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Cholate-soluble and -insoluble iron binding components of rabbit duodenal brush-border membrane. Relevance to Fe^{2+} uptake by brush-border membrane vesicles

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Fe^{2+} uptake by brush-border membrane vesicles from rabbit duodenum has been investigated and found to show similar qualitative properties to those previously demonstrated with mouse proximal intestine brush-border membrane vesicles (Simpson, R.J. and Peters, T.J. (1986) Biochim. Biophys. Acta 856, 109–114). In particular, a relatively low affinity ($K_{m_{(app)}}$ approx. 83 μM), NaCl and pH sensitive transport component is present. The disruption of $^{59}Fe^{2+}$-laden vesicles with sodium cholate, followed by gel filtration or centrifugal analysis reveals that cholate insoluble material ($M_r > 10^6$) is the major destination for $^{59}Fe^{2+}$ taken up by intact vesicles. Analysis of cholate extracts for Fe^{2+} binding ability reveals a single high-capacity (49.8 ± 15.6 nmol / mg vesicle protein (S.E., $n = 3$)), high-affinity ($K_{d_{(app)}}$ less then 5 μM) binding component with an M_r equivalent to approx. 10^4 on gel filtration in the presence of cholate. This binding component is extracted into chloroform / methanol (2:1, v / v) is relatively heat and protease resistant and thus appears to be a lipid.

Introduction

Understanding of the biochemistry of iron absorption is currently lacking. In particular, the mechanism and regulation of the transport of inorganic iron across the brush-border membrane is ill understood.

Cox and O'Donnell ([1–3], have investigated this process by fractionating $^{59}Fe^{2+}$-labelled rabbit duodenal brush-border membrane vesicles. They found a detergent soluble $^{59}Fe^{2+}$-labelled glycoprotein. No demonstration of membrane carrier activity has been made for this material and no Fe^{2+} binding properties of the purified material were reported. The significance of this component of the brush-border membrane in the transport of Fe^{2+} by brush-border membrane vesicles must be re-interpreted in the light of recent studies that have revealed the composite nature of Fe^{2+} uptake by brush-border membrane vesicles [4,5]. We recently demonstrated that Fe^{2+} transport by mouse brush-border membrane in vitro is mediated by a low-affinity ($K_{m_{(app)}}$ 60–90 μM), NaCl-sensitive process with a pH optimum of 6.8–6.9. We observed that sodium cholate extracts of mouse brush-border membrane vesicles contain pH- and NaCl-sensitive Fe^{2+} binding components [5].

Mouse is, however, a poor source of material for the purification of putative Fe^{2+} carriers from brush-border membrane. This is because of small quantities available and because frozen material was found to be unsuitable for iron uptake studies [6]. Frozen rabbit brush-border membranes vesicles have been used successfully for iron uptake studies [7].

Abbreviation: Hepes: 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

In this paper rabbit duodenal brush-border membrane vesicles prepared from frozen gut and stored frozen are shown to exhibit Fe^{2+} uptake properties similar to those previously reported for fresh mouse and rabbit duodenal vesicles. Cholate-soluble and -insoluble Fe^{2+} binding components of the vesicles are examined for Fe^{2+} binding both in situ and in their isolated forms.

Materials and Methods

Materials. Sepharose CL6B and Sephadex G-50 (medium) were obtained from Pharmacia (Uppsala, Sweden), sodium cholate was obtained from Sigma and used without further purification. Chloroform and methanol (Analar grade, BDH) were redistilled before use. Cyanocobalamin was from Duncan Flockhart&Co. Ltd. (Greenford, U.K.), horse spleen ferritin was from Calbiochem (La Jolla, CA, U.S.A.) and Blue dextran 2000 was from Pharmacia. Other reagents or solvents were Analar grade from Sigma or BDH. Sagatal (sodium pentabarbitone B.P.) was from May and Baker Ltd. (Dagenham, U.K.). Proteases were from Sigma (Type XXI, from *Streptomyces griseus* (Pronase), Type X (Thermolysin) and Chymotrypsin).

General Methods. Protein concentrations were determined by the modified Lowry method [8]. Protease digestion was performed in 0.1 M mannitol, 0.1 M NaCl, 20 mM Na-Hepes (pH 7.4) at 37°C and for thermolysin, 1 mM $CaCl_2$ was added. Electron microscopy was performed as described in Ref. 6.

Preparation of brush-border membrane vesicles. Brush-border membrane vesicles were prepared from frozen rabbit duodenum by the method of Kessler et al. [9] essentially as described by Simpson and Peters [6]. The proximal 50 cm of small intestine was removed within 30 min of death from rabbits, killed by an overdose of sodium pentabarbitone. The segment was flushed with 100 ml of ice-cold 0.15 M NaCl then stored at −70°C. Brush-border membrane vesicles were prepared typically from approx. 20 g of tissue (1 duodenum) which was thawed in 300 mM mannitol, 12 mM Na-Hepes (pH. 7.1). The duodenum was cut open and spread, mucosal surface upwards, on a cold plastic dish. Mucus and adherent food particles were removed by blotting with tissue paper. The mucosa was scraped off with a glass slide and suspended in 150 ml of ice-cold 50 mM mannitol, 2 mM Na-Hepes (pH 7.1). The mixture was homogenized for 2 min at full speed in a pre-cooled Waring blender. Subsequent procedures were conducted at 0–4°C. Solid $MgCl_2$ (10 mM final concentration) was added to the homogenate which was gently stirred for 20 min before centrifugation at $3000 \times g$ for 10 min. The supernatant was recentrifuged for 30 min at $40000 \times g$ and the resulting vesicle pellet was suspended, by repeated passage throught a 21 gauge needle, in 80 ml of resuspension buffer (0.1 M mannitol, 0.1 M NaCl, 0.1 mM $MgSO_4$, 20 mM Hepes (pH 7.4), filtered through a 0.22 μm Millipore filter before use). The suspension was centrifuged for 15 min at $6000 \times g$ and the supernatant recentrifuged at $40000 \times g$ for 30 min to sediment the brush-border membrane vesicles. The final vesicle pellet was resuspended as above in 1–2 ml of resuspension buffer to a protein concentration of 10–20 mg/ml. Similar procedures to this have been widely used for the preparation of brush-border vesicles for transport studies [6,7,9]. These preparations are typically 13–20-fold enriched in brush-border marker enzymes. Vesicle suspensions were used immediately or stored at −70°C.

$^{59}Fe^{2+}$-labelling of vesicles and preparation of cholate extracts. Vesicle suspensions were thawed at 37°C and were incubated for 5 or 60 min at 37°C with 91 μM $^{59}Fe^{2+}$, 1.8 mM sodium ascorbate in resuspension buffer or the same buffer without $MgSO_4$. Vesicles were collected by centrifuging for 30 min at $40000 \times g$ and resuspended in 6 ml of ice-cold 0.15 M NaCl followed by recentrifugation. The vesicle pellet was resuspended in resuspension buffer to a protein concentration of approx. 10 mg/ml. Solid sodium cholate was added to give a final concentration of 10% (w/v). After vortexing, the solution was found to clarify within approx. 2 min. The volume change accompanying this cholate solubilization process was less than 5%. The resulting mixture was either immediately applied to Sepharose CL6B columns or centrifuged for 1 h at $165000 \times g$. The supernatant (cholate extract) and pellet (cholate-insoluble material) were separated and this pellet was dispersed (passage through 21 gauge needle) in the same volume of resuspension buffer as the original

mixture occupied. The cholate extract contained approx. half (54.2 ± 4.4% (m + S.E., n = 7)) of recovered protein (recovery, 91 ± 9% (m ± S.E., n = 7)). Extracts and pellets were stored at −20°C if not used immediately. All centrifugation and extraction procedures were performed at 0–4°C.

Gel filtration analysis of cholate-solubilised vesicles and extracts. Gel filtration of solubilised vesicles and cholate extracts was performed on Sepharose CL6B (85 × 1.6 cm) and Sephadex G-50 (47 × 1 cm) columns equilibrated and eluted (13 ml/h and 17 ml/h, respectively) with 0.15 M NaCl, 11 mM sodium cholate, 10 mM Na-Hepes (pH 7.0 at 20°C). Fractions of 3.25 ml and 1.4 ml were collected from the Sepharose and Sephadex columns, respectively, and these were counted for ^{59}Fe (Beckman Gamma-7000) or assayed for Fe^{2+} binding (see below). Absorbance was monitored at 280 nm with a Uvicord S (LKB, Bramma, Sweden). Markers used to calibrate the columns were ferritin, cytochrome *c*, cyanocobalamin, Phenol red, Blue dextran 2000 and $^{59}Fe^{2+}$/ascorbate (1 : 20, molar ratio) solutions. Column fractions were concentrated by ultrafiltration and flow dialysed over a YM-2 filter (Amicon, Stonehouse, Glos., U.K.) with an Amicon UM2 ultrafiltration cell and stored at −20°C. All above procedures were performed at 4–6°C.

$^{59}Fe^{2+}$ uptake and binding measured by Millipore filtration. Vesicle $^{59}Fe^{2+}$ uptake was determined as described previously [4,5]. Briefly, vesicles were incubated at 37°C with $^{59}Fe^{2+}$-Na ascorbate (molar ratio 1 : 20) in 0.1 M mannitol, 0.1 M NaCl, 20 mM Na-Hepes (final pH 7.25). Uptake was terminated by rapid filtration (0.22 μm filter, Millipore filtration manifold catalogue No. XX2702550) of 50 μl of incubation mixture. The filter was immediately washed with 10 ml of ice-cold 0.1 mM Fe^{2+}, 2 mM sodium ascorbate, 0.15 M NaCl.

Fe^{2+} binding by column fractions or extracts was studied in the same medium with final cholate concentrations of less than 5.5 mM. The quantity of Fe^{2+} binding material was adjusted to yield uptake values of less than 10–20% of total medium radioactivity. Retention of ^{59}Fe-labelled proteins and column fractions by the filters was tested by incubation in 10 vol. of the same incubation medium (without $^{59}Fe^{2+}$-ascorbate) followed by filtration and washing as above. Radioactivity retained by filters was determined by gamma counting in a Beckman Gamma-7000. Previous studies [5] have shown that cholate-soluble Fe^{2+}-binding components of mouse proximal intestine brush-border membrane vesicles are retained by Millipore GSWP filters, provided low medium cholate concentrations are employed (less than approx. 0.25%).

$^{59}Fe^{2+}$ uptake by cholate-insoluble vesicle components measured by centrifugation. Vesicles or resuspended cholate insoluble material were incubated with $^{59}Fe^{2+}$-ascorbate as for filtration assays (final volume 50 μl) for 5 or 60 min at 37°C in 10 ml polycarbonate ultracentrifuge tubes. Solid sodium cholate was added (10% (w/v)) and the tubes vortexed. The tubes were immediately spun for 1 h at 165 000 × g at 4°C. The supernatant was removed and the tube carefully washed with distilled water (50 μl) without disturbing the pellet. Tube (with pellet) and supernatant (plus washings) were counted as above for ^{59}Fe.

Results and Discussion

$^{59}Fe^{2+}$ uptake by frozen-stored rabbit duodenal brush-border membrane vesicles

Fig. 1A shows Fe^{2+} uptake by frozen-stored rabbit duodenal brush-border membrane vesicles as a function of time. Quantitatively, uptake is similar to that reported for fresh vesicles [1,2]. Overall uptake can be divided into a small, rapid, NaCl-insensitive and relatively pH-insensitive uptake and a large, slower, more pH-sensitive component. We have shown, with mouse brush-border membrane vesicles, that the larger component of uptake is consistent with a membrane transport process by the vesicles with intravesicular binding of Fe^{2+} [5].

Fig. 1B shows initial Fe^{2+} uptake by vesicles at two medium Fe^{2+} concentrations as a function of pH. The variability in uptake rates by different vesicle preparations can be noted by comparing Figs. 1A and 1B. This variability has been noted with fresh mouse vesicles [4] and can be seen in previously published data obtained from fresh rabbit vesicles [1,2]. Qualitative observations are, however, highly reproducible. The pH optimum (7.0) in overall uptake is the same as was reported

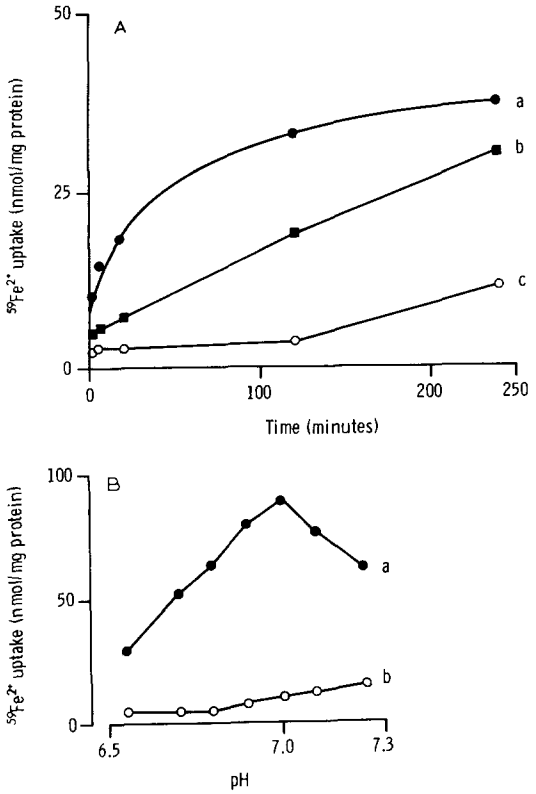

Fig. 1. $^{59}Fe^{2+}$ uptake by frozen-stored rabbit duodenal brush-border membrane vesicles. (A) Vesicles were prepared from frozen rabbit duodenum as described in Materials and Methods and assayed for $^{59}Fe^{2+}$ uptake by Millipore filtration [4]. (a) medium was 91 μM $^{59}Fe^{2+}$, 1.8 mM sodium ascorbate, 0.1 M NaCl, 0.1 M mannitol, 20 mM Hepes (final pH 7.25). Incubation was at 37°C, (b) as (a) except the final pH was 6.55, (c) as (a) except the final assay contained 1.8 M NaCl. (B) Uptake was determined after 1 min incubation as in (Aa) except the final pH was varied as in Ref. 5. (b) as (a) except $^{59}Fe^{2+}$ was 9.1 μM and sodium ascorbate 0.18 mM.

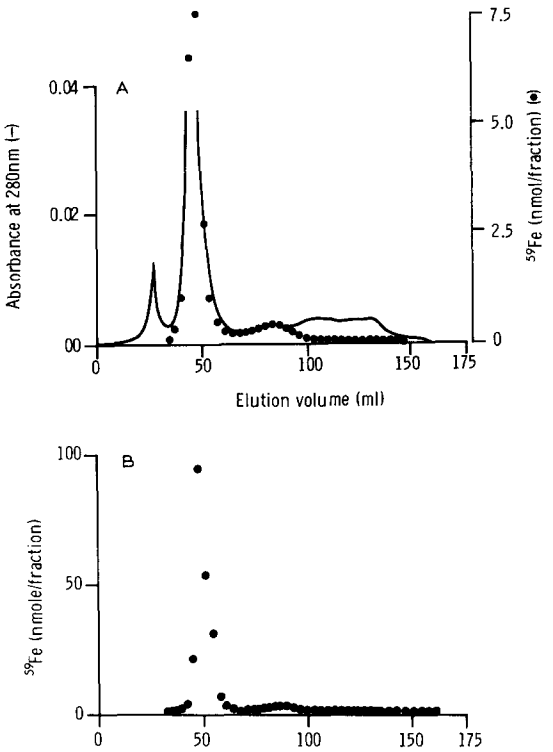

Fig. 2. Gel filtration analysis of cholate-disrupted rabbit brush-border membrane vesicles. (A) Vesicles were prepared and $^{59}Fe^{2+}$ labelled as described in Materials and Methods (incubation time 5 min). Vesicles, 0.5 ml, (4.6 mg protein) were labelled and, after washing, suspended in 0.5 ml of resuspension buffer (see Materials and Methods), sodium cholate, 50 mg, was added and the mixture vortexed and applied to a 85×1.6 cm Sepharose CL6B column equilibrated and eluted with 0.15 M NaCl, 11 mM sodium cholate, 10 mM Hepes (pH 7.0). (B) As (A) except the incubation time during $^{59}Fe^{2+}$ labelling was 60 min and the vesicle protein concentration was reduced to 0.85 mg/ml, with an 11-fold larger incubation volume, during the labelling. Subsequent procedures were identical to (2A).

for fresh rabbit duodenal brush-border membrane vesicles [1] and is approx. 0.1 unit higher than reported for mouse vesicles [5]. High-affinity uptake (curve b, Fig. 1B) shows no pH optimum, as was noted with mouse vesicles. Studies of the dependence of uptake on medium Fe^{2+}, in the presence and absence of NaCl, reveal that NaCl-sensitive uptake has a $K_{m_{(app)}}$ of approx. 80 μM, similar to mouse vesicles [4]. Thus, all the major conclusions derived from extensive studies with mouse brush-border membrane vesicles can be obtained with frozen-stored rabbit brush-border membrane vesicles.

Studies with cholate extracts of mouse proximal intestine brush-border membrane vesicles revealed cholate extractable Fe^{2+} binding components [5]. We therefore set out to disrupt rabbit brush-border membrane vesicles, with and without prior $^{59}Fe^{2+}$ labelling and search for $^{59}Fe^{2+}$-labelled and $^{59}Fe^{2+}$-binding components of brush-border membrane vesicles.

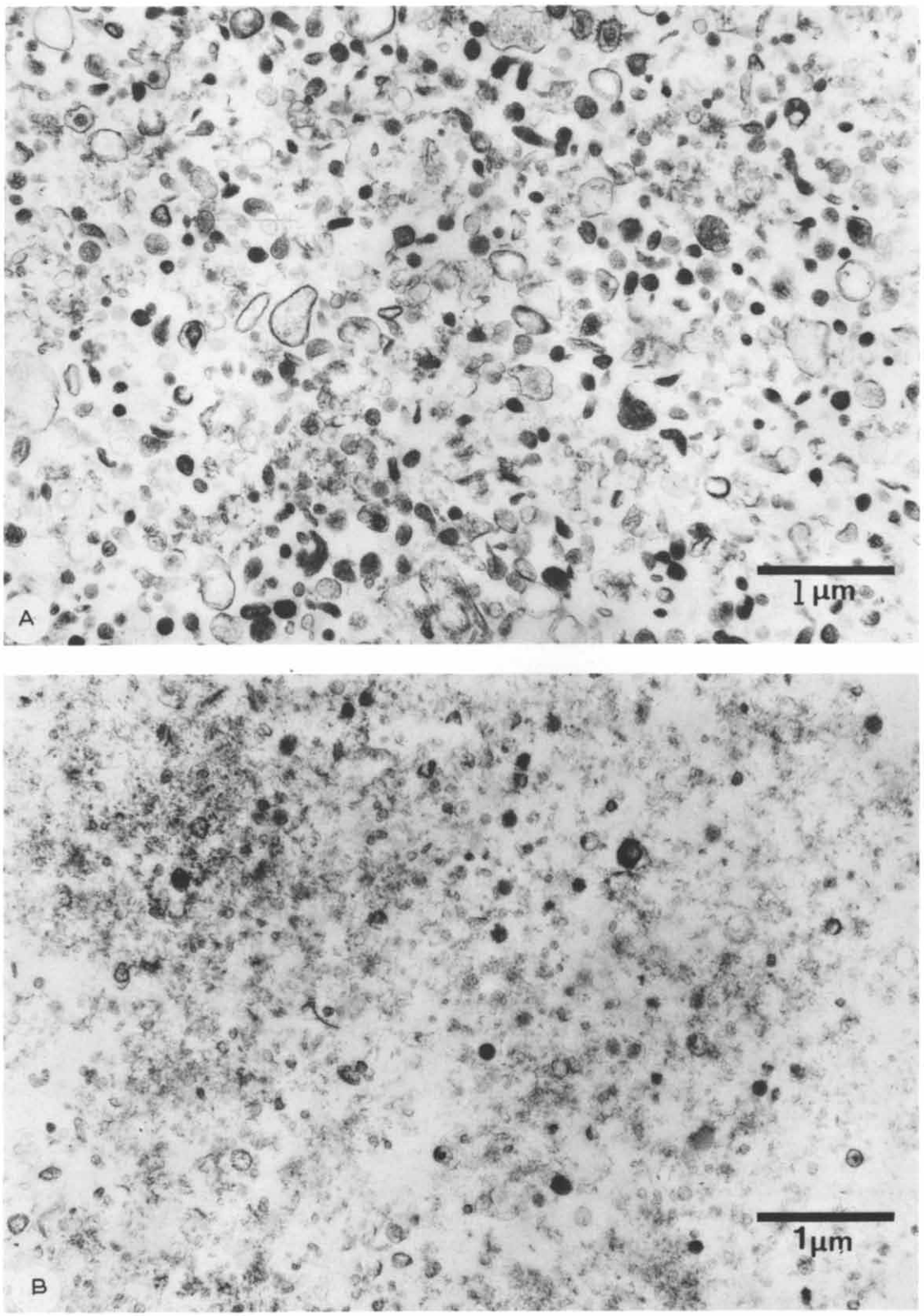

Fig. 3. Electron micrographs of A) intact rabbit brush-border vesicles preparation and (b) cholate-insoluble material. Vesicles and 165000 × *g* cholate extract pellet were prepared as in Materials and Methods. Fixing and staining with uranyl acetate was as described in Ref. 6.

Fractionation of $^{59}Fe^{2+}$-labelled brush-border membrane vesicles

Fig. 2 shows the elution profile of ^{59}Fe from vesicles incubated for short (Fig. 2A) and long Fig. 2B) periods with $^{59}Fe^{2+}$ prior to cholate disruption and fractionation on Sepharose CL6B. The A_{280nm} profiles for each experiment were similar. Comparison of Figs. 2A and 2B reveals that as the Fe^{2+} uptake proceeds, the very high molecular weight ^{59}Fe-labelled peak increases relative to the other labelled components. Electron micrographs of this material and also of $165000 \times g$ pellets from cholate extractions reveal microvillus cores (Fig. 3). Also present are larger aggregates and occasional nondisrupted vesicles.

When centrifuged cholate extracts of ^{59}Fe-labelled vesicles were similarly chromatographed, a similar profile was obtained except that the large peak at approx. 48 ml elution volume was considerably reduced and was smaller than the peak at 85 ml. The major included ^{59}Fe-labelled peak of radioactivity (85 ml) had the elution position of horse spleen ferritin but elutes significantly earlier than the major Triton X-100 extractable ^{59}Fe peak reported by O'Donnell and Cox [3]. Ferritin contamination of brush-border membrane vesicle preparations would not be surprising and it is possible that ferritin is present inside brush-border membrane vesicles.

Column recoveries of $^{59}Fe^{2+}$ from such experiments were high ($72 \pm 6\%$ S.E., $n = 3$) but consistently lower than recoveries obtained when high molecular weight ^{59}Fe-labelled material was rechromatographed on the same column (88–92% recovery) or when ^{59}Fe-labelled ferritin was chromatographed alone (100% recovery).

The high recovery of radioactivity from the ^{59}Fe-labelled vesicles suggests that the high molecular weight material contains the major $^{59}Fe^{2+}$ destination site(s) in the vesicle Fe^{2+} uptake process. The fact that the recoveries nevertheless differ significantly from 100% ($p < 0.05$) and are lower than rechromatographed column eluates suggests that other Fe^{2+} binding components are present which either do not elute from the Sepharose CL6B or lose their iron during the chromatography. Sepharose CL6B retains a high proportion (more than 90%) of ^{59}Fe-$^{2+}$-ascorbate (1 : 20) when quantities similar to those employed for Fig. 2A run as a marker. Any Fe^{2+}-binding material with a dissociation constant in the μM range would be expected to lose its Fe^{2+}, due to mass action effects, on gel filtration chromatography. This dissociated Fe^{2+} would be likely to adsorb to the column, given the low recovery of $^{59}Fe^{2+}$-ascorbate. It is therefore necessary, particularly when searching for possible Fe^{2+} carrier moieties which may not have high ($K_{d(app)}$ less than 1 μM) affinity for Fe^{2+}, to perform Fe^{2+} binding studies rather than just searching for Fe^{2+}-labelled sites.

Fe^{2+}-binding components of cholate-disrupted brush-border membrane vesicles

Fig. 4 shows the profile of $^{59}Fe^{2+}$ binding by column fractions (assayed by Millipore filtration after dilution of the cholate) from cholate-solubilised vesicle preparations. The predominant $^{59}Fe^{2+}$-binding peak occurs at a relatively low apparent molecular weight. The marker cytochrome *c* (M_r 12500) elutes at a similar position (129 ml) to this peak.

The use of a filter binding assay to determine Fe^{2+}-binding material depends on the assumption that proteins and other components of vesicles are retained by the filters. Studies with ^{59}Fe-labelled

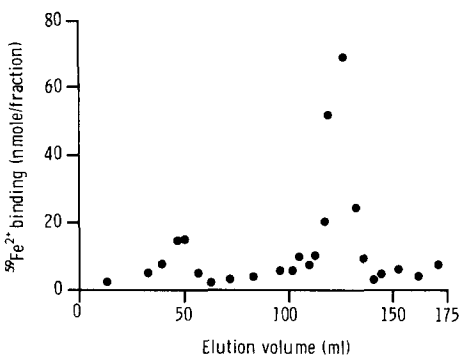

Fig. 4. $^{59}Fe^{2+}$ binding by gel filtration fractions from cholate-disrupted brush-border membrane vesicles. Unlabelled vesicles (1 ml, 9.1 mg protein) were disrupted with 100 mg of sodium cholate and loaded on a column as in Fig. 2. $^{59}Fe^{2+}$ binding to column fractions was determined after incubation of 5 μl of column fraction with 50 μl of 100 μM $^{59}Fe^{2+}$, 2 mM sodium ascorbate, 0.1 M NaCl, 0.1 M mannitol, 20 mM Hepes (final pH 7.25) for 10 min at 37°C. Mixture was applied to a 0.22 μm Millipore filter, washed and counted as for vesicle uptake studies (see Materials and Methods).

ferritin, lactoferrin and transferrin revealed that these proteins were retained between 50 and 100% by 0.22 μm Millipore filters, provided protein and cholate concentrations were kept low, similar to those employed in the assays for Fig. 4. Retention of ^{59}Fe-labelled vesicle components from experiments such as in Fig. 2 were examined in model $^{59}Fe^{2+}$ binding experiments. The predominant ^{59}Fe-labelled site was $91 \pm 4\%$ ($M \pm$ S.E., $n = 6$) retained. The minor peak (85 ml on Fig. 2) was at least 65% retained. Recovery of the main $^{59}Fe^{2+}$-binding component (Fig. 4) was more difficult to assess. However, experiments where limiting amounts of $^{59}Fe^{2+}$ were incubated with the Fe^{2+} binding fractions showed that retention was at least 40%. These values allow a semi-quantitative analysis to be performed.

In order to further investigate the Fe^{2+}-binding components of the vesicles, analysis of cholate extracts by Sephadex G-50 was undertaken. This gel filtration matrix was found to give better recoveries of $^{59}Fe^{2+}$-ascorbate, when chromatographed as a marker, than Sepharose CL6B (65% compared with 4%, respectively).

Fig. 5A shows the ^{59}Fe-labelling profile of a cholate extract of vesicles labelled as for Fig. 2A. Total recovery of ^{59}Fe (i.e. column eluate plus cholate insoluble material) was close to 100% (mean of three experiments, $99 \pm 5\%$ (S.E.)). The additional recovered ^{59}Fe (compared to Sepharose CL6B analyses) elutes in the same position (38 ml) as $^{59}Fe^{2+}$-ascorbate/incubation mixture chromatographed without vesicles.

Fig. 5B shows the $^{59}Fe^{2+}$-binding profile for a similar experiment except that the prior ^{59}Fe-labelling was omitted. The single Fe^{2+}-binding peak again elutes at a similar position to the M_r 12 500 marker cytochrome *c* (22 ml). The recovery of $^{59}Fe^{2+}$-binding capacity could be measured by assay of the original extract for $^{59}Fe^{2+}$ binding under similar conditions of final assay cholate concentration. The recovery of binding capacity of extracts in the low molecular weight peak was again high ($78 \pm 15\%$, S.E. ($n = 6$)), suggesting that the predominant Fe^{2+} binding site in cholate extracts of brush-border membrane vesicles is the material of apparent M_r of approximately 10 000.

In another series of experiments, cholate extracts were labelled with varying amounts of

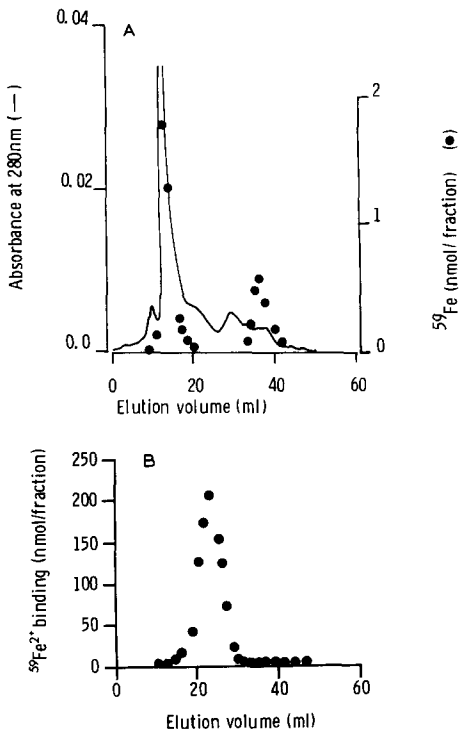

Fig. 5. Sephadex G-50 gel filtration analysis of cholate extracts of rabbit brush-border membrane vesicles. (A) Cholate extract (165 000 × *g* supernatant, 10% cholate, w/v) from vesicles labelled and washed as in Fig. 2A were prepared as in Materials and Methods. Extract (0.5 ml, 6.15 nmol $^{59}Fe^{2+}$) was applied to a 47 × 1 cm Sephadex G-50 column equilibrated and eluted with 0.15 M NaCl, 11 mM sodium cholate, 10 mM Na-Hepes (pH 7.0). (B) As (A) except that 1 ml of vesicle extract was applied to the column without preincubation with $^{59}Fe^{2+}$. $^{59}Fe^{2+}$ binding was determined as in Fig. 4.

$^{59}Fe^{2+}$-ascorbate, then fractionated on the Sephadex G-50 column. It was found that under no circumstances did significant amounts of ^{59}Fe appear in the region 20–35 ml. Assay of these fractions for ^{59}Fe binding revealed that the $^{59}Fe^{2+}$-binding material was still present. This suggests that the low molecular weight $^{59}Fe^{2+}$-binding material dissociates on gel filtration chromatography.

The characteristics of the low molecular weight $^{59}Fe^{2+}$-binding material

Fig. 6 shows the $^{59}Fe^{2+}$ uptake time-course for the low molecular weight and cholate-insoluble fractions. The uptake by low molecular weight

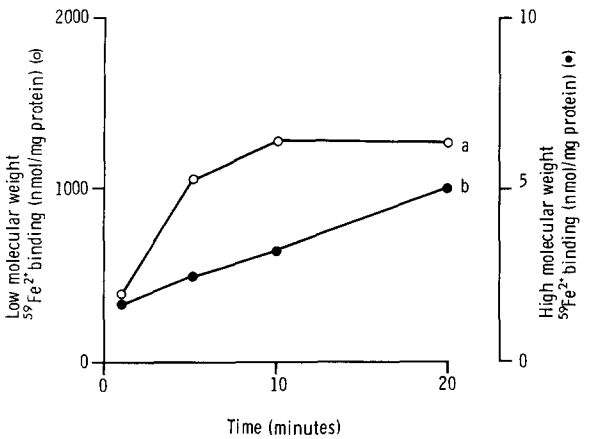

Fig. 6. $^{59}Fe^{2+}$ uptake by low and high-molecular weight vesicle components. Column fractions from Sepharose CL6B fractionation (a) 127 ml (low-molecular weight) and (b) 48 ml (high-molecular weight component) were incubated and Fe^{2+} uptake determined as described in Fig. 4.

material shows a rapid time-course, similar to cholate extracts of mouse brush-border membrane vesicles [5]. The time-course is consistent with a binding process. Uptake by cholate-insoluble material (Fig. 6) is slow after a small initial rapid component, and the linear phase continues for at least 1 h. Fig. 7 shows the pH dependence of the binding by low molecular weight material. Different preparations of low molecular weight material showed optima in the range 7.0–7.2. Fig. 8 shows that binding by this material is characterised by relatively high affinity ($K_{d_{(app)}}$ less than 5 μM).

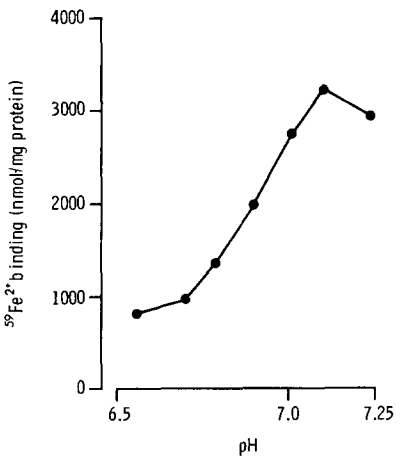

Fig. 7. pH dependence of $^{59}Fe^{2+}$ binding by cholate-soluble component of brush-border membrane vesicles. Fractions 21–31 ml from Sephadex G-50 fractionation of cholate extracts were combined and concentrated to 0.5 ml by ultrafiltration and flow dialysed against 10 vol. of 0.15 M NaCl (YM-2 filter). Uptake determinations were performed with material diluted 10-fold in resuspension buffer and incubated for 10 min in media as in Fig. 1B. Uptake was determined by Millipore filtration as in Fig. 6.

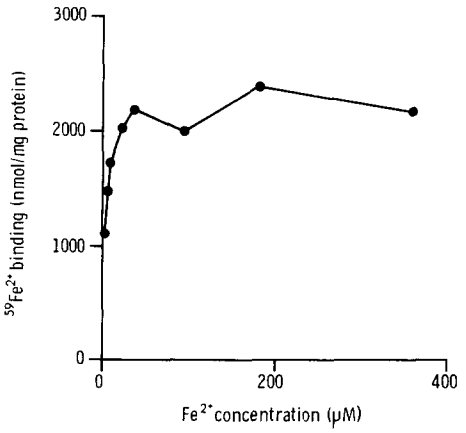

Fig. 8. Dependence of $^{59}Fe^{2+}$ uptake by cholate-soluble $^{59}Fe^{2+}$-binding material on medium $^{59}Fe^{2+}$ concentration. $^{59}Fe^{2+}$-binding material, prepared and diluted as in Fig. 7, was incubated for 10 min at 37°C with various medium $^{59}Fe^{2+}$ concentrations and constant $^{59}Fe^{2+}$/ascorbate ratios of 1:20. Uptake was determined as in Fig. 4.

Nature of the low molecular weight $^{59}Fe^{2+}$-binding material

The material was relatively stable to heat treatment, binding being decreased by less than 10% on boiling for 5 min. Prolonged heating (30 min at 80°C) led to a greater loss of binding (53 ± 15% (mean ± S.E., $n = 3$)). Treatment with proteases failed to obtain 100% degradation. The most effective protease of three tested (chymotrypsin, thermolysin and pronase) was thermolysin, which produced little loss in binding after 19 h incubation at 37°C (mean 27 ± 18% (S.E., $n = 4$) loss with 450 μg/ml thermolysin. The concentration of this broad specificity protease employed here is very high (compare Ref. 10) and the loss of binding incomplete. The Fe^{2+}-binding material showed no distinct band on sodium dodecylsulphate polyacrylamide gel electrophoresis (15% gel, 0.1% sodium dodecylsulphate, reducing conditions) after staining with Coomassie blue. A broad, diffuse staining at lower molecular weight than bovine pancreatic trypsin inhibitor (M_r approx. 6500)

was noted. The Fe^{2+}-binding material was insensitive to sulphydryl reagent, with less than 5% loss in binding after 30 min incubation with 0.9 mM *p*-chloromercuribenzene-sulphonate at 37°C (pH 7.25). This, combined with the apparent inability of metallothionein to bind Fe^{2+} [11] indicates that it is unlikely that this protein accounts for the low molecular weight $^{59}Fe^{2+}$-binding material.

The material appears to be a hydrophobic component in that removal of detergent by passage down Sephadex G-50 (in the absence of cholate) resulted in aggregation, the $^{59}Fe^{2+}$-binding material appearing at the void volume of the column. Marked loss of $^{59}Fe^{2+}$ binding capacity was produced by chloroform/methanol extraction (greater than 80% loss after 3 extractions with 1 vol. of 2:1 chloroform/methanol). When chloroform/methanol extracts were evaporated under N_2 and resuspended in the column buffer (0.15 M NaCl, 10 mM Hepes (pH 7.0), 11 mM sodium cholate) by sonication, the $^{59}Fe^{2+}$-binding material was fully recovered (111 ± 8% recovery, mean ± S.E. for three experiments) suggesting the material may be a lipid. The apparent molecular weight of 10000 presumably represents that of a mixed cholate/lipid micelle.

The significance of $^{59}Fe^{2+}$ binding by cholate-extractable material in $^{59}Fe^{2+}$ uptake by intact brush-border membrane vesicles

As noted above, cholate-extract-bound $^{59}Fe^{2+}$ appears to be dissociated on gel filtration chromatography. Our experiments show that the majority of vesicle-bound $^{59}Fe^{2+}$ is in cholate-insoluble material. Fig. 6 suggests that, on cholate solubilisation, the uptake rate by cholate insoluble material is relatively slow. In order to further investigate the relationship of $^{59}Fe^{2+}$ uptake by cholate-insoluble material and the low molecular weight material in intact vesicles, $^{59}Fe^{2+}$ uptake by cholate-insoluble material was investigated by centrifugal analysis (Table I).

These experiments show that cholate-solubilised, but unfractionated, vesicles behave in a similar manner to the separated cholate-soluble and -insoluble components, that is, the ^{59}Fe is taken up relatively slowly by cholate-insoluble material. This suggests that the slow uptake noted by cholate-insoluble material in Fig. 6 is not due to aggregation effects brought about the removal of cholate and lipid. It is also clear that intact vesicles behave differently from cholate-treated vesicles. In particular, ^{59}Fe rapidly accumulates in the cholate-insoluble site(s) in intact vesicles. It should be noted that these differences are not due to low recovery of cholate insoluble material.

These observations suggest that $^{59}Fe^{2+}$ uptake by intact vesicles is a complicated process of transport followed by binding inside the vesicles which is not explained by the separated binding components, even when re-mixed. It is, however, possible that dilution effects, caused by the opening of vesicles by cholate, are important. Alternatively, it may be that separation of Fe^{2+} from ascorbate by the intact vesicle membrane, allow-

TABLE I

$^{59}Fe^{2+}$ UPTAKE BY CHOLATE-INSOLUBLE VESICLE COMPONENTS

Experiments. (a) Vesicles were incubated for the indicated time at 37°C with 90 μM $^{59}Fe^{2+}$, 1.8 mM sodium ascorbate, 0.1 M NaCl, 0.1 M mannitol, 20 mM Na-Hepes (final pH 7.25). After incubation, vesicles were solubilised with 10% cholate and fractionated as described in Methods. (b) Cholate-insoluble material, prepared from the same vesicles as (a) and resuspended in the same buffer were incubated, treated with cholate and centrifuged as for (a). (c) Vesicles were incubated and treated as for (a) except that cholate was added before the $^{59}Fe^{2+}$-labelling incubation was performed. In the case of (b) the data were expressed relative to the original vesicle protein concentration prior to cholate extraction. Recovery of cholate-insoluble material which had been ^{59}Fe-labelled in situ in intact vesicles was 81 ± 8% (S.E., $n = 5$) after control incubation (lacking ^{59}Fe) and centrifugation. Total recovery of ^{59}Fe in these experiments was 100.1 ± 0.4% (S.E., $n = 13$).

Experiment	Incubation time (min)	Uptake of $^{59}Fe^{2+}$ in cholate-insoluble material (nmol per mg vesicle protein)
(a) Intact vesicles	5	30.4 ± 2.0 (3)
	60	56.7 ± 6.7 (4)
(b) Cholate-insoluble material	5	8.0 ± 2.3 (3) *
	60	17.6 ± 3.6 (3) **
(c) Cholate-disrupted vesicles	5	8.4 ± 1.5 (3) *

* $p < 0.01$ compared with intact vesicles ($t = 5$ min).
** $p < 0.01$ compared with intact vesicles ($t = 60$ min) (mean ± S.E. for n experiments).

ing oxidation of Fe^{2+} to Fe^{3+}, is important.

It is clear, however, that removal of cholate-soluble vesicle components markedly reduces the uptake rate by the cholate-insoluble residue and the major Fe^{2+}-binding material removed by cholate may thus be responsible for Fe^{2+} transport across the brush-border membrane. The identity of this cholate extractable lipid is currently under investigation.

Conclusions

Fe^{2+} uptake by brush-border membrane vesicles may be mediated by a low molecular weight, cholate- and chloroform/methanol-extractable membrane component. The ultimate destination for Fe^{2+} taken up by vesicles is bound to cholate-insoluble vesicle components.

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