(2)

Studies on the Effects of Dietary Casein on Bone and Mineral Metabolism

Kin-ya Ashida

1997

Contents

Chapter 1

Introduction

Skeletal development starts at early stage of embryo and its activity continues throughout life. The longitudinal growth of long bone almost stopped after puberty. The mature bone, however, is not inactive tissue. Bone was continuously destroyed and formed. The bone metabolism is affected by various factors, e.g., age, mechanical stress and nutritional status. It is needless to say that skeleton plays a role to support whole body and its components, and to induce exercise with skeletal muscle. The fragile bone reduces physical activities of animals. The bone growth itself is important in animal production because skeletal growth is a dominant factor to decide body mass and physical constitution.

Bone has another physiologically important function to maintain calcium homeostasis. Therefore, bone metabolism is closely related to calcium status. Some of nutritional factors influence bone metabolism via the changes in calcium metabolism. Milk and milk products are known to be an excellent calcium source. The reasons exist in high availability of calcium as well as high content of the mineral. The absorbability of calcium from food sources is largely determined by other food components (Heaney et al. 1990). In this case, other milk components may contribute to high availability of milk calcium.

In this thesis, the nutritional regulation of bone metabolism was studied through investigating the dietary effects of casein, major milk protein, on calcium bioavailability and bone status in rats and chicks.

Chapter 2

Literature Review

2.1 Bone Metabolism

2.1.1 Growth of Long Bone

2.1.1.1 Longitudinal Growth

A long bone is anatomically divided into two parts, the ends of the bone, epiphysis and the center, diaphysis. The ends of diaphysis where it joins the epiphysis are especially termed metaphysis. In a growing animal, the epiphysis is separated from the diaphysis by a cartilage tissue, growth plate. The longitudinal extension of long bone is induced by the process of endochondral ossification in the growth plate. The growth plate consists of layers of cartilage containing different stages of chondrocyte which are well-classified as resting zone, proliferating zone, maturing zone and calcifying zone. Some cells of resting zone proliferate and form into columns. These cells secrete cartilaginous matrix into maturation. The cells hypertrophy and the newly formed cartilage is calcified. And then, the calcified cartilage is invaded by blood vessels and resorbed by multinucleated cells, chondroclasts. In the space of resorbed cartilage, bone is formed by osteoblasts. These events continuously occur in the growth plate, leading to longitudinal extention of long bones.

2.1.1.2 Lateral Growth

Lateral growth of long bone is achieved by intramembranous ossification. The outside surface of long bone is covered with a membrane, periosteum. Osteoblasts, which are derived from the periosteum, additionally formed new bone on the surface of existing bone. Although the long bone lateral grows by this event, the appropriate thickness of

cortical bone is maintained by bone resorption of osteoclasts on the endosteal surface.

2.1.2 Bone Remodeling

The bone, which is formed by the endochondral ossification or the intramembranous ossification, is sequentially remodeled. The surface or inside of bone is partially resorbed by osteoclasts. The loss of resorbed bone is normally refilled with new bone formed by osteoblasts. This bone remodeling occurs overall bone throughout the life.

2.2 Calcium Metabolism

2.2.1 Calcium Dynamics

Ingested calcium is taken in from the intestine in monogastric animals and birds. Intestinal calcium absorption is taken place by two processes. One is an active, saturable transport which occurs by energy-dependent mechanism by transcellular route. The other is an passive, non-saturable transport that occurs by simple diffusion or solvent drag mechanism by paracellular route. The former is limited in the proximal intestine, especially duodenum, while the latter appears throughout the intestine (Bronner et al. 1986). The passive transport is usually predominant in calcium absorption in animals fed adequate calcium. In the cases of low calcium intake and growing, however, the active transport is facilitated by vitamin D.

The absorbed calcium from the intestine enters an exchangeable calcium pool, i. e., serum calcium. Serum constantly exchanges calcium with tissues, especially with a skeletal tissue. More than 99% of calcium in an animal body exists in the skeletal tissue. A part of serum calcium is excreted into feces with undigested calcium through the gastrointestinal tract and into urine through the kidney. In addition, some serum calcium is also mobilized into fetuses in pregnant mammals, into milk in lactating mammals and into

eggs in laying birds.

2.2.2 Calcium Homeostasis and Bone

Calcium ion is necessary to maintain physiological cell function. Therefore, the concentration of extracellular calcium of normal animals and birds is constantly controlled within a narrow range by calcium regulating hormones, i.e., parathyroid hormone (PTH), calcitonin and 1,25-dihydroxychorecalciferol (active vitamin D_3, 1,25-$(OH)_2D_3$). When the concentration of serum calcium is beyond an appropriate value, thyroid gland secretes calcitonin into blood. Calcitonin directly acts osteoclasts and suppresses the activity of the cells and thus decrease the calcium flux from bone to serum to reduce serum calcium. When the concentration of serum calcium falls, parathyroid grand secretes PTH. The mechanism of its action to raise serum calcium is multiple and complex. Parathyroid hormone acts osteoblasts and stimulates calcium release by osteoclastic bone resorption via regulatory system of osteoblasts. Parathyroid hormone also acts kidney and directly increase renal tubular reabsorption of calcium. Furthermore, the hormone increases the activity of 1α-hydroxylase in kidney and stimulates the production of 1,25-$(OH)_2D_3$. The increment of serum 1,25-$(OH)_2D_3$ enhances intestinal calcium absorption and renal tubular reabsorption of calcium by facilitating the active calcium transport. In bone, the induction of osteoclast is also stimulated by 1,25-$(OH)_2D_3$.

2.3 Dietary Casein in Bone and Mineral Metabolism

Milk and milk products are known to be an excellent source of calcium. The reasons exist in high availability of calcium as well as high content of the mineral (Buchowski 1989). Milk contains almost all nutrients, i.e., protein, carbohydrate, lipid, vitamins and minerals, as it is an only dietary source of newborns. In addition, some already-

known physiologically active proteins or peptides and steroids exist in milk. For example, a considerable amount of parathyroid hormone-related protein, which has similar effects to parathyroid hormone, in bovine and human milk may play a role of regulation of calcium metabolism in sucklings (Barlet and Davicco 1990, Law et al. 1990, Otsubo et al. 1990, Ratcliffe et al. 1990). On the other hand, the macro nutrients in milk-specific forms are thought to act functionally on calcium metabolism. Lactose, major carbohydrate in milk, is well-known to stimulate intestinal calcium absorption (Dupuis & Fournier 1963, Lengemann 1959). Casein, one of the components of milk protein, is also specified and commonly found in mammalian milk. The content of casein is about 80% of cow's milk protein and about 35% of human's. Bovine casein consists of four portion, α_{s1}-, α_{s2}-, β- and κ-caseins. Many functional peptides have been found in the hydrolysate of bovine casein (Schlimme & Meisel 1995, Totsuka and Kaminogawa 1992), as shown in Table 2-1. It is suggested that casein supplies physiological regulator besides nutrient.

2.3.1 Casein Phosphopeptides

2.3.1.1 Structure and Calcium-binding Property

Some casein phosphopeptides (CPP) result from proteinase-digestion of whole casein (Table 2-1). The primary structures of main CPP fragments from in vitro trypsin-digested β-casein (Dumas et al. 1971, Manson and Annan 1971) and α_{s1}-casein (Mercier et al. 1971) are as follows:

β-casein phosphopeptide

H_2N-Arg-Glu-Leu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu-

SerP-Leu-SerP-SerP-SerP-Glu-Glu-Ser-Ile-Thr-Agr-OH

α_{s1}-casein phosphopeptide

H_2N-Asp-Ile-Gly-SerP-Glu-SerP-Thr-Glu-Asp-Glu-Ala-Met-Glu-

Asp-Ile-Lys-Glu-Met-Glu-SerP-Ile-SerP-SerP-SerP-Glu-Glu-Ile-

Val-Pro-Asn-SerP-Val-Gln-Glu-Lys-OH

The β-casein phosphopeptide and the α_{s1}-casein phosphopeptide contain four and seven phosphorylated serine residues, respectively. The structure of the three consecutive phosphoserine residues followed by the two consecutive glutamic acid residues is common to both CPP.

Manson and Annan (1971) predicted that the consecutive phosphoserine residues of β-casein could have some biological significances. Baumy et al. (1989) showed the calcium-binding property of each of four phosphoserine residues of CPP (1-25) from β-casein using ^{31}P NMR.

2.3.1.2 Calcium Solubilization

Reeves & Latour (1958) at first clarified a physicochemical property of CPP. They prepared crude phosphopeptides from pancreatic casein hydrolysate and demonstrated that these peptides sequestered calcium phosphate in the buffered solution with pH 7 to 10.5. A similar in vitro effect of inhibiting the formation of insoluble calcium phosphate was thereafter confirmed in crude CPP from bovine whole casein hydrolyzed by trypsin (Berrocal 1989) and purified CPP from bovine whole (Gerber and Jost 1986) and bovine β-casein hydrolysate (Sato et al 1986, 1991). It was noted that this solubilizing property was induced by the binding of calcium ion to phosphorylated residues of CPP because CPP lost the property of calcium binding and solubilizing by the dephosphorylation of CPP (Berrocal 1989, Gerber and Jost 1986). Furthermore, when rats ingested purified CPP from whole casein, the stimulation of calcium solubility was found

in the luminal contents of rat small intestine (Nagasawa et al. 1991, Hirayama et al. 1992, Lee et al. 1992). This suggestion was supported by the finding that a part of ingested CPP remained with phosphorylated structure in the intestine (Kasai et al. 1995, Hirayama et al. 1992).

2.3.1.3 Calcium Absorption and Utilization

Mykkänen & Wasserman (1980) studied the effect of CPP on intestinal calcium absorption by using the in vitro technique of everted gut sac and the in situ technique of ligated duodenal loop of chicks. They showed that the intestinal transport of calcium was increased by the supplementation of CPP fraction of whole casein hydrolysate both in the in vitro and the in situ studies. They additionally suggested that vitamin-D-independent mechanism of calcium transport was enhanced by CPP from the results of positive effect of CPP in the intestine of rachitic chicks as well as normal chicks. Sato et al. (1986) also observed that the supplementation of purified CPP from β-casein, compared to peptides from soy protein, increased calcium absorption in the ligated ileal loop and calcium uptake by femur in in situ studies of rat. Since the stimulated calcium transport by CPP was accompanied by an increase of calcium solubility in the lumen, they concluded that CPP enhances calcium absorption from the small intestine by increasing the concentration of soluble calcium, resulting in the increment of skeletal calcium deposition. Thereafter, Li et al. (1989) confirmed the indirect effect of CPP on the intestinal calcium absorption through denying the direct effect estimated by the alteration of electrical parameter and the mucosal-to-serosal and serosal-to-mucosal flux of calcium in the rat ileal tissue. They suggested that the physiological role of CPP is only the inhibition of the precipitation of phosphate-calcium salts, based on additional finding of inefficient absorption of calcium bound to CPP. The increase in the calcium solubility and

calcium absorption in the postprandially-ligated ileal loop was observed in the rats orally supplemented with CPP (Kitts et al 1992, Yuan & Kitts 1991).

Although the simulative effects of CPP on calcium solubility and intestinal calcium absorption have been constantly observed in the in vitro and in situ studies, the in vivo effect of CPP on intestinal calcium absorption or calcium utilization is not clear. In the early works, the efficiency of CPP in bone calcification or skeletal calcium uptake was reported. Mellander (1950) observed that dietary supplementation of CPP-calcium complex to rachitic children promoted bone calcification without administration of vitamin D. Patrick & Bacon (1957) also reported that orally supplied calcium as CPP salt was more efficiently deposited in rat femur than calcium as inorganic salts with or without supplementation of vitamin D. They failed, however, to observe such the stimulatory effect of CPP in chick.

Recently, some reports showed negative effects of CPP on intestinal calcium absorption and bone mineralization. Pointillart and Guéguen (1989) reported that the dietary supplementation of CPP did not influence apparent absorption and retention of calcium and bone parameters in growing pigs fed adequate calcium. Scholz-Ahrens et al. (1990) reported that the feeding of crude CPP did not increase apparent absorption and retention of calcium in vitamin-D-deficient weaning rats fed adequate calcium. Brommage et al. (1991) showed that fractional calcium absorption of digestive tract was not altered by partial replacing whey protein with CPP as protein source in adult female rats. Yuan & Kitts (1991) reported that the supplementation of CPP to diet containing adequate calcium and isolated soy protein as protein source did not influence apparent calcium absorption and bone calcium content in growing rats, in spite of increasing luminal calcium solubility and fractional calcium absorption in the ligated ileal loop.

On the other hand, the other recent reports supported the early findings. Lee et

al. (1992) consistently demonstrated the effect of dietary CPP on calcium utilization in the balance studies using weaning rats fed mildly low calcium diets containing isolated soy protein as protein source. They observed that the 0.123% to 3.5% supplementation of purified CPP from whole casein improved apparent absorption and retention of calcium accompanied by a rise in soluble calcium contents of the small intestine. Simultaneously, the bone weight and calcium content increased in rats fed the diets containing CPP at 1.75% to 3.5%. Matsui et al. (1994) observed higher calcium content and lower tartrate-resistant acid phosphatase activity in ectopic bone induced by decalcified bone matrix implantation in rats fed a low-calcium diet supplemented with 0.5% semi-purified CPP from whole casein than in rats fed a low-calcium diet without CPP. Although they did not directly estimate intestinal calcium absorption, they suggested that dietary CPP depressed bone resorption by increasing the amount of available calcium, with resulting prevention of bone calcium loss. Heaney et al. reported that the supplementation of semi-purified CPP from whole casein enhanced fractional absorption of co-ingested calcium in postmenoposal women with lower basal absorptive performance but not in those with higher performance. Tsuchita et al. (1993) observed that dietary supplementation of purified CPP from β-casein stimulated the improvement in apparent absorption immediately after ovariectomized young rats with calcium deficiency were fed on diets containing calcium at 0.5%, but not 0.3% or 0.1%. The facilitative effect of any dietary contents of CPP, however, was observed during 7 to 9 days or 26 to 28 days of the feeding period. Goto et al. (1995) investigated the effect of dietary supplementation of semi-purified CPP from whole casein, at high dose of 10% as major source of calcium and phosphorus, on the balance of calcium, phosphorus and magnesium in growing male and female rats. They observed an increase in apparent absorption and retention of calcium in male rats. This supplementation of CPP also increased apparent absorption and

retention of phosphorus and apparent absorption of magnesium and decreased urinary phosphorus excretion in both male and female rats. Other studies (Tsuchita et al. 1995, 1996) also showed a decrease in urinary phosphorus excretion owing to an increase in renal tubular reabsorption rate of phosphorus, in male growing and aged ovariectomized rats fed similar diets containing large amount of CPP. In these studies, the supplementation of CPP significantly increased bone weight and bone mineral density of femur and humerus in growing rats and bone mineral density of femur in ovariectomized rats without significant changes in calcium metabolism. These studies suggested that dietary CPP could influence bone status through some mechanisms except for calcium utilization, including phosphorus metabolism. The stimulatory effect of CPP on bone mineralization was also suggested in birds. Kusuhara et al. (1992) reported that dietary supplementation of crude CPP from whole casein stimulated the calcification of hypertrophic chondrocyte zone of epiphyseal growth plate in growing broilers.

Although the reasons why the results obtained from previous in vivo studies was often inconsistent have not been fully resolved, the effect of ingested CPP was thought to be multiple and dependent on experimental conditions including doses, purification and chemical structure of supplemented CPP, the composition of diets, sex, age and nutritional status of served animals, and the method or procedure of evaluation.

2.3.1.4 In Vivo Formation of CPP and Calcium Absorption

Casein phosphopeptides are formed by gastrointestinal digestion in vivo as well as by enzymatic hydrolysis in vitro. Naito et al. (1972) obtained a fraction containing a large amount of phosphopeptides from the small intestinal contents of rats fed casein diets. This fraction showed high calcium-binding activity. Therefore, they suggested a physiological significance of phosphopeptide formation during the digestion of casein in

the intestinal tract in calcium absorption. Naito and Suzuki (1974) confirmed the formation of CPP in the contents of small intestine of rats fed diets containing β-casein. Lee et al. (1979) observed higher contents of soluble calcium in the lower, but not upper, small intestine of rats fed a casein diet than those fed a egg albumin diet. They confirmed the formation of macrophosphopeptides in the small intestinal lumen of rats fed a 20% casein diet and then demonstrated the higher solubility of calcium and phosphorus in the contents of distal small intestine in rats fed a diet containing casein as a sole source of protein than in those fed diets containing egg albumin, isolated soy protein or amino acid mixture instead of casein (Lee et al. 1980). Furthermore, they investigated the effect of the intestinal calcium absorption in rats fed casein using in situ techniques (Lee et al. 1983). The rats fed a diet containing 20% casein showed higher fractional absorption of orally supplemented calcium in ligated segments of distal small intestine, accompanied by higher solubility of luminal calcium, than those fed a diet containing an equivalent amino acid mixture. The transport of calcium directly injected into the ligated segments to the portal vein was also higher in rats fed the casein diet. Nagasawa et al. (1991) observed higher fractional and apparent absorption of calcium in ligated ileal loops with higher content of inorganic phosphate in rats fed casein than in those fed whey protein or isolated soy protein. Sato et al. (1983) observed that the simulative effect of casein on luminal calcium solubilization and intestinal calcium absorption was lost by the dephosphorylation of casein and thus concluded that the specic effect of dietary casein on the enhancement of calcium absorption is essentially due to the phosphates in CPP which are formed during luminal proteolysis of casein molecules. These studies indicated that ingested casein can form CPP during intestinal digestion and contribute intestinal calcium absorption by inhibiting the formation of insoluble calcium-phosphate complex in the distal small intestine. The in vivo formation of CPP was proved in minipigs as well as in

rats (Meisel & Frister 1988, 1989).

Nevertheless, the effects of dietary casein on intestinal absorption or balance of calcium have not been clarified in vivo. Brommage et al. (1991) reported that increasing the level of dietary casein from 9% to 27% slightly increased fractional calcium absorption in adult female rats fed a low-calcium diet. Scholz-Ahrens et al. (1990) reported that there were no significant differences between casein and whey protein as dietary protein in femoral weight and calcium content in weaning minipigs fed low calcium diets and in apparent absorption and retention of calcium in vitamin-D-deficient weaning rats fed adequate calcium.

2.3.2 Other Peptides

It is generally known that osteoblasts and chondrocytes are derived from the same progenitor cells as fibroblasts , and osteoclasts are derived from the same progenitor cells as macrophages. Peptides, which stimulated the DNA synthesis of BALB/c3T3 cells, fibroblastic cells derived from mouse in vitro, were found in tryptic hydrolysates of human (Azuma et al. 1989) and bovine (Nagaune et al. 1989) β-casein. The peptides which increased the number of phagocytotic macrophage in vitro were also founded in tryptic hydrolysates of human (Parker et al. 1989) and bovine β-casein (Berthou et al. 1989). These peptides may directly influence the activity of bone cells, if they are absorbed structurally intact through the gastrointestine.

Table 2-1. Functional peptides derived from bovine casein.

Bioactivity and nomenclature	Origin	Reference
Opioid-agonistic peptides		
β-casomorphin-4-amide (morphiceptin)	β(60-63)	Chang et al. 1981, 1985
β-casomorphin-5	β(60-64)	Henschen et al. 1979
β-casomorphin-7	β(60-66)	Brantl et al. 1979, Henschen et al. 1979
β-casomorphin-11	β(60-70)	Meisel & Frister 1986
exorphin (α-casomorphin)	α_{s1}(90-96)	Loukas et al. 1983, Zioudrou et al. 1979
Opioid-antagonistic peptides		
casoxin 6	κ(33-38)	Yoshikawa et al. 1986
casoxin A	κ(35-41)/(35-42)	Chiba et al. 1989
casoxin B	κ(57-60)	Chiba et al. 1989
casoxin C	κ(25-34)	Chiba et al. 1989
Immunostimulating peptide		
α_{s1}-casein fragment	α_{s1}(194-199)	Migliore-Samour & Jollès 1988
β-casein fragment	β(63-68)	Migliore-Samour & Jollès 1988
β-casein fragment	β(191-193)	Berthou et al. 1987
Angiotensin I-converting enzyme-inhibitory peptides		
casokinin (CEI_5)	α_{s1}(23-27)	Maruyama et al. 1985
casokinin (CEI_{12})	α_{s1}(23-34)	Maruyama & Suzuki 1985
casokinin ($CEI_{\alpha C6}$)	α_{s1}(194-199)	Maruyama et al. 1987
casokinin ($CEI_{\beta 7}$)	β(177-183)	Maruyama et al. 1985

(Continued)

(Continued, Table 2-1)

Cell-growth-stimulating peptides		
(casokinin ($CEI_{\beta 7}$))	β(177-183)	Nagaune et al. 1989
Mineral-binding peptides		
α-casein phosphopeptide	α_{s1}(43-79)	Lee et al. 1983
	α_{s1}(43-58)	Gerber & Jost 1986
	α_{s1}(59-79)	Gerber & Jost 1986
	α_{s1}(66-74)	Meisel and Frister 1988, 1989
	α_{s2}(46-70)	Gerber & Jost 1986
β-casein phosphopeptide	β(1-25)	Lee at al. 1983
	β(1-28)	Gerber & Jost 1986
Antithrombotic peptides		
casoplatelin	κ(106-116)	Fiat et al. 1989
Calmodulin-binding peptides		
-	α_{s2}(164-179)	Kizawa et al. 1995
-	α_{s2}(183-206)	Kizawa et al. 1995
-	α_{s2}(183-207)	Kizawa et al. 1995

Chapter 3

Effect of a High Casein Diet on Bone and Mineral Metabolism in Rats

INTRODUCTION

Although high protein diets have been well established to induce an increase in urinary calciumin in rats (Bell et al. 1975, Funaba et al. 1989, Whiting & Draper 1980, 1981) as well as in men (Allen et al. 1979a, 1979b, Schuette et al. 1980, Sherman 1920), the effect on calcium absorption is less clear. Some reports showed that calcium absorption was stimulated by high protein diets (Engstrom and DeLuca 1963, Walker and Linkswiler 1972), while others did not (Allen et al. 1979b, Kim and Linkswiler 1979, Howe and Beecher 1981). The discrepancy on calcium absorption is thought to be caused by the differences of used protein sources, animal age and feeding duration.

On the other hand, magnesium metabolism has been less investigated than calcium metabolism in animals under high protein intake. Although it was reported in early times that high protein intake enhanced magnesium absorption in human (McCance et al. 1942), the effect of high protein intake on magnesium absorption has not been confirmed (Kitano et al. 1987). In addition, high protein feeding often induces an increase of urinary magnesium excretion in human (McCance et al. 1942), rats (Sterck et al. 1992, Verbeek et al. 1993, Zhang & Beynen 1992) and cattle (Wang & Beede 1990).

When a casein diet is ingested, CPP are formed in the lumen of distal small intestine (Lee et al. 1980). These peptides have abilities to bind calcium and other cations. Lee et al. (1980 and 1983) suggested from an in situ study that the formed CPP inhibited formation and precipitation of insoluble calcium salts and stimulated calcium absorption. However, the in vivo effect of ingested casein on intestinal calcium absorption is controversial.

This study was conducted to examine the effect of long-term feeding of a high

casein diet on calcium and magnesium metabolism and bone integrity in rats.

MATERIALS & METHODS

Sixty-two weaning male Wistar rats, aged 28 days, were used. Animals were housed individually in hanging stainless steel cages under controlled conditions with constant temperature (24-26°C) and a 12 hour light : 12 hour darkness cycle. All rats were fed a control diet containing 20% casein (Table 3-1) for 7 days. Then, the animals were divided into two dietary groups and fed either the control diet or a high casein diet containing 40% casein. Both the control diet and the high casein diet contained 15.4 MJ/kg metabolizable energy, 0.61% calcium, 0.56% phosphorus, 0.05% magnesium, which satisfied the requirements for growing rats (National Research Council 1978). The diets and water were given freely.

Each dietary group was further divided into four groups, and intake and excretion of calcium, phosphorus and magnesium in feces and urine were measured individually for 120 hours on either day 1-5, 21-25, 61- 65 or 161-165. Furthermore, on either day 20, 40, 80 or 180, 7 to 8 animals of each group were killed under pentobarbital sodium anesthesia, and the samples of plasma and right tibias were collected.

Feed and fecal samples were digested by nitric acid and perchlonic acid. Calcium, phosphorus and magnesium in urine and in the digested samples were measured. Plasma was deproteinized by 10% trichloroacetic acid and its calcium, phosphorus and magnesium concentrations were measured. After tibias were free of adherent tissue, they were dried at 135°C for 2 hours and then ashed at 600°C for 24 hours. The ash was dissolved in 1 mol/l hydrochloric acid, and calcium and magnesium concentrations of the solution were measured. Calcium and magnesium were analyzed by atomic absorption spectrophotometry. Phosphorus was analyzed by the method of Gomori (1942).

Apparent intestinal absorption of each mineral was calculated as the intake minus the fecal excretion. The retention was calculated as the apparent absorption minus the urinary excretion. Urinary excretion, apparent absorption and retention of each mineral were expressed as the percentage of intake.

Data were analyzed by two-way ANOVA using the GLM procedure of the Statistical Analysis System program (SAS Institute Inc.) to test the effect of diet, time and diet × time interaction. When the diet × time interactions were significant, means of all eight groups were compared by Fisher's least significant difference test. When the effects of time, but not the diet × time interactions, were significant, least square means of each time group were compared by Fisher's least significant difference test. Significant levels of all the statistical tests were set at $p<0.05$.

RESULTS

Feed intake and the intakes of calcium, phosphorus and magnesium in the high casein group were not significantly different from those in the control group in each period of balance tests. Body weight was similar between the two dietary groups throughout the experiment (Figure 3-1).

Urinary calcium excretion was significantly higher in the high casein group than in the control group (Table 3-2). Apparent absorption and retention of calcium were also significantly higher in the high casein group than in the control group, and the absorption and retention in both groups was noteworthily lower in the latter two periods than in the former two periods.

Apparent absorption and retention of phosphorus were significantly higher, but urinary phosphorus excretion was significantly lower in the high casein group than in the control group (Table 3-3). While urinary phosphorus excretion gradually increased in both

groups during the experiment, apparent absorption and retention decreased.

Urinary magnesium excretion was higher in rats fed the high casein diet than in rats fed the control diet (Table 3-4). Urinary magnesium excretion similarly changed in both dietary groups, i.e., the urinary excretion decreased from day 1-5 to day 21-25 and then gradually increased. Apparent magnesium absorption was significantly higher in the high casein group than in the control group. Apparent absorption and retention of magnesium continued to decrease throughout the experiment in both dietary groups. However, the reduction of apparent absorption and retention of magnesium was more remarkable in the control group than in the high casein group in the latter two collection periods. As a result, the difference of magnesium absorption between the two dietary groups was significant on day 61-65 and day 161-165, but not on day 1-5 and day 21-25. Although magnesium retention was gradually decreased in both dietary groups, it was significantly higher in the high casein group than in the control group on day 161-165, but not the other periods. Rats fed the high casein diet kept a positive retention even in the last period although the negative retention was observed in rats fed the control diet.

There was no significant difference in plasma concentrations of calcium, inorganic phosphorus and magnesium between the two dietary groups (Table 3-5). Plasma calcium and inorganic phosphorus concentrations gradually decreased during the experiment in both groups. On the other hand, plasma magnesium concentration did not change during the experiment.

Tibial dry weight and specific gravity were significantly higher in the high casein group than in the control group (Table 3-6). The wet weight also tended to be higher ($p<0.10$) in the high casein group. These parameters of tibia significantly increased with age in both the dietary groups.

Tibial calcium content in the high casein group was not significantly different from

that of the control group. The magnesium content was significantly higher in the high casein group than in the control group. The calcium content continued to increase significantly throughout the experiment. The magnesium content significantly increased up to day 80 and then reached the plateau.

DISCUSSION

The high casein diet significantly increased urinary excretion, apparent absorption and retention of calcium. The increase in urinary calcium excretion has been demonstrated in rats fed high protein diets composed by not only casein but also other protein sources (Calvo et al. 1982, Whiting & Draper 1980, Zang & Beynen 1992). This protein-induced calciuria is thought to be caused by a reduction of renal reabsorption of calcium (Whiting & Draper 1980) besides an increase in glomerular filtration rate (Allen et al. 1979a, Schuettcet al. 1980). The increased calcium excretion in urine affected less calcium retention because urine was a quite minor rout of calcium excretion. Therefore, the increment of apparent calcium absorption resulted in increasing calcium retention in the high casein group.

High casein intake did not increase calcium absorption in some previous balance studies (Allen & Hall 1978, Graves & Wolinsky 1980, Shenolikar & Rao 1968, Zhang & Beynen 1992). Howe & Beecher (1981) observed that a high casein diet increased apparent calcium absorption in weaning rats only at week 2, 5 and 6 during 7 weeks of balance study. Verbeek et al. (1993) also observed an increase in apparent calcium absorption in 3-week-old rats fed a high casein diet for 2 weeks, but not in 7-week-old rats fed a similar high casein diet for 2 or 3 weeks. These findings may indicate that the stimulatory effect of high casein intake on calcium absorption is temporary. All these results, however, were obtained from relatively short-term studies. In this study, the stimulatory effect of the high casein diet on apparent calcium absorption seemed to be weaker on day 21-25 than on day 1-5 although the

statistics did not prove such the fact. The increment of apparent calcium absorption, however, was suspended in the later periods in the high casein group.

On the other hand, no increase in calcium absorption has been observed in balance tests of rats fed a high protein diet produced by other protein sources (Funaba et al. 1989, Sterck et al. 1992, Zhang & Beynen 1992). Therefore, the increased apparent calcium absorption by high casein intake is thought to be due to the used protein source. When rats ingest a casein diet, CPP are formed in the lumen of distal small intestine (Lee et al. 1980). The peptides are known to stimulate intestinal calcium absorption by inhibiting the formation of insoluble calcium salts with phosphate. Verbeek et al. (1993) showed that the increment of apparent absorption was accompanied by an increase in the amount of soluble luminal calcium in ileum in rats fed a high casein diet. The increase in apparent calcium absorption by high casein intake may be explained by the stimulatory effect of CPP on intestinal calcium absorption.

The high casein diet increased phosphorus retention by both increasing the apparent absorption and decreasing the urinary excretion. Although the effect of high protein intake itself on phosphorus absorption has not been clear, an increase in apparent phosphorus absorption has been often observed in rats fed high casein diet (Graves & Wolinsky 1980, Howe & Beecher 1981, Verbeek et al. 1993, Zhang & Beynen 1992). The increase in apparent phosphorus absorption may be also due to such the action of CPP because CPP stimulates phosphorus solubility by the same manner as calcium. Otherwise, the increased apparent absorption of phosphorus may be derived from the difference in absorbability between inoganic and organic phosphorus as Howe & Beecher (1981) suggested. Casein includes much phosphorus as called "phosphoprotein". Although both the experimental diets contained same amount of phosphorus, its origin was quite different. The increment of apparent absorption and urinary excreation of phosphorus might be partly due to a decrease in

the endogenous loss because the increased bone mass enhanced the demand of the mineral.

The high casein diet increased apparent absorption and urinary excretion of magnesium. The intensity of the effect on the urinary excretion was almost constant through the experiment, while that on the apparent absorption was modulated by the feeding duration. That is, the stimulatory effect of high casein diet on the apparent absorption was less clear before day 21-25 and thereafter, was growing with the extension of feeding period. In result, the rate of an decrease in apparent magnesium absorption with time was more moderate in the high casein group than in the control group. The high casein diet suppressed the reduction of apparent magnesium absorption in aged rats. Magnesium retention was not changed by the high casein diet in early period, while the high casein diet increased the retention in the late period.

Zhang & Beynen (1992) observed an increase in apparent magnesium absorption in rats fed a high casein diet, but not in rats fed a high soybean protein diet or a high cod meal diet. The efficiency of magnesium absorption is known to be largely dependent on the solubility of luminal magnesium (Brink et al. 1992, Hardwick et al. 1991). It was reported that a high casein diet increased magnesium solubility in ileal lumen (Verbeek et al. 1993) Goto et al. (1995) reported that dietary CPP stimulated apparent magnesium absorption in growing rats. The high casein diet might increase the solubility of magnesium through the action of CPP and improve magnesium absorption, in particular, in aged rats whose intestinal function is on the decline.

The increment of urinary magnesium excretion has been demonstrated in rats fed high casein diets (Petito & Evans, 1984, Verbeek et al. 1993, Whiting & Draper 1980, Zhang & Beynen 1992) as well as other high protein diets (Calvo et al. 1982, Sterck et al. 1992, Whiting & Draper 1980, Zhang & Beynen 1992). Although the mechanisms of increased magnesium loss in urine by high protein intake is not known, the increase in apparent

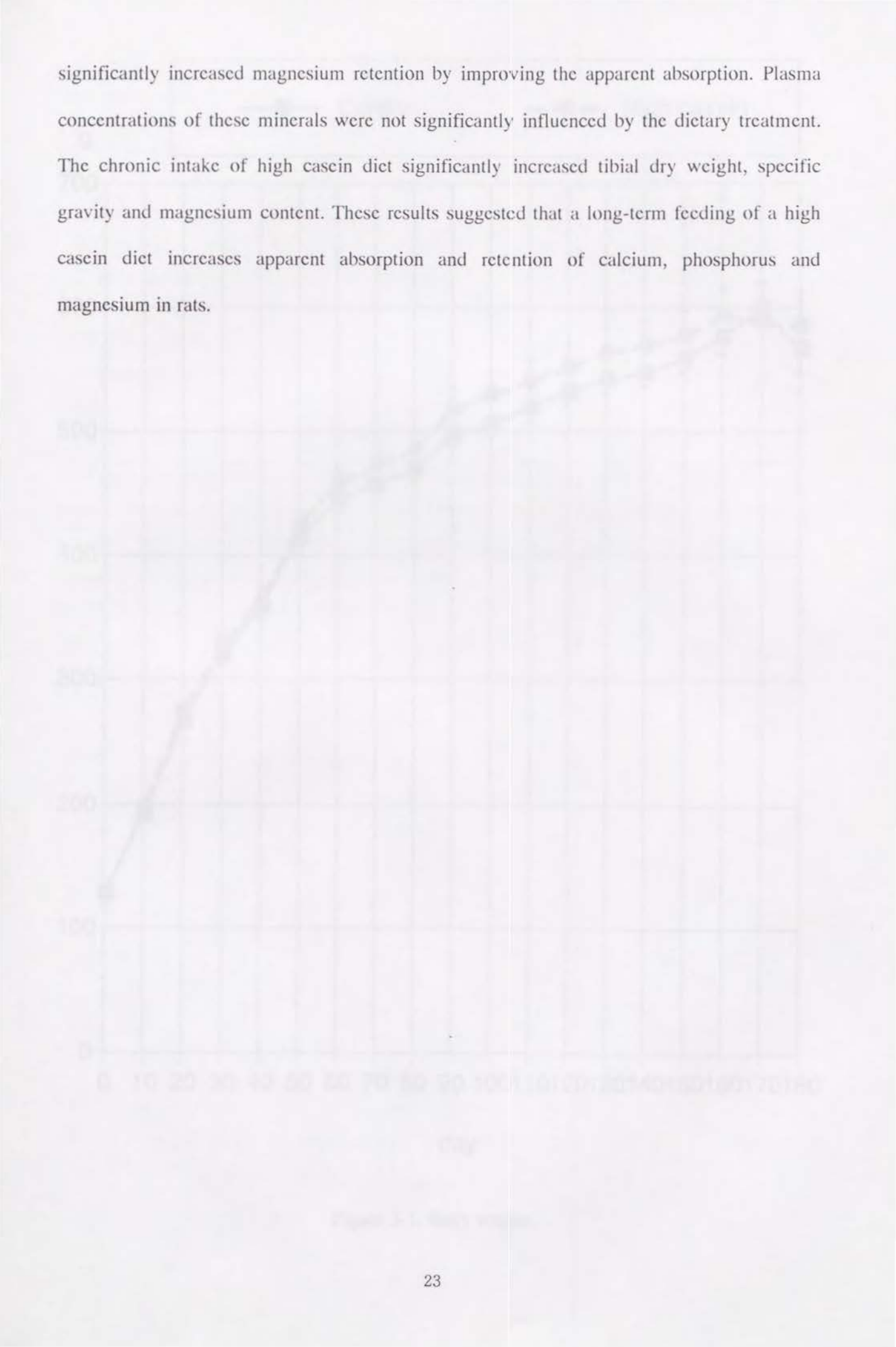
magnesium absorption may partly cause the increase in urinary magnesium excretion in this study. An increase in glomerular filtration rate induced by high protein ingestion may be involved as in the case of calcium.

Calcium and phosphorus are major inorganic components of bone. Next to these minerals, magnesium is an important mineral in bone. The high casein diet increased tibial dry weight and specific gravity. Such the increment of bone mass was thought to be caused by the improved retention of calcium, phosphorus and magnesium. Bone also plays as a storage of these minerals in the body. Therefore, the increase in bone mass and bone magnesium content strongly suggested that high casein intake increase bioavailability of calcium, phosphorus and magnesium in rats.

SUMMARY

The chronic effects of high casein intake on calcium, phosphorus and magnesium balance were investigated in growing rats. Male Wistar rats, aged 28 days, were fed a control diet (20% casein) for 7 days. And then, the animals were divided into two dietary groups and fed either the control diet or a high casein diet (40% casein). Both the two experimental diets contained 0.61% calcium, 0.56% phosphorus and 0.05% magnesium, which satisfied the requirements for growing rats. Balance tests were performed during day 1-5, 21-25, 61-65 and 161-165 of the experiment, and serum and tibias were collected on day 20, 40, 80 or 180. There was no significant difference in feed intake and body weight gain throughout the experiment. The high casein diet significantly increased calcium retention by stimulating the apparent absorption, irrespectively of the test periods. Phosphorus retention was also continually increased by the high casein diet through an increase in the apparent absorption besides a decrease in the urinary excretion. The high casein diet did not significantly influence magnesium retention in the early three periods. In the last periods, however, the diet

significantly increased magnesium retention by improving the apparent absorption. Plasma concentrations of these minerals were not significantly influenced by the dietary treatment. The chronic intake of high casein diet significantly increased tibial dry weight, specific gravity and magnesium content. These results suggested that a long-term feeding of a high casein diet increases apparent absorption and retention of calcium, phosphorus and magnesium in rats.



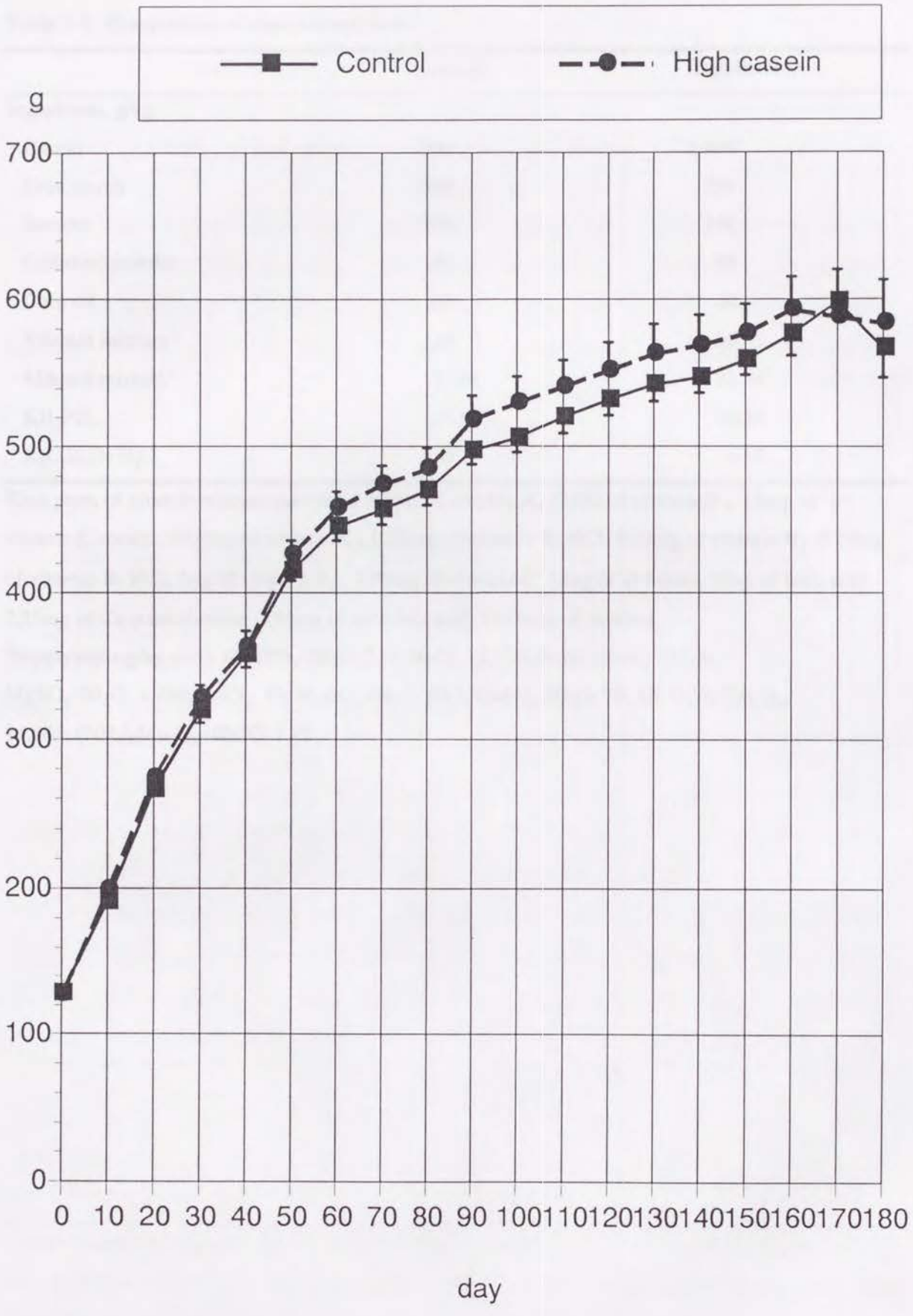


Figure 3-1. Body weight.

Table 3-1. Composition of experimental diets

	Control	High casein
Ingredients, g/kg		
Casein	200	400
Corn starch	300	200
Sucrose	290	190
Cellulose powder	80	80
Corn oil	60	60
Vitamin mixture[1]	20	20
Mineral mixture[2]	32.84	32.84
KH_2PO_4	17.16	10.59
$K_3C_6H_5O_7 \cdot H_2O$	0	6.57

[1]One gram of vitamin mixture contained 466IU of vitamin A, 233IU of vitamin D_3, 12mg of vitamin E acetate, 0.06mg of vitamin K_3, 0.59mg of vitamin B_1 HCl, 0.59mg of vitamin B_2, 0.29mg of vitamin B_6 HCl, 2μg of vitamin B_{12}, 5.88mg of vitamin C, 10μg of D-biotin, 20μg of folic acid, 2.35mg of Ca-pantothenate, 2.94mg of nicotinic acid, 11.76mg of inositol.

[2]Supplying(mg/kg diet): $CaHPO_4 \cdot 2H_2O$, 215; NaCl, 12,530; ferric citrate, 311.5; $MgSO_4 \cdot 7H_2O$, 4,990; $ZnCl_2$, 10; $MnSO_4 \cdot 4H_2O$, 60.5; $CuSO_4 \cdot 5H_2O$, 78; KI, 0.25; $CaCO_3$, 14,645; $(NH_4)_6Mo_7O_{24} \cdot 4H2O$, 1.25.

Table 3-2. Effect of a high casein diet on calcium balance

	Urinary excretion		Absorption		Retention	
Day	Control	High casein	Control	High casein	Control	High casein
			g/100g calcium intake			
1-5	0.42±0.06(8)^{AB}	0.96±0.12(8)	49.0±1.5(8)^{A}	59.6±2.1(8)	48.6±1.4(8)^{A}	58.7±2.1(8)
21-25	0.39±0.07(7)^{C}	0.45±0.06(7)	43.7±2.5(7)^{B}	44.3±1.4(7)	43.3±2.4(7)^{B}	43.8±1.4(7)
61-65	0.34±0.05(8)^{BC}	0.61±0.09(8)	18.8±2.5(8)^{C}	23.3±1.9(8)	18.5±2.5(8)^{C}	22.7±1.9(8)
161-165	0.47±0.05(8)^{A}	1.07±0.24(8)	13.3±5.1(8)^{C}	21.9±1.8(8)	12.8±5.2(8)^{C}	20.9±1.8(8)
			Statistical significance			
Diet	**		**		**	
Time	**		**		**	
Diet × Time	NS		NS		NS	

Values are means±SEM(n). Statistical significance of two-way ANOVA: **, $p<0.01$; NS, not significant ($p>0.05$). Different capital letters indicate significant differences among least square means of each time ($p<0.05$).

Table 3-3. Effect of a high casein diet on phosphorus balance

	Urinary excretion		Absorption		Retention	
Day	Control	High casein	Control	High casein	Control	High casein
			g/100g phosphorus intake			
1-5	29.7±0.9(8)^{C}	29.1±1.0(8)	76.7±0.8(8)^{A}	80.8±1.0(8)	47.1±1.2(8)^{A}	51.7±1.4(8)
21-25	29.1±0.7(7)^{C}	23.4±0.7(7)	67.4±1.7(7)^{B}	65.7±0.8(7)	38.3±2.1(7)^{B}	42.3±1.3(7)
61-65	36.5±1.3(8)^{B}	34.0±1.3(8)	55.3±1.9(8)^{C}	59.0±1.3(8)	18.9±1.1(8)^{C}	25.0±1.8(8)
161-165	41.1±3.5(8)^{A}	36.9±0.7(8)	48.0±2.9(8)^{D}	53.5±1.3(8)	6.9±6.1(8)^{D}	16.6±1.4(8)
			Statistical significance			
Diet	**		*		**	
Time	**		**		**	
Diet × Time	NS		NS		NS	

Values are means±SEM(n). Statistical significance of two-way ANOVA: *, $p<0.05$; **, $p<0.01$; NS, not significant ($p>0.05$). Different capital letters indicate significant differences among least square means of each time ($p<0.05$).

Table 3-4. Effect of a high casein diet on magnesium balance

	Urinary excretion		Absorption		Retention	
Day	Control	High casein	Control	High casein	Control	High casein
			g/100g magnesium intake			
1-5	12.1±1.9(8)^{C}	23.8±1.1(8)	61.1±1.8(8)^{a}	67.2±1.3(8)^{a}	49.0±3.0(8)^{a}	43.4±2.0(8)^{ab}
21-25	7.9±2.0(7)^{D}	3.8±1.8(7)	48.7±2.2(7)^{bc}	50.7±1.9(7)^{b}	40.7±3.4(7)^{ab}	36.9±2.7(7)^{b}
61-65	21.3±3.0(8)^{B}	28.2±1.9(8)	35.9±2.7(8)^{d}	45.9±2.7(8)^{bc}	14.6±2.5(8)^{cd}	17.8±1.8(8)^{c}
161-165	30.9±2.3(8)^{A}	35.0±3.4(8)	19.1±5.5(8)^{e}	41.8±2.1(8)^{cd}	-11.8±6.4(8)^{e}	6.8±2.5(8)^{d}
			Statistical significance			
Diet		**		**		NS
Time		**		**		**
Diet × Time		NS		**		**

Values are means±SEM(n). Statistical significance of two-way ANOVA: **, $p<0.01$; NS, not significant ($p>0.05$). Different capital and small letters indicate significant differences among least square means of each time group and means of individual group, respectively ($p<0.05$).

Table 3-5. Effect of a high casein diet on mineral concentrations of plasma

	Calcium		Inorganic phosphorus		Magnesium	
Day	Control	High casein	Control	High casein	Control	High casein
			mg/100 ml			
20	10.20±0.22(7)^{A}	10.11±0.16(6)	9.19±0.60(7)^{A}	9.01±0.31(6)	1.89±0.15(7)	1.79±0.04(6)
40	9.93±0.16(6)^{AB}	9.96±0.10(7)	7.62±0.37(6)^{B}	7.66±0.13(7)	1.84±0.05(6)	1.83±0.06(7)
80	9.87±0.07(6)^{AB}	9.92±0.09(8)	7.19±0.56(6)^{B}	7.22±0.29(8)	2.08±0.18(6)	1.66±0.08(8)
180	9.72±0.15(4)^{B}	9.62±0.12(4)	4.56±0.44(4)^{C}	5.37±0.48(4)	1.56±0.04(4)	1.66±0.03(4)
			Statistical significance			
Diet	NS		NS		NS	
Time	*		**		NS	
Diet × Time	NS		NS		NS	

Values are means±SEM(n). Statistical significance of two-way ANOVA: *, $p<0.05$; **, $p<0.01$; NS, not significant ($p>0.05$). Different capital letters indicate significant differences among least square means of each time ($p<0.05$).

Table 3-6. Effect of a high casein diet on tibial weight and specific gravity

	Wet weight		Dry weight		Specific gravity	
Day	Control	High casein	Control	High casein	Control	High casein
	mg		mg		g/cm^3	
20	301±11(6)$^{\wedge}$	300±13(4)	212±7(6)$^{\wedge}$	214±7(4)	1.15±0.01(6)$^{\wedge}$	1.17±0.02(4)
40	428±11(7)^{B}	437±14(7)	325±10(7)^{B}	329±9(7)	1.42±0.01(7)^{B}	1.43±0.01(7)
80	634±17(7)^{C}	673±29(8)	467±9(7)^{C}	491±20(8)	1.50±0.01(7)^{C}	1.51±0.01(8)
180	709±19(8)^{D}	771±23(8)	533±15(8)^{D}	579±17(8)	1.58±0.01(8)^{D}	1.62±0.01(8)
	Statistical significance					
Diet	NS		*		**	
Time	**		**		**	
Diet × Time	NS		NS		NS	

Values are means±SEM(n). Statistical significance of two-way ANOVA: *, $p<0.05$; **, $p<0.01$; NS, not significant ($p>0.05$). Different capital letters indicate significant differences among least square means of each time ($p<0.05$).

Table 3-7. Effect of a high casein diet on tibial contents of calcium and magnesium

	Calcium		Magnesium	
Day	Control	High casein	Control	High casein
	mg		mg	
20	60±3(6)$^{\wedge}$	58±4(4)	1.18±0.06(6)$^{\wedge}$	1.19±0.09(4)
40	96±2(7)^{B}	101±3(7)	1.53±0.05(7)^{B}	1.57±0.06(7)
80	120±4(7)^{C}	127±6(8)	2.23±0.06(7)^{C}	2.52±0.13(8)
180	127±5(8)^{D}	134±6(8)	2.08±0.08(8)^{C}	2.48±0.11(8)
	Statistical significance			
Diet	NS		**	
Time	**		**	
Diet × Time	NS		NS	

Values are means±SEM(n). Statistical significance of two-way ANOVA: **, $p<0.01$; NS, not significant ($p>0.05$). Different capital and small letters indicate significant differences among least square means of each time ($p<0.05$).

Chapter 4

Effect of Dietary Peptides from Casein Hydrolysate on Bone Growth in Rats

INTRODUCTION

Milk provides all nutrients which is necessary for newborn growth. Recently, many functional peptides have been found in milk protein hydrolysates (Schlimme & Meisel 1995, Totsuka & Kaminogawa 1992). It is suggested that milk proteins play a role as physiological regulators as well as nutrient sources providing nitrogen and amino acids. Casein phosphopeptides (CPP) derived from trypsin-digested bovine α_{s1}- and β-casein are known to stimulate intestinal calcium absorption (Naito 1986). These peptides are, therefore, thought to contribute to high availability of milk calcium. It is not clear, however, whether CPP influences bone which has 99% of body calcium. Although the trypsin-hydrolysates of casein contains other known or unknown peptides, their effects on bone growth have not been investigated. This study was conducted to investigate the effect of casein peptides on bone growth in rats.

MATERIALS AND METHODS

Peptide products tested in this study were whole hydrolysate (CPP-I) and its component fractions (CPP-III; BP-I; BP-II) of trypsin-digested whole casein (Figure 4-1). A whole hydrolysate of trypsin-digested whole casein (CPP-I) was divided into three fractions, a phosphopeptides-rich fraction (CPP-III) and the other two fractions (BP-I and BP-II). Their preparation was according to the method of production of commercially available semi-purified CPP (Meiji CPP-III®, Meiji Seika Kaisha, Ltd., Tokyo, Japan). The chemical properties of the four peptide products are shown in Table 4-1. Forty eight male Wistar-strain rats aged 6 week were divided into six groups. One group was killed on that day and the liver,

kidneys, spleen and tibias of each rat were taken. All the animals of other groups were individually housed in stainless steel cages in a room with controlled temperature (24°C) and lighting (12-hour light/12-hour darkness). The five dietary groups were assigned to a control diet or either of four peptide diets containing CPP-I, CPP-III, BP-I and BP-II (Table 4-2), and fed each diet and water ad libitum for 30 days. These diets satisfied the nutritional requirements of calcium, phosphorus and magnesium. Body weight was measured every 10 days and the amount of ingested feed was recorded during the last 5 days of the experiment. At the end of the feeding trial, the animals of the five groups were killed and their organs were dissected out.

The livers, kidneys and spleens were weighted immediately after the collection. The right tibias were analyzed for physical parameters, i. e., length, diaphyseal diameter, wet weight, volume and specific gravity. The volume and the specific gravity was determined on an Archimedean theorem. The dry weight of right tibias were then measured after drying at 135°C for two hours. After the tibias were ashed at 600°C for 24 hours, thier crude ash contents were determined.

After the feed samples digested by nitric acid and perchloric acid and tibial ash were dissolved in hydrochloric acid, the concentrations of calcium and magnesium in the solution were analyzed by atomic absorption spectrophotometry and phosphorus concentrations were determined by the method of Gomori (1942).

The left tibias were fixed in 10 % formalin, decalcified with EDTA, dehydrated through sequential changes of 70% to 100% ethanol, and embedded in paraffin. The 5μm section of longitudinal direction were stained with hematoxylin and eosin, and then histologically measured the width of epiphyseal growth plate and the content of trabecular bone.

The significant differences between means of each group were determined by

Student's t-test. The level of significance was preset at $p<0.05$.

RESULTS

Feed intake was slightly, but significantly, lower in the CPP-I group than the control group (Table 4-3). Initial and final body weight, and dairy gain of body weight during the experiment were not significantly different between the control group and each of the peptide groups. Spleen weight was significantly lower in the CPP-I group than in the control and CPP-III group (Table 4-4). The weights of liver and kidney were similar in all the dietary groups.

The CPP-III group showed significantly higher tibial length than the control group (Table 4-5). The length of the other peptide groups was not significantly different from that of the control group. The width and the wet weight were not significantly different among the dietary groups. Only the BP-I group in the peptide groups showed significantly higher tibial volume than the control group. The specific gravity of the CPP-I, BP-I and BP-II groups, but not CPP-III group, was significant lower than that of the control group.

Tibial dry weight was significantly higher in only the BP-I group than in the control group (Table 4-6). There was no significant difference in total content of tibial crude ash among the dietary groups, while the concentration based on tibial dry weight was significantly lower in the CPP-I and BP-I groups than in the control and CPP-III groups. Furthermore, the CPP-I group showed significantly lower concentration of phosphorus than the other dietary groups. The BP-I group also showed lower concentrations of calcium and magnesium than the control and CPP-III groups.

Total width of epiphyseal growth plate of tibia, the width of resting and proliferating zone and the width of mature and calcifying zone were not significantly different between the control group and each of the peptide groups (Table 4-7). The ratio of

trabecular bone volume in metaphyseal cavity of tibia was significantly lower in the BP-II group than in the other dietary groups.

DISSCUSSION

Casein phosphopeptides are thought to stimulate intestinal calcium absorption by preventing the formation of insoluble precipitaion of calcium and phosphate. It was reported that the feeding of CPP increased apparent calcium absorption and femoral weight and calcium content in rats fed insufficient calcium (Lee et al. 1992). On the other hand, the supplementation of CPP to diets containing adequate calcium often failed to increase intestinal calcium absorption in rats (Brommage et al. 1991, Scholz-Ahrens et al. 1990, Yuan & Kitts, 1991). Lee et al. (1992) suggested that CPP contribute less to intestinal calcium absorption in animals ingesting an adequate amoun of calcium. In the present study, dietary CPP as CPP-III and CPP-I did not increase tibial weight and calcium concentration in rats fed adequate calcium. The absence of effect may be due to no increase in calcium absorption.

The supplementation of CPP-III, however, increased tibial length. Kusuhara et al. (1992) showed that the feeding of CPP-I to broilers stimulated the calcification of and shortened the width of hypertrophy zone. Neither CPP-I nor CPP-III, however, influenced the width of epiphyseal growth plate in this study. Matsui et al. (1994) observed increments in alkaline phosphatase activity and calcium contents of ectopic bone induced through endochondral ossification by decalcified bone matrix implantation and suggested an possibility that CPP or its fragments directly act on bone. In this study, CPP or trace peptides in CPP-III fraction might influence bone formation after cartilage calcification in epiphyseal growth plate by unknown mechanism of action, except an increment in calcium bioavailability.

The supplementation of CPP-I, BP-I and BP-II similarly decreased tibial specific

gravity. The mechanisms of action on bone, however, seem to be different among CPP-I, BP-I and BP-II. Dietary BP-I increased tibial volume and dry weight, and decreased the concentrations of ash, calcium and magnesium. Therefore, the decrease in specific gravity in the BP-I group could be due to the greater growth of volume and relatively mild growth of wet weight. The lower concentration of ash or minerals also could be due to the greater growth of dry weight and relatively mild mineralization. Mineral utilization might possibly be suppressed by BP-I. There is a possibility from these data that dietary BP-I stimulates bone mass growth followed by less mineralization.

Dietary BP-II decreased trabecular bone volume of tibia although it did not influence tibial whole weight and mineral concentrations. The lower specific gravity of the BP-II group could be responsible for the suppression of the trabecular bone volume. It is difficult to understand the mechanism of action of BP-II only on trabecular bone. It has been generally thought that a reduction of available calcium induces a decrease in trabecular bone more quickly rather than cortical bone. It will be related to the difference in the rate of osteoclastic bone resorption to maintain calcium homeostasis. Because osteoclasts more easily approach trabecular bone owing to its larger surface than cortical bone. The decrease in trabecular bone volume might indicate the stimulation of bone resorption in the BP-II group.

The decrease in specific gravity in the CPP-I group could be partly explained by the effects of BP-I or BP-II. While, neither an increase in tibial volume nor a decrease in trabecular bone volume were observed in the CPP-I group. Although both CPP-I and BP-I decreased tibial concentration of crude ash, the decrease in crude ash was associated with an decrease in phosphorus concentration, but not calcium and magnesium concentration, in the CPP-I group. The lower feed intake might affect tibial specific gravity in the CPP-I group. The suppression of feed intake may be due to the actions of casomorphins in CPP-I. Casomorphins are opioid peptides derived from bovine β-casein and directly suppress

intestinal motility. Digesta retention, which is induced by this effect, could result in a decrease in feed intake. This speculation may be supported by the report of Putman et al. (1996). They observed that the gastric infusion of opioid antagonists blocked the suppressive effect of the oral supplemention with casein on feed intake. These effects of CPP-I on bone and feed intake were not fully followed by the effects of its fractions, suggesting that several functional peptides of CPP-I acts synergistically or has interactions each other.

SUMMARY

This study was conducted to investigate the effect of dietary peptides derived casein on bone growth in rats. A whole hydrolysate of trypsin-digested whole casein (CPP-I) was divided into three fractions, a phosphopeptides-rich fraction (CPP-III) and the other two fractions (BP-I and BP-II). Five groups of 6-week-old male rats were fed a control diet (casein-peptide-free) or either of diets containing CPP-I, CPP-III, BP-I and BP-II for 30 days. All the diets satisfied the recommended level of calcium, phosphorus and magnesium. The CPP-I group showed significantly lower specific gravity of tibia and the concentrations of crude ash and phosphorus than the control group. The decrement of tibial specific gravity was also observed in the BP-I and BP-II group. The decrement of crude ash concentration was observed in the BP-I group. Neither of three groups fed peptide fractions showed the lower phosphorus concentration of tibia. In addition, only the CPP-III group showed significantly greater longitudinal growth of tibia. The BP-I group showed significantly higher tibial volume and dry weight and lower concentrations of calcium and magnesium. The BP-II group showed lower trabecular bone volume in the tibial cavity, without changes in mineralization. Consequently, the findings in CPP-I feeding did not fully agree with those in either feeding of its component fractions. These results suggested that several peptides derived from casein hydrolysates influence bone growth by themselves or synergistically in

rats.

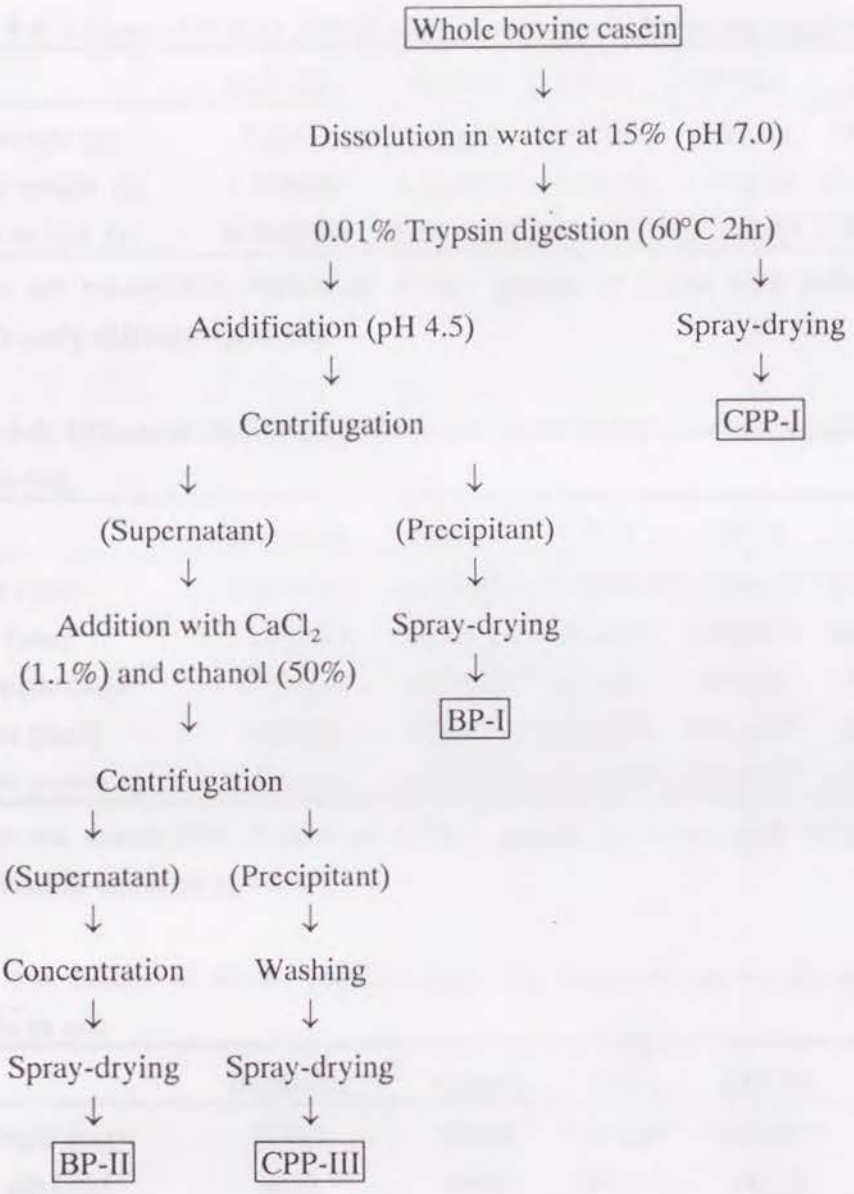


Figure 4-1. Preparation of casein peptide products

Table 4-1. Properties of casein peptide products

	CPP-I	CPP-III	BP-I	BP-II
Dry matter (%)	95.5	94.7	97.9	93.4
Crude protein (%)	79.9	75.7	86.1	70.8
Calcium (%)	0	4.8	0	1.6
Phosphorus (%)	0.7	2.7	0.5	0.2
CPP (%)	(12-15)	88.4	5.1	5.8
Calcium solubilizability (mg Ca/0.2mg)	0.097	0.108	0.026	0.030

Table 4-4. Effects of dietary peptides from casein hydrolysate on organ weight in rats

	Beginning	Control	CPP-I	CPP-III	BP-I	BP-II
Liver weight (g)	7.2±0.5	15.3±1.2	14.7±0.3	15.1±1.4	14.7±1.3	14.8±1.8
Kidney weight (g)	1.46±0.09	2.50±0.19	2.44±0.18	2.73±0.35	2.52±0.28	2.47±0.28
Spleen weight (g)	0.50±0.06	0.70 ± 0.09^{a}	0.59 ± 0.03^{b}	0.65 ± 0.02^{a}	0.70 ± 0.17^{ab}	0.62 ± 0.09^{ab}

Values are means±SD. Values of dietary groups in a row with different superscripts are significantly different ($p<0.05$).

Table 4-5. Effects of dietary peptides from casein hydrolysate on physical parameters of right tibia in rats

	Beginning	Control	CPP-I	CPP-III	BP-I	BP-II
Length (mm)	27.12±0.47	35.25 ± 0.32^{b}	35.50 ± 0.54^{ab}	35.68 ± 0.31^{a}	35.77 ± 1.02^{ab}	35.33 ± 0.88^{ab}
Width (mm)	2.24±0.10	3.03±0.09	3.09±0.21	3.02±0.26	3.03±0.16	3.08±0.16
Wet weight (mg)	239±12	462±12	457±30	450±38	470±15	450±23
Volume (mm^3)	187±10	329 ± 6^{b}	330 ± 23^{ab}	321 ± 27^{ab}	342 ± 11^{a}	329 ± 18^{ab}
Specific gravity (g/cm^3)	1.28±0.02	1.41 ± 0.02^{a}	1.39 ± 0.02^{bc}	1.40 ± 0.02^{ab}	1.38 ± 0.01^{c}	1.37 ± 0.02^{c}

Values are means±SD. Values of dietary groups in a row with different superscripts are significantly different ($p<0.05$).

Table 4-6. Effects of dietary peptides from casein hydrolysate on dry weight and ash content of tibia in rats

	Beginning	Control	CPP-I	CPP-III	BP-I	BP-II
Dry weight (mg)	109±5	252 ± 8^{b}	253 ± 20^{ab}	248 ± 21^{ab}	264 ± 6^{a}	254 ± 11^{ab}
Crude ash (mg)	68±3	165±5	161±13	162±15	169±3	164±8
Crude ash (mg/g DW)	622±5	655 ± 3^{a}	639 ± 4^{b}	653 ± 9^{a}	641 ± 8^{b}	646 ± 13^{ab}
Ca (mg/g DW)	224±6	236 ± 3^{ab}	231 ± 5^{bc}	238 ± 5^{a}	230 ± 3^{c}	231 ± 7^{bc}
P (mg/g DW)	110±4	112 ± 1^{a}	109 ± 2^{b}	112 ± 1^{a}	112 ± 2^{a}	112 ± 2^{a}
Mg (mg/g DW)	4.10±0.12	4.52 ± 0.07^{a}	4.41 ± 0.11^{ab}	4.54 ± 0.09^{a}	4.34 ± 0.18^{b}	4.40 ± 0.12^{ab}

Values are means±SD. Values of dietary groups in a row with different superscripts are significantly different ($p<0.05$).

Table 4-7. Effects of dietary peptides from casein hydrolysate on growth plate thickness and trabecular bone volume of tibial proximal metaphysis in rats

	Control	CPP-I	CPP-III	BP-I	BP-II
Growth plate					
Resting and proliferating zone (mm)	0.25 ± 0.06^{ab}	0.22 ± 0.00^{ab}	0.22 ± 0.02^{b}	0.25 ± 0.05^{ab}	0.24 ± 0.02^{a}
Maturing and calcifying zone (mm)	0.15 ± 0.05^{ab}	0.13 ± 0.01^{ab}	0.12 ± 0.01^{ab}	0.16 ± 0.04^{a}	0.12 ± 0.03^{b}
Total (mm)	0.40±0.10	0.35±0.01	0.34±0.04	0.40±0.07	0.36±0.04
Trabecular bone volume (%)	28.1 ± 2.3^{a}	27.3 ± 2.8^{a}	27.5 ± 3.1^{a}	27.4 ± 3.1^{a}	23.9 ± 2.0^{b}

Values are means±SD. Values of dietary groups in a row with different superscripts are significantly different ($p<0.05$).

Chapter 5

Effect of Dietary Casein Phosphopeptides on Bone Growth in Weaning Rats

INTRODUCTION

Milk is superior to other feeds in calcium availability. Therefore, sudden switch from milk to solid diets may induce transient calcium deficiency at weaning of suckling animals. It was reported that early weaning suppressed bone growth accompanied by the restriction of calcium absorption in carves (Funaba et al. 1996). Casein phosphopeptides (CPP) derived from trypsin-digested bovine α_{s1}- and β-casein are known to stimulate intestinal calcium absorption. These peptides are therefore thought to contribute to high availability of milk calcium. This study was conducted to investigate the effect of dietary CPP on postweaning bone growth in rats.

MATERIALS AND METHODS

Sixteen male Wistar-strain rats were forcibly weaned at 3 weeks old and individually moved in stainless steel cages in a room with controlled temperature (24°C) and lighting (12-hour light/12-hour darkness). These animals were divided into two groups and fed a control diet or a CPP diet containing commercially available semi-purified CPP (Meiji CPP-III®, Meiji Seika Kaisha, Ltd., Tokyo, Japan) at 3% (Table 5-1). Each diet contained 0.66-0.68% calcium and 0.61-0.62% phosphorus. The diets and water were given ad libitum. After three weeks of feeding trial, all the rats were killed by blood collection from abdominal artery under pentobarbital sodium anesthesia. And then, the livers, kidneys, spleens and tibias of each rat were dissected out. Body weight was measured every 7 days and the amount of ingested feed was recorded during the last 5 days in the experiment.

The livers, kidneys and spleens were weighted immediately after the collection.

Blood was heparinized and centrifuged at 2500 rpm at 4°C for 15 minutes, and serum was separated within one hour after the blood collection. The right tibias were analyzed for physical parameters, i. e., length, diaphyseal diameter, wet weight, volume and specific gravity. The volume and the specific gravity was determined on an Archimedean theorem. The dry weight of right tibias were then measured after drying at 135°C for two hours. After the tibias were ashed at 600°C for 24 hours, crude ash contents in them were determined.

After the feed samples were digested by nitric acid and perchloric acid and dissolved in hydrochloric acid, calcium concentration was analyzed by atomic absorption spectrophotometry and phosphorus concentration was determined by the method of Gomori (1942). Serum concentrations of parathyroid hormone (PTH) were measured by immunoradiometric assay detecting N-terminal regions using a commercial kit (Rat PTH immunoradiometric assay kit, Nichols Institute Diagnostics, USA). Serum calcitonin concentrations were measured by radioimmunoassay using a commercial kit (Calcitonin RIA "Mitsubishi", Mitsubishi Petrochemical Co., Tokyo, Japan).

The significance of difference between the control and the CPP groups was determined by Student's t-test.

RESULTS

Feed intake, initial and final body weight and dairy gain of body weight during the experiment were not significantly different ($p>0.05$) between the control group and the CPP group (Table 5-2). The weights of liver, kidney and spleen in the CPP group were similar to those in the control group (Table 5-3).

Serum concentrations of PTH and calcitonin in the CPP group were not significantly different from those in the control group (Table 5-4).

Tibial physical parameters, i. e., length, width, wet weight, volume and specific

gravity, were not significantly influenced by the diets (Table 5-5). The dry weight and the content of crude ash, however, were significantly higher ($p<0.01$) in the CPP group than in the control group. The concentration of crude ash in tibial dry tissue was not significantly different between the two groups.

DISSCUSSION

Casein phosphopeptides have calcium-binding capacity due to its phosphorylated serine residues (Baumy et al. 1989) and make soluble complex with calcium. It has been suggested that CPP increase the solubility of calcium by inhibiting the formation of insoluble calcium salt in distal small intestine and stimulate intestinal calcium absorption by the passive transport in in situ study of rats (Kitts et al. 1992, Sato et al. 1986).

Dietary CPP, however, failed to increase intestinal calcium absorption in rats fed an adequate amount of calcium in previous studies (Brommage et al. 1991, Scholz-Ahrens et al. 1990, Yuan & Kitts 1991). The supplementation of CPP to a diet containing an adequate amount of calcium did not influence tibial weight and the content of crude ash and calcium in 6-week-old rats (See Chapter 4). When the amount of dietary calcium satisfies the needs of amimal, an increase in soluble luminal calcium by CPP will contribute little net calcium absorption.

In the present study, dietary supplementation of CPP increased tibial dry weight and ash content in weaning rats although the experimental diets contained the recommended amount of calcium. Calcium may be mainly absorbed by a passive transport in suckling animals because the availability of milk calcium is quite high. On the other hand, young animals fed normal solid diets with lower calcium availability than milk must absorbed much calcium by an active calcium transport. It is thought that intestinal calcium absorption is reduced in weaning animals until they adapt the solid diet. The reduction of calcium

absorption may greatly affect bone because bone growth needs much calcium. Funaba et al. (1996) reported that restriction of apparent absorption and retention of calcium was accompanied by the suppression of bone growth at early weaning of calves. In this study, ingested CPP could increase bone dry weight and ash content at weaning by improving intestinal calcium absorption by passive transport until the active transport developed to adapt the solid diets. Perhaps the adaptation of intestine was completed at the end of experiment because PTH concentration of serum in the control group was moderate. Therefore, the hormonal levels couldn't explain the results in tibia.

SUMMARY

Although CPP are known to stimulating intestinal calcium absorption, effects of the peptides on bone status has not been clarified. This study was conducted to investigate the effects of CPP on bone growth in weaning rats. After 16 male rats were weaned at 3 weeks old, they were divided into 2 groups and fed a control diet or a CPP diet for 3 weeks. The two diets contained similar amounts of calcium and phosphorus, which satisfied the recommended levels. These dietary treatments did not influenced body weight gain and the weights of liver, kidney and spleen. The CPP diet significantly increased tibial dry weight and ash content. These results suggested that dietary supplementation of CPP beneficially influenced bone growth in weaning rats, probably through improvement of calcium utilization at weaning.

Table 5-1. Composition of diets (%)

	Control	CPP
Ingredients		
CPP-III	0	3.0
Isolated soybean protein	20.0	17.0
Sucrose	49.35	49.90
DL-Methionine	0.5	0.5
Corn starch	15	15
Cellulose	5	5
Corn oil	5	5
Mineral mixture[1]	3.5	3.5
Vitamin mixture[2]	1	1
Choline chloride	0.1	0.1
$CaHPO_4 \cdot 2H_2O$	0.45	0
$CaCO_3$	0.10	0
Chemical composition[3]		
Calcium	0.68	0.66
Phosphorus	0.62	0.61

[1]AIN-76 mineral mixture (American Institute of Nutrition 1977)

[2]AIN-76 vitamin mixture (American Institute of Nutrition 1977)

[3]Author's analysis

Table 5-2. Effects of casein phosphopeptides (CPP) on feed intake, body weight, body weight gain and organ weight in weaning rats

	Control	CPP
Feed intake (g/day)	19.8±1.8	18.8±2.4
Initial body weight (g)	67±11	67±6
Final body weight (g)	223±16	220±22
Body weight gain (g/day)	7.4±0.6	7.1±0.8

Values are means±SD.

Table 5-3. Effects of casein phosphopeptides (CPP) on wet weight of liver, kidney and spleen in weaning rats

	Control	CPP
Liver (g)	13.2±0.7	12.1±2.1
Kidney (g)	1.98±0.22	1.93±0.23
Spleen (g)	0.68±0.08	0.66±0.14

Values are means±SD.

Table 5-4. Effects of casein phosphopeptides (CPP) on serum concentrations of PTH and calcitonin in weaning rats

	Control	CPP
PTH (pg/ml)	29.1±12.9	43.2±19.5
Calcitonin (pg/ml)	30.2±7.7	28.9±7.4

Values are means±SD.

Table 5-5. Effects of casein phosphopeptides (CPP) on parameters of right tibia in weaning rats

	Control	CPP
Length (mm)	30.38±0.87	29.98±0.43
Width (mm)	2.42±0.16	2.45±0.08
Wet weight (mg)	310±16	314±34
Volume (mm^3)	243±16	248±21
Specific gravity (g/cm^3)	1.28±0.04	1.27±0.05
Dry weight (mg)	139±8	150±5**
Crude ash (mg)	90±5	98±3**
Crude ash (mg/g DW)	647±8	653±8

Values are means±SD. **:Significantly different from control group ($p<0.01$).

Chapter 6

Effects of Dietary Casein Phosphopeptides on Mineral Metabolism in Rats Fed an Inadequate Amount of Calcium

INTRODUCTION

Milk and milk products have been reported to be excellent sources of calcium due to a large amount of available calcium. The high calcium availability of milk have been attributed to lactose (Sato et al. 1983, Shortt & Flynn 1991, Yuan & Kitts 1991) and casein phosphopeptides (CPP) (Lee et al. 1980, Nagasawa et al. 1991, Naito et al. 1972). The phosphopeptides are produced by enzymatic digestion of casein in vitro or during the digestion of casein in the small intestine (Mellander 1950, Naito et al. 1972, Naito & Suzuki 1974). The peptides include some phosphoserine residues and have capacity to bind to bivalent metal ions (Blakeborough et al. 1983, Brule et al. 1982, Dickson & Perkins 1971, Harzer & Kauer 1982, Lönnerdal et al. 1985). Therefore, CPP can prevent the precipitation of insoluble calcium-phosphate precipitates in the digestive tract by forming soluble complex with calcium (Li et al. 1989). It has been reported that CPP improve calcium absorption in vitro (Mykkänen & Wasserman 1980) and in situ (Kitts et al. 1992, Mykkänen & Wasserman 1980, Sato et al. 1986). Lee et al. (1992) reported that CPP increased apparent absorption of calcium and its retention in rats. However, the effects of CPP have not been clear on calcium absorption in vivo studies (Brommage et al. 1991, Kitts et al. 1992, Lee et al. 1992, Pointillart & Guëguen 1989, Scholz-Ahrens et al. 1990). The present study ascertained the effects of CPP on calcium availability in rats. Additionally, CPP have a possibility to improve absorption of other minerals. This study was also investigated the effect of CPP on absorption of phosphorus, magnesium and zinc in rats.

MATERIALS AND METHODS

A commercial CPP product (Meiji CPP-III®, Meiji Seika Kaisha, Ltd., Tokyo, Japan) was used in this study. It was produced by semi-purification of tryptic hydrolysate of whole bovine casein (see Fig. 4-1). Four experimental diets supplemented with CPP at 0, 0.1, 0.3 and 0.5 % were prepared according to the formulations shown in Table 6-1. All the diets commonly included isolated soybean protein as protein source and contained almost same amount of calcium, phosphorus, magnesium and zinc. The concentrations of calcium and zinc were slightly below the recommended levels for growing rats (National Research Council 1978) and the ratio of calcium and phosphorus was about 1 to 2.

Twelve eight female Wistar rats aged 6 week were individually housed in stainless steel metabolic cages in a room with controlled temperature (22°C), relative humidity (60%) and lighting (12-hour light/12-hour darkness). The animals were randomly allotted to 4 dietary groups of 7 animals each and freely given the experimental diets and distilled water for 28 days. From 3 days after the initiation of feeding trial, feed intake was recorded daily, and feces and urine were collected for 5 days. All the animals were exsanguinated under pentobarbital anesthesia at the end of the feeding trial and then the right femurs were collected.

Diets, feces and femurs were digested by nitric acid and perchloric acid and dissolved in 0.1 mol/l hydrochloric acid for mineral analysis. The concentrations of calcium, magnesium and zinc in the solutions and diluted urine were measured with an atomic absorption spectrophotometry. The phosphorus concentration was determined by the method of Gomori (1942).

Apparent mineral absorption was calculated as intake minus fecal excretion and was expressed as the percentage of intake. Mineral retention was calculated as intake minus fecal and urine excretion, and expressed as the percentage of intake.

Means of each CPP group were compared with those of the control (CPP-free) group by Fisher's least significant difference test. The level of significance was set at $p<0.05$.

RESULTS

The supplementation of CPP did not significantly influence feed intake and body weight gain (Table 6-2). Apparent absorption and retention of calcium were significantly ($p<0.05$) higher in the 0.3% CPP group than in the control group (Table 6-3). Apparent absorption and retention of calcium in the 0.5% CPP group tended to be higher than the control group ($p<0.10$). Furthermore, the 0.5% CPP group showed significantly higher apparent absorption ($p<0.05$) and retention ($p<0.01$) of phosphorus than the control group. Magnesium retention was significantly higher in the 0.3% CPP group ($p<0.01$) and the 0.5% group ($p<0.05$) than in the control group. There were no significant differences, however, in apparent absorption and urinary excretion of magnesium between the control group and either of the CPP groups. Apparent absorption and retention of zinc were significantly ($p<0.05$) higher in the 0.3% and 0.5% CPP groups than in the control group.

Calcium concentration in femur was significantly ($p<0.05$) higher in the 0.3% CPP group than in the control group (Table 6-4). There was a tendency ($p<0.10$) that femoral calcium concentration in the 0.5% CPP group also was higher than that in the control group. Femoral dry weight and crude ash content and the concentrations of phosphorus, magnesium and zinc did not differ between the control group and either CPP group.

DISCUSSION

Dietary supplementation of CPP at 0.3% and more increased apparent absorption and retention of calcium and femoral calcium concentration in rats fed 0.4% calcium diets. These results are in agreement with those of Lee et al. (1992). They reported that CPP

supplemented from 0.125% to 3.5% in 3.5% calcium diets increased apparent absorption and retention. On the other hand, there were some reports that CPP did not influence on calcium absorption in animals fed sufficient amounts of calcium (Brommage et al. 1991, Pointillart & Guégen 1989, Scholz-Ahrens et al. 1990, Yuan & Kitts 1991). Because dietary calcium concentration in the present study was 80% of the recommendation of National Research Council (1978), it was thought that the amount of calcium ingested by the rats was slightly below the requirement. Under such the condition, CPP could efficiently improve calcium absorption owing to its inhibitory effect on calcium precipitation. When an amount of dietary calcium fills the need, CPP could not facilitate calcium absorption any more. Because CPP can't directly influence the intestine (Li et al. 1989) and animals will not absorb excess calcium through the regulation of calcium absorption.

The supplementation of 0.5% CPP increased apparent absorption and retention of phosphorus. There have been a few reports which the effects of CPP on phosphorus availability are studied. Pointillart and Guëguen (1989) reported that a diet containing 5% of CPP increased phosphorus absorption, but not phosphorus retention in pigs. Tsuchita et al. (1995) and Goto et al. (1995) reported that diet containing 9 to 10 % of CPP increased femoral phosphorus in rats but did not affect phosphorus absorption. However, dietary phosphorus used by Pointillart and Gueguen (1989), Tsuchita et al. (1995), and Goto et al. (1995) in their studies were originated from CPP not from the inorganic salts. The results of the present study suggested that CPP might improve absorption of inorganic phosphorus directly. On the other hand, Gill et al. (1959) described that calcium and phosphorus interfered the absorption of the other mineral each other by forming insoluble calcium phosphate. Casein phosphopeptides may increase phosphorus absorption indirectly because CPP inhibit the precipitation of calcium phosphate (Mellander 1963, Reeves & Latour 1958). Phosphors absorption might be improved through the increase of calcium absorption in the

0.5% CPP group. However, phosphorus absorption in the 0.3% CPP group did not increase irrespective of the increase of calcium absorption. The reason of this is not clear.

The supplementations of 0.3% and 0.5% CPP increased magnesium retention. The change in apparent absorption and urinary excretion of magnesium was not clear enough to explain the increment of the retention. The increment of magnesium retention might be a result of a decrease in endogenous excretion of magnesium owing to an increase in body requirement accompanied by the increment of calcium and phosphorus retention. Because magnesium is one of components of bone ash beside calcium and phosphorus.

Rats fed the control diet and the 0.1% CPP diet showed poor availability of zinc. Dietary zinc content was less than the content recommended by National Research Council. Furthermore, soybean products have an adverse effect on zinc availability due to a large amount of phytic acid (Forbes et al. 1979, Lei et al. 1993, Lo et al. 1981). The substance in isolated soybean protein may have inhibited zinc availability in the control and 0.1% CPP groups. Apparent absorption and retention of zinc were increased by the supplementation of 0.3% and 0.5% CPP. Casein phosphopeptides have serine phosphate residues, which bind avidly to not only calcium but also other divalent metal ions (Blakeborough et al. 1983, Brulé et al. 1982, Dickson and Perkins 1971, Harzer and Kauer 1982, Lönnerdal et al. 1985). Dietary CPP, therefore, may have improved zinc availability by forming soluble complex with zinc. Platt et al. (1987) suggested that milk had a protective effect on the precipitation of zinc induced by phytate. Zinc in soybean protein was reported to be less available to chicken (O'Dell et al. 1960), pigs (Oberleas et al. 1962), and rats (Forbes & Yohe 1960) than that in casein. Higher zinc availability of casein may be not only due to phytate-free, but also to the existence of CPP. Therefore, CPP may be important in increasing the availability of zinc in animals fed diets containing phytate.

SUMMARY

Effects of casein phosphopeptides (CPP) on mineral availability were examined in rats. Animals were fed a control (CPP-free) diet or either of diets containing 0.1%, 0.3% and 0.5% CPP. All diets contained the same amounts of calcium (4 g/kg), phosphorus (8 g/kg), magnesium (0.6 g/kg) and zinc (10 mg/kg). Apparent absorption and retention of calcium were significantly ($p<0.05$) higher in rats fed the 0.3% CPP diet than rats fed the control diet. In addition, the 0.3% CPP diet significantly increased femoral calcium concentration compared to the control diet. Rats fed the 0.5% CPP diet had a tendency ($p<0.10$) to increase apparent absorption, retention and femoral content of calcium compared with rats fed the control diet. Furthermore, the 0.5% CPP diet significantly increased apparent absorption and retention of phosphorus. Rats fed the 0.3% and 0.5% CPP diets showed significantly higher apparent absorption and retention of zinc compared with rats fed the control diet. These two CPP diets also increased magnesium retention. These results suggest that CPP improved availability of not only calcium but also phosphorus, magnesium and zinc.

Table 6-1. Composition of experimental diets

	Dietary CPP level, %			
	0	0.1	0.3	0.5
Ingredient, g/kg				
Isolated soybean protein	200	199	198	196
Casein phosphopeptides	0	1	3	5
Starch	646.34	646.56	646.09	646.52
Corn oil	50	50	50	50
Cellulose	5	5	5	5
Vitamin mixture[1]	20	20	20	20
Mineral mixture[2]	40	40	40	40
Trace elements mixture[3]	2	2	2	2
Choline chloride	1	1	1	1
$CaCO_3$	5.96	5.84	5.61	5.38
KH_2PO_4	29.7	29.6	29.3	29.1
Chemical analysis				
Calcium, g/kg	3.7	3.7	4.0	4.0
Phosphorus, g/kg	7.8	8.1	7.3	8.9
Magnesium, g/kg	0.59	0.57	0.62	0.60
Zinc, mg/kg	10.2	10.4	11.0	10.5

[1]One gram of vitamin mixture contains 466IU of vitamin A, 233IU of vitamin D_3, 12mg of vitamin E acetate, 0.06mg of vitamin K_3, 0.59mg of vitamin B_1 HCl, 0.59mg of vitamin B_2, 0.29mg of vitamin B_6 HCl, 2μg of vitamin B_{12}, 5.88mg of vitamin C, 10μg of D-biotin, 20μg of folic acid, 2.35mg of Ca-pantothenate, 2.94mg of nicotinic acid, 11.76mg of inositol.

[2]Supplying(g/kg diet): $CaHPO_4$, 5.43; NaCl, 10; $MgSO_4 \cdot 7H_2O$, 4.

[3]Supplying(g/kg diet): $FeSO_4 \cdot 7H_2O$, 112; $CuSO_4 \cdot 5H_2O$, 40; $MnSO_4 \cdot 4H_2O$, 28; $ZnSO_4 \cdot 7H_2O$, 30; $Ca(IO_3)_3$, 0.28; $CoSO_4 \cdot 7H_2O$, 0.1.

Table 6-2. Effects of casein phosphopeptides (CPP) on feed intake and body weight in rats fed an inadequate amount of calcium[1]

	Dietary CPP level, %			
	0	0.1	0.3	0.5
Feed intake, g/d	15.7 ± 0.7	15.2 ± 0.4	15.0 ± 0.7	15.6 ± 0.4
Initial body weight, g	191 ± 4	190 ± 3	190 ± 3	189 ± 4
Final body weight, g	243 ± 6	237 ± 7	240 ± 6	243 ± 5
Body weight gain, g	51.5 ± 4.3	46.6 ± 4.6	50.3 ± 3.5	54.3 ± 5.0

[1]Values are means ± SEM.

Table 6-3. Effects of casein phosphopeptides (CPP) on mineral balances in rats fed an inadequate amount of calcium[1]

	Dietary CPP level, %			
	0	0.1	0.3	0.5
Calcium				
Intake, mg/d	58.1 ± 2.5	56.2 ± 1.5	60.0 ± 3.0	62.5 ± 1.6
Fecal excretion, mg/d	30.6 ± 1.5	29.9 ± 1.7	27.5 ± 1.7	29.0 ± 1.3
Urinary excretion, mg/d	0.17 ± 0.01	0.20 ± 0.01*	0.17 ± 0.01	0.17 ± 0.01
Apparent absorption, %	49.0 ± 2.3	48.9 ± 0.9	54.2 ± 1.7*	53.5 ± 1.8
Retention, %	48.7 ± 2.3	48.5 ± 0.9	53.9 ± 1.6*	53.2 ± 1.8
Phosphorus				
Intake, mg/d	122 ± 5	123 ± 3	110 ± 5	139 ± 4*
Fecal excretion, mg/d	20.6 ± 1.1	19.0 ± 1.7	18.1 ± 1.1	19.1 ± 0.5
Urinary excretion, mg/d	105 ± 6	103±3	96 ± 4	100 ± 4
Apparent absorption, %	83.7 ± 1.0	85.2 ± 0.9	83.4 ± 0.8	86.2 ± 0.4*
Retention, %	0.46 ± 2.18	5.39 ± 1.70	-4.13 ± 1.71	12.6 ± 1.1**
Magnesium				
Intake, mg/d	9.26 ± 0.41	8.65 ± 0.23	9.30 ± 0.46	9.37 ± 0.25
Fecal excretion, mg/d	5.04 ± 0.30	4.72 ± 0.37	4.31 ± 0.27	4.61 ± 0.21
Urinary excretion, mg/d	3.03 ± 0.10	2.94 ± 0.13	2.73 ± 0.08	2.77 ± 0.17
Apparent absorption, %	47.7 ± 2.0	47.9 ± 2.2	53.7 ± 1.7	50.8 ± 2.1
Retention, %	15.4 ± 2.1	13.3 ± 0.9	24.3 ± 0.8**	21.2 ± 2.1*
Zinc				
Intake, μg/d	162 ± 7	158 ± 4	165 ± 8	164 ± 4
Fecal excretion, μg/d	187 ± 15	179 ± 16	148 ± 8*	151 ± 9*
Urinary excretion, μg/d	48 ± 3	54 ± 3	42 ± 2	43 ± 4
Apparent absorption, %	-14.2 ± 12.0	-8.4 ± 6.5	9.5 ± 5.2*	8.2 ± 3.6*
Retention, %	-48.8 ± 15.3	-41.2 ± 7.6	-16.3 ± 6.5*	-16.4 ± 4.2*

[1]Values are means ± SEM. Means with asterisks are significantly different from the control (CPP-free) group (*: P<0.05, **: P<0.01).

Table 6-4. Effects of casein phosphopeptides (CPP) on dry weight, crude ash weight and mineral contents of femur in rats fed an inadequate amount of calcium[1]

	Dietary CPP level , %			
	0	0.1	0.3	0.5
Dry weight, mg	424 ± 11	414 ± 5	418 ± 9	406 ± 5
Crude ash, mg	251 ± 8	235 ± 7	249 ± 5	247 ± 6
Calcium, mg/g dry tissue	202 ± 3	204 ± 2	212 ± 3*	208 ± 2
Phosphorus, mg/g dry tissue	110 ± 2	111 ± 1	113 ± 2	112 ± 1
Magnesium, mg/g dry tissue	4.38 ± 0.12	4.31 ± 0.10	4.41 ± 0.09	4.39 ± 0.07
Zinc, μg/g dry tissue	172 ± 7	177 ± 8	169 ± 5	158 ± 5

[1]Values are means ± SEM. Means with asterisks are significantly different from the control (CPP-free) group ($P<0.05$).

Chapter 7

Effect of Dietary Casein Phosphopeptides on Metabolism of Ectopic Bone Induced by Decalcified Bone Matrix in Rats

INTRODUCTION

Casein phosphopeptides (CPP) are derived from trypsin-digested bovine α_{s1}- and β-casein. Ingested CPP increase luminal calcium solubility in the distal small intestine by inhibiting the formation of insoluble calcium salts due to their calcium-binding capacity. In a series of this study, it was found that CPP increased apparent calcium absorption and femoral calcium contents in rats fed an inadequate amount of calcium. The weight of femur itself, however, was not significantly influenced by CPP although bone is highly calcified tissue. The lack of significant change in skeletal integrity may be explained by relative stability of bone.

This study was designed to evaluate the effect of dietary CPP on the ectopic bone formation induced by the demineralized bone matrix in growing rats. In addition, whether the form of supplemented CPP influences their effects was examined.

MATERIALS AND METHODS

Demineralized bone matrix powder was prepared by the method of Huggins et al. (1970) The diaphysis of long bones of Wistar-strain rats were cleaned of the bone marrow and the adherent soft tissue. The bones were washed with water and then crushed and sieved to obtain particles of 200-400 μm. The powder was demineralized by 0.5 mol/liter HCl for 3 hours, and then washed in distilled water for 2 hours. The demineralized bone powder was subsequently defatted with ethyl ether.

Two kinds of CPP products with different forms, calcium-bound (CaCPP) and

calcium-free (Ca-free CPP), were prepared from a tryptic digest of bovine whole casein by an ethanol precipitation method (Tsuchita et al., 1993). The calcium-free product additionally received the treatment of calcium removal (Tsuchita et al., 1996). The calcium concentration of CaCPP and Ca-free CPP were 70 mg/g and trace, respectively.

Twenty-one male Wistar-strain rats being about 300 g body weight were individually housed in stainless steel cages. These animals were divided into 3 groups and fed a control diet or either of two CPP diets containing CaCPP or Ca-free CPP at 0.5% (Table 7-1). Each diet contained 0.26-0.27% calcium and 0.50-0.53% phosphorus. The dietary level of calcium was under the recommended level of 0.5% for growing rats. The diets and water were given ad libitum. One week after the initiation of feeding trial, each animal was subcutaneously implanted with gelatin capsules (No. 5, Eli Lilly, Indianapolis, USA) containing approximately 30 mg of the demineralized bone matrix powder under diethyl ether anesthesia.

Fourteen day after the implantation, all the rats were killed by blood collection from abdominal artery. And then, implants were dissected out from each animal. Blood was heparinized and centrifuged at 2500 rpm at 4°C for 15 minutes, and serum was separated. Two implants in each rat were free of adherent tissue for measuring calcium content and enzyme activities. For measuring enzyme activities, one cleaned implant in each rat was homogenized in 0.15 mol/liter NaCl containing 3 mmol/liter NaHCO3 at 4°C by Ultra disperser (Yamato Scientific Co. Tokyo, Japan) with 32G generator. The homogenates were centrifuged for 30 minutes at 4500 x g at 4°C and the activities of alkaline (EC 3.1.3.1) and tartrate-resistant acid (EC 3.1.3.2) phosphatase, and protein concentration in the supernatants were determined. Protein concentration was measured by the method of Lowry et al. (1951). The activity of the enzymes was determined by the amount of p-nitrophenol production from p-nitrophenylphosphate and 100 mmol/liter sodium carbonate buffer (pH 9.8). The solution

for the assay of tartrate-resistant acid phosphatase activity (TR-ACP) was 6.7 mmol/liter disodium p-nitrophenylphosphate, 50 mmol/liter sodium citrate buffer (pH 4.9) with 20 mmol/liter sodium tartrate. The other cleaned implant from each rat was ashed by nitric and perchloric acids and calcium content in the solution was measured by an atomic absorption spectrophotometry.

After feed samples were digested by nitric acid and perchloric acid and dissolved in hydrochloric acid and serum was deproteinized by 10% trichloroacetic acid, calcium concentration of these solutions was analyzed by atomic absorption spectrophotometry and phosphorus concentration was determined by the method of Gomori (1942).

The significance of difference among the dietary groups was determined by Student's t-test ($p<0.05$).

RESULTS

Initial and final body weights of rats were similar among the three dietary groups (Table 7-2). Serum concentrations of calcium and inorganic phosphorus were also not significantly influenced by the dietary treatments (Table 7-3).

Calcium content of implants was significantly higher in both the CPP groups than in the control group (Table 4). The TR-ACP activity was significantly lower in the CaCPP group, but not in the Ca-free group, than in the control group. The activity of implants in the CaCPP group was also significantly lower than in the Ca-free CPP group. Although ALP activity in implants in either of the CPP groups was not significantly different from that in the control group, the activity was significantly lower in the CaCPP group than in the Ca-free CPP group.

DISSCUSSION

Both the CaCPP and Ca-free CPP diets similarly increased calcium content of implants compared to the control diet. The enzyme activities in implants, however, were significantly different between the two CPP groups. The two kinds of CPP products seemed to increase calcium content in implants by different mechanisms of action.

The CaCPP diet significantly reduced TR-ACP activity in implants, and did not influence the ALP activity. These results in CaCPP are in agreement with the report of Matsui et al. (1994). They showed that calcium-bound CPP supplementation increased calcium content and decreased in TR-ACP activity in implants incubated for 21 days. As their suggestion, the increase in calcium content of implants could be partly due to a suppression of bone resorption, because TR-ACP activity is known to be related to bone resorption. A low calcium diet generally increases bone resorption. And Matsui et al. (1994) also observed higher TR-ACP activity in 14-day-implants in rats fed a low calcium diet than in rats fed a normal calcium diet. Perhaps, the suppression of TR-ACP activity in the Ca-CPP group may be explained by an improvement of calcium utilization, which is derived from the simulative effect of CPP on intestinal calcium absorption.

On the other hand, Ca-free CPP also increased calcium content of implants although the TR-ACP activity as well as the ALP activity were not significantly different between the Ca-free group and the control group. Both the two enzyme activities, however, were significantly higher in the Ca-free group than in the CaCPP group. Therefore, Ca-free CPP might increase the calcium content of implamts by increasing the ALP activity instead of decreasing the TR-ACP activity. Because ALP is known to be related to bone calcification. There is no firm evidence, however, that CPP increase ALP activity in skeletal tissue.

However, the increase in calcium content might not be related to the enzyme activities in the Ca-free CPP group. It could be speculated that CPP stimulated calcification by increasing the amount of available calcium for bone through an increase in intestinal

calcium absorption or another ways. If it is postulated that CPP or its fragments are absorbed from the intestine with a calcium-binding property, this possibility could be supported by the finding from an in vitro study that the supplementation of CPP to medium stimulated calcification of cultured embryonic rat bone.

SUMMARY

Although CPP are known to stimulate intestinal calcium absorption, effects of the peptides on bone status have not been clarified. This study was conducted to investigate the effects of CPP on ectopic bone formation induced by demineralized bone matrix in growing rats. In general preparations of CPP, the peptides bind much calcium in the process of isolation from hydrolyzed casein. The influence of the calcium-bound form of CPP on its action was additionally examined in this study. Twenty-one male growing rats were divided into 3 groups and fed a control diet without CPP or one of two CPP diets containing calcium-bound CPP (CaCPP) or calcium-free CPP (Ca-free CPP). These diets similarly contained 0.26 % calcium and 0.52% phosphorus. The dietary concentration of calcium was under the recommended level of 0.6% for growing rats. One week after the initiation of feeding for the experimental diets, demineralized bone matrix powder was subcutaneously implanted in all the animals. These implants were harvested 2 weeks after the implantation and evaluated calcium content, alkaline phosphatase (ALP) activity being a marker for bone calcification and tartrate-resistant acid phosphatase (TR-ACP) activity being a marker for bone resorption. These dietary treatments did not influenced body weight gain. Both the CaCPP and Ca-free CPP diets similarly increased calcium content of implants compared to the control diet. The CaCPP diet, but not the Ca-free diet, significantly reduced TR-ACP activity in implants. There were no significant differences in the ALP activity between the control group and the CaCPP group or the Ca-free CPP group. Each enzyme activity in implants, however, was

significantly different between the two CPP groups. These results suggested that dietary CPP with or without bound Ca stimulate bone calcification in growing rats fed insufficient calcium. There seems to be different mechanisms in the action of the two forms of CPP.

Table 7-1. Composition of diets (%)

	Control	Ca-CPP	Ca-free CPP
Ingredients			
Calcium-bound CPP	0	0.5	0
Calcium-free CPP	0	0	0.5
Isolated soybean protein	20.0	19.61	19.586
Corn starch	66.003	66.146	66.082
Corn oil	5	5	5
Cellulose	0.5	0.5	0.5
Vitamin mixture[1]	2	2	2
Macro mineral mixture[2]	4	4	4
Micro mineral mixture[3]	0.2	0.2	0.2
$CaCO_3$	0.374	0.285	0.374
KH_2PO_4	1.823	1.759	1.758
Chemical composition[4]			
Calcium	0.26	0.27	0.27
Phosphorus	0.52	0.53	0.50
Crude protein	19.6	18.7	18.5

[1]Containing the following per gram: vitamin A, 500 IU; vitamin D_3, 100 IU; dl-α-tocopheryl acetate, 5 mg: vitamin K_3, 5.2 mg; vitamin B_1·HCl, 1.2 mg; vitamin B_2, 4 mg; vitamin B_6·HCl, 0.8 mg; vitamin B_{12}, 0.5 μg; vitamin C, 30 mg; d-biotin, 20 μg; folic acid, 200 μg; calcium pantothenate, 5 mg; p-aminobenzoic acid, 5 mg; nicotic acid, 6 mg; inositol, 6 mg; choline chloride, 200 mg.

[2]Containing the following per gram: NaCl, 250 mg; $MgSO_4 \cdot 7H_2O$, 100 mg; $CaHPO_4$, 135.7 mg.

[3]Containing the following per gram: $FeSO_4 \cdot 7H_2O$, 112 mg; $CuSO_4 \cdot 5H_2O$, 40 mg; $MnSO_4 \cdot 4H_2O$, 28 mg; $ZnSO_4 \cdot 7H_2O$, 30 mg; $Ca(IO_3)_3$, 0.28 mg; $CoSO_4 \cdot 7H_2O$, 0.1 mg.

[4]Author's analysis.

Table 7-2. Effects of dietary casein phosphopeptides (CPP) on body weight gain in rats

	Control	Ca-CPP	Ca-free CPP
Initial body weight (g)	302±16	305±16	303±11
Final body weight (g)	368±22	365±17	382±20

Values are means±SD.

Table 7-3. Effects of dietary casein phosphopeptides (CPP) on serum concentration of calcium and inorganic phosphorus in rats

	Control	Ca-CPP	Ca-free CPP
Calcium (mg/100 ml)	9.03±0.41	9.18±0.39	9.33±0.13
Inorganic phosphorus (mg/100 ml)	5.13±0.37	4.94±0.54	5.17±0.59

Values are means±SD.

Table 7-4. Effects of dietary casein phosphopeptides (CPP) on calcium content and enzyme activities of implants in rats

	Control	Ca-CPP	Ca-free CPP
Calcium (mg/g wet tissue)	6.8 ± 2.0^{b}	19.5 ± 6.6^{a}	16.8 ± 9.7^{a}
TR-ACP (units/mg protein)	2.02 ± 0.18^{a}	1.50 ± 0.17^{b}	1.86 ± 0.08^{a}
ALP (units/mg protein)	8.15 ± 1.17^{ab}	7.19 ± 1.30^{b}	9.33 ± 1.06^{a}

Values are means±SD. One unit of enzyme activity was defined as 1 μmol of p-nitrophenol production for 30 minuets at 37°C. Values of dietary groups in a row with different superscripts are significantly different ($p<0.05$).

Chapter 8

Effects of Dietary Casein Phosphopeptides and Calcium Levels on Eggshell Quality and Bone Status in Laying Hens

INTRODUCTION

Laying hens need much calcium to produce eggshell. Eggshell calcium is derived from feed and bone. A shortage of dietary calcium not only lowers eggshell quality but also accelerates a decrease in skeletal calcium. While, excess calcium in a diet is also harmful to egg production (Gutowska & Parkhurst 1942). Therefore, it is important to increase calcium availability as well as to feed an adequate amount of calcium.

Milk is known to have much available calcium. One of the reasons for the high calcium availability of milk is accounted by casein, which is major protein of cow's milk. Lee et al. (1979, 1980, 1983) and Naito et al. (1972) reported that ingested casein produced phosphopeptides with high affinity for calcium in a small intestine and thus increased calcium solubility, resulting in contributing to an intestinal calcium absorption in rats. We also reported that an elevation of casein level in a diet increased apparent calcium absorption in rats (Ashida et al. 1994). Casein phosphopeptides (CPP) are also produced in vitro by trypsin digestion of bovine casein. The peptides have been shown to prevent the precipitation of calcium in rat small intestine (Hirayama et al. 1992b, Lee et al. 1992). Lee et al. (1992) reported that dietary supplementation of CPP improved apparent calcium absorption in rats. The stimulatory effect of CPP on intestinal absorption of calcium has been demonstrated in chicks (Mykkänen & Wasserman 1980).

In the present study, it was investigated whether dietary CPP was useful for eggshell quality and bone status of laying hens.

MATERIALS AND METHODS

We used commercially available CPP (Meiji CPP-III®, Meiji Seika Kaisha, Ltd., Saitama), which derived from the tryptic hydrolysate of whole bovine casein (Hirayama et al. 1992a), in this study. Feeding trial was performed in the farm of Shiga Prefectural Junior College, Kusatsu-shi, from May 10 to June 28 in 1993. Sixty White Leghorn laying hens which were in the eighth month after the first laying were individually housed in stainless-steel cages in a room without controlling ambient temperature and lightning. Feeds and water were given ad libitum. They were divided into two groups and preliminarily fed diets containing 3.4% or 1.5% calcium without CPP (Table 8-1) for one week. Then, at Week 0 of the experiment, each group was further divided into three groups and fed each of the 3.4% and 1.5% calcium diets supplied with 0, 0.5 or 1.0% CPP for six weeks. Each group was fed from the common feeder during this period. After feed samples were digested by nitric acid and perchloric acid, calcium and phosphorus contents were analyzed by atomic absorption spectrophotometry (AA-782, Nippon Jarrell-Ash, Kyoto) and by the method of Gomori (1942), respectively.

The amount of ingested feed per group, and the number and the weight of laid eggs per hen were recorded during the experiment, and the egg production and feed conversion were calculated. Eggs were collected from only hens laying at Week 0, 3 and 6, and the physical parameters of eggshell, i.e., breaking strength, dry weight, thickness and specific gravity, were measured. The breaking strength was measured by the analyzer of eggshell breaking strength (Eggshell intensity tester, Fujihira Kogyo, Tokyo). The specific gravity was determined on an Archimedean theorem.

Blood was collected with heparinized tubes from the wing vein in the morning at Week 0, 3 and 6. Immediately after the collection of blood, the ionized calcium concentration of whole blood was determined by a calcium ion analyzer (Sera-252, Horiba Ltd., Kyoto).

Randomly-selected five hens fed each diet were killed by decapitation at the end of the experiment. Their tibiae were dissected and adhering tissues were removed. After the tibiae were dried at 135°C for two hours, dry weight and specific gravity were measured. The specific gravity of tibia was determined by the same method as that of eggshell. After the tibiae were ashed at 600°C for 24 hours, ash content was measured.

Data were analyzed by two-way ANOVA using the General Linear Model procedure of SAS® (SAS Institute 1985) after the discard of outlying observations by the method of Smirnoff-Grubbs (Grubbs 1950). The overall effects of dietary levels of calcium and CPP and their interaction were tested. When the interaction was significant, significant differences among the means of each group were determined by Duncan's new multiple range test (Duncan 1955). A significant level of all the statistical tests were set at $p<0.05$.

RESULTS

The dietary levels of calcium and CPP did not systemically affect egg production of laying hens (Table 8-2). The egg production was, however, significantly lower in hens fed the 0.5% CPP diet in the 1.5% calcium group than in the other hens. Feed intake was similar in hens fed each of the experimental diets. Hence the hens fed the 0.5% CPP diet in the 1.5% calcium group showed relatively higher feed conversion than the other hens.

The blood concentration of ionized calcium was significantly decreased by the reduction of dietary calcium throughout the experiment (Table 8-3). The dietary levels of CPP did not affect the ionized calcium concentration irrespective of dietary calcium level at Week 3 and 6.

The reduction of dietary calcium level significantly decreased the breaking strength, the dry weight, the thickness and the specific gravity of eggshell almost throughout the experiment (Table 8-4). The effect of CPP supplementation on the specific gravity differed

with the dietary calcium levels at Week 6 as shown by the significant interaction between the effects of calcium and CPP levels. While the specific gravity was significantly higher in hens fed both the 0.5% and 1.0% CPP diets than in those fed the CPP-free diet in the 1.5% calcium group, there was no significant difference among the dietary levels of CPP in the 3.4% calcium group. The supplementation of CPP did not affect the specific gravity at Week 3 and the breaking strength, the dry weight and the thickness at Week 3 and 6, irrespective of dietary calcium levels.

The reduction of dietary calcium level also decreased the dry weight, the ash content and the specific gravity of tibia at the end of the experiment (Table 8-5). The significant interaction between the effects of dietary calcium and CPP levels showed that dietary calcium level affected the action of dietary CPP on the ash content and the specific gravity. The ash content was significantly higher in hens fed the 0.5% CPP diet than in hens fed the CPP-free diet in the 3.4% calcium group. The specific gravity was also significantly higher in hens fed the 0.5% and 1.0% CPP diets than in hens fed the CPP-free diet in the normal calcium group. Nevertheless, the tibial ash content and specific gravity of hens fed the diets supplied with CPP were not different from those of hens fed the CPP-free diet in the normal calcium group. The supplementation of CPP did not significantly affect the tibial dry weight of hens fed either normal calcium diets or low calcium diets.

DISSCUSSION

Bone is an important tissue for calcium store and the regulation of extracellular calcium concentration. Laying fowls need considerable calcium for forming eggshell. Since the amount of dietary calcium absorbed from the digestive tract is not enough to form eggshell, eggshell is supplied with calcium from bone. During eggshell calcification, bone resorption is activated and skeletal calcium is mobilized. Farmer et al. (1986) indicated that

eggshell weight linearly or quadratically decreased as dietary calcium level was lowered from 3.75 % to 0.08 % and the contribution of bone calcium to eggshell formation quadratically increased. Bolden & Jensen (1985) reported that a low calcium diet reduced egg-breaking strength and tibial bone ash. The present study showed that the 1.5 % calcium diet decreased eggshell quality and the concentration of blood ionized calcium with the reduction of bone dry weight, ash content and specific gravity. The lost of bone mass would result from stimulated bone resorption to mobilize calcium for eggshell formation and the maintenance of calcium homeostasis in hens fed the low calcium diet.

Dietary CPP recovered the specific gravity of eggshell reduced by the low calcium diet after six weeks. The absence of significant effect in the other parameters of eggshell may be caused by their great individual variations per group. The increment of the specific gravity might be partly due to the unaccountable decrease in egg production in hens fed the 0.5% CPP diet in the low calcium group. The specific gravity was higher in hens fed the 1.0% CPP diet than in those fed the CPP-free diet, although egg production was similar. The improvement of eggshell quality could be caused by an increase in the availability of dietary calcium because CPP has been reported to enhance intestinal calcium absorption (Lee et al. 1992, Sato et al. 1986).

Tibial characteristics were not affected by the supplementation of CPP in hens fed the low calcium diet. Although CPP supplementation to the low calcium diet improved eggshell quality as mentioned above, the improvement was not complete. Eggshell calcium would be still highly dependent on skeletal calcium in hens fed the low calcium diet with CPP. Therefore, it was thought that the amount of available calcium increased by CPP supplementation was not be enough much to suppress bone resorption in hens fed the low calcium diet.

The supplementation of CPP affected bone but not eggshell in hens fed the normal

calcium diet. The dietary level of 3.4% calcium, which satisfies the recommendation for calcium requirement of laying hens (National Research Council, 1994), was seemed to be enough high to form eggshell. Therefore, more calcium might not be necessary for eggshell production. Eggshell calcification would not be stimulated furthermore even if calcium absorption had been increased by CPP. It was reported that dietary CPP improved bone weight and calcium content in rats fed a low calcium diet (Hirabayashi et al. 1993, Lee et al. 1992). In addition, Matsui et al. (1994) showed that the CPP supplementation suppressed bone resorption in decalcified bone matrix implanted in rats fed a low calcium diet. A reduction of bone resorption is thought to increase bone mass and calcium content of hens fed the normal calcium diet containing CPP. Dietary CPP could suppress the mobilization of skeletal calcium through its stimulatory effect on intestinal calcium absorption. While, there is another possibility independent of calcium absorption. Kusuhara et al. (1992) demonstrated that dietary CPP stimulated the calcification of epiphyseal cartilage in growing broilers fed an enough amount of calcium. Matsui et al. (1994) observed that feeding of CPP increased alkaline phosphatase activity, which is involved in skeletal calcification, in the ectopic bone formation induced by bone matrix implantation in rats. Furthermore, CPP added in culture medium stimulated calcification of in vitro cultured embryonic rat bone (Berber & Jost 1986). The improvement of bone status in hens fed CPP might be explained by the stimulatory effect of CPP on bone calcification. Bone calcium content usually decreases even in laying hens fed a normal diet as they continue to produce eggs. The shortage of skeletal calcium results in a reduction of eggshell quality and egg production. Accordingly, the effects of dietary CPP are expected to appear in eggshell quality through the improvement of bone status in hens fed an enough amount of calcium when the experiment is prolonged.

The dietary CPP affected bone or eggshell without any change in the levels of blood ionized calcium. Because the blood concentration of ionized calcium was strictly controlled

by the hormones to maintain calcium homeostasis, the effect of CPP on calcium metabolism was too mild to change the ionized calcium levels in blood. The improvement of calcium utilization by CPP, however, might affect eggshell and bone through an increase in calcium-ligand complex in blood or changes in calcium-regulating hormones.

In conclusion, this study suggested that dietary CPP was useful in improving eggshell quality and bone status in laying hens and the effects of CPP could differed with the amount of calcium intake.

SUMMARY

This study was conducted to evaluate the effects of dietary casein phosphopeptides (CPP) on eggshell quality and bone status in laying hens fed low or normal calcium diets. Sixty laying hens were fed 1.5% or 3.4% calcium diets without CPP supplementation for one week. Then they were fed 3.4% or 1.5% calcium diets containing CPP at 0%, 0.5% or 1.0% for six weeks. In hens fed the 3.4% calcium diet, the supplementation with 0.5% or 1.0% CPP significantly ($p<0.05$) increased tibial ash content and specific gravity. However, CPP supplementation did not affect eggshell quality in these hens. The reduction of dietary calcium to 1.5% decreased the breaking strength, the dry weight, the thickness and the specific gravity of eggshell. Tibial weight, ash content and specific gravity were also reduced by feeding the low calcium diet. Although CPP supplementation did not affect the tibial parameters in hens fed the 1.5% calcium diet, 0.5% and 1.0% CPP supplied to the 1.5% calcium diet significantly increased the eggshell specific gravity . These results suggested that dietary CPP could increase bone mass and ash content of laying hens fed a normal calcium diet and improve eggshell quality of those fed a low calcium diet.

Table 8-1. Composition of experimental diets

	3.4% Calcium			1.5% Calcium		
Ingredients, %	0% CPP[1]	0.5% CPP	1.0% CPP	0% CPP	0.5% CPP	1.0% CPP
CPP[1]	0	0.5	1.0	0	0.5	1.0
Corn	56.4	56.4	56.4	56.4	56.4	56.4
Soybean meal	20.0	20.0	20.0	20.0	20.0	20.0
Fish meal	5.0	5.0	5.0	5.0	5.0	5.0
Alfalfa meal	4.0	4.0	4.0	4.0	4.0	4.0
Soybean oil	1.5	1.5	1.5	1.5	1.5	1.5
Cellulose	0	0	0	4.9	4.9	4.9
$CaCO_3$	7.3	7.3	7.3	2.4	2.4	2.4
NaCl	0.4	0.4	0.4	0.4	0.4	0.4
$CaHPO_4$	1.1	1.1	1.1	1.1	1.1	1.1
Premixture[2]	0.3	0.3	0.3	0.3	0.3	0.3
Corn starch	4.0	3.5	3.0	4.0	3.5	3.0
Chemical composition						
ME[3], Mcal/kg	2.80	2.80	2.80	2.80	2.80	2.80
Crude protein[3], %	18.5	18.9	19.3	18.5	18.9	19.3
Calcium[4], %	3.47	3.44	3.72	1.69	1.64	1.64
Phosphorus[4], %	0.61	0.62	0.66	0.62	0.64	0.65

[1]CPP=casein phosphopeptides (Meiji CPP-III®, Meiji Seika Kaisha, LTD., Tokyo).

[2]Provide the following per kg diet: Cu, 15 mg; Zn, 120 mg; Mn, 120 mg; I, 0.9 mg; vitamin A (retinyl acetate), 12,000 IU; vitamin D_3, 4,800 IU; vitamin E (dl-α-tocopheryl acetate), 9 IU; vitamin B_2, 9 mg; vitamin B_{12}, 6 μg; d-pantothenic acid, 6 mg; niacin, 30 mg; folic acid, 0.15 mg; choline, 210 mg; vitamin K (menadione sodium bisulfite), 12 mg.

[3]Calculated analysis.

[4]Authors' analysis.

Table 8-2. Effects of dietary casein phosphopeptides (CPP) and calcium levels on egg production, feed intake and feed conversion in laying hens

Dietary calcium, %	3.4			1.5			Probabilities[1]		
Dietary CPP, %	0	0.5	1.0	0	0.5	1.0	Ca	CPP	Ca×CPP
Egg production[2], %	75.7±3.1[a] (10)	79.1±2.2[a] (10)	76.4±3.6[a] (10)	77.6±3.9[a] (10)	64.0±3.3[b] (8)	77.7±4.1[a] (8)	0.160	0.209	0.026
Feed intake[3], g/d	118	120	116	114	117	117			
Feed conversion[3], g:g	2.30	2.29	2.31	2.29	2.78	2.25			

[1]Significance probabilities of effects based on ANOVA: Ca=dietary calcium levels; CPP=dietary CPP levels; Ca×CPP=interaction between the effects of dietary CPP and calcium levels.

[2]Values are means±SEM(n). Values in the same row with different superscript letters are significantly different ($p<0.05$).

[3]Data were not individually estimated. Therefore, ANOVA was not applied.

Table 8-3. Effects of dietary casein phosphopeptides (CPP) and calcium levels on blood ionized calcium concentrations in laying hens [1]

Dietary calcium, %	3.4			1.5			Probabilities[2]		
Dietary CPP, %	0	0.5	1.0	0	0.5	1.0	Ca	CPP	Ca×CPP
Blood ionized calcium concentrations, mmol/L									
Week 0	1.54±0.02 (30)	-	-	1.42±0.02 (30)	-	-	<0.001	-	-
Week 3	1.50±0.04 (10)	1.44±0.02 (10)	1.45±0.02 (10)	1.34±0.03 (10)	1.39±0.05 (10)	1.35±0.03 (9)	<0.001	0.802	0.222
Week 6	1.49±0.04 (10)	1.47±0.04 (10)	1.42±0.03 (10)	1.32±0.03 (10)	1.36±0.03 (10)	1.27±0.03 (10)	<0.001	0.066	0.678

[1]Values are means±SEM(n). Values in the same row with different superscript letters are significantly different ($p<0.05$).

[2]Significance probabilities of effects based on ANOVA: Ca=dietary calcium levels; CPP=dietary CPP levels; Ca×CPP=interaction between the effects of dietary CPP and calcium levels.

Table 8-4. Effects of dietary casein phosphopeptides (CPP) and calcium levels on breaking strength, dry weight, thickness, specific gravity of eggshell in laying hens[1]

Dietary calcium, %	3.4			1.5			Probabilities[2]		
Dietary CPP, %	0	0.5	1.0	0	0.5	1.0	Ca	CPP	Ca×CPP
Breaking strength, kg/cm^2									
Week 0	3.12±0.12 (28)	-	-	2.87±0.10 (30)	-	-	0.115	-	-
Week 3	2.83±0.20 (10)	3.25±0.28 (10)	3.06±0.10 (10)	2.51±0.18 (9)	2.54±0.17 (6)	2.80±0.16 (9)	0.011	0.346	0.514
Week 6	2.92±0.20 (9)	3.10±0.20 (8)	2.67±0.15 (9)	2.23±0.16 (8)	2.70±0.19 (6)	2.51±0.10 (7)	0.005	0.128	0.288
Dry weight, g/100g egg weight									
Week 0	8.18±0.05 (28)	-	-	7.49±0.13 (30)	-	-	<0.001	-	-
Week 3	8.13±0.23 (10)	8.30±0.31 (10)	8.39±0.17 (10)	7.31±0.16 (9)	7.58±0.15 (6)	7.63±0.19 (9)	<0.001	0.385	0.978
Week 6	8.23±0.16 (10)	8.41±0.16 (9)	8.59±0.17 (10)	7.21±0.16 (8)	7.60±0.22 (7)	7.66±0.19 (7)	<0.001	0.068	0.846
Thickness, μm									
Week 0	356±5 (28)	-	-	324±5 (30)	-	-	<0.001		
Week 3	344±8 (10)	352±13 (10)	356±7 (10)	336±6 (9)	345±6 (6)	347±12 (9)	0.311	0.422	0.995
Week 6	359±8 (10)	344±8 (9)	367±10 (10)	329±3 (8)	322±11 (7)	331±9 (7)	<0.001	0.189	0.730

(Continued)

(Continued, Table 4)

Specific gravity, g/cm^3									
Week 0	2.42±0.02 (28)	-	-	2.33±0.02 (30)	-	-	0.003	-	-
Week 3	2.31±0.01 (10)	2.30±0.02 (10)	2.32±0.02 (10)	2.02±0.02 (9)	2.06±0.02 (6)	2.11±0.04 (9)	<0.001	0.095	0.233
Week 6	2.12±0.01[a] (10)	2.10±0.01[a] (9)	2.13±0.02[a] (10)	2.01±0.01[b] (8)	2.11±0.02[a] (7)	2.11±0.02[a] (7)	0.003	0.003	0.001

[1]Values are means±SEM(n). Values in the same row with different superscript letters are significantly different ($p<0.05$).

[2]Significance probabilities of effects based on ANOVA: Ca=dietary calcium levels; CPP=dietary CPP levels; Ca×CPP=interaction between the effects of dietary CPP and calcium levels.

Table 8-5. Effects of dietary casein phosphopeptides (CPP) and calcium levels on dry weight, ash content and specific gravity of tibia in laying hens[1]

Dietary calcium, %	3.4			1.5			Probabilities[2]		
Dietary CPP, %	0	0.5	1.0	0	0.5	1.0	Ca	CPP	Ca×CPP
Dry weight, g	5.45±0.31 (5)	6.36±0.22 (4)	5.37±0.42 (5)	3.94±0.15 (3)	4.10±0.18 (5)	4.50±0.28 (4)	<0.001	0.252	0.088
Ash content, mg/g dry weight	501±11^{b} (5)	587±19^{a} (4)	529±34^{ab} (5)	526±16^{ab} (3)	490±7^{b} (5)	494±13^{b} (4)	0.044	0.347	0.028
Specific gravity, g/cm^3	0.96±0.02^{b} (5)	1.11±0.04^{a} (4)	1.09±0.04^{a} (5)	0.79±0.04^{c} (3)	0.81±0.00^{c} (5)	0.77±0.02^{c} (4)	<0.001	0.038	0.046

[1]Values are means±SEM(n). Values in the same row with different superscript letters are significantly different ($p<0.05$).

[2]Significance probabilities of effects based on ANOVA: Ca=dietary calcium levels; CPP=dietary CPP levels; Ca×CPP=interaction between the effects of dietary CPP and calcium levels.

Chapter 9

General Discussion and Conclusion

The effects of dietary casein on mineral metabolism and bone status in rats and hens were studied in a series of this study.

The effect of dietary casein level on mineral utilization was investigated in balance study and bone analysis during 180-day-feeding trial of a high casein diet in growing rats. The high casein diet increased calcium retention by stimulating the apparent absorption throughout the experiment. Phosphorus retention was also continually increased by the high casein diet through an increase in the apparent absorption besides a decrease in the urinary excretion. The high casein diet did not significantly influence magnesium retention in the early period. In the late period, however, the diet significantly increased magnesium retention by improving the apparent absorption. The chronic intake of high casein diet increased tibial dry weight, specific gravity and magnesium content. These results suggest that a long-term feeding of a high casein diet increases the utilization of calcium, phosphorus and magnesium and bone mass in rats.

Many functional peptides have been found in casein hydrolysates. Casein phosphopeptides (CPP) derived from trypsin-digested bovine α_{s1}- and β-casein are known to stimulate intestinal calcium absorption. The effect of dietary peptides derived from casein on bone growth was investigated in rats. A whole hydrolysate of trypsin-digested whole casein was divided into three fractions, and the other two fractions. These peptide fractions were supplemented into diets containing the recommended level of calcium, phosphorus and magnesium. Six-week-old male rats were fed these diets for 30 days. Only phosphopeptides-rich fraction showed significantly greater longitudinal growth of tibia. The other peptide fractions or a whole hydrolysate adversely affected the tibial integrity or mineral contents.

Therefore, it is suggested that casein hydrolysates involve several functional peptides related to bone metabolism and they alone or synergistically influence bone growth in rats.

The effects of CPP on bone growth was investigated in weaning rats. Three-week-old rats were fed a CPP diet containing adequate calcium. The dietary treatments did not influence body weight gain and liver, kidney and spleen weight. The CPP diet significantly increased tibial dry weight and ash content. It was suggested that dietary supplementation of CPP beneficially influences bone growth in weaning rats.

The effects of CPP on mineral availability were examined in rats fed insufficient calcium. Dietary CPP improved apparent intestinal absorption, retention and femoral content of calcium. The peptides also increased apparent absorption and retention of phosphorus and zinc. These results suggested that CPP increase the availability of calcium, phosphorus and zinc by improving intestinal absorption of these minerals.

The effects of CPP on bone metabolism were investigated using a short-term in vivo bone model. The supplementation of CPP to the low calcium diet increased calcium content of ectopic bone induced by demineralized bone matrix implantation. These results suggested that dietary CPP stimulate bone calcification in growing rats fed insufficient calcium.

The effects of dietary CPP on eggshell quality and bone status were evaluated in laying hens fed low or normal calcium diets. Dietary CPP significantly increased tibial ash content and specific gravity in hens fed normal calcium diets. However, CPP supplementation did not affect eggshell quality in these hens. The reduction of dietary calcium to 1.5% decreased the breaking strength, the dry weight, the thickness and the specific gravity of eggshell. Tibial weight, ash content and specific gravity were also reduced by feeding the low calcium diet. The supplementation of CPP did not affect the tibial parameters but significantly increased the eggshell specific gravity in hens fed the low calcium diets. These results suggested that dietary CPP could increase bone mass and ash content of laying hens

fed a normal calcium diet and improve eggshell quality of those fed a low calcium diet.

In conclusion, the action of high casein intake to bone status and calcium and phosphorus metabolism was thought to be partly explained by that of CPP. Dietary CPP appear to act on bone especially in cases of calcium shortage, i. e., under the situation of low calcium intake and weaning in rats, and laying egg in hens.

Acknowledgment

I would like to express my appreciation to Professor Dr. H. Yano for his full discussion, suggestion and encouragement throughout the course of this study. I also would like to express my sincere thanks to Professor Emeritus Dr. R. Kawashima for his giving me an opportunity to start this study in this laboratory.

I should express much thanks to Associate Professor Dr. T. Matsui for his patient guidance, benefit suggestion and encouragement through my research. I also can not forget Dr. F. Yano, Associate Professor in Kinki University now, for her much encouragement and a number of help through my research.

I would like to thank Dr. M. Funaba, Lecturer in Azabu University now, for his helpful guidance at the beginning of this study and encouragement throughout my research. Special thanks are given to Mrs. M. Hirabayashi for her generous cooperation with my research and much discussion in this study. I also wish to thank many other postgraduate students and colleague in the Laboratory of Animal Nutrition, staffs in the Department of Animal Science, and staffs and postgraduate students in the Laboratory of Animal Resources, Department of Tropical Agriculture for their help.

Finally, I would like to thank my parents for their financial support and encouragement throughout the course of this study.

Allen, L. H., Hall, T. E. (1978) Calcium metabolism, intestinal calcium-binding protein, and bone growth of rats fed high protein diets. J. Nutr. 108: 967-972.

Allen, L. H., Bartlett, R. S., Block, G. D. (1979a) Reduction of renal calcium reabsorption in man by consumption of dietary protein. J. Nutr. 109: 1345-1350.

Allen, L. H., Oddoye, E. A., Margen, S. (1979b) Protein-induced hypercalciuria: A longer term study. Am. J. Clin. Nutr. 32: 741-749.

American Institute of Nutrition (1977) Report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies. J. Nutr. 107: 1340-1348.

Azuma, N., Nageue, S., Ishino, Y., Mori, H., Kaminogawa, S., Yamauchi, K. (1989) DNA-synthesis stimulating peptides from human β-casein. Agric. Biol. Chem. 53: 2631-2634.

Barlet, J. P., Davicco, M. J. (1990) Parathyroid hormone-related peptide (PTHrP). Reprod. Nutr. Dev. 30: 639-651.

Baumy, J.-J., Guenot, P., Sinbandhit, S., Brule, G. (1989) Study of calcium binding to phosphoserine residues of β-casein and its phosphopeptide (1-25) by ^{31}P NMR. J. Dairy Res. 56: 403-409.

Bell, R. R., Engelmann, D. T., Sie, T.-L., Draper, H. H. (1975) Effect of a high protein intake on calcium metabolism in the rat. J. Nutr. 105: 475-483.

Berrocal, R., Chanton, S., Juillerat, M. A., Pavillard, B., Scherz, J.- C., Jost, R. (1989) Tryptic phosphopeptides from whole casein. II. Physicochemical properties related to the solubilization of calcium. J. Dairy Res. 56: 335-341.

Berthou, J., Migliore-Samour, D., Lifchitz, A., Delettrè, J., Floc'h, F., Jollès P. (1987) Immunostimulating properties and three-dimensional structure of two peptides from human and cow caseins. FEBS Lett. 218: 55-58.

Blakeborough, P., Salter, D. N., Gurr, M. I. (1983) Zinc binding in cow's milk and human milk. Biochem. J. 209: 505-512.

Bolden, S. L., Jensen, L. S. (1985) The effect of marginal levels of calcium, fish meal, torula yeast and alfalfa meal on feed intake, hepatic lipid accumulation, plasma estradiol, and egg shell quality among laying hens. Poult. Sci., 64: 937-946.

Brantl, V., Teschemacher, H., Henschen, A., Lottspeich, F. (1979) Novel opioid peptides derived from casein (β-casomorphins). I. Isolation from bovine casein peptone. Hoppe-Seyler's Z. Physiol. Chem. 360: 1211-1216.

Brink, E. J., Beynen, A. C., Dekker, P. R., Van Beresteijn, E. C. H., Van der Meer, R. (1992) Interaction of calcium and phosphate decreases ileal magnesium solubility and apparent magnesium absorption in rats. J. Nutr. 122: 580-586.

Brommage, R., Juillerat, M. A., Lost, R. (1991) Influence of casein phosphopeptides and lactulose on intestinal calcium absorption in adult female rats. Lait 71: 173-180.

Bronner, F., Pansu, D., Stein, W. D. (1986) An analysis of intestinal calcium transport across the rat intestine. Am. J. Physiol. 250: G561-G569.

Brule, G., Roger, L., Fauquant, J., Piot, M. (1982) Phosphopeptides from casein-based material. United States Patent No. 4358465.

Buchowski, M. S., Sowizral, K. C., Lengemann, F. W., Campen, D. V., Miller, D. D. (1989) A comparison of intrinsic and extrinsic tracer methods for estimating calcium bioavailability to rats from dairy foods. J. Nutr. 119: 228-234.

Calvo, M. S., Bell, R. R., Forbes, R. M. (1982) Effect of protein-induced calciuria on calcium metabolism and bone status in adult rats. J. Nutr. 112: 401-1413.

Chang, K.-J., Killian, A., Hazum, E., Cuatrecasas, P., Chang, J.-K. (1981) Morphiceptin (NH_4-Tyr-Pro-Phe-Pro-$CONH_2$): A potent and specific agonist for morphine (μ) receptors. Science 212: 75-77.

Chang, K.-J., Su, Y. F., Brent, D. A., Chang, J.-K. (1985) Isolation of a specific μ-opiate receptor peptide, morphiceptin, from an enzymatic digest of milk proteins. J. Biol. Chem. 260: 9706-9712.

Chiba, H., Tani, F., Yoshikawa, M. (1989) Opioid antagonist peptides derived from κ-casein. J. Dairy Res. 56: 363-366.

Dickson, I. R., Perkins, D. J. (1971) Studies on the interactions between purified bovine caseins and alkaline-earth metal ions. Biochem. J. 124: 235-240.

Dumas, B. R., Brignon, G., Grosclaude, F. and Mercier, J.-C. (1971) Structure primaire de la caséine β bovine. Enchaînement de 32 résidus d'amino-acides de la partie NH_2-terminale. Eur. J. Biochem. 20: 264-268.

Duncan, D. B. (1955) Multiple range and multiple F tests. Biometrics, 11: 1-42.

Dupuis, Y., Fournier, P. (1963) Lactose and the absorption of calcium and strontium. In: The transfer of calcium and strontium across biological membranes (Wasserman, R. H., ed.), pp265-276. Academic Press, New York, NY, USA.

Engstrom, G. W., DeLuca, H. F. (1963) Effect of egg white diets on calcium metabolism in the rat. J. Nutr. 81: 218-222.

Farmer, M., Roland, D. A., Sr., Clark, A. J. (1986) Influence of dietary calcium on bone calcium utilization. Poult. Sci., 65: 337-344.

Forbes, R. M., Weingartner, K. E., Parker, H. M., Bell, R. R., Erdman, J. W., Jr. (1979) Bioavailability to rats of zinc, magnesium and calcium in casein-, egg-, and soy protein-containing diets. J. Nutr. 109: 1652-1660.

Forbes, R. M., Yohe, M. (1960) Zinc requirement and balance studies with the rat. J. Nutr. 70: 53-57.

Funaba, M., Saito, S., Kagiyama, K., Iriki, T., Abe, M. (1996) Bone growth rather than myofibrillar protein turnover is strongly affected by nutritional restriction at early weaning

of calves. J. Nutr. 126: 898-905.

Funaba, M., Yano, H., Kawashima, R. (1989) Effect of high protein diet and sodium bicarbonate supplementation on calcium metabolism in rats. J. Nutr. Sci. Vitaminol. 35: 495-503.

Gerber, H. W., Jost, R. (1986) Casein phosphopeptides: their effect on calcification of in vitro cultured embyonic rat bone. Calcif. Tissue Int. 38: 350-357.

Gill, W. B., Finlayson, B., Vermeulen, C. W. (1959) Prevention of urinary calcium in rats by adding calcium to the diets: Experimental urolithiasis XVI. J. Yrol. 82: 256.

Gomori, G. (1942) A modification of the calorimetric phosphorus determination for use with photometric colorimeter. J. Lab. Clin. Med. 27: 955-962.

Goto, T., Yonehara, Y., Tsuchita, H., Kuwata, T. (1995) Availability of calcium (Ca) and phosphorus (P) from casein phosphopeptides (CPP) in growing rats. J. Jpn. Soc. Nutr. Food Sci. 48: 195-202.

Graves, K. L., Wolinsky, I. (1980) Calcium and phosphorus metabolism in pregnant rats ingesting a high protein diet. J. Nutr. 110: 2420-2432.

Grubbs, F. E. (1950) Sample criteria for testing outlying observations. Ann. Math. Stat. 21: 27-58.

Gutowska, M. S., Parkhurst, R. T. (1942) Studies in mineral nutrition of laying hens II. Excess of calcium in the diet. Poult. Sci., 21: 321-328.

Hardwick, L. L., Jones, M. R., Brautbar, N., Lee, D. B. N. (1991) Magnesium absorption: Mechanisms and the influence of vitamin D, calcium and phosphate. J. Nutr. 121: 13-23.

Harzer, G., Kauer, H. (1982) Binding of zinc to casein. Am. J. Clin. Nutr. 35: 981-987.

Heaney, R. P., Recker, R. R., Weaver, C. M. (1990) Absorbability of calcium sources: the limited role of solubility. Calcif. Tissue Int. 46: 300-304.

Heaney, R. P., Saito, Y., Orimo, H. (1994) Effect of caseinphosphopeptide on absorbability

of co-ingested calcium in normal postmenopausal woman. J. Bone Miner. Met. 12: 77-81.

Henschen, A., Lottspeich, F., Brantl, V., Teschemacher, H. (1979) Novel opioid peptides derived from casein (β-casomorphins). II. Structure of active components from bovine casein peptone. Hoppe-Seyler's Z. Physiol. Chem. 360: 1217-1224.

Hirayama, M., Toyota, K., Yamaguchi, G., Hidaka, H., Naito, H. (1992a) HPLC analysis of commercial casein phosphopeptides. Biosci. Biotech. Biochem. 56: 1126-1128.

Hirayama, M., Toyota, K., Hidaka, H., Naito, H. (1992b) Phosphopeptides in rat intestinal digesting casein phosphopeptides. Biosci. Biotech. Biochem. 56: 1128- 1129.

Howe, J. C., Beecher, G. R. (1981) Effect of dietary protein and phosphorus levels on calcium and phosphorus metabolism of the young, fast growing rat. J. Nutr. 111: 708-720.

Huggins, C., Wiseman, S., Reddi, A. H. (1970) Transformation of fibroblasts by allogeneic and xenogeneic transplants of demineralized tooth and bone. J. Exp. Med., 132: 1250-1258.

Jollès, P., Lévy-Toledano, S., Fiat, A.-M., Soria, C., Gillessen, D., Thomaidis, A., Dunn, F. W., Caen, J. P. (1986) Analogy between fibrinogen and casein. Effect of an undecapeptide isolated from κ-casein on platelet function. Eur. J. Biochem. 158: 379-382.

Kasai, T., Iwasaki, R., Tanaka, M., Kiriyama, S. (1995) Caseinphosphopeptides (CPP) in feces and contents in digestive tract of rats fed casein and CPP preparations. Biosci. Biotech. Biochem. 59: 26-30.

Kim, Y., Linkswiler, H. M. (1979) Effect of level of protein intake on calcium metabolism and on parathyroid and renal function in the adult human male. J. Nutr. 109: 1399-1404.

Kitano, T., Azami, S., Esasi, T. (1987) Effect of high protein intake on magnesium balance. JJSMgR. 6: 133-138.

Kitts, D. D., Yuan, Y. V., Nagasawa, T., Moriya, Y. (1992) Effect of casein, casein phosphopeptides and calcium intake on ileal ^{45}Ca disappearance and temporal systolic blood pressure in spontaneously hypertensive rats. Br. J. Nutr. 68: 765-781.

Kizawa, K., Naganuma, K., Murakami, U. (1995) Calmodulin-binding peptides isolated from α-casein peptone. J. Dairy Res. 62: 587-592.

Kusuhara, S., Hori, E., Watanabe, E., Saito, Y. (1992) Calcification of broiler epiphyseal cartilage using casein phosphopeptide. Jpn. Poult. Sci., 29: 254-261.

Law, F. M. K., Moate P. J., Leaver, D. D., Diefenbach-Jagger, H., Grill, V., Ho, P. W. M., Martin, T. J. (1991) Parathyroid hormon-related protein in milk and its correlation with bovine milk calcium. J. Endocrinol. 128: 21-26.

Lee, Y. S., Noguchi, T., Naito, H. (1979) An enhanced intestinal absorption of calcium in the rat directly attributed to dietary casein. Agric. Biol. Chem. 43: 2009-2011.

Lee, Y. S., Noguchi, T., Naito, H. (1980) Phosphopeptides and soluble calcium in the small intestine of rats given a casein diet. Br. J. Nutr. 43: 457-467.

Lee, Y. S., Noguchi, T., Naito, H. (1983) Intestinal absorption of calcium in rats given diets containing casein or amino acid mixture: The role of casein phosphopeptides. Br. J. Nutr., 49: 67-76.

Lee, Y. S., Park, G., Naito, H. (1992) Supplemental effect of casein phosphopeptides (CPP) on the calcium balance of growing rats. J. Jpn. Soc. Nutr. Food Sci. 45: 333-338.

Lei, X., Ku, P. K., Miller, E. R., Ullrey, D. E., Yokoyama, M. T. (1993) Supplemental microbial phytase improves bioavailability of dietary zinc to weaning pigs. J. Nutr. 123: 1117-1123.

Lengemann, F. W. (1959) The site of action of lactose in the enhancement of calcium utilization. J. Nutr. 69: 23-27.

Li, Y., Tomé, D., Desjeux, J. F. (1989) Indirect effect of casein phosphopeptides on calcium absorption in rat ileum in vitro. Reprod. Nutr. Develop. 29: 227-233.

Lo, G. S., Dettle, S. L., Steinke, F. H., Hopkins, D. L. (1981) Effect of phytic acid : zinc molar ratio and isolated soybean protein on zinc bioavailability. J. Nutr. 111: 2223-2235.

Lönnerdal, B., Keen, C. L., Hurley, L. S. (1985) Manganaese binding proteins in human and cow's milk. Am. J. Clin. Nutr. 41: 550-559.

Loukas, S., Varoucha, D., Zioudrou, C., Streaty, R. A., Klee, W. A. (1983) Opioid Activities and structures of α-casein-derived exorphins. Biochemistry 22-4567-4573.

Manson, W., Annan, W. D. (1971) The structure of a phosphopeptide derived from β-casein. Arch. Biochem. Biophys. 145: 16-26.

Maruyama, S., Mitachi, H., Awaya, J., Kurono, M., Tomizuka, N., Suzuki, H. (1987) Angiotensin I-converting enzyme inhibitory activity of the C-terminal hexapeptides of α_{s1}-casein. Agric. Biol. Chem. 51: 2557-2561.

Maruyama, S., Nakagomi, K., Tomizuka, N., Suzuki, H. (1985) Angiotensin I-converting enzyme inhibitor derived from an enzymatic hydrolysate of casein. II. Isolation and bradykinin-potentiating activity on the uterus and the ileum of rats. Agric. Biol. Chem. 49: 1405-1409.

Maruyama, S., Suzuki, H. (1982) A peptide inhibitor of angiotensin I converting enzyme in the tryptic hydrolysate of casein. Agric. Biol. Chem. 46: 1393-1394.

Matsui, T., Yano, H., Awano, T., Harumoto, T., Saito, Y. (1994) The influences of casein phosphopeptides on metabolism of ectopic bone induced by decalcified bone matrix implantation in rats. J. Nutr. Sci. Vitaminol. 40: 137-145.

McCance, R. A., Widdowson, E. M., Lehmann, H (1942) The effect of protein intake on the absorption of calcium and magnesium. Biochem. 36: 686-691.

Meisel, H. (1986) Chemical characterization of opioid activity of an exorphin isolated from in vivo digests of casein. FEBS lett. 196: 223-227.

Meisel, H., Frister, H. (1988) Chemical characterization of a caseinophosphopeptide isolated from in vivo digests of a casein diet. Biol. Chem. Hoppe-Seyler 369: 1275-1279.

Meisel, H., Frister, H. (1989) Chemical characterization of bioactive peptides from in vivo

digests of casein. J. Dairy Res. 56: 343-349.

Mellander, O. (1950) The physiological importance of the casein phosphopeptide calcium salts. II. Peroral calcium dosage of infants. Acta Soc. Med. Upsal. 55: 247-255.

Mellander, O. (1963) Phosphopeptides: Chemical properties and their possible role in the intestinal absorption of metals. In: The transfer of calcium and strontium across biological membranes (Wasserman, R. H., ed.), pp265-276. Academic Press, New York, NY, USA.

Mercier, J.-C., Grosclaude F., Ribadeau-Dumas B. (1971) Strucure primaire de la caséine α_{s1}-bovine. Séquence complète. Eur. J. Biochem. 23: 41-51.

Migliore-Samour, D., Jollès, P. (1988) Casein, a prohormone with an immunomodulating role for the newborn. Experientia 44: 188-193.

Mykkänen, H. M., Wasserman, R. H. (1980) Enhanced absorption of casein phosphopeptides in rachitic and normal chicks. J. Nutr. 110: 2141-2148.

Nagasawa, T., Yuan, Y. V., Kitts, D. D. (1991) Casein phosphopeptides enhance paracellular calcium absorption but do not alter temporal blood pressure in normotensive rats. Nutr. Res. 11: 819-830.

Nagaune, S., Azuma, N., Ishino, Y., Mori, H., Kaminogawa, S., Yamauchi, K. (1989) DNA-synthesis stimulating peptides from bovine β-casein. Agric. Biol. Chem. 53: 3275-3278.

Naito, H. (1986) The mechanism of enhancement in intestinal calcium absorption with phosphopeptides derived during casein digestion. J. Jpn. Soc. Nutr. Food Sci. 39: 433-439.

Naito, H., Kawakami, A., Imamura, T. (1972) In vivo formation of phosphopeptide with calcium-binding property in the small intestinal tract of the rat fed on casein. Agr. Biol. Chem. 36: 409-415.

Naito, H., Suzuki, H. (1974) Further evidence for the formation in vivo of phosphopeptide in the intestinal lumen from dietary β-casein. Agr. Biol. Chem. 38: 1543-1545.

National Research Council (1978) Nutrient requirements of laboratory animals. 3rd rev. ed.

National Academy Press, Washington, DC, USA.

National Research Council (1994) Nutrient requirements of poultry. 9th rev. ed. National Academy Press, Washington, DC, USA.

Oberleas, D., Muhrer, M. E., O'Dell, B. E. (1962) Effects of phytic acid on zinc availability and parakeratosis in swine. J. Anim. Sci. 21: 57-61.

O'Dell, B. L., Savage, J. E. (1960) Effect of phytic acid on zinc availability. Proc. Soc. Exp. Biol. Med. 103: 304-306.

Otsubo, K., Nagasaki, K., Oue, C., Kuranami, M., Honda, S., Yamaguchi, K. (1990) Parathyroid hormone-related protein in bovine milk products. Igaku no ayumi 152: 457-458.

Parker, F., Migliore-Samour, D., Floc'h, F., Zerial, A., Werner, G. H., Jollès, J., Casaretto, M., Zahn, H., Jollès P. (1984) Immunostimulating hexapeptide from human casein: amino acid sequence, synthesis and biological properties. Eur. J. Biochem. 145: 677-682.

Patrick, H., Bacon, J. A. (1957) The effect of vitamin D upon bone mineralization of Ca^{45} and Sr^{89} as chlorides and as phosphopeptides. J. Biol. Chem. 228: 569-572.

Petito, S. L., Evans, J. L. (1984) Calcium status of the growing rat as affected by diet acidity from ammonium chloride, phosphate and protein. J. Nutr. 114: 1049-1059.

Platt, S. R., Nadeau, D. B., Gifford, S. R., Clydesdale, F. M. (1987) Protective effect of milk on mineral precipitation by sodium phytate. J. Food Sci. 52: 240-241.

Pointillart, A., Guéguen, L. (1989) Effect of casein phosphopeptides on calcium and phosphorus utilization in growing pigs. Reprod. Nutr. Dev. 29: 477-486.

Putman, L. R., Froetschel, M. A., Barb, C. R., Azain, M. J., Edwards, G. L. (1996) Opioid and cholecystokinin antagonist block casein induced satiety in the meal-fed pig. J. Dairy Sci. 79 (Suppl. 1): 106 (Abs. D82).

Ratcliffe, W. A., Green, E., Emly, J., Norbury, S., Lindsay, M., Heath, D. A., Ratcliffe, J. G. (1990) Identification and partial characterization of parathyroid hormone-related protein in

human and bovine milk. J. Endocrinol. 127: 167-176.

Reeves, R. E., Latour, N. G. (1958) Calcium phosphate sequestering phosphopeptides from casein. Science 128: 472.

SAS Institute (1985) SAS® User's Guide: Statistics. Version 5 Edition. SAS Institute Inc., Cary, NC, USA.

Sato, R., Noguchi, T., Naito, H. (1983) The necessity for the phosphate portion of casein molecules to enhance Ca absorption from the small intestine. Agr. Biol. Chem. 47: 2415-2417.

Sato, R., Noguchi, T., Naito, H. (1986) Casein phosphopeptide (CPP) enhances calcium absorption from the ligated segment of rat small intestine. J. Nutr. Sci. Vitaminol. 32: 67-76.

Sato, R., Shindo, M., Gunshin, H., Noguchi, T., Naito, H. (1991) Characterization of phosphopeptide derived from bovine β-casein: an inhibitor to intra-intestinal precipitation of calcium phosphate. Biochim. Biophys. Acta 1077: 413-415.

Schlimme, E., Meisel, H. (1995) Bioactive peptides derived from milk proteins. Structural, physiological and analytical aspects. Die Nahrung 39: 1-20.

Scholz-Ahrens, K. E., Kopra, N., Barth, C. A. (1990) Effect of casein phosphopeptides on utilization of calcium in minipig and vitamin-D-deficient rats. Z. Ernährungswiss. 29: 295-298.

Schuette, S. A., Zemel, M. B., Linkswiler, H. M. (1980) Studies on the mechanism of protein-induced hypercalciuria in older men and women. J. Nutr. 110: 305-315.

Shenolikar, I. S., Rao, B. S. N. (1968) Influence of dietary protein on calcium metabolism in young rats. Ind. Jour. Med. Res. 56: 1412-1422.

Sherman, H. C. (1920) Calcium requirement in man. J. Biol. Chem. 44: 21-27.

Shortt, C., Flynn, A. (1991) Effect of dietary lactose on salt-mediated changes in mineral metabolism and bone composition in the rat. Br. J. Nutr. 66: 73-81.

Stah, B. G., Belonje, B., Paquet, A. (1990) The lack of effect of synthetic phosphoseryl peptide on calcium absorption by the rat. Nutr. Res. 10: 1331-1336.

Sterck, J. G. H., Ritskes-Hoitinga, J., Beynen, A. C. (1992) Inhibitory effect of high protein intake on nephrocalcinogenesis in female rats. Br. J. Nutr. 67: 223-233.

Totsuka, M., Kaminogawa, S. (1992) Physiological functions of the ingredients of animal products. Anim. Sci. technol. (Jpn.) 63: 867-877.

Tsuchita, H., Goto, T., Shimizu, T., Yonehara, Y., Kuwata, T. (1996) Dietary casein phosphopeptides prevent bone loss in aged ovariectomized rats. J. Nutr. 126: 86-93.

Tsuchita, H., Goto, T., Yonehara, Y., Kuwata, T. (1995) Calcium and phosphorus availavility from casein phosphopeptides in male growing rats. Nutr. Res. 15: 1657-1667.

Tuchita, H., Sekiguchi, I., Kuwata, T., Igarashi, C., Ezawa, I. (1993) The effect of casein phosphopeptides on calcium utilization in young ovariectomized rats. Z. Ernährungswiss. 32: 121-130.

Verbeek, M. J. F., Van den Berg, G. J., Lemmens, A. G., Beynen, A. C. (1993) High protein intake raises apparent but not true magnesium absorption in rats. J. Nutr. 123: 1880-1887.

Walker, R. M., Linkswiler, H. M. (1972) Calcium retention in the adult human male as affected by protein intake. J. Nutr. 102: 1297-1302.

Wang, C., Beede, D. K. (1990) Effects of supplemental protein on acid-base status and calcium metabolism of nonlactating Jersey cows. J. Dairy Sci. 73: 3178-3186.

Whiting, S. J., Draper, H. H. (1980) The role of sulfate calciuria of high protein diets in adult rats. J. Nutr. 110: 212-222.

Whiting, S. J., Draper, H. H. (1981) Effect of chronic high protein feeding on bone composition in the adult rat. J. Nutr. 112: 178-183.

Yoshikawa, M., Tani, F., Ashikaga, T., Yoshimura T., Chiba, H. (1986) Purification and characterization of an opioid antagonist from a peptic digest of bovine κ-casein. Agric. Biol.

Chem. 50: 2951-2954.

Yuan, Y. V., Kitts, D. D. (1991) Confirmation of calcium absorption and femoral utilization in spontaneously hypertensive rats fed casein phosphopeptide supplemented diets. Nutr. Res. 11: 1257-1272.

Zhang, X., Beynen, A. C. (1992) Increasing intake of soybean protein or casein, but not cod meal, reduces nephrocalcinosis in female rats. J. Nutr. 122: 2218-2225.

Zioudrou, C., Streaty, R. A., Klee, W. A. (1979) Opioid peptides derived from food proteins. J. Biol. Chem. 254: 2446-2449.