like DFO ($K_r \approx 10^{10}$) (29) or EDTA ($K_r \approx 10^{14}$) (30). In the presence of high concentrations (5 mM EDTA or 200 μM DFO), all Fe(II) was removed from the vesicles so that isotope exchange experiments could not be performed. It is not known why the strong chelators did not support isotope exchange at lower concentrations when enough Fe(II) remained bound to the vesicles so that exchange experiments could be carried out. However, the inability to support Fe(II) exchange may be related to the very low off-rate constants which are likely

\[ K_r = \frac{[Fe^{3+}]}{[Fe^{2+}]} \]

2The $K_r$ for dithiothreitol was estimated from its coordination chemistry in comparison to 2,3-dimercapto-1-propanol, EDTA, and DFO. In addition, since the rate-limiting step in multidentate complex formation with an aquaion is primarily determined by the initial dissociation of a water molecule (water exchange rate for Fe(II) = $3.2 \times 10^{-6}$ s⁻¹ (31), we anticipate that the on-rate constants for all ligands would be similar (personal discussion with Dr. F. Urbach, Professor of Chemistry, Case Western Reserve University).
Iron Transport across Brush-Border Membranes

Iron Transport across Vesicle Membranes by a Carrier-mediated Process—Isotope exchange at equilibrium has been particularly successful (18) in studying substrate translocation across membrane vesicles because measured rates can be interpreted in terms of kinetic models under well defined transmembrane conditions (32). The demonstration of saturation under such conditions provides evidence for a carrier-mediated transport process. Using vesicles derived from iron-deficient mice, saturation kinetics could be demonstrated providing strong evidence for a membrane carrier-mediated iron transport process. This conclusion also implies that iron must be transported into the vesicle with a large proportion of binding sites inside the membrane. This transport process is documented only for the 29% of iron remaining in the vesicles after exposure to dithiothreitol. Whether this process applies to the iron pool that is removable by chelating agents is unproven. The documented transport pathway for moving Fe(II) across the brush-border could be very important in overall iron absorption since it is under obvious biological regulation.

The fact that saturation occurred at lower Fe(II) concentrations in iron-deficient mice than in normal suggests that there may be some qualitative difference in the membrane iron recognition process in iron deficiency. This difference is also suggested by the rapid uptake kinetics and sharp plateau which do not inherently describe Michaelis-Menten kinetics, but may suggest some cooperativity in membrane transport of iron when biological availability has been markedly diminished.

Our results suggest that the uptake of Fe(II) by the brush-border membrane appears to be a 2-fold process. The first step is the binding of Fe(II) to apparent high affinity binding sites on the membrane surface and transport (carrier-mediated) of Fe(II) across the membrane. This transport step is under biological regulation. The second step in vitro is the subsequent binding (chelation) of the iron on the inside of the membrane in a reversible process in the presence of a suitable chelating agent (dithiothreitol, EDTA, DFO), and the iron is transported out of the vesicle. In vivo, intact mucosal cells, the second step is likely to show apparent reversibility as iron is further metabolized by intracellular mechanisms and not available for transport out of the luminal surface of the mucosal cell.

REFERENCES

1. Bothwell, T. H., Pirizio-Kroli, G., and Finch, C. A. (1958) J. Lab. Clin. Med. 51, 24
2. Manis, J. G., and Schachter, B. (1962) Am. J. Physiol. 203, 73–90
3. Nathanson, M. H., McLaren, G. D., Saitel, G. M., and Muir, W. A. (1983) Fed. Proc. 42, 826
4. Greenberger, N. J., Balsezak, S. P., and Ackerman, G. A. (1969) J. Clin. Invest. 48, 711-721
5. Kimmich, G. A. (1970) Biochemistry 9, 3659–3668
6. Kimmich, G. A. (1970) Biochemistry 9, 3669–3677
7. Peppeelli, J. E., Edwards, H. A., and Bennerman, R. M. (1982) Blood 60, 635–641
8. Hopfer, U., Nelson, K., Perrotto, J., and Isselbacher, K. J. (1973) J. Biol. Chem. 248, 25–32
9. Easahm, E. J., Bell, D. L., and Douglas, A. P. (1977) Biochem. J. 164, 289–294
10. Cox, T. M., and O’Donnell, M. W. (1981) Biochem. J. 194, 753–759
11. Cox, T. M., and O’Donnell, M. W. (1980) Int. J. Biochem. 1, 446–454
12. Marx, J. M. J., and Aisen, P. (1981) Biochem. Biophys. Acta 649, 297–304
13. Schmitz, J., Preiser, H., Maestracci, D., Ghoosh, B. K., Ceder, J. J., and Crane, R. K. (1973) Biochem. Biophys. Acta 323, 98–112
14. Dahlqvist, A. (1967) Anal. Biochem. 22, 99–107
15. Forstner G., Sabesin, S. M., and Isselbacher, K. J. (1983) J. Biol. Chem. 258, 265–275
16. Taqui Khan, M. M., and Martell, A. E. (1967) J. Am. Soc. Chem. 89, 4176–4185
17. Hopfer, U. (1977) J. Supramol. Struct. 7, 1–13
18. Lucke, H., Wurbren, H., Menge, H., and Murer, H. (1978) Pfluegers Arch. Eur. J. Physiol. 373, 243–248
19. Aisen, P., Liebman, A., and Zweier, J. (1978) J. Biol. Chem. 253, 1930–1937
20. Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Irazya, S., and Singh, R. M. M. (1966) Biochemistry 5, 467–477
21. Dowdle, E. B., Schacter, D., and Schniker, H. (1960) Am. J. Physiol. 188, 699
22. Jacobs, T., Offwell, T. H., and Carlton, R. W. (1966) Am. J. Physiol. 210, 694–700
23. Kimmer, C. L., Mukagerjee, T., and Deller, D. J. (1973) J. Am. J. Dis. 18, 781–791
24. Huebner, H. A., Huebner, E., and Rummel, W. (1975) in Iron Metabolism and Its Disorders (Kiefer, H., ed) p. 13, Excerpta Medica, Amsterdam
25. Hemmeplardh, D., and Morgan, E. H. (1974) Biochem. Biophys. Acta 373, 84–99
26. Huebner, H. A., Huebner, E., Cabe, E., Rummel, W., and Finch, C. A. (1983) Blood 61, 283–290
27. May, P. M., and Williams, D. R. (1978) in Metal Ions in Biological Systems (Sigel, H. ed) Vol. 7, p. 29, Marcel Dekker, New York
28. Anderegg, G., L’Epplathenier, F., and Schwarzenbach, G. (1963) Helv. Chim. Acta 46, 1461
29. Anderegg, G. (1964) Helv. Chim. Acta 47, 1801
30. Margenon, D. W., Cayley, G. R., Weatherburn, D. C., and Pagenkopf, G. K. (1978) ACS Monogr. 27, 174
31. Hopfer, U. (1981) Fed. Proc. 40, 2480–2485
Iron transport across brush-border membranes from normal and iron-deficient mouse upper small intestine.
W A Muir, U Hopfer and M King

J. Biol. Chem. 1984, 259:4896-4903.

Access the most updated version of this article at http://www.jbc.org/content/259/8/4896

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/259/8/4896.full.html#ref-list-1