The effect of high intakes of casein and casein phosphopeptide on calcium absorption in the rat

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The effect of the level or source of dietary protein or protein-derived peptides on Ca absorption is not well understood. We determined, therefore, the influence of habitual dietary casein level, meal casein and meal casein phosphopeptide (CPP) on Ca absorption in the rat. True fractional Ca absorption was investigated in male 7-week-old rats, Wistar strain, in three separate studies using a faecal ^{47}Sc : ^{47}Ca ratio method. In studies A and C, rats (*n* 8 per group) were fed on a purified diet containing 200 g casein/kg for 2 weeks. Rats were then given a ^{47}Ca-labelled meal (10 g) containing (per kg) either 0, 100, 200, or 300 g casein (study A) or 0, 100, 200, 350 or 500 g CPP (study C). In study B, rats (*n* 24 per group) were fed on a purified diet containing (per kg) either 200, 350 or 500 g casein for 2 weeks. Each group was then further randomized into three groups (*n* 8 per group) and given a ^{47}Ca-labelled meal (10 g of the same diet) containing (per kg) either 200, 350 or 500 g casein. Ca absorption from a meal was unaffected by increasing meal casein concentration from 0 to 300 g/kg (study A), but was increased with a meal casein content of 500 g/kg (study B). Fractional Ca absorption decreased with increasing usual dietary casein intake in the range 200–500 g/kg (study B), suggesting intestinal adaptation. Ca absorption was unaffected by inclusion of 100 g CPP/kg in a single meal but was significantly ($P < 0.001$) reduced by 200, 350 and 500 g CPP/kg meal, with no evident dose-relationship. Thus, while Ca absorption was enhanced by high-casein meals, the mechanism remains unclear.

Calcium absorption: Casein: Casein phosphopeptide

The effect of the level or source of dietary protein or protein-derived peptides on Ca absorption is not well understood. Ca balance studies in human subjects have shown that the amount of dietary protein has no effect on apparent Ca absorption (Walker & Linkswiler, 1972; Allen *et al.* 1979; Hegsted *et al.* 1981; Spencer *et al.* 1983; Zemel, 1988). Similarly, studies in rats have shown that feeding high levels of protein in the diet ranging from 180 to 360 g/kg has no effect on apparent Ca absorption (Allen & Hall, 1978; Howe & Beecher, 1981). However, it is possible that adaptation of the active Ca transport absorptive process to high-casein diets could explain the lack of effect of casein on apparent Ca absorption in these metabolic balance studies. Furthermore, the design of these studies does not allow for investigation of the effect of protein on true Ca absorption, as endogenous losses of Ca are not accounted for.

There have been few studies on the effect of individual proteins on true Ca absorption from single meals. Brommage *et al.* (1991) reported that true fractional Ca absorption from a single meal in rats was increased by 26 % when the casein content of the meal was increased from 90 to 270 g/kg, representing a range of about 45–135 % of the requirement. However, Carrié *et al.* (1994) showed that Ca absorption from a liquid meal in healthy male subjects was unaffected by inclusion of 20 g of casein, egg albumin or whey protein in the meal, which is equivalent to about 50 % of the recommendation.

The early work of Mellander and colleagues demonstrated that, in the absence of vitamin D, casein phosphopeptides (CPP) derived from the intestinal digestion of casein enhanced bone calcification in rachitic children (Mellander, 1950) and chicks (Mellander & Olsson, 1956). CPP have also been shown to have beneficial effects on bone in rats (Lee *et al.* 1992; Tsuchita *et al.* 1993, 1996). Such CPP have the capacity to chelate Ca and to prevent the precipitation of Ca phosphate salts (Berrocal *et al.* 1989), thereby increasing the amount of soluble intraluminal Ca available for absorption across the mucosa (Kitts & Yuan, 1992). Although the

influence of CPP on Ca absorption in the rat and chick intestine has been demonstrated using *in situ* and *in vitro* models (rat intestine: Lee *et al.* 1979, 1980; Sato *et al.* 1986; Nagasawa *et al.* 1991; Yuan & Kitts, 1991; Kitts *et al.* 1992; chick intestine: Mykkanen & Wasserman, 1980), studies in intact animals have produced conflicting results. Some studies have failed to find a significant enhancement in Ca absorption with the addition of CPP to the diets (in pigs: Pointillart & Guégen, 1989; in vitamin D replete minipigs: Scholz-Ahrens *et al.* 1990; in vitamin D replete and deficient rats: Shah *et al.* 1990; Brommage *et al.* 1991; Yuan & Kitts, 1991; Kopra *et al.* 1992). However, only one of these (Brommage *et al.* 1991) has investigated the effect of CPP on Ca absorption from a meal. Others have reported that Ca absorption in rats was enhanced by the addition of CPP to the diets (Lee *et al.* 1992; Tsuchita *et al.* 1993; Hansen *et al.* 1996; Saito *et al.* 1998) or to aqueous phytate-containing Ca solutions (Hansen *et al.* 1996).

Controversy also exists in terms of the effect of CPP on Ca absorption in humans. Two studies in healthy adult subjects reported no enhancement by CPP of fractional Ca absorption from single meals (Hansen *et al.* 1997*a*, *b*). However, it should be noted that in the study by Hansen *et al.* (1997*b*), CPP addition to a rice-based infant cereal meal significantly increased the total quantity of Ca absorbed (i.e. mg Ca). Furthermore, Heaney *et al.* (1994) reported that CPP administration was associated with better absorption of co-ingested Ca by postmenopausal women with low basal absorptive performance. Therefore, the effect of CPP on Ca absorption from a single meal remains unclear.

The objective of the present studies was to determine the influence of habitual dietary casein level, meal casein and meal CPP on Ca absorption in the rat.

Materials and methods

Preparation of casein phosphopeptide

Sodium caseinate (862 g protein/kg) was obtained from Dairygold Ltd, Mitchelstown, Co. Cork, Ireland. Ca determination kits and Chelex-100 were obtained from Sigma Chemical Co. Ltd, Poole, Dorset, UK. A sodium caseinate hydrolysate was prepared using bioprotease N100L (*Bacillus subtilis*, Batch no. S9806499, a gift from Quest International, Carragaline, Cork, Ireland) as described by McDonagh & FitzGerald (1998). CPP were enriched from hydrolysate using the Ca aggregation and ultrafiltration protocols as outlined by Brule *et al.* (1982). Ca determination and CPP decalcification was performed as described by McDonagh & FitzGerald (1998). The organic P content of the CPP, as measured according to International Dairy Federation (1990) provisional standard 42B, was 34·9 g/kg powder. The Na content of the CPP, measured using a flame photometric method after wet acid digestion, was 22 g/kg powder. The protein content (N equivalent) of the CPP, measured using the Kjeldahl method (International Dairy Federation, 1993), was 892 g/kg powder and the N : P value was 4·0 (for more information about the purity and composition of the CPP used in the present study see McDonagh & FitzGerald, 1998).

Table 1. Composition of the modified AIN-76 diet (American Institute of Nutrition, 1977)

Ingredient[*]	Content (g/kg)
Casein	200·0
DL-Methionine	3·0
Maize starch	150·0
Sucrose	487·5
Fibre	50·0
Maize oil	50·0
AIN mineral mix†	35·0
AIN vitamin mix‡	10·0
Calcium carbonate	12·5
Choline bitartrate	2·0

[*] Sources of ingredients: casein (sodium caseinate), Kerrymore Milk Products Ltd, Listowel, Co. Kerry, Ireland; DL-methionine, Rhone Poulenc, Animal Nutrition, Commentry, France; maize starch, Cagill Bergen op Zoom, The Netherlands; sucrose, Irish Sugar plc, Sugar Division, Athy Road, Carlow, Ireland; fibre, Avicel microcrystalline cellulose, N.F., FMC International, Food and Pharmaceutical Products Division, Little Island, Co. Cork, Ireland; maize oil, St. Bernard's brand, Dunnes Stores Ltd, 67 Stephen's Green, Upper Dublin 8, Ireland; choline bitartrate, Brown and Gilmore, Carrigaline East, Co. Cork, Ireland.

† Contained (g/kg): potassium dihydrogen phosphate 376, dipotassium hydrogen phosphate 160, sodium chloride 74, magnesium oxide 24, manganous carbonate 3·5, ferric citrate 6, zinc carbonate 1·6, cupric carbonate 0·3, potassium iodate 0·01, sodium selenite 0·01, chromium potassium sulphate 0·55, sucrose 354.

‡ Contained (per kg): nicotinic acid 3 g, calcium pantothenate 1·6 g, riboflavin 600 mg, thiamin-HCl 600 mg, pyridoxine-HCl 700 mg, folic acid 200 μg, biotin 20 μg, cyanocobalamin 1 mg, cholecalciferol 2·5 mg, menaquinone 5·0 mg, retinyl palmitate 120 mg, DL-α tocopheryl acetate 5000 mg.

Preparation of rat diets

A modified AIN-76 purified diet (American Institute of Nutrition, 1977) was used in the present study. The mineral mix was modified by replacing $CaHPO_4$ with $CaCO_3$ as the sole source of Ca and by including KH_2PO_4 and K_2HPO_4 to supply the P requirement (Table 1). $CaCO_3$ was used in the diets, as the Ca source included in the test meals was $CaCO_3$ uniformly labelled with ^{47}Ca (see later).

Preparation of ^{47}calcium-labelled meals

Labelled $CaCO_3$ was prepared by mixing ^{47}Ca (as $^{47}CaCl_2$ in NaCl (9 g/l), specific activity 7·9 GBq/g; Forskningscenter Riso, 4000 Roskilde, Denmark) with 2 M-$CaCl_2$, addition of a slight molar excess of Na_2CO_3 to precipitate $CaCO_3$, and washing the precipitate on a filter, followed by drying at 100°. ^{47}Ca-labelled meals containing 5 g Ca/kg were prepared by substituting ^{47}Ca-labelled $CaCO_3$ for $CaCO_3$ and adding 0·1 g Sc/kg as $ScCl_3$ (Aldrich Chemical Co., Milwaukee, WI, USA) (as a carrier for ^{47}Sc) in the AIN-76 diet outlined in Table 1.

^{47}Calcium absorption studies

Three studies were carried out using male rats, 7-weeks-old, Wistar strain, which were obtained from the Biological Services Unit, University College, Cork, Ireland. In study A, thirty-two rats (average weight 201 g), were fed *ad libitum* on a purified diet (AIN-76) containing (g/kg): Ca as $CaCO_3$ 5·0, P 4·0, casein as sodium caseinate 200 (Kerrymore Milk Products Ltd., Listowel, Co. Kerry, Ireland) and given distilled water *ad libitum* for 14 d. Feed

was provided at 17.00 hours each day. On the fifteenth day rats were randomized by weight into four groups of eight rats each and placed in individual cages with a grid-floor and a facility for separate collection of faeces and urine. After fasting for 10 h (09.00–19.00 hours), animals were given overnight (19.00–09.00) a radiolabelled meal (10 g AIN-76 diet), containing (per kg), either 0, 100, 200, or 300 g casein as the sole protein source (in replacement of sucrose), 5·0 g Ca as ^{47}Ca-labelled CaCO$_3$ (0·037 MBq ^{47}Ca/10 g meal) and 0·2 g of the food-dye Fast Green FCF (Sigma Chemical Co. Ltd). The dye permitted easy identification of the radioactive faeces. On the following morning the radio-labelled meal was removed and any remaining feed was weighed, and after 4 h, the rats were replaced on the AIN-76 diet to which they had been adapted.

In study B, seventy-two rats (average weight 209 g) were randomized by weight into three groups of twenty-four rats each and fed *ad libitum* on a purified diet (AIN-76) containing (g/kg): Ca as CaCO$_3$ 5·0, P 4·0 and no additional or 150 or 300 of additional casein (in replacement of sucrose) (representing a total casein content of 200, 350 and 500 g/kg diet respectively), for 14 d. On the fifteenth day each group was then further randomized into three groups of eight rats each (average weight 230 g). As described for study A, the animals were placed in individual cages, fasted and given overnight a radiolabelled meal (10 g), but in this case containing (per kg), either no additional casein or 150 or 300 g additional casein (in replacement of sucrose) (representing a total casein content of 200, 350 and 500 g/kg meal respectively) and 0·2 g Fast Green FCF (Sigma Chemical Co Ltd). As in study A, on the following morning consumption of the radiolabelled meal was monitored and the animals replaced on the AIN-76 diet to which they had been adapted.

Study C was similar in design to study A, except that in this study forty-eight rats (average weight 193 g) were randomized by weight into six groups of eight rats each and fed overnight on a radiolabelled meal (10 g) consisting of either a modified casein-free AIN-76 diet, containing CPP at a level of 0, 100, 200, 350 or 500 g/kg diet as the sole protein source (in replacement of sucrose), or a control AIN-76 diet containing 200 g casein/kg, and 0·2 g Fast Green FCF (Sigma Chemical Co. Ltd) per kg diet. Based on the theoretical yield of CPP from casein of approximately 100 g/kg (Kopra *et al.* 1992) it can be estimated that the diets containing 100, 200, 350 or 500 g CPP/kg respectively, represented about two-, four-, seven- and ten-fold the CPP content of the diet containing 500 g casein/kg in study B. Furthermore, in the study of Tsuchita *et al.* (1996) a positive effect on bone (i.e. prevention of bone loss in aged ovari-ectomized rats) was observed with CPP added at a level of 125 g/kg diet. As in study A, on the following morning consumption of the radiolabelled meal was monitored and the animals replaced on the AIN-76 diet to which they had been adapted.

In all three studies Ca absorption was determined by modification of the ^{47}Sc : ^{47}Ca ratio method of Brommage & Binacua (1991) and McCredie *et al.* (1984). This method is based on the fact that ^{47}Ca decays to ^{47}Sc which is not absorbed by the adult rat intestine and thus acts as an inert marker for estimation of the intestinal absorption of ^{47}Ca. In this ^{47}Sc : ^{47}Ca ratio method, quantitative collections of green-coloured faeces were carried out daily for 3 d imme-diately after administration of the radiolabelled meals. ^{47}Ca and ^{47}Sc in the daily faecal samples and in radiolabelled meal samples were determined in a well γ-counter (Compugamma, LKB Wallac, LKB Instruments Ltd, South Croyden, Surrey) using energy ranges of 144–178 keV and 1144–1295 keV for ^{47}Sc and ^{47}Ca respectively. Using these counting windows neither isotope contributes to the counts for the other and complete discrimination between the isotopes is achieved.

Fractional absorption of ^{47}Ca was calculated using the following equation:

Fractional absorption (%) =

$$\frac{(^{47}Sc : ^{47}Ca)_{faeces} - (^{47}Sc : ^{47}Ca)_{meal}}{(^{47}Sc : ^{47}Ca)_{faeces} - (^{47}Sc : ^{47}Ca)_{meal} + (^{47}Sc : ^{47}Ca)_{meal} \times e_{t=0}^a},$$

where $e^a = e^{(\lambda\, Ca - \lambda\, Sc) \times t}$ (t is the time elapsed after initial administration of isotope (t=0), $\lambda = \log\, 2/t_{0.5}$, $t_{0.5}$ is the radioactive half-life of the element ($t_{0.5}$ for ^{47}Sc and ^{47}Ca are 3·43 and 4·53 d respectively).

In order to obtain an estimate of urinary losses of ^{47}Ca, complete urine collections were made for each group of animals (in study B) for each of the first 3 d after feeding the radiolabelled meal.

Endogenous loss of ^{47}Ca in faeces was calculated from the slope of the plot of log ^{47}Ca retention *v.* time, from day 3 to day 7 after feeding the radiolabelled meal, estimated from the loss of ^{47}Ca in quantitative faecal collections.

At the end of the ^{47}Ca absorption study (study C), all animals were killed by over-exposure to diethyl ether and femora (from the right side of each animal) were harvested, cleaned of adhering soft tissue and ^{47}Ca was determined.

Statistical methods

Data are presented as means with their standard errors. ^{47}Ca absorption data was subjected to one-way ANOVA (in studies A and C) with variation attributed to meal casein content and meal CPP content respectively, and to two-way ANOVA (in study B) with variation attributed to dietary casein content and meal casein content (Snedecor & Cochran, 1967). Urinary ^{47}Ca data (from study B) was subjected to two-way ANOVA with variation attributed to dietary casein content and meal casein content. Femur ^{47}Ca deposition data (from study C) was subjected to one-way ANOVA with variation attributed to meal CPP content. To follow up the ANOVA, all pairs of means were compared by the method of least significant difference (Snedecor & Cochran, 1967).

Results

The effect of meal casein content on fractional absorption of meal Ca is shown in Tables 2 and 3. In study A, increasing meal casein content from 0 to 300 g/kg had no effect on Ca absorption in 9-week-old male rats. Two-way ANOVA of the data (from study B) showed that fractional absorption of meal Ca was significantly affected by previous dietary casein intake and meal casein content (in the range 200–500 g/kg meal), and that the influence of one of these variables was dependent on the other, as evident by the significant two-way interaction between dietary casein and

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Table 2. Effect of meal casein content on fractional absorption of [47]calcium from a meal in 9-week-old rats (study A)*

(Mean values with their standard errors)

| Meal casein (g/kg) | *n* | [47]Ca absorption (%) | |
		Mean	SE
0 (protein-free)	8	44·1	2·5
100	8	42·3	2·4
200	8	43·5	3·4
300	8	47·8	1·7
ANOVA (one-way); *P* value		0·47	

* For details of diets and procedures, see Table 1 and pp. 674–675.

meal casein on Ca absorption (Table 3). More specifically, fractional absorption of meal Ca was significantly increased at the 500 g/kg level of meal casein content compared with the meals containing 200 or 350 g casein/kg meal. This

increase was more marked in animals previously adapted to diets containing 200 g casein/kg diet. Fractional absorption of meal Ca decreased with previous dietary casein intake and this decrease was greater for high-casein meals.

The effect of dietary casein intake and meal casein content on cumulative urinary [47]Ca loss in male rats is shown in Table 3. Over the first 3 d, 1·5–3·2 % of the absorbed [47]Ca dose (only about 1 % of the administered dose) appeared in urine. Increasing previous dietary casein content significantly increased urinary [47]Ca loss while increasing meal casein content had no effect.

The effect of CPP content in a meal on fractional absorption of meal Ca and on [47]Ca deposition in bone (femur) in 9-week-old rats is shown in Table 4. Increasing the concentration of CPP in the meal from 0 to 100 g/kg had no effect on Ca absorption but CPP at 200, 350 and 500 g/kg significantly reduced fractional Ca absorption relative to the protein-free meal in a non-dose related manner.

Table 3. Effect of dietary casein intake and meal casein content on fractional [47]calcium absorption and urinary [47]calcium loss in 9-week-old rats (study B)*

(Mean values with the pooled standard error of the mean)

| Dietary casein (g/kg) | Meal casein (g/kg) | *n* | [47]Ca | |
			Absorption (%)	Urinary loss (%)†
200	200	8	39·0	1·9
	350	8	44·4	1·5
	500	8	59·6	2·0
350	200	8	36·6	2·0
	350	8	35·8	2·4
	500	8	41·8	3·0
500	200	8	29·5	2·6
	350	8	29·8	3·2
	500	8	39·5	2·7
Pooled SEM			2·3	0·1
Significance (*P*) of variance ratio, effect of:				
Dietary casein			<0·001	0·003
Meal casein			<0·001	0·37
Dietary casein × meal casein			0·03	0·33
Least significant difference (*P* < 0·05)			6·6	0·9

* For details of diets and procedures, see Table 1 and pp. 674–675.
† Representing cumulative urinary [47]Ca loss over first 3 d after administration of isotope, expressed as a percentage of absorbed [47]Ca.

Table 4. Effect of casein phosphopeptide (CPP) content on fractional absorption of [47]calcium from a meal and [47]calcium deposition in femur in 9-week-old male rats (study C)*

(Mean values with their standard errors)

| Meal | CPP content (g/kg) | *n* | [47]Ca absorption (%) | | Bone [47]Ca | | | |
| | | | | | Administered dose (%) | | Absorbed dose (%) | |
			Mean	SE	Mean	SE	Mean	SE
AIN-76†	0	8	35·0	4·5	1·6	0·1	3·7	0·3
CPP - 0 (control)	0	8	37·3	2·0	1·5	0·1	3·6	0·2
CPP - 100	100	8	32·5	2·6	1·3	0·1	3·7	0·1
CPP - 200	200	8	24·8‡§	1·4	1·1‡§	0·1	3·8	0·2
CPP - 350	350	8	18·7‡§	2·3	0·9‡§	0·1	4·4	0·5
CPP - 500	500	8	27·7§	2·4	1·0‡§	0·1	3·0	0·4
ANOVA (one-way); *P* value			<0·001		<0·001		0·10	

* For details of diets and procedures, see Table 1 and pp. 674–675.
† AIN-76 control meal contains 200 g casein/kg diet as the sole protein source.
‡ Mean values were significantly different from those of the AIN-76 control meal (by least significant differences): *P* < 0·05.
§ Mean values were significantly different from those of the protein-free meal (by least significant differences): *P* < 0·05.

Increasing the concentration of CPP in the meal from 0 to 100 g/kg had no significant effect on [47]Ca deposition (expressed as percentage administered or percentage absorbed dose) in femurs of 9-week-old rats relative to the protein-free meal. However, increasing the CPP content further to 200, 350 or 500 g/kg meal significantly reduced the percentage of administered (but not the percentage of absorbed) [47]Ca dose which was deposited in bone relative to the protein-free meal (Table 4).

Endogenous loss of [47]Ca in faeces was negligible (less than 0·3 % per d) in each of the studies. Furthermore, there were no significant differences in endogenous loss of [47]Ca in faeces between the different dietary treatments in any of the three studies.

Discussion

The rat was used in the present series of studies because the absorption mechanisms for Ca are similar in rats and in human subjects (Bronner, 1987; Norman, 1990) and a number of dietary and physiological factors affect Ca absorption similarly in the two species (e.g. age, pregnancy, lactation, 1,25-dihydroxycholecalciferol, oxalic acid, phytate: Mahoney & Hendricks, 1985; Brommage & Binacua, 1991; Cashman & Flynn, 1996), although some exceptions to this have also been reported (e.g. lactose, phosphate: Mahoney & Hendricks, 1978; Hegsted et al. 1981; Tremaine et al. 1986; Miller, 1989).

Effect of meal protein content

In part B of the present study, fractional absorption of Ca from a meal was significantly affected by the casein content of the meal and also by the casein content of the usual diet, with a significant interaction between these two variables (as shown by two-way ANOVA). For example, increasing the casein content of a meal from 200 to 500 g/kg meal significantly increased fractional Ca absorption (by 53 %) in rats previously adapted for 2 weeks on a diet containing 200 g casein and 5 g Ca/kg diet. On the other hand, increasing the casein content of the meal from 200 to 350 g/kg had no significant enhancing effect on Ca absorption. Similarly, there was no effect of meal casein content in the lower range 0–300 g/kg on Ca absorption (part A of the present study), unlike the study of Brommage et al. (1991) which showed that fractional Ca absorption from a single meal, in rats previously adapted for 3 weeks on a diet containing 2 g Ca and 180 g casein/kg diet, was increased by 26 % when the casein content of the meal was increased from 90 to 270 g/kg meal. This discrepancy between the studies may be influenced by the lower habitual Ca intake and meal Ca content used in the study of Brommage et al. (1991). It may be possible that an enhancing effect of casein on Ca absorption at lower levels (up to 270 g/kg meal) is more evident under conditions of marginal dietary Ca levels as used in the study by Brommage et al. (1991).

Effect of dietary protein content

While fractional Ca absorption was enhanced by high-casein meals in the present study, continued feeding of a high-casein diet over 2 weeks resulted in adaptation, leading to a reduction in the efficiency of Ca absorption. This reduction in efficiency of Ca absorption with increased dietary casein intake was, most likely, effected through a down-regulation of the vitamin D-dependent active transcellular route of Ca absorption, similar to that observed when dietary Ca intake is increased (Ireland & Fordtran, 1973; Pansu et al. 1981; Cashman & Flynn, 1996). Although not measured, one could postulate that it is likely that the initial enhancement in the efficiency of Ca absorption by high-casein meals leads to an increase in plasma ionized-Ca concentration, which in turn results in a decrease in plasma parathyroid hormone levels and consequently, to a decrease in 1,25-dihydroxycholecalciferol levels in plasma. Reduced plasma 1,25-dihydroxycholecalciferol levels result in lower production of the intestinal Ca binding protein, calbindin D9-K, which reduces the rate of active transcellular Ca transport. However, it should be emphasized that high-protein diets have been shown to cause other gastrointestinal adaptive effects. For example, high-protein diets (500 g casein/kg diet) fed over 2–3 weeks have been shown to significantly increase the rate of gastric emptying in rats (Shi et al. 1997), and this might influence Ca absorption.

Such adaptative responses may explain the lack of effect of casein on apparent Ca absorption in metabolic balance studies. For example, Allen & Hall (1978) found no effect of casein on apparent Ca absorption in rats when the casein content of a diet (containing 6 g Ca/kg) was increased from 180 to 360 g/kg for 4 weeks. Similarly, Howe & Beecher (1981) reported that apparent Ca absorption was unaffected by increasing the casein content of a diet (containing 9 g Ca/kg) from 250 to 450 g/kg diet for 7 weeks. More recently, Yuan & Kitts (1994) found that apparent Ca absorption was similar in rats fed on either 60 or 200 g casein and 5 g Ca/kg diet for 6 weeks.

Effect of meal casein phosphopeptide content

The mechanism of casein-induced enhancement of Ca absorption is unclear. CPP, formed by proteolytic digestion of casein, have been shown to enhance Ca absorption in some studies with intact rats (Lee et al. 1992; Tsuchita et al. 1993; Hansen et al. 1996; Saito et al. 1998). However, the findings of the present study showed that increasing the concentration of CPP in a single meal from 0 to 100 g/kg meal had no effect on fractional absorption of meal Ca relative to the protein-free meal. Based on the theoretical yield of CPP from casein of approximately 10 g/kg (Kopra et al. 1992) it can be estimated that the diet containing 100 g CPP/kg contained about twice the CPP content of the diet containing 500 g casein/kg in study B which was shown to stimulate Ca absorption. These findings may suggest that the enhancing effect of high-casein meals on Ca absorption is not due to its CPP yield in the gastrointestinal tract. However, as the doses of CPP used in the present study were at least twice the CPP content of the diet containing 500 g casein/kg, the possibility of an enhancing effect of CPP at lower doses cannot be excluded. It should be noted, however, that several studies which have examined the effect of lower doses of dietary CPP (in the range 18–86 g/kg) on apparent Ca absorption in the rat have also reported a lack of

effect (Pointillart & Guégen, 1989; Shah *et al.* 1990; Yuan & Kitts, 1991; Kopra *et al.* 1992). Furthermore, Brommage *et al.* (1991) failed to find any significant effect of CPP on true intestinal Ca absorption in rats which were pre-adapted on a diet containing 180 g casein and 5 g Ca/kg diet and fed a single meal containing whey protein alone or with CPP at a level of either 38 or 76 g/kg meal.

Saito *et al.* (1998) have recently suggested that, based on their findings which showed that CPP supplementation (in the range 0·7–3·5 g CPP/kg diet) enhanced Ca absorption under conditions of marginal dietary Ca (3·5 g/kg), the Ca content of the diet might be an important factor in determining the effect of CPP on Ca absorption. However, while some studies which have found an effect of CPP on Ca absorption have fed marginal Ca levels (3·5 g/kg diet; Saito *et al.* 1998), others have used adequate Ca levels (5 g Ca/kg (Tsuchita *et al.* 1993)). Furthermore, studies which have failed to find an effect of CPP on Ca absorption have fed both high and low levels of dietary Ca (1, 3 and 5 g Ca/kg (Tsuchita *et al.* 1993), 3 and 9 g Ca/kg (Kopra *et al.* 1990)).

Similar controversy exists in reports from studies on human subjects. For example, Hansen *et al.* (1997a) found that Ca absorption from high- or low-phytate meals was not significantly influenced by the addition of CPP in healthy adult subjects. The same group also reported that fractional Ca absorption was not affected by CPP added to a rice-based infant cereal or a whole-grain infant cereal in healthy adult subjects (Hansen *et al.* 1997b), but CPP addition to a rice-based infant cereal meal significantly increased the total quantity of Ca absorbed (i.e. mg Ca). Furthermore, Heaney *et al.* (1994) reported that CPP administration was associated with better absorption of co-ingested Ca by postmenopausal women with low basal absorptive performance.

Our findings *in vivo* contrast with those from studies that demonstrated an enhancement of Ca absorption by CPP in the rat intestine using *in vitro* and *in situ* techniques (Lee *et al.* 1979, 1980; Sato *et al.* 1986; Nagasawa *et al.* 1991; Yuan & Kitts, 1991). One possible explanation for this could be that the CPP do not remain intact long enough in the intestine *in vivo* (Srinivasan & Rao, 1979; Van der Meer *et al.* 1988). They may be dephosphorylated by the intestinal phosphatase (*E.C.* 3.1.3.1), making the peptides more susceptible to proteolysis. However, *in vivo* rat studies, in which CPP had been given orally, showed that CPP remained in the ileum 1 h after intubation (Brommage *et al.* 1991). It should also be noted that the Ca-binding properties of CPP may differ significantly between preparations (McDonagh & FitzGerald, 1998) and this could influence the interaction of Ca and CPP in the intestine. For example, Tsuchita *et al.* (1996) found that decalcified CPP was less effective in preventing ovariectomy-induced bone loss in aged rats compared with a Ca-containing CPP.

The reasons for the reduction in fractional Ca absorption with the high-CPP containing meals in the present study is unclear. Interestingly, Hansen *et al.* (1997a) found that addition of 1000 mg CPP to a low-phytate–high-Ca meal significantly reduced fractional Ca absorption in human adult subjects when compared with that from the same meal type but containing 0 or 250 mg CPP. The authors suggested that the reduction in fractional Ca absorption may have been due to the Ca contribution from added CPP (i.e.

1000 mg CPP supplied 21 % additional Ca above that in the control diet). However, this could not have been the case in the present study as the CPP were de-calcified and therefore provided no extra Ca in the meals. It may be that the lower fractional absorption of Ca observed in the present study when CPP were added in high amounts was due to an impaired release of Ca ions from the CPP–Ca complexes at the intestinal lumen. Li *et al.* (1989), in a study of intestinal Ca transport in rat ileum, found that the CPP–Ca complex was not absorbed and remained on the mucosal side of the tissue. Another possibility is that the reduction in fractional Ca absorption with the high-CPP-containing meals is due to the increased P : Ca ratio from 0·8 : 1 to 4·3 : 1 as a consequence of the increasing amounts of CPP from 0 to 500 g/kg meal. Howe & Beecher (1981) found that Ca absorption in rats was significantly reduced with increasing dietary P content. Similarly, Mahoney & Hendricks (1978) reported that changing the P : Ca ratio from 1 : 1 to 2 : 1 by addition of P to diets led to significantly decreased levels of Ca absorption.

It could be that the effect of casein on Ca absorption may be due to a general effect of protein as suggested by Brommage *et al.* (1991) who observed a stimulatory effect on Ca absorption in rats by both soyabean protein and casein.

The effect of CPP on ^{47}Ca deposition in bone expressed as a percentage of administered dose reflects the effect of CPP on ^{47}Ca absorption, in that bone ^{47}Ca uptake was unaffected by meal CPP at a level of 100 g/kg but was reduced by meal CPP in the range 200–500 g/kg. However, bone ^{47}Ca uptake, expressed as a percentage of absorbed dose, was unaffected by any level of CPP indicating that CPP had no direct acute effect on bone calcification.

Urinary ^{47}calcium loss

Overall, urinary ^{47}Ca loss was small, with only about 1 % of the absorbed dose lost in urine in the first 3 d after isotope administration. These findings are similar to those of McCredie *et al.* (1984) and Cashman & Flynn (1996), who reported that only 1·7–3·9 % of the ^{47}Ca administered to young growing rats as either an oral dose of $CaCl_2$ or in the AIN-76 diet respectively, appeared in urine within 2–3 d. When urinary ^{47}Ca loss was expressed as percentage of absorbed dose, it was found that increasing dietary casein, but not meal casein content, significantly increased the urinary ^{47}Ca losses. This effect of dietary casein on urinary ^{47}Ca excretion is consistent with the findings of other studies which showed that urinary Ca is significantly increased in rats fed on high-casein diets (Allen & Hall, 1978; Whiting & Draper, 1980; Howe & Beecher, 1981). A similar effect of dietary protein on urinary Ca loss is observed in human subjects (Allen *et al.* 1979; Hegsted *et al.* 1981; Mahalko *et al.* 1983; Pannemans *et al.* 1997) and has been attributed to an increased glomerular filtration rate and a decreased tubular Ca reabsorption caused by catabolism of S-containing amino acids native to the protein (Kim & Linkswiler, 1979; Schuette *et al.* 1980).

Conclusions

The present studies have shown that Ca absorption was enhanced by high-casein meals (i.e. 500 g/kg diet) in rats,

but at high-dietary casein intakes Ca absorption efficiency was reduced, probably due to either adaptation in the active transcellular Ca transport or an acceleration in the rate of gastric emptying. Ca absorption from a meal was unaffected by inclusion of 100 g CPP/kg in a single meal but was significantly reduced by CPP added at levels of 200, 350 and 500 g/kg meal, with no evident dose-relationship. Thus, while Ca absorption was enhanced by high-casein meals, the mechanism remains unclear. The effects of casein and CPP on Ca absorption demonstrated in the present studies in rats may have significance for human nutrition but these findings would need to be confirmed in studies on human subjects.

Acknowledgement

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