

The effect of casein phosphopeptides on calcium absorption from calcium-fortified milk in growing rats

H. Tsuchita*, T. Suzuki, and T. Kuwata

Nutritional Science Research Institute, Meiji Milk Products Co. Ltd, 1-21-3 Sakaecho, Higashimurayama, Tokyo 189-8530, Japan

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The effect of casein phosphopeptides (CPP) prepared from bovine casein by enzymatic hydrolysis (extrinsic CPP) on Ca absorption from Ca-fortified milk was studied in young male rats, in comparison with that produced from casein in the small intestine (intrinsic CPP). The gastrointestinal Ca disappearance (Ca ingested – (gastric Ca + intestinal Ca)) was calculated as an indirect measurement of Ca absorption. After being fasted overnight, the animals were given 2.0 ml Ca-fortified milk (30 g fat, 35 g protein, 2.7 g Ca/kg) without or with 1.0 mg extrinsic CPP/ml, by gastric intubation. The intestinal soluble Ca level after 15 min and the gastrointestinal Ca disappearance after 15 and 30 min in the rats given Ca-fortified milk with 1.0 mg extrinsic CPP/ml were significantly higher than these figures in the rats given Ca-fortified milk without CPP ($P < 0.05$). When the rats were given unfortified milk (1.35 g Ca/kg) in another reference experiment, no significant effect on intestinal soluble Ca and gastrointestinal Ca disappearance was apparent from the addition of CPP to milk. Ca availability was estimated by measuring ^{45}Ca -deposits in the bones of rats 48 h after being given 2.0 ml Ca-fortified milk labelled with ^{45}Ca (180 kBq/2 ml) with or without 0.25 mg CPP/ml. The levels of ^{45}Ca radioactivity of the femur and tibia from the rats given Ca-fortified milk with extrinsic CPP were significantly higher than those from the control group ($P < 0.05$). These results suggest that the addition of CPP to Ca-fortified milk could increase Ca absorption by growing rats mainly from CaCO_3 added to the milk. The mechanism of CPP related to the interaction of CPP and Ca in the gastrointestinal tract is discussed.

Calcium absorption: Casein phosphopeptide: Milk

Dairy foods are the main source of Ca in the diet. Milk and milk products are well known for their good Ca availability in man: (Recker & Heaney, 1985; Recker *et al.* 1988) and in experimental animals (Wong & LaCroix, 1980; Buchowski & Miller, 1990). The good availability of Ca from milk could partly reflect the enhancing effect of lactose (Miller, 1989) and casein phosphopeptides (CPP) on Ca absorption. In addition, colloidal Ca and the Ca:P ratio may be involved in the good Ca availability from milk. Dairy product manufacturers are now marketing high-Ca milk in response to the increased publicity of this mineral (Tunick, 1987). In this case, an enhancing effect of lactose or CPP in Ca-fortified milk on Ca absorption could be expected. However, the contribution of these components to the Ca availability of Ca-fortified milk has not been clarified.

When rats were fed with a bovine-casein diet, Ca absorption was enhanced due to CPP derived from digestion of the protein (intrinsic CPP; Naito *et al.*, 1972; Naito & Suzuki, 1974). Feeding CPP prepared from bovine casein by enzymatic hydrolysis (extrinsic CPP) has also shown an enhancing effect on Ca absorption by animals (Lee *et al.* 1992; Tsuchita *et al.* 1993). The effect of extrinsic CPP on Ca absorption could reflect the Ca-solubilizing action which inhibits the formation of insoluble calcium phosphate in the intestine (Sato *et al.* 1986). However, some reports have not shown such an effect of extrinsic CPP on Ca absorption (Brommage *et al.* 1991; Yuan & Kitts, 1991). This discrepancy might have originated from differences in the experimental design such as the dietary Ca level or the Ca status of the animals. In addition, the extent to which the interaction between

Abbreviation: CPP, casein phosphopeptides.

* **Corresponding author:** Dr H. Tsuchita, fax +81 42 395 1829, email hiroschi_tsuchita@meiji-milk.com

extrinsic CPP and Ca occurs in the gastrointestinal tract may be a key factor for this inconsistency. Since the physico-chemical state of Ca in fluid milk is not homogenous (Holt, 1985), different interactions between CPP and the mineral during the digestion of Ca-fortified milk and unfortified milk may occur. In this context, the interaction between extrinsic CPP and Ca should be clarified in both the small intestine and in the stomach. The aim of this present study is to evaluate the effect of extrinsic CPP on Ca absorption from Ca-fortified milk and unfortified milk in growing male rats, focusing on the difference in the effects of intrinsic CPP and extrinsic CPP. We conducted experiments to: (1) clarify the Ca-solubilizing effect of extrinsic CPP in the stomach as well as in the intestine; (2) assess the level of extrinsic CPP; and (3) estimate the effect of extrinsic CPP on Ca availability.

Materials and methods

Milk preparation

Milk samples were prepared by dissolving skimmed-milk powder in recombined milk made from skimmed milk and cream (30 g milk fat, 35 g milk protein and 52 g lactose/kg). Ca and/or CPP was added or not according to the experimental protocols (Table 1). A colloidal solution of CaCO₃ was stabilized by adding gum arabic before mixing with milk. The concentration of gum arabic in the milk was 0.1 mg/ml. CPP was dispersed with skimmed-milk powder and dissolved in milk. CPP was a commercial product prepared from a tryptic hydrolysate of bovine whole casein (CPP-III; Meiji Seika Kaisha, Ltd, Tokyo, Japan), whose content of phosphopeptides was estimated to be 860 g/kg DM by an HPLC analysis (Saito *et al.* 1998) and the N:P ratio was 8.0. The content of Ca in each milk sample was as follows: unfortified milk 1.35 mg/ml, Ca-fortified milk 2.7 mg/ml (Table 1). The samples of milk used in Experiment 4 were prepared by adding 50 µl ⁴⁵CaCl₂ in water (specific activity 36 kBq/µl, Ca content 55 ng/µl; NEM Life Science Products, Inc., Boston, MA, USA) to 20.0 ml Ca-fortified milk with or without CPP, the solution being held overnight whilst being continuously stirred at 10°C.

Animals

Male Sprague-Dawley rats (6-week-old; Japan SLC, Shizuoka, Japan) were housed in individual Al cages in a temperature-controlled (23 ± 2°C) room with 50 ± 10 % humidity and a 12 hour light–dark cycle, and were fed on a

stock diet (CE-7; Clea Japan, Tokyo, Japan) for a 10 d adaptation period. The composition of the stock diet was as follows: Ca 1050 mg/kg, P 930 mg/kg, crude protein 176 g/kg. The care of the rats in this study conformed with *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1985).

Experimental procedures

Four experiments were carried out: Experiment 1 and 2 were performed to clarify the Ca-solubilizing effect of extrinsic CPP in Ca-fortified milk or unfortified milk; Experiment 3, to assess the effect of the level of extrinsic CPP on Ca absorption; and Experiment 4, to evaluate the effect of extrinsic CPP on Ca availability by using ⁴⁵Ca.

Experiment 1. After being fasted overnight (11 ± 2 h), sixty-three rats were assigned to two groups of twenty-eight animals each and to one group of seven animals, each group having a similar mean body weight of 217 (SD 3.1) g. One group of twenty-eight animals was given 2.0 ml Ca-fortified milk with added CPP (1.0 mg/ml) by gastric intubation. At times of 15, 30, 60 and 120 min after the administration, seven animals were chosen at random to measure the Ca concentration in their gastrointestinal contents. The other group of twenty-eight animals was given 2.0 ml Ca-fortified milk without CPP orally, and treated similarly. A group of seven animals was not given anything and was treated similarly to act as a control group. Animals randomly chosen at each time were anaesthetized by inhalation of isoflurane (1.5–3.5 % in a mixture of O₂–CO₂ (95:5, v/v)). An incision was made in the abdomen, and the cardia, pylorus and ileocaecum were ligated. The stomach and small intestine were excised, and the contents were recovered by flushing the interior with 5.0 ml distilled deionized water.

Experiment 2. Another batch of sixty-three rats was subjected to the same procedure as that in Experiment 1, except that the milk samples were not fortified with CaCO₃ (unfortified milk).

Experiment 3. Four groups of fourteen rats each were given 2.0 ml Ca-fortified milk with different levels of CPP orally (Table 1). After this administration (30 and 60 min), seven animals were randomly chosen from each group to measure the Ca concentration in the gastrointestinal contents.

Experiment 4. Two groups of seven rats each were given orally 2.0 ml of one of the Ca-fortified milk samples labelled with ⁴⁵Ca, to which CPP had been added (0.25 mg/ml) or not (Table 1). Blood samples (200 µl) were taken from the tail vein by making a small incision at 20 and

Table 1. Composition of milk samples

Experiment no.	Milk*	CPP (mg/ml)	Time point (min)†
1	Ca-fortified	0, 1.0	15, 30, 60, 120
2	Unfortified	0, 1.0	15, 30, 60, 120
3	Ca-fortified	0, 0.25, 0.5, 1.0	30, 60
4	Ca-fortified	0, 0.25	20, 40, 60, 180, 360, 540, 1440, 2040

CPP, casein phosphopeptides.

* The Ca content of Ca-fortified milk and unfortified milk was 2.7 mg/ml and 1.35 mg/ml respectively.

† Time point at which gastrointestinal Ca was measured (*n* 7 at each time point).

40 min and at 1, 3, 6, 9, 24 and 34 h after the administration to determine the ^{45}Ca radioactivity. The incision wound was treated with surgical adhesive after the blood sampling. The animals were killed by overdosing with sodium pentobarbital 48 h after administering the radioisotope, and the right femur and tibia were excised to determine the ^{45}Ca radioactivity.

Analytical methods

The gastric and intestinal contents were centrifuged (15 000 *g*, 20 min). The precipitate was dried and ashed overnight in a muffle furnace at 600°C (insoluble Ca). The ashed sample was then dissolved in 1.0 M-HNO₃ for Ca determination by an atomic absorption spectrophotometer (AA-6500F; Shimadzu, Kyoto, Japan), the concentration of Ca in the supernatant being directly determined by the instrument (soluble Ca). The bone samples were digested with a mixture of 15:7 M-HNO₃ (three volumes) and 9.2 M-HClO₄ (one volume) at 200°C for 2 h. The radioactivity of ^{45}Ca in the plasma and digested bone was measured by a liquid scintillation analyser (TRI-CARB 2700TR; Packard Japan, Tokyo, Japan).

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 content 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.

Statistical analysis

Data reported in the figures and tables are expressed as mean values with their standard errors. In all experiments, one-way ANOVA with a subsequent Bonferroni test was used at each time point to test for any significant differences in the mean values of the experimental groups ($P < 0.05$). Data from Experiments 1, 2 and 3 were separately analysed by two-way ANOVA, with CPP addition and time as the main effect. Data from Experiment 4 were analysed by ANOVA for repeated measures. The analysis was performed by using StatView version 4.0 statistical software (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Gastric emptying

In Experiment 1, the gastric Ca level in the rats given Ca-fortified milk with added CPP was significantly lower than that in the rats given Ca-fortified milk without CPP at the time points of 15 and 30 min ($P < 0.05$; Fig. 1). The addition of CPP to Ca-fortified milk significantly modified the gastric Ca content ($P < 0.05$). The gastric insoluble Ca level in the rats given Ca-fortified milk with added CPP was significantly lower than that in the rats given

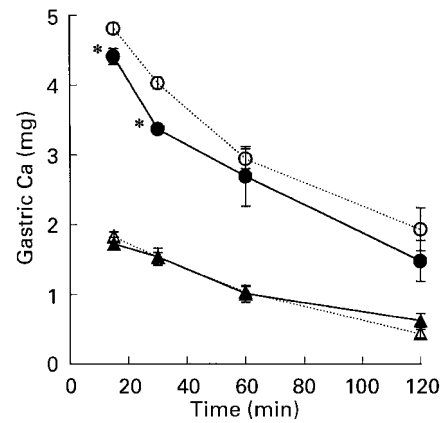


Fig. 1. Total Ca in the gastric contents from rats given Ca-fortified milk with 1.0 mg casein phosphopeptides (CPP)/ml (●) or without CPP (○) in Experiment 1 and unfortified milk with CPP (▲) or without CPP (△) in Experiment 2. For details of composition of milks see Table 1 and p. 6, and for procedures see p. 6. Values are means for seven rats per group with their standard errors represented by vertical bars. The values from the rats not given milk were as follows (mg): Experiment 1, 0.130 (SE 0.009); Experiment 2, 0.053 (SE 0.014). Mean values were significantly lower than those of the control group at the same time point in Experiment 1: * $P < 0.05$. Data were analysed separately in each experiment by two-way ANOVA. In Experiment 1: CPP effect, $P < 0.05$; time effect, $P < 0.05$; CPP × time interaction, NS. In Experiment 2: CPP effect, NS; time effect, $P < 0.05$; CPP × time interaction, NS.

Ca-fortified milk without CPP at the time points of 15 and 30 min ($P < 0.05$; data not shown). Since the amount of Ca ingested by each animal was constant (5.4 mg/animal in Experiment 1, and 2.7 mg/animal in experiment 2), a low level of gastric Ca indicates a high level of gastric emptying of Ca. In contrast, when the rats were given unfortified milk in Experiment 2, the addition of CPP did not affect the gastric Ca level (Fig. 1).

Intestinal soluble calcium

The amount of soluble Ca in the intestinal contents increased with increasing time after the ingestion of the milk samples. Only in Experiment 1 was the intestinal soluble Ca level in the rats given Ca-fortified milk with CPP significantly higher than that in the rats given Ca-fortified milk without CPP 15 min after the ingestion (Fig. 2). Although a similar trend was apparent at the time points of 30, 60 and 120 min, the addition of CPP to Ca-fortified milk did not significantly affect the level of intestinal soluble Ca (Fig. 2). The intestinal insoluble Ca level in the rats given Ca-fortified milk with CPP was similar to that in the rats given Ca-fortified milk without CPP 15 and 30 min after the ingestion, but tended to be higher after 60 min (data not shown). When the rats were given unfortified milk in Experiment 2, the addition of CPP had no significant effect on the amount of soluble Ca (Fig. 2).

Gastrointestinal calcium disappearance

The gastrointestinal Ca disappearance in the rats given Ca-fortified milk with CPP was significantly higher than that in the rats given Ca-fortified milk without CPP 15 and 30 min

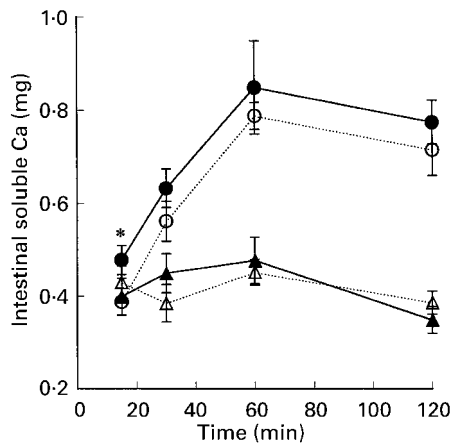


Fig. 2. Soluble Ca in the small intestinal contents from rats given Ca-fortified milk with 1.0 mg casein phosphopeptides (CPP)/ml (●) or without CPP (○) in Experiment 1 and unfortified milk with CPP (▲) or without CPP (△) in Experiment 2. For details of composition of milks see Table 1 and p. 6, and for procedures see p. 6. Values are means for seven rats per group with their standard errors represented by vertical bars. The values from the rats not given milk were as follows (mg): Experiment 1, 0.196 (SE 0.017); Experiment 2, 0.159 (SE 0.026). Mean value was significantly higher than that of the control group at the same time point in Experiment 1: * $P < 0.05$. Data were analysed separately in each experiment by two-way ANOVA. In Experiment 1: CPP effect, NS; time effect, $P < 0.05$; CPP \times time interaction, NS. In Experiment 2: CPP effect, NS; time effect, NS; CPP \times time interaction, NS.

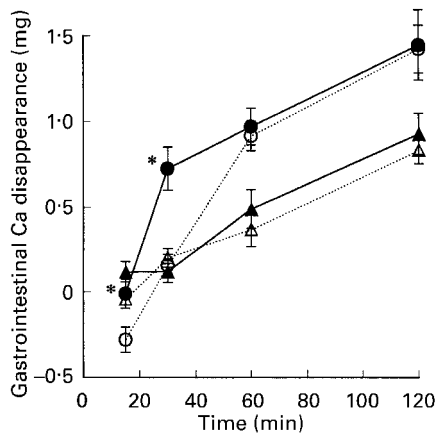


Fig. 3. Gastrointestinal Ca disappearance by rats given Ca-fortified milk with 1.0 mg casein phosphopeptides (CPP)/ml (●) or without CPP (○) in Experiment 1 and unfortified milk with CPP (▲) or without CPP (△) in Experiment 2. For details of composition of milks see Table 1 and p. 6, and for procedures see p. 6. Values are means for seven rats per group with their standard errors represented by vertical bars. The values from the rats not given milk were as follows (mg): Experiment 1, -0.427 (SE 0.047); Experiment 2, -0.474 (SE 0.227). Mean values were significantly higher than those of the control group at the same time point in Experiment 1: * $P < 0.05$. Data were analysed separately in each experiment by two-way ANOVA. In Experiment 1: CPP effect, $P < 0.05$; time effect, $P < 0.05$; CPP \times time interaction, NS. In Experiment 2: CPP effect, NS; time effect, $P < 0.05$; CPP \times time interaction, NS.

Table 2. Gastrointestinal calcium disappearance by rats given calcium-fortified milk at 30 min and 60 min after ingesting milk* (Mean values with their standard errors for seven rats per group)

CPP level (mg/ml)	Gastrointestinal Ca disappearance (mg)			
	30 min		60 min	
	Mean	SE	Mean	SE
0	-0.254 ^a	0.166	0.286 ^a	0.181
0.25	0.242 ^b	0.085	0.788 ^b	0.076
0.5	0.213 ^b	0.071	0.692 ^b	0.122
1.0	0.253 ^b	0.064	0.744 ^b	0.080

CPP, casein phosphopeptides.

* For details of composition of milks see Table 1 and p. 6, and for procedures see p. 6.

^{a,b}Mean values within a column with unlike superscript letters were significantly different ($P < 0.05$).

after the ingestion ($P < 0.05$; Fig. 3). The addition of CPP to Ca-fortified milk significantly affected the gastrointestinal Ca disappearance ($P < 0.05$). In contrast, when the rats were given unfortified milk, the addition of CPP had no significant effect on the gastrointestinal Ca disappearance (Fig. 3).

The gastrointestinal Ca disappearance in the rats given Ca-fortified milk with 0.25, 0.5 or 1.0 mg CPP/ml was significantly higher than that in the animals given this milk without CPP 30 and 60 min after the ingestion ($P < 0.05$; Table 2). However, there was no significant difference related to the level of added CPP among the three groups given samples with CPP addition.

⁴⁵Ca in the blood and bones

The radioactivity of plasma ⁴⁵Ca from the rats given Ca-fortified milk with 0.25 mg CPP/ml tended to be slightly higher than that from the animals given milk without CPP 20 and 40 min, and 1, 3 and 6 h after the ingestion, although there was no statistical significance in the difference (Fig. 4). There was also no significant difference in the area under the curve among the groups (data not shown).

The radioactivity of the femur and tibia from the rats given Ca-fortified milk with CPP was significantly higher than that from the control group ($P < 0.05$; Table 3).

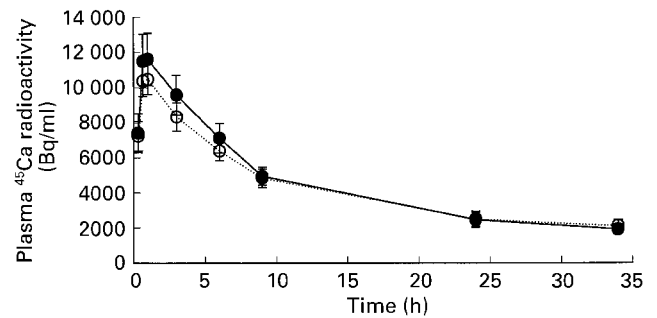


Fig. 4. Plasma ⁴⁵Ca radioactivity from rats given Ca-fortified milk with 0.25 mg casein phosphopeptides (CPP) (●) or without CPP (○). For details of composition of milks see Table 1 and p. 6, and for procedures see pp. 6–7. Values are means for seven rats per group with their standard errors represented by vertical bars. Data were analysed by two-way ANOVA: CPP effect, NS; time effect, $P < 0.05$; CPP \times time interaction, NS.

Table 3. ^{45}Ca radioactivity in the right femur and tibia from rats given calcium-fortified milk†
(Mean values with their standard errors for seven rats per group)

	^{45}Ca radioactivity ($\times 10^4$ Bq/g bone)			
	Femur		Tibia	
	Mean	SE	Mean	SE
Control	51.7	2.5	49.6	2.6
CPP (0.25 mg/ml)	61.8*	3.2	60.2*	3.1

CPP, casein phosphopeptides.

Mean values were significant different from corresponding control values:
* $P < 0.05$.

† For details of composition of milks see Table 1 and p. 6, and for procedure see pp. 6–7.

Discussion

The present data indicate that the addition of CPP to Ca-fortified milk could increase Ca absorption by young male rats. In contrast, no effect of extrinsic CPP on Ca absorption was apparent when the animals were given unfortified milk. This suggests that extrinsic CPP enhanced the Ca absorption mainly from CaCO_3 added to the milk. The gastrointestinal Ca disappearance was observed at an earlier time after the ingestion of Ca-fortified milk due to the effect of extrinsic CPP: the gastrointestinal Ca disappearance 15 and 30 min after the rats had been given Ca-fortified milk with 1.0 mg CPP/ml was significantly greater than that after the rats had been given Ca-fortified milk without CPP ($P < 0.05$). Although the gastrointestinal Ca disappearance does not equal the apparent Ca absorption by metabolic measurement, the higher gastrointestinal Ca disappearance could have been associated with an increase in Ca absorption. The radioactivity of ^{45}Ca in the femur and tibia excised 48 h after the ingestion of milk was significantly higher in the rats given Ca-fortified milk with 0.25 mg CPP/ml than in the rats given Ca-fortified milk ($P < 0.05$), which indicates increased Ca availability (Buchowski *et al.* 1989). The radioactivity of plasma ^{45}Ca in the rats given Ca-fortified milk with 0.25 mg CPP/ml was slightly higher than that in the rats given control milk up to 6 h after the ingestion, but no significant difference could be detected at each time point ($P < 0.05$). This might have been caused by low exchangeability of the tracer in Ca-fortified milk. The relative exchangeability of the extrinsic tracer in bovine milk was 70 % (Yamanouchi & Yoneda, 1977). In addition, half the amount of Ca in Ca-fortified milk was insoluble CaCO_3 , which suggests a lower exchangeability. Therefore, the radioactivity of plasma ^{45}Ca was over-estimated (Buchowski *et al.* 1989), and the effect of extrinsic CPP might not have been clearly shown.

Since the gastrointestinal Ca disappearance was calculated by the amount of Ca remaining in the gastrointestinal tract, the value included the amount of Ca that had moved into the caecum. The caecum has been reported as a highly efficient absorptive site (Favus, 1985), and Ca absorption across the caecum could have physiological importance in regulating intestinal Ca homeostasis in rats (Karbach & Feldmeier, 1993). The radioactivity of plasma ^{45}Ca from the rats given Ca-fortified milk with CPP tended to be

higher than that from the rats given control milk up to 6 h after the ingestion. This suggests that the extrinsic CPP might have remained in the caecum as well as in the small intestine until at least 6 h after ingesting the milk. The presence of CPP in the caecum is supported by the report that part of the CPP formed in the small intestine was detected in the faeces from rats that had been fed on a casein-based diet (Pelissier *et al.* 1981; Kasai *et al.* 1992). Further experiments are needed to elucidate the digestibility of extrinsic CPP and its influence on Ca absorption in the caecum.

Among many reports on Ca absorption from dairy products (Smith *et al.* 1985; Sheikh *et al.* 1987; Recker *et al.* 1988; Nickel *et al.* 1996), there has been little data presented on Ca-fortified milk. In a study using the double-label stable isotope technique in human subjects, no clear difference was apparent in the efficiency of Ca absorption between skimmed milk and Ca-enriched skimmed milk (Fairweather-Tait *et al.* 1989). In the present study, insoluble CaCO_3 was suspended in milk by adding a hydrocolloid to prevent precipitation. When some hydrocolloids were added to milk products, the additives had no effect on the solubility of Ca after *in vitro* digestion (Marin & Zee, 1992). This evidence suggests that hydrocolloids added to Ca-fortified milk would have been unlikely to affect Ca absorption in the present results.

Approximately one-third of the Ca in bovine milk is present in the serum phase as free Ca^{2+} or is predominantly complexed by citrate and phosphate. The remaining two-thirds is partly incorporated in micellar calcium phosphate and partly bound directly to casein (Holt, 1985). Such physico-chemical characteristics of Ca in milk would affect the interaction with CPP. When being digested in the stomach, diffusible or free Ca^{2+} will be released from unfortified milk as well as CaCO_3 . However, the gastric Ca in rats given unfortified milk was not affected by the addition of CPP in Experiment 2. This indicates that there was little interaction between CPP and the Ca released from unfortified milk, especially during the first 60 min of the experiment. One reason for this would be that CPP added to the milk could be emptied from the stomach faster than Ca was liberated from milk (Holt & Hukins, 1991). Alternatively, diffusible Ca released from unfortified milk might have been emptied from the stomach in a stable matrix with the protein hydrolysate, resulting in no interaction with extrinsic CPP. A mineral-rich peptide fraction including phosphopeptides has been recovered from the tryptic hydrolysate of bovine casein micelles (Gagnaire *et al.* 1996), although the gastric emptying characteristics were not referred to. The increase in gastric emptying of Ca by the addition of extrinsic CPP to Ca-fortified milk could have been due to the effect of CPP on increasing soluble Ca in the stomach. Increasing soluble Ca is the key mechanism of CPP to augment Ca absorption from the small intestine. Therefore, it is effective for CPP to move simultaneously with Ca from the stomach to the small intestine, because the interaction between CPP and Ca would easily take place in the small intestine. When all dietary Ca was bound to CPP in advance, good Ca availability has been shown (Tsuchita *et al.* 1996), where any interference with any interaction between CPP and Ca from the digestion of other

food ingredients would have been reduced. It is suggested that the effect of extrinsic CPP on Ca absorption could depend on the degree of interaction occurring between Ca and CPP in the stomach as well as in the small intestine.

The present result using growing rats should not be directly extrapolated to man, and dietary intervention studies are needed to evaluate the addition of CPP to Ca-fortified milk and other milk products. Improvement of the availability of Ca from milk products may be of help for those people with low Ca absorption (Heaney *et al.* 1994).

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