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THE KINETICS OF IRON UPTAKE *IN VITRO* BY HUMAN DUODENAL MUCOSA: STUDIES IN NORMAL SUBJECTS*

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SUMMARY

1. A method for determining initial rates of unidirectional radio-Fe uptake from a ferric chelate of nitrilotriacetic acid by human duodenal biopsy specimens *in vitro* has been devised. [^{57}Co]cyanocobalamin was used as an extracellular fluid marker, and was shown to give results in close agreement with other markers.

2. Uptake was linear for up to 20 min and exhibited saturation kinetics over the concentration range 18–450 μmole/l.

3. In the presence of 2:4 dinitrophenol and fluoride, uptake was reduced by approxi-50%, indicating dependence on metabolic energy.

4. Uptake of Fe was markedly diminished at reduced incubation temperatures, demonstrating a high activation energy for the uptake process.

5. Many of the criteria for the demonstration that the initial uptake of Fe depends on an active transport mechanism have been fulfilled.

6. The apparent distribution volume of ^{14}C-labelled nitrilotriacetate chelate did not exceed the extracellular fluid space, suggesting that Fe is transferred to specific receptors on the enterocyte.

The findings are discussed in relation to the possibility that uptake may be a rate-controlling step for the regulation of net intestinal absorption of Fe in man.

INTRODUCTION

The intestinal absorption of Fe is unique in that it alone is responsible for the maintenance of body iron homoeostasis (McCance & Widdowson, 1937). Although the regulatory capacity for iron absorption is considered to reside in the epithelial cells lining the lumen of the proximal small intestine, the location of the control step has not been defined. Possible sites include undirectional influx across the brush border membrane, processing by intracellular organelles or transport molecules, and net flux across the baso-lateral membranes to the portal plasma. Experimental data obtained with segments of rodent intestine incubated with radio-iron *in vitro* or *in vivo* (Dowdle, Schachter & Schenker, 1960; Jacobs, Bothwell & Charlton, 1966) have suggested that the control of absorption is exerted by an active transport process possessing saturation kinetics, but little is known of this process in man. Alterations

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in net Fe absorption occur in Fe deficiency and in the inborn error of metabolism, primary haemochromatosis (Williams, Manenti, Williams & Pitcher, 1966). Thus detailed studies of the transport processes involved in Fe absorption by the human intestine are necessary before the precise nature of these alterations can be determined.

The present experiments were designed to investigate the characteristics of unidirectional Fe uptake by human duodenal mucosa incubated with radio-Fe *in vitro*. The technique employs a non-absorbable marker for extracellular fluid so that uptake of radio-Fe by the mucosa can be corrected for any adherent mucosal medium. The method also allows the tissue to be incubated for short periods to determine initial rates of Fe influx across the brush border.

METHODS

The broad principles for valid measurement of rates of unidirectional uptake into intestinal tissue have been defined by Sallee, Wilson & Dietschy (1972). A non-absorbed, extracellular marker must be used to correct for contamination by adherent incubation medium; if radio-labelled probe and marker molecules are used, it must be possible to determine their separate radioactivities in tissue homogenates; the tissue incubation period should be short enough to determine initial rates of uptake without the development of significant intracellular concentration gradients, but also sufficient to permit equilibration of the extracellular fluid marker; finally, corrected values for tissue uptake should be linear with respect to time. The present technique was designed to satisfy these criteria and, as far as possible, to overcome inconstancies in the solubility and redox state of Fe within a freely oxygenated system.

Tissue specimens. The mucosal biopsy specimens were taken from either healthy volunteers or patients undergoing investigation for possible digestive disease. Body Fe status, as reflected by blood haemoglobin concentration, red cell indicies, transferrin saturation, as well as serum ferritin levels, was normal in all individual studied (Dacie & Lewis, 1975). A Crosby suction biopsy capsule was guided into the distal duodenum under fluoscopic control, and specimens of mucosa weighing 15–60 mg were obtained. In every case histological examination showed normal human duodenal mucosa. The studies were approved by the local ethical committee.

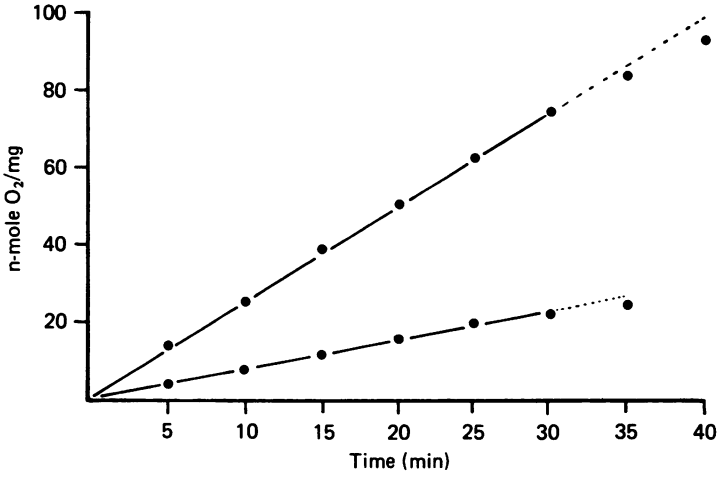
Incubation technique. The incubation buffer was designed to minimize the formation of insoluble Fe complexes. N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (HEPES), at a concentration of 16 m-mole/l. was the chosen buffering agent (Good, Winget, Winter, Connolly, Izawa & Singh, 1966), and in addition the medium contained 125 mM-NaCl, 3·5 mM-KCl, 1 mM-$CaCl_2$, 10 mM-$MgSO_4$ and 10 mM-D-glucose. After adjustment to pH 7·4 with 0·1 M-NaOH, the osmolality was 290 ± 5 m-osmole/l. Immediately after collection, the biopsy specimens were plunged into ice-cold, oxygenated HEPES buffer for transport to the laboratory. Here, after rinsing to remove mucus, the tissue was cut into portions weighing approximately 2 mg and pre-incubated for 30 sec at 37 °C in tubes containing 5 ml. buffer. To avoid adsorption of Fe^{3+} ions onto glass, polythene ware was used throughout. Oxygenation was achieved by bubbling humidified 100 % O_2 directly into the medium. ^{59}Fe ferric nitrilotriacetate [Fe^{3+}-$(NTA)_2$] was used at final concentrations of 18–450 μmole/l. in HEPES buffer. The Fe chelate was prepared by the method of Bates, Billups & Saltman (1967) with $^{59}FeCl_3$ (IFS 1, sp. activity 3–20 mc/mg Fe, Amersham Radiochemical Ltd, Amersham, England) and a molar excess of nitrilotriacetate. [^{57}Co]cyanocobalamin (CT12, sp. activity 10–20 $\mu c/\mu g$, Amersham Radiochemical Ltd) was also added to a final concentration of 5 n-mole/l.

Incubation was stopped by removing the tissue, blotting and rinsing in 2 ml. ice-cold buffer. After re-blotting, it was weighed on a torsion balance and the radioactivity of a 0·5 ml. homogenate or aliquot of diluted medium was measured in a twin channel gamma counter (NaI crystal, Gammamatic MK2, Nuclear Enterprises). The contributions of each isotope to the emission was determined by channel ratio analysis using pure ^{59}Fe and ^{57}Co sources on each occasion. In experiments in which [^{14}C]polyethylene glycol (PEG 4000) and [^{14}C-1]nitrilotriacetic acid were used, the β-emission was determined in a liquid scintillation counter and, where

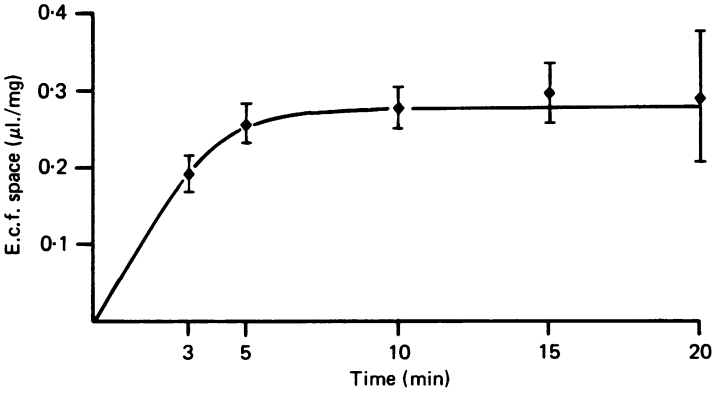
applicable, quench correction by the automatic external standard technique was calculated. Appropriate corrections for contamination in the ^{14}C channel by β-emission from ^{59}Fe and ^{57}Co were made after determining the γ/β spill-over ratio from standards of known activity. The [^{14}C]PEG 4000 and [^{14}C-1]NTA were obtained from Amersham Radiochemical Ltd.

Except where stated, the incubation period for the kinetic studies was 5 min. Where appropriate, tissue protein was measured fluorimetrically (Peters, Batt, Heath & Tilleray, 1976) with crystalline bovine serum albumin as standard. Uptake was expressed as p-mole Fe/mg tissue. min, and was calculated thus:

$$\text{uptake} = \frac{\dfrac{^{59}\text{Fe in tissue (cpm)}}{^{59}\text{Fe cpm per } \mu\text{l. medium}} - \dfrac{^{57}\text{Co in tissue (cpm)}}{^{57}\text{Co cpm per } \mu\text{l. medium}} \times \text{p-mole Fe per } \mu\text{l. medium}}{\text{tissue wet weight (mg)}}$$



Text-fig. 1. Time course for O_2 consumption. Two biopsy specimens were incubated with stirring in 10 ml. HEPES buffer equilibrated with atmospheric O_2 at 37 °C before submersion under oil. O_2 consumption was determined after measurement of pO_2 in aliquots of buffer removed at intervals using a Radiometer D616 O_2 electrode.



Text-fig. 2. Time course for equilibrium of [^{57}Co]cyanocobalamin as e.c.f. marker. Biopsy specimens from nine to seventeen control subjects incubated in the presence of radio-Fe. Each point shows mean ± S.E. of the mean.

RESULTS

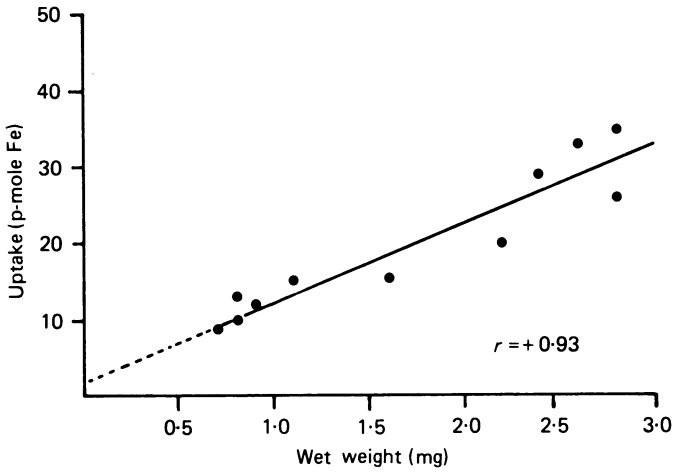
Validation of the methods. Examination by light and electron microscopy after incubation showed no evidence of any structural damage or oedema in the mucosal specimens (Pls. 1 and 2). Complete biochemical integrity was demonstrated by the determination of O_2 consumption during incubation of biopsy specimens *in vitro* using an O_2 electrode. O_2 utilization remained linear for at least 25 min as shown in Text-figure 1. Text-figure 2 shows the relationship of extracellular fluid space (e.c.f.) to incubation time *in vitro*: the [^{57}Co]cyanocobalamin equilibrated within 5 min. The mean e.c.f. volume measured by this technique in 427 biopsy specimens from sixty individuals was $18{\cdot}3 \pm 11{\cdot}5\,\%$ (S.D. of an observation) of mucosal wet weight. This is in close agreement with the [^{14}C]inulin space of $15{\cdot}5 \pm 6{\cdot}1\,\%$ for human intestinal biopsies reported by Thier, Segal, Fox, Blair & Rosenberg (1965). A direct comparison between two e.c.f. markers measured in the same system simultaneously in twenty-five paired biopsies from four individuals was undertaken. E.c.f. measured with [^{57}Co]cyanocobalamin was $14{\cdot}7 \pm 2{\cdot}3\,\%$ of wet weight (S.E. of mean), and with [^{14}C]PEG 4000 (2–10 μmole/l.), $12{\cdot}9 \pm 2{\cdot}5\,\%$. No significant difference between these two means was detected by Student's paired t test. It is noteworthy that there was a significant correlation ($r = +0{\cdot}56$, $P < 0{\cdot}01$) between e.c.f. volume and wet weight of tissue in any individual.

Text-fig. 3 shows the relationship between weight of tissue and corrected uptake of Fe after 10 min incubation at a medium concentration of 18 μmole/l. There is a close correlation between uptake and the amount of tissue as measured by wet weight ($r = +0.93$), and the intercept is not significantly different from zero. In addition, a highly significant correlation between wet weight and protein content of tissue after homogenization was found ($r = +0{\cdot}91$, $P < 0{\cdot}001$, $n = 21$). These observations validate mucosal wet weight as a means of expressing uptake of Fe in this system.

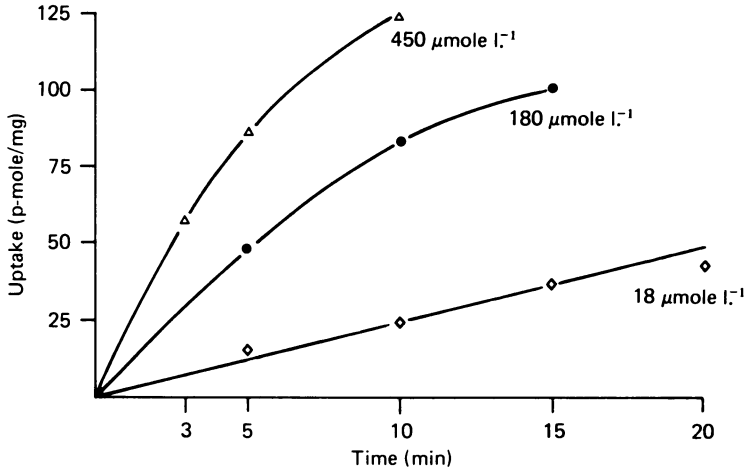
The time course for corrected uptake of Fe at three different Fe concentrations is shown in Text-fig. 4. At the lower concentration, uptake was linear for 20 min. Some reduction in net uptake is seen after 10 min at the higher concentrations, and in subsequent studies of uptake kinetics an incubation period of 5 min was chosen.

The rate of Fe uptake as a function of concentration. The effect of increasing medium Fe concentrations over the range 18–450 μmole/l. on uptake is shown in Text-fig 5. Uptake shows saturation kinetics within the concentration range studied. Using the Eadie-Hofstee plot (Hofstee, 1952) and linear regression analysis by the method of least-squares, values for the apparent K_t and V_{max} were derived from these concentration-velocity studies. In nine experiments the mean V_{max} ($\pm$ S.E. of mean) was $9{\cdot}6 \pm 1{\cdot}5$ p-mole Fe/mg.min and the apparent K_t 127 ± 42 μmole/l., demonstrating saturation within a physiological concentration range (Jacobs & Miles, 1969).

The effect of metabolic inhibitors. In the presence of 0·1 mM 2:4 dinitrophenol, an inhibitor of oxidative phosphorylation, and 10 mM-NaF as an inhibitor of glycolysis, Fe uptake was reduced by a mean of 55 %. In preliminary experiments, addition of either DNP or fluoride alone to the incubation buffer resulted in only 15–30 % inhibition. Control uptake of Fe^{3+}–$(NTA)_2$ (18 μmole/l) after incubation for 10 min at 37 °C was $33 \pm 8{\cdot}4$ p-mole/mg (S.E. of mean), while uptake by paired samples in the presence of the two inhibitors was $15 \pm 2{\cdot}4$ ($n = 6$); the difference between these



Text-fig. 3. Relation between wet weight of biopsy tissue and corrected Fe uptake. Specimens from two individuals were incubated for 10 min at an Fe concentration of 18 μmole/l.

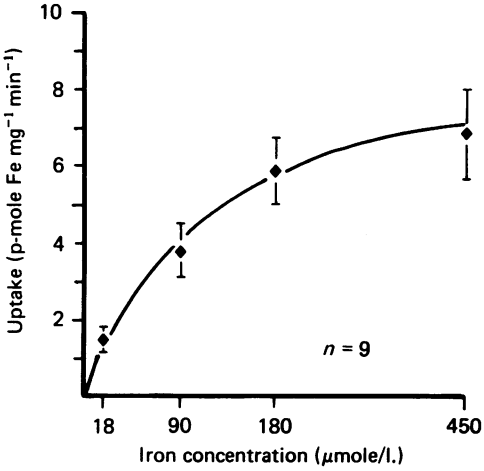


Text-fig. 4. Time course for iron uptake at three different medium Fe concentrations. Points represent mean uptake rates of two specimens from each individual.

values being significant at $P < 0{\cdot}05$ according to the Wilcoxon paired differences test. At the inhibitor concentrations used, there was no significant change in the tissue e.c.f. This indicates that there were no gross changes in membrane permeability to account for the observed inhibition of Fe uptake, which is therefore attributed to a reduction in the available supply of metabolic energy necessary for iron transport. However, membrane integrity was altered in the presence of 1 mM-DNP, as evidenced by an increased e.c.f. volume and a greatly increased apparent uptake of Fe.

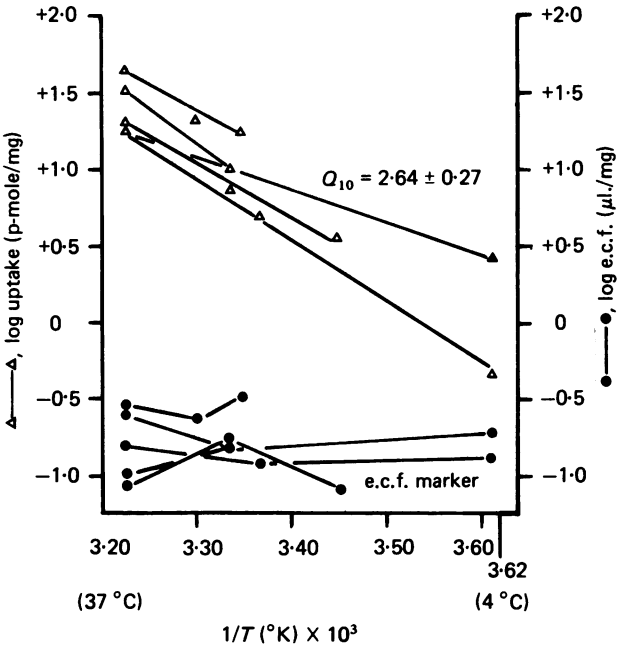
The influence of incubation temperature on iron uptake. Reduction of the incubation temperature in the range 37–4 °C diminished iron uptake significantly, although it was without effect on the measured e.c.f. In Text figure 6 the data from five such experiments are shown plotted on an Arrhenius diagram. The steep slope reflects the

high activation energy for the uptake process, which is also evident from the mean Q_{10} of 2·64 (37–27 °C), indicating marked temperature dependence, as has been previously found for over-all Fe absorption in the guinea-pig intestine (Jacobi, Pfleger & Rummel, 1956). This is an important charcteristic of active transport processes (Kotyk & Janáček, 1970).



Text-fig. 5. Relation between uptake velocity and medium iron concentration. The points represent the mean ± S.E. of nine subjects after 5 min incubation, each experiment being performed in duplicate.

Studies with radiolabelled nitrilotriacetate. In order to investigate whether or not the intact Fe chelate Fe^{3+}-$(NTA)_2$ passed across the brush border membrane of the mucosal epithelial cells, studies using chelates of 'cold' Fe with [1-^{14}C]NTA were undertaken. At Fe concentrations of 18–450 μmole/l. and chelate concentrations of 36–900 μmole/l., the apparent distribution volume of [1–^{14}C]NTA was compared with the extracellular fluid volume as measured with [^{57}Co]cyanocobalamin after 5 min incubation. In paired samples from four subjects distribution volumes of 0·141 ± 0·096 μl./mg (S.D. of an observation) and 0·147 ± 0·058 μl./mg were obtained for the radiolabelled chelate and extracellular fluid marker, respectively (n = 28). No significant difference between the two fluid spaces was found on paired statistical analysis. Furthermore, in two experiments where it was possible to determine the distribution of ^{59}Fe, [1–^{14}C]NTA and [^{57}Co]cyanocobalamin in fourteen biopsy specimens, the apparent distribution volume of the ^{59}Fe was almost an order of magnitude greater at 1·18 ± 0·44 μl./mg than that of both the respective radiolabelled chelate and the e.c.f. marker. These results imply that the Fe is transferred from the chelate to receptors at the brush border membrane, and that the intact chelate is excluded from mucosal epithelial cells. To exclude possible artifacts related to the use of synthetic chelates, Fe uptake was studied in the same system using [^{59}Fe]ferric citrate as a donator. In duplicate samples from two subjects incubated with 18 μmole/l. iron, uptake was linear for 20 min. The mean rate of uptake was 0·9 p-mole Fe/mg.min and may be compared with that obtained when nitrilotriacetate was used (1·7 ± 0·8 p-mole/mg.min; S.D. of an observation).



Text-fig. 6. Effect of incubation temperature on Fe uptake and e.c.f. space: Arrhenius diagram. The points represent the mean of two or three determinations from each individual. Results of five separate experiments are shown. Conditions: 10 min incubation; iron 18 μmole/l., 4–37 °C.

DISCUSSION

In this system, biopsy specimens of human duodenal mucosa remain viable for at least 20 min. This is verified by the linear time course for Fe uptake and O_2 consumption, as well as the action of metabolic inhibitors and the effects of reductions in incubation temperature. Radioactive vitamin B_{12} has been used *in vivo*, and is a suitable marker for extracellular fluid in the duodenum (Schütz & Reizenstein, 1963). Our studies confirm that it is also an excellent marker for *in vitro* experiments. The technique allows uptake of the Fe probe to be calculated after only a brief exposure, thus avoiding artefacts due to backward flux from intracellular accumulations. The data suggest that initial rates of Fe influx were determined when 5 min incubations were used.

The presentation of the iron as a Fe^{3+} chelate of nitrilotriacetic acid enables it to be maintained in a soluble oxidized state. The observation that the intact chelate is excluded from the intracellular compartment is noteworthy since it resembles closely the release and uptake of Fe *in vivo* from intraluminal food Fe complexes. Stable Fe^{3+}-NTA chelates (affinity constant 10^{8}M) are known to give up Fe rapidly to apotransferrin *in vitro* (binding constant 10^{24} M) (Bates *et al.* 1967) and this, taken with the experimental findings, points to the existence of high affinity receptors for iron in the enterocyte membrane (Hübers, Hübers, Forth, Leopold & Rummel, 1971). The remote possibility that the [^{14}C]NTA has been metabolised to freely diffusible products which escape detection within the tissue can be largely discounted by the

findings of Michael & Wakim (1971), who detected less than 1 % of the isotopic dose in the expired CO_2 of rats given parenteral [^{14}C]NTA. These authors also found that ^{14}C-labelled disodium NTA was poorly absorbed by the rabbit and the Rhesus monkey. In the present experiments, the mucosa was exposed to the chelate only very briefly, and significant catabolism would not be expected.

Unidirectional uptake of iron by human duodenal mucosa has many of the features of active transport (Wilson, 1962). The process shows both saturation kinetics and a dependence on metabolically derived energy. Furthermore, when the apparent intracellular concentration of Fe after 20 min incubation at the lower medium concentrations was calculated by the method of Crane & Mandelstam (1960), apparent 'distribution ratios' greater than unity (4·9 $\pm$ 1·5; S.E. mean) were obtained; although this suggests that iron may be accumulated against a concentration gradient, it is unlikely that free ionised Fe exists within the cytoplasm, and so this observation cannot *per se* be construed as direct evidence for active transport.

It is possible that the kinetic characteristics revealed in this study could have been the result of simple binding of radioactive Fe to surface receptors on the brush border membrane. This interpretation can be excluded on the grounds that simple surface binding would be expected to be more or less instantaneous and not incremental over several minutes, as was observed. The rate of Fe uptake in this system is slower by several orders of magnitude than would be expected for binding. Indeed, simple surface binding would not show any dependence upon metabolic energy, and such striking temperature sensitivity would be exceptional. Moreover, autoradiography after 10 min incubation with $^{55}Fe^{3+}$-$(NTA)_2$ (Orlic, 1968) showed no concentration of grains on the brush border membranes; radioactivity was detected throughout the epithelium. Finally, extensive subcellular localization studies employing density gradient centrifugation failed to show brush border Fe binding in this system (Cox & Peters, unpublished results).

It is probable that the regulatory step for intestinal absorption of Fe is accessible to metabolic control mechanisms and is a component of a co-ordinated, energy-requiring transport process. The work of Jacobi *et al.* (1956), Dowdle *et al.* (1960) and Jacobs *et al.* (1966) support the view that over-all intestinal absorption involves active transport; however, positive evidence for its location in the intracellular sequence of transport events is lacking (Manis & Schachter, 1962). On the other hand, the experiments of Pollack (1968), who perfused loops of duodenum from normal and iron-deficient dogs with radio-Fe solutions and collected portal plasma at timed intervals, have shown that the final transfer from mucosa to plasma cannot be rate-limiting for absorption, as has been proposed by Conrad & Crosby (1963). Although studies on unidirectional Fe uptake by rodent intestine have been reported previously (Acheson & Schultz, 1972; Sheehan, 1976) conflicting results concerning its regulatory significance were obtained, possibly due to species differences. Acheson & Schultz (1972) showed that haemorrhagic anaemia was associated with increased initial rates of uptake into segments of rabbit duodenum incubated with radio-Fe *in vitro*, and that this effect was abolished by prior dosing with parenteral Fe. However, Sheehan (1976), who used a similar system to study rat intestine, failed to confirm these findings, and in addition found no evidence of active or even carrier-mediated transport.

The use of the present technique in man has enabled the kinetics of Fe uptake by the small intestinal mucosa to be characterized in health. An application of this method to the study of patients with disordered Fe metabolism or with Fe deficiency should help to clarify the role of the intestinal mucosa in the regulation of iron balance (Cox & Peters, 1978).

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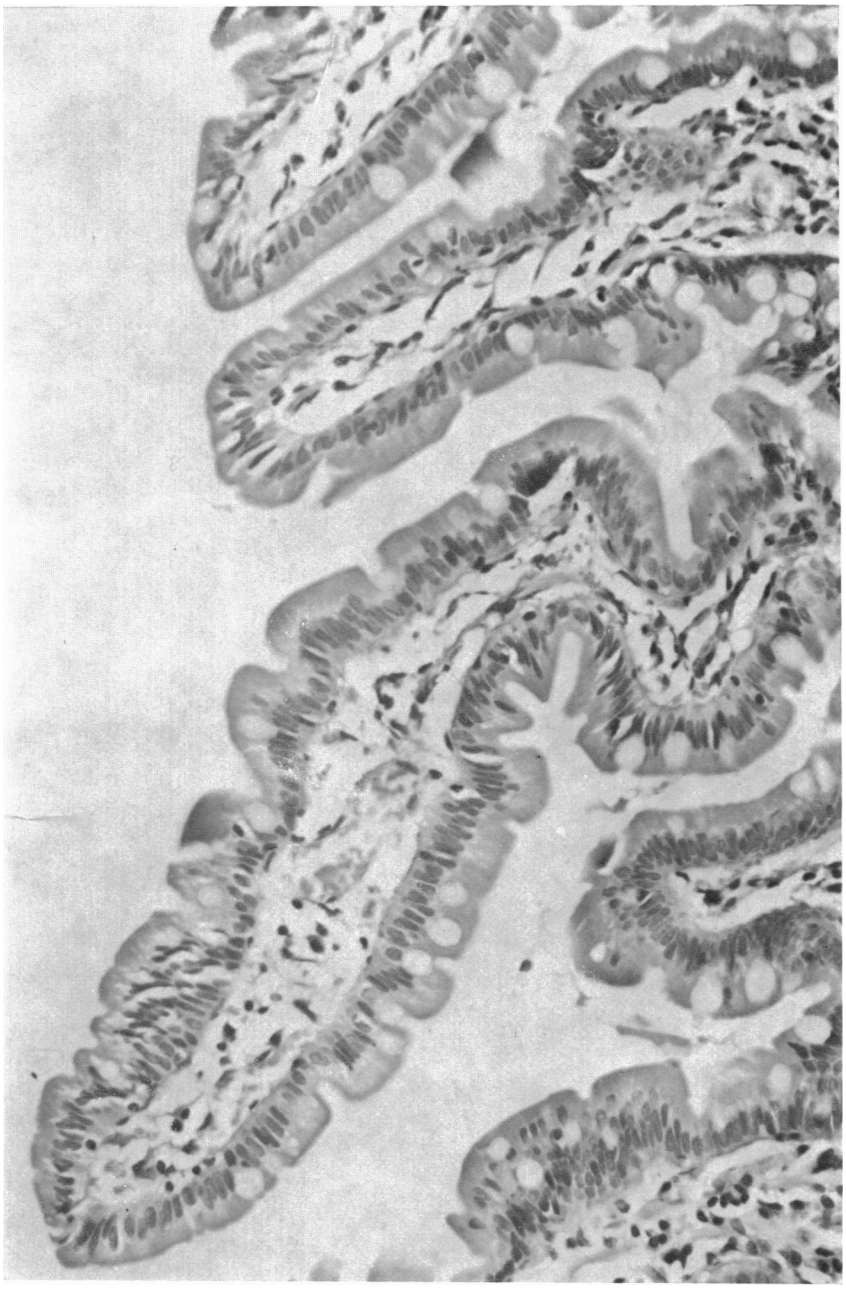
EXPLANATION OF PLATES

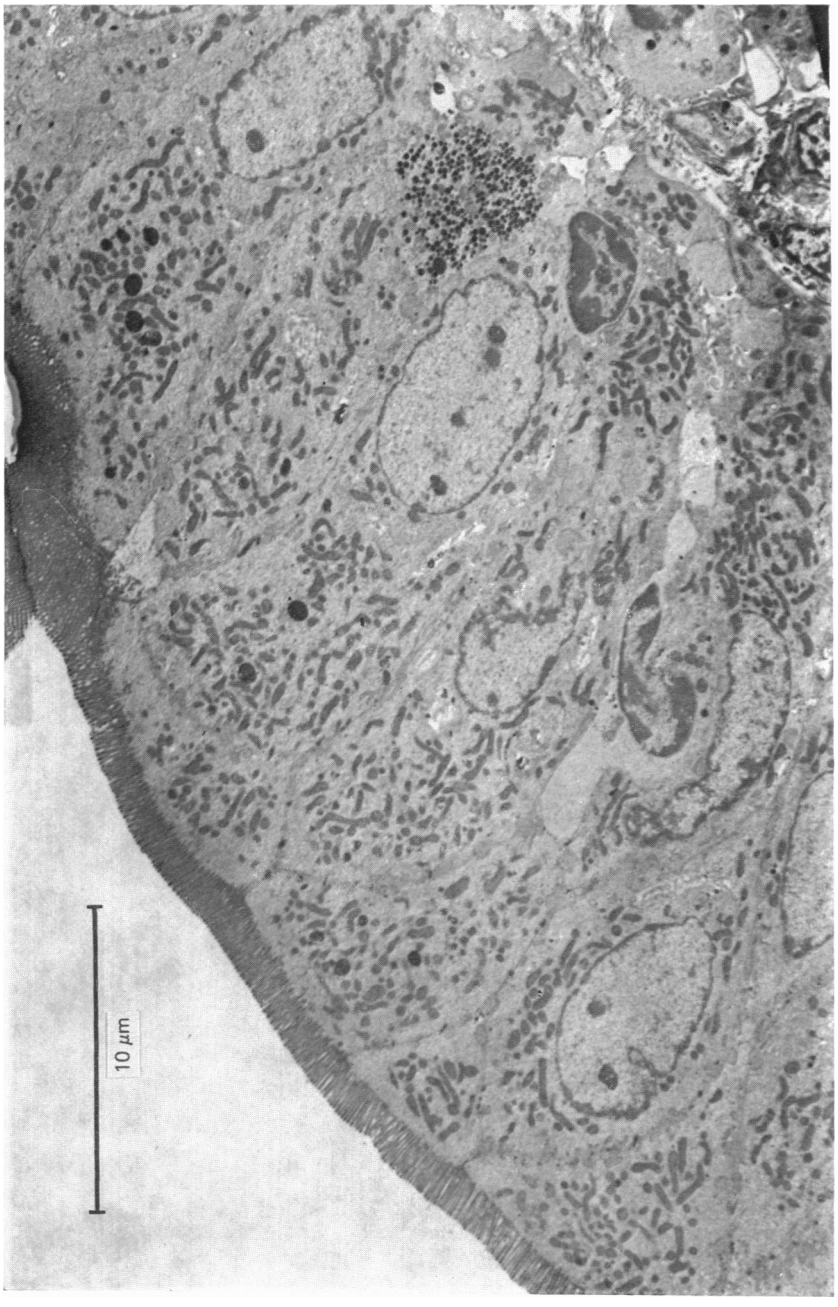
PLATE 1

High power micrograph (× 400) of human duodenal villi after incubation for 10 min with radio-Fe *in vitro*. Haematoxylin and eosin stain.

PLATE 2

Electron micrograph (× 4000) of mucosal epithelium after incubation for 10 min with radio-Fe *in vitro*. Osmium fixation and uranyl acetate stain.





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