

Effects of bioactive substances in milk on mineral and trace element metabolism with special reference to casein phosphopeptides

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Bioactivity of phosphopeptides yielded after tryptic hydrolysis of casein (CPP) was reported more than 50 years ago when CPP were found to improve calcium balance in rachitic newborns. Several investigations have been carried out to study the effects of CPP mainly on calcium metabolism but also on other minerals like iron and zinc. Most of the experiments were *in vitro* studies or short-term experiments like the effects of CPP after single meals or their effect on mineral disappearance from intestinal everted sac or ligated loop. Investigations on calcium balance were also mainly short term, i.e. 3–4 weeks, and mainly done in rats. A few experiments have been carried out in minipigs, an animal model that is closer to the human than the rat. Studies in human were rare and short term. To date a variety of other peptides have been isolated after enzymatic hydrolysis, and some have been investigated for bioactivity, with equivocal findings. Bioactivity of phosphopeptides seemed to be more obvious when investigations were done *in vitro* or short term. Results were less clear in metabolic balance studies, especially under physiological conditions. The composition of the basal diet, i.e. content of calcium and phytate, or the protein source had a significant impact on the effect of phosphopeptides. It was concluded that phosphopeptides revealed positive effects on mineral solubility and absorbability, and bone mineralisation under certain experimental conditions. Accordingly they could have a beneficial effect on bone health for some groups of the population.

Phosphopeptides: Calcium absorption: Bone health

Potential physiological action – short-term experiments

Background

Information on bioactive substances in milk which influence mineral metabolism is based on observations by Mellander (1947, 1950, 1963). Mellander (1950) incubated casein with pepsin and pancreatic juice. A fraction of peptides was yielded that were resistant to further degradation by proteolytic enzymes. The peptides were shown to contain phosphoserine-rich sequences and increase calcium balance by about 39–78 % in rachitic newborns. Later, several authors demonstrated that phosphopeptides from tryptic digestion of casein (CPP) had a high affinity to calcium ions *in vitro* (Reeves & Latour, 1958; Naito *et al.* 1972; Naito & Suzuki, 1974). However, when calcium binding was studied in the presence of CPP using an Ussing chamber no effect was seen (Li *et al.* 1989).

CPP liberation

Naito and co-workers reported that CPP were liberated *in vivo* after feeding β -casein to rats (Naito *et al.* 1972; Naito & Suzuki, 1974). It was found that liberation of CPP

depended on digestion time of the meal. After feeding 200 g casein/kg diet, liberation of CPP in the small intestine of rats occurred at 2.5 and 5.5 h, but not at 13.5 and 25.5 h postprandially. The simultaneous feeding of casein and a basal diet proved to be necessary to release CPP in these experiments (Lee *et al.* 1980). Feeding casein alone (Lee *et al.* 1980), or the amino acid mixture equivalent to 200 g casein/kg diet (Lee *et al.* 1983), did not have this effect.

Effect of intact protein on indicators of calcium absorption

Dietary casein, when compared to egg albumin augmented the level of ^{45}Ca in the soluble fraction of chyme of the small intestine (Lee *et al.* 1979). The rate of disappearance of ^{45}Ca from the intestinal ligated loop was highest in rats that had been fed on casein when compared to gluten, gelatine, dephosphorylated casein (Sato *et al.* 1983) whey protein or soy protein isolate (Nagasawa *et al.* 1991; Kitts *et al.* 1992). From the work of Sato *et al.* (1983) it became clear that the phosphorylated form of casein was a prerequisite, and therefore might be the cause for the enhancing effect of this protein on calcium solubility compared to other proteins.

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Effect of isolated CPP on indicators of calcium absorption

Mykkänen & Wasserman (1980) observed a dose dependency of CPP. They demonstrated that the increase in duodenal ^{47}Ca absorption by CPP was more prominent in rachitic chicks than in control animals using an *in situ* experimental approach (ligated loop). Moreover, this effect was dose-dependent and was obvious only with a 1 mM, but not with a 25 mM solution of CPP. Such an effect was not observed if a tryptic hydrolysate of bovine serum albumin or different quantities of phosphate were injected. When different doses of CPP were incubated *in vitro* (everted sac method), higher amounts of calcium were transported out of the ileal lumen in a dose-dependent manner. Since the stimulatory effect on calcium absorption was not vitamin D-dependent, the authors concluded that CPP acted on the passive absorption pathway. The formation of soluble complexes of CPP with calcium, prevented precipitation of calcium phosphates and consequently raised the amount of soluble calcium (Mykkänen & Wasserman, 1980). This assumption was confirmed in rats (Lee *et al.* 1979). The peptic hydrolysate of casein was twice more efficient than that of soy protein in raising the solubility of calcium *in vitro* (Sato *et al.* 1986).

Sato *et al.* (1986) isolated CPP from chyme and injected them together with a $^{45}\text{CaCl}_2$ solution into the ligated loop of rats. After 2 h of incubation the rate of disappearance of calcium was four times and the rate of calcium incorporation into bone three times higher than in the control group. In addition, dietary calcium was absorbed 15 % more efficiently than endogenous calcium.

Effect on mineral incorporation into bone

Beside the effect on calcium absorption, dietary casein or CPP also stimulated calcium incorporation into bone. In the rat, instilled $^{45}\text{CaCl}_2$ disappeared sooner from the intestinal ligated loop and accumulated more efficiently in the femur when casein was fed compared to soy isolate (Sato *et al.* 1986). In rachitic chicks ^{47}Ca accumulation in the tibia was higher in the presence of CPP (Mykkänen & Wasserman, 1980).

In young male rats fed on a diet containing 0.4 % calcium and as little as 0.5 % CPP for 3 weeks, ectopic bone induced in decalcified bone matrix implants was analysed (Matsui *et al.* 1994). In this model for bone mineralisation a higher content of calcium, lower values for concentration of tartrate-resistant acid phosphatase and less osteoclasts in implants were shown (Matsui *et al.* 1994). In another experiment, CPP increased calcification of cultured embryonic rat bone explants and calcium solubility in the culture medium (Gerber & Jost, 1986). These findings suggest suppression of bone resorption and stimulation of bone mineralisation by CPP.

CPP and cariogenicity

Apart from a stimulatory effect of milk protein-derived phosphopeptides on mineral absorption and thereby on bone mineralisation, some dairy products, especially cheese, had an anticariogenic effect in animal and human

caries models (Rosen *et al.* 1984; Harper *et al.* 1986; Silva *et al.* 1986, 1987). This effect was attributed to the phosphoprotein casein and to the calcium phosphate content of cheese (Silva *et al.* 1987). A direct positive effect of CPP on remineralisation of resected molar slab models was demonstrated by Reynolds (1997). CPP stabilised the calcium phosphate in solution and thus maintained a high concentration gradient of these minerals, a prerequisite for high rates of enamel remineralisation. In addition it was shown that CPP reduced adhesion of cariogenic microbes (*Streptococcus mutans*) (Schüpbach *et al.* 1996). Phosphopeptides are also present in human saliva (Minaguchi *et al.* 1988; Madapallimattam & Bennick, 1990). Interestingly, they were able to inhibit hydroxyapatite formation, and could thus prevent undesired mineral formation on the tooth surface.

Interaction between diet and CPP

CPP increased calcium absorption from ligated ileal loops more markedly when added to a diet that contained soy protein isolate in place of casein (Kitts *et al.* 1992), indicating that dietary casein *per se* must have had a stimulating effect on calcium solubility and thus disappearance from the gut.

In the rat pup, not only calcium but also zinc absorption was improved after gastric intubation of Ca-containing CPP in the presence of phytate. No effect was found when a Na-containing CPP preparation or casein or whey protein was added (Hansen *et al.* 1996). Further, the authors tested two types of infant formulas based on oat or soy. Zinc absorption was highest when the formula based on oat contained Ca-CPP and was significantly improved when Na-CPP, casein or whey protein was added to the oat-based formula. When the formula was based on soy, only Na-CPP was effective (no values were given for the effect of Ca-CPP). Calcium absorption from oat-based diet was improved if Ca-CPP or casein was added. From the soy-based diet only supplementation with Ca-CPP improved calcium absorption whereas Na-CPP reduced calcium absorption significantly. Zinc binding and uptake was higher after incubating Caco-2 cell culture with low concentrations of CPP, in contrast to high concentrations, which had an inhibitory effect (Hansen *et al.* 1996).

In summary, the published data on the effect of CPP/casein on mineral solubility and absorption were inconsistent, which may partly be due to the diversity of experimental approaches. Nevertheless, CPP may have a potential to improve mineral balance of certain groups of the population that might be at risk for intakes below the recommended levels.

Meanwhile several potentially bioactive casein phosphopeptides have been described with different contents of phosphorylated serine residues. Gagnaire *et al.* (1996) were able to identify 14 different CPP after tryptic hydrolysis, ultracentrifugation, reversed-phase HPLC on-line electrospray ion source-mass spectrometry. Four CPP were isolated from α_{S1} -, two from α_{S2} -, and four from β -casein. Several different commercial protease preparations from bacterial, fungal, plant and animal origin can be used to yield CPP (McDonagh & FitzGerald, 1998).

The presence of a diversity of CPP after tryptic hydrolysis of casein, the observation that such CPP increased soluble calcium and calcium absorption from the ligated loop (Yuan & Kitts, 1991), and exacerbated calcium incorporation into bone *in vivo* (Sato *et al.* 1986) pointed to an exciting potential of such phosphopeptides. The detection of CPP in the chyme of the small intestine of rats (Lee *et al.* 1980) or pigs (Meisel & Frister, 1988) and in the faeces of rats (Kasai *et al.* 1992, 1995) following feeding of casein are compatible with bioactivity of CPP. It becomes obvious that nutrition studies are now required to establish physiological action *in vivo* and that only long-term studies can confirm improvement of not only calcium absorption but also calcium bioavailability. Therefore strengthening of bone under long-term conditions, i.e. adaptation by hormonal regulation, should be the parameter to indicate health benefits.

Examples of established *in vivo* functions – long-term experiments

Effect of intact protein

When rats were fed on casein compared to whey protein in the presence of phytate for 24 weeks, calcium retention was significantly higher (Scholz-Ahrens *et al.* 1993). This effect was obvious only in older adult rats, at the age of 29 weeks, after 24 weeks on diets. Stimulation of calcium retention was not observed when rats were young and rapidly growing, at the age of 11 weeks; after 6 weeks on diets or in younger adult rats (at the age of 20 weeks, after 16 weeks on diets). In older rats the capacity for calcium absorption and retention is lower than in younger animals (Scholz-Ahrens *et al.* 1996), probably because of reduced demands but also possibly due to decreased kidney and other organ function. It needs to be established whether aged subjects with presumably higher demands but reduced organ function, like postmenopausal women, would preferably benefit from CPP.

The minipig is well known as an animal model that is very close to the human, particularly with respect to nutrition physiology. Three experiments were performed in this species to study two milk proteins with different potential for CPP release and their effect on calcium and bone metabolism (Scholz-Ahrens *et al.* 1989, 1990a, b, c, 1991). In study one, 8-week-old weanling miniature piglets were fed a semisynthetic diet with 15 % protein either as acid casein or whey protein concentrate for 12 weeks. During this time a doubling of weight was achieved. After 8 weeks calcium retention was significantly lower and plasma parathyroid hormone (PTH) tended strongly to be higher in minipigs fed casein compared to animals on whey protein concentrate (Table 1). After 12 weeks, the fraction of soluble calcium/total calcium in the chyme of small intestine was significantly lower whereas plasma concentration of calcium and PTH were significantly higher following casein compared to whey protein (Table 1, Scholz-Ahrens *et al.* 1989, 1990a). Body weight gain, calcium excretion with faeces or urine, apparent calcium absorption and plasma concentration of calcitonin were not different (Scholz-Ahrens *et al.* 1989). There was no

Table 1. Effect of dietary casein and whey protein concentrate on parameters of Ca metabolism in growing minipigs on 0.3 % dietary Ca

		Casein		WPC	
		Mean	SEM	Mean	SEM
After 8 weeks					
Ca-retention	g/18 d	11.54	0.02	13.08*	0.10
Plasma					
Ca	mmol/l	2.72	0.05	2.75	0.02
PTH	nmol/l	0.12	0.02	0.07	0.01
After 12 weeks					
Soluble Ca/total Ca†		2.60	0.71	6.90*	0.71
Plasma					
Ca	mmol/l	2.60	0.06	2.39*	0.03
PTH	nmol/l	0.14	0.02	0.09*	0.01

PTH, parathyroid hormone; WPC, whey protein concentrate.

* $P < 0.05$; $n = 8$.

† In the chyme of distal small intestine.

significant difference in one of the parameters measured in femora such as wet weight, ash content, calcium concentration, bone density (g hydroxylapatite/cm³) measured by X-ray or bending moment between the dietary groups (Scholz-Ahrens *et al.* 1990b). The lower portion of soluble calcium in the small intestine at the end of the experiment after 12 weeks thus may not reflect less solubilisation. It may indicate an adaptation process towards faster absorption following casein. Nevertheless, the sum of single metabolic changes in pigs fed casein compared to whey protein concentrate had no persisting effect on bone mineralisation.

A second study was performed with identical study design except for dietary calcium content, which was 0.8 %. Plasma ionic or total calcium, organic phosphate and alkaline phosphatase, soluble calcium in chyme of the distal small intestine were not different between the two diets (not shown). Higher plasma concentration of PTH after feeding a casein-containing diet was confirmed, at least in tendency ($P < 0.1$, Scholz-Ahrens *et al.* 1990c, Table 2). Concentrations of 25-(OH)-vitamin D₃ were significantly higher in minipigs on diets containing casein compared to whey protein concentrate, but no effect of diet was seen on X-ray density of trabecular bone of femoral neck ($P = 0.5$, Table 2). In that study (Scholz-Ahrens *et al.* 1990c) calcium retention was not different between groups, with 1.09 ± 0.08 g/d when casein was fed, and 1.15 ± 0.08 g/d in animals on whey protein (mean \pm SEM).

Table 2. Effect of dietary casein and whey protein concentrate on parameters of Ca metabolism in growing minipigs on 0.8 % dietary Ca

		Casein		WPC	
		Mean	SEM	Mean	SEM
After 12 weeks					
Plasma					
PTH	nmol/l	0.18	0.03	0.11*	0.01
25(OH)D ₃	nmol/l	342	21	262†	22
X-ray bone density	mg/cm ³	297	13	312	21

PTH parathyroid hormone; WPC, whey protein concentrate.

* $P < 0.1$.

† $P < 0.05$, $n = 8$.

Table 3. Effect of dietary casein and whey protein concentrate on parameters of Ca metabolism in growing minipigs on diets containing 0.8% dietary Ca and lacking vitamin D

		Casein		WPC	
		Mean	SEM	Mean	SEM
After 8 weeks					
Plasma					
25(OH)D ₃	nmol/l	39.5	3.1	30.7*	2.6
After 12 weeks					
Plasma					
1,25(OH) ₂ D ₃	pmol/l	82.3	16.3	129.2*	14.8
Femur Ca	mg/g	188	2.37	179*	2.70

WPC, whey protein concentrate.

* $P < 0.05$; $n = 19$.

The third study was similar to study 2 except that the animals were vitamin D-depleted (Scholz-Ahrens *et al.* 1991). After 8 weeks plasma concentrations of calcidiol were significantly higher following casein compared to whey protein concentrate (Table 3). After 12 weeks significantly lower plasma concentrations of calcitriol (1,25(OH)₂D₃) were observed in the minipigs on casein. There was no difference in calcium balance or calcium content in trabecular bone of lumbar vertebra after 12 weeks. However, calcium concentration in the femur was slightly but significantly higher when casein was fed (Table 3). The higher calcidiol concentration indicates an increased capacity for calcium absorption. Thus, the increased femur calcification following casein-derived CPP might be mediated *via* a rise in plasma calcidiol concentration as the initial step. The improved femur mineralisation is compatible with reduced plasma calcitriol concentrations if lower calcitriol indicated a reduced bone turnover. The way in which different dietary proteins could have triggered this effect could not be explained. The vitamin D content of each protein preparation was not different (both below detectable level).

The authors concluded from the experiments in minipigs (Scholz-Ahrens *et al.* 1989; 1990a, b, c, 1991) that the effect of casein-derived CPP on calcium balance and bone performance was small in minipigs, an animal model that is closer to humans than the rat. Changes in calcium metabolism by CPP were detectable only at lower dietary calcium or if vitamin D supply was insufficient. The latter effect was in agreement with that reported by Mykkänen & Wasserman (1980) in vitamin D-deficient chicken. Therefore, CPP might be of minor relevance under physiological conditions, when counterbalance by hormonal regulation occurs. Nevertheless, a possible effect of isolated CPP or special CPP preparations could not be excluded especially if an optimum ratio of CPP and dietary calcium are used (Saito *et al.* 1998).

Effect of CPP preparations

Two experiments have been carried out in rats that investigated the effect of the concentrated CPP preparation, kindly provided by Sopharga, France, which was given for 7 weeks (Kopra *et al.* 1992). The CPP was characterised by Brulé *et al.* (1982) in more detail in a United States patent.

The diets contained 0.8% calcium, 0.6% phosphorus and 1.8% CPP. In vitamin D-replete (experiment 1) or in vitamin D-deficient rats (experiment 2) there was no effect of different milk proteins (casein *v.* whey protein) or of CPP, when added to the whey containing diet, on calcium balance, dry weight, ash, calcium, or phosphorus contents of femora (Kopra *et al.* 1992). The results are in accordance with those reported by Yuan & Kitts (1991) after feeding of CPP to rats for 10 weeks. In spite of higher ⁴⁵Ca absorption from the ligated loop, calcium balance was not improved.

In contrast, Tsuchita *et al.* (1995) observed in male growing rats significantly higher serum osteocalcin and reduced urinary cAMP when calcium-containing phosphopeptides (Ca-CPP) were included into a diet compared to a control group based on egg albumin. Considering this finding, they suggested Ca-CPP stimulated bone formation. Femur and humerus weights were significantly higher. However, this was not due to a better mineralisation but to other, organic components. Calcium, magnesium and phosphorus contents in humerus and calcium and magnesium content in femur were not different. Phosphorus content was higher but only in femur. Bone mineral density (BMD) was higher at three out of nine sites analysed at the excised femur and two out of nine at the humerus. This beneficial effect on bone density was only achieved by Ca-CPP, and not when a preparation free of calcium (Ca-free CPP) was given. In the Ca-free CPP diet calcium was included as CaCO₃. It remains questionable whether Ca-CPP promoted bone formation by way of the 9%, but non-significantly higher phosphorus retention, as reported by Tsuchita *et al.* (1995). It appears more probable that the increase in plasma alkaline phosphatase, serum osteocalcin, and renal tubular reabsorption rate, and the lower urinary cAMP following Ca-CPP reflected metabolic responses to the difference in phosphorus absorption which was 16% lower (although non-significant) in the diet containing Ca-CPP.

Tsuchita *et al.* (1993a) did not observe changes in calcium balance by CPP in ovariectomised (OVX) rats. Results are also in accordance with those in aged OVX rats, that did not develop higher femoral weight, ash, calcium or phosphorus, bone mineral content (BMC) or BMD of lumbar vertebrae (Tsuchita *et al.* 1996). The BMD of three out of nine segments of femur was higher after feeding of Ca-CPP. Loss of BMD *in vivo* was not prevented significantly before week 16. No positive effect on bone performance was observed with a CPP preparation free of calcium but enriched with CaCO₃ (Ca-free CPP). On the contrary, animals on diets containing Ca-free CPP lost almost 20% of their body weight (Tsuchita *et al.* 1996). The authors did not report the feed consumption. Therefore, it remains speculative as to whether a lower feed intake, lower biological value of the protein, part of that special CPP preparation, or other ingredients were responsible for the negative effect on body weight and presumably BMD *in vivo*. Other studies in animal models did not show a significant beneficial effect of CPP on calcium absorption or bone metabolism *in vivo* (Brommage *et al.* 1991; Pointillart & Guéguen, 1989).

It was reported that not only metabolism of calcium but also of other minerals and other aspects of mineral status

may be influenced by CPP. In young iron-deficient rats CPP-bound iron affected some parameters of iron status and metabolism positively, such as mean cell volume, haemoglobin and haematocrit, and some negatively, such as urine iron (Ait-Oukhatar *et al.* 1997). Other parameters like iron absorption or red blood cells were not affected. The authors concluded that binding iron to CPP seemed to improve its bioavailability, a finding recently confirmed by Pérès *et al.* (1999), and hasten the cure of iron deficiency in the young rat (Ait-Oukhatar *et al.* 1997). Another micronutrient (zinc) was found to bind to casein-derived CPP and to casein as long as it was not dephosphorylated (Harzer & Kauer, 1982).

Phosphopeptides other than CPP

Some authors suggested that not only the casein fraction is a source of bioactive components with regard to bone metabolism, but also whey (Tsuchita *et al.* 1993b). They compared two different whey mineral complexes (WMC I and WMC II) differing in sodium, potassium, chloride and protein content or CaCO₃ in a feeding trial in growing rats. There was no difference in calcium and phosphorus retention, urinary hydroxyproline excretion (a parameter of bone resorption) between different calcium sources either at 0.3% or 0.6% dietary calcium. WMC II induced only a small and temporary decrease of urinary cAMP (a parameter of PTH function), higher activities of alkaline phosphatase, calcium concentration in femur, and bone density in humerus when dietary calcium content was high. The authors speculated that this effect could be mediated by some small nitrogenous compounds that deserve further investigation or by trace lipids occurring in WMC after processing. In particular, the lipid fraction of WMC II was rich in oestradiol and calcitriol and raised the plasma concentration of these steroids in OVX rats. Furthermore, calcium absorption, femur calcium and humerus bone density were higher (Tsuchita & Kuwata, 1995).

Potential or established health benefits

Most of the findings in the literature that dealt with the mineral absorption-stimulating effect of CPP were based on *in vitro*, *in situ*, cell culture or single meal studies. The majority of studies were done in rats. A summary of those results provided considerable evidence for a potential effect of casein-derived phosphopeptides to improve mineral absorption. This potential was not limited to calcium but was also valid for zinc and iron, and possibly other elements that had not been investigated until now.

Only a few investigations have been performed as long-term experiments. However, only a long-term experimental approach allows extrapolation from beneficial action of CPP to physiological significance in terms of human health. *In vitro* studies and short-term *in situ* or single meal experiments are excellent and important approaches for studying the influence of dose, type of CPP preparation, basal dietary calcium (habitual calcium), protein source, meal effect and interaction with other nutrients or diet components or to compare physiological reactions in different animal species. Regarding bone health, however,

such studies are of limited relevance as long as no improvement on bone performance, such as bone mineralisation, density, or stability, was shown. It became obvious from the long-term experiments in animal models, that adaptation *via* hormonal regulation may lead to a zero effect on bone in spite of transient improvements in some parameters. Controlled long-term experiments in humans are difficult to perform. Hansen *et al.* (1997) investigated in single meal experiments the effect of CPP on calcium and zinc absorption from infant foods given to twenty two young adult individuals. When whole body retention of radioisotopes was measured, calcium and zinc absorptions were improved from rice-based cereal, whereas no effect was seen from whole-grain cereal. In osteoporotic women with low basal absorptive capacity CPP incorporation improved calcium absorption in a single meal experiment (Heaney *et al.* 1994).

Conclusion

It is obvious that long-term studies in humans are needed if conclusions are to be drawn from the evidence of specific effects of phosphopeptides seen *in vitro*, *in situ* and in single meal experiments. Bone density or other parameters of bone quality should be monitored if claims on bone health are to be made. There is a need for further studies in order to confirm beneficial effects on human health in general or for certain groups of population.

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