Iron-Transport Characteristics of Vesicles of Brush-Border and Basolateral Plasma Membrane from the Rat Enterocyte

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Vesicles of brush-border and basolateral plasma membrane were prepared from enterocytes of the rat small intestine. The separateness of these two varieties of plasma membrane was confirmed by appropriate enzyme assays. The uptake of Fe^{2+} by these membrane vesicles was studied, and the results suggest differences between the two types of membrane in both the amount of Fe^{2+} taken up and in the rate of uptake. At low (up to $3\text{ }\mu\text{M}$) concentrations of Fe^{2+} , uptake by both membrane types showed evidence of saturation and could be blocked with the thiol inactivator *N*-ethylmaleimide. The studies suggest that Fe^{2+} is taken into an osmotically active space by a process of facilitated diffusion at low concentrations, but that at higher concentrations the process appeared to obey first-order kinetics. The data provide further evidence for the existence of functional polarity in the epithelial cell of the small intestine.

There is considerable support for the hypothesis that the enterocyte is the prime regulator of iron homeostasis by increasing or decreasing absorption (Van Campen, 1974). The mechanisms involved in this remain to be determined, but may well be related to control of membrane-transport function. Under normal circumstances iron is absorbed in the upper small intestine, but this is probably mostly due to the fact that iron is present in available forms at these sites rather than to any unique properties intrinsic to the upper intestinal mucosa. Manis & Schachter (1962) have suggested that iron, in physiological amounts, is absorbed by an active-transport mechanism consisting of two main steps: (1) initial mucosal uptake of iron and (2) subsequent transfer of iron into the blood. Such studies suggest that this two-step mechanism reflects functional polarity of the enterocyte, which could be related to differences in the transport properties for iron possessed by the plasma membrane of the apical and basolateral parts of the cell. It has been shown (Douglas *et al.*, 1972; Fujita *et al.*, 1972) that there are distinct differences between the plasma membrane of the enterocyte brush border and of the basolateral surface, one being rich in disaccharidases and the

other rich in ouabain-sensitive ATPase. § Hopfer *et al.* (1975) have shown a difference in transport properties with respect to D-glucose between these two varieties of plasma membrane. Therefore in the light of these considerations we have performed studies of iron transport using plasma membrane prepared from opposite ends of the rat enterocyte in an attempt to relate the membrane properties to the observations on iron absorption made in intact animals.

Experimental

Materials

Adult Wistar-strain rats, each weighing 200–300 g, were used. $^{59}\text{FeSO}_4$ was obtained from New England Nuclear Corp., Boston, MA, U.S.A., and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ from The Radiochemical Centre, Amersham, Bucks., U.K. Non-radioactive substrates used for enzyme assays were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Dextran 110 was from Pharmacia, Uppsala, Sweden, and all other chemicals were from BDH, Poole, Dorset, U.K., and were of analytical grade.

Preparation of membranes

Isolated epithelial cells. These were prepared as described previously (Douglas *et al.*, 1972). The cells were collected into ice-cold $0.15\text{ M-NaCl}/0.8\text{ mM-dithiothreitol}$ adjusted to pH 7.4 with 0.15 M-NaHCO_3 , and harvested by centrifuging for 2 min at

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§ Abbreviation: ATPase, adenosine triphosphatase.

500g. The pellet was resuspended in 40 ml of this solution four or five times until a clear supernatant resulted and phase-contrast microscopy revealed few bacteria, parasites and ova.

Basolateral plasma membranes. These were prepared by the method of Douglas *et al.* (1972) with a Parr pressure bomb (Parr Instrument Co., Moline, IL, U.S.A.) for nitrogen-cavitation homogenization of the cells. Subcellular fractionation was performed as described previously (Douglas *et al.*, 1972) and final separation of basolateral plasma membranes from those of the endoplasmic reticulum was achieved on a discontinuous dextran gradient, density 1.075 g/ml. The pure basolateral membrane material was washed once at 105 000g for 30 min and resuspended in the appropriate buffer.

Microvillus plasma membranes. Purified brush borders were prepared from intestinal scrapings by the method of Forstner *et al.* (1968). Their method of further purification of the microvillus membrane was modified since EDTA, used in the original method, is a known chelator of iron and would have been entrapped during vesiculation and have interfered with subsequent studies. Therefore, after passage through Pyrex glass wool and centrifuging at 500g for 10 min, the pellet of brush borders was resuspended in 30 ml of 25 mM-NaCl/1.0 mM-Tris/HCl, pH 8.1, and disrupted in the Parr pressure bomb at a pressure of 3450 kPa (500 lbf/in²) for 30 min. The mixture was then centrifuged at 1800g for 15 min to yield a sediment and a supernatant fraction. The latter contained the membrane fraction, and the membranes were harvested by centrifugation at 105 000g for 30 min and then resuspended in the appropriate buffer by repeated passage through a no. 25-gauge needle.

Analytical methods and enzyme assays

All fractions for assay were stored at -40°C until required. Protein was determined by a modification of the Lowry method (Hartree, 1972), with crystalline bovine serum albumin as the standard. Sucrase (EC 3.2.1.26) was determined by the two-step method of Dalquist (1964), and alkaline phosphatase (EC 3.1.3.1) with β -glycerophosphate as the substrate (Douglas *et al.*, 1972). ATPase (EC 3.6.1.3) was assayed by the method of Avruch & Wallach (1971).

Electron microscopy

A portion of each of the final membrane preparations was examined by electron microscopy as previously described (Douglas *et al.*, 1972).

Uptake method

The uptake of ⁵⁹Fe by the membranes was studied by using a modification of the method of Hopfer *et al.* (1973). The membranes (50–200 µg/ml) were incubated in a water bath with ⁵⁹FeSO₄ (sp. radio-

activity 13–19 mCi/mg) sufficient to give approx. 500 000 c.p.m./ml. Uptake of iron was terminated by removing a sample (10–20 µg of protein) and immediately diluting with a 50-fold excess of ice-cold buffer (see below), collecting the membranes on a washed Millipore filter (HA025, pore size 0.45 µm) and washing once with 5 ml of the same buffer. The amount of radioactivity retained on the Millipore filter was subsequently determined by using an auto-gamma counter.

Initial experiments were performed in 0.15 M-NaCl/0.01 M-NaH₂PO₄/0.01 M-Na₂HPO₄ buffer, pH 7.4, because this was found to give the least retention of ⁵⁹Fe by the filters (<1%) and buffered FeSO₄ satisfactorily. Subsequent addition of 8.0 mM-ascorbic acid resulted in about 80% increase in uptake by the membranes. Because other workers (Manis & Schachter, 1962) have used a Tris buffer and shown it to be more efficient than phosphate buffers in studies of iron transport by gut sacs, and Manis (1973) found that iron was stable in the bivalent form for 1 h at 37°C in 2.0 mM-Tris/HCl, pH 6.0, this latter buffer was used in later experiments, also resulting in a further 70% increase in membrane uptake although associated with up to 20% retention of ⁵⁹Fe by the filters.

In all experiments controls were run in identical incubation media but without membrane in order to correct for filter uptake and possible precipitation of iron salts. Measurements were always made in at least triplicate on the same preparation and were within 8% of the mean. The absolute amount of iron uptake varied by up to a factor of two between different preparations, and results of single experiments are therefore presented unless stated otherwise. However, all experiments were performed at least three times with similar results. Preliminary studies showed that, after preparation of membranes, the vesicles could be kept for up to 24 h at 4°C without loss of uptake being demonstrable. After this time, uptake was sometimes decreased dramatically, presumably owing to vesicle leakage, and therefore all uptake experiments were performed within 24 h of membrane preparation.

Results

Characterization of plasma-membrane preparations

Electron-microscope appearances of both membrane types showed highly purified membrane material having the typical trilaminar 'unit membrane' structure. In both instances most had formed into closed vesicles. The vesicles of microvillus membrane could be clearly distinguished from those of basolateral membrane by the presence of adherent glycocalyx material.

The purity and yield of membranes was followed

as a routine by measuring the activity of sucrase and alkaline phosphatase marker enzymes.

The final microvillus-membrane preparation contained 15% of the sucrase activity and 17% of the alkaline phosphatase activity that was present in the starting homogenate, giving specific activities relative to those in the starting homogenate (1.00) of 2.22 and 4.08 respectively, which are similar to those found by other workers (Forstner *et al.*, 1968; Hopfer *et al.*, 1973). The specific activity of sucrase was 58 times that found in the preparations of basolateral membrane.

The final basolateral-membrane preparation contained 0.1% of the sucrase activity and 6% of the alkaline phosphatase and Na^+, K^+ -dependent ATPase that were present in the original homogenate. The specific activities, relative to those in the starting homogenate (1.00), for these three enzymes were 0.04, 3.14 and 6.12 respectively, which are comparable with those found by other workers (Douglas *et al.*, 1972; Fujita *et al.*, 1972; Murer *et al.*, 1974).

Fig. 1. Uptake of $^{59}\text{FeSO}_4$ by brush-border (●) and basolateral (○) membrane

Results are expressed as means $\pm$ S.E.M. of nine separate experiments with each variety of membrane. Uptake method was as described in the text; the medium was identical for all experiments (final concn.): NaCl (0.1 M), phosphate (6 mM), $^{59}\text{FeSO}_4$ (1 μM). Temperature was 37°C, and pH 7.4.

Uptake of $^{59}\text{FeSO}_4$

The uptake of Fe^{2+} by both types of membrane is shown in Fig. 1. There are marked differences between the two membrane types both in amount taken up per mg of protein and in the rate of uptake. Basolateral membranes reached equilibrium after 10 min and took up twice as much Fe^{2+} as did microvillus membranes. The latter membranes, however, reached equilibrium in 2–5 min. With the Millipore-filtration technique it was not possible to measure initial rates of uptake, since it took approx. 1 min to filter, wash and replace with a new filter. However, it was estimated that microvillus membranes attained half-maximal uptake between 10 and 20 s, whereas this time was 40–60 s for basolateral membranes. The rate of uptake by both membrane types was decreased by lowering the temperature of the incubation (Fig. 2), although the basolateral membranes appeared to be more sensitive to this procedure.

When membranes were preloaded with ^{59}Fe and then diluted into an iron-free medium, the Fe^{2+} was released (Fig. 3). There did not appear to be any difference in the rate of this release by the two membrane types.

The effect of different concentrations of Fe^{2+} on subsequent uptake is shown in Fig. 4. Non-radioactive FeSO_4 was used to alter the concentration of Fe^{2+} in the incubation medium. With low concentrations (less than 3 μM) there appears to be a saturation effect, which is similar with both varieties of membrane. With higher concentrations (up to 50 mM), however, the graph became linear, suggesting that first-order kinetics then apply.

Distinction between binding of ^{59}Fe to membrane and ^{59}Fe transport into an intravesicular aqueous space

Several authors (Greenberger *et al.*, 1969; Kimber *et al.*, 1973) have reported binding of iron to isolated intestinal brush borders, and therefore experiments

Fig. 2. Effect of temperature on uptake of $^{59}\text{FeSO}_4$ by enterocyte plasma membrane: (a) brush border; (b) basolateral. Results shown are the means of triplicate estimations in single experiments. Medium was identical with that used in Fig. 1. Uptake of ^{59}Fe is given as a percentage of its initial activity.

were carried out to examine the effect of the intravesicular space on ^{59}Fe uptake. Since osmotic forces influence the size of the intravesicular compartment, it is possible to prevent collapse of this space by entrapping an impermeant solute and subsequently alter the intravesicular volume by changing the osmolarity of the incubation medium. By using

Fig. 3. Release of ^{59}Fe from preloaded membrane vesicles. The membranes (●, brush border; ○, basolateral) were preloaded with $2\mu\text{M}$ - $^{59}\text{FeSO}_4$ by incubation at this iron concn. for 30 min at 37°C . Other solute concns. were: NaCl (0.1 M), phosphate (6 mM), ascorbic acid (6 mM), pH 7.4. At zero time a portion of preloaded membrane was diluted 10-fold into the same buffer which did not contain iron, and iron remaining in the membrane was determined at subsequent time-intervals in the usual manner. Values are calculated relative to an undiluted control taken at zero time.

the impermeant carbohydrate solute cellobiose thus to alter the osmolarity, iron uptake was (Fig. 5) shown to be inversely proportional to the osmolarity of the incubation medium. Further, if the graph is extrapolated to infinite medium osmolarity (or zero space), no uptake would then occur. These observations were made with both microvillus membranes and with basolateral membranes. Therefore, under the conditions of these experiments, the measured ^{59}Fe uptake could be accounted for completely by transport into the intravesicular space and not by binding to the outside of the membrane.

Fig. 5. Effect of osmolarity of medium on uptake of iron by enterocyte plasma membrane

Membranes (●, brush border; ○, basolateral) were prepared in cellobiose (0.1 M). Uptake was measured from a medium with the following final composition: NaCl (0.01 M), phosphate (6 mM), $^{59}\text{FeSO}_4$ (1.0 μM) and sufficient cellobiose to give the indicated osmolarity (shown as inverse osmolarity). The osmolarity was calculated as the sum of contributions from all compounds, assuming ideal behaviour. Vertical bars indicate the s.e.m. of three experiments made with the same preparation of membranes.

Fig. 4. Effect of iron concentration on uptake by enterocyte plasma membranes: ●, brush border; ○, basolateral. Medium contained (final concns.): NaCl (0.1 M), phosphate (6 mM), ascorbic acid (6 mM) and $^{59}\text{FeSO}_4$ at the indicated concn. Results shown are the means of triplicate estimations in single experiments.

Fig. 6. Inhibition of iron uptake by the thiol inactivator *N*-ethylmaleimide

Results shown (uptake as percentage of initial activity) are the means of triplicate estimations in single experiments after 20 min incubation at 37°C. Medium contained: NaCl (100 mM), phosphate (6 mM), ascorbic acid (6 mM), $^{59}\text{FeSO}_4$ (1 μM), pH 7.4. *N*-Ethylmaleimide (NEM) was present at a concn. of 1 mM. Unshaded histogram, basolateral membranes; shaded histogram, brush border.

Inhibition studies

The experiments described above were performed in the absence of any ATP or added oxygen. When an ATP-recycling system (Mukhtar *et al.*, 1975) was added there was no demonstrable difference in uptake by either membrane type. Similarly inhibition of glycolysis (5 mM-NaF) and oxidative phosphorylation (20 μM -2,4-dinitrophenol) produced no decrease in uptake. However, in the presence of 1.0 mM-*N*-ethylmaleimide (which is a thiol inactivator), and at all concentrations of Fe^{2+} up to 3.0 μM , there was a 90% decrease in uptake (Fig. 6). This inhibition by *N*-ethylmaleimide did not occur at iron concentrations greater than 3 μM when, as indicated above, uptake was linear with respect to concentration.

Discussion

The present data represent the first attempt to characterize the transport mechanism of iron across isolated plasma-membrane vesicles of the enterocyte. Electron-microscopic and enzyme studies suggest that the two types of membrane are pure and distinct. The microvillus plasma membrane was rich in sucrase activity and shown by microscopic examination to be associated with the glycocalyx, whereas the basolateral membrane was rich in Na^+ , K^+ -ATPase and was similar in microscopic appearance to that described earlier (Douglas *et al.*, 1972).

The literature concerning the absorptive mechanism for iron has been confusing. Several workers (Dowdle *et al.*, 1960; Manis & Schachter, 1962;

Jacobs *et al.*, 1966) have determined that absorption was dependent on oxidative phosphorylation. However, these experiments were done with intact cells and it is possible that the intracellular pathway of iron is dependent on this system rather than the mechanism of transport of iron across the plasma membrane of the cell. This concept is supported by the work of Greenberger *et al.* (1969) and of Kimber *et al.* (1973), who found no evidence of oxygen- or glucose-dependence of iron uptake by isolated brush borders. There is, however, good evidence that the transport of iron involves a saturable process. Smith & Pannacciulli (1958) showed that increasing concentrations of iron, although resulting in increased uptake, were associated with a fall in the percentage uptake, suggestive of competition for binding sites by iron molecules. Gitlin & Cruchaud (1962) suggested from their kinetic studies that absorption at doses in the physiological range was mediated by enzyme or carrier, but that simple diffusion operated at higher iron concentrations.

The present uptake data indicate that iron is being transported into an osmotically active space. No evidence was found for this process being dependent on oxidative phosphorylation, and the saturation effect observed at concentrations up to 3 μM suggests that uptake is by facilitated diffusion. A role for thiol groups is indicated by the observation that this uptake could be blocked by *N*-ethylmaleimide. Of particular interest, and confirming the work of Gitlin & Cruchaud (1962), was the finding that, with higher concentrations of iron, uptake assumed a linear rate with respect to concentration and was no longer blocked by *N*-ethylmaleimide, suggesting that a passive process such as simple diffusion was then operating. However, as Forth & Rummel (1973) point out, for chemical reasons free diffusion seems unlikely and at these higher (probably non-physiological) concentrations non-specific binding sites of nearly unlimited capacity appear likely to determine the absorption rate.

The difference in uptake between the two membrane types is more difficult to explain. The method used to measure uptake was not suitable for initial kinetic studies and no attempt was made to estimate the intravesicular volume. It is possible that the two membrane types produced different-sized vesicles, although homogenization by nitrogen cavitation tends to result in uniformly small vesicles by comparison with other methods (Steck & Wallach, 1970). The length of time to prepare the membranes was different, being 6 h for microvillus membranes and 30 h for basolateral membranes. However, any decay of transport properties with time would reasonably be expected to occur more markedly in the basolateral membranes and so would perhaps tend to diminish the observed difference, in favour of increased uptake by these membranes. A further

factor attesting to the validity of our observations relates to the known structural differences between the two varieties of plasma membrane studied. The microvillus plasma membrane, as distinct from the plasma membrane of the rest of the enterocyte, is associated with more glycoprotein (the glycocalyx) and is thicker than the lateral plasma membrane (Sjostrand, 1963; Fujita *et al.*, 1972). Thus the protein content of a 'unit' of microvillus membrane is probably higher than that of a 'unit' of basolateral membrane and, since the present results are expressed in relation to protein content, this will tend to diminish the difference that we have observed.

Because the microvillus plasma membrane forms about 80% of the total external surface area of the enterocyte it may 'need' fewer binding sites per unit area to transport the iron presented to it. The present observations are consistent with previous work (Manis & Schachter, 1962), suggesting differences between uptake of iron by the enterocyte and the subsequent exit of iron from the enterocyte. They also confirm that the enterocyte is characterized by a functional polarity.

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References

- Avruch, J. & Wallach, D. F. H. (1971) *Biochim. Biophys. Acta* **233**, 334–347
- Dalquist, A. (1964) *Anal. Biochem.* **7**, 18–25
- Douglas, A. P., Kerley, R. & Isselbacher, K. J. (1972) *Biochem. J.* **128**, 1329–1338
- Dowdle, E. B., Schachter, D. & Schenker, H. (1960) *Am. J. Physiol.* **198**, 609–613
- Forstner, G. G., Sabesin, S. M. & Isselbacher, K. J. (1968) *Biochem. J.* **106**, 381–390
- Forth, W. & Rummel, W. (1973) *Physiol. Rev.* **53**, 724–792
- Fujita, M., Ohta, H., Kawai, K., Matsui, H. & Nakao, M. (1972) *Biochim. Biophys. Acta* **274**, 336–347
- Gitlin, D. & Cruchaud, A. (1962) *J. Clin. Invest.* **41**, 344–350
- Greenberger, N. J., Balcerzak, S. P. & Ackerman, G. A. (1969) *J. Lab. Clin. Med.* **73**, 711–721
- Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422–427
- Hopfer, U., Nelson, K., Perrotto, J. & Isselbacher, K. J. (1973) *J. Biol. Chem.* **248**, 25–32
- Hopfer, U., Sigrist-Nelson, K. & Murer, H. (1975) *Ann. N.Y. Acad. Sci.* **264**, 414–426
- Jacobs, P., Bothwell, T. H. & Charlton, R. W. (1966) *Am. J. Physiol.* **210**, 694–700
- Kimber, C. L., Mukherjee, T. & Deller, D. J. (1973) *Am. J. Dig. Dis.* **18**, 781–791
- Manis, J. G. (1973) *Proc. Soc. Exp. Biol. Med.* **144**, 1025–1029
- Manis, J. G. & Schachter, D. (1962) *Am. J. Physiol.* **203**, 73–80
- Mukhtar, E. D., Smith, B. R., Pyle, G. A., Hall, R. & Vice, P. (1975) *Lancet* **ii**, 713–716
- Murer, H., Hopfer, U., Kinne-Saffran, E. & Kinne, R. (1974) *Biochim. Biophys. Acta* **345**, 170–179
- Sjostrand, F. S. (1963) *J. Ultrastruct. Res.* **8**, 517–541
- Smith, M. D. & Pannacchiulli, I. M. (1958) *Br. J. Haematol.* **4**, 428–434
- Steck, T. L. & Wallach, D. F. H. (1970) *Methods Cancer Res.* **5**, 93–153
- Van Campen, D. (1974) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **33**, 100–105