Mechanism underlying the inhibitory effect of high calcium carbonate intake on iron bioavailability from ferrous sulphate in anaemic rats

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The influence of high $CaCO_3$ intake on the bioavailability of Fe from $FeSO_4$ was assessed during Fe repletion of rats with Fe-deficiency-induced anaemia. Fe-deficient rats with a mean blood haemoglobin concentration of 4·1 mmol/l were fed on purified Fe-adequate diets containing either 6·2 or 25·0 g $CaCO_3$/kg (ten rats per group). Haemoglobin repletion after 14 d was significantly depressed by high $CaCO_3$ intake (9·5 *v.* 9·8 mmol/l for high and low $CaCO_3$ intake respectively; $P = 0·03$), as was apparent Fe retention (367 *v.* 552 μg/d during days 5–7, $P < 0·001$; 146 *v.* 196 μg/d during days 19–21, $P < 0·001$). The concentration of Fe in the liquid phase of the proximal half of the small intestine was significantly lower in the high-$CaCO_3$ group (3·71 *v.* 5·20 μg/g digesta; $P = 0·02$). Mucosal uptake and mucosal transfer of Fe were determined with orally administered ^{59}Fe and Cr as a non-absorbable marker. Mucosal transfer was significantly diminished by $CaCO_3$ loading (90 *v.* 100% of mucosal uptake; $P = 0·04$), whereas mucosal uptake was not. ^{59}Fe retention values at 14 d after administration were not significantly different (57·6 *v.* 51·9%; $P = 0·14$). Fe contents of liver and spleen were significantly decreased by high compared with low $CaCO_3$ intake (879 *v.* 590 μg Fe in liver, $P < 0·001$; 92 *v.* 63 μg Fe in spleen, $P < 0·001$). It is concluded that high intake of $CaCO_3$ depresses Fe bioavailability in rats. The $CaCO_3$-induced decrease in Fe solubility in the digesta probably was associated with an increased efficiency of mucosal Fe uptake so that the amount of mucosal uptake remained unaltered. The $CaCO_3$-induced decrease in Fe transfer through the mucosal cytoplasm and/or basolateral membrane may have been responsible for the concurrent decrease in Fe bioavailability.

Non-haem Fe: Calcium carbonate intake

The term Fe bioavailability is frequently used but not defined by common consent. It is defined by Fairweather-Tait (1987) as 'a measure of the proportion of the total (Fe) in a food or diet that is digested, absorbed and metabolized by normal pathways', and as a result different methods for the estimation of Fe bioavailability are applied. More limited definitions are used also (Van Dokkum, 1992). Fe bioavailability may be assessed using the so-called rat haemoglobin(Hb)-repletion bioassay, which is the official method of the Association of Official Analytical Chemists (Fritz *et al.* 1974; Association of Official Analytical Chemists, 1990). This assay involves the determination of the increase in blood Hb concentrations in rats with Fe-deficiency-induced anaemia after the administration of a diet containing the Fe compound under study relative to the reference source of Fe, i.e. $FeSO_4$.

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Supplemental $CaCO_3$ has been shown to inhibit Fe absorption in humans when it was consumed together with food (Dawson-Hughes *et al.* 1986; Cook *et al.* 1991). The inhibitory effect of $CaCO_3$ on Fe bioavailability has been demonstrated also in various Hb-repletion bioassays with rats (Freeman & Ivy, 1942; Chapman & Campbell, 1957; Prather & Miller, 1992). Dietary $CaCl_2$ has been shown by Barton *et al.* (1983) to decrease Fe absorption in Fe-deficient rats, the effect being caused by the cation. Barton *et al.* (1983) suggested that part of the Ca effect is located at the microvillus membrane, but that Ca also blocks Fe release by the enterocyte. Further support for inhibition of intramucosal Fe transport by Ca comes from human studies. The inhibitory effect of Ca on Fe absorption appeared to be equal for haem- and non-haem-Fe, whereas it is known that these Fe sources have separate pathways until they reach the cytoplasm of the mucosal cells (Hallberg *et al.* 1991, 1993). Minute amounts of Ca are needed to elicit an effect, indicating that competition between Fe and Ca at the level of the apical receptor is unlikely (Hallberg *et al.* 1992).

In the present rat Hb-repletion bioassay we evaluated the effect of $CaCO_3$ on the various steps from oral uptake of Fe to its incorporation into haem. Thus, we measured intestinal Fe solubility, as well as apparent Fe retention. With separate measurements of mucosal Fe uptake and mucosal Fe transfer we were able to address the possibility that $CaCO_3$ inhibits the intramucosal transport of Fe. Since the Hb-repletion bioassay involves the use of an anaemic rat model it was considered relevant to measure blood Hb repletion and other haematological variables and liver and spleen Fe concentrations as affected by $CaCO_3$ loading.

MATERIALS AND METHODS

The experimental protocol was approved by the Animal Experiments Committee of the Department of Laboratory Animal Science.

Animals, housing and diets

Weanling, male Wistar (U:WU) rats, aged about 3 weeks, were used. To induce anaemia, all rats went through a pre-experimental period of 4 weeks (Fig. 1), during which they received demineralized water and a purified diet low in Fe (Table 1). Feed and water were given *ad lib.* During the first 3 weeks the rats were housed in groups of four or five animals in Macrolon Type III cages (UNO BV., Zevenaar, The Netherlands), containing a layer of sawdust which was free of Fe. At 1 week before the end of the pre-experimental period the rats were transferred to metabolism cages (Tecniplast Gazzada, Buguggiate, Italy) in which they were housed individually. After the pre-experimental period (day 0 of the experiment) the rats were divided into two groups of eleven animals each such that the distributions of body weights and blood Hb values were similar for the two groups. The metabolism cages were placed in randomized position in a room with controlled temperature (20–22°) and lighting (light period 07.00–19.00 hours).

During the experimental period of Hb repletion the rats had free access to demineralized water and a purified diet containing either half (6·2 g/kg; low $CaCO_3$) or double (25·0 g/kg; high $CaCO_3$) the recommended amount of Ca as $CaCO_3$ (National Research Council, 1978). Both diets were made adequate in Fe by the addition of $FeSO_4.7H_2O$ (Table 1). The diets, which were in powdered form, were stored at 4° until used for feeding. Feed intake was recorded daily, water intake was recorded during the two balance periods (see p. 112), and all animals were weighed weekly. The experimental period lasted 23 d. One rat died due to complications of blood sampling, and one rat died of unknown cause, so that the results relate to ten rats per dietary group.

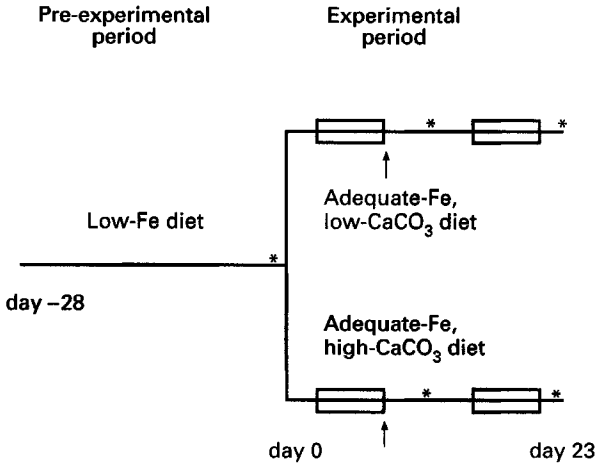

Fig. 1. Experimental design of the study. After a pre-experimental period of 28 d the animals were allocated to one of two Fe-sufficient diets with either a low (6·2 g/kg) or high (25·0 g/kg) level of $CaCO_3$. Blood was withdrawn on days 0, 14 and 23 to determine haematological variables; Fe balance measurements were performed by excreta collection during days 5–7 and days 19–21, ^{59}Fe was administered on day 8 to assess mucosal uptake and mucosal transfer of Fe and to determine apparent Fe retention, and the rats were killed on day 23 to remove the small intestine, liver and spleen. □, Balance periods (days 5–7 and 19–21); *, orbital puncture on days 0, 14 and 23; ↑, ^{59}Fe administered orally. For details of diets, see Table 1.

Table 1. *Composition of the diets*

	Pre-experimental	Low $CaCO_3$	High $CaCO_3$
Variable ingredients (/kg diet)			
Glucose (g)	704·3	710·6	691·8
$CaCO_3$ (g)	12·5	6·2	25·0
$FeSO_4.7H_2O$ (mg)	—	174	174
Constant ingredients* (g)	283·2	283·2	283·2
Chemical analysis			
Fe (mg/kg)	7·5	36·8	37·7

* The constant ingredients consisted of (g): casein 151, maize oil 25, coconut fat 25, cellulose 30, $MgCO_3$ 1·4, $NaH_2PO_4.2H_2O$ 20·1, KCl 1·0, $KHCO_3$ 7·7, Fe-free mineral premix 10, vitamin premix 12. The mineral premix consisted of (mg): MnO_2 79·0, $CuSO_4.5H_2O$ 15·7, $ZnSO_4.H_2O$ 33·0, $Na_2SeO_3.5H_2O$ 0·3, $NiSO_4.6H_2O$ 13·0, $CrCl_3.6H_2O$ 1·5, NaF 2·0, $SnCl_2.H_2O$ 1·9, KI 0·2, NH_4VO_3 0·2, maize meal 9853·2. The vitamin premix consisted of (mg): thiamin 4, pteroyl monoglutamic acid 1, riboflavin 3, biotin 2, nicotinamide 20, menadione 0·05, DL-calcium pantothenate 17·8, DL-α tocopheryl acetate 60, pyridoxine 6, retinyl acetate and retinyl palmitate 8 (1200 retinol equivalents), cyanocobalamin 50, cholecalciferol 2 (1000 IU), choline chloride 2000, maize meal 9826·15.

Haematological tests

On the last day of the pre-experimental period and on days 14 and 23 of the experiment, blood was drawn by orbital puncture while the rats were under light diethyl ether anaesthesia. About 0·8 ml blood was collected from each rat in a heparinized tube. Hb and mean cell volume (MCV) were determined using the Sysmex K1000 (Automated Hematology Analyzer; Toa Medical Electronics Co. Ltd., Kobe, Japan). Plasma was obtained by low-speed centrifugation and Fe and total Fe-binding capacity (TIBC) were determined using commercial kits (Iron FZ Test and IBC Test, ROCHE; Hoffmann-La

Roche & Co. Ltd. Diagnostica, Basle, Switzerland) and the COBAS-BIO auto-analyser (Hoffmann-La Roche BV, Mijdrecht, The Netherlands).

Iron analysis in faeces and urine

Faeces and urine of each rat were collected separately and quantitatively during days 5–7 and 19–21 of the experiment. Cages and collection tubes had been rinsed with 0·1 M-HCl and demineralized water before use. The faeces and urine collected daily were pooled for each rat during each balance period. Pooled faeces were weighed, dried (60°, 48 h), ashed (500°, 17 h), dissolved in 6 M-HCl and analysed for Fe with atomic absorption spectrometry (Varian AA-475; Varian Techtron, Springvale, Australia). Urinary pH was determined, and Fe was measured in undiluted urine with atomic absorption spectrometry.

Measurements using ^{59}Fe

Immediately after the first balance period (days 5–7) in all rats feed was withheld for 2 h. The rats then received orally a ^{59}Fe-labelled suspension of diet. [^{59}Fe]ferric citrate was reduced to Fe^{2+} using an equimolar amount of ascorbic acid. This solution was mixed with demineralized water and each of the experimental diets, to which 0·2 g $CrCl_3$/kg was added as non-absorbable marker (water–diet 1:1, w/w). Both suspensions were swirled continuously while the doses to be administered to the rats were taken. Samples of approximately 2 g were administered orally to each rat using an olive-tipped oroesophageal needle. With the administration of suspension, each rat ingested about 55 kBq ^{59}Fe and a total Fe dose of 40 μg.

Rats were then restrained in a Perspex cylinder to be placed in a cylindrical Pb castle with an internal diameter of 160 mm, a height of 530 mm, and a NaI-scintillation crystal at the bottom. A horizontal opening in the Pb castle allowed placement of the cylinder with the rat inside above the scintillation crystal. Radioactivity in each rat was measured using a gamma-scanner (Automatic Scanner mod. DS4/4S; Tracerlab Ltd., Weybridge, Surrey) immediately after radiotracer administration and restraining. Baseline radioactivity was corrected for background radiation and the resulting value set at 100%.

Whole-body radioactivity was measured again at days 1, 2, 3, 4, 5, 8, 11, and 14 after radiotracer administration. Faeces and urine were collected separately and quantitatively on those days and also measured for ^{59}Fe. All values for radioactivity were corrected for radioisotope decay and also for day-to-day variation of the scanner by using a Ra source. Recovery of the administered ^{59}Fe in faeces and urine collected for 14 d plus residual whole-body radioactivity was 99·4 (SE 1·4)% (n 20).

Cr as $CrCl_3$ was administered together with ^{59}Fe to measure mucosal uptake and mucosal transfer of Fe. The technique described by Marx (1979) was applied, except that stable instead of radioactive Cr was used. Faeces were dried (60°, 48 h), ashed (500°, 17 h), dissolved in 6 M-HCl and Cr was then analysed by atomic absorption spectrometry. Faecal Cr excretion was measured also before administration of the test dose to determine background levels. The accurate amount of Cr ingested with the oral dose was calculated as the analysed amount excreted during 11 d after dosing minus baseline Cr excretion. Cr in the form of $CrCl_3$ can be considered an inert indicator (Donaldson & Barreras, 1966) with the same transit time as Fe (Marx *et al.* 1980). The proportion of administered Cr not excreted in faeces after 48 h reflects the amount present in the lumen of the gastrointestinal tract at that time. The amount of lumen Fe equals the amount ingested minus the sum of the amount excreted in the faeces and that which crossed the apical membrane of the mucosal cells, which is referred to as mucosal uptake. Reliable measurements of mucosal Fe uptake have to be made within 48 h after ingestion of the test dose (Marx, 1979), because

of the short life-span of the intestinal mucosal cells. Mucosal uptake can be calculated as follows (Marx, 1979):

$$\text{mucosal } {}^{59}\text{Fe uptake}$$
$$(\%\text{ of administered dose}) = \frac{[{}^{59}\text{Fe}_{\text{body2}} - \text{Cr}_{\text{body2}}]}{[100 - \text{Cr}_{\text{body2}}]} \times 100,$$

where ${}^{59}\text{Fe}_{\text{body2}}$ is ^{59}Fe not excreted in faeces after 2 d expressed as a percentage of administered dose, and Cr_{body2} is lumen Cr after 2 d, expressed as a percentage of the total Cr excretion within 14 d, corrected for background Cr.

The amount of Fe that crossed the basolateral membrane of the mucosal cells (mucosal transfer) was calculated as follows:

$$\text{mucosal Fe transfer}$$
$$(\text{proportion of mucosal uptake}) = \frac{{}^{59}\text{Fe}_{\text{body14}}}{\text{mucosal } {}^{59}\text{Fe uptake after 2 d}},$$

where ${}^{59}\text{Fe}_{\text{body14}}$ is ^{59}Fe not excreted in faeces after 14 d expressed as a percentage of administered dose.

To assess apparent Fe absorption, the ^{59}Fe-retention data for each rat were plotted on a logarithmic scale (y axis) *v.* the period (d) after administration of the test dose on an ordinal scale (x axis). Apparent ^{59}Fe absorptions were the y-intercept values after extrapolation of the linear part (2–14 d post administration) of the retention curves (Heth & Hoekstra, 1965).

Iron analysis in digesta

Fe solubility in the contents of the small intestine was measured because soluble rather than insoluble Fe may be available for absorption (Brouwer *et al.* 1993) and because the small intestine is the site of Fe absorption (Barton *et al.* 1983).

On day 22, feed was withheld for 22 h with 10 min intervals between successive animals to standardize their nutritional status for the moment of digesta collection. Demineralized water was provided *ad lib.* After the fasting period, 10 g of its feed was supplied to each animal, allowing a period of 3 h to eat it. The rats were then anaesthetized with diethyl ether, subjected to orbital puncture and subsequently exsanguinated by aortic puncture. The entire small intestine was removed and divided into a proximal and a distal part of equal length. Digesta were collected and separated into a liquid and a solid phase by centrifugation, after which pH was determined in the liquid fraction (Brouwer *et al.* 1993). Fe in the digesta fractions was measured using a commercial kit (Iron FZ Test) and the COBAS-BIO auto-analyser.

Iron analysis in liver and spleen

Liver and spleen were removed, weighed and frozen at −20° until analysis. A portion of liver (about 2 g) and the total spleen were dried overnight (105°) and then ashed for 17 h at 500°. The ash was dissolved in 6 M-HCl and Fe measured.

Statistical analysis

The data within each dietary group were found to be normally distributed according to the Kolmogorov–Smirnov test. As the variables whose values were used to allocate diets to rats appeared to have no effect on the responses, the observations were treated as if the diets had been allocated completely at random to the rats. Differences between groups were tested for statistical significance with Student's t test. Covariate analysis was performed on selected data as indicated. Pearson correlation coefficients between selected individual measurements were calculated. The pre-set level of significance was $P < 0{\cdot}05$. All statistical

analyses were carried out using a SPSS computer program (Statistical Package for Social Sciences, 1988).

RESULTS

Growth, feed and water intake

Feed intakes were slightly, but significantly, higher in the high-$CaCO_3$ group (Table 2). The higher feed intake did not influence growth performance, which could have been related to the somewhat lower energy density of the high-$CaCO_3$ diet. Water intake and relative weights of liver and spleen did not differ between the two groups.

Haematological variables

After receiving the Fe-deficient diet for 4 weeks the rats were anaemic, with a mean blood Hb concentration of 4·1 mmol/l (66 g/l). Other haematological values were also consistent with a microcytic hypochromic anaemia (Table 3). MCV and plasma Fe were low and TIBC was high, consistent with Fe-deficiency-induced anaemia in rats (McCall *et al.* 1962; Yokoi *et al.* 1991).

The level of Hb increased after feeding the Fe-sufficient diets. The high $CaCO_3$ concentration in the Fe-sufficient diet slightly, but significantly, inhibited Hb repletion after 14 and 23 d. MCV increased after transfer to the Fe-sufficient diets, but to a significantly lesser extent in the high-$CaCO_3$ group. Plasma Fe increased, whereas TIBC decreased after the anaemic rats were transferred to the diets adequate in Fe. Plasma Fe was significantly higher in the high-$CaCO_3$ group after 14 and 23 d, and TIBC was higher after 14 d.

Iron balance

Table 4 shows that Fe intake in the high-$CaCO_3$ group was consistently higher than that in the low-$CaCO_3$ group. This group difference was caused by the higher feed intake (Table 2) and the slightly higher dietary Fe concentration (Table 1) for rats in the high-$CaCO_3$ group. The high dietary $CaCO_3$ concentration caused a significantly higher faecal Fe excretion after days 5–7. After days 19–21, faecal Fe excretion tended ($P = 0{\cdot}07$) to be higher in the high-$CaCO_3$ group. Apparent Fe retention after days 5–7 and 19–21 was significantly lower in rats fed on the high-$CaCO_3$ diet. The use of Fe intake as co-variate had no essential impact on the levels of statistical significance. In both groups the apparent retention of Fe decreased with time. Urinary Fe excretion was negligible when compared with faecal Fe excretion.

To check the well-known action of dietary $CaCO_3$ as a urinary alkalinizer we measured urinary pH. As would be anticipated, mean urinary pH was significantly higher in the high-$CaCO_3$ group. The values for the high-$CaCO_3$ *v.* low-$CaCO_3$ group were: 7·56 (SE 0·08) *v.* 6·19 (SE 0·04) for days 5–7 ($P < 0{\cdot}001$) and 7·80 (SE 0·23) *v.* 6·81 (SE 0·26) for days 19–21 ($P = 0{\cdot}01$).

Measurements with ^{59}Fe

Retention of ^{59}Fe was consistently lower in the high-$CaCO_3$ group but only during the first 2 d after administration of the radiotracer was the reduction statistically significant, the values after 2 d being 73·5 (SE 3·0) and 62·6 (SE 2·5) % ($P = 0{\cdot}012$). Fe retention after 14 d tended ($P = 0{\cdot}14$) to be lower in the high-$CaCO_3$ group (Table 5). Mucosal Fe uptake was similar for the two groups (Table 5), but mucosal transfer was significantly lower in the high-$CaCO_3$ group.

Calculation of the apparent absorption according to the method of Heth & Hoekstra (1965) resulted in values of 74 (SE 3·3) and 65 (SE 2·6) % for the rats given the low- and high-$CaCO_3$ diets respectively (n 10; $P = 0{\cdot}03$).

Table 2. *Feed and water intake and body and organ weights in rats with iron-deficiency-induced anaemia and subsequently fed for 23 d on Fe-sufficient diets either low (6·2 g/kg) or high (25·0 g/kg) in calcium carbonate*†

(Mean values with their standard errors for ten rats per group)

Diet...	Low $CaCO_3$		High $CaCO_3$	
	Mean	SE	Mean	SE
Feed intake (g/d)	16·0	0·3	17·0*	0·2
Water intake (g/d)	19·3	1·8	23·2	3·3
Body wt (g)				
Initial (day 0)	168·9	3·1	171·6	1·9
Final (day 23)	241·3	3·9	247·5	3·4
Organ wt (g/kg body wt)				
Liver	33·7	0·5	33·2	0·3
Spleen	1·8	0·1	1·8	0·1

Mean value was significantly different from that for low-$CaCO_3$ group (two-tailed Student's *t* test): * $P < 0·05$.
† For details of diets and procedures, see Table 1 and pp. 110–113.

Table 3. *Haematological variables in rats with iron-deficiency-induced anaemia and subsequently fed on Fe-sufficient diets either low (6·2 g/kg) or high (25·0 g/kg) in calcium carbonate*†

(Mean values with their standard errors for ten rats per group)

Diet...		Low $CaCO_3$		High $CaCO_3$	
Variable	Time-point (day)	Mean	SE	Mean	SE
Hb (mmol/l)‡	0	4·1	0·0	4·1	0·1
	14	9·8	0·1	9·5*	0·1
	23	10·3	0·1	9·8**	0·0
MCV (fl)	0	44·4	0·4	44·5	0·5
	14	73·3	0·9	64·2***	1·6
	23	70·1	0·9	63·3***	1·0
Plasma Fe (μmol/l)	0	5·7	0·3	6·0	0·3
	14	44·2	1·4	56·0**	3·4
	23	23·0	0·9	30·2*	2·8
TIBC (μmol/l)	0	158	3	159	2
	14	92	3	103**	2
	23	79	2	84	2

Hb, haemoglobin; MCV, mean cell volume; TIBC, total Fe-binding capacity.
Mean values were significantly different from those for low-$CaCO_3$ group (two-tailed Student's *t* test: * $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
† For details of diets and procedures, see Table 1 and pp. 110–112.
‡ 1 mmol Hb/l = 16·1 g Hb/l.

Iron in digesta

After 22 h of fasting, the rats in the low-$CaCO_3$ group ate 5·51 (SE 0·40) g of feed within 3 h and those in the high-$CaCO_3$ group ate 6·09 (SE 0·35) g. This difference in feed intake was not statistically significant (n 10; $P = 0·29$).

The rats fed on the high-$CaCO_3$ diet had a smaller liquid phase in the proximal and the distal half of the small intestine (Table 6). Only one measurement was possible for lumen

Table 4. *Apparent iron retention in rats with Fe-deficiency-induced anaemia and subsequently fed on Fe-sufficient diets either low (6·2 g/kg) or high (25·0 g/kg) in calcium carbonate*†

(Mean values with their standard errors for ten rats per group)

Diet...		Low $CaCO_3$		High $CaCO_3$	
Variable	Time-point (day)	Mean	SE	Mean	SE
Fe intake (μg/d)	5–7	657	14	729**	10
	19–21	568	13	627**	12
Faecal Fe (μg/d)	5–7	102	13	359***	6
	19–21	369	24	478	14
Urinary Fe (μg/d)	5–7	3·0	0·3	2·6	0·2
	19–21	2·1	0·2	2·2	0·2
Apparent Fe retention‡ (μg/d)	5–7	552	10	367***	12
	19–21	196	21	146***	11
Apparent Fe retention‡ (% of intake)	5–7	84	2	50***	1
	19–21	35	4	23*	2

Mean values were significantly different from those for low-$CaCO_3$ group (two-tailed Student's *t* test): * $P < 0·05$; ** $P < 0·01$, *** $P < 0·001$.

† For details of diets and procedures, see Table 1 and pp. 110–113.

‡ Calculated as Fe intake minus faecal excretion minus urinary excretion.

Table 5. *Mucosal uptake, mucosal transfer and retention of* ^{59}*Fe in rats with Fe-deficiency-induced anaemia and subsequently fed on Fe-sufficient diets either low (6·2 g/kg) or high (25·0 g/kg) in calcium carbonate*†

(Mean values with their standard errors for ten rats per group)

Diet...	Low $CaCO_3$		High $CaCO_3$	
	Mean	SE	Mean	SE
Mucosal ^{59}Fe uptake (% of administered dose)	59·0	4·7	57·9	2·8
Mucosal ^{59}Fe transfer (proportion of mucosal uptake)	1·00	0·04	0·90*	0·02
^{59}Fe retention after 14 d (% of administered dose)	57·6	2·9	51·9	2·2

Mean value was significantly different from that for low-$CaCO_3$ group (two-tailed Student's *t* test): * $P < 0·05$.

† For details of diets and procedures, see Table 1 and pp. 110–113.

pH in the proximal intestine for the high-$CaCO_3$ group because the amounts of liquid digesta were minute. In the distal half of the intestine lumen pH was significantly higher in the high-$CaCO_3$ group. Solid-phase weights did not differ between the dietary groups (Table 6).

The amount and concentration of Fe in the liquid phase of the proximal intestine were significantly lower for the high-$CaCO_3$ group. The Fe concentration in the liquid phase of the distal intestine was similar for the two groups. For the distal intestine about 97% of the Fe was in the solid phase for both groups, whereas for the proximal intestine it was 86% for the high-$CaCO_3$ group and 79% for the low-$CaCO_3$ group.

Iron in liver and spleen

Fe stores in liver and spleen were significantly smaller after feeding the high-$CaCO_3$ diet compared with low-$CaCO_3$ diet (Table 7).

Table 6. *Iron contents in digesta of rats with Fe-deficiency-induced anaemia and subsequently fed on Fe-sufficient diets either low (6·2 g/kg) or high (25·0 g/kg) in calcium carbonate*†

(Mean values with their standard errors for no. of rats per group shown)

Diet...	Low $CaCO_3$			High $CaCO_3$		
	Mean	SE	*n*	Mean	SE	*n*
Proximal intestine						
Liquid-phase wt (mg)	211	16	10	134**	20	9
Liquid-phase pH	6·20	0·07	7	6·31	—	1
Solid-phase wt (mg)	26	5	10	29	5	9
Fe:						
Amount in liquid phase (μg)	1·10	0·11	10	0·49**	0·10	5
Amount in solid phase (μg)	5·19	1·13	10	3·46	0·54	5
Concentration in liquid phase (μg/g)	5·20	0·31	10	3·71*	0·47	6
Distal intestine						
Liquid-phase wt (mg)	506	28	10	385**	20	10
Liquid-phase pH	6·29	0·09	10	7·09***	0·11	10
Solid-phase wt (mg)	106	7	10	104	3	10
Fe:						
Amount in liquid phase (μg)	1·40	0·13	10	1·03*	0·05	10
Amount in solid phase (μg)	40·94	4·11	10	32·01	1·03	10
Concentration in liquid phase (μg/g)	2·76	0·20	10	2·69	0·10	10

Mean values were significantly different from those for low-$CaCO_3$ group (two-tailed Student's *t* test): * $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.

† For details of diets and procedures, see Table 1 and pp. 110–113.

Table 7. *Iron contents in liver and spleen of rats with Fe-deficiency-induced anaemia and subsequently fed on Fe-sufficient diets either low (6·2 g/kg) or high (25·0 g/kg) in calcium carbonate*†

(Mean values with their standard errors for ten rats per group)

Diet...	Low $CaCO_3$		High $CaCO_3$	
	Mean	SE	Mean	SE
Fe concentration (μg/g dry wt)				
Liver	363	14	241***	9
Spleen	891	59	586***	24
Total Fe (μg)				
Liver	879	33	590***	20
Spleen	92	4	63***	2

Mean values were significantly different from those for low-$CaCO_3$ group (two-tailed Student's *t* test): *** $P < 0·001$.

† For details of diets and procedures, see Table 1 and pp. 110–113.

Correlations between selected measures

In an attempt to identify determinants of Hb repletion in the rat Hb-repletion bioassay, correlations between Hb levels after 14 d and the other estimates of Fe bioavailability were calculated for individual rats within dietary groups (*n* 10). Correlation coefficients for the low- and high-$CaCO_3$ groups respectively were 0·16 and 0·21 for apparent Fe retention (μg/d), 0·23 and 0·46 for ^{59}Fe retention (% of dose), 0·19 and 0·25 (*n* 6) for Fe

concentration in the liquid phase of proximal intestinal digesta (μg/g), 0·01 and 0·49 for liver Fe (μg), and −0·18 and −0·29 for spleen Fe (μg). All correlations failed to reach statistical significance ($P > 0·1$), and the explained variance was always below 25%.

DISCUSSION

Fe repletion with the high-$CaCO_3$ diet compared with the low-$CaCO_3$ diet depressed Hb repletion in the rats. The concentration of Hb was slightly, but significantly, lower after feeding the high-$CaCO_3$ diet. This corroborates findings of earlier work (Freeman & Ivy, 1942; Chapman & Campbell, 1957; Prather & Miller, 1992). The anaemic-rat model displays a very high efficiency of Fe absorption in order to utilize optimally dietary Fe. It could be suggested that the inhibitory effect of $CaCO_3$ on Fe bioavailability is smaller in anaemic rats than in normal rats. Thus, the anaemic-rat model used may be relatively insensitive to dietary components and Fe sources affecting Fe bioavailability.

The decrease in Fe bioavailability after $CaCO_3$ loading, as illustrated by the depressed Hb repletion, appears to be caused by interference with Fe absorption. Apparent Fe absorption, which essentially equals retention because urinary Fe excretion is negligible, was markedly lowered by extra $CaCO_3$ in the diet. This was demonstrated both by means of the balance study and by ^{59}Fe administration. The calculated apparent absorption values from the ^{59}Fe study refer to day 8 and are expected to be intermediate between those for days 5–7 and days 19–21 from the Fe balance data (Table 4). This held true for the low-$CaCO_3$ group, but in the high-$CaCO_3$ group the apparent absorption for day 8 was not lower than that for days 5–7. It seems that in the high-$CaCO_3$ group Fe absorption was maintained at a higher level for a longer period of time.

Dietary Ca:Fe and the levels of Ca and Fe may determine whether or not Ca inhibits Fe absorption (Barton *et al.* 1983; Hallberg *et al.* 1992). With Ca:Fe ratios up to 100:1, Barton *et al.* (1983) did not find inhibition of Fe absorption when 1 μM-$FeCl_2$ was injected into gastrointestinal loops of rats. A dose-related effect of Ca emerged when 1 mM-$FeCl_2$ and Ca:Fe ratios between 10:1 and 100:1 were used. In the present study the Fe concentrations in the liquid phase of the digesta were in the range of 50–100 μM, which appeared appropriate to elicit the inhibitory effect of Ca on Fe absorption. Different dietary Ca:Fe ratios and different levels of these elements used in various studies (Barton *et al.* 1983; Hallberg *et al.* 1992), including this one, may have caused the different degrees of Ca-induced inhibition of Fe absorption that were seen.

The proximal part of the small intestine is the major site of Fe absorption in rats (Barton *et al.* 1983) and man (Wheby, 1970). The concentration of Fe in the liquid phase of the digesta in the proximal, but not in the distal, half of the small intestine was significantly lowered by high $CaCO_3$ intake. In the light of the concept that soluble rather than insoluble non-haem-Fe in the intestinal lumen is available for absorption, the $CaCO_3$-induced decrease in soluble Fe concentration in the proximal intestine explains the decrease in apparent absorption. The question then arises of why ingested $CaCO_3$ reduces the amount of soluble Fe. It is possible that $CaCO_3$ raised the mean pH in the proximal intestine as it did in the distal intestine. The rise in pH may be caused by the extra Ca ions in the digesta forming $Ca(OH)_2$. An increase in pH of digesta may lead to reduced Fe solubility. In the proximal intestine the solid-phase Fe:liquid-phase Fe ratio was 7·82 (SE 1·47, n 5) in rats fed on the high-$CaCO_3$ diet *v.* 4·38 (SE 0·67, n 10) in rats fed on the low-$CaCO_3$ diet, the difference being statistically significant ($P = 0·03$). Thus, $CaCO_3$ feeding and the resultant increase in pH of the digesta may shift the equilibrium of soluble and insoluble Fe towards the formation of insoluble Fe. $CaCO_3$-loading reduced the total amount of Fe in the small intestine. The amount of Fe consumed after the 3 h feeding period was similar for the low-

$CaCO_3$ and high-$CaCO_3$ groups. Possibly the extra $CaCO_3$ had decreased the transit time of the digesta, resulting in a lower Fe content.

The Fe absorption process can be divided into mucosal uptake, i.e. transport across the apical membrane of mucosal cells, and mucosal transfer, i.e. transport through the mucosal cytoplasm and across the basolateral membrane into the plasma (Bothwell *et al.* 1979; Marx, 1979). With the use of orally administered ^{59}Fe, and Cr as non-absorbable marker, we determined mucosal uptake and transfer of Fe. $CaCO_3$-loading was found not to influence mucosal Fe uptake. This observation was unexpected because the $CaCO_3$-induced decrease in Fe solubility in the intestinal lumen would be expected to cause a decrease in mucosal Fe uptake. However, apart from intestinal Fe solubility, mucosal uptake may be regulated also by Fe status. Low hepatic Fe concentrations, which point to a diminished Fe status, are associated with a high efficiency of Fe absorption (Sijtsma *et al.* 1993). Perhaps the effects on mucosal Fe uptake of the reduction in intestinal Fe solubility and lower Fe status in the high-$CaCO_3$ group had cancelled each other out. In the rats fed on the low-$CaCO_3$ diet the impact of the higher Fe solubility may have overruled that of their better Fe status. In this context the lower amount of soluble Fe in the rats given the high-$CaCO_3$ diet did contribute to the lower degree of Fe absorption in these animals.

Mucosal Fe transfer was significantly lowered by the high dietary $CaCO_3$ concentration. Thus, it seems that the inhibition of apparent Fe absorption seen after $CaCO_3$ feeding reflected a decrease in Fe transport within the mucosal cells and/or across the basolateral membrane. An explanation for this intramucosal inhibition cannot yet be given. Hallberg *et al.* (1993) found that the inhibitory effect of high Ca intake was equal for haem- and non-haem-Fe absorption in man. Since the mucosal uptake mechanism differs for haem- and non-haem-Fe, it would imply that dietary Ca interferes with the transport of Fe through the mucosal cell. Studies on the effect of added phytate or ascorbic acid on eventual intralumen Ca–Fe interactions gave additional proof that this interaction was not located in the gastrointestinal tract (Hallberg *et al.* 1992). This possibility is supported by the present findings.

The correlations for individual rats within dietary groups between Hb repletion and apparent Fe retention, ^{59}Fe retention, soluble intestinal Fe, liver Fe or spleen Fe, would suggest that Hb repletion is not strongly influenced by the selected indicators of Fe bioavailability. The correlation coefficients were not higher than 0·49 and failed to reach statistical significance. However, the true correlation coefficients may be higher than the observed ones. Intra-individual differences in the measured values will only degrade the correlation coefficients. The direct relationship between soluble intestinal Fe and Hb repletion will be influenced by the efficiency of Fe absorption, which is inversely correlated with Fe status (Sijtsma *et al.* 1993). The amount of Fe absorbed probably depends on the combination of Fe availability for absorption and the activity of the mucosal Fe absorption mechanism. Thus, although differences in intestinal Fe solubility may determine differences in Fe bioavailability, Fe solubility is expected not to be closely associated with the degree of Hb repletion in the rat bioassay. Furthermore, Hb repletion may be determined partly at the post-absorptive stage, e.g. mucosal transfer, which will further diminish any correlation between Fe solubility or mucosal Fe uptake and Hb repletion. These considerations should be taken into account in the development of *in vitro* alternatives to the Hb-repletion bioassay. Current *in vitro* methods basically provide a certain measure of Fe solubility (Miller *et al.* 1981).

In summary, it can be concluded that high $CaCO_3$ intake depresses Fe bioavailability in rats at least at two stages: a decrease in Fe solubility in the intestinal digesta, accompanied by an inhibition of the mucosal transfer of Fe through the mucosal cytoplasm and/or at the basolateral membrane.

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