

## Studies on calcium absorption from milk using a double-label stable isotope technique

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Calcium absorption was measured in ten male volunteers from skimmed milk, Ca-enriched skimmed milk or watercress (*Nasturtium officinale*) soup. The foods were labelled extrinsically with 30 mg <sup>44</sup>Ca. Shortly after consuming the labelled meal, each subject was given an intravenous injection of 3 mg <sup>42</sup>Ca. Fractional absorption from the oral dose was determined from plasma and urine samples collected 24–72 h later, using fast atom bombardment mass spectrometry to measure isotope ratios. The values for urine and plasma were in good agreement. Mean percentage absorption was 45.5 (SEM 1.9) % from the skimmed milk, 35.7 (SEM 4.7) % from the Ca-enriched milk and 27.4 (SEM 1.9) % from the watercress soup. The effect of consuming 568 ml (1 pint) Ca-enriched milk each day for 4 weeks on the efficiency of absorption of Ca was studied. Although there was no statistically significant difference between Ca absorption before and after the supplementation period, the results were considered to be somewhat inconclusive due to the small number of subjects and wide individual variation in Ca absorption.

### Calcium absorption: Milk calcium: Double-label stable isotope technique

Calcium in milk is generally considered to have a higher bioavailability than that from cereals and vegetables, but there is little evidence to substantiate this. The present study was designed to compare Ca absorption from skimmed milk and a Ca-enriched skimmed milk (Vital®; Express Foods Group) with watercress (*Nasturtium officinale*) soup, using the stable isotope <sup>44</sup>Ca to label the endogenous Ca in the food, and at the same time administering <sup>42</sup>Ca intravenously in order to measure true Ca absorption from the oral dose (DeGrazia *et al.* 1965).

Although the amount of Ca available for absorption undoubtedly differs between individual foods and diets, the situation is further complicated by the fact that intestinal adaptation may take place in response to the level of Ca intake in terms of a changed efficiency of absorption (Malm, 1953; Birge *et al.* 1969). The absorption study on milk was repeated after each subject had consumed 568 ml (1 pint) Vital (skimmed milk enriched with calcium gluconate, providing approximately 1000 mg Ca extra/d; Express Foods Group, South Ruislip, Middx) each day over a 4-week period, the objective being to determine whether or not the increased Ca intake had an effect on the efficiency of Ca absorption.

### MATERIALS AND METHODS

Ten adult male volunteers (mean age 35.1 (SEM 2.9) years, clothed weight 70.0 (SEM 2.0) kg; height 1.773 (SEM 0.021) m) were recruited from the staff at the Institute of Food Research, Norwich Laboratory, and their habitual Ca intake estimated by means of a locally developed questionnaire, similar to that used by Nelson *et al.* (1988).

Table 1. *Composition of watercress (Nasturtium officinale) soup (0.524 g calcium/kg)*

Ingredients	Amount (g/portion)	Ca content (% of total Ca)
Watercress	66	76
Parsnips	20	6
Carrots	20	8
Onions	20	5
Potatoes	66	4
Vegetable stock	58	1

The subjects were divided into two equal groups with similar mean Ca intakes and, following an overnight fast, each subject was given 250 g watercress soup (containing 131 mg Ca, Table 1), to which had been added 30 mg  $^{44}\text{Ca}$ . The subjects refrained from eating for a further 4 h during which time (2–3 h after the meal, that is when the plasma  $^{44}\text{Ca}$  was at its highest level) they were given a 3 mg intravenous (i.v.) injection of  $^{42}\text{Ca}$  into the antecubital vein. The following morning a 10 ml blood sample and a saliva sample were taken for analysis. Urine collections (24 h) were carried out for 6 d commencing immediately after the isotopically labelled meal.

On a separate occasion the previously described procedure was repeated but instead of the labelled meal five of the subjects were given 136 ml skimmed milk (containing 150 mg Ca), and the other five 83 ml Vital, a commercial skimmed milk enriched with calcium gluconate (containing 154 mg Ca); an additional 30 mg  $^{44}\text{Ca}$  were added to each portion of milk. The soup and milk were given in random order to eliminate any effects of time, and the subjects did not all participate on the same day. Each subject was then given 568 ml (1 pint) Vital (1050 mg Ca) to consume daily, in addition to their normal diet, for a period of 4 weeks. They were asked to continue their usual dietary regimen, as identified from the Ca questionnaire, and to avoid any high-Ca foods that were not a regular part of their diet. At the end of the 4 weeks, the Ca absorption study from milk was repeated.

#### *Validation of use of extrinsic label for Ca*

Fresh intact watercress plants (Fobdown Farm, New Arlingford, Hants) were placed in deionized water and half were intrinsically labelled with  $^{47}\text{Ca}$  ( $\text{CaCl}_2$ ; Amersham International plc, Amersham, Bucks) by adding the radioisotope to the water and harvesting the leaf and upper stem (which contained approximately 8% of the  $^{47}\text{Ca}$  content of the plant) 3 d later. The watercress was homogenized with deionized water (20 g fresh leaves plus upper stem with 60 ml water) and 5 ml homogenate were given by gastric intubation to sixteen rats.

Extrinsically labelled portions of the watercress were prepared in the same way by adding the isotope to unlabelled watercress homogenate just before intubation. The percentage retention of the  $^{47}\text{Ca}$  was measured by whole body counting using a small animal gamma counter (NE8112, NE Technology Ltd, Beenham, Berks) 7 d post-dosing, as described previously (Fairweather-Tait & Wright, 1985). There was no significant difference between the groups given extrinsically labelled watercress (Ca retention 80.3 (SEM 1.2)%) and those given intrinsically labelled watercress (Ca retention 76.3 (SEM 2.2)%).

#### *Preparation and administration of stable isotopes*

Two enriched isotopes were obtained from Oak Ridge National Laboratory (Tennessee, USA) as calcium carbonate:  $^{42}\text{Ca}$  (94.10 atom %) and  $^{44}\text{Ca}$  (98.78 atom %).

The  $^{44}\text{Ca}$  solution was prepared by dissolving the calcium carbonate in concentrated Aristar hydrochloric acid (2.5 g  $\text{CaCO}_3$  plus 5 ml HCl), adjusting the pH to 6.0 with 1 M-sodium hydroxide and making the solution up to a final volume of 100 ml. The  $^{44}\text{Ca}$  solution for injection was prepared in a similar manner and 5-ml portions were dispensed into ampoules and sterilized. The  $^{42}\text{Ca}$  concentration of the solution for injection was 0.701 mg/ml. The exact quantity of isotope given to each subject was measured, in the case of the  $^{44}\text{Ca}$  by weighing the dispensed volume (approximately 3 ml/dose) and in the case of the i.v.  $^{42}\text{Ca}$  by weighing the loaded syringe before and after injection (approximately 5 ml/dose).

#### *Atomic absorption spectroscopy (AAS)*

The Ca concentration of the test meals, enriched solutions of  $^{44}\text{Ca}$  and  $^{42}\text{Ca}$ , plasma and saliva 24 h after the test meals, and 24 h urine collections 2 and 3 d post-dosing were measured.

(a) The milks were subjected to a wet-digestion procedure in which 5-ml portions (in triplicate) were heated at  $100^\circ$  for 20 min with 25 ml concentrated Analar nitric acid, followed by dropwise addition of hydrogen peroxide. The solution was transferred to a volumetric flask and made up to volume with distilled water. Further dilutions were made to provide a final Ca concentration of between 1 and  $6\ \mu\text{g/ml}$ .

(b) Subsamples of the watercress soup were dried and 5-g (in quadruplicate) portions were heated to  $480^\circ$  for 48 h, the ash taken up in hot concentrated Analar HCl, and the solution made up to volume with distilled water, and diluted as described previously.

Lanthanum chloride was added to all solutions before making up to final volume to provide each sample undergoing AAS with an  $\text{LaCl}_2$  concentration of 10 g/l. Certified reference materials (National Bureau of Standards, Office of Standard Reference Materials, Washington, DC, USA) were also analysed for Ca to confirm analytical accuracy.

#### *Fast atom bombardment (FAB) mass spectrometry*

Blood was collected in heparinized tubes, centrifuged, the plasma removed and trichloroacetic acid (35 g/l; TCA) added at a ratio of 3 parts:1 part plasma. The mixture was allowed to stand for 15 min at  $4^\circ$ , centrifuged and the deproteinized supernatant fraction removed.

The deproteinized plasma (1 ml portions) was evaporated to dryness under an infra-red lamp and redissolved with  $100\ \mu\text{l}$  2 M-Aristar  $\text{HNO}_3$ . The urine was deproteinized using TCA as described previously, and the saliva by adding a few drops of concentrated Aristar HCl. The urine Ca was concentrated by precipitating the Ca as oxalate (Jiang & Smith, 1987) by adding 2.5 ml saturated ammonium oxalate solution to 2.5 ml urine, mixing, centrifuging and discarding the supernatant fraction. The oxalate was redissolved in  $100\ \mu\text{l}$  1 M-HCl.

Samples and standards were dispensed in  $2\ \mu\text{l}$  portions onto the central area of the copper probe tip of the direct insertion probe and dried in a vacuum lock before being inserted into the FAB source of the MS80 RFA mass spectrometer (Kratos Analytical Instruments, Manchester) operating at a resolution of 3000. The precise masses of various Ca isotopes from  $2\ \mu\text{l}$  calcium nitrate ('SpectroSol'; BDH, Poole, Dorset) were used to calibrate the electric sector scan of the mass range 40–44 daltons. The mass spectrometer was scanned five times and the next seventy-five consecutive scans of 3 s duration were recorded and averaged. Each scan consisted of four 0.15 s settling times between sweeping the 'lock mass' at  $m/z$  39.9626 of the  $^{40}\text{Ca}$  isotope in 0.3 s and then switching sequentially to  $m/z$  43.9555,  $m/z$  41.9586 and  $m/z$  39.9626 for 0.7 s each. Each sample was analysed three times and the results averaged. Enriched samples were 'bracketed' with runs of

unenriched samples. The  $^{44}\text{Ca} : ^{40}\text{Ca}$  and  $^{42}\text{Ca} : ^{40}\text{Ca}$  ratios were determined by calculating the area under the peaks by computer.

#### *Calculation of Ca absorption and statistical treatment*

Assuming that both the orally and i.v. administered isotopes were metabolized at the same rate once equilibrium was reached, absorption from the oral dose was calculated in plasma, saliva and urine according to the following equation (Yergey *et al.* 1987):

$$\text{percentage absorption} = \frac{(\text{na } ^{44}\text{Ca}) (^{42}\text{Ca i.v.}) \times \Delta \% \text{ XS } ^{44}\text{Ca} \times 100}{(\text{na } ^{42}\text{Ca}) (^{44}\text{Ca oral}) \times \Delta \% \text{ XS } ^{42}\text{Ca}}$$

where na is the ratio of the two Ca isotopes in nature, i.v. and oral refer to the exact dose given, and  $\Delta \% \text{ XS}$  represents the degree to which a particular ratio differs from natural levels.

Paired *t* tests were used to compare percentage Ca absorption (measured in 24 h plasma) from the skimmed milk, Vital and watercress soup. The initial and final absorption values from both milks were also compared by paired *t* tests. The relation between percentage absorption calculated from 24 h plasma and from days 2 and 3 urine, and 24 h saliva were examined by regression analysis.

#### *Ethical consideration*

The study was approved by the Institute of Food Research Ethical Committee.

### RESULTS

The Ca intake of the subjects, as estimated from the questionnaire, is shown in Table 2. There was quite a wide range in intake, from 500 to 1800 mg/d, with approximately 60% of the total Ca being provided by dairy products.

The percentage absorption of Ca from the two milks and watercress soup when measured in plasma 24 h post-dosing is shown in Fig. 1. The mean absorption for all ten subjects from the soup was 27.4 (SEM 1.9)%, for the five subjects given skimmed milk 45.5 (SEM 1.9)% and for the five given Vital 35.7 (SEM 4.7)%. When absorptions from milk and watercress soup were compared in each group of five subjects, it was shown that the Ca was less well absorbed from watercress soup than from skimmed milk ( $P < 0.001$ ), but there was no statistically significant difference between watercress soup and Vital, probably due to the wide range of absorption values seen in the Vital group, nor was there any difference between percentage Ca absorption from skimmed milk and Vital.

The relation between saliva and plasma, collected 24 h post-dosing, is shown in Fig. 2. The two sets of absorption values were related ( $R=0.69$ ,  $P < 0.01$ ), but there was some discrepancy between the theoretical (1:1 ratio) and the fitted lines. Fig. 3 shows that there was a better agreement between Ca absorption estimated from 24 h plasma and urine

Table 2. Calcium intake (mg/d) of ten male subjects, estimated by questionnaire

(Mean values with their standard errors and range)

	Mean	SEM	Range
Dairy sources	723	114	168-1366
Non-dairy sources	484	111	148-1046
Total Ca	1207	137	498-1835

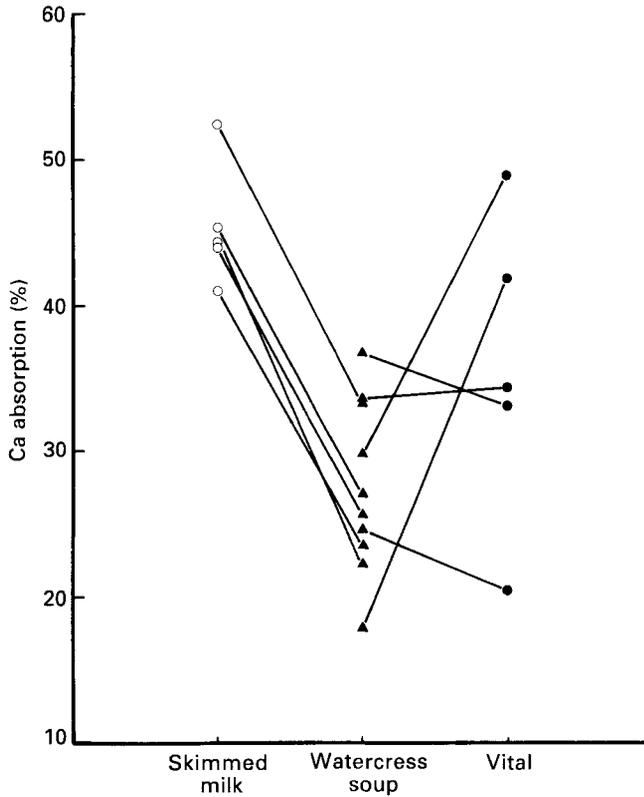


Fig. 1. Calcium absorption (%) from skimmed milk (○), Ca-fortified milk (Vital; Express Foods Group) (●), and watercress (*Nasturtium officinale*) soup (▲) in ten adult men as measured from blood plasma collected 24 h post-dosing using a double-label stable isotope technique (for details, see p. 380).

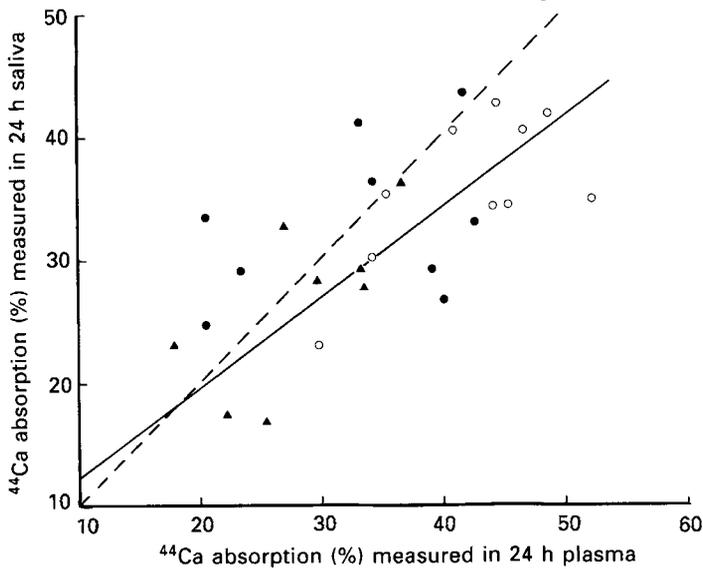


Fig. 2. Calcium absorption (%) measured from saliva and plasma collected 24 h post-dosing, from skimmed milk (○), Ca-fortified milk (Vital; Express Foods Group) (●), and watercress (*Nasturtium officinale*) soup (▲). For details of procedures, see p. 380. (---), Perfect (1:1) agreement; (—), fitted to the values using regression analysis.

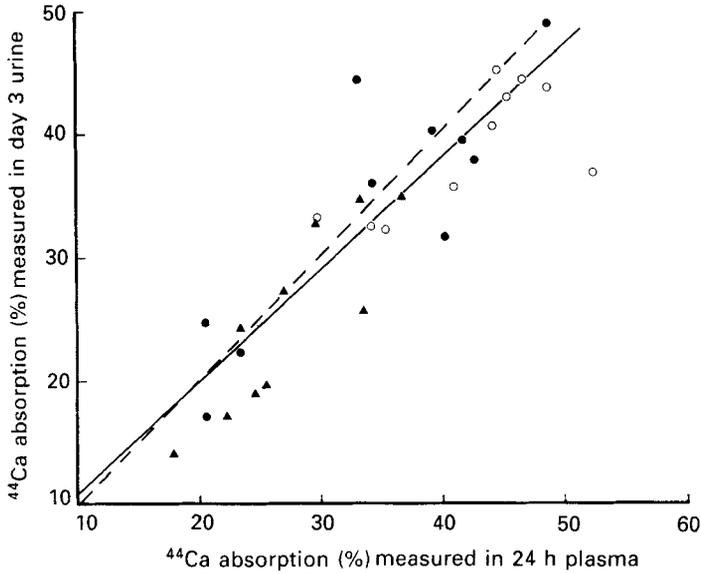


Fig. 3. Calcium absorption (%) measured from urine collected 3 d and plasma 24 h post-dosing, from skimmed milk (○), Ca-fortified milk (Vital; Express Foods Group) (●), and watercress (*Nasturtium officinale*) soup (▲). For details of procedures, see p. 380. (---) Perfect (1:1) agreement; (—), fitted to the values using regression analysis.

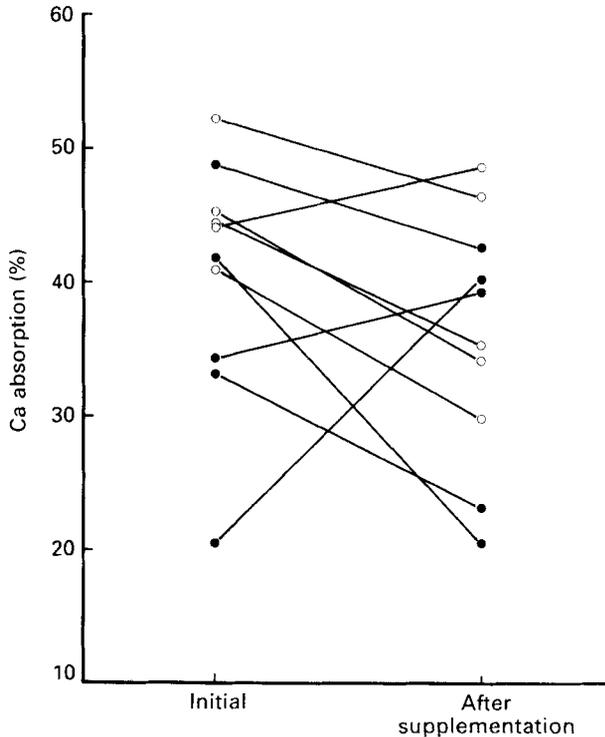


Fig. 4. Effect of milk supplementation on percentage calcium absorption in men from skimmed milk (○) and Ca-fortified milk (Vital; Express Foods Group) (●) before and after supplementation with 568 ml (1 pint) Vital daily for 4 weeks. For details of procedures, see p. 380.

collected 3 d post-dosing ( $R0.88$ ,  $P < 0.001$ ). Similar results were found with day 2 urine ( $R0.86$ ,  $P < 0.001$ ), but these values are not shown.

There appeared to be no significant effect of consuming 1 pint Vital daily for 4 weeks on the efficiency of Ca absorption. The results for the subjects are shown in Fig. 4. Mean initial absorption for the ten men was 40.6 (SEM 2.9) % and final absorption 36.1 (SEM 3.0) %. The mean response was similar for both types of milk used for the test meal.

#### DISCUSSION

A reliable estimate of Ca absorption may be obtained using combined oral and i.v. tracers, as first developed by DeGrazia *et al.* (1965) using  $^{45}\text{Ca}$  and  $^{47}\text{Ca}$ . Using radioisotopes, fractional intestinal Ca absorption can be determined from the ratio of the specific activities of oral  $^{47}\text{Ca}$ :i.v. administered Ca in a plasma or urine sample taken 24 h after simultaneous administration of the two isotopes. This technique circumvents the necessity for complete metabolic balances and is, therefore, simpler, more attractive, and not prone to the errors inevitably associated with faecal collections. Unlike the single-label techniques (which measure apparent absorption because re-excreted tracer cannot be distinguished from unabsorbed tracer) the results obtained from the double-label technique are a measure of true Ca absorption. The method has been shown to yield results that are comparable with methods such as whole body counting (Roth & Werner, 1985).

More recently, because of increasing awareness of the hazards of ionizing radiation, efforts have been made to use stable isotopes instead of radioisotopes wherever possible. Thus, Yergey *et al.* (1987) used an oral dose of  $^{44}\text{Ca}$  (0.2–0.5 mg/kg body-weight) coupled with an intravenous dose of  $^{42}\text{Ca}$  (0.02–0.1 mg/kg body-weight) to measure fractional Ca absorption in two premature infants and one adult from a commercial milk formula. They employed thermal-ionization mass spectrometry to quantify the isotopic enrichment in sequential urine samples, and found that the time required to obtain constant absorption results was longer (about 24 h) in the adult than in the infants (about 10 h). This they thought might be explained by differences in intestinal transit time. They have recently published further information from this study, using  $^{46}\text{Ca}$  instead of  $^{42}\text{Ca}$ , and discuss both the thermal ionization mass spectrometry methodology and the double-label technique (Hillman *et al.* 1988). Lehmann & Kessler (1982) used a similar procedure (one healthy volunteer given 184 mg  $^{44}\text{Ca}$  oral dose, and 27 mg  $^{42}\text{Ca}$  i.v.) in a preliminary study with field desorption mass spectrometry, and found reasonably good agreement between percentage Ca absorption calculated from plasma, urine and saliva samples taken more than 24 h after the oral dose. The results of the present study also showed agreement between plasma, urine and saliva, and if one accepts that plasma is the most appropriate body fluid in which to measure isotopic enrichment, the next best was urine collected 2–3 d post-dosing. The latter has the advantage of being less invasive and more suitable for studies where blood sampling is best avoided, as in infants, or where it is impracticable or difficult to organize.

The amounts and timing of isotope administration are an important consideration of the experimental design because, unlike radioisotopes, stable isotopes are not strictly tracers. Because they are naturally present in all tissues and foods, the dose administered must be large enough to cause measurable enrichment in the samples to be analysed. Regarding the oral dose, until more experimental information is available, it must be assumed that the added isotope behaves in a similar fashion to the Ca contained in the test food or Ca-containing substance. The rat study described in the present paper demonstrated the validity of using an extrinsic label to measure endogenous Ca in watercress. It was not possible to validate the use of an extrinsic label for milk. The preparation of intrinsically labelled cows' milk is extremely expensive and for this reason, coupled with time

constraints, it was not possible to carry out a study along the lines of that described for watercress. However, the validity of using an extrinsic label to measure Ca absorption from milk is currently under investigation by Weaver *et al.* (1988) who have previously shown that extrinsic labels can be used for low-oxalate (e.g. milk) but not high-oxalate food (Weaver *et al.* 1987). Preliminary data indicate that extrinsic labelling techniques are valid (Martin *et al.* 1989). In a similar study, in which spinach and milk were compared, Heaney *et al.* (1988) labelled the spinach intrinsically and the milk extrinsically, but did not discuss the labelling aspect of the methodology. Other studies in the literature report the use of radio-Ca as an extrinsic label for milk (Kocián *et al.* 1973; Recker *et al.* 1988). Regarding the intravenous dose, several studies have used amounts of isotope in the region of 30 mg, which has been calculated to be equivalent to 2% of the rapidly miscible component of the Ca pool (Heaney & Skillman, 1971). Thus the dose used in the present study (approximately 3 mg  $^{42}\text{Ca}$ ), an order of magnitude less than many other studies, is unlikely to perturb steady state conditions. The appropriate time of administration of the i.v. dose cannot be identified with any certainty. Ideally the i.v. administered isotope should be present in the blood at the same time as the orally administered isotope but this is not possible because the latter is absorbed from the intestine over an extended time period. Since orally administered Ca isotope appears to reach a maximum in the blood 2–3 h after ingestion (Kocián *et al.* 1973) we chose, like Lehmann & Kessler (1982), to give the i.v. dose 2–3 h after the test meal in the hope that most of the two isotopes would be metabolized together.

The use of FAB mass spectrometry to measure inorganic isotopes is a very recent development. Smith (1983) was the first to use this method for Ca isotopes, and in a methodology paper has subsequently reported its use for measuring fractional true absorption of Ca by the double isotope technique (Smith *et al.* 1985). In the absence of a dedicated inorganic mass spectrometer, FAB offers an alternative method of measuring inorganic stable isotopes, but is less sensitive and generally less accurate than thermal ionization. It does, however, have the advantage of requiring less rigorous sample preparation (Pratt *et al.* 1987).

Differences in bioavailability of Ca from a variety of foods were reported more than 60 years ago in metabolic balance studies on children (Sherman & Hawley, 1922), when milk was shown to be superior to vegetables with respect to Ca retention. There is very little information in the literature to support the widely held belief that the Ca in milk is particularly well absorbed by adults. Using  $^{47}\text{Ca}$  as an extrinsic label in milk, Kocián *et al.* (1973) found apparent absorption (measured by whole body counting 7 d post-dosing) to be about 40% in five healthy males, which agrees quite well with our value of 45% for skimmed milk. Recker *et al.* (1988), using  $^{45}\text{Ca}$  as an extrinsic label, found that Ca absorption in post-menopausal women from milk products (whole milk, chocolate milk, yoghurt, imitation milk, cheese) was similar to calcium carbonate, with means ranging from 22 to 27% from a 25 mg dose. The present experiment re-affirms the results of the original balance studies on dairy and plant foods in that the Ca in skimmed milk was better absorbed than that in watercress soup. The reason for this is unclear but may be attributable to substances present in the watercress soup that bind Ca thereby rendering it less absorbable, e.g. oxalate (Pingle & Ramasastri, 1978) and fibre (Kelsay *et al.* 1979), plus the fact that certain components of milk have been implicated as enhancers of Ca absorption, e.g. lactose (Charley & Saltman, 1963) and casein (Lee *et al.* 1979).

With regard to the milk enriched with calcium gluconate, it has been shown that different Ca salts are absorbed with differing degrees of efficiency. For example, Nicar & Pak (1985) found that calcium citrate had a higher bioavailability than calcium carbonate when given at doses of 1000 mg, and Spencer *et al.* (1966) showed that lactate was superior to gluconate. Using an oral Ca challenge of Ca salts in milk, Goddard *et al.* (1986) found that

the peak effects of calcium gluconate were reached before carbonate and lactate, indicating a difference in metabolic response. The present experiment did not show a significant difference between calcium gluconate-enriched milk and skimmed milk. There was a wide variation in percentage Ca absorption between the individuals which, coupled with the small numbers in the groups, makes it impossible to reach a firm conclusion regarding the bioavailability of calcium in Vital. In particular one subject absorbed a particularly small fraction of Ca from Vital (approximately 20%) on the first occasion, but absorbed double this amount on the second occasion.

Finally, the medium-term effects of consuming a higher Ca diet on absorption efficiency were addressed. Adaptation to differing Ca intakes has been shown to occur in man, whereby fractional Ca absorption is increased by a low-Ca diet and reduced by a high-Ca diet (Malm, 1958; Heaney *et al.* 1975), including milk supplementation, over 2 years (Recker & Heaney, 1985). The adaptation is thought to occur primarily at the site of the ileum (Norman *et al.* 1981). The male subjects in the present study all appeared to have adequate intakes of Ca, and when given 568 ml (1 pint) Vital daily, their intakes approximately doubled. Over the 4-week period, this extra Ca appeared to have little or no effect on the efficiency of absorption of Ca. Seven subjects had a reduced absorption, but in three subjects fractional absorption rose. It is possible that the time period was too short for any adaptive response to have manifested itself, and it is not possible to predict what the effects would be over a longer time period.

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