Studies on the binding of iron by rabbit intestinal microvillus membranes

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1. ^{59}Fe binding by microvillus membranes purified from rabbit intestine was studied by means of a microfiltration procedure. 2. Binding activity from ferrous ascorbate chelates was 100-fold greater than from ferric chelates of citrate and nitrilotriacetate. Dual-label experiments indicated dissociation of iron complexes before binding to the membranes. 3. Binding was inhibited at low incubation temperatures and was optimal at neutral pH. 4. Binding activity was reduced in ileal preparations when compared with membranes prepared from proximal intestine. 5. Initial binding velocity followed saturation kinetics over the range 45–450 μM -iron: it was weakly inhibited in the presence of excess Co^{2+} and V^{3+} . 6. The data provide additional evidence for high-affinity iron-binding sites on the intestinal microvillus membrane and indicate properties that may reflect the functional significance of the binding step in the absorption pathway for iron.

Iron is an abundant and essential nutrient, but, owing to its reactivity and the nature of its solution chemistry, its biological availability is poor. Indeed, the remarkable prevalence of iron deficiency in all human populations and certain domestic animals (Underwood, 1971) is an indication that concentrations of available iron are often critically low.

Problems of availability and transfer of iron are partially overcome in living organisms by the synthesis of high-affinity iron-binding proteins which serve to deliver iron to cells and tissues; the transferrin proteins and iron-transport systems in bacterial membranes (Langman *et al.*, 1972) are examples of such adaptations. However, in the vertebrate intestine, poor bioavailability of dietary iron poses special difficulties, since this organ is alone responsible for obtaining the total requirements for body iron from the environment. It is therefore likely that an analogous high-affinity system exists to facilitate incorporation of iron from the gut lumen.

Luminal fluid is in direct contact with the apical, microvillus membrane of the absorptive epithelium, and it has been suggested (Hübers *et al.*, 1971; Cox *et al.*, 1979) that this membrane contains specific binding sites for iron and iron complexes. Avid binding to this surface membrane would promote efficient initial extraction of iron from the luminal contents and permit direct delivery to

transport moieties involved in its subsequent passage across the epithelium (Hübers *et al.*, 1976).

The aims of the present studies were to examine preparations of purified intestinal microvillus membranes for iron-binding activity, to investigate the kinetic characteristics of this process, and to determine its functional significance in relation to the chemistry and absorption physiology of iron.

Experimental

Preparation of membranes

Male New Zealand White rabbits fed pelleted rabbit chow (230 mg of Fe/kg; Labsure, Poole, Dorset, U.K.) for 4–6 weeks were allowed water only for 36 h before death. The animals were killed by injecting 30 ml of air intravenously. Except where stated, the proximal 50 cm of intestine was removed, flushed with chilled neutral 0.15 M-NaCl, opened and blotted. Mucosa, obtained by scraping, was used to prepare microvillus membranes by the method of Schmitz *et al.* (1973). Tris was replaced by Hepes (sodium salt) in the hypo-osmotic homogenizing buffer and all subsequent procedures were performed at 0–4°C. The microvillus membrane pellet (P_2) was suspended, except where stated otherwise, in an iso-osmotic incubation buffer (see below) by repeated passage through a 21-gauge needle.

Enzyme assays

Sucrase (EC 3.2.1.48) was determined in whole homogenates and membrane suspensions by the

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

method of Dahlqvist (1968); likewise succinate dehydrogenase (EC 1.3.99.1) was assayed by the method of Prospero (1974). ($\text{Na}^+ + \text{K}^+$)-ATPase (EC 3.6.1.3) was determined as described by Quigley & Gotterer (1972). These enzymes were used as markers for microvillus, mitochondrial and basolateral membranes respectively. Protein was measured by the fluorescamine method as modified by Van Frank (1975), with crystalline bovine serum albumin as standard (Amour Pharmaceutical Co., Phoenix, Az, U.S.A.).

⁵⁹Fe uptake assay

Uptake was determined, within 2 h of membrane preparation, in the presence of sodium salts of competing chelates: ascorbate, citrate and nitrilotriacetate were used at a fixed concentration of 10 mM. Membranes (final protein concn. 0.04–0.09 mg/ml) were normally incubated in Hepes incubation buffer at pH 7.35 [20 mM-Hepes (sodium salt)/130 mM-NaCl/3.5 mM-KCl/1 mM-MgCl₂/1 mM-CaCl₂]. This buffer contained freshly-made chelates prepared by the method of Bates *et al.* (1967). ⁵⁹Fe (IFS 1; The Radiochemical Centre, Amersham, Bucks., U.K.) was equilibrated with sufficient FeCl₃ in 0.1 M-HCl to give, after chelate formation and the addition of a 100-volume excess of incubation buffer, 50–500 μM -chelate with a specific radioactivity of 5–50 $\mu\text{Ci}/\mu\text{g}$ -atom of Fe. Incubations were started by rapidly mixing 50–100 μl of membrane suspension with sufficient medium at 37°C to give a final volume of 1 ml. They were stopped by diluting them 10-fold with ice-cold Hepes buffer and filtering through moistened 0.45 μm Millipore filters (HAWP 25). The filters were washed twice with 10 ml of buffer. The procedure has been shown in this laboratory and elsewhere (Hopfer *et al.*, 1973) to recover particulate sucrase activity quantitatively. Radioactivity from ⁵⁹Fe retained by the filters was determined with 10% efficiency in a well-type γ -radioactivity counter. Radioactivity in the medium was measured after applying 50 μl to a dry filter. Radioactivity non-specifically adherent to the filters was determined after filtering suitably diluted media without membranes and washing the

filters; these values (less than 0.1% of the radioactivity in the medium) were subtracted from all corresponding experimental values. This minimal retention of radioactivity from the medium did not change significantly over the course of the incubations and testifies to the stability of the chelate systems employed. Results of representative single experiments are shown, since up to 2-fold variation in uptake was observed on different days between different membrane preparations. However, within experiments, errors were generally small; replicate determinations were normally within 5% of mean values. Qualitatively identical results to those illustrated were observed on at least three occasions with different membrane preparations, unless otherwise stated. Statistical significances were evaluated by analysis of variance.

Results and discussion

Purification of microvillus membranes

Microvillus membranes isolated from the intestine by this method form right-side out vesicles spontaneously; they are thus ideally suitable for binding or transport studies (Kenny & Booth, 1978). Purification data are set out in Table 1, where it can be seen that our membrane pellet, which contained only 1–3% of homogenate protein, possesses approx. one-quarter of the total sucrase activity. Sucrase was enriched 15–20 fold, but marker enzymes for mitochondrial and plasma membranes were not enriched. Our purifications are similar to those of Schmitz *et al.* (1973), who found only minimal contamination of their membrane pellet by other organelle markers.

Time course for membrane iron uptake and effect of different chelates

Iron uptake by microvillus membranes over the concentration range 10–450 μM -iron chelate was time-dependent for 30–60 min at 37°C (Figs. 1 and 4). During this period, less than 10% of radioactivity in the medium was lost on filtration of the incubation mixture. Uptake studies carried out after 70 min pre-incubation gave results within 5% of

Table 1. Purification of microvillus membranes from rabbit intestine
Values are means $\pm$ S.E.M. for the numbers of determinations in parentheses.

Fraction	Specific activity of:		
	Sucrase (27) (units/mg of protein)	Succinate dehydrogenase (6) (units/mg of protein)	(Na^+/K^+)-ATPase (3) (μg -atom of P/h per mg of protein)
Whole homogenate	2.69 $\pm$ 0.27	68.9 $\pm$ 14.9	2.81 $\pm$ 1.23
Membrane pellet	41.1 $\pm$ 5.2	28.8 $\pm$ 14.4	2.13 $\pm$ 1.13
Enrichment factor	17.5 $\pm$ 1.6	0.4 $\pm$ 0.1	0.9 $\pm$ 0.2
Yield (%)	22.8 $\pm$ 2.6	1.2 $\pm$ 0.4	4.7 $\pm$ 1.7

Fig. 1. *Chelate-mediated iron binding to membranes* (a) Shows the time course for membrane incorporation of iron presented as Fe(II) ascorbate (■), Fe(III) nitrilotriacetate (Δ), and Fe(III) citrate (●). Ligand was present at $90\mu\text{M}$, with 110-fold excess of chelator. Single point determinations employing $50\mu\text{g}$ of membrane protein/ml are shown; the scale on the vertical axis is amplified 100-fold, as shown, for the ferric chelates. (b) Shows the time course for incorporation of radiolabelled components of the Fe(II)-ascorbate complex. Results of parallel incubations employing either ^{59}Fe -ascorbate (■) or ^{56}Fe -L-[carboxy- ^{14}C]ascorbate (□) complex at $90\mu\text{M}$ in presence of 110-fold excess of ascorbate are shown. Incubations contained $70\mu\text{g}$ of membrane protein and either $0.3\mu\text{Ci}$ of ^{14}C or $0.5\mu\text{Ci}$ of ^{59}Fe in 1 ml. ^{14}C retained by filters was assayed in a liquid-scintillation counter at 85% efficiency.

divalent ascorbate complex. It is likely that both the thermodynamic stability constants of these different complexes [which for the ferric chelates are very great (Sillén, 1971)] and the kinetic lability of the individual chelates (Bates *et al.*, 1967) interact to produce these effects. Such factors in the present system would override differences in the redox state of the metal. Oxidation state probably influences iron availability for intestinal absorption solely by affecting its intraluminal solubility (Forth & Rummel, 1973).

Further experiments were carried out to determine whether or not the ascorbate complex is taken up intact, or dissociates to yield its iron at the membrane surface (Fig. 1b). In parallel incubations in which ^{56}Fe -L-[carboxy- ^{14}C]ascorbate and ^{59}Fe -ascorbate were employed, no uptake of the ^{14}C was detected, although membrane incorporation of ^{59}Fe was again confirmed ($P < 0.01$). In subsequent experiments, membrane uptake of ^{59}Fe was therefore determined in the presence of 10 mM-ascorbate.

Binding versus transport of iron

In view of the tendency of membrane preparations to vesiculate, it was important to distinguish binding from transport of iron into a membrane-bound space. When equilibrium uptake was examined in the presence of increasing concentrations in the medium of the resistant and impermeant disaccharide D-(+)-cellobiose as described by Hopper *et al.* (1973), iron uptake was unaffected. This observation strongly suggests that, in the present system, uptake was a manifestation of iron binding rather than transfer to an osmotically active space. This view is further supported by the finding that iron incorporation by $100\mu\text{l}$ of membrane suspension, added to give final protein concentrations in excess of those normally employed (approx. $200\mu\text{g}/\text{ml}$), was up to 3-fold greater than the quantity of iron in $100\mu\text{l}$ of medium, indicating uptake above equilibrium concentrations. In the absence of an energy source this is strong presumptive evidence for binding of iron to membrane components, since simple transport under these circumstances would not be expected to progress against an electrochemical gradient.

Relation between iron binding and membrane concentration

In order to validate membrane protein as a suitable measure for expression of iron binding by microvillus membranes, and further to show that binding was directly proportional to membrane concentration, iron incorporation by samples of serially-diluted membranes was determined after short incubations. As illustrated in Fig. 2, iron binding by a given preparation of membranes was linearly dependent upon protein concentration over

values obtained with freshly prepared membranes and fresh medium, indicating that the exponential decrease in the velocity of the metal association observed with time was due neither to deterioration of the separate components, nor to significant depletion of iron chelate. As shown in Fig. 1(a), differences were observed in the availability of iron presented as ferrous or ferric chelates. The membrane uptake from ferric chelates of citrate and nitrilotriacetate was less than 1% of that from the

Fig. 2. Dependence of binding on membrane concentration

Single-point determinations of iron incorporation are shown after 5 min incubation in the presence of $90\mu\text{M}$ -ferrous ascorbate at 37°C . Bound iron is plotted against the amount of membrane protein present in 1 ml of the incubation mixture. The correlation coefficient (r) was $+0.998$.

Fig. 3. Influence of pH of the medium on initial binding velocity

Portions ($50\mu\text{l}$) of membrane suspension containing $70\mu\text{g}$ of membrane protein were mixed briskly with $950\mu\text{l}$ of buffer at 37°C and incubated for 60 s. Media were buffered with piperazine systems in the presence of $130\text{ mM-NaCl}/3.5\text{ mM-KCl}/1\text{ mM-MgCl}_2$ and contained Fe(II)-ascorbate complex at $90\mu\text{M}$. Media below pH 6.8 and above pH 8.0 were buffered with $20\text{ mM-piperazine/HCl}$; media in the range pH 6.8–8.0 were buffered with 20 mM-Hepes (sodium salt). The pH of the medium was determined after final preparation of the chelate; it was not affected by addition of the membrane suspension.

a wide range $3\text{--}135\mu\text{g}$ of protein/ml), with intercepts near zero. These data confirm the efficient recovery of small quantities of membrane by the microfiltration procedure and illustrate the degree of reproducibility of binding determinations within individual experiments.

Effects of pH and decreased incubation temperature

The relation between initial rates of iron binding and medium pH was studied in a single experiment, the results of which are shown in Fig. 3. The activity curve indicates that H^+ concentration markedly affects membrane iron binding with an optimum at, or near, neutrality. The symmetry of the curve, with a sharp peak at pH 7, suggests that the effects represent biological changes induced in the membrane; the alterations are not those expected from chemical influences on the iron-ascorbate complex. Moreover, the optimum pH of binding activity lies closely within the normal intraluminal pH range. The data are in accord with the recent studies of iron uptake by isolated rat intestinal epithelial cells, where an optimum near pH 7 was also found (Savin & Cook, 1978).

Membrane iron binding was temperature-sensitive. Decrease of the incubation temperature to 10°C produced striking decreases in both the rate and extent of binding compared with control incubations

at 37°C [$P < 0.01$; Fig. 4(a)]. A similar sensitivity to incubation temperature has been reported for overall iron absorption in the guinea pig (Jacobi *et al.*, 1956) and also for unidirectional uptake of iron into human duodenal mucosa (Cox & Peters, 1979). Moreover, glucose binding to isolated intestinal brush borders is also dependent upon temperature (Eicholz *et al.*, 1968).

Effects of location on membrane binding activity

Microvillus membranes prepared identically from distal rabbit intestine exhibited considerably less iron-binding activity than did membranes from corresponding proximal segments (Fig. 4b). As expected, no consistent differences in microvillar marker enzyme distributions were detected in membranes prepared from different regions; in three experiments, mean enrichment factors for sucrose were 14.8 and 16.8-fold in proximal and distal membranes, respectively, although both the rate and extent of binding were lower in distal preparations ($P < 0.001$). These data are of particular relevance because of the localization of the capacity for iron absorption mainly to proximal intestinal segments both *in vivo* and *in vitro* (Brown &

Fig. 4. *Effect of temperature and location on iron binding* (a) Shows the influence of decreased incubation temperature on iron binding. Portions (100 μ l) of membrane suspension, containing 40 μ g of protein, were incubated with 10 μ M-Fe(II)-ascorbate complex at 37°C (Δ) or 10°C ($\blacksquare$). (b) The effects of location are shown in parallel determinations of binding by membranes prepared from either the proximal 20 cm of mucosa (Δ) or the distal 20 cm of ileal mucosa ($\blacktriangle$). The final concentration of Fe(II)-ascorbate complex was 10 μ M.

Fig. 5. *Kinetics of iron binding and the effects of transition metal ions*

Initial binding velocity was determined after incubation of 50 μ g of membrane protein for 60 s in the presence of 45–450 μ M-Fe(II)-ascorbate complex ($\bullet$) in a final volume of 1 ml. Parallel determinations were undertaken with the addition of CoCl_2 ($\blacksquare$) or VOSO_4 ($\blacklozenge$) to give final concentrations of 900 μ M.

were judged to be greater than 90% of theoretical initial values. Binding rates at a range of physiological concentrations (10–450 μ M) followed saturation kinetics, as shown in Fig. 5. This points to the existence of a limited number of binding sites for iron in the membrane. Similar kinetic behaviour was observed in the presence of ferric nitrilotriacetate as a donating agent (results not shown), and suggests that the iron binding sites possess high affinity for both iron (II) and iron (III). In seven separate experiments with ascorbate, kinetic analysis indicated an apparent maximum binding velocity of 66.8 ± 15 (S.E.M.) ng-atom/min per mg of protein. Half-maximal velocities were reached at iron concentrations of 186 ± 37 μ M. These data would appear to be compatible with the view that binding of iron to microvillus membranes is related to its transport across the brush border; there is a close similarity between the K_m for binding and the K_t for unidirectional uptake into both human (Cox & Peters, 1979) and rabbit (T. M. Cox, unpublished work) duodenal mucosa *in vitro* (100–200 μ M).

Previous workers have reported similarities in the handling of certain transition ions in the intestinal pathway for absorption (see Forth & Rummel, 1973). Preliminary time-course experiments in the present system indicated that iron binding could be weakly inhibited by the addition of Co^{2+} to the incubation medium. Further kinetic studies with other transition ions were carried out and are shown together in Fig. 5.

In the presence of 900 μ M- Co^{2+} or $-\text{V}^{3+}$ small

Justus, 1958; Wheby, 1970). The present findings are in agreement with the reports of Greenberger *et al.* (1969) and Hübers *et al.* (1971) who found in the rat that iron uptake by whole brush borders was greater in membranes isolated from jejunal segments when compared with membranes from ileal segments.

Studies on the kinetics of iron binding

Initial rates of iron binding were assessed after 60 s incubations. Binding velocity decreased exponentially with time, and determinations after 60 s

but significant ($P < 0.05$) inhibitions of iron-binding velocity were observed at all concentrations of iron. For V^{3+} , kinetic analysis revealed a 60% increase in the apparent K_m for iron binding, indicating weak competitive inhibition, (K_i approx. 1.5 mM). In the presence of excess Co^{2+} a smaller decrease in binding rate was also seen, but the percentage inhibition generally increased at higher iron concentrations. The results suggest a mixed inhibitory action with K_i approx. 2 mM. Cobalt at this concentration is known to stimulate brush border endopeptidases (Kenny, 1977), and an analogous effect on membrane proteinases, combined with the general effects of divalent cations on the integrity of isolated brush borders (Miller & Crane, 1961), could accelerate membrane autolysis. Such actions on membrane integrity in the present experiments with cobalt may have accounted for its mixed inhibitory effects on iron-binding kinetics.

The weak inhibitory effects of other related transition ions on iron-binding activity, in contrast to the powerful 'self-inhibition' of ^{59}Fe uptake by saturation with non-radioactive iron, strongly suggest that the microvillus membrane contains high-affinity metal-binding sites with a relative specificity 10-fold more avid for iron than for V^{3+} or Co^{2+} in this system. The data support and extend previous binding studies by Hübers *et al.* (1971) who showed that ^{59}Fe bound to rat jejunal brush borders, unlike cobalt, was not removed by repeated washing; such differences were not seen in preparations of ileal membranes.

Other workers have carried out iron-uptake studies with preparations of whole brush border membranes; binding, as opposed to transport, was assumed, and kinetic effects have not been reported (Greenberger *et al.*, 1969; Hübers *et al.*, 1971; Kimber *et al.*, 1973; Savin & Cook, 1978). The first three groups employed EDTA in their preparative procedures and neither Greenberger *et al.* (1969) nor Hübers *et al.* (1971) used other agents to maintain iron in a soluble form. Colloidal iron may be generated in this way. Savin & Cook (1978) carried out limited studies with brush borders from rat intestine and showed that, in the presence of Tris, membrane uptake accounted for more than 50% of iron uptake by isolated epithelial cells.

Iron-uptake studies by preparations of rat microvillus and plasma membranes were reported by Eastham *et al.* (1977). This group showed unequivocally that iron was transported into vesicular spaces, but the purity of their microvillus preparation is called into question by low enrichment factors for sucrase (2.2-fold). Moreover, these workers observed considerable retention of radioactivity by their filters in the absence of membranes indicating significant aggregation of iron in the medium.

The present experiments provide evidence for the

existence on the rabbit microvillus membrane of binding sites for iron which show specificity and considerable affinity for this metal. Iron was bound to membranes even in the presence of powerful chelating agents; binding was time-dependent and showed both temperature- and pH-sensitivity. Binding activity was also found to be decreased in membranes prepared from the ileum compared with those isolated from proximal mucosa. Moreover, binding rates followed saturation kinetics over a physiological range of concentrations of iron and were only weakly decreased by other transition ions present in excess. Evidence has also been presented that the ligand-chelate complex dissociates at the membrane level and that only the iron remains bound. These characteristics, in conjunction with the similarities between the K_m for membrane binding and the K_i of mucosal iron uptake, suggest that there may be an operational link between the initial binding step and other transport processes involved in iron absorption.

Note Added in Proof (24 November 1980)

Since submitting this paper we have demonstrated that microvillus iron binding is enhanced in experimental iron deficiency [*Biochem. Internat.* (1980) **1**, 446–454].

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References

- Bates, G. W., Billups, C. & Saltman, P. (1967) *J. Biol. Chem.* **242**, 2810–2815
- Brown, E. B. & Justus, B. W. (1958) *Am. J. Physiol.* **194**, 319–326
- Cox, T. M. & Peters, T. J. (1979) *J. Physiol. (London)* **289**, 469–478
- Cox, T. M., Mazurier, J., Spik, G., Montreuil, J. & Peters, T. J. (1979) *Biochim. Biophys. Acta* **588**, 120–128
- Dahlqvist, A. (1968) *Anal. Biochem.* **22**, 99–107
- Eastham, E. J., Bell, J. I. & Douglas, A. P. (1977) *Biochem. J.* **164**, 289–294
- Eicholz, A., Howell, K. E. & Crane, R. K. (1968) *Fed. Proc. Fed. Am. Soc. Expt. Biol.* **27**, 643
- Forth, W. & Rummel, W. (1973) *Physiol. Rev.* **53**, 724–792
- Greenberger, N. J., Balcerzak, S. P. & Ackerman, G. A. (1969) *J. Lab. Clin. Med.* **73**, 711–721
- Hopfer, U., Nelson, K., Perrotto, J. & Isselbacher, K. J. (1973) *J. Biol. Chem.* **248**, 25–32
- Hübers, H., Hübers, E., Forth, W., Leopold, G. & Rummel, W. (1971) *Acta Pharmacol. Toxicol.* **29**, Suppl. 4, 22

- Hübers, H., Hübers, E., Rummel, W. & Crichton, R. (1976) *Eur. J. Biochem.* **66**, 447–455
- Jacobi, H., Pfleger, K. & Rummel, W. (1956) *Arch. Exptl. Pathol. Pharmacol.* **229**, 198–206
- Kenny, A. J. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 393–444, North-Holland Publishing Co., Amsterdam, New York and Oxford
- Kenny, A. J. & Booth, A. G. (1978) *Essays Biochem.* **14**, 1–44
- Kimber, C. L., Mukherjee, T. & Deller, D. J. (1973) *Am. J. Dig. Dis.* **18**, 781–791
- Langman, L., Young, I. G., Frost, G. E., Rosenberg, H. & Gibson, F. (1972) *J. Bacteriol.* **112**, 1142–1149
- Miller, D. & Crane, R. K. (1961) *Biochim. Biophys. Acta* **52**, 293–298
- Prospero, T. D. (1974) in *Methodological Developments in Biochemistry* (Reid, E., ed.), vol. 4, pp. 411–412, Longman, New York
- Quigley, J. P. & Gotterer, G. S. (1972) *Biochim. Biophys. Acta* **255**, 107–113
- Savin, M. A. & Cook, J. D. (1978) *Gastroenterology* **75**, 688–694
- Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B. K., Cerda, J. J. & Crane, R. K. (1973) *Biochim. Biophys. Acta* **323**, 98–112
- Sillén, L. G. (1971) *Chem. Soc. Spec. Publ.* **25 Suppl. 1**
- Underwood, E. J. (1971) *Trace Elements in Human and Animal Nutrition*, 3rd edn., pp. 14–56, Academic Press, New York and London
- Van Frank, R. M. (1975) *Anal. Biochem.* **65**, 552–555
- Wheby, M. S. (1970) *Scand. J. Haematol.* **7**, 56–62