Studies on Fe Complexes Produced by Yeast. IV. Mechanism of Fe Transport from an Fe(II)–Oligosaccharide Complex across the Mucosal Membrane of the Rat Intestine

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The mechanism of Fe transport across the rat duodenal membrane from an Fe(II)–oligosaccharide complex (designated Bl-c) produced in wine by yeast and showing high hematopoietic activity in rats was examined. The Fe uptake from Bl-c by brush border membrane (BBM) vesicles isolated from the rat intestine was based mainly on the Fe binding to membrane components which were suggested to be inside the vesicles. Evidence that Fe was transported into the vesicles by a special transport system other than simple diffusion was obtained by observing saturation kinetics under conditions of isotope exchange, and temperature and pH dependence. This Fe uptake was not inhibited by any metal ions tested, including inorganic Fe(II), and the BBM vesicles from the duodenum had a higher Fe uptake than those from the other parts of the small intestine. Furthermore, the BBM vesicles isolated from rats with Fe deficiency showed a significantly increased Fe uptake. The K_m value for Bl-c uptake was 0.16 mM, lower than the values for $FeSO_4$ and ferrous ascorbate. These results suggest that a special transport system selective for Bl-c may be present on the mucosal membrane. Bl-c was taken up by the BBM vesicles in the form of Fe–oligosaccharide complex. From the preloaded vesicles, Bl-c was released temperature- and pH-dependently. The fractionation of the intestinal mucosa used in *in situ* and *in vitro* absorption experiments suggests that the Fe transport across the BBM by a special transport process is the initial step of the intestinal absorption of Fe from Bl-c and that Fe is transferred into a soluble fraction of the mucosal cells in the form of a complex with oligosaccharide.

Keywords ferrous complex; oligosaccharide; mucosal transport; ferrous absorption mechanism; brush border membrane vesicle

Introduction

We have investigated the gastrointestinal absorption of Fe from an Fe(II)–oligosaccharide complex (designated Bl-c according to the order of fractionation). Our previous studies have demonstrated that (i) this complex, produced in wine by *Saccharomyces cerevisiae*, showed high gastrointestinal Fe absorption and incorporation of Fe into hemoglobin when it was administered orally to rats[1]; (ii) it had a molecular weight of around 1500, and its ligand was composed of a uronic acid and neutral sugars; (iii) it had high Fe solubility even in alkaline aqueous solution and was stable to oxidation of Fe(II) and to degradation of the complex structure; and (iv) the high intestinal Fe absorption from Bl-c might be attributed to its high solubility in the intestines (luminal factor) and to its characteristics in the transport process across the intestinal membrane (mucosal factors).[2,3] A special Fe transport system was suggested by which Fe from Bl-c may be transported to the serosal side in the form of Fe(II)–oligosaccharide complex,[3] but the details of the mechanism remain unclear.

Recently, isolated brush border membrane (BBM) vesicles have frequently been used for studies of transport mechanisms of substances in the intestinal mucosa, including studies on the Fe absorption mechanism.[4-8,9] Some studies have suggested the presence of Fe-specific binding sites on the BBM whose binding activity reflects the Fe demand of the animal,[4,5,7] and some others have referred to the specific Fe transport system of BBM vesicles.[8,9] In the present study, we examined Fe uptake from Bl-c by the rat intestinal BBM vesicles in order to elucidate the mechanism of Fe transport from Bl-c across the intestinal mucosa.

Materials and Methods

Preparation of ^{59}Fe-Labeled Fe Complex Wine containing ^{59}Fe-labeled Fe complexes was prepared through fermentation by *Saccharomyces cerevisiae* of ^{59}Fe-labeled $FeCl_3$ (New England Nuclear Corp.)-supplemented grape juice, and the fraction designated Bl-c was separated and purified by column chromatography with Amberlite XAD-2 (Rohm & Haas Co.) and Sephadex G-15 and G-50 (Pharmacia Fine Chemicals, Inc.) successively, as reported previously.[1]

Animals Six-week-old male Jcl:SD rats (CLEA Japan, Inc. (CLEA)) were given a pelleted diet (CLEA Stock Diet CE-2, CLEA; Fe content, 315 μg/g). Four- or 5-week-old male rats of the same strain were fed an Fe-deficient diet (CLEA; Fe content, 20 μg/g) and distilled water for 1 or 2 weeks prior to the experiment.

Fe Uptake by Small Intestinal BBM Vesicles (i) Preparation of BBM Vesicles: Normal rats deprived of food overnight were sacrificed by dislocation of cervical vertebrae, and the duodenum, jejunum, or ileum was excised and everted. The mucosa was scraped, in an ice bath, from the excised intestine, and BBM vesicles were prepared by the Mg precipitation method according to Christiansen and Carlsen.[10] Mucosal scrapings were suspended in a buffer solution consisting of 50 mM mannitol and 2 mM Hepes buffer (pH 7.1) and homogenized at the top speed for 2 min by the use of a Nissei homogenizer (model BM-2) with cooling. Solid $MgSO_4$ was added to 10 mM homogenate, and the mixture was maintained on ice for 20 min. After centrifugation for 15 min at 3000 × *g*, the supernatant was recentrifuged for 30 min at 27000 × *g*. The pellet obtained was suspended in a resuspension buffer solution consisting of 0.1 M mannitol, 0.1 M NaCl, 0.1 mM $MgSO_4$, and 20 mM Hepes buffer (pH 7.4). After rehomogenization and centrifugation for 20 min at 6000 × *g*, the supernatant was recentrifuged for 30 min at 27000 × *g*. The resulting pellet containing BBM vesicles was mixed with the cold resuspension buffer and suspended by repeated passage through a 25-gauge needle. The vesicle suspension (5 mg protein/ml) was used for experiments within 24 h after preparation.

The purity of the vesicle preparation was checked by measurements of its specific activities of sucrase, a marker enzyme of BBM,[11] and ouabain-sensitive ($Na^+ + K^+$)-adenosine triphosphatase(ATPase), a marker enzyme of basolateral plasma membrane.[12] The vesicle preparation was further examined by electron microscopy.

(ii) Uptake Experiment: ^{59}Fe-labeled Bl-c and Fe compounds ($FeSO_4$ and ferrous ascorbate) labeled with ^{59}Fe according to the previously reported method[1] were incubated with the BBM vesicles, and the Fe uptake from each compound by the vesicles was examined by the rapid filtration method. The incubation medium having the composition of 0.1 M NaCl, 0.1 M mannitol, and 20 mM Hepes buffer (pH 7.0) was used unless otherwise stated. The final concentration of the vesicles was 500 μg

protein/ml. Each mixture, 100 μl, was incubated at 37 °C, and the uptake was terminated with 5 ml of the ice-cold incubation medium. The mixture was immediately allowed to pass through a cellulose acetate filter (0.45 μm; Advantec Co.). The filter was washed with 5 ml of the ice-cold incubation medium, and ^{59}Fe in the filter was determined. The determined value was corrected by subtracting the amount of ^{59}Fe adsorbed on the filter (less than 1% of the added ^{59}Fe) obtained beforehand with the mixture of the same composition without the vesicles.

(iii) Release of ^{59}Fe from the ^{59}Fe-Preloaded Vesicles: After the stop solution was added to the vesicle preparation incubated in the medium containing ^{59} Fe-Bl-c for 60 min as described above, the mixture was centrifuged at 27000 × *g* for 20 min at 4 °C to collect vesicles. The collected vesicles were resuspended by adding the same incubation medium as above (pH 7.0 or 9.0) or the incubation medium adjusted to pH 5.0 with Mes buffer instead of Hepes buffer. The suspension was incubated at 4 or 37 °C and subjected to rapid filtration to determine ^{59}Fe activity remaining in the vesicles. Separately, the incubated suspension was centrifuged for 20 min at 27000 × *g*, the supernatant with or without glycosidase treatment[3] (1-h incubation at 30 °C with 10 units each of α-amylase and α-glucosidase from *Bacillus* species and yeast, respectively (Sigma Chemical Co.)) was applied to a Sephadex G-25 column (Pharmacia), and Fe eluted with 0.154 M NaCl–20 mM Hepes buffer (pH 7.0) was examined to determine its chemical form.

Fractionation of the Duodenal Mucosa Subjected to *in Situ* and *in Vitro* Fe Absorption Fe absorption studies were carried out with the ^{59}Fe–Fe compounds *in situ* (ligated duodenal loop method) and *in vitro* (everted duodenal sac method), as reported previously.[3] In the *in situ* experiment, a ligated duodenal loop was prepared from each rat under Na pentobarbital anesthesia, and 0.5 ml of ^{59}Fe–Fe compound solution was injected. In the *in vitro* experiment, 0.5 ml of the serosal medium (0.1 M NaCl, 5 mM KCl, 0.1 mM $MgSO_4$, 0.3% glucose, and 40 mM Hepes buffer (pH 7.0)) was injected into an everted rat duodenal sac, which was then incubated at 37 °C for 30 min in the mucosal medium containing ^{59}Fe–Bl-c (4 ml). The composition of the mucosal medium used was the same as that of the serosal medium. In both experiments, the duodenal tissue was removed after a certain period of time and washed well with cold physiological saline. The mucosa was scraped from each tissue and washed three times with cold saline. The preparation was processed by the Mg precipitation method and centrifuged at 27000 × *g*, and the radioactivities in the collected supernatant and BBM vesicles were determined. The supernatant was analyzed by gel filtration on Sephadex G-200 and G-50 columns (Pharmacia).

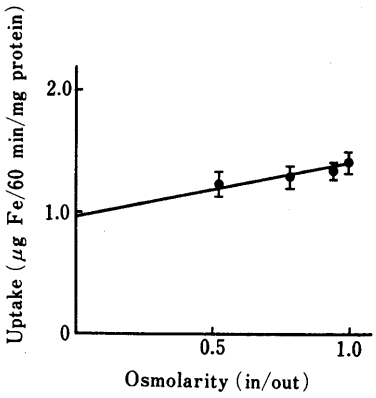

Fig. 1. Effect of Medium Osmolarity on Fe Uptake from Bl-c by Rat Duodenal BBM Vesicles

Initial Bl-c concentration, 11.2 μg Fe/ml (200 μM); mannitol concentration, 0.1 M (in) and 0.1—0.5 M (out). Each point with a bar represents the mean ± S.E. (n = 3).

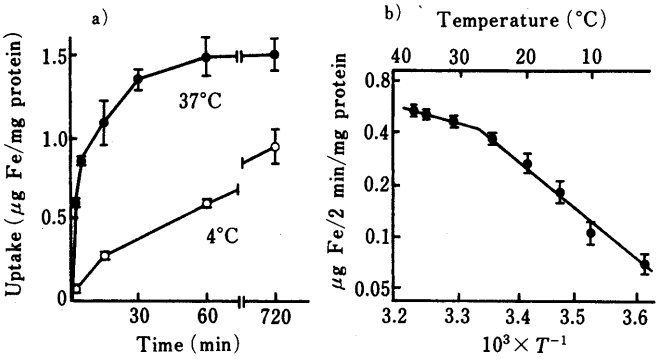

Fig. 2. Temperature Dependence of Fe Uptake from Bl-c by Rat Duodenal BBM Vesicles

a) Time courses; *b*) Arrhenius plot. Initial Bl-c concentration, 11.2 μg Fe/ml (200 μM). Each point with a bar represents the mean ± S.E. (n = 3).

Results

Characterization of BBM Vesicles The specific sucrase activity of the BBM vesicles prepared was about 20 times that of the first homogenate, and the specific ouabain-sensitive ($Na^+ + K^+$)-ATPase activity was 1/18 of the first homogenate value, indicating a highly purified BBM vesicle preparation. Electron microscopy revealed that the preparation exclusively contained closed, spherical, and right-side out vesicles similar to those described by Kessler *et al.*[13] and that each vesicle was predominantly filled with core proteins.

Characterization of Fe Uptake by BBM Vesicles 1) Osmolarity Dependence The value of intravesicular volume depends on the osmolarity of the medium.[13,14] So the effects of osmolarity on Fe uptake from Bl-c by the duodenal BBM vesicles were examined by varying the mannitol concentration in the incubation medium, to distinguish between transport of Fe from Bl-c into the osmotically active, intravesicular space and binding to the membrane components. As shown in Fig. 1, the Fe uptake was only slightly affected by the osmotic changes. The amounts of Fe taken up by the vesicles were far larger than the amounts of transported Fe estimated from the previously reported intravesicular space (1—1.5 μl/mg protein) in the BBM vesicles prepared by this method.[14] These results indicate that most of the Fe taken up by the BBM vesicles was bound to the membrane components (including uptake into the hydrophobic layer or inside the vesicles).[9,14]

2) Temperature Dependence Figure 2 shows the effects of temperature on the Fe uptake from Bl-c by the duodenal BBM vesicles. At 37 °C, the Fe uptake proceeded rapidly with time and almost reached equilibrium at 60 min. At 4 °C, the uptake process was markedly slow and did not reach equilibrium even at 720 min. In the Arrhenius plot of the Fe uptake at 2 min after the start of incubation (Fig. 2-b), the uptake showed a biphasic pattern with the turning point around 27 °C, and the apparent activation energies required for the respective phases were calculated to be 5.3 and 12.7 kcal/mol.

3) Concentration Dependence Effects of Bl-c and Fe compound concentrations on Fe uptake by the duodenal BBM vesicles are expressed as a Lineweaver–Burk plot in Fig. 3. The Fe uptake from Bl-c showed apparent saturation, and the K_m and V_{max} values were 9.2 μg Fe/ml (0.16 mM) and 0.22 μg Fe (3.9 nmol)/mg protein/min, respectively. On the other hand, the K_m values for $FeSO_4$ and ferrous ascorbate were 0.69 and 0.77 mM, respectively.

4) pH Dependence Figure 4 shows the Fe uptake from Bl-c by the duodenal BBM vesicles at various pH values of the incubation medium. At both 2 and 60 min after the start of incubation, the amount of Fe taken up reached the maximum level around pH 6 and decreased at alkaline pH.

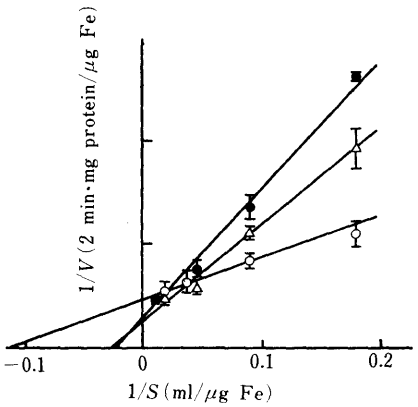

Fig. 3. Effect of Fe Compound Concentration on Fe Uptake by Rat Duodenal BBM Vesicles

○, Bl-c; ●, ferrous ascorbate; △, $FeSO_4$. Each point with a bar represents the mean ± S.E. ($n=3$).

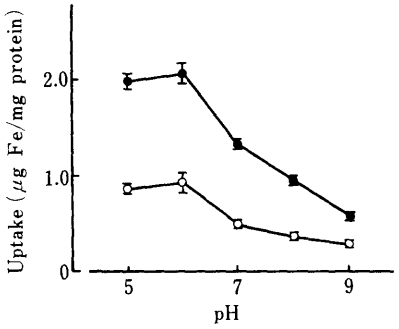

Fig. 4. Effect of pH on Fe Uptake from Bl-c by Rat Duodenal BBM Vesicles

Initial Bl-c concentration, 11.2 μg Fe/ml (200 μM). ○, 2 min; ●, 60 min after incubation at $pH_{in}=7.4$. Each point with a bar represents the mean ± S.E. ($n=3$).

TABLE I. Effect of pH Gradient on Fe Uptake from Bl-c by the Rat Duodenal BBM Vesicles

pH[a]		Fe uptake
In	Out	(μg Fe/2 min/mg protein)
6.5	6.5	0.53 ± 0.01
6.5	7.4	0.40 ± 0.05
7.4	6.5	0.63 ± 0.07
7.4	7.4	0.49 ± 0.01
7.4	6.5 + FCCP[b]	0.44 ± 0.08
7.4	7.4 + FCCP[b]	0.51 ± 0.01

Each value represents the mean ± S.E. ($n=3$). *a*) pH inside and outside the vesicles. *b*) FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), 10 μM, was added to the outside medium.

The effects on the Fe uptake of a pH gradient between the inside and outside of the duodenal BBM vesicles were also examined (Table I). The pH inside the vesicles (pH_{in}), as well as the pH outside the vesicles (pH_{out}), affected the Fe uptake, and the highest Fe uptake was obtained when the pH_{in} was larger than the pH_{out}. The increase of Fe uptake by this pH gradient was diminished by the addition of a protonophore (FCCP).

5) Effects of Metal Ions Figure 5 shows the effects of metal ions added to the incubation medium on Fe uptake from Bl-c and $FeSO_4$ by the duodenal BBM vesicles. The addition of $FeSO_4$, ferrous ascorbate, $CoCl_2$, $MnCl_2$, or

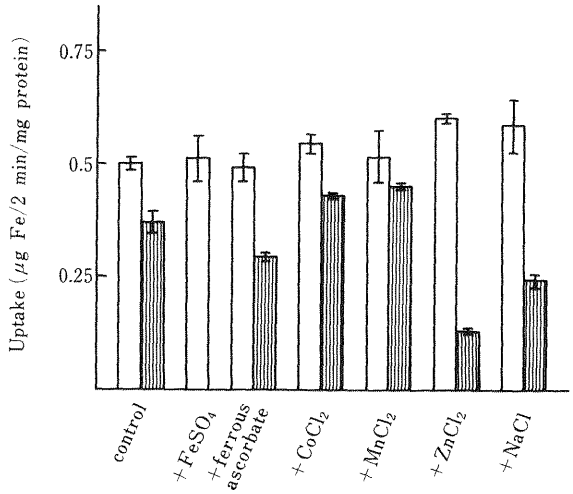

Fig. 5. Effect of Metal Ions on Fe Uptake from Bl-c and $FeSO_4$ by Rat Duodenal BBM Vesicles

□, Bl-c; ▤, $FeSO_4$. Initial Bl-c or $FeSO_4$ concentration, 11.2 μg Fe/ml (200 μM); metal ion concentration, 1 mM, except NaCl concentration (0.225 M). Each column or point with a bar represents the mean ± S.E. ($n=3$).

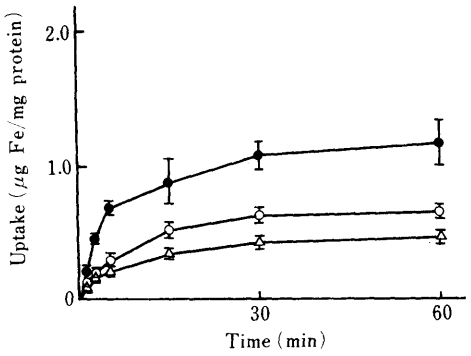

Fig. 6. Fe Uptake from Bl-c by BBM Vesicles Prepared from Different Parts of the Rat Small Intestine

●—●, duodenum; ○—○, jejunum; △—△, ileum. Initial Bl-c concentration, 11.2 μg Fe/ml (200 μM). Each point with a bar represents the mean ± S.E. ($n=3$).

$ZnCl_2$ at 1 mM exerted little influence on the Fe uptake from Bl-c. Besides, the addition of NaCl at a high concentration of 0.225 M did not inhibit the Fe uptake from Bl-c. On the other hand, the Fe uptake from $FeSO_4$ was decreased by the addition of ferrous ascorbate, $ZnCl_2$ or NaCl. Under these incubation conditions, no significant degradation of Bl-c or Fe exchange between Bl-c and Fe compounds was observed.

6) Fe Uptake by BBM Vesicles Prepared from Different Parts of the Small Intestine Figure 6 shows the time courses of Fe uptake from Bl-c by BBM vesicles prepared, at the same time, from the duodenum, jejunum, and ileum. Among these 3 parts, the duodenum showed the highest Fe uptake both in the initial phase and at equilibrium.

7) Effect of Fe Deficiency Figure 7 shows the Fe uptake from Bl-c by the BBM vesicles prepared from the duodenum of Fe-deficient rats, compared with that by the duodenal vesicles from normal rats. The Fe uptake tended to increase according to the degree of Fe deficiency.

8) Fe Release from the Bl-c-Preloaded BBM Vesicles The duodenal BBM vesicles preloaded with Bl-c were collected and incubated in media of pH 5.0—9.0 to examine

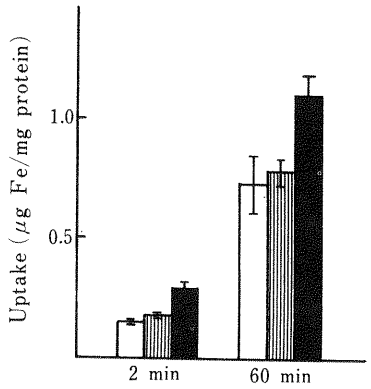

Fig. 7. Effect of Fe Deficiency on Fe Uptake from Bl-c by Rat Duodenal BBM Vesicles

□, normal rat (Hematocrit 44.1%); ▤, Fe-deficient rat (35.6%); ■, Fe-deficient rat (22.6%). Initial Bl-c concentration, 11.2 μg Fe/ml (200 μM). Each column with a bar represents the mean ± S.E. ($n=3$).

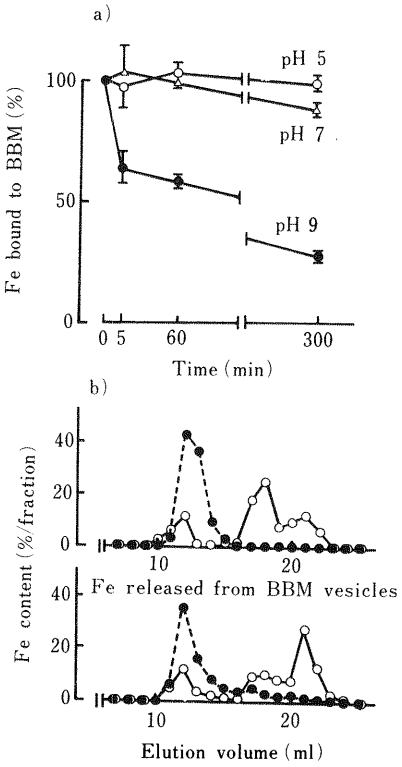

Fig. 8. Fe Release from Bl-c-Preloaded Rat Duodenal BBM Vesicles

a) Fe release from Bl-c-preloaded vesicles at pH 5.0—9.0; each point with a bar represents the mean ± S.E. ($n=3$). ○—○, pH 5; △—△, pH 7; ●—●, pH 10. b) Gel filtration on Sephadex G-25 of Bl-c and the incubation medium containing Fe released from the Bl-c-preloaded BBM vesicles at pH 9.0; ●, before enzymatic treatment; ○, after treatment with glycosidases (α-amylase (10 units) + α-glucosidase (10 units)).

Fe release from the vesicles. As shown in Fig. 8-a, the Fe release was slow at pH 5.0 and 7.0, while it was rapid at pH 9.0. The Fe release from the vesicles was temperature-dependent, and little Fe was released at 4 °C at any pH (data not shown).

To examine the chemical form of the Fe released at pH 9.0, the medium containing the released Fe was subjected, without further treatment or after glycosidase treatment, to a Sephadex G-25 column (Fig. 8-b). The elution position of the released Fe was identical to that of intact Bl-c. The

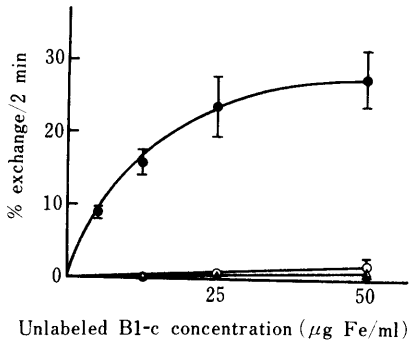

Fig. 9. Fe Exchange Rate in Bl-c-Preloaded Rat Duodenal BBM Vesicles

The rate of isotope exchange between ^{59}Fe-Bl-c in preloaded vesicles and unlabeled Bl-c (●), ferrous ascorbate (○), and $FeSO_4$ (△) (5—50 μg Fe/ml) was determined. Each point with a bar represents the mean ± S.E. ($n=3$—4).

TABLE II. Fe Contents in Fractions of Rat Duodenal Mucosal Cells[a] Which Took Up Fe from ^{59}Fe-Bl-c *in Situ* (Ligated Rat Duodenal Loop Method)

Fraction	Time after injection (min)			
	2	10	60	360
Total mucosal cell				
(ng Fe)	810 ± 45	1351 ± 204	1188 ± 181	1037 ± 99
(ng Fe/mg protein)	58.6 ± 4.8	97.7 ± 27.2	91.2 ± 14.9	77.2 ± 1.6
Purified BBM				
(ng Fe)	75 ± 31	88 ± 3	89 ± 1	53 ± 2
(ng Fe/mg protein)	62.9 ± 4.0	62.8 ± 16.1	60.3 ± 2.9	34.6 ± 6.8
Supernatant				
(ng Fe)	299 ± 21	338 ± 104	538 ± 243	342 ± 109
(ng Fe/mg protein)	30.4 ± 5.1	37.0 ± 6.5	52.5 ± 17.6	39.4 ± 14.3
Residual precipitate				
(ng Fe)	462 ± 241	930 ± 232	524 ± 71	526 ± 188
(ng Fe/mg protein)	59.2 ± 25.4	132.4 ± 34.8	96.9 ± 1.3	76.4 ± 17.9

Each value represents the mean ± S.E. ($n=3$). *a*) The mucosal cells were collected from a ligated rat duodenal loop (5 cm in length) at the indicated time points after ^{59}Fe-Bl-c (20 μg Fe) was injected into the loop.

elution pattern of Fe from the glycosidase-treated medium also almost corresponded to that of the fragment formed by the cleavage of the ligand (oligosaccharide) from intact Bl-c.

Effect of the Addition of Unlabeled Bl-c on Fe Release (Isotope Exchange with ^{59}Fe-Bl-c) The BBM vesicles preloaded with ^{59}Fe-Bl-c were collected and incubated in media containing various concentrations of unlabeled Bl-c, $FeSO_4$, or ferrous ascorbate to examine the isotope exchange (Fig. 9). The isotope exchange rate was saturated at high concentrations of unlabeled Bl-c, while the exchange rates with $FeSO_4$ and ferrous ascorbate were very low. In the case of the BBM vesicles preloaded with ^{59}Fe-ferrous ascorbate or ^{59}Fe-$FeSO_4$, the exchange rate with unlabeled Bl-c was quite low. These findings suggest that the Fe taken up from Bl-c may be located inside the vesicles and that a carrier-mediated process[9,15] selective for Bl-c may be involved in this exchange.

Roles of the BBM in *in Situ* and *in Vitro* Fe Absorption from Bl-c 1) Fe Distribution in the Intestinal Mucosa after *in Situ* Fe Absorption When ^{59}Fe-Bl-c was injected into the ligated rat duodenal loop, ^{59}Fe eliminated from the loop was rapidly taken up by the intestinal tissue (mainly

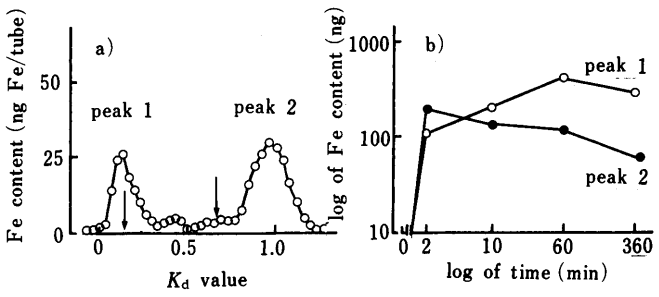

Fig. 10. Analysis of the Soluble Fraction of the Homogenized, ^{59}Fe-Bl-c-Loaded Rat Duodenal Mucosal Cells

a) Gel filtration on Sephadex G-200 of the soluble fraction obtained at 2 min after Bl-c injection into the loop. b) Time courses of amounts of peak substances. ^{59}Fe-Bl-c (20 μg Fe/loop) was injected into a ligated rat duodenal loop, the mucosa was excised and homogenized, and the supernatant obtained by centrifugation of the homogenate was used as the sample (soluble fraction). Arrows show the positions of the peaks which appeared on gel filtration of the soluble fraction prepared from the $FeSO_4$-loaded mucosal cells.

distributed in the mucosal layer) and then transferred into the body (bloodstream), as reported previously.[3]

The Fe-distributed mucosa was excised at specified time points and homogenized. Each homogenate was centrifuged at 3000 × *g* and then at 27000 × *g* to purify BBM, and Fe contents in the purified BBM, supernatant, and residual precipitate (obtained by the first, low-speed centrifugation) were determined (Table II). The Fe content in the purified BBM showed the highest value at 10 min after Bl-c injection into the loop and declined thereafter. By contrast, the Fe content in the soluble fraction (supernatant) peaked later (at 60 min). The yield of the BBM based on the sucrase activity was as low as less than 30%, and most of the rest of the BBM remained in the residual precipitate.

The soluble fraction at each time point was subjected to gel filtration on a Sephadex G-200 column (Fig. 10a). Each fraction showed two peaks, one around $k_d \fallingdotseq 0.1$ (peak 1) and the other around $K_d \fallingdotseq 1.0$ (peak 2). Peak 1 increased in amount gradually after Bl-c injection and reached the maximum at 60 min, while peak 2 already attained the maximum at 2 min and declined thereafter (Fig. 10b). The elution position of peak 1 was almost identical to that of ferritin (molecular weight, 450000), which has been reported to form stable Fe pools inside the intestinal mucosa.[16] Peak 2 passed through the dialysis membrane, and the elution patterns on Sephadex G-50 of Fe from this peak substance treated and nontreated with glycosidases were almost the same as those of Fe from intact Bl-c. When the soluble fraction of the homogenized, $FeSO_4$-loaded mucosa was similarly prepared and subjected to gel filtration, two peaks appeared (as shown by arrows in Fig. 10a). One peak corresponded to peak 1 in the Bl-c administration experiment, and the other peak was small and appeared around $K_d \fallingdotseq 0.7$, near the elution position of transferrin (molecular weight, 76000); however, little Fe was eluted at the position corresponding to that of peak 2 in the Bl-c administration experiment.

2) Fe Transfer from the Intestinal Mucosa after *in Vitro* Fe Absorption When an everted rat duodenal sac was incubated in the mucosal medium containing ^{59}Fe–Bl-c, Fe was smoothyl taken up by the tissue and subsequently transferred into the serosal medium as *in situ*. The mucosa was excised from the duodenal sac, processed as above, and Fe contents in the purified BBM, supernatant, and residual precipitate were determined (Table III). The Fe content per mg protein was the highest in the purified BBM (shown as the control value in Table III). When the duodenal tissue was denatured by heating, Fe distribution to the BBM (binding) markedly decreased, and the Fe content in the soluble fraction (supernatant) was also less than half the control value; as a result, the Fe transfer into the serosal medium was nearly completely inhibited. When the Bl-c-preloaded everted gut was washed well and incubated in the Fe-free medium, the Fe bound to the BBM decreased. Instead, the Fe content in the soluble fraction increased, and Fe transfer into the serosal medium was newly noted.

TABLE III. Fe Contents in Fractions of Rat Duodenal Mucosa Which Took Up Fe from ^{59}Fe-Bl-c *in Vitro* (Everted Rat Duodenal Sac Method)

Sample	Fe content (ng Fe/fraction)[a]			Fe transfer into serosal medium (ng Fe/ml serosal medium)
	BBM	Supernatant	Residual precipitate	
Control[b]	267 (73.1)	431 (33.7)	5312 (51.3)	324 ± 72[c]
Heated gut sac[d]	12 (20.3)	183 (42.4)	3941 (48.8)	2 ± 0
Bl-c-preloaded gut sac[e]	192 (49.6)	953 (59.7)	4814 (46.5)	78 ± 38

a) Fe content in each fraction of the pooled mucosal cells from 3 animals. The figure in parentheses is the Fe content (ng Fe) per mg protein. *b*) The everted gut sac was incubated for 30 min under the following conditions: initial ^{59}Fe-Bl-c concentration in mucosal medium, 5.6 μg Fe/ml; and composition of medium, 0.1 M NaCl, 5 mM KCl, 0.1 mM $MgSO_4$, 0.3% glucose, 40 mM Hepes buffer (pH 7.0). *c*) Mean ± S.E. (n=3). *d*) The everted gut sac was heated at 110 °C for 5 min and then incubated for 30 min under the same conditions as *b*). *e*) The everted gut sac was preloaded with ^{59}Fe-Bl-c under the same conditions as *b*) and then incubated in the Bl-c-free medium for 30 min.

Discussion

The mechanism of Fe transport across the intestinal mucosa remains uncertain in detail, but it has been reported that (i) a special transport system, other than passive diffusion, may be involved,[3,17-20] and (ii) the first step in the system is the binding of Fe to specific sites on the surface of the BBM.[4,5,7] Recent studies on the Fe transport into BBM vesicles have suggested that Fe(II) is transported through a specific carrier-mediated process and accumulated in the intravesicular space.[8,9] In the present study, the Fe uptake from the Fe(II)–oligosaccharide complex (Bl-c) by the rat duodenal BBM vesicles was not affected by the osmotic difference between the inside and outside of the vesicles, and this finding suggests that Fe taken up by the BBM vesicles may be mainly bound to membrane components (including uptake into the hydrophobic layer or inside the vesicles). This Fe uptake showed a biphasic pattern characteristic of a special transport system[21] in the Arrhenius plot, with a turning point around 27 °C, and the Fe uptake by the BBM vesicles was facilitated by the pH gradient (in > out). Furthermore, evidence for Fe translocation inside the vesicles by a special transport system included the saturation phenomenon in the isotope exchange experiment.

Our previous absorption experiments performed *in situ* (ligated rat duodenal loop method) and *in vitro* (everted rat duodenal sac method) revealed that (i) the Fe tissue uptake and transfer from Bl-c to the serosal side depended on the temperature and pH, (ii) these tended to saturate at high

concentrations, and (iii) the upper part of the small intestine showed the highest Fe absorption.[3] These Fe absorption characteristics were in good accordance with the present results on the Fe uptake from Bl-c by the rat intestinal BBM vesicles. In addition, the Fe uptake by the BBM vesicles was not inhibited by any metal ions tested, including inorganic Fe(II), and the isotope exchange was observed selectively with unlabeled Bl-c, but not with $FeSO_4$ or ferrous ascorbate. These findings suggest that the BBM contains a transport system selective for Bl-c and that the state of Fe transported into the vesicles from Bl-c may be different from the states of Fe in ferrous ascorbate and $FeSO_4$. However, further investigation should be made in order to establish finally whether the transport system on the BBM for Bl-c is independent of that for inorganic Fe(II) or not.

When the role of the BBM in Fe absorption from Bl-c was examined with the fractions of duodenal mucosal cells subjected to the *in situ* and *in vitro* Fe absorption experiments, Fe taken up first by the BBM was transferred *via* the soluble fraction to the serosal side. The Fe uptake and transfer to the BBM were diminished by heating, and this suggests that a protein carrier may be involved in these processes.

As described above, the Fe taken up by the BBM vesicles from Bl-c was estimated to be located inside the vesicles. This Fe taken up by the vesicles is considered to be in the form of a complex with oligosaccharide, based on the result that the Fe released from the Bl-c-preloaded BBM was an Fe–oligosaccharide complex similar to Bl-c. This conclusion is supported by the result of the selective isotope exchange experiment. Furthermore, peak 2 which appeared on gel filtration of the soluble fraction of the duodenal mucosa used for the *in situ* experiment and seems to be involved in Fe transfer into the serosal side, was also an Fe–oligosaccharide complex. These findings suggest that Bl-c may be transported across the BBM in the form of a complex with oligosaccharide, and this suggestion is supported by the previous result that Bl-c in the everted gut sac experiment was transferred into the serosal medium in the form of a complex by a special transport system.[3]

Fe absorption studies with inorganic Fe salts have disclosed that the absorbed Fe is present in the intestinal mucosal cells, bound to ferritin and transferrin-like proteins.[14,22-24] The present study, however, revealed no peak to show that Fe from Bl-c was bound to transferrin-like proteins. This difference in Fe form in the intestinal mucosa between Bl-c and inorganic Fe salts, as well as the low K_m value for the Fe uptake from Bl-c by the BBM vesicles, may be reflected in the higher rate of Fe transfer from Bl-c than that from inorganic Fe salts.[3]

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