Journal of Dairy Research (2007) **74** 356–366. © *Proprietors of Journal of Dairy Research 2007*
doi:10.1017/S0022029907002725 *First published online 26 July 2007 Printed in the United Kingdom*

Influence of bovine and caprine casein phosphopeptides differing in α_{s1}-casein content in determining the absorption of calcium from bovine and caprine calcium-fortified milks in rats

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Received 23 May 2005 and accepted for publication 8 January 2007

Bovine and caprine milks have a similar overall gross composition, but vary considerably in the ratios of their casein components. These differences cause significant changes in the ability of caseins to bind and stabilize calcium (Ca). It might be expected that these *in vitro* variations, which are thought to be due to differences in casein phosphopeptides (CPP) content, could lead to *in vivo* differences in the digestion and absorption of Ca. To test this hypothesis three milks with different casein ratios [bovine (B), caprine high in α_{s1}-casein (CH) and caprine low in α_{s1}-casein (CL)] were compared with regard to Ca absorption and deposition in growing male rats. For comparison, each milk was Ca-fortified (BCa-milk, CHCa-milk, and CLCa-milk) and CPP, prepared by enzymatic hydrolysis from the respective caseins (extrinsic CPP), were added to both native and Ca-milks. The effects of added CPP (extrinsic) could then be compared with intrinsic CPP released from the gastrointestinal digestion of caseins. Total gastric Ca was sampled at 15, 30 and 60 min after ingestion. No differences were found among the native milks with or without CPP, but the Ca from all Ca-milks (regardless of casein type) appeared to clear the stomach more rapidly and this was enhanced by the extrinsic CPP. The total intestinal Ca was not different among the native milks ± CPP, however, it rose more rapidly with Ca fortification, and was higher at 30 min for all CPP-Ca-milks. At 60 min the total intestinal Ca level fell for the CPP-Ca-milks while all others continued to rise. These observations suggest that the CPP in Ca-milks enhance gastric clearance and uptake from the intestine. Ca availability from BCa-milk, CHCa-milk, and CLCa-milk with and without CPP was estimated by both plasma and femur uptake of ^{45}Ca. Ca availability was enhanced at 5 h in the plasma in each case by added CPP. In all cases CPP stimulated Ca availability in the femur, but the CL-CPP was higher ($P<0{\cdot}05$) than that of either CH-CPP or B-CPP (extrinsic CPP). Based on the results of this study we can conclude that the addition of CPP will have beneficial effect on the absorption of Ca in growing rats from $CaCO_3$ added to bovine and caprine milks.

Keywords: calcium absorption, casein phosphopeptides, bovine, caprine, milk.

Dairy products represent an excellent source of calcium (Ca), and several studies have demonstrated positive linkages between dairy sources of Ca and greater bone mass measurements or reduced fracture rates (Weaver, 1992; Infante & Tormo, 2000; Volek, 2003; Fisher et al. 2004; Matkovic et al. 2004). Dairy products are thought to improve bone mass and reduce fracture via enhanced intestinal Ca absorption as well as increased Ca retention by bone. However, less well documented is the positive effect of Ca fortification of dairy sources on the mineral status of bone (Moyer-Mileur et al. 1992; Tsuchita et al. 2001; Kato et al. 2002; Du et al. 2004; Mora-Gutierrez et al. 2007). This is important as foods fortified with Ca are being used more often by consumers (Nicklas, 2003) because of the perceived health benefits (Tunick, 1987; Berner et al. 1990; Zemel & Miller, 2004).

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Several factors have been suggested to be responsible for the pronounced bioavailability of Ca from milk and dairy products. These include: the unique nature of the casein transport complexes (the casein micelles; Farrell et al. 2006), an optimal Ca:P ratio (Cámara-Martos & Amaro-López, 2002), lactose binding (Cámara-Martos & Amaro-López, 2002), and facilitation of uptake by casein phosphopeptides (CPP) released from enzymatic digests of whole casein during food processing (Combes et al. 2002) or gastrointestinal digestion (Tsuchita et al. 2001; Kato et al. 2002; Mora-Gutierrez et al. 2007).

Caprine milk differs from bovine milk in several physicochemical characteristics and in its technological behaviour (Ambrosoli et al. 1988; Remeuf et al. 1995; Mora-Gutierrez & Farrell, 2001). The proportions of the α_s-casein fractions are in a large part responsible for the differences observed between the two milks. In addition, a recent study compared unfortified (native) and Ca-fortified bovine and caprine cheeses in terms of Ca absorption and deposition in growing male rats (Mora-Gutierrez et al. 2007). In the latter study the caprine milk used was low in α_{s1}-, but high in α_{s2}-casein. Significant differences in Ca absorption were found among the cheeses with the data yielding the pattern: caprine Ca-fortified>caprine=bovine Ca-fortified>bovine>control. The enhanced Ca absorption resulted in the production of higher bone mass and correspondingly increased resistance to bone fracture in the femoral bones of rats (Mora-Gutierrez et al. 2007). It could be speculated that CPP generated during the cheese manufacturing process enhanced the observed Ca absorption and femoral bone deposition. However, definitive effects of the proportions of α_s-casein fractions on Ca absorption from caprine Ca-fortified milks and their derived dairy products have not been established, nor have the effects of added caprine CPP been studied.

In the present work, we describe for the first time the in vivo gastric digestion of bovine and caprine milks differing in α_{s1}-casein content [high (CH-milk) and low (CL-milk) in α_{s1}-casein] in Ca absorption and femoral bone deposition in growing male rats. Second, the effect of Ca fortification from $CaCO_3$ was studied on absorption and femoral bone deposition. In addition the effects of added CPP (purified from each milk type) were tested on Ca uptake, both with and without fortification with Ca.

Materials and Methods

Preparation of bovine and caprine milks

Bovine milk (B) was obtained from an individual Jersey cow, whereas caprine milks were obtained from individual French-Alpine goats at the International Dairy Goat Research Center, Prairie View A&M University, Prairie View, Texas, USA. The caprine milks (C) were screened for high (H) and low (L) content of α_{s1}-casein by RP-HPLC (Waters, Milford, Massachusetts 01757, USA) according to methods described by Mora-Gutierrez et al. (1991), this yields the terms (CH) and (CL) respectively. The cow and goats were in mid-lactation. All milk samples were skimmed by centrifugation at 4000 ***g*** for 30 min at 4 °C. The skimmed milks were analysed by SDS-PAGE and densitometry (Basch et al. 1989) to quantify the different fractions of caseins.

In addition, skim milk samples were analysed (AOAC, 1995) to determine total protein, lactose, calcium, and vitamin D. The phosphorus content and the ratio of casein to whey protein in all skimmed milk samples were determined according to methods described by Cerbulis & Farrell (1976) and Basch et al. (1985), respectively.

Preparation of bovine and caprine casein phosphopeptides

The bovine and two caprine whole caseins, characterized by a high (H) and a low (L) α_{s1}-casein content, were obtained by isoelectric precipitation of the individual skimmed milk samples described above. The bovine CPP (B-CPP), caprine CPP high in α_{s1}-casein (CH-CPP), and caprine CPP low in α_{s1}-casein (CL-CPP) were prepared from a tryptic digest of bovine and caprine whole caseins by an ethanol precipitation method (Tsuchita et al. 1993). The CPP content was determined by HPLC (Hirayama et al. 1992). Calcium was removed from the bovine and caprine CPP solutions by treatment with a cation-exchange resin (Supelco, Bellefonte, Pennsylvania 16823, USA). These Ca-free bovine and caprine CPP solutions were lyophilized. The concentrations of Ca in the Ca-free B-CPP, CH-CPP and CL-CPP were determined by flame atomic absorption spectrophotometry after dry digestion (Varian Instrument SpectrAA 55, Walnut Creek, California 94598, USA). Molar weights of the B-CPP, CH-CPP and CL-CPP were determined (Smyth & FitzGerald, 1997). Other CPP analyses were performed according to AOAC (1995).

Preparation of bovine and caprine Ca-fortified milks

A colloidal solution of 0·67% (w/v) $CaCO_3$ was stabilized by adding 0·01% (w/v) gum arabic before mixing with skimmed milk to form Ca-fortified milks (BCa-milk, CHCa-milk, CLCa-milk) containing 2·7 mg Ca/ml and 0·1 mg gum arabic/ml (gum arabic uniformly suspends the added Ca and other nutrients in the system ensuring even and controlled dosage). The 2·7 mg Ca/ml level of fortification was followed as a model of fortification frequently seen in the United States in which Ca is added to foods per serving, i.e., an amount based on the Ca content of a cup (240 ml) of milk (300 mg) or an amount intended to provide 100% of the U.S. Daily Value (1000 mg). B-CPP, CH-CPP and CL-CPP derived from in vitro tryptic digestion of bovine and caprine whole caseins (extrinsic CPP) were dissolved in the respective BCa-milk, CHCa-milk, and CLCa-milk at doses 0·25, 0·5, and 1·0 mg/ml. These doses of CPP are sufficient to promote Ca absorption from BCa-milk in growing rats (Tsuchita et al. 2001).

https://doi.org/10.1017/S0022029907002725 Published online by Cambridge University Press

Rats

Male Sprague-Dawley rats (Harlan Teklad, Madison, Wisconsin 53744, USA), weighing 110 g upon arrival, were housed individually in stainless steel mesh bottom cages in a temperature-controlled (23 ± 2 °C) room with 45 ± 10% humidity and a 12-h light-dark cycle. The rats of all experimental and the control groups were fed the diet of the American Institute of Nutrition (AIN-76, American Institute of Nutrition, 1977). The composition of the AIN-76 diet in terms of Ca, P, and protein was 5200 mg/kg, 4000 mg/kg, and 200 g/kg, respectively. The rats of the Ca-fortified control groups were fed a Ca-fortified AIN-76 diet. The Ca content of the Ca-fortified AIN-76 diet was 7900 mg/kg. Animals had free access to food and double deionized water (Milli-Q Biocel, Ultrapure Water System, Millipore Corporation, Billerica, Massachusetts 01821, USA) for 10 d. Prior to the oral administration of the milk samples, rats were starved overnight (12 ± 2 h), but were allowed free access to double deionized water. The care of the rats conformed to the Guidelines on the Use of Living Animals in Scientific Investigations (Biological Council, 1987).

After 10 d the animals were allotted by body weight to two controls [(no added milk), (no added milk+2·7 g dietary Ca/kg)] and twelve experimental groups (B-milk, CH-milk, CL-milk, BCa-milk, CHCa-milk, CLCa-milk) with and without added CPP. Each control and treatment group consisted of seven rats. Each rat had an average body weight of 140 g.

Ca availability studies

Assessment of Ca concentration in the gastrointestinal tract at a fixed extrinsic CPP concentration. For each milk (B, CL and CH) seven rats received the native milk and seven received the native milk with added CPP, whereas seven rats received the Ca-milk and seven more received the latter with added CPP. The CPP concentration was 1·0 mg/ml. Controls did not receive milk, but one control group had added 2·7 g dietary Ca/kg, whereas the other did not receive dietary Ca. The milks were administered as a 2 ml bolus by gastric intubation, without the use of anaesthesia. At 15, 30 and 60 min after the administration of the milk samples, all rats were anesthetized by inhalation of halothane (Sigma Chemical Co., St. Louis, Missouri 63178, USA). An incision was made in the abdomen and forceps were placed on the cardiac, pyloric and ileo-cecal junctions to avoid displacement of the contents of one compartment into another. Once ligated, the stomach and small intestine were excised and the contents were recovered by washing the interior with 5 ml double deionized water. The gastric and intestinal contents were homogenized and centrifuged (15 000 ***g***, 20 min). The precipitate was dried and ashed overnight in a muffle furnace at 550 °C (insoluble Ca). The ashed sample was then dissolved in 1 M-HNO_3 for Ca determination by atomic absorption spectrophotometry (Varian Instrument SpectrAA 55). Calcium in the supernatant was measured in triplicate by atomic absorption spectrophotometry (soluble Ca).

Assessment of Ca concentration in the gastrointestinal tract of rats at variable extrinsic CPP concentration. For each Ca-milk (BCa-milk, CHCa-milk, and CLCa-milk) four groups of seven rats each were administered orally 2 ml of one of the Ca-fortified milk samples with variable concentrations of CPP (0·0, 0·25, 0·5 or 1·0 mg/ml). Animals were sacrificed at 30 and 60 min after administration of the samples. The Ca concentration in the gastrointestinal contents was measured as described above.

Calculation of the calcium variables

The level of gastric Ca was calculated as the sum of gastric insoluble Ca and gastric soluble Ca. The gastrointestinal Ca disappearance was calculated from the amount of Ca in the gastrointestinal contents as follows: gastrointestinal Ca disappearance = Ca ingested − (gastric Ca + intestinal Ca), where intestinal Ca is the sum of insoluble Ca and soluble Ca in the intestinal contents.

Preparation of ^{45}Ca radio-labelled fortified milks

Samples of radio-labelled BCa-milk, CHCa-milk and CLCa-milk were prepared as follows: an aliquot of Ca-fortified milk samples (20 ml) with or without CPP was mixed with 50 µl $^{45}CaCl_2$ (specific activity 36 kBq/µl, Ca content 55 ng/µl; NEN Life Science Products, Inc., Boston, Massachusetts 02118, USA), and incubated at 4 °C for 24 h with continuous stirring.

Measurement of ^{45}Ca-radioactivity in the blood plasma and femoral bone

For each Ca-milk (BCa-milk, CHCa-milk, and CLCa-milk), seven rats were administered orally 2 ml of one of the Ca-fortified milk samples labelled with ^{45}Ca, and three other groups of seven received the same Ca-milks to which B-CPP, CH-CPP or CL-CPP had been added (0·25 mg/ml). Blood samples (200 µl) were drawn from the tail vein at 5, 10 and 20 h after the administration to measure ^{45}Ca-radioactivity in the blood plasma. Surgical adhesive was placed over the incision after blood collection. All rats were then killed by overdosing with an intraperitoneal injection of 5 mg sodium pentobarbital (Sigma) per 100 g body weight 48 h after the ingestion of Ca-fortified milk samples containing the radioisotope, and the right femur was excised. The bone samples were digested with a mixture of 15·7 M-HNO_3 (three volumes) and 9·2 M-$HClO_4$ (one volume) at 200 °C for 2 h. The radioactivity of ^{45}Ca in the digested bone was measured with a Beckman LS 1800 scintillation counter (Beckman Instruments, Inc., Fullerton, California 92834, USA).

https://doi.org/10.1017/S0022029907002725 Published online by Cambridge University Press

Statistical analysis

The following statistical models were used to study the total gastric Ca content, the intestinal soluble Ca content, the intestinal total Ca content, and the gastrointestinal Ca disappearance, respectively, using the PROC general linear model procedure of SAS (SAS Institute, 1999):

$$Y = \mu + D_i + T_k + (D*T)_{ik} + \varepsilon_{ik}$$

$$Y = \mu + D_i + T_k + C_j + (D*T)_{ik} + + (C*T)_{jk} + (C*D)_{ij} + (C*D*T)_{ijk} + \varepsilon_{ijk}$$

Where Y = mean value of each variable tested, μ = the population mean, D_i = treatment (i = 1 to 14; 1 = control, 2 = control+Ca, 3 = B-milk, 4 = B-milk+B-CPP, 5 = BCa-milk, 6 = BCa-milk+B-CPP, 7 = CH-milk, 8 = CH-milk+CH-CPP, 9 = CHCa-milk, 10 = CHCa-milk+CH-CPP; 11 = CL-milk, 12 = CL-milk+CL-CPP, 13 = CLCa-milk, and 14 = CLCa-milk+CL-CPP), T_k = time (k = 1 to 3; 1 = 15 min, 2 = 30 min, and 3 = 60 min), D*T = interaction of treatment and time, C*T = interaction of CPP concentration and time, C*D*T = interaction of concentration, treatment, and time ε = error term, the random variable assumed to be normally distributed with mean equal to zero and constant variance.

Duncan multiple range test was used to compare means that showed significant variation ($P<0{\cdot}05$).

The results of ^{45}Ca radioactivity measurements in plasma and femoral bones were tested statistically by one-way analysis of variance (ANOVA), followed by Duncan's test to compare means that showed significant variation ($P<0{\cdot}05$):

$$Y = \mu + D_i + \varepsilon_i$$

Where D_i = treatment (i = 1 to 6, 1 = BCa-milk, 2 = BCa-milk+B-CPP, 3 = CHCa-milk, 4 = CHCa-milk+CH-CPP, 5 = CLCa-milk, and 6 = CLCa-milk+CL−CPP), Y and , = as defined above.

Nonlinear regression analysis of gastric emptying data

The decrease in total gastric Ca (gastric emptying) appeared to follow a nonlinear process and so as a provisional model, the data were fitted with an exponential decay function using the program Origin 7·5 from OriginLab (Northampton, MA, USA). The equation used to follow the change in total gastric Ca with time was:

$$Ca_{total} = A_1 + A_2 e^{-kt} \quad [1]$$

Here A_1 and A_2 are fitting parameters for the change of Ca with time (t) and k represents the apparent rate constant. Iterative fitting was carried out stepwise until chi square reached a minimum value, usually 0·01% indicating a good fit to the equation. From the k value of Eqn 1, an apparent half time ($T_{1/2}$) for Ca disappearance was calculated from:

$$T_{1/2} = 0{\cdot}693/k \quad [2]$$

Table 1. (**a**) Composition of bovine (B) and caprine (C) milks high (CH) and low (CL) in α_{s1}-casein. (**b**) Composition of casein phosphopeptides (CPP) purified from bovine and caprine milks high and low in α_{s1}-casein

Values are means ± SD for $n = 3$

a. Milk components	B-milk	CH-milk	CL-milk
Total protein (g/kg)	32·9 ± 0·2	33·1 ± 0·1	32·8 ± 0·1
α_{s2}-Casein (%)	12·1 ± 0·3	9·2 ± 0·3	29·2 ± 0·3
α_{s1}-Casein (%)	39·5 ± 0·4	25·1 ± 0·3	5·9 ± 0·1
β-Casein (%)	37·2 ± 0·5	51·6 ± 0·5	50·5 ± 0·6
κ-Casein (%)	11·2 ± 0·2	13·8 ± 0·1	14·4 ± 0·2
Lactose (g/kg)	46·9 ± 0·1	45·6 ± 0·3	45·3 ± 0·1
Calcium (mg/kg)	1243 ± 1	1250 ± 1	1251 ± 1
Phosphorous (mg/kg)	1029 ± 1	1034 ± 1	1032 ± 2
Casein/whey	5·32 ± 0·4	5·51 ± 0·2	5·49 ± 0·4
Vitamin D (IU)	24·1 ± 0·1	25·3 ± 0·1	24·7 ± 0·1
b. CPP components	B-CPP	CH-CPP	CL-CPP
Total CPP (g/kg)	890 ± 1	881 ± 3	907 ± 1
Calcium (mg/kg)	48·1 ± 0·1	47·3 ± 0·1	49·4 ± 0·2
Molecular weight (g/mol)	4109	3875	3899
Dry matter (g/kg)	936·1 ± 0·5	929·4 ± 0·3	927·2 ± 0·2
Total nitrogen (g/kg)	112·0 ± 0·2	110·8 ± 0·1	109·8 ± 0·1
Phosphorous (g/kg)	25·5 ± 0·4	26·8 ± 0·1	26·7 ± 0·3
N/P	4·4	4·1	4·1
Ca/CPP μg/mg	0·054	0·054	0·054

Results

The native bovine and caprine milks used in this study had similar compositions in terms of total protein, lactose, and calcium (Table 1a). Some minor differences were observed in the phosphorus and vitamin D content of the native bovine and caprine milks, however, these milks differed considerably in casein composition (Table 1a). The composition of bovine and caprine CPP is presented in Table 1b. Minor differences were noted between the different components of bovine and caprine CPP. Note the low level of Ca in the CPP due to the ion exchange treatment, thus the CPP contribute negligible amounts of Ca to the milk samples.

Gastric emptying

As would be expected, all of the milk products increased the total gastric Ca levels of all rats at all times sampled, relative to the control or the control with added dietary Ca (Table 2). Addition of the respective CPP to the milks had no effect on total gastric Ca resulting from any

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Table 2. Total gastric Ca content (mg), measured at intervals during 1 h post intubation, in rats administered bovine and caprine native milks and Ca-fortified milks with casein phosphopeptides (CPP) at 1·0 mg/ml or without CPP[1,2]

Values are means ± SEM for $n=7$

	Time (min)			
Treatments[3]	0	15	30	60
B-milk	$2·53 \pm 0·13^{be}$	$1·91 \pm 0·13^{cf}$	$1·70 \pm 0·13^{cf}$	$0·96 \pm 0·13^{bg}$
B-milk+B-CPP	$2·53 \pm 0·13^{be}$	$1·93 \pm 0·13^{cf}$	$1·67 \pm 0·13^{cf}$	$0·92 \pm 0·13^{bg}$
BCa-milk	$7·78 \pm 0·13^{ae}$	$4·71 \pm 0·13^{af}$	$4·04 \pm 0·13^{ag}$	$3·00 \pm 0·13^{ah}$
BCa-milk+B-CPP	$7·78 \pm 0·13^{ae}$	$4·20 \pm 0·13^{bf}$	$3·09 \pm 0·13^{bg}$	$2·89 \pm 0·13^{ah}$
CH-milk	$2·53 \pm 0·13^{be}$	$1·94 \pm 0·13^{cf}$	$1·64 \pm 0·13^{cf}$	$0·95 \pm 0·13^{bg}$
CH-milk+CH-CPP	$2·53 \pm 0·13^{be}$	$1·94 \pm 0·13^{cf}$	$1·63 \pm 0·13^{cf}$	$0·93 \pm 0·13^{bg}$
CHCa-milk	$7·60 \pm 0·13^{ae}$	$4·61 \pm 0·13^{af}$	$3·96 \pm 0·13^{ag}$	$2·87 \pm 0·13^{ah}$
CHCa-milk+CH-CPP	$7·60 \pm 0·13^{ae}$	$4·23 \pm 0·13^{bf}$	$3·09 \pm 0·13^{bg}$	$2·81 \pm 0·13^{ag}$
CL-milk	$2·53 \pm 0·13^{be}$	$1·96 \pm 0·13^{cf}$	$1·63 \pm 0·13^{cf}$	$0·95 \pm 0·13^{bg}$
CL-milk+CL-CPP	$2·53 \pm 0·13^{be}$	$1·93 \pm 0·13^{cf}$	$1·64 \pm 0·13^{cf}$	$0·93 \pm 0·13^{bg}$
CLCa-milk	$7·58 \pm 0·13^{ae}$	$4·60 \pm 0·13^{af}$	$3·96 \pm 0·13^{ag}$	$2·89 \pm 0·13^{ah}$
CLCa-milk+CL-CPP	$7·58 \pm 0·13^{ae}$	$4·00 \pm 0·13^{bf}$	$2·97 \pm 0·13^{bg}$	$2·80 \pm 0·13^{ah}$
Control	$0·01 \pm 0·13^{c}$	$0·08 \pm 0·13^{d}$	$0·06 \pm 0·13^{d}$	$0·05 \pm 0·13^{c}$
Ca-control	$0·08 \pm 0·13^{c}$	$0·15 \pm 0·13^{d}$	$0·14 \pm 0·13^{d}$	$0·09 \pm 0·13^{c}$

[1]A dose of CPP at 1·0 mg/ml promotes Ca absorption from BCa-milk in rats (Tsuchita et al. 2001)
[2]The total Ca administered by intubation was taken to be the zero time value
[3]B-milk, CH-milk and CL-milk as defined in Table 1a, the CPP are defined in Table 1b and Ca-milk is Ca fortified milk
[a,b,c,d]Means within the same column without common superscripts are significantly different ($P<0·05$)
[e,f,g,h]Means within the same row without common superscripts are significantly different ($P<0·05$)

of the milk samples, in essence all of the data from the milks±CPP form a rather uniform set of six experiments. Addition of Ca to each milk sample caused a significant rise in the total gastric Ca levels relative to the milks alone, but there were no significant differences among the Ca-fortified milks. However, the total gastric Ca levels in the rats administered BCa-milk, CHCa-milk and CLCa-milk with their respective added CPP (1 mg/ml) were significantly lower ($P<0·05$) than in the rats administered BCa-milk, CHCa-milk, and CLCa-milk without CPP at 15 and 30 min after ingestion of the milks (Table 2). Here all Ca-milks form a data set of three experiments, while the+CPP Ca-milks form another data set. These changes were fitted with the exponential decay model given in Eqn 1 (note that the total doses of Ca given to each set of rats as given in Table 2 can be taken as the zero time total gastric Ca). The data fit well to the decay model (chi square=0·01%). Focusing on the apparent $T_{1/2}$ for each set (Eqn 2) the half time for Ca gastric loss of the native milks is 58·2±3·8 min. Interestingly the addition of Ca not only results in higher gastric Ca, but also a more rapid emptying with $T_{1/2}$ equal to 11·4±0·3 min for all Ca-milks. The addition of CPP decreases the $T_{1/2}$ to 8·0±0·6 min for all Ca-milks with CPP. Since the same amount of each milk was given to all rats in each of the Ca-fortified milks, it would be reasonable to assume that a lower level of total gastric Ca indicates a more rapid gastric emptying of Ca in the Ca-fortified bovine and caprine milks containing extrinsic CPP.

Intestinal soluble calcium

It has been postulated that Ca uptake is related to the intestinal soluble Ca levels (Tsuchita et al. 2001). Here all of the native milk products increased the intestinal soluble Ca content of all rats at all times sampled relative to the control or the control with Ca (Table 3a), and addition of the CPP to the milks had no significant effect on intestinal soluble Ca content resulting from any of the milk samples. For the Ca-fortified milk samples only changes related to increased Ca dosage occurred as seen in Table 3a. In addition, CPP had no effects on the soluble Ca levels, relative to Ca fortification alone.

Intestinal total calcium

When the total intestinal Ca was considered, again all of the native milk products increased the total intestinal Ca content of all rats at all times sampled relative to the control or the control with added dietary Ca (Table 3b), and taking the time averaged total intestinal Ca of the controls as a zero time value, it can be seen that all milk values increased similarly with time regardless of milk type (B-milk, CH-milk, CL-milk) or the presence of CPP. Addition of Ca to each milk sample did cause a significant rise in the intestinal total Ca content at 15, 30 and 60 min relative to the milks alone; again this was independent of milk type as seen in Table 3b for the Ca-fortified milks. Similarly, the amount of total Ca intestinal content of rats administered BCa-milk, CHCa-milk, and CLCa-milk with

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Table 3. (**a**) Intestinal soluble Ca (mg) content and (**b**) intestinal total Ca content (mg) in rats administered bovine and caprine Ca-fortified and native milks with casein phosphopeptides (CPP) at 1·0 mg/ml or without CPP[1, 2]

Values are means ± SEM for $n=7$

a. Soluble Ca	Time (min)		
Treatments[3]	15	30	60
B-milk	0·44 ± 0·03^{ab}	0·44 ± 0·03^{b}	0·47 ± 0·03^{b}
B-milk+B-CPP	0·45 ± 0·03^{a}	0·46 ± 0·03^{b}	0·48 ± 0·03^{b}
BCa-milk	0·37 ± 0·03^{bd}	0·56 ± 0·03^{ae}	0·77 ± 0·03^{af}
BCa-milk+B-CPP	0·42 ± 0·03^{abd}	0·58 ± 0·03^{ae}	0·80 ± 0·03^{af}
CH-milk	0·43 ± 0·03^{ab}	0·43 ± 0·03^{b}	0·45 ± 0·03^{b}
CH-milk+CH-CPP	0·44 ± 0·03^{ab}	0·45 ± 0·03^{b}	0·47 ± 0·03^{b}
CHCa-milk	0·38 ± 0·03^{abd}	0·54 ± 0·03^{ae}	0·76 ± 0·03^{af}
CHCa-milk+CH-CPP	0·45 ± 0·03^{ad}	0·59 ± 0·03^{ae}	0·79 ± 0·03^{af}
CL-milk	0·44 ± 0·03^{ab}	0·46 ± 0·03^{b}	0·46 ± 0·03^{b}
CL-milk+CL-CPP	0·45 ± 0·03^{a}	0·46 ± 0·03^{b}	0·48 ± 0·03^{b}
CLCa-milk	0·39 ± 0·03^{abd}	0·58 ± 0·03^{ae}	0·78 ± 0·03^{af}
CLCa-milk+CL-CPP	0·49 ± 0·03^{ad}	0·60 ± 0·03^{ae}	0·82 ± 0·03^{af}
Control	0·18 ± 0·03^{c}	0·18 ± 0·03^{c}	0·19 ± 0·03^{c}
Ca-control	0·20 ± 0·03^{c}	0·21 ± 0·03^{c}	0·26 ± 0·03^{c}
b. Total Ca			
B-milk	0·70 ± 0·03^{ae}	0·71 ± 0·03^{ae}	1·21 ± 0·03^{af}
B-milk+B-CPP	0·69 ± 0·03^{ae}	0·76 ± 0·03^{ae}	1·24 ± 0·03^{af}
BCa-milk	3·47 ± 0·03^{be}	4·00 ± 0·03^{bf}	4·50 ± 0·03^{bg}
BCa-milk+B-CPP	3·47 ± 0·03^{be}	4·41 ± 0·03^{cf}	4·12 ± 0·03^{cg}
CH-milk	0·68 ± 0·03^{ae}	0·79 ± 0·03^{ae}	1·21 ± 0·03^{af}
CH-milk+CH-CPP	0·68 ± 0·03^{ae}	0·79 ± 0·03^{ae}	1·23 ± 0·03^{af}
CHCa-milk	3·39 ± 0·03^{be}	3·90 ± 0·03^{bf}	4·45 ± 0·03^{bg}
CHCa-milk+ CH-CPP	3·25 ± 0·03^{be}	4·22 ± 0·03^{cf}	4·02 ± 0·03^{cg}
CL-milk	0·65 ± 0·03^{ae}	0·79 ± 0·03^{ae}	1·21 ± 0·03^{af}
CL-milk+CL-CPP	0·70 ± 0·03^{ae}	0·78 ± 0·03^{ae}	1·22 ± 0·03^{af}
CLCa-milk	3·37 ± 0·03^{be}	3·87 ± 0·03^{bf}	4·42 ± 0·03^{bg}
CLCa-milk+CPP	3·46 ± 0·03^{be}	4·32 ± 0·03^{cf}	4·01 ± 0·03^{cf}
Control	0·42 ± 0·03^{ce}	0·15 ± 0·03^{df}	0·05 ± 0·03^{dg}
Ca-control	0·40 ± 0·03^{ce}	0·33 ± 0·03de	0·26 ± 0·03^{df}

[1]A dose of CPP at 1·0 mg/ml promotes Ca absorption from BCa-milk in rats (Tsuchita et al. 2001)
[2]The time averaged total intestinal Ca for the controls of 0·24 mg (no Ca given) was taken as the intestinal zero time value for all samples
[3]B-milk, CH-milk and CL-milk as defined in Table 1a, the CPP are defined in Table 1b and Ca-milk is Ca fortified milk
[a,b,c,d]Means within the same column, separately for Tables 3a and 3b, without common superscripts are significantly different ($P<0·05$)
[e,f,g]Means within the same row without common superscripts are significantly different ($P<0·05$)

added CPP increased at 15 and 30 min but the values at 30 min time were greater in the presence of CPP (Table 3b). Most interestingly at 60 min, the total intestinal Ca decreased for the Ca-fortified milks in the presence of CPP, while these values continued to increase in the absence of CPP. This indicates that the CPP not only facilitated gastric emptying (Table 2) but facilitated intestinal uptake especially at 60 min.

Table 4. Gastrointestinal Ca disappearance (mg) in rats administered bovine and caprine Ca-fortified and native milks with casein phosphopeptides (CPP) at 1·0 mg/ml or without CPP at 60 min[1]

Values are means ± sem for $n=7$

Treatments[2]	Gastrointestinal Ca disappearance (mg)
B-milk	0·36 ± 0·01^{b}
B-milk+B-CPP	0·37 ± 0·01^{b}
BCa-milk	0·28 ± 0·01^{c}
BCa-milk+BCPP	0·77 ± 0·01^{a}
CH-milk	0·37 ± 0·01^{b}
CH-milk+CH-CPP	0·37 ± 0·01^{b}
CHCa-milk	0·28 ± 0·01^{c}
CHCa-milk+CH-CPP	0·77 ± 0·01^{a}
CL-milk	0·37 ± 0·01^{b}
CL-milk+CL-CPP	0·38 ± 0·01^{b}
CLCa-milk	0·27 ± 0·01^{c}
CLCa-milk+CL-CPP	0·77 ± 0·01^{a}
Control	−0·10 ± 0·01^{e}
Ca-control	0·18 ± 0·01^{d}

[1]A dose of CPP at 1·0 mg/ml promotes Ca absorption from BCa-milk in rats (Tsuchita et al. (2001)
[2]B-milk, CH-milk and CL-milk as defined in Table 1a, the CPP are defined in Table 1b, and Ca-milk is Ca fortified milk
[a,b,c,d,e]Means without common superscripts are significantly different ($P<0·05$)

Gastrointestinal calcium disappearance

Gastrointestinal Ca disappearance is an indirect measurement of Ca absorption. This value was calculated at 60 min because the data in Table 3 indicated the greatest possible differences might occur there. There were no significant differences among all of the milks (B-milk, CH-milk, CL-milk) at 60 min but all were significantly greater relative to the control and even the control with added dietary Ca (Table 4). The addition of the respective CPP to the milks had no significant effect, suggesting that absorption of native Ca in milks appears optimal. Curiously addition of Ca to each milk sample appeared to cause a decrease in gastrointestinal disappearance at 60 min suggesting that Ca fortification with $CaCO_3$ may alter the Ca/P ratio of the milks and apparently temporarily lower absorption. However, with respect to the gastrointestinal Ca disappearance, we found that a significantly different ($P<0·05$) amount of Ca disappeared at 60 min from the gastrointestinal tract of the rats in which CPP was added with BCa-milk, CHCa-milk, and CLCa-milk than in any other group of rats. The three different types of CPP (B-CPP, CH-CPP, and CL-CPP) did not differ in terms of gastrointestinal Ca disappearance. Thus, the apparent absorption of Ca related to these peptides was similar in all three milks and agrees with the change in total intestinal Ca seen above. Note again, all types of CPP equally stimulated gastrointestinal Ca disappearance (Table 4). As noted above, in the absence of CPP, but with Ca

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Table 5. Gastrointestinal Ca disappearance (mg) in rats administered bovine and caprine Ca-fortified milks with 4 levels of casein phosphopeptides (CPP) (mg/ml) at 30 and 60 min after ingestion[1,2]

Values are means ± SEM for $n=7$

	Gastrointestinal Ca disappearance (mg)					
	30 min			60 min		
CPP (mg/ml)	BCa-milk	CHCa-milk	CLCa-milk	BCa-milk	CHCa-milk	CLCa-milk
0	$-0{\cdot}262 \pm 0{\cdot}004^{be}$	$-0{\cdot}259 \pm 0{\cdot}004^{be}$	$-0{\cdot}252 \pm 0{\cdot}004^{be}$	$0{\cdot}284 \pm 0{\cdot}004^{ae}$	$0{\cdot}277 \pm 0{\cdot}004^{ae}$	$0{\cdot}274 \pm 0{\cdot}004^{ae}$
0·25	$0{\cdot}280 \pm 0{\cdot}004^{cd}$	$0{\cdot}288 \pm 0{\cdot}004^{bcd}$	$0{\cdot}293 \pm 0{\cdot}004^{bd}$	$0{\cdot}778 \pm 0{\cdot}004^{ad}$	$0{\cdot}780 \pm 0{\cdot}004^{ad}$	$0{\cdot}785 \pm 0{\cdot}004^{ad}$
0·5	$0{\cdot}283 \pm 0{\cdot}004^{bd}$	$0{\cdot}286 \pm 0{\cdot}004^{bd}$	$0{\cdot}291 \pm 0{\cdot}004^{bd}$	$0{\cdot}775 \pm 0{\cdot}004^{ad}$	$0{\cdot}775 \pm 0{\cdot}004^{ad}$	$0{\cdot}779 \pm 0{\cdot}004^{ad}$
1·0	$0{\cdot}279 \pm 0{\cdot}004^{cd}$	$0{\cdot}285 \pm 0{\cdot}004^{bcd}$	$0{\cdot}292 \pm 0{\cdot}004^{bd}$	$0{\cdot}765 \pm 0{\cdot}004^{ad}$	$0{\cdot}771 \pm 0{\cdot}004^{ad}$	$0{\cdot}774 \pm 0{\cdot}004^{ad}$

[1]Doses of CPP at 0·25, 0·5, and 1·0 mg/ml promote Ca absorption from BCa-milk in rats (Tsuchita et al. 2001)
[2]B-milk, CH-milk and CL-milk as defined in Table 1a, the CPP are defined in Table 1b, and Ca-milk is Ca fortified milk
[a,b,c]Means in the same row without common superscripts are significantly different ($P<0{\cdot}05$)
[d,e]Means in the same column without common superscripts are significantly different ($P<0{\cdot}05$)

Table 6. Plasma ^{45}Ca radioactivity (B_q/ml) in rats administered bovine and caprine Ca-fortified milks with casein phosphopeptides (CPP) at 0·25 mg/ml or without CPP[1]

Values are means ± SEM for $n=7$

	Time (h)		
Treatments[2]	5	10	20
BCa-milk	$7300 \pm 63{\cdot}9^{bc}$	$5320 \pm 63{\cdot}9^{d}$	$4019 \pm 63{\cdot}9^{e}$
BCa-milk+B-CPP	$7592 \pm 63{\cdot}9^{ac}$	$5427 \pm 63{\cdot}9^{d}$	$4024 \pm 63{\cdot}9^{e}$
CHCa-milk	$7329 \pm 63{\cdot}9^{bc}$	$5325 \pm 63{\cdot}9^{d}$	$4015 \pm 63{\cdot}9^{e}$
CHCa-milk+CH-CPP	$7617 \pm 63{\cdot}9^{ac}$	$5418 \pm 63{\cdot}9^{d}$	$4015 \pm 63{\cdot}9^{e}$
CLCa-milk	$7350 \pm 63{\cdot}9^{bc}$	$5332 \pm 63{\cdot}9^{d}$	$4020 \pm 63{\cdot}9^{e}$
CLCa-milk+CL-CPP	$7632 \pm 63{\cdot}9^{ac}$	$5421 \pm 63{\cdot}9^{d}$	$4025 \pm 63{\cdot}9^{e}$

[1]A dose of CPP at 0·25 mg/ml promotes Ca absorption from BCa-milk in rats (Tsuchita et al. 2001)
[2]B-milk, CH-milk and CL-milk as defined in Table 1a, the CPP are defined in Table 1b, and Ca-milk is Ca fortified milk
[a,b]Means within the same column without common superscripts are significantly different ($P<0{\cdot}05$)
[c,d,e]Means within the same row without common superscripts are significantly different ($P<0{\cdot}05$)

fortification, gastrointestinal disappearance was minimal at 15 min and rose significantly at 30 and 60 min signifying a gradual uptake of Ca relative to the control and even the control with Ca (Table 4). The addition of the respective CPP at only 0·25 mg/ml caused a significant increase (almost three fold) in Ca gastrointestinal disappearance over the Ca-milks without CPP (Table 5). Added CPP above this amount showed no dose dependency; apparently the ratio of CPP to $CaCO_3$ has an optimal limit which is quite small. All of the data at this juncture point to enhanced uptake of extrinsic Ca driven by the CPP.

^{45}Ca in the blood plasma

The data in Table 6 showed that 5 h after administration of radioactivity labelled BCa-, CHCa-, and CLCa-milk+CPP (0·25 mg/ml), the ^{45}Ca-radioactivity in the whole circulating plasma was significantly higher ($P<0{\cdot}05$) than in those groups of rats administered BCa-, CHCa-, and CLCa-milk – CPP. However, at 10 and 20 h after the ingestion of the different diets there was no significant difference in the level of ^{45}Ca radioactivity in the plasma of rats. This is in accord with the data presented above for the gastrointestinal disappearance of Ca in Ca-fortified milks. Table 3b shows that, in the absence of CPP, after 1 h the total intestinal Ca is still increasing for these samples, whereas the CPP facilitated a significant decrease in total intestinal Ca at 60 min. The effect of CPP even at 0·25 mg/ml apparently lingers for up to 5 h (Table 6). The level of ^{45}Ca-radioactivity in the blood plasma decreased ($P<0{\cdot}05$) between 5, 10, and 20 h after ingestion in all rats receiving control or experimental diets indicating most likely the combination of uptake from the blood and isotopic dilution.

Femoral bone incorporation of ^{45}Ca

^{45}Ca-radioactivity of femoral bones in rats fed BCa-, CHCa-, and CLCa-milk were significantly ($P<0{\cdot}05$) affected by CPP addition (0·25 mg/ml) and type of CPP added to the diet (Table 7). CLCa-milk containing extrinsic CL-CPP (highest α_{s2}-casein) had the highest ^{45}Ca retention in femoral bone and CHCa-milk containing extrinsic CH-CPP (lowest α_{s2}-casein) had the second highest level, while BCa-milk containing extrinsic B-CPP had the lowest level of the three Ca-milks. This indicates that extrinsic CL-CPP was superior in Ca absorption in the femur than the other types of extrinsic CPP. As a whole the two different types of intrinsic CPP from CHCa-milk and CLCa-milk were better, than or at least as effective, in terms of Ca absorption as intrinsic CPP produced from BCa-milk.

Discussion

Milk and dairy products represent the chief source of calcium in the typical Western diet. The phosphoserine

https://doi.org/10.1017/S0022029907002725 Published online by Cambridge University Press

Table 7. ^{45}Ca radioactivity in the right femur from rats administered bovine and caprine Ca-fortified milks with casein phosphopeptides (CPP) at 0·25 mg/ml or without CPP[1]

Values are means ± SEM for $n=7$

Treatments[4]	^{45}Ca radioactivity[2] ($\times 10^4$ Bq/g bone)	ΔCPP[3]	% increase
BCa-milk	50·8 ± 1·4^{c}		
BCa-milk+ B-CPP	61·2 ± 1·4^{b}	10·4	20
CHCa-milk	53·9 ± 1·4^{c}		
CHCa-milk+ CH-CPP	62·5 ± 1·4^{ab}	8·6	16
CLCa-milk	54·4 ± 1·4^{c}		
CLCa-milk+ CL-CPP	65·6 ± 1·4^{a}	11·2	21

[1]A dose of CPP at 0·25 mg/ml promotes Ca absorption from BCa-milk in rats (Tsuchita et al. 2001)
[2]The mean values of milks (with added CPP) were significantly different from corresponding milks (without added CPP) values
[3]ΔCPP = CPP milk – control milk
[4]B-milk, CH-milk and CL-milk as defined in Table 1a, the CPP are defined in Table 1b, and Ca-milk is Ca fortified milk
[a,b,c]Means without common superscripts are significantly different ($P<0·05$)

groups in the bovine and caprine casein subunits are the primary sites for Ca binding (Mora-Gutierrez et al. 1993; Farrell, 1999), and while Ca is mostly bound to casein, it is also included in casein micelles as an inorganic calcium-phosphate matrix (colloidal calcium phosphate) (Bak et al. 2001, Farrell et al. 2006). In vitro, Ca binds differentially to the various casein fractions (α_{s2}->α_{s1}->β->κ-casein) so this could influence Ca absorption in rats, particularly because of the compositional differences noted in Table 1a. Indeed Mora-Gutierrez et al. (2007) have shown that a caprine cheese low in α_{s1}-casein (high in α_{s2}-) increased Ca uptake and deposition relative to a similarly prepared bovine cheese. It was suggested that CPP formed during cheese manufacture or digestion might facilitate the Ca uptake. Soluble low molecular weight Ca-peptide complexes formed during digestion of BCa-milk (intrinsic CPP) are thought to be partially responsible for Ca absorption from milk (Tsuchita et al. 2001). These researchers also found a significant enhancement on Ca absorption from BCa-milk when adding CPP that was prepared in vitro from bovine whole casein by enzymatic hydrolysis (extrinsic CPP). As noted above because of the intrinsic difference in bovine and caprine caseins, a difference in Ca absorption might be expected as they differ significantly in their potential phosphopeptides.

The results of this study demonstrate that the addition of B-CPP, CH-CPP and CL-CPP to their respective native milks did not increase Ca absorption in young male rats. This is supported by two recent studies on humans by López-Huertas et al. (2006) and Teucher et al. (2006). However in our studies, the CPP aided Ca absorption of the extrinsic Ca of Ca-fortified milks. This conclusion is based on the findings that (a) no effect of extrinsic CPP on Ca absorption was evident when the animals were administered native milks (b) the presence of extrinsic CPP facilitated gastric loss of total Ca for the Ca-fortified milks and significantly decreased its apparent half time of disappearance and (c) the apparent loss of intestinal total Ca was accelerated by the presence of the CPP in the Ca-fortified milks; no significant species differences were observed among the two caprine milks and bovine milk with regard to changes in total gastrointestinal Ca with time. In this study, CPP did not increase the apparent level of soluble Ca as had previously been reported by Tsuchita et al. (2001) but as noted above the CPP affected the change in total Ca in the fortified milks. So once in the caecum, which is a highly efficient absorptive intestinal site (Favus, 1985), CPP may exert beneficial effects on Ca absorption from BCa-milk as proposed by Tsuchita et al. (2001) and from CHCa-milk and CLCa-milk as was shown in this study. Moreover, the CPP effect is immediate and readily observed in the stomach (15 min) and persists for up to 1 h.

The CPP played a role in the overall gastrointestinal disappearance at 60 min in the Ca-fortified milks (Table 5). The effect of CPP in the intestinal disappearance is quite notable since the 0·25 mg/ml, as calculated from Table 1b, is 125 nmole of peptides representing 0·8 μmoles of peptide phosphate. However, as seen in Table 5 for all Ca-milks the average amount of Ca transferred at 60 min with added CPP is 0·5 mg Ca or 12·5 μmole. This represents a ratio of 100 Ca: 1 CPP or 16 Ca for every peptide bound P. The CPP effect is in a limited sense catalytic for the uptake of Ca.

Supporting the gastrointestinal loss data is the fact that the radioactivity of plasma ^{45}Ca values were significantly ($P<0·05$) higher 5 h after the administration of BCa-milk, CHCa-, and CLCa-milk+CPP (extrinsic CPP) than in the group of rats in which CPP was not added (intrinsic CPP). The immediate effect of CPP on apparent uptake (15 to 30 min) could account for rapid uptake of the label into the plasma.

The radioactivity of ^{45}Ca in the femoral bones excised 48 h after the ingestion of the Ca-fortified milks was significantly higher in the group of rats given BCa-, CHCa-, and CLCa-milk with 0·25 mg CPP/ml (extrinsic CPP) than in the rats given Ca-fortified milks without added CPP (intrinsic CPP) ($P<0·05$). CL-CPP (5·9% α_{s1}-casein of total) produced greater ($P<0·05$) increases in Ca availability than B-CPP (39·5% α_{s1}-casein of total). This CL-CPP contains proportionately greater α_{s2}-casein (29·2% α_{s2}-casein of total), which binds more calcium than α_{s1}-casein (Kitts, 1994) and contains several phosphoserine clusters (Farrell et al. 2004). The addition of CH-CPP (25·1% α_{s1}-casein of total) also enhanced Ca availability from CHCa-milk in rats but was comparable with that of B-CPP suggesting that Ca availability depends on the ratio of α_{s2}- to α_{s1}-casein. CPP addition to BCa-, CHCa-, and CLCa-milk markedly increased Ca availability in rats ($P<0·05$),

https://doi.org/10.1017/S0022029907002725 Published online by Cambridge University Press

which is in agreement with the previous report on B-CPP by Tsuchita et al. (2001). This has been ascribed to the effect of B-CPP (extrinsic CPP) on increasing the level of soluble Ca in the digestive tract (Lee et al. 1992).

The extrinsic Ca source used in this study was $CaCO_3$. In several animal studies, Ca absorption from dairy products containing bovine milk (Buchowski & Miller, 1990) and caprine milk (Buchowski et al. 1989) was similar to control groups containing $CaCl_2$ or $CaCO_3$. Many Ca-fortified dairy products are supplemented with $CaCO_3$, which is a readily absorbable Ca source. Since the solubility of $CaCO_3$ in water is low, it is suspended in milk by adding a hydrocolloid (i.e., carrageenan, gum arabic) to prevent precipitation. These hydrocolloid stabilizers do not have an effect on the solubility of Ca after in vitro digestion (Marin & Zee, 1992). Therefore, it is unlikely that the gum arabic added to the bovine and caprine Ca-fortified milks of the present study would have an effect on Ca absorption. The solubility of the $CaCO_3$ also impinges on the use of extrinsic radio labelled minerals which involves a very controversial assumption that the extrinsic label has fully equilibrated (or exchanged) with the non-labelled intrinsic minerals of the foods being tested (Turnlund, 1991; Nickel et al. 1996). The $CaCO_3$ would be slow to exchange with the label, but Aoki et al. (1988) has shown that a good deal of the milk Ca is also hard to exchange. However, after ingestion the pH of the stomach and later that of the intestine would alter all of the initial equilibria. Note that the inorganic calcium-phosphate complex of milk would be more like brushite at low pH and more like apatite at higher pH (Bak et al. 2001). As the CPP had no effect on the milk only samples, it is likely that the CPP may be acting directly on the extrinsic $CaCO_3$. However, CPP behaves differently in humans with other Ca additives such as Ca lactate (López-Huertas et al. 2006; Teucher et al. 2006).

A key issue relative to the positive effect of CPP in calcium absorption is the CPP/Ca weight/weight ratio. Thus a CPP/Ca ratio equal to 15 has been associated with optimum Ca transport in the distal small intestine of rats in vitro (Erba et al. 2002). Studies by Tsuchita et al. (2001) demonstrated that the addition of CPP to BCa-milk increased gastrointestinal Ca disappearance in rats in vivo, but the effect was not related to the dose of CPP added in a CPP/Ca ratio between 0·1 and 0·4. In our study the theoretical CPP/Ca ratios for tryptic digestion in the native milks can be calculated from Table 1a and Farrell et al. (2004). These are 3·4 for B-milk, 3·0 for CH-milk and 3·6 for the CL-milk; addition of the respective CPP raises them to 4·2, 3·8 and 4·4 respectively. Here no effects were observed for added CPP as seen in Tables 2 through 4. In contrast positive responses were observed when B-, CH- and CL-CPP were added to their respective Ca-fortified milks. If we consider only the extrinsic CPP and the extrinsic $CaCO_3$, then the CPP/Ca ratio was 0·9. This low number argues for a direct catalytic effect of the extrinsic CPP on the extrinsic $CaCO_3$ as proposed above.

The type of meal ingested is also a key consideration in achieving optimum Ca absorption levels at different CPP/Ca ratios. Thus Ca absorption has been reported to increase with fermented dairy products (Kärkkäinen et al. 1997; Talbot et al. 1999), while Hansen et al. (1997) conclude that the slow release of newly formed CPP throughout the digestive tract may have a greater effect than preformed CPP resulting from fermentation. Results from our rat study with bovine and caprine Ca-fortified cheeses (Mora-Gutierrez et al. 2007) and the ones reported in this study with bovine and caprine Ca-fortified milks clearly suggest that B-, CH- and CL-CPP, in particular CL-CPP (highest α_{s2}-casein), exert a positive effect on Ca absorption regardless of CPP/Ca ratios, dose of CPP, and/or Ca-fortified dairy source (i.e., Ca-fortified cheese, Ca-fortified milk). These findings may be of importance in population groups who are under conditions of marginal Ca intake (Saito et al. 1998). However, the importance of bovine and caprine CPP produced during gastrointestinal digestion of bovine and caprine Ca-fortified milk (this study) or during cheesemaking from bovine and caprine Ca-fortified milk (Mora-Gutierrez et al. 2007) in human nutrition can only be obtained through absorption studies in humans.

In conclusion, these results are indicative of the overall beneficial effect of B- and C-CPP (extrinsic CPP) on facilitating gastric loss of extrinsic calcium and its intestinal uptake from the digestive tract. Only one difference between the bovine and the two caprine Ca-fortified milks was found with regard to Ca deposition in the femur. It is widely believed that Ca must be soluble to be absorbed but the level of intestinal soluble Ca did not increase with added CPP. Thus a key mechanism of CPP to enhance Ca absorption from the small intestine may lie in its ability to facilitate transport of the less soluble forms of Ca. Even though studies in humans are necessary to confirm these results, a formulation of bovine or caprine milk fortified with $CaCO_3$ and with added CPP may provide a useful approach to prevent Ca deficiency (Heaney et al. 1994).

Financial support for this research was provided in part by grant No. 01-35503-10992 of the USDA, National Research Initiative Competitive Grants Program.

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