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Effects of D-Fructose on the Uptake of Iron by the Intestinal Brush-Border Membrane Vesicles from Rats.

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We have studied the iron uptake by the purified brush-border membrane vesicles (BBMVs) to determine the effect of fructose on the absorption of iron. BBMVs were prepared by the modified calcium precipitation method. The degree of purification was routinely assessed by the marker enzyme, alkaline phosphatase, and the functional integrity was tested by D-[1-^{3}H]glucose uptake. The appearance of membrane vesicles was shown by transmission electron microscopy (TEM). The uptakes of complexes of labeled iron [^{59}Fe] with fructose and ascorbate were measured with a rapid filtration technique. The uptake rate and pattern of the two iron-complexes, Fe(III)-fructose and Fe(III)-ascorbate, were also observed. A typical overshooting uptake of D-glucose was observed with peak value of 2~3 times higher concentration than that at equilibrium. This result was similar to other studies with BBMVs. TEM showed that the size of BBMVs was uniform and we can hardly find any contaminants. Fe(III)-fructose has the higher value of Vmax and the lower value of Km than those of Fe(III)-ascorbate, respectively. It may be concluded that D-fructose is more effective in promoting the iron absorption than ascorbate.

Keyworks—Iron, Fructose, Ascorbic acid, Fe(III)-fructose, Fe(III)-ascorbate, Brush-border membrane vesicles.

Introduction

Intestinal absorption of iron is the major pathway for regulating the quantity of body iron stores.[1] Nathanson and McLaren[2] suggested that iron uptake from intestinal lumen into the mucosa was the rate-limiting step in iron absorption and that this rate increased in iron deficiency. The most widely accepted mechanism for the intestinal absorption of iron is carrier-mediated transport. It is currently believed that absorption of iron can occur at any level of the gastrointestinal tract from the stomach distally.[3-7] However, absorption is greatest in the duodenum and progressively less in a descending gradient.[8-10] Divalent ferrous iron is absorbed better than trivalent form.[11-12]

It is well known that solubility of ferric iron in an aqueous solution is essentially nil above pH 4,[13-14] due to the formation of ferric oxyhydroxide. It is also known that a major factor in iron absorption is its solubility, which is easily seen from the fact that soluble (or chelated) iron is absorbed much more readily than insoluble oxides.[15] Conrad and Schade[14] reported that ascorbate forms a soluble chelate with ferric chloride at an acid pH. Saltman et al[16-17] reported on the chemical properties of chelates of iron with fructose and other reducing sugars, all of which were extremely stable and soluble over a wide range of pH encountered in the gastrointestinal tract.

Purified brush-border membrane vesicles(BB-

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MVs) constitute a relatively simple experimental system suited to investigate the uptake step without interference from whole cells, in particular, the intracellular iron pool, cellular metabolism, protein synthesis and the exit step across the basolateral plasma membrane.[18-19] Only a few studies of iron transport using BBMVs have been published.[20-23]

The present study on iron uptake by rat intestinal BBMVs was designed by the expectation that D-fructose might enhance the iron absorption. Based on many reports[3, 14, 24, 25] that ascorbate promotes iron absorption, we have chosen the ascorbate as a reference substance. In this study, we investigated the effects of fructose on the uptake of iron by rat intestinal BBMVs compared to those of ascorbate. We also checked the functional integrities of BBMVs.

Materials and Methods

Materials and Equipments

D-[1-^{3}H]glucose (specific activity, 3.0 Ci/mmol) and $^{59}FeCl_3$ (specific activity, 0.5 Ci/mmol) were obtained from Amersham (Arlington Height, IL, USA). HEPES and Tris were from Sigma (St. Louis, MO, USA). All the other chemicals were of analytical grade and used without further purification. All solutions used in the preparation of the membranes and in the uptake studies were prepared with re-distilled deionized water.

UV-spectrophotometer (UVIKON 930) used in enzyme and protein assay was purchased from KONTRON Inst. (Zurich, Switzerland). Gamma counter (COBRA 5002) and liquid scintillation counter (TRI-CARB 4000) were from PACKARD Inst. (Dowers Grove, IL, USA). The Millipore filters used in the uptake studies had a pore size of 0.2 μm and diameters of 2.5 mm.

Preparation of BBMVs

The BBMVs were prepared from the entire small intestine of three male rats (KIST : Wistar) weighing 220−260 g. The isolation procedure was based on the divalent cation precipitation method of Kessler *et al*[26]. This method was slightly modified to remove the contaminants consisted of cell debris. All steps were performed on the ice bath.

Rats were killed by cervical dislocation. The intestines were immediately removed and rinsed with ice-cold saline. The intestines were everted by a long wire and smoothly blotted by soft tissue to remove mucus and then brush-border were scraped by thin plastics to be adjustable of a gap. The scrapings were made to be a 5 % homogenate by adding homogenization solution consisting of 0.1 M D-mannitol and 1 mM Tris/HEPES, pH 7.4 and homogenized by warning blender homogenizer for 2 min at maximum speed. One molar concentration of $CaCl_2$ was added to a final concentration of 20 mM and the homogenate was stirred for 2 min and allowed to stand for 20 min to precipitate intracellular organelles and basolateral plasma membrane. The homogenate was centrifuged for 15 min at 4,200 rpm in a refrigerated centrifuge (Beckman J-6B, Beckman Inst., California, USA) at 4℃. The supernatant was centrifuged for 30 min at 17,500 rpm in a high speed centrifuge (Centrikon T-124, KONTRON Inst., Zurich, Switzerland). The resulting pellets were suspended in 30 ml of loading solution consisting of 100 mM mannitol, 100 mM HEPES/Tris, pH 7.5 and homogenized in a glass-Teflon homogenizer. Ten millimolar concentration of $CaCl_2$ was added to a final concentration of 0.05 mM and centrifuged for 15 min at 4,200 rpm. The supernatant was centrifuged for 30 min at 17,500 rpm. The final pellets were resuspended in a 3 ml of loading solution by passage through a 25 gauge needle. BBMVs were deep frozen until used.

Enzyme and Protein Assay of BBMVs

The degree of purification of BBMVs was routinely assessed from the enrichment of alkaline phosphatase in the final preparations compared

to the 5% homogenate. Alkaline phosphatase activity was determined by the method of Forstner *et al.*[27]

Protein was assayed according to the method of Lowry *et al*[28] with bovine serum albumin as the standard.

Electron Microscopy of BBMVs

Negative staining method; A formvar-coated copper grid was allowed to float on a drop of the BBMVs suspension (approx. 5 mg protein/m*l*) for 5 min. After removing excess liquid, the grid was then brought in contact with an aqueous solution of uranyl acetate (1 %) for 30 sec. The grids were then blotted dry and examined in an electron microscope (Carl Zeiss 109, Carl Zeiss Co., West Germany).

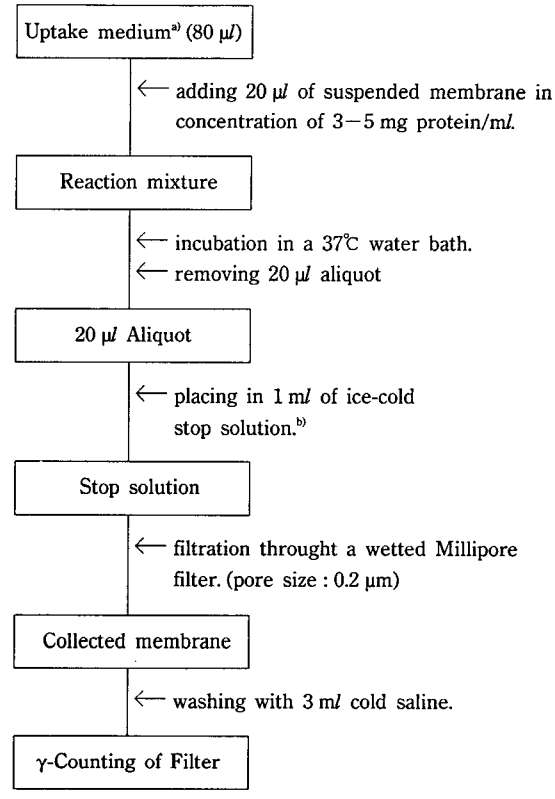
Preparations of Iron-Complexes

The complex of iron with ascorbate, Fe (III)-ascorbate, was prepared by the method of Conrad and Schade.[14] The complex of iron with fructose, Fe (III)-fructose, was prepared by adding a 1000 -fold molar excess of fructose (5 mM) to $FeCl_3$ solution (5 μM) containing trace amounts of $^{59}FeCl_3$ (0.0002 μCi/μ*l*) according to the method of Marx and Aisen.[23] After 10 min, the pH of the two iron-complex solutions was adjusted to 7.4 with 2 N NaOH.

Uptake by BBMVs

The uptakes of the two iron-complexes and D-glucose were studied employing the rapid Millipore filtration technique.[23]

Uptake of D-glucose served to assess the functional integrity of BBMVs. The membrane vesicles at a final concentration of 3~5 mg protein per m*l* were incubated at 25℃ in 80μ*l* of uptake medium consisting of 1 mM D-glucose containing D-[^{3}H]glucose (0.05 μCi/μ*l*), D-mannitol (0.1 M), Tris/Hepes (0.1 M), NaCl (0.1 M), pH 7.4, in which initial inward Na^+ gradient is 100 mM/0 mM for outside/inside. Twenty microliters of vortexed vesicle suspension was added to 80 μ*l* uptake medium in a shaking waterbath. The reaction was



Scheme 1—Uptake Procedures of Iron-Complexes.

a) Uptake medium : 40 μ*l* of solution B and 40 μ*l* of iron-complex solution. solution B composition : 0.1 M mannitol, 0.1 M NaCl, 40 mM Tris/HEPES (pH 7.4) and 2mM D-glucose

b) Ice-cold stop solution : 0.1 mM $FeCl_3$ and 100 mM fructose (or ascorbic acid) adjusted to pH 7.0 with NaOH

terminated at desired times by removal of a 20 μ*l* aliquot from the medium after brief vortexing, which was first diluted with a 50-fold excess of an ice-cold stop solution (1 m*l*) and then immediately filtered on a prewetted Millipore filter (pore size, 0.2 μm) and washed three times with 1 m*l* of stop solution. The filters were solubilized in a Filter-Count® (Amersham, Arlington Height, IL, USA) and radioactivity was measured with a liquid scintillation counter (Packard, Dowers Grove, IL, USA). All the results were corrected for nonspecific binding to the filters by dealing with a medium without membrane vesicles. To

confirm the dependence of D-glucose uptake on Na^+ ion, the uptake experiment was performed in a medium without NaCl, in which initial inward Na^+ gradient is 0 mM/0 mM for outside/inside.

As presented in scheme 1, the uptake procedure of iron-complexes was identical with those of D-glucose. The concentration dependence of iron-complexes binding to BBMVs was studied using incubation media with iron concentration ranging from 7.5 to 210 μM. Each medium with different concentration of iron-complexes was incubated at 37℃ for 2 min. The remaining procedure was the same as the above methods of D-glucose uptake. The results were analyzed by a Lineweaver-Burk plot.

Results and Discussion

Characterization of the Prepared Membrane Vesicles

Purity

The degree of purification of the prepared me mbrane vesicles was routinely assessed by the marker enzyme such as alkaline phosphatase, which was mostly localized in the intestinal brush-border membranes. The specific activity of alkaline phosphatase in the final prepared membrane vesicles was increased 16-fold over the homogenate of intestinal scrapings (Table I). This implies that the prepared membranes were very well isolated as brush-border membranes from the starting materials.

Functional integrity

Table I—*Specific activities of Alkaline Phosphatase in the Prepared Membranes*

Fraction	Specific activity[a] (units/mg protein)
Homogenates	0.1293 ± 0.03
BBMVs	2.1256 ± 0.51

[a]Each value represents the mean± S.E. of eight prepara tions.

To test for resealing of the membrane as ve sicles, their transport competence for Na gradient-driven glucose uptake was measured. A typical overshooting uptake was observed with peak values of 2−3 times the equilibrium. Fig. 1 showed that the presence of sodium ions increased the initial rate as well as the extent of D-glucose uptake, which was very specific for microvillous membrane.[29] Therefore, it may be concluded that the vesicles were sealed and oriented right-side out and that the prepared membranes were originated from the brush-border membranes, not from the basolateral plasma membarnes.

Electron microscopic appearance

Electron microscopic appearance of the prepared membrane vesicles is that of a highly purified brush-border membrane fraction (Fig. 2). We observed that the rather uniform size and shape of vesicles were formed and the fuzzy contours of the outer surface of vesicles could be found with the negative staining method. Most of the membranes are of a vesicular form with a size approximately that of a microvillus. Fibrous core

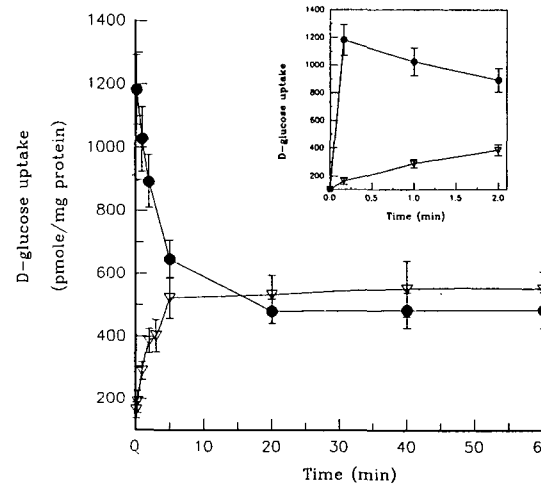


Figure 1—D-glucose uptake by brush-border membrane vesicles.

Keys : ●; uptake medium with initial inward Na^+ gradient for outside/inside (100 mM/0 mM), ▽; uptake medium with initial inward Na^+ gradient for outside/inside (0 mM/0 mM).

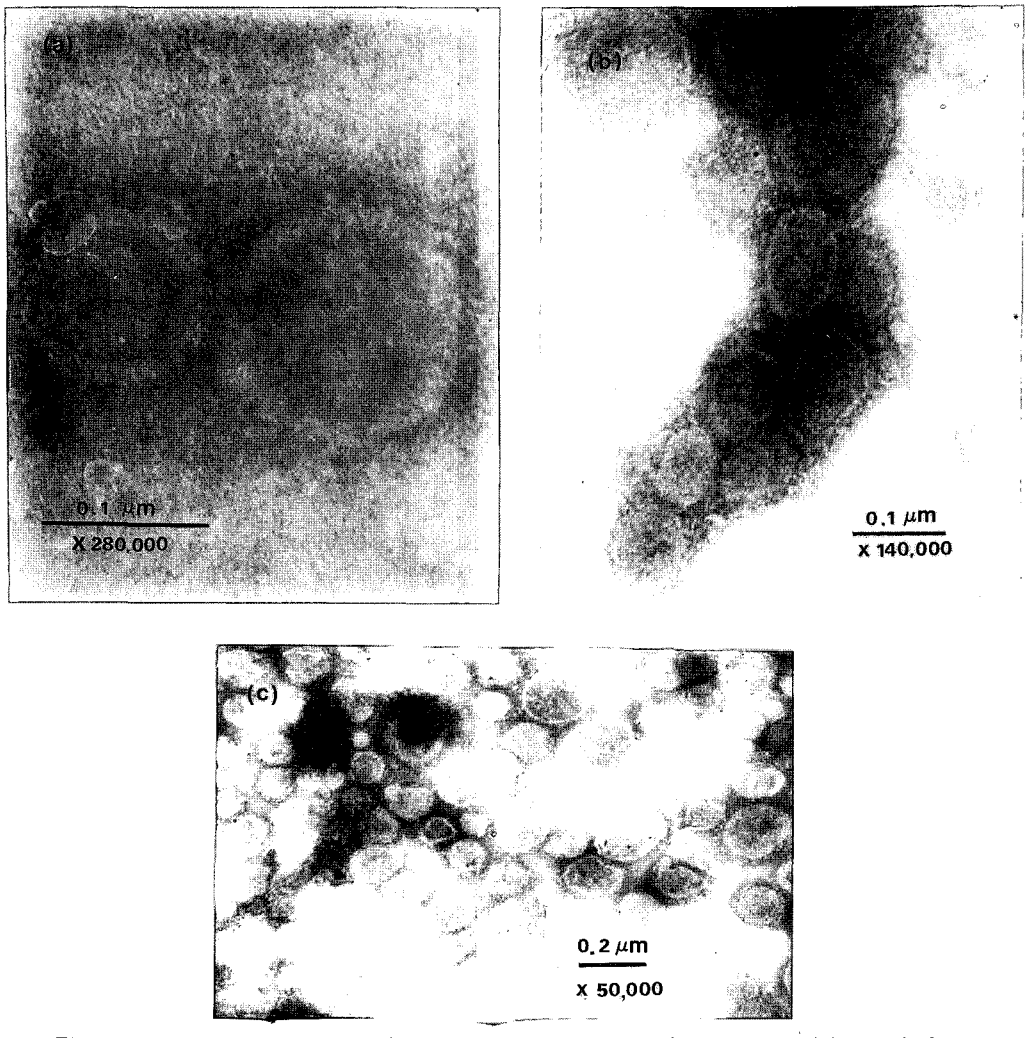


Figure 2—Electron microscopic appearances of the final prepared vesicles with negative staining method.

materials and any other contaminants were virtually absent. So it was proven that the modified calcium precipitation method was useful for the preparation of BBMVs.

Uptake of Iron-Complexes

As shown in fig. 3, there is more rapid initial uptake in Fe(III)-fructose than in Fe(III)-ascorbate, which are leveled off at about 5 min at 37℃.

The absorption of iron depends not only on factors related to microvillus and basolateral membrane but also on intraluminal and intracellular factors. Purified microvillous membrane vesicles provided a system for investigating solely the interaction of iron with intestinal mucosal membranes. The uptake of iron appears to be a 2-phase process. The first step is the binding of iron to apparent high affinity binding sites on the membrane surface and the second is the carrier-mediated transport of iron across the membrane that is under biological regulation. We found no transmembrane transport of iron. However, the iron uptake and/or binding in the prepared intestinal membrane vesicles followed the saturation kinetics, that is, the concentration dependent profile was observed. K_m and V_{max} values for the two iron-complexes was estimated by a Linewea-

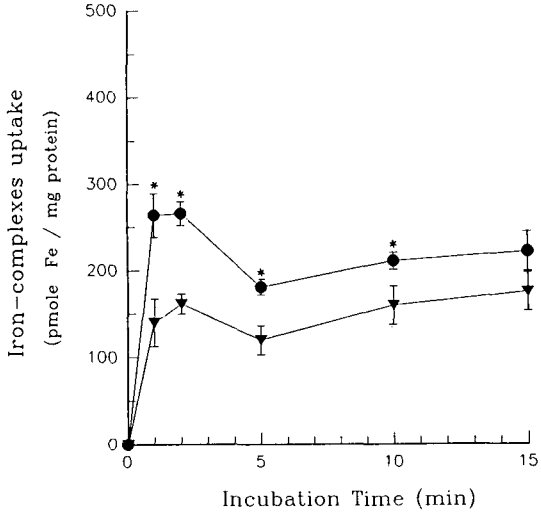


Figure 3—Iron-complexes uptake by brush-border membrane vesicles.
Keys : ●; Fe (III)-fructose, ▼; Fe (III)-ascorbate, *p<0.05.

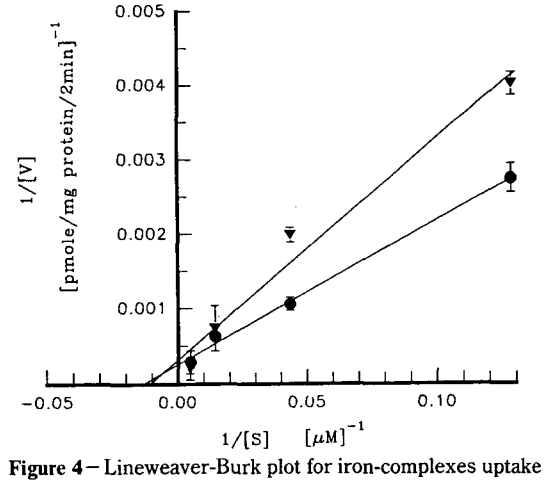


Figure 4—Lineweaver-Burk plot for iron-complexes uptake by brush-border membrane vesicles.
Keys : ●; Fe (III)-fructose, ▼; Fe (III)-ascorbate.

ver-Burk plot (Fig. 4). Table II shows that the K_m values of Fe (III)-fructose and Fe (III)-ascorbate are 76.0 μM and 93.6 μM, respectively. Fe (III)-fructose has the higher value of V_{max} and the lower value of K_m than those of Fe (III)-ascorbate. Based on this observation, we could estimate the relative contributions of the two chelating agents to the absorption of iron. That is, it seems that D-fructose forms a more effective chelate or complex with iron than ascorbate and that it may enhance the solubility and/or the binding force(or affinity) to carrier proteins.

Table II—*V_{max} and K_m values for Iron-Complexes*

Iron-complexes	V_{max} (pmole/mg protein/2 min)	K_m (μM)
Fe (III)-fructose	3929.27	75.99
Fe (III)-ascorbate	3150.60	93.57

Conclusions

In this study, the effects of fructose on the uptake of iron by rat intestinal BBMVs compared to those of ascorbate and functional integrities of BBMVs were investigated. Results obtained can be summarized as follows;

1. Specific activity test of alkaline phosphatase, D-glucose uptake and electron microscopy showed that BBMVs prepared by the modified calcium precipitation method have been highly purified and retain the functional transport system.

2. Initial and equilibrium uptake rate of Fe (III)-fructose are larger than those of Fe(III)-ascorbate.

3. Fe(III)-fructose has the higher value of V_{max} and the lower value of K_m than those of Fe(III)-ascorbate.

These observations suggest that D-fructose is more effective in promoting the iron absorption than ascorbate.

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