Title: The Nutritional and Physiological Role of Calcitonin on Calcium and Phosphorus Metabolism in Sheep

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CHAPTER 1 Introduction

Calcium and phosphorus are nutritionally important elements in animals. More than 99% of calcium and about 80% of phosphorus exist in the skeletal tissue where calcium and phosphorus make crystallized hydroxyapatite. And the crystal makes bone hard and protects bone against a mechanical stress. Calcium existing in soft tissues acts as a cofactor of some enzymes, as a second messenger of hormones and as a neuro-muscular transmitter. Calcium also needs contraction of muscles and exocytosis of some cells. Phosphorus is indispensable element in energy metabolism in almost all organisms because glycolysis starts at phosphate binding to glucose and produced energy stores as high-energy phosphate bound in adenosine triphosphate and creatine phosphate. And many coenzymes contain phosphorus. Furthermore phosphate is one of the components of nucleotides.

Calcium metabolism closely relates to phosphorus metabolism and all of calcium regulating factors directly or indirectly affect phosphorus metabolism. Extra cellular calcium concentrations were regulated rigidly by parathyroid hormone, vitamin D3 and calcitonin. It is very important to study the mechanism of calcium and phosphorus regulation in animal bodies from the viewpoint of animal growth, milk production and animal health.

It is well known that calcitonin, which is secreted from the thyroid gland possesses hypocalcemic activities in various
vertebrates (1,2,3). Munson et al. (4) found that calcitonin deficiency increased plasma calcium concentrations in pigs. And it was indicated that oral calcium load increased plasma calcium concentrations in thyroidectomized rats more than in sham operated ones (5). Furthermore, Kalu (6) showed that serum calcium concentrations were increased after feeding in thyroidectomized rats but were not changed in sham operated animals. From these results, it was proposed that one of the physiological role of calcitonin was prevention of postprandial hypercalcemia which was induced by acute absorption of calcium from the small intestine.

In ruminants, however, some workers indicated that thyroidectomy little affects serum calcium concentrations (7,8). Because ruminants have the rumen which can retain much digesta, it is thought that digesta moves more slowly to the lower digestive tract where calcium was mainly absorbed. It is natural to consider that the postprandial acute calcium absorption may hardly occur in ruminants.

It is widely known that calcitonin affect renal electrolytes excretion. Kimura and Ogata (9) proposed that hyperphosphaturemia was the most authentic and noticeable action of calcitonin in rats. On the other hand, Braithwaite (10) indicated that urinary loss of phosphorus was 4.2% of total loss of phosphorus in sheep. But Reddy et al. (11) reported that loss of phosphorus in urine was 30% in rats. These reports suggested that the importance of urinary phosphorus excretion in phosphorus metabolism was different between sheep and rats.

There is a possibility that physiological role of calcitonin
in ruminants differ from that in monogastric animals. The thesis was to study the nutritional and physiological role of calcitonin on calcium and phosphorus metabolism in sheep.
CHAPTER 2 Review of Literature

a) History of Studies about Calcitonin

Calcitonin was discovered and named by Copp et al. (12) in 1961 which was based on the observation that perfusion of calcium into the dog thyroid gland and parathyroid gland complex reduced plasma calcium concentrations. And they proposed that the parathyroid gland secreted not only parathyroid hormone but also a hypocalcemic factor, i.e., calcitonin. Two years later, Hirsch et al. (13) found that plasma calcium concentrations were decreased by cautery of the parathyroid gland within 30 min in rats, though the surgical removal of the parathyroid gland resulted slow decrement in plasma calcium concentrations. And they suggested that the cautery of the parathyroid gland induced to secretion of hypocalcemic factors from the thyroid gland. Then they succeeded in extracting thyrocalcitonin (calcitonin) from the thyroid gland of rats from which were removed the parathyroid gland (1).

The histological study of Bussolati and Pearse (14) showed that calcitonin was produced by the C-cell derived from the ultimobranchial gland. In mammals, the C-cell developed in the ultimobranchial body of the embryo and invaded the thyroid gland and the parathyroid gland (14). On the other hand, the ultimobranchial body resided as a separate gland in lower vertebrates (15). Copp et al. (15) and Tauber et al. (16) independently extracted calcitonin from the ultimobranchial gland.
of chick and dog-fish shark.

Though the extracts of the ultimobranchial gland reduced plasma calcium concentrations in rats (1), hypocalcemic effect of calcitonins was still controversial in lower vertebrates (17).

b) **Structure and Assay of Calcitonin**

Calcitonin has been isolated and purified from the ultimobranchial glands of eel (18) and salmon (19) and from the thyroid glands of porcine (20), bovine (21), sheep (22), rats (23) and human (24). Amino acid sequence of calcitonin from these species has been determined and it was clarified that all of them were composed of 32 amino acid residues, had a disulfide bridge between the 1st and the 7th amino acid residue, contained prolineamide in a carboxyl terminal and identify the 1st, 3rd, 4th, 5th, 6th, 7th, 9th, 28th and 32nd amino acid residues (25).

The other amino acid residues were widely different among species in a standpoint of physical characteristics, i.e., acidic, basic or aromatic (26). The variability of amino acid residues allows for considerable differences among them in terms of chemical properties, for example, the salmon calcitonin elutes earlier than the other calcitonins on gel filtrations (27).

Yamauchi and Orimo (25) proposed that calcitonins were classified into three groups according to differences of amino acid sequence of hormones as shown in Table 2-1. The classification was consist with the differences of biological and immunological activities (25).

It is well known that there is an active portion in many kind
### Table 2-1 Classification of Calcitonins

<table>
<thead>
<tr>
<th>Type</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmon</td>
<td>Salmon (I, II, III), Eel, Chicken</td>
</tr>
<tr>
<td>Human</td>
<td>Human, Rat</td>
</tr>
<tr>
<td>Porcine</td>
<td>Porcine, Bovine, Ovine</td>
</tr>
</tbody>
</table>

### Table 2-2 Immunoreactivities of Calcitonins

<table>
<thead>
<tr>
<th>Calcitonin</th>
<th>Antiserum against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Salmon</td>
</tr>
<tr>
<td>Salmon</td>
<td>++</td>
</tr>
<tr>
<td>Chicken</td>
<td>±</td>
</tr>
<tr>
<td>Human</td>
<td>-</td>
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<td>Rat</td>
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<td>Porcine</td>
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<td>Ovine</td>
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</table>
of hormones and that a fragment containing an active portion can represent the activity perfectly. On the other hand, any fragments of porcine calcitonin could not be active (24). Furthermore the prolineamide in the carboxyl terminal of the hormone was necessary for its activities (24) and calcitonin which was severed a disulfide bridge between the 1st and the 7th cysteine residues, could not decrease serum calcium concentrations (28). From these results, it was suggested that the whole molecule of calcitonin was necessary for the biological activity.

The bioassay of calcitonin has been based on the hypocalcemic activities in rats injected with calcitonins (1). It is prescribed that 10 mi.u. of calcitonin is the amount which reduces 10% of serum calcium concentrations 1 hour after injection into rats weighing about 150 g and being fasted for 24 hours (29).

Recently Talmage et al. (30) proposed that a hypocalcemic activity might be only a secondary manifestation of a more basic function and the bioassay on the basis of hypocalcemic activity did not reflect true activity. By this assay system, salmon calcitonin could not decrease serum calcium concentrations in salmon itself but was more effective than the same amount of rat calcitonin for rats.

Many workers reported the suppressive effect of calcitonin on bone resorption in various vertebrates. And Raisz et al. (31) and Orimo et al. (32) used this suppressive action for the bioassay of calcitonin in which 45-calcium was injected into pregnant rats in order to label fetal bone with 45-calcium then
fetal bone were isolated and incubated. The 45-calcium released into medium from bone became a quantitative index of bone resorption and it was shown that there was close correlation between the amount of calcitonin added to the medium and 45-calcium release. By this system, calcitonin contents could be detected.

These bioassay systems could detect calcitonin content ranging from 1.7 to 15 mi.u. which was much more than physiological calcitonin content in blood. However it was impossible to measure calcitonin concentrations in serum or plasma.

Deftos et al. (33) reported a radioimmunoassay of porcine calcitonin. They injected with only porcine thyroid extracts into guinea pigs 6-8 times to obtain antiserum. But their system could not detect serum calcitonin concentrations. In 1968, Tashjian et al. (34) succeeded in detection of serum calcitonin concentrations in pigs by a radioimmunoassay. Guinea pigs were immunized by porcine calcitonin bound to rabbit serum albumin which was emulsified with Freund's complete adjuvant. This immunization produced antibodies being useful for the radioimmunoassay of calcitonin. From these results, it was suggested that porcine calcitonin had not a powerful immunogenic activities and that conjugation to carrier increased immunogenic activities in porcine calcitonin.

Applying the method of Tashjian et al. (34), radioimmunoassay of calcitonin of human (35), rat (36), sheep (37), salmon (38) and eel (38) were developed and these radioimmunoassay systems indicated that immunological specificity of species. For instance, antiserum against porcine calcitonin did not respond
eel calcitonin. These results were shown in Table 2-2. And the immunological specificity was consistent with the differences of structure of calcitonin.

Recently it was developed that concentrations of several hormones in blood were detected by receptor assays using specific binding of hormones to membrane receptors. A hormone, which can bind receptors, may have biological activities. Though a radioimmunoassay detected only immunological activities.

Marx et al. (39) reported that the kidney had the specific receptor for calcitonin and they tried to develop a receptor assay using a purified membrane receptor from the kidney. However the sensitivity of this assay was not enough to measure serum concentrations in blood.

c) Secretion of Calcitonin

As mentioned previously, Copp et al. (12) suggested that the increment of calcitonin secretion were induced by the rise of calcium concentrations in blood. Care et al. (40) indicated that rates of calcitonin secretion from the thyroid gland fluctuated ranging from 5 to 30 mi.u./hour/kg body weight in an ewe when serum calcium concentrations varied from 8 to 12 mg/100 ml. And there was an intimate correlation (r=0.80-0.99) between serum calcium concentrations and calcitonin secreting rates.

Radde et al. (41) showed that an intravenous magnesium load reduced serum calcium concentrations in intact rats but thyroidectomy inhibited the hypocalcemia induced by magnesium load. From these results, they suggested that hypermagnesemia increased calcitonin secretion. However, Care et al. (42) found
that the stimulatory effect of hypermagnesemia on calcitonin secretion was usually less than the secretion caused by an equimolar increment in plasma calcium concentrations using a radioimmunoassay of calcitonin. And they suggested that changes in plasma magnesium concentrations did not influence calcitonin secretion under a normal conditions.

It is natural that parathyroid hormone increase serum calcium concentrations which stimulates calcitonin secretion. Furthermore Gittes (43) suggested that the parathyroid gland secreted thyrocalcitonin (calcitonin) releasing factor which increased the secretion rate of calcitonin. However, the direct effect of the parathyroid gland on calcitonin secretion is now denied by many workers (44,45).

It was reported that glucagon administration decreased serum calcium concentrations by way of increasing calcitonin secretion (46). However, physiological level of glucagon could not affect calcitonin secretion (47).

Cooper et al. (48) found that calcium load into the jejunum stimulated calcitonin secretion without the increment in serum calcium concentrations and they suggested that gastrin increased calcitonin secretion. And then they verified that calcitonin secretion increased when physiological dose of gastrin was infused into the thyroid gland (49). Furthermore Care et al. (50) and Cooper et al. (49) independently showed that cholecystokinin was also potent calcitonin releasing agent in pigs and that natural gastrin and synthetic analog such as pentagastrin could increase calcitonin secretion.
Some workers demonstrated that beta-adrenergic antagonists (51) and alpha-adrenergic antagonists (52) inhibited calcitonin secretion and Talmage et al. (30) suggested that calcitonin secretion was regulated by various neurotransmitters as well as by a number of humoral and paracrine factors.

Roos et al. (53) indicated that levels of calcitonin in blood were higher in female than in male rats. And plasma calcitonin concentrations varied according to the reproductive cycles, i.e., serum calcitonin concentrations increased during gestation and lactation and the lowest values were reached during estrous (54). However, the mechanism of relationship between calcitonin secretion and estrous cycles has not been clear.

Recently Deftos et al. (55) found that antiserum against calcitonin could bind the intermediate and the anterior pituitary lobes. They suggested that this intracellular material was calcitonin related substance and that it might be part of the 31 kilo dalton precursor protein known to give rise to ACTH, beta-lipotropin, beta-endorphin and enkephalin. And many workers (56,57) confirmed the existence of calcitonin like immunoreactivities in the anterior pituitary cells. Talmage et al. (30) proposed that the peptide like thyroidial calcitonin might be related to feeding behavior.

d) Action of Calcitonin

1) Kidney

In 1967, Rasmussen et al. (58) indicated that porcine calcitonin infusion led to a transient hyperphosphaturia and decreased excretion of urinary calcium and magnesium without a
change in glomerular filtration in rats. On the other hand, Barlet (2) found that infusion of porcine, salmon and human calcitonin at a physiological level increased urinary calcium and phosphorus excretion but decreased urinary magnesium excretion in sheep. Kimura and Orimo (9) found that urinary calcium, magnesium and phosphorus excretion were increased by a large dose of salmon calcitonin injection (1-1.3 i.u./rat) but phosphorus excretion in urine was increased and calcium excretion was decreased by small dose injection (2 mi.u./rat) in young rats.

The effect of calcitonin on urinary calcium and magnesium excretion were obscure and results were fluctuated by the difference of kind, dose and purity of calcitonin, method of administration and animals used (9). However, the hyperphosphaturic effect of calcitonin was generally observed in almost all experiments.

It is well known that calcitonin does not affect glomerular filtration rate. Furthermore calcitonin inhibits reabsorption of phosphorus at the renal proximal convoluted tubule because calcitonin can increases urinary phosphorus excretion even if animals were administrated by a functional inhibitor of the distal renal tubule (59).

The kidney seems to be an endocrine gland of active vitamin-D₃, i.e., 1,25-dihydroxyvitamin-D₃ (60). Galante (61) suggested that calcitonin might increase the activity of 25-hydroxyvitamin-D₃ 1-alpha-hydroxylase in the kidney, though an inhibitory effect also reported (62). The effect of calcitonin on activation of vitamin-D₃ is now controversial.
Suda et al. (63) found that calcium concentrations ranging from 0.05-0.2 mM were correlated to the 1-alpha-hydroxylase activities but higher concentrations of calcium (0.3-0.5 mM) inhibited the activities in mitochondrial suspensions of the kidney. Borle (64) showed that calcitonin increased cytoplasmic concentrations of calcium by means of the inhibition of calcium efflux from cells in the kidney. There is a possibility that the changed calcium concentrations in cytoplasm by calcitonin affect changes of the hydroxylase activities in the kidney.

Calcitonin stimulates parathyroid hormone secretion by means of reduction of serum calcium concentrations and parathyroid hormone is the major activator of the 25-hydroxyvitamin-D3 1-alpha-hydroxylase in the kidney (65). Calcitonin induced the decrease in serum phosphorus concentrations which stimulated vitamin-D3 activation (66). It is natural to consider that calcitonin indirectly, at least, increased 1,25-dihydroxyvitamin-D3 synthesis.

2) Bone

In 1965, Friedman et al. (67) found that the stimulatory effect of parathyroid hormone on bone resorption in fetal bone of rats were inhibited by calcitonin in vitro. Rasmussen et al. (58) showed that the increased urinary hydroxyproline excretion by parathyroid hormone administration were disappeared by calcitonin injection in rats. Because hydroxyproline is one of the unique amino acids of collagen and about 60% of collagen in a whole body exists in bones (68), Rasmussen et al. (58) suggested that calcitonin inhibited bone resorption in the presence of
parathyroid hormone in vivo. Cohn and Wong (69) indicated that calcitonin inhibited hyaluronate synthesis, acid phosphatase activities and mineral/matrix resorption stimulated by parathyroid hormone in isolated osteoclast like cells in vitro. These results indicated that stimulative effect of parathyroid hormone on osteoclastic bone resorption was inhibited by calcitonin in vivo and in vitro.

On the other hand, calcitonin could reduce serum calcium concentrations in parathyroidectomized rats (1). And some workers suggested that the action of calcitonin on bone resorption was also observed in the absence of parathyroid hormone (67,70,71).

The mechanism of decreased bone resorption by calcitonin was not well known. Histological studies suggested that the suppressive effect of calcitonin on osteoclastic bone resorption was brought about by the decrease of osteoclastic number, the reduction of ruffled borders of osteoclasts and the inhibition of the cytoplasmic motility of osteoclast in vitro (72).

Talmage (30) suggested that osteoclastic bone resorption reduced by calcitonin might be important in bone remodeling because calcitonin treatment could increase in bone density and ultimobranchial gland ectomy decreased bone density in submammalian species (17). However, an influence of calcitonin on physiological bone remodeling in mammals have not yet been established.

It has been suggested that the rapid calcium flux system between blood and bone fluid transported calcium at least 20 fold more than bone formation and bone resorption (74). Grubb et al.
found that calcitonin inhibited 45-calcium efflux from bone fluid within minutes but calcitonin had no direct effect on calcium influx. Furthermore Norimatsu et al. (76) proposed that calcium efflux from bone fluid to blood (extra cellular fluid) was regulated by the lining cell-osteocyte complex on the bone surface. And they suggested that the reduction of calcium efflux from bone fluid was most likely to the cause of the hypocalcemic effect of calcitonin.

The mechanism of regulation of calcium efflux was not clear. But there is a interesting suggestion that calcitonin decreased cytoplasmic calcium content in bone cell through permeability of calcium ion which may be related to the stimulation or restriction of several enzymic actions. The changes of enzymic activities might regulate calcium efflux from bone fluid (30). Recently Vanderwiel et al. (77) suggested that calcitonin stimulated extracellular accumulation of phosphorus in regions adjacent to bone surface using electron microprobe analysis. The increase of phosphorus in bone fluid can stimulate binding phosphorus to calcium in the physicochemical nature which may reduce calcium efflux from bone fluid.

The effect of calcitonin on bone formation and bone growth are still controversial. McWhinné (78) suggested that adequate dose of calcitonin injection increased bone alkaline phosphatase activity which was related to bone calcification and the gain of bone length was stimulated by calcitonin injection in the femur of chick embryo. And Orimo et al. (79) indicated that calcitonin directly increased alkaline pyrophosphatase activities in the
tibia of thyroparathyroidectomized rats. Because Russel et al. (80) proposed that inorganic pyrophosphate overshadowed bone hydroxyapatite crystal surface and inhibited the growth of bone crystal, it was considerable that calcitonin might stimulate bone formation through the removal of pyrophosphate from bone crystal surface.

On the other hand, Cohn and Wong (69) indicated that collagen synthesis and bone alkaline phosphatase activities of osteoblast was not affected by calcitonin administration in vitro. Furthermore, Milhaud and Mookhar (81) showed that calcitonin significantly reduced deposition of calcium salt using 45-calcium kinetics. And Baylink et al. (73) histologically suggested that calcitonin decreased bone and bone matrix formation and that the magnitude of the decrease in bone formation was 24% and that of matrix formation was 22%. In addition, they found that the decrease in bone resorption by calcitonin was 68%. Because bone formation was coupled with bone resorption, the suppressive effect of calcitonin on bone formation were indirect action of calcitonin, i.e., the inhibition of bone formation was induced by calcitonin restricting bone resorption.

3) Gastro-Intestinal Tract

Though the digestive tract is important in calcium and phosphorus absorption, the effect of calcitonin on calcium absorption is still obscure and the effect on phosphorus absorption is little known.

It was reported that the gastro-intestinal tract was not necessary for the hypocalcemic action of calcitonin because
calcitonin administration reduced serum calcium concentrations in gastroenterectomized rats as same as in normal ones (82). Krawitt (83) did not find any significant change in calcium absorption which was evaluated by the disappearance of 45-calcium from the small intestine after calcitonin injection in rats. Some investigators indicated that intestinal calcium absorption was elevated by calcitonin administration both in vitro and vivo (81,84).

On the other hand, by measuring the disappearance of 45-calcium from the small intestine and the efflux of 45-calcium to the portal vein in situ, Olson et al. (85) indicated that the large dose (500 mi.u./rat) of calcitonin infusion increased calcium absorption but the small dose (10 mi.u./rat) of calcitonin decreased calcium absorption within 4 hours and they suggested that physiological level of calcitonin inhibited calcium absorption and remarkable hypocalcemia induced by pharmacological level of calcitonin increased calcium absorption. Barlet (86) indicated that calcium and phosphorus excretion in feces increased after long term infusion of physiological level of calcitonin in sheep. And Swaminathan et al. (87) suggested that calcitonin indirectly inhibited calcium absorption because calcium absorption, measured by the disappearance of 45-calcium from the thyli-vela loop, was reduced 2 days after the beginning of calcitonin infusion in pigs. And they hypothesized that calcitonin inhibited vitamin-D₃ activation in the kidney and the reduction of 1,25-dihydroxyvitamin-D₃ decreased calcium absorption from the small intestine.

It is known that calcitonin injection reduces secretion of
gastrin and gastric acid in man (88). Furthermore Hufner et al. (89) reported that calcitonin injection decreased pancreatic enzymes secretion and inhibited the contraction of the gallbladder. Yamaguchi (90) showed that calcitonin injection increased bile calcium and phosphorus excretion without the reduction of bile flow rate in thyroparathyroidectomized rats. He suggested that the hypocalcemic effect of calcitonin was due to increasing in the excretion of calcium via bile because the ligature of bile duct prevented a hypocalcemic action of calcitonin. Meyer and Meyer (91) reported that liver phosphorus content was increased by calcitonin injection in thyroparathyroidectomized rats which might induce the increment in bilary phosphorus excretion. Gray et al. (92) found that calcitonin administration affect water and electrolytes transferation in the small intestine in man.

4) Other Organs

Rizzo and Goltzman (93) reported that specific and saturable recepte sites for calcitonin were widely distributed in the central nervous systems of the rats and were predominant in the hypothalamus.

Freed et al. (94) found that subcutaneous single injection of calcitonin could suppress food intake for 48-72 hours in rats. Furthermore they showed that pharmacological dose of calcitonin were necessary for reducing food intake when rats were administrated calcitonin subcutaneously but a lower dose of calcitonin was effective when animals were given calcitonin intracerebroventricularly (94). However Talmage et al. (30)
suggested that calcitonin levels circulating under normal condition did not affect feeding behavior because the amount of calcitonin enough to act on the brain could not pass the blood-brain barrier physiologically.

As previously mentioned, calcitonin like peptide were found in the anterior pituitary cell (56,57). And the peptide might regulate feeding behavior in stead of thyroidial calcitonin (30).

However, many workers still propose that calcitonin secreted from the thyroid gland affects the brain (94,95). The functions of calcitonin and calcitonin like peptide in the brain are new aspects for calcitonin studies.

It was reported that parathyroid hormone stimulated mitosis, DNA synthesis and cell proliferation in the thymus and the bone marrow and that this action was inhibited by calcitonin in rats (96). Furthermore Rixon et al. (97) suggested that calcitonin was necessary for the regenerative response of the liver which were partially ectomized because calcitonin deficiency severely reduced the regenerative activity which was measured by 3H-thymidine incorporation to DNA and a small dose of calcitonin injection recovered the activity in calcitonin deficient animals.

e) Physiological Role of Calcitonin

It was originary suggested by Copp et al. (12) that calcitonin administration reduced plasma calcium concentrations and that the role of calcitonin was to regulate calcium concentrations in extracellular fluid within a narrow range of fluctuation. Hypocalcemia induced by calcitonin was reported in the European
eel (98) but was not found in some amphibians and birds of which calcitonin induced hypocalcemia in rats (99).

A large dose of calcitonin injection which severely reduced serum calcium concentrations in young rats produced little hypocalcemia in old rats (100). However, hyperphosphaturic action of calcitonin was found also in old rats and several other actions of calcitonin were not affected by aging (101). From these results, Talmage et al. (30) proposed that the hypocalcemic effect of calcitonin might be only a secondary manifestation of more basic function.

Munson et al. (4) reported that thyroidectomy of fasted pigs increased plasma calcium concentrations. Gray and Munson (5) indicated that oral calcium load increased plasma calcium concentrations more markedly in thyroidectomized rats than in sham operated ones. Furthermore Barlet (102) found that calcitonin inhibited hypercalcemia induced by 1,25-dihydroxyvitamin-D$_3$ injection. And the antihypercalcemic action did not change with increasing age of rats. It is established that one of calcitonin roles is an antihypercalcemic action.

Milhaud et al. (103) and Kalu (6) found that plasma calcium concentrations were increased in thyroidectomized rats but were not changed in intact ones after feeding and they suggested that the physiological role of calcitonin was in the prevention of postprandial hypercalcemia which was induced by acute calcium absorption from the small intestine.

Talmage et al. (104) found that postprandial renal calcium excretion was less in sham rats than in thyroidectomized ones
when animals were fed a diet containing normal calcium but
urinary calcium loss was more in sham rats than in
thyroidectomized ones after the animals were fed the calcium free
diet. These results suggested that postprandial urinary calcium
excretion was primary related to the calcium content of the last
meal in thyroidectomized rats but, in intact rats, urinary
calcium excretion was affected by the amount of calcium intake in
the preceding days. It had been hypothesized that calcitonin
secretion during feeding led to conserve dietary calcium in the
bone to provide a source of calcium for the usage during fasting
period. The hypothesis was confirmed by the report of Vanderwirk
and Talmage (105) that calcium containing compound increased in
the lining cell-osteocyte units on the endosteal surface in
intact rats 3 hours after feeding and the compound disappeared at
the next 12 hours after decreasing gradually. They reported that
the compound was found neither in thyroidectomized rats nor in
intact rats fed calcium free diet.

It was suggested that calcitonin was more effective in
lactating rats than in nonlactating ones because the hypocalcemic
effect of calcitonin was much greater in the thyroidectomized
lactating rats than in thyroidectomized nonlactating ones (106).
Garel et al. (37) indicated that serum calcitonin concentrations
increased in pregnant and lactating sheep and the same results
were found in rats and women (107). It is well known that a
substantial amount of calcium were lost into milk and fetus.
Barlet (108) indicated that calcitonin might protect the bone
against the loss of calcium which was suspected to occur during
lactation in goats. Hirsch and Hagaman (109) suggested that a
single cycle of pregnancy and lactation proceed normally in the deficiency of calcitonin but multiple cycles were impaired by the absence of calcitonin in rats.

f) Studies in Ruminants

There were a few studies about calcitonin in ruminants. In 1957, Stott and Smith (8) suggested that thyroidectomy did not affect serum calcium concentrations in cows and the same results were shown in sheep by Nelson (7). However, in these experiment, thyroxine was not supplied in spite of thyroidectomy which induced not only calcitonin deficiency but also the lack of thyroxine secretion. And thyroxine was known to affect bone metabolism which was closely related to the action of calcitonin (110). Furthermore these studies were not counted on the influence of feeding on fluctuation of serum calcium concentrations after thyroidectomy though it was reported that thyroidectomy increased plasma calcium concentrations after feeding in rats (6).

On the other hand, Inskeep et al. (111) showed that intraperitoneal load of calcium increased serum calcium concentrations more remarkably in thyroidectomized sheep than in intact ones and calcitonin inhibited hypercalcemia induced by activated vitamin-D3 (102). From these results, it was indicated that calcitonin could possess antihypercalcemic action also in ruminants. In 1972, Barlet (2) indicated that calcitonin infusion decreased serum calcium concentrations and increased in urinary calcium and phosphorus excretion in sheep.

It was found that serum calcitonin concentrations were
increased by pregnancy and lactation in sheep and that calcitonin deficiency substantially decreased bone calcium during lactation in sheep (108).

As mentioned previously, it was indicated that excretion of calcium and phosphorus in feces were increased in sheep by continuous calcitonin infusion which was suggested that calcitonin inhibited absorption of calcium and phosphorus in ruminants (86). However, the experiment could not be shown the effect of calcitonin on the true absorption of calcium and phosphorus but that on apparent absorption because calcitonin may affect endogenous secretion of calcium and phosphorus into the gastro-intestinal tract.
CHAPTER 3 Determination of Serum Calcitonin Concentrations by Radioimmunoassay

Many investigators assumed that thyroidectomy induced calcitonin deficiency (4,5,6,103). However, C-cell which produces calcitonin in the thyroid gland may exist in the parathyroid gland because it is reported that C-cell developing in the ultimobranchial body of the embryo invades the thyroid gland and the parathyroid gland in mammals (14). In addition, recently Nakanishi et al. (112) indicated that the bovine 31k pituitary peptid contained the amino acid sequence similar to calcitonin in the N-terminal which was suggested that calcitonin related substance was produced in the pituitary gland. The detection of serum calcitonin concentrations was required for the substantiation that calcitonin was not secreted in thyroidectomized animals.

There are several bioassay systems of calcitonin but the sensitivity of these systems are rather low and calcitonin concentrations in blood can not be detected by bioassays (1,31,32). In 1969, Tashjian et al. (34) succeeded in detecting serum calcitonin concentrations in pigs by a radioimmunoassay. As shown in Table 2-1, calcitonins were classified into 3 groups from the stand point of the difference in amino acid sequence and each class had immunological specificity (25). And Garel et al. (37) measured ovine plasma calcitonin concentrations using a porcine radioimmunoassay system in 1974. This experiment was to
study on the analytical procedure of serum calcitonin concentrations in sheep by a new porcine radioimmunoassay system and also to examine whether thyroidectomized sheep secreted calcitonin or not.

Materials and Methods

1) Production of antiserum

Porcine calcitonin (Armour Pharmaceutical Company, Eastbourne, England; 73 i.u./mg) and the same content of bovine serum albumin were bound by the method of Reichlin et al. (113). Six guinea pigs were injected subcutaneously with the porcine calcitonin bound to bovine albumin (3 i.u. in 0.8% NaCl) which was emulsified with the same volume of Freund's complete adjuvant (Nakarai Chem. Ltd., Kyoto) at 2 week intervals.

Blood was collected from each animal by cardiac puncture under light ether anesthesia 10 days after injections. And the antisera were stored at -20°C.

2) Iodination and purification

Porcine calcitonin was labeled with the chloramine-T method of Greenwood et al. (114). According to a conventional method, 125-I labeled calcitonin was purified by Sephadex G-25 fine eluted with 0.05M phosphate buffer(pH 7.5). And then labeled calcitonin was further purified by Dowex ion-exchange resin 1X10 (Muromachi Kagaku, Kyoto) eluted with 0.1N HCl. Furthermore the labeled calcitonin were purified by Sephadex G-50 fine eluted with 0.05M phosphate buffer(pH 7.5).

Radioactivity of every fractionated tube was countered by well gammar counter (Aloka, Chigago, USA).
3) Binding activity of labeled calcitonin to antisera

Several antisera were diluted to 1:1000, 1:5000, 1:7500, 1:10000 and 1:20000 with 0.05M phosphate buffer (pH 7.5). The diluted antisera and the diluent were incubated with labeled calcitonin (10000 cpm/0.1ml) at 4°C for 24 hours. The final volume was 0.5 ml (0.1 ml labeled porcine calcitonin + 0.1 ml diluted antiserum + 0.3 ml diluent). After incubation, calcitonin binding to antibody and free calcitonin was separated by the chacol coated dextran method.

4) Plotting the standard curve

Porcine calcitonin (Armour Pharmaceutical Company, Eastbourne, England, 73 i.u./mg) was used as standard in the assay. Dilutions in 0.05 M phosphate buffer (pH 7.5) were prepared in the concentration ranging to 10-500000 pg/ml.

Two methods of incubation were tried to plot standard curve, i.e., nonsaturated incubation and saturated ones. As shown in Table 3-1, the optimal diluted antiserum and standard solutions were mixed and incubated for 24 hours prior to adding labeled calcitonin then the mixture of all solutions were incubated for 48 hours with the nonsaturated method. On the other hand, all of solution were mixed at the same time and then the mixture was incubated for 48 hours with the saturation method.

The sensitivity was ensured by student's t test.

5) Separation of bound calcitonin from free calcitonin

Numerous separation method have been reported but some of these techniques had some difficulties in handling with a large number of samples. In this experiment, the methods which could
Table 3-1 Method of Plotting the Standard Curve

<table>
<thead>
<tr>
<th>Saturated Method</th>
<th>Nonsaturated method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted antiserum 100 µl</td>
<td>Diluted antiserum 100 µl</td>
</tr>
<tr>
<td>Standards 100 µl</td>
<td>Standards 100 µl</td>
</tr>
<tr>
<td>$^{125}$I labeled Calcitonin 100 µl</td>
<td>Phosphate buffer 200 µl</td>
</tr>
<tr>
<td>Phosphate buffer 200 µl</td>
<td>Incubation for 24 hours at 4°C</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubation for 48 hours at 4°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Incubation for 24 hours at 4°C</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Addition of $^{125}$I labeled calcitonin 100 µl</td>
</tr>
<tr>
<td></td>
<td>Incubation for 24 hours at 4°C</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcitonin binding to antibody and free calcitonin were separated by the chaoel coated dextran method</td>
<td></td>
</tr>
</tbody>
</table>


operate many samples at the same time, i.e., the dextran-charcol method (34), the talc method (37) and the double antibody method (150 using anti guinea pig IgG serum of goat) were tried. Radio activities absorbed by charcoal, talc or the second antibody were detected by an auto well gamma counter (Aloka, Chicago, USA).

6) Radioimmunoassay of serum calcitonin concentrations in sheep

As shown in Table 3-2, the radioimmunoassay has been done. And 24-48 hours of preincubation and 24-96 hours of incubation were used. The effects of protease inhibitor addition on the sensitivity of radioimmunoassay system were also examined. The diluent was prepared as 1 ml of the final solution contained 100 ki.u. of kallikrein trypsin inhibitor (Trasylor, Beringer Manheim Yamanouchi, Tokyo)

7) Animal preparation

Three intact sheep and 3 thyroidectomized sheep (ectomized 8 months before the experiment), weighing about 40 kg, were used. Every sheep was injected intravenously with 1.26 mM CaCl per kg dissolved in 0.6 ml of distilled water within 1 minute. Blood samples were collected before and 30 minutes after the injection. And serum calcium and calcitonin concentrations were measured.

Results and Discussion

Labeled calcitonin was initially purified by filtration on Sephadex G-25 fine (Fig. 3-1). One major peak eluted from the column at fraction number 10 which had higher radioactivity (18X100000 cpm/5 drops). Then the other peak was found at fraction number 23, which had rather high radioactivity (5X100000 cpm/5 drops). It seems that the first peak is 125-I binding to
Table 3-2 Method of Radioimmunoassay

<table>
<thead>
<tr>
<th>Diluted antiserum</th>
<th>100 µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard or sample</td>
<td>100 µl</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>100 µl</td>
</tr>
<tr>
<td>Trypsin inhibitor</td>
<td>100 Ki.u.</td>
</tr>
</tbody>
</table>

Incubation for 48 hours at 4°C

Addition of $^{125}I$ labeled
100 µl

Incubation for 96 hours at 4°C

Addition of serum
(to standard)
or phosphate buffer
(to sample)

Calcitonin binding to antibody and and free calcitonin were separated by the chaco coated dextran method
Fig. 3-1 Gel filtration of $^{125}$I labeled calcitonin on Sephadex G-25 (fine)
Column size; 1X15 cm
Eluted solution; 0.05M Phosphate buffer (pH 7.5)

Fig. 3-2 Ion exchange chromatography of Sephadex G-25 purified calcitonin which was labeled by $^{125}$I on Dowex 1X10
Column size; 1X5 cm
Eluted solution; 0.1N HCl
Fig. 3-3 Gel filtration of Dowex 1X10 purified calcitonin which was labeled by $^{125}$I on Sephadex G-50 (fine)
Column size; 1X30 cm
Eluted solution; 0.05M Phosphate buffer (pH 7.5)

Fig. 3-4 Binding capacity of labeled calcitonin against diluted antisera
The ratios of bound to free labeled calcitonin was shown on the vertical axis.
The dilution of antisera was shown on the horizontal axis.
calcitonin and the second peak is free 125-I. The fraction number 10 to 13 were used by the further purification.

As shown in Fig. 3-2, the mixture of the 10th to 13th fractions were applied to Dowex 1X10. About 70% of the radioactivity was unabsorbed and eluted from the column. Because Dowex 1X10 absorbed cations in acidic states, only 125-I binding to calcitonin could elute.

The next step was purification of calcitonin by Sephadex G-50 fine (Fig. 3-3). The first sharp peak was eluted at the 21st fraction and the second peak was at the 34th fraction which was rather broad and had lower radioactivities. From the elution time, it was clear that the 1st peak was 125-I binding to calcitonin and the second was 125-I binding to calcitonin fragments which was produced by calcitonin degradation during the labeling procedures.

Four immunized guinea pigs produced antisera which could bind labeled calcitonin at a dilution of 1:1000. But the antiserum which could be useful for the radioimmunoassay at a dilution of 1:5000-1:10000 was produced by a guinea pig after 7 times of immunization. The serum were used at a dilution of 1:8000 for further studies (Fig. 3-4).

Two standard curves produced by the saturation and the nonsaturation methods are illustrated in Fig 3-5. In the saturation method, More than 100 pg/ml of porcine calcitonin was detected. On the other hand, about 50 pg/ml of calcitonin could be measured by nonsaturation method. The bound/free ratio was significantly (P<0.01) different between blank and 5 pg (50
Fig. 3-5 Standard curves in the saturation (O) and the nonsaturation (●) methods of radioimmunoassay. The ratios of bound to free labeled calcitonin was shown on the vertical axis. The amount of standard calcitonin was shown in the horizontal axis.
Fig. 3-6 The comparison of the separation methods of bound calcitonin and free calcitonin.

(O); Charcol dextran method.

(Δ); Talc method

(□); Second antibody method

The ratios of bound to free labeled calcitonin was shown on the vertical axis.
The amount of standard calcitonin was shown in the horizontal axis.
Table 3-3 Effect of calcium load on serum calcitonin concentrations in thyroidectomized sheep (pg/ml)

<table>
<thead>
<tr>
<th></th>
<th>Before load</th>
<th>After load</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>105</td>
<td>820</td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>1020</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>910</td>
</tr>
<tr>
<td>Thyroidectomy</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D. values were less than 50 pg/ml.

Table 3-4 Effect of calcium load on serum calcium concentrations in thyroidectomized sheep (mg/100ml)

<table>
<thead>
<tr>
<th></th>
<th>Before load</th>
<th>After load</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>9.27</td>
<td>15.09</td>
</tr>
<tr>
<td></td>
<td>8.61</td>
<td>12.38</td>
</tr>
<tr>
<td></td>
<td>9.51</td>
<td>11.89</td>
</tr>
<tr>
<td>Thyroidectomy</td>
<td>9.68</td>
<td>13.61</td>
</tr>
<tr>
<td></td>
<td>8.29</td>
<td>14.47</td>
</tr>
<tr>
<td></td>
<td>8.90</td>
<td>13.04</td>
</tr>
</tbody>
</table>
pg/ml) of calcitonin in the nonsaturation method.

Fig. 3-6 showed that the dextran charcol method was the most suitable for this assay. And the second antibody method did not separated 125-I binding to calcitonin from free 125-I.

As shown in Table 3-3, serum calcitonin concentrations were not detected by 24 hours of preincubation and 24-48 hours of incubation. By 48 hours of incubation and 48-72 hours of incubation, serum calcitonin concentrations in some samples could be detected however coefficients of variation were rather high and serum calcitonin in a sample could not be detected. On the other hand, all serum calcitonin concentrations became higher and coefficients of variation became lower by the addition of the protease inhibitor. From these results, 48 hours of preincubation and 96 hours of incubation were used in the presence of the protease inhibitor.

As shown in Table 3-4, serum calcitonin concentrations belonged to about 50-100 pg/ml in intact sheep. However, in thyroidectomized sheep, serum calcitonin concentrations were not detected by the assay system. Serum calcium concentrations were increased by the calcium load in intact sheep as well as in thyroidectomized one. On the other hand, serum calcitonin concentrations were increased and reached to 600-1000 pg/ml in intact sheep but were not detected in thyroidectomized animals after calcium load.

It was suggested that the thyroidectomy induced the disappearance of endogenous calcitonin secretion in sheep. If the parathyroid gland or the pituitary gland secreted calcitonin, the amount of calcitonin secretion was very low and the secretion
from these glands might not be affected by the increase in serum calcium concentrations.

Summary

To measure serum calcitonin concentrations, a radioimmunoassay system has been developed. Guinea pigs were injected with porcine calcitonin which was bound to bovine serum albumine and was emulsified with Freund's complete adjuvand to produce antisera.

125I-labeled calcitonin was purified by Sephadex G-25, Dowex 1X10 and Sephadex G-50. From the test of binding activity of labeled calcitonin to antisera, it was shown that one of the sera was useful for the radioimmunoassay at a dilution of 1:5000-1:10000. Antiserum, sample and diluent were mixed and then preincubated for 48 hours. Then the labeled calcitonin (10000 cpm) were added. The solution was mixed and incubated for 96 hours. The incubation mixture was contained 100 ki.u. of protease inhibitor. Calcitonin bound to antibody and free calcitonin were separated by dextran-charcol method. By this assay system, 50 pg/ml of calcitonin concentrations were detected.

Serum calcitonin concentrations were 50-100 pg/ml in intact sheep. Serum calcitonin concentrations were increased and reached to 600-900 pg/ml by intravenous calcium load. However, serum calcitonin concentrations were not detected in thyroidectomized sheep even after the calcium load.

It was suggested that thyroidectomy induced calcitonin deficiency in sheep.
CHAPTER 4 Effect of Thyroidectomy and Thyroparathyroidectomy on Calcium and Phosphorus Metabolism

It was shown that calcitonin deficiency induced by thyroidectomy increased serum calcium concentrations in rats and pigs (4). Kalu (6) suggested that a physiological role of calcitonin was an inhibition of postprandial hypercalcemia which might be induced by the elevation of calcium absorption from the digestive tract.

On the other hand, some workers showed that serum calcium concentrations had little changed after thyroidectomy in adult sheep (7) and cows (8). Though thyroxine affects bone metabolism (110) and one of the major target of calcitonin was bone (30, 67, 76, 77), thyroxine therapy was not provided in these reports. Furthermore it was known that action of calcitonin on calcium metabolism became weaker along with aging (100).

It is well known that parathyroid hormone is the antagonist against calcitonin in calcium metabolism. And the decrease in calcium concentrations in blood has been shown by thyroparathyroidectomy in rats (116), goats (117) and sheep (7). Kalu et al. (118) indicated that the lowering of calcium concentrations in blood plasma in thyroparathyroidectomized rats was resulted from the decrease of bone resorption and the increase of calcium excretion in urine. While Payne and Sanson (119) reported that serum calcium and phosphorus concentrations lowered after thyroparathyroidectomy though thyroparathyroid-
ectomy enhanced to release minerals from the bone and scarcely increased urinary calcium excretion in goats.

The experiment was to study the effects of thyroidectomy and thyroparathyroidectomy on calcium and phosphorus metabolism in adult sheep with thyroxine administration and to study the effect of feeding on daily fluctuation of serum calcium concentrations in lambs.

Materials and Methods

Experiment 1

Ten adult sheep, weighing about 40 kg, were used. All animals were kept in metabolism cages and given the diet shown in Table 4-1 at a level of 2% of body weight at 9:00 a.m. daily. Water was available at all times. Three wethers were thyroidectomized and 4 animals were thyroparathyroidectomized by Nelson's method (7) 4 hours after feeding. The other 3 sheep were intact.

Thyroidectomized and thyroparathyroidectomized animals were injected intramuscularly with 2.5 mg of L-thyroxine (Nakarai Chemicals, Ltd., Kyoto) dissolved in corn oil once a week. The first time of injection was one week after surgery. Serum thyroxine concentrations were measured by a radioimmunoassay (120) on the 3rd and 6th day after L-thyroxine injection in order to examine the effect of thyroxine injection.

Blood samples were collected from the jugular vein before and 0.5, 1, 2, 4, 8 and 12 hours after surgery. From the next day of the surgery to the 21st day after surgery, blood samples were obtained 4 hours after feeding daily. Serum calcium and phosphorus concentrations and serum free hydroxyproline
Table 4-1 Composition of Diet

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orchardgrass hay</td>
<td>40</td>
</tr>
<tr>
<td>Ground barley</td>
<td>30</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>20</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>8.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.0</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>0.5</td>
</tr>
</tbody>
</table>

% of air dry matter

Table 4-2 Serum thyroxine concentrations (µg/100ml) after L-thyroxine injection in thyroidectomized and intact sheep

<table>
<thead>
<tr>
<th>Thyroxine concentrations after injection</th>
<th>3rd day</th>
<th>6th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>6.8 ± 1.8</td>
<td>7.2 ± 0.8</td>
</tr>
<tr>
<td>Intact</td>
<td>7.2 ± 1.9</td>
<td>7.4 ± 1.4</td>
</tr>
</tbody>
</table>

Mean ± SE.
TX means thyroidectomy
concentrations as an index of bone resorption were determined. During the last 2 days of this experiment, urinary samples were collected from 24 hours collection period in all sheep to determine calcium and phosphorus excretion in urine.

Experiment 2

Two lambs, weighing about 16 kg (6 months old) were used. The animals were kept in metabolism cages under 12 hours light and 12 hours dark condition. Thyroidectomy and feeding was in accordance with experiment 1. Blood samples were collected from the jugular vein before and the 1st day, the 1st week and the 2nd week after the surgery at 2 hours intervals.

Serum and urinary calcium concentrations were measured by an atomic absorption spectrophotometry, phosphorus concentrations were determined by the method of Gomori (121), serum free hydroxyproline concentrations by the method of Bergmann and Loxley (122) and urinary creatinine concentrations by the method of Stelgens (123). Statistical differences were evaluated by student's t test.

Results and Discussion

As shown in Table 4-2, serum thyroxine concentrations were little change after the L-thyroxine injection and were not different from the values in intact sheep. It seems that the dose and the procedure of L-thyroxine injection were enough to supply thyroid hormone to wethers which were ectomized the thyroid gland.

Fig 4-1 shows the changes of serum calcium concentration after
Fig. 4-1 Changes of serum calcium concentrations after thyroidectomy (●) and thyroparathyroidectomy (○). The values are shown as the percentages of preoperative serum calcium concentration. Each point represents means ± SE.
thyroidectomy and thyroparathyroidectomy as percentages of the preoperative value. Serum calcium concentrations began to decrease from 12 hours after thyroparathyroidectomy. The concentrations on the 2nd day after thyroparathyroidectomy lowered at the level of 72% of the value before the operation, then elevated gradually and recovered to the preoperative value on the 21st day. In the report by Stott and Smith (124), the recovery of serum calcium level was found 3 or 4 weeks after thyroparathyroidectomy in nonlactating cows. Payne and Charnings (117) showed the occurrence of hypocalcemia after thyroparathyroidectomy in growing goats but they did not find the recovery of serum calcium concentrations as shown in the present experiment. Nelson et al. (125) demonstrated that the rate of decrease in serum calcium was rapid in young thyroparathyroidectomized sheep while adult sheep were more tolerant to thyroparathyroidectomy, being capable of correcting hypocalcemia.

In thyroidectomized sheep, serum calcium concentrations were temporarily decreased after surgery and recovered soon. After then, an obvious change was not found in serum calcium concentrations through the experimental period. The temporary reduction of serum calcium concentrations after thyroidectomy might be due to the momentary secretion of calcitonin which would be induced by the surgical procedure. Kaplan et al. (126) observed the similar phenomenon in man.

The results that serum calcium concentrations had little change in thyroidectomized sheep after the evanescent lowering agreed with the Nelson's report (7) used adult sheep without
Fig. 4-2 Effect of feeding on serum calcium concentrations in thyroidectomized lamb.
(a) Before operation, (b) the 1st day after thyroidectomy,
(c) the 1st week after thyroidectomy,
(d) the 2nd week after thyroidectomy.
Arrows indicated feeding.
Values were expressed as means for 2 samples.
thyroxine supplement.

As shown in Fig. 4-2, obvious changes in serum calcium concentrations were not found in also thyroidectomized lambs which was inconsistent with the reports of Kalu (6) and Talmage et al. (127) that feeding substantially increased serum calcium concentrations in thyroidectomized rats. They proposed that one of the physiological role of calcitonin was a prevention of postprandial hypercalcemia which was induced by the rapid calcium absorption from the digestive tract.

However, the increase of calcium absorption after feeding might not occur in sheep because of slow movement of digesta from the rumen to the lower digestive tract. It seems that calcitonin is not necessary to counteract a rapid calcium absorption after feeding in ruminants.

As presented in Fig. 4-3, serum phosphorus concentrations began to decrease soon after thyroparathyroidectomy and reached 65% of the preoperative value 24 hours after the surgery. Then serum phosphorus concentrations remained constantly for next 3 days and began to decrease again. On the 6th day after thyroparathyroidectomy, serum phosphorus concentrations reached 42% of the preoperative value. The reduction of serum phosphorus concentrations in thyroparathyroidectomized sheep might be owing to the deficiency of parathyroid hormone. The results supported the indications by Georgievskii (128) and Care et al. (129).

In thyroidectomized wethers, serum phosphorus concentrations decreased after the operation but recovered within 24 hours after the surgery. Then there was little change until the end of the
Fig. 4-3 Changes of serum phosphorus concentrations after thyroidectomy (●) and thyroparathyroidectomy (○).
The values are shown as the percentages of preoperative serum phosphorus concentration.
Each point represents means ± SE.

Fig. 4-4 Changes of serum hydroxyproline concentrations after thyroidectomy (●) and thyroparathyroidectomy (○).
The values are shown as the percentage of preoperative serum hydroxyproline concentration.
Each point represents means ± SE.
experiment. As shown in chapter 10, serum phosphorus concentrations tended to decrease after feeding in sheep. The reduction of phosphorus concentrations until 12 hours after thyroidectomy might be due to feeding because the animals were given the diet 4 hours before the surgery. Talmage et al. (130) postulated that one of the main roles of calcitonin was to prevent the increase of plasma phosphorus concentrations. However, in this experiment, calcitonin deficiency did not affect serum phosphorus concentrations of sheep.

Fig. 4-4 shows the change of serum hydroxyproline concentrations after surgery. When sheep were thyroidectomized, serum hydroxyproline tended to reduce a little extent on the 1st day and recovered 2 weeks after the operation. The reduction of hydroxyproline concentrations on the 1st day might be owing to the increased secretion of calcitonin during the operation. It was observed in chapter 10 that serum hydroxyproline concentrations decreased after feeding in thyroidectomized and intact wethers. The reduction on the 1st day was partly due to feeding in thyroidectomized sheep. In rats, bone resorption was enhanced by calcitonin deficiency (131). However, the increase in serum hydroxyproline concentrations was not found in thyroidectomized sheep.

Serum hydroxyproline concentrations were rapidly lowered after thyroparathyroidectomy and attained to the level of 42% of the initial value on the 2nd day after the operation. After then, serum hydroxyproline concentrations began to elevate and recovered to the initial level on the 14th day after thyroparathyroidectomy. Serum hydroxyproline concentrations in thyropara-
thyroidectomized sheep decreased more remarkable than in thyroidectomized sheep. The difference suggested that an acute deficiency of parathyroid hormone reduced bone resorption. Kalu et al. (118) reported that the deficiency of parathyroid hormone did not depress the bone resorption within 3 hours but the bone resorption decreased on the next day of thyroparathyroidectomy in rats. They used urinary hydroxyproline excretion as an index of bone resorption. Urinary hydroxyproline excretion may change more slowly than serum hydroxyproline concentrations when bone resorption is decreased.

The recovery of serum hydroxyproline and calcium concentrations in thyroparathyroidectomized sheep was found at the same time as the 2nd reduction of serum phosphorus concentrations. Fleish and Neuman (132) reported that there was a reciprocal relationship between serum calcium and phosphorus concentrations from the standpoint of physico-chemical nature. The reduction of serum phosphorus concentrations might partly contribute to the recovery of serum calcium concentrations.

It was shown by Tanaka and Deluca (66) that a reduction of serum phosphorus concentrations stimulated the activation of 25-hydroxyvitamin-D₃ to 1,25-dihydroxyvitamin-D₃. Boris et al. (133) found the enhancement of bone resorption by 1,25-dihydroxyvitamin-D₃ injection in thyroparathyroidectomized rats. Therefore, it could be considerable that the decrease of serum phosphorus concentrations contributed to recover bone resorption through a role of 1,25-dihydroxyvitamin-D₃ in thyroparathyroidectomized sheep.
Fig. 4-5 presents a relationship between serum calcium concentrations and serum hydroxyproline concentrations in thyroparathyroidectomized wethers. A significant correlation was found between serum calcium and hydroxyproline concentrations \((P<0.05)\). The changes of serum calcium concentrations appeared to be closely associated with bone resorption in thyroparathyroidectomized sheep.

Ratios of urinary calcium and phosphorus concentrations to urinary creatinine are presented in Table 4-3. Urinary calcium excretion in thyroidectomized sheep had a trend to be higher than in intact sheep. The infusion of calcitonin into rats led to the decrease in excretion of urinary calcium \((58)\). However, Kimura and Ogata \((9)\) reported that calcium excretion in urine increased when 1 i.u. of calcitonin was injected but decreased in the case of 2 mi.u. of calcitonin injection in rats. It could be postulated from the results of this experiment that calcitonin deficiency elevated urinary calcium excretion in sheep. Urinary calcium excretion in thyroparathyroidectomized sheep were higher than those in thyroidectomized and intact sheep. It was in agreement with the suggestion that increased renal tubular reabsorption of calcium has been considered one of the classical effects of parathyroid hormone \((134)\).

Urinary phosphorus excretion in thyroidectomized sheep were much lower than in thyroparathyroidectomized and intact one. It has been observed that the infusion of calcitonin significantly increase urinary phosphorus excretion in sheep \((2)\) and rats \((58)\). The decrease in urinary phosphorus excretion was due to the deficiency of calcitonin in thyroidectomized sheep.
Fig. 4-5 Relationship between serum calcium and serum hydroxyproline concentrations after thyroparathyroidectomy.

\[ Y = 2.56X + 3.64 \]
\[ r = 0.71 \ (P < 0.05) \]
Table 4-3 Urinary calcium and phosphorus excretion in thyroidectomized, thyroparathyroidectomized and intact sheep

<table>
<thead>
<tr>
<th></th>
<th>TX</th>
<th>TPTX</th>
<th>Intact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium/Creatinine</td>
<td>4.62 ± 1.24^a</td>
<td>7.21 ± 0.16^b</td>
<td>1.09 ± 0.06^a</td>
</tr>
<tr>
<td>Phosphorus/Creatinine</td>
<td>2.2 ± 0.6^c</td>
<td>165.2 ± 52.9^d</td>
<td>184.5 ± 19.1^d</td>
</tr>
</tbody>
</table>

Mean ± SE.
TX means thyroidectomy and TPTX means thyroparathyroidectomy.

^a,b ; Means in the same line with different superscript letters differ significantly (P<0.05)

^c,d ; Means in the same line with different superscript letters differ significantly (P<0.01).
Increased renal phosphorus excretion is one of the best known effects of parathyroid hormone (134). However, urinary phosphorus excretion did not decrease in thyroparathyroidectomized wethers in this experiment. The reason why a reduction of urinary phosphorus excretion was not found in calcitonin and parathyroid hormone deficient animals has not been known well. It could be considered at least that phosphorus reabsorption in the kidney lowered because urinary phosphorus excretion had little change nevertheless serum phosphorus concentrations decreased in thyroparathyroidectomized sheep. Since ruminants have a characteristic metabolic route of phosphorus such as saliva, the effect of calcitonin and parathyroid hormone on phosphorus metabolism may be different when compared to those in nonruminants.

Summary

The experiment was to study the effects of thyroidectomy and thyroparathyroidectomy on serum concentrations and urinary excretions of calcium and phosphorus in sheep. There was no obvious changes in serum calcium and phosphorus concentrations after thyroidectomy in adult sheep. And feeding did not affect serum calcium concentrations in thyroidectomized lambs. Serum calcium and hydroxyproline concentrations decreased 2 days after thyroparathyroidectomy and recovered 1 week after. Since there was a close relationship between serum calcium concentrations and serum hydroxyproline, it could be considered that deficiency of parathyroid hormone reduced serum calcium concentrations through the decrease in bone resorption in sheep. The recovery of serum
calcium and hydroxyproline concentrations might be dependent on adaptation against the long term deficiency of parathyroid hormone, that is, the activation of vitamin-D₃ induced by low serum phosphorus concentrations. Serum phosphorus concentrations continued to decreased after thyroparathyroidectomy. Deficiency of parathyroid hormone might result in the lowering of serum phosphorus concentrations.

Urinary calcium excretion in thyroidectomized sheep had a trend to be higher than in intact sheep. Thyroparathyroidectomized animals excreted more calcium via urine than in thyroidectomized ones. Urinary phosphorus excretion were much lower in thyroidectomized sheep when compared to intact and thyroparathyroidectomized ones. These results indicated that calcitonin deficiency brought about the promotion of urinary calcium excretion and the reduction of urinary phosphorus excretion. And parathyroid hormone deficiency enhanced urinary calcium loss in sheep.
CHAPTER 5 Effect of Oral Calcium Load on Calcium and Phosphorus Metabolism

It was suggested that a physiological role of calcitonin was to prevent a postprandial hypercalcemia caused by rapid calcium absorption because serum calcium concentrations were increased after feeding in thyroidectomized rats (6). Gray and Munson (5) indicated that oral calcium load increased serum calcium concentrations more remarkable in thyroidectomized rats than in sham operated ones. And they suggested that one of roles of calcitonin was anti-hypercalcemic action.

On the other hand, as shown in chapter 4, thyroidectomy did not change serum calcium concentrations in sheep and a postprandial hypercalcemia was not found in thyroidectomized ones. However, Inskeep and Kenny (111) indicated that serum calcium concentrations were higher in thyroidectomized sheep than in intact ones after intraperitoneal calcium load and Barlet (102) found that calcitonin inhibited hypercalcemia induced by activated vitamin-D₃ in sheep. This study was to investigate the function of calcitonin in calcium and phosphorus metabolism during oral calcium load in thyroidectomized and sham operated sheep.

Materials and Methods

Six young sheep, weighing about 25 kg, were used. All animals were kept in metabolism cages. Three wethers were
Table 5-1 Composition of diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Normal Ca diet</th>
<th>High Ca diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orchardgrass hay</td>
<td>60 %</td>
<td>58.4 %</td>
</tr>
<tr>
<td>Ground barley</td>
<td>30</td>
<td>29.2</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>9.8</td>
<td>9.6</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>0.2</td>
<td>2.8</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.37</td>
<td>1.31</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.33</td>
<td>0.33</td>
</tr>
</tbody>
</table>

% of air dry matter
thyroidectomized and the other 3 were performed sham operation. All sheep were given a normal diet until 6 days after operations and then given a high calcium diet during 6 days at a level of 2% of body weight at 9:00 a.m. daily. The compositions of these diets were shown in Table 5-1. Water was available at all times. Thyroidectomized animals were injected intramuscularly with 0.25 mg of L-thyroxine (Nakarai Chemicals Ltd., Kyoto) dissolved in corn oil daily.

Blood and urine were collected on the last day of normal calcium diet period, the 1st, 3rd, 6th day of high calcium diet period. Blood samples were collected 8 hours after feeding. Urine collection were made over 24 hour period after feeding. Serum and urinary calcium concentrations were measured by an atomic absorption spectrophotometry, serum and urinary phosphorus concentrations by the method of Gomori (121), serum parathyroid hormone levels were determined by a radioimmunoassay (135) and serum free hydroxyproline concentrations were measured by the method of Bergmann and Loxley (122). Urinary total hydroxyproline concentrations were determined by the method of Koevoet (136) and urinary polypeptide hydroxyproline concentrations were measured by the method of Krane et al. (137). Statistical differences were evaluated by student's t test.

Results and Discussion

As shown in Table 5-2, serum calcium concentrations were significantly (P<0.01) increased by the calcium load in thyroidectomized sheep and the increase in serum calcium concentrations was more remarkable (P<0.05) in thyroidectomized
Table 5-2 Effect of oral calcium load on serum calcium and phosphorus concentrations in sheep

<table>
<thead>
<tr>
<th>Day of Calcium load</th>
<th>Serum Calcium (mg/100ml)</th>
<th>Serum Phosphorus (mg/100ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>TX</td>
<td></td>
</tr>
<tr>
<td>Before Calcium load</td>
<td>9.52 ± 0.29</td>
<td>6.82 ± 0.41</td>
</tr>
<tr>
<td>1st</td>
<td>9.59 ± 0.21</td>
<td>5.81 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>10.00 ± 0.11</td>
<td>6.65 ± 0.07^a</td>
</tr>
<tr>
<td>3rd</td>
<td>9.60 ± 0.12</td>
<td>5.17 ± 0.43*</td>
</tr>
<tr>
<td></td>
<td>10.26 ± 0.18*^a</td>
<td>4.62 ± 0.31*</td>
</tr>
<tr>
<td>6th</td>
<td>9.81 ± 0.15</td>
<td>5.11 ± 0.49*</td>
</tr>
<tr>
<td></td>
<td>10.69 ± 0.13**^a</td>
<td>4.99 ± 0.33*</td>
</tr>
</tbody>
</table>

Mean ± SE.
Significantly different (*; P<0.05, **; P<0.01) from the value before calcium load.
Significantly different (a; P<0.05, b; p<0.01) from Sham sheep.
Sham means sham operated and TX means thyroidectomized.
Table 5-3 Effect of oral calcium load on bone resorption and bone formation indices in sheep

<table>
<thead>
<tr>
<th></th>
<th>Serum Free Hyp (mg/l)</th>
<th>Urinary Total Hyp excretion (mg/day)</th>
<th>Urinary polypeptid Hyp excretion (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>1.96 ± 0.12</td>
<td>141 ± 16</td>
<td>7.94 ± 1.53</td>
</tr>
<tr>
<td><strong>Calcium load</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TX</td>
<td>2.14 ± 0.58</td>
<td>143 ± 12</td>
<td>8.10 ± 1.38</td>
</tr>
<tr>
<td><strong>Day of Calcium load</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>1st</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>1.60 ± 0.05</td>
<td>136 ± 12</td>
<td>8.70 ± 1.16</td>
</tr>
<tr>
<td>TX</td>
<td>2.16 ± 0.49</td>
<td>138 ± 6</td>
<td>8.74 ± 1.31</td>
</tr>
<tr>
<td><strong>3rd</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>1.31 ± 0.10*</td>
<td>90 ± 3</td>
<td>9.75 ± 1.79</td>
</tr>
<tr>
<td>TX</td>
<td>2.06 ± 0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>109 ± 11</td>
<td>8.77 ± 1.15</td>
</tr>
<tr>
<td><strong>6th</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>0.45 ± 0.16**</td>
<td>72 ± 7*</td>
<td>13.15 ± 1.17*</td>
</tr>
<tr>
<td>TX</td>
<td>1.85 ± 0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.91 ± 1.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Mean ± SE.

Significantly different (*; P<0.05, **; P<0.01) from the value before calcium load.

Significantly different (a; P<0.05, b; P<0.01) from Sham sheep.

Sham means sham operated and TX means thyroidectomized.
sheep than in sham operated ones on the 3rd and the 6th day of calcium load. Gray and Munson (5) reported that a calcium load by stomach tube produced hypercalcemia in thyroidectomized rats but not in intact ones. It was shown in the present experiment that calcitonin regulated physiologically serum calcium concentrations in sheep as well as in monogastric animals.

Serum phosphorus concentrations were substantially ($P<0.05$) decreased by loading calcium in both sham operated and thyroidectomized sheep. Yano et al. (138) reported that a feeding of high calcium diet inhibited phosphorus absorption from the digestive tract owing to the reduction of phosphorus solubility in the gut. As described later, bone resorption had a trend to be reduced by the calcium load in all sheep. Therefore the reduction of serum phosphorus concentrations might be due to the inhibition of phosphorus absorption from the gut and/or the decrease of phosphorus releasing from the bone.

Serum free hydroxyproline concentrations were significantly ($P<0.01$) decreased by giving the high calcium diet on sham operated sheep (Table 5-3). A similar trend was found in thyroidectomized animals but the response was not statistically significant. Thus serum free hydroxyproline levels were significantly lower in sham operated sheep than in thyroidectomized ones ($P<0.05$ on the 3rd day; $P<0.01$ on the 6th day of calcium load). Both of urinary total hydroxyproline excretion and serum free hydroxyproline concentrations were similarly changed by the treatment. Total hydroxyproline excretion in urine were significantly ($P<0.05$) decreased by calcium loading in sham operated sheep. A modest decrease in
total hydroxyproline excretion was induced by the calcium load in thyroidectomized animals but the reduction was not statistically significant. The excretion of total hydroxyproline was, thus, significantly less (P<0.05) in sham operated sheep comparing with that in thyroidectomized ones on the 6th day of calcium load. Kalu et al. (131) indicated that calcitonin deficiency increased the release of calcium from the bone in rats. Rasmussen et al. (58) also reported that calcitonin inhibited bone resorption caused by parathyroid hormone because calcitonin injection counteracted the increased urinary total hydroxyproline excretion by parathyroid hormone in rats. The results in this experiment that serum free hydroxyproline concentrations and urinary total hydroxyproline excretion went down obviously in sham operated sheep in comparison with thyroidectomized ones suggested that the increase of calcitonin secretion lowered bone resorption in sheep fed the high calcium diet.

On the other hand, it was considerable that calcitonin deficiency might scarcely affect bone resorption since the indices of bone resorption were not different between sham operated and thyroidectomized sheep given the normal diet. The indices of bone resorption tended to be decreased by the calcium load even in thyroidectomized sheep. It appeared that there might be another factor than calcitonin reducing bone resorption. It is well known that parathyroid hormone increases bone resorption in various animals. The slight reduction of serum parathyroid hormone levels caused by calcium load might partly contribute to the decrease in bone resorption in thyroidectomized animals.

In both groups, serum parathyroid hormone concentrations were
gradually lowered by the calcium load (Fig. 5-1). It is established that parathyroid hormone secretion is primarily regulated by serum calcium concentrations (139). The reduction of serum parathyroid hormone levels might be closely correlated with the increase in serum calcium concentrations also in sheep. On the other hand serum calcium concentrations were little different between sham operated and thyroidectomized sheep before the calcium load, while serum parathyroid hormone levels tended to be higher in sham operated sheep than in thyroidectomized ones. It might be possible that parathyroid hormone secretion were partly reduced in thyroidectomized sheep because of the removal of the parathyroid gland in the thyroid gland.

Krane et al. (137) reported that the urinary polypeptide containing hydroxyproline was a part of collagen precursors and that the amount of urinary polypeptide hydroxyproline excretion serve as an index of bone collagen synthesis because the elevation of polypeptide hydroxyproline excretion in urine was found in patients with some skeletal disorders stimulating bone formation. In the present investigation, urinary excretion of polypeptide hydroxyproline were significantly (P<0.05) increased by the calcium load in sham operated wethers, while the increase was not statistically significant in thyroidectomized sheep (Table 5-3).

The effect of calcitonin on bone formation has been controversial. McWhinnie (78) reported that calcitonin stimulated bone formation in chick embryo and Gaillard (140) observed that calcitonin accelerated bone formation in vitro. Orimo et al. (79) suggested that calcitonin activated bone pyrophosphatase which
Fig. 5-1 Effect of oral calcium load on serum parathyroid hormone concentrations in thyroidectomized sheep (●) and sham operated ones (○). Values were indicated means ± SE.

a; Significantly (P<0.05) different from the value before calcium load.
might stimulate bone calcification through a reduction of pyrophosphate in the skeleton. On the other hand, Baylink et al. (73) reported that calcitonin inhibited bone formation in thyroparathyroidectomized rats. Cohn and Wong (69) postulated that calcitonin did not increase bone formation because calcitonin could not counteract the reduction of bone alkaline phosphatase activities and that of collagen processing (prolyl hydroxylase activities) induced by parathyroid hormone in osteoblast-like cell in vitro. It was suggested from this experiment, that calcitonin enhanced bone collagen synthesis in vivo because urinary polypeptide hydroxyproline excretion were more in sham operated sheep than in thyroidectomized ones during the calcium load.

As shown in Table 5-4, urinary calcium excretion kept higher in thyroidectomized sheep than in sham operated animals throughout the experimental period. The increase of calcium excretion was also more obvious in thyroidectomized sheep comparing with sham operated ones. The results were coincident with the report by Talmage and Grubb (141) using rats. It was shown that bone resorption became less and bone formation became more in sham operated sheep than in thyroidectomized ones, i.e., calcium retained less in thyroidectomized sheep than in sham operated ones. As indicated in chapter 6, calcium absorption tended to be higher in thyroidectomized wethers than in intact ones. Therefore it could be considered that calcitonin decreased urinary calcium excretion by means of the increment in bone calcium and the decrement in calcium absorption.

On the other hand, Barlet (2) observed that calcitonin
Table 5-4 Effect of oral calcium load on urinary calcium and phosphorus excretion in sheep

<table>
<thead>
<tr>
<th></th>
<th>Urinary Calcium (mg/day)</th>
<th>Urinary Phosphorus (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Calcium load</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>9.2 ± 4.1</td>
<td>7.89 ± 0.91</td>
</tr>
<tr>
<td>TX</td>
<td>25.8 ± 3.8(^a)</td>
<td>2.53 ± 0.59(^b)</td>
</tr>
<tr>
<td>Day of Calcium load</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>10.5 ± 4.8(^b)</td>
<td>4.86 ± 0.61(^*)</td>
</tr>
<tr>
<td>TX</td>
<td>58.7 ± 9.1(^*)</td>
<td>2.70 ± 0.45(^a)</td>
</tr>
<tr>
<td>3rd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>12.9 ± 7.4(^a)</td>
<td>2.34 ± 0.48(^**)</td>
</tr>
<tr>
<td>TX</td>
<td>47.2 ± 6.9(^a)</td>
<td>2.20 ± 0.38</td>
</tr>
<tr>
<td>6th</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>17.9 ± 4.8(^a)</td>
<td>2.19 ± 0.60(^**)</td>
</tr>
<tr>
<td>TX</td>
<td>35.5 ± 3.2(^a)</td>
<td>2.13 ± 0.85</td>
</tr>
</tbody>
</table>

Mean ± SE.
Significantly different (*; P<0.05, **; P<0.01) from the value before calcium load.
Significantly different (a, P<0.05, b; P<0.01) from Sham sheep.
Sham means sham operated and TX means thyroidectomized.
administration increased urinary calcium excretion in sheep. Although calcitonin might reduce renal reabsorption of calcium, a role of calcitonin on the kidney might be limited in comparison with those on the other organs, i.e., the skeleton and the gut. The present study sustained the suggestion of Talmage that one of the physiological role of calcitonin was calcium storage in the bone. It is natural to consider that the increase of urinary calcium excretion can contribute to prevent a rise of serum calcium concentrations in calcitonin deficient sheep.

Urinary phosphorus excretion were significantly higher (P<0.01) in sham operated sheep than in thyroidectomized ones given the normal diet. The results were in agreement with the indication of Barlet (2) that calcitonin enhanced urinary phosphorus excretion in sheep. Urinary phosphorus excretion were significantly (P<0.05) decreased by the calcium load in sham operated sheep, whereas the calcium load brought about slight decreases in phosphorus excretion in thyroidectomized sheep. As same as urinary calcium excretion, the reduced phosphorus excretion by the calcium load in sham operated wethers might be closely correlated to the decrease in bone resorption and the increase in bone formation. And the stimulative effect of calcitonin on phosphorus storage in the bone which was coupling with the conservation of calcium overcame the hyperphosphaturic action of calcitonin.

Summary

This study was to investigate a role of calcitonin on calcium
and phosphorus metabolism in thyroidectomized and sham operated sheep orally loaded calcium. Though serum calcium concentrations and urinary calcium excretion tended to be increased by the calcium load in all sheep, the increase was more remarkable in thyroidectomized sheep than in sham operated ones.

Serum phosphorus concentrations were decreased in all sheep as the high calcium diet was given. Urinary phosphorus excretion were substantially decreased by the calcium load in sham operated sheep and were slightly decreased in thyroidectomized animals.

The calcium load reduced serum free hydroxyproline concentrations, urinary total hydroxyproline excretion and serum parathyroid hormone concentrations in sham operated sheep. The elevation of urinary polypeptide hydroxyproline excretion was more remarkable in sham operated sheep than in thyroidectomized ones. These results suggested that calcitonin played a role to decrease bone resorption and to increase bone formation when a high calcium diet was fed to sheep. The role of calcitonin on the kidney might be limited in comparison with that on the skeleton in sheep fed a high calcium diet. The reduction of serum parathyroid hormone concentrations, which was induced by the calcium load, might be contribute to the decrease in bone resorption.
CHAPTER 6 Suppressive Effect of Calcitonin on Absorption of Calcium and Phosphorus

The digestive tract is important for calcium and phosphorus metabolism. However, the effect of calcitonin on calcium absorption is still controversial and the effect on phosphorus absorption is little known. Krawitt (83) did not find any significant changes in calcium absorption after calcitonin injection in rats but Olson et al. (85) indicated that physiological level of calcitonin infusion immediately inhibited calcium absorption in rats. On the other hand, Swaminathan et al. (87) suggested that calcitonin indirectly affected calcium absorption because calcium absorption was reduced 2 days after calcitonin infusion in pigs. Barlet (86) found that apparent absorption of calcium and phosphorus were decreased by long term infusion of calcitonin in sheep. The experiment was to investigate the effect of calcitonin on calcium and phosphorus absorption in sheep.

Materials and Methods

Six adult sheep, weighing about 40 kg were used. Three sheep were thyroidectomized 3 months before the experiment and the others were intact. Every thyroidectomized sheep was injected with 2.5 mg of L-thyroxine (Nakarai Chemicals, Ltd., Kyoto) once a week dissolved in corn oil in order to supply thyroid hormone. Every animal was surgically prepared with a carotid loop and a
portal venous catheter. A siliconized catheter was inserted via branch of the omasum-abomasum vein into the portal vein, then a ligature was tightened around the vessel. The animals were used for the experiment 2 weeks after the surgery. All animals were given a diet containing 0.41% calcium and 0.40% phosphorus (Table 6-1) at a level of 2% of body weight daily. Water was available at all times. Each thyroidectomized sheep was injected intramuscularly with 1 i.u./kg body weight of porcine calcitonin (Armour Pharmaceutical Company Ltd., East Bourne, England, 80 i.u./mg protein) dissolved in 16% gelatin and each intact sheep was injected with the vehicle at feeding time.

Blood samples were collected before feeding and 1, 2, 4 and 8 hours after feeding on the day of the vehicle injection and the day of calcitonin injection in thyroidectomized sheep. At the same time, blood samples were also obtained from intact sheep.

Hemoglobin content was determined by the method of Drabkin and Austine (142) to correct water content in blood. Blood serum was separated and stored at -14°C for analysis. Serum calcium concentrations were measured by an atomic absorption spectrophotometry and serum phosphorus concentrations by Gomori's method (121). Calcium and phosphorus absorption were estimated by a veno-arterial blood difference technique. Statistical differences were evaluated by student's t test.

Results

Table 6-2 shows serum calcium and phosphorus concentrations in arterial blood before feeding. There was no significant difference in serum calcium and phosphorus concentrations between
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orchardgrass hay</td>
<td>60</td>
</tr>
<tr>
<td>Ground barley</td>
<td>30</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>9.8</td>
</tr>
<tr>
<td>CaCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.2</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.37</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.33</td>
</tr>
</tbody>
</table>

% of air dry matter

Table 6-2 Serum calcium and phosphorus concentrations before feeding

<table>
<thead>
<tr>
<th>Item</th>
<th>Intact</th>
<th>Thyroidectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>8.14 ± 0.10</td>
<td>8.44 ± 0.36</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>4.72 ± 0.12</td>
<td>4.58 ± 0.14</td>
</tr>
</tbody>
</table>

Results were expressed as means ± SE. for 12 samples.
thyroidectomized and intact sheep.

Fig. 6-1 shows postprandial changes in serum calcium and phosphorus concentrations in arterial blood. Little change was found in serum calcium concentrations in either thyroidectomized sheep or intact ones injected with vehicle. In thyroidectomized sheep injected with calcitonin, serum calcium concentrations began to decrease 1 hour after feeding (calcitonin injection) and reached 76% of the initial calcium level 8 hours after feeding.

Though serum phosphorus concentrations significantly (P<0.05) increased 2 hours after feeding, the concentrations tended to decrease 4 hours after feeding in intact sheep. Serum phosphorus concentrations also decreased after feeding in thyroidectomized sheep whether calcitonin were injected or not. The reduction in serum phosphorus was more remarkable in thyroidectomized animals injected with calcitonin than in thyroidectomized and intact ones which were not injected with calcitonin.

Fig. 6-2 shows differences in veno-arterial blood calcium and phosphorus concentrations. A significant (P<0.05) increase in calcium absorption was found in thyroidectomized animals injected with vehicle 8 hours after feeding. And calcium absorption was decreased significantly (P<0.01) in thyroidectomized sheep injected with calcitonin.

Differences in veno-arterial blood phosphorus concentrations were less in thyroidectomized animals than in intact ones before feeding. The differences in phosphorus absorption between thyroidectomized and intact sheep became smaller after feeding whether thyroidectomized sheep were injected with calcitonin or
Fig. 6-1 Changes of serum calcium (a) and phosphorus (b) concentrations of arterial blood after feeding in intact (○), thyroidectomized (■) and thyroidectomized sheep injected with calcitonin (▲). Values were indicated means ± SE of % of before feeding for 6 samples. Arrows indicated feeding and injection. 
a; Significantly (P<0.05) different from intact sheep.  
b; Significantly (P<0.01) different from intact sheep.
Fig. 6-2 Portal venous - carotid arterial blood differences of calcium (a) and phosphorus (b) in intact (O), thyroidectomized (▲) and thyroidectomized sheep injected with calcitonin (■). Values were indicated means ± SE for 6 samples. Arrows indicated feeding and injection.

a; Significantly (P< 0.05) different from intact sheep.

b; Significantly (P< 0.01) different from intact sheep.
Discussion

The results showing that calcium absorption in thyroidectomized animals was greater than in intact sheep 8 hours after feeding and calcium absorption was immediately decreased by calcitonin injection indicated that calcitonin directly influenced calcium absorption in sheep. Olson et al. (85) also found acute reduction of calcium absorption by calcitonin infusion using an in vitro rat intestinal perfusion technique. On the other hand, Swaminathan et al. (87) found that calcium absorption began to be reduced 2 days after calcitonin infusion using a Thiry-Vella loop technique in swine and suggested that calcitonin had no direct effect on calcium absorption but calcitonin affect calcium absorption by way of the inhibition of 1,25-dihydroxyvitamin-D₃ formation which was well known to being the major stimulator of calcium absorption in the intestine (143). However, the suggestion that calcitonin affect calcium absorption by way of the inhibition of vitamin-D₃ activation was suspected because it was reported that calcitonin did not suppress vitamin-D₃ activation (61) and Olson et al. (85) found that the acute stimulative effect of 1,25-dihydroxyvitamin-D₃ on calcium absorption were inhibited by calcitonin infusion. The difference between these results may be due to the technique used in estimating calcium absorption. In the Thiry-Vella loop technique, calcium absorption was measured by the disappearance of calcium from the intestinal lumen. On the other hand, calcium absorption was estimated by the outflow of calcium to the portal vein using
the veno-arterial blood difference method and the in vitro intestinal perfusion technique.

Halloran and Deluca (144) suggested that there were 2 independent mechanisms related to the stimulative effect of vitamin-D₃ on calcium absorption because single injection of 1,25-dihydroxyvitamin-D₃ demonstrated biphasic increase of calcium absorption, i.e., the acute stimulation occurred 2 hours after the injection and the late one was induced after 24 hours.

The mechanism of calcium absorption was consist of the influx of calcium across the brush border membrane and the efflux across the basal lateral membrane. Because it is well known that calcium concentrations in cytosol is much lower than calcium levels in extra cellular fluid and in the luminal fluid of the intestine (145), the influx of calcium could be occurred by the diffusion and the efflux was induced by the active transport. From these reports, it is suggested that calcitonin may rapidly inhibit the efflux of calcium from the cytosol to the blood which was induced by active transport and then reduced the influx from the intestinal lumen over a long term. Yamaguchi (90) demonstrated the increase in calcium excretion via bile when calcitonin was injected into rats. It is possible that calcium absorption from the gut is depressed but calcium concentrations in the intestinal lumen are increased by the calcitonin injection.

Serum phosphorus concentrations were temporarily elevated 2 hours after feeding in intact sheep but the elevation of serum phosphorus concentrations was not found in thyroidectomized animals whether calcitonin was injected or not. The increase in
serum phosphorus levels might be caused by phosphorus absorption from the gut in intact sheep because phosphorus absorption was much greater in intact sheep than in thyroidectomized sheep before feeding. The finding that phosphorus absorption was not affected by calcitonin injection in thyroidectomized animals indicated that calcitonin did not seriously affect phosphorus absorption in sheep. The reasons why there was so little phosphorus absorption before feeding and that it tended to increase after feeding in thyroidectomized sheep was not clear. However it was reported that endogenous phosphorus excretion plays an important role in phosphorus metabolism of ruminants (145). It is possible that the capacity for phosphorus absorption was not different between thyroidectomized sheep and intact ones though the amount of phosphorus inflow via saliva or bile was less in thyroidectomized sheep than in intact animals.

Summary

This study was carried out to determine the effect of calcitonin on calcium and phosphorus absorption in sheep. Three thyroidectomized and 3 intact sheep, which were prepared with a carotid loop and a portal venous catheter, were used. Thyroidectomized sheep were injected intramuscularly with porcine calcitonin at feeding time. Serum calcium concentrations did not change in intact and thyroidectomized sheep after feeding but calcium veno-arterial blood differences in thyroidectomized sheep were higher than in intact ones 8 hours after feeding. Serum calcium concentrations and calcium veno-arterial blood differences were decreased by calcitonin injection into
thyroidectomized sheep.

Calcitonin injection also reduced serum phosphorus concentrations. Phosphorus veno-arterial blood differences in thyroidectomized sheep were much less than in intact ones before feeding although the blood differences were not affected by calcitonin injection in thyroidectomized animals.

The results indicated that calcitonin directly decreased calcium absorption which caused a reduction in serum calcium concentrations, and that calcitonin injection did not directly affect phosphorus absorption in thyroidectomized sheep.
CHAPTER 7 Effect of Calcitonin on Bile Excretion of Calcium and Phosphorus

It was suggested that the hypocalcemia induced by calcitonin was partly due to the increment in excretion of calcium via bile duct because calcitonin injection (80 mi.u./100 g body weight) increased bile calcium excretion in thyroparathyroidectomized rats (90). However, it might be afraid that the dose of calcitonin injection into rats was pharmacological level because circulating calcitonin in a rat of 150 g was calculated as approximately 1 mi.u. (85). Different effects of calcitonin on urinary mineral excretion (9) and intestinal calcium absorption (85) were observed between rats administrated with a large amount of calcitonin and ones administrated with a small amount of calcitonin.

Some workers indicated that a substantial amount of phosphorus were excreted into the small intestine in sheep (145,146). While the endocrine regulation of phosphorus excretion into the gut has not been clarified yet. In chapter 6, it was proposed that thyroidectomy inhibited endogenous loss of phosphorus into the gut before feeding in sheep.

This experiment was to study the role of calcitonin on calcium and phosphorus excretion via bile in sheep which were infused with calcitonin at a physiological level.
Materials and Methods

Six young sheep, weighing about 30 kg, were used. All animals were fed the diet shown in Table 7-1 at a level of 2% of body weight daily. Water was available at all times. Three sheep were thyroidectomized 3 months before the experiment and the other 3 were sham operated. In order to supply thyroid hormone, every thyroidectomized sheep was injected intramuscularly with 2.5 mg of L-thyroxine (Nakarai Chemicals Ltd., Kyoto) dissolved in corn oil once a week.

After 24 hours of feeding, the experiment was conducted under pentobarbitone sodium (Pitman-Moore, Washington, U.S.A) anesthesia. The abdomen was opened by a right side incision. The bile duct was fitted with a re-entrant cannula of siliconized tube (2 mm of inside diameter) and a ligature was tightened around the bile duct. A siliconized catheter was inserted into the jugular vein to collect blood and to infuse calcitonin or vehicle (Fig. 7-1). Thyroidectomized sheep were infused with porcine calcitonin (Armor Pharmaceutical Company Ltd., Eastbourne, England; 74 i.u./mg protein) dissolved in 0.8% NaCl (4 mi.u./ml) at a rate of 20 mi.u./kg body weight/hour for 5 hours. Care et al. (147) indicated that calcitonin secretion rate from the thyroid gland was shown to fluctuate between 5 and 30 mi.u./kg body weight/hour in an ewe when serum calcium concentrations varied between 8 and 12 mg/100 ml. On the other hand, sham operated sheep were infused with 0.8% NaCl (vehicle). The bile samples were collected for 10 min every 1 hour in thyroidectomized and sham sheep.
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orchardgrass hay</td>
<td>59.7</td>
</tr>
<tr>
<td>Ground barley</td>
<td>30.0</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>9.8</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.3</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>0.2</td>
</tr>
</tbody>
</table>

% of air dry matter
Fig. 7-1 Surgical procedure of biliary re-entrant cannula.
Calcium concentrations in bile and serum were measured by an atomic absorption spectrophotometry and inorganic phosphorus concentrations were determined by the method of Fiske and Subbarow (148). Lipid phosphorus in bile was measured by the method of Zilversmit and Davis (149). Statistical differences were evaluated by student's t test.

Results

As shown in Table 7-2, bile secretion rate tended to be reduced in both thyroidectomized and sham operated sheep and the rate was significantly (P<0.05) decreased in sham operated animals 4 hours after the beginning of vehicle infusion. However, there was no significant difference in secretion rate of bile between thyroidectomized and sham operated sheep.

Bile calcium excretion were more in sham operated sheep than in thyroidectomized ones before infusion (P<0.05). The significant reduction of bile calcium excretion was found in sham operated sheep (P<0.01) but not observed in thyroidectomized ones. And bile calcium excretion was more in thyroidectomized sheep infused with calcitonin than in sham operated ones infused with vehicle 4 hours after the beginning of infusion (P<0.05).

Bile inorganic phosphorus excretion was increased in thyroidectomized animals during the experiment. A significant difference between thyroidectomized sheep infused with calcitonin and sham operated ones infused with vehicle was found in bile inorganic phosphorus excretion 4 hours after the beginning of infusion.

Bile lipid phosphorus secretion had a trend to be decreased
Table 7-2 Effect of calcitonin infusion on rate of bile secretion, bile calcium and phosphorus excretion in sheep

<table>
<thead>
<tr>
<th></th>
<th>Before infusion</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile secretion</td>
<td>Sham</td>
<td>4.02 ± 0.75</td>
<td>3.53 ± 1.13</td>
<td>3.27 ± 1.05</td>
<td>3.00 ± 1.11</td>
<td>2.45 ± 0.81*</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>3.03 ± 0.89</td>
<td>2.66 ± 0.46</td>
<td>2.85 ± 0.71</td>
<td>2.46 ± 2.51</td>
<td>2.51 ± 0.79</td>
</tr>
<tr>
<td>Bile calcium</td>
<td>Sham</td>
<td>499 ± 21</td>
<td>367 ± 89</td>
<td>256 ± 144</td>
<td>325 ± 83*</td>
<td>207 ± 46*</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>338 ± 53a</td>
<td>306 ± 25</td>
<td>325 ± 24</td>
<td>321 ± 53</td>
<td>355 ± 22a</td>
</tr>
<tr>
<td>Bile inorganic phosphorus</td>
<td>Sham</td>
<td>75 ± 16</td>
<td>73 ± 14</td>
<td>85 ± 18</td>
<td>85 ± 29</td>
<td>83 ± 14</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>68 ± 9</td>
<td>61 ± 14</td>
<td>90 ± 19</td>
<td>101 ± 21</td>
<td>130 ± 20*</td>
</tr>
<tr>
<td>Bile lipid phosphorus</td>
<td>Sham</td>
<td>1.20 ± 0.07</td>
<td>1.20 ± 0.28</td>
<td>1.10 ± 0.16</td>
<td>1.09 ± 0.15</td>
<td>0.97 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>TX</td>
<td>1.28 ± 0.17</td>
<td>1.11 ± 0.15</td>
<td>1.10 ± 0.15</td>
<td>1.10 ± 0.35</td>
<td>1.19 ± 0.67</td>
</tr>
</tbody>
</table>

Values were indicated means ± SD.
Sham means sham operated sheep and TX means thyroidectomized ones.
Sham operated sheep were infused with vehicle and thyroidectomized ones were infused with calcitonin.
*; Significantly different (P< 0.05) from the value before infusion.
**; Significantly different (P< 0.01) from the value before infusion.
a; Significantly (P< 0.05) different from sham operated sheep.
and the reduction was significant (P<0.05) in both groups 4 hours after the beginning of infusion. However, the secretion rate of lipid phosphorus were not different between thyroidectomized and sham operated wethers during the experiment.

As shown in Table 7-3, serum calcium concentrations were slightly higher in thyroidectomized sheep than in sham ones before infusion. While the concentrations were significantly (P<0.05) reduced in thyroidectomized sheep though not changed in sham ones throughout the experiment. At the 5th hour of infusion, serum calcium concentrations were significantly (P<0.05) lower in thyroidectomized animals than in sham operated ones.

Serum inorganic phosphorus concentrations appeared to be increased in sham operated sheep, on the contrary, serum inorganic phosphorus were substantially decreased by calcitonin infusion in thyroidectomized sheep. The differences of serum inorganic phosphorus concentrations between thyroidectomized and sham operated sheep were significant (P<0.05) after 2 hours of the beginning of infusion.

Discussion

Serum calcium and inorganic phosphorus concentrations in thyroidectomized sheep were decreased by calcitonin infusion which was thought to be as much as physiological secretion rate in a normal ewe. Therefore, the results showed that calcitonin reduced serum calcium and inorganic phosphorus concentrations even though the hormone infused into wethers at a physiological secretion rate.
Table 7-3 Effect of calcitonin infusion on serum calcium and phosphorus concentrations in sheep

<table>
<thead>
<tr>
<th></th>
<th>Before infusion</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum calcium (mg/100ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>8.01 ± 0.55</td>
<td>7.87 ± 0.58</td>
<td>8.38 ± 0.48</td>
<td>8.13 ± 0.61</td>
<td>7.87 ± 0.44</td>
<td>7.95 ± 0.36</td>
</tr>
<tr>
<td>TX</td>
<td>9.03 ± 0.45</td>
<td>8.65 ± 0.55</td>
<td>8.09 ± 0.34*</td>
<td>7.96 ± 0.51*</td>
<td>7.51 ± 0.37*</td>
<td>7.20 ± 0.12*b</td>
</tr>
<tr>
<td>Serum phosphorus (mg/100ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>5.01 ± 0.38</td>
<td>5.27 ± 0.56</td>
<td>5.82 ± 0.67</td>
<td>5.75 ± 0.65</td>
<td>6.15 ± 0.50</td>
<td>6.07 ± 0.64</td>
</tr>
<tr>
<td>TX</td>
<td>4.97 ± 0.25</td>
<td>4.59 ± 0.53</td>
<td>4.12 ± 0.58a</td>
<td>4.07 ± 0.41a</td>
<td>4.01 ± 0.35a</td>
<td>4.13 ± 0.38ab</td>
</tr>
</tbody>
</table>

Values were indicated means ± SD.
Sham means sham operated sheep and TX means thyroidectomized ones.
Sham operated sheep were infused with vehicle and thyroidectomized ones were infused with calcitonin.

* ; Significantly (P< 0.05) different from the value before infusion.
\% ; Significantly (P< 0.01) different from the value before infusion.
a ; Significantly (P< 0.05) different from sham operated sheep.
b ; Significantly (P< 0.01) different from sham operated sheep.
The secretion rate of bile was not different between thyroidectomized animals and sham operated ones. Hufner et al. (89) indicated that calcitonin reduced the gallbladder contraction and decreased bile secretion rate in man. However, the results from the present experiment using sheep could not support their report.

Although there was no reduction in bile calcium excretion in thyroidectomized wethers infused with calcitonin, bile calcium excretion were decreased in sham operated ones. It could be considerable that calcitonin enhanced calcium excretion via bile in sheep. The rate of bile calcium excretion was 196 µg/10 min in sham operated sheep and was 363 µg/10 min in thyroidectomized ones 5 hours after the beginning of infusion. Recently, Yamaguchi (90) also reported that calcitonin injection increased bile calcium excretion in thyroparathyroidectomized rats. Barlet (2) indicated that urinary calcium excretion was 27 mg/day, i.e., 188 µg/10 min in sheep infused with vehicle and was 54 mg/day, i.e., 375 µg/10 min in sheep infused with calcitonin. It could be considerable that both of urine and bile excretion induced the reduction of serum calcium concentrations. Therefore the role of calcitonin on bile calcium excretion would be as important as that on urinary calcium excretion in sheep. It was not clear why bile calcium excretion was reduced in sham operated sheep. But it may be possible that the decrease of bile calcium excretion rate is owing to the reduction of bile secretion rate which may be induced by pentobarbitone anesthesia in sham operated sheep.

The elevation of bile inorganic phosphorus excretion was more in thyroidectomized sheep infused with calcitonin than in sham
operated ones infused with vehicle. The results indicated that calcitonin increased inorganic phosphorus excretion via bile. It was reported by Talmage et al. (130) that calcitonin decreased plasma phosphate concentrations by moving this ion out of extra cellular fluid such as blood plasma. And Meyer and Meyer (91) reported that liver phosphate was increased by calcitonin injection in thyroparathyroidectomized rats. It might be hypothesized that inorganic phosphorus were exuded from the hepatic cell to bile by means of the increase in inorganic phosphorus concentrations in the hepatic cell when calcitonin was administrated.

Barlet (2) indicated that inorganic phosphorus excretion in urine was 12 mg/day, i.e., 83 μg/10 min in sheep infused with vehicle and was 38 mg/day, i.e., 264 μg/10 min in sheep infused with calcitonin. It was shown in the present experiment that bile inorganic phosphorus excretion was reached 85 μg/10 min in sham operated sheep infused with vehicle and 120 μg/10 min in thyrodiectomized ones infused with calcitonin 5 hours after the beginning of infusion. It could be considered that calcitonin acted on the liver as same as the kidney in stand point of calcium and inorganic phosphorus excretion in sheep.

Bile lipid phosphorus secretion had a trend to decrease in both thyroidectomized and sham operated sheep during the experiment. The secretion of lipid phosphorus was about 10 fold more than the excretion of bile inorganic phosphorus. If the substantial amount of phospholipid which secreted into the intestine had been lost via feces, sheep should become phosphorus
deficiency. However, Adams and Heath (150) suggested that the large amount of phospholipid which entered the duodenum via bile might facilitate the uptake of fat into the cells of the intestinal mucosa. It would be natural to consider that most of all phospholipid secreted into the intestine was reabsorbed in sheep because sheep did not become phosphorus deficiency notwithstanding the large amount of lipid phosphorus secretion via bile.

Summary

The bile duct was fitted with a re-entrant cannula in thyroidectomized and sham operated sheep. Under pentobarbital anesthesia, porcine calcitonin were infused into thyroidectomized wethers at a physiological secretion rate (20 mi.u./kg body weight/hour) and sham operated ones were infused with vehicle alone. Serum calcium and phosphorus concentrations were decreased and bile inorganic phosphorus excretion were increased by calcitonin infusion. Calcium excretion via bile were decreased in sham operated animals infused with vehicle but were not changed in thyroidectomized sheep infused with calcitonin. Lipid phosphorus secretion via bile tended to be decreased in both thyroidectomized and sham operated sheep. The rates of bile calcium and inorganic phosphorus excretion were almost the same as those of urinary excretion. Therefore, it was suggested that calcitonin increased calcium and inorganic phosphorus excretion via bile which were as important as urinary excretion of these minerals.
CHAPTER 8 Effect of Calcitonin on Salivary Phosphorus and Calcium Excretion

In chapter 6, it was suggested that calcitonin deficiency reduced the amount of phosphorus inflow into the gut in sheep because phosphorus absorption was much less in thyroidectomized sheep than intact ones before feeding.

As shown in chapter 7, calcitonin infusion increased phosphorus excretion via bile but bile phosphorus excretion was not different between in thyroidectomized wethers and in sham operated ones before infusions.

It is well known that salivary phosphorus excretion plays an important role in phosphorus metabolism of ruminants (145). This experiment was therefore to study the effect of calcitonin on phosphorus and calcium excretion in the saliva.

Materials and Methods

Six young sheep, weighing about 30 kg, were used. All animals were fed a diet shown in Table 7-1 at a level of 2% of body weight daily. Water was available at all times. Three sheep were thyroidectomized and the other 3 were sham operated. In order to supply thyroid hormone every thyroidectomized sheep was injected intramuscularly with 2.5 mg of L-thyroxine (Nakarai Chemicals Ltd., Kyoto) dissolved in corn oil once a week.

The experiment was conducted under pentobarbitone sodium anesthesia (Pitman-Moore, Washington, U.S.A.) 24 hours after
feeding. The left parotid duct was cannulated with a siliconized tube which was inserted from the salivary duct on the jaw. A siliconized catheter was inserted into the jugular vein to collect blood samples and to infuse calcitonin or vehicle. Thyroidectomized animals were infused with porcine calcitonin (Armour Pharmaceutical Company, Eastbourne, England; 80 i.u./mg protein) dissolved in 0.8% NaCl (4 mi.u./ml) at a rate of 30 mi.u./hour per kg body weight for 5 hours, which was within the physiological rate of secretion of calcitonin. Care et al. (147) indicated that rates of secretion of calcitonin from the thyroid gland fluctuated ranging from 5 to 30 mi.u./hour per kg body weight in an ewe when serum calcium concentrations varied from 8 to 12 mg/100 ml. Sham operated sheep were infused with vehicle only. Salivary samples were collected for 10 min in every hour from all sheep.

Calcium concentrations in the saliva and serum were measured by atomic absorption spectrophotometry and inorganic phosphorus concentrations were determined by the method of Fiske and Subbarow. (148). Serum parathyroid hormone concentrations were measured by a radioimmunoassay using porcine parathyroid hormone (148). Salivary cyclic AMP concentrations were measured by the method of Honma et al. (151). Statistical differences were evaluated by student's t test.

Results

Serum phosphorus concentrations tended to increase in sham operated sheep but to decrease in thyroidectomized one during infusion. (Fig. 8-1a) The difference in serum phosphorus
Fig. 8-1 Effect of calcitonin infusion on serum concentrations (a) and salivary excretion (b) of phosphorus in sheep. Thyroidectomized sheep (○; open columns) were infused with porcine calcitonin. Sham operated sheep (●; hatched columns) were infused with vehicle. Values were indicated means ± SD for three sheep. *; P<0.05, **; P<0.01 compared with the value before infusion. +; P<0.05, ++; P<0.01 compared with the value from sham operated sheep.
concentration between thyroidectomized sheep and sham operated ones became significant (P<0.05) after 2 hours of infusion. As shown in Fig. 8-1b, thyroidectomized sheep excreted less phosphorus in the saliva than sham operated sheep before infusion (P<0.05). A significant (P<0.05) increase of phosphorus excretion in the saliva was found after 1 hour of calcitonin infusion. The excretion continued to increase up to 5 hours after the beginning of infusion in thyroidectomized sheep, though it scarcely changed in sham operated animals. As a result, after 4 hours and 5 hours of infusion thyroidectomized sheep infused with calcitonin excreted significantly (P<0.05) more phosphorus in the saliva than sham operated ones infused with vehicle.

Serum calcium concentrations were slightly higher in thyroidectomized sheep than in sham operated ones before infusion. (Fig. 8-2a) Serum calcium concentrations decreased significantly (P<0.05) after 2 hours of calcitonin infusion in thyroidectomized sheep and became significantly (P<0.01) lower in thyroidectomized animals than in sham operated ones after 5 hours of infusion.

Salivary calcium excretion in thyroidectomized and sham operated sheep was not different before infusion. The excretion was decreased by calcitonin infusion in thyroidectomized sheep accompanied by a reduction in serum calcium concentrations. On the other hand, salivary calcium excretion hardly changed in sham operated sheep during the experiment.

Serum parathyroid hormone concentrations tended to be lower in thyroidectomized sheep than in sham operated ones before infusion.
Fig. 8-2 Effect of calcitonin infusion on serum concentrations (a) and salivary excretion (b) of calcium in sheep.

Thyroidectomized sheep (○; open columns) were infused with porcine calcitonin.

Sham operated sheep (●; hatched columns) were infused with vehicle.

Values were indicated means ± SD for three sheep. *;P<0.05, **;P<0.01 compared with the value before infusion.

+;P<0.05, ++;P<0.01 compared with the value from sham operated sheep.
Fig. 8-3 Effect of calcitonin infusion on serum parathyroid hormone concentrations in sheep.

Thyroidectomized sheep (O) were infused with porcine calcitonin.
Sham operated sheep (●) were infused with vehicle.
Values were indicated means ± SD for three sheep.

*; P<0.05 compared with the value before infusion.
++; P<0.05 compared with the value from sham operated sheep.
<table>
<thead>
<tr>
<th></th>
<th>Before infusion</th>
<th>Infusion, hours</th>
<th>Infusion, hours</th>
<th>Infusion, hours</th>
<th>Infusion, hours</th>
<th>Infusion, hours</th>
<th>Infusion, hours</th>
<th>Infusion, hours</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saliva secretion (ml/10min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>3.44 ± 0.30</td>
<td>3.52 ± 0.40</td>
<td>3.50 ± 0.44</td>
<td>3.17 ± 0.25</td>
<td>2.87 ± 0.39</td>
<td>3.74 ± 0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TX</td>
<td>3.72 ± 0.13</td>
<td>3.53 ± 0.92</td>
<td>3.97 ± 0.21</td>
<td>3.42 ± 0.12</td>
<td>3.28 ± 0.26</td>
<td>3.53 ± 0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salivary cyclic AMP (pmol/10min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>46.7 ± 6.8</td>
<td>31.2 ± 5.7</td>
<td>27.3 ± 7.2</td>
<td>54.0 ± 14.2</td>
<td>29.4 ± 6.4</td>
<td>51.9 ± 11.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TX</td>
<td>33.3 ± 3.2</td>
<td>42.4 ± 13.9</td>
<td>30.0 ± 10.5</td>
<td>34.1 ± 12.0</td>
<td>54.0 ± 6.5</td>
<td>37.5 ± 5.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values were indicated means ± SD.
Sham means sham operated sheep and TX means thyroidectomized sheep.
Sham operated sheep were infused with vehicle and thyroidectomized ones were infused with calcitonin.
(Fig. 8-3). While serum parathyroid hormone concentrations were significantly (P<0.05) raised after 3 hours of calcitonin infusion in thyroidectomized sheep, vehicle infusion did not appear to affect the concentration in sham operated sheep.

As shown in Table 8-1, salivary secretion rate in sham operated sheep was 3.44 ± 0.30 ml/10 min and in thyroidectomized sheep it was 3.72 ± 0.13 ml/10 min and no significant change occurred during infusion.

Salivary cyclic AMP excretion was 46.7 ± 6.8 pmol/10 min in sham operated sheep and 37.3 ± 3.2 pmol/10 min in thyroidectomized ones. There was no significant change in the excretion of the cyclic nucleotide. Salivary cyclic AMP concentrations were 9-12 nmol/l, which were almost the same as those reported in blood plasma by Steiner et al. (152).

Discussion

Calcitonin did not affect salivary secretion rates in sheep. However, it has been found that calcitonin injection reduces gastric acid secretion, pancreatic enzyme and bile secretion in man. Thus the effect of calcitonin on the salivary gland may differ from that on the other exocrine glands in the gastrointestinal tract.

Salivary phosphorus excretion was lower in thyroidectomized animals than in sham operated ones before infusion and was increased by calcitonin infusion in thyroidectomized sheep. Tomas (152) suggested that parathyroid hormone increased salivary phosphorus excretion in sheep. There is a possibility that hypocalcemia induced by calcitonin infusion, stimulates
parathyroid hormone secretion and then the increment in serum parathyroid hormone concentrations might enhance salivary phosphorus excretion in sheep. However, in our experiment, salivary phosphorus excretion increased before the increment in serum parathyroid hormone concentrations and the changes of phosphorus excretion in the saliva was not closely associated with the changes in serum parathyroid hormone concentrations. It could be concluded therefore that calcitonin acted directly on the salivary gland to increase the excretion of salivary phosphorus in sheep.

In chapter 6, it was found that phosphorus absorption was less in thyroidectomized sheep than in intact ones before feeding and suggested that calcitonin deficiency reduced the amount of phosphorus inflow into the gut. It appears likely that the decrease of salivary phosphorus excretion by calcitonin deficiency reduces phosphorus content in the gut, contributing thereby to the decrease in phosphorus absorption in sheep.

Barlet (2) indicated that urinary phosphorus excretion was 12 mg/day (i.e. 83 μg/10 min) in sheep infused with vehicle and 38 mg/day (i.e. 264 μg/10 min) in animals infused with calcitonin. On the other hand, salivary phosphorus excretion was 1.3 mg/10 min in sham operated sheep infused with vehicle and 1.9 mg/10 min in thyroidectomized animals infused with calcitonin after 5 hours of infusion. It is clear from these results that salivary phosphorus excretion is very important in phosphorus homeostasis and the action of calcitonin on hypophosphatemia is in part dependent on the increment of salivary phosphorus excretion in
sheep.

After the reduction of serum calcium concentrations, salivary calcium excretion decreased in thyroidectomized sheep infused with calcitonin. The decrease in salivary calcium excretion may be due to the reduction of serum calcium concentrations in sheep.

Summary

Three thyroidectomized sheep were infused with intravenously with porcine calcitonin at a rate of 30 mi.u./hour per kg body weight and 3 sham operated ones were infused with vehicle for 5 hours. Saliva was collected from the left parotid duct by cannulation for 10 min in every hour.

Salivary secretion rates were not changed in either thyroidectomized or sham operated sheep throughout the experiment. Before infusion, salivary phosphorus excretion was less in thyroidectomized sheep than in sham operated ones. Calcitonin infusion increased salivary phosphorus excretion and decreased serum phosphorus concentrations in thyroidectomized sheep. Vehicle infusion did not affect salivary phosphorus excretion in sham operated sheep.

Serum concentrations and salivary excretion of calcium were decreased by calcitonin infusion into thyroidectomized sheep but were not changed in sham operated sheep infused with vehicle.

Calcitonin infusion increased serum parathyroid hormone concentrations in thyroidectomized sheep after the decrease in serum calcium concentrations. However, vehicle infusion did not affect serum parathyroid hormone concentrations in sham operated sheep. There was little change of cyclic AMP excretion during the
experiment in either thyroidectomized or sham operated animals.

It is concluded that calcitonin increases salivary phosphorus excretion in sheep.
It is well known that calcitonin affects urinary mineral excretion. Rasmussen et al. (58) indicated that calcitonin decreased urinary calcium excretion and increased phosphorus excretion in rats. Barlet (2) reported that calcitonin administration increased urinary calcium and phosphorus excretion in sheep. And several investigators obtained different results about the effect of calcitonin on urinary calcium excretion (154,155). From these results, it is clear that the effect of calcitonin on urinary calcium excretion is fluctuated by the difference of kind, dose and purity of calcitonin, method of administration and animal specise.

On the other hand, calcitonin deficiency increased urinary calcium excretion (chapter 4) and the increment in urinary calcium excretion might be inhibited and hypophosphaturemia was stimulated by the presence of calcitonin during oral calcium load in sheep (chapter 5).

The present study was designed to investigate the effect of several doses of calcitonin on urinary calcium and phosphorus excretion.

Materials and Methods

Six young sheep weighing about 25 kg were used. All animals were kept in metabolism cages. Three wethers were thyroid-
ectomized and the other 3 were performed sham operation 2 weeks before the experiment. All sheep were given a diet shown in Table 7-1 at a level of 2% of body weight daily. Water was available at all times. Thyroidectomized animals were injected intramuscularly with 0.25 mg of L-thyroxine (Nakarai Chemicals Ltd., Kyoto) dissolved in corn oil daily. And thyroidectomized sheep were injected intramuscularly with 0, 0.25, 0.5 and 1 i.u./kg body weight of porcine calcitonin (Armor Pharmaceutical Company Ltd., Eastbourne England; 80 i.u./mg protein) dissolved in 16% gelatin at 6 day intervals at feeding time. At the same time, sham operated sheep were injected with vehicle.

Blood samples were collected 8 hours after injection on the days of calcitonin or vehicle administration. And urine collection were made over 24 hours after injection. Serum and urine calcium concentrations were measured by an atomic absorption spectrophotometry and serum and urinary phosphorus concentrations were by the method of Gomori (121). Serum parathyroid hormone levels were determined by a radioimmunoassay using bovine parathyroid hormone (135). Statistical differences were evaluated by student's t test.

Results and Discussion

As shown in Table 9-1, serum calcium concentrations tended to be decreased by calcitonin injection in thyroidectomized sheep and the reduction was significant (P<0.05) when animals were injected with 1 i.u./kg body weight of calcitonin. Serum phosphorus concentrations were also decreased by calcitonin injection while the significant difference were not found between
Table 9-1 Effect of calcitonin injection on serum calcium and phosphorus excretion in thyroidectomized sheep

<table>
<thead>
<tr>
<th></th>
<th>Amount of Calcitonin Injection (i.u./kg)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Serum Calcium</td>
<td>TX</td>
</tr>
<tr>
<td>mg/100ml</td>
<td>9.68 ± 0.11</td>
</tr>
<tr>
<td>Serum Phosphorus</td>
<td>TX</td>
</tr>
<tr>
<td>mg/100ml</td>
<td>6.60 ± 1.04</td>
</tr>
</tbody>
</table>

Values were indicated means ± SD for 3 sheep.
TX means thyroidectomized sheep which were injected with calcitonin.
Sham means sham operated sheep which were injected with vehicle
*; P<0.05, **; P<0.01 compared with the value when sheep were injected
with 0 i.u./kg of calcitonin (vehicle)
b; P<0.01 compared with the value from sham operated sheep.
calcitonin injection group and vehicle injection group. It seemed that the large fluctuation of serum phosphorus concentrations masked the hypophosphatemic effect of calcitonin in the present experiment.

As shown in Fig. 9-1, serum parathyroid hormone concentrations tended to be higher in sham operated sheep than in thyroidectomized ones which was described in chapter 5. By the larger dose of calcitonin administrations (0.5 and 1 i.u./kg body weight), serum parathyroid hormone levels were significantly \((P<0.05)\) increased in according to the reduction of serum calcium concentrations in thyroidectomized sheep. It was known that hypocalcemia induced the secretion of parathyroid hormone.

Fig. 9-2 shows the effect of calcitonin on the daily urine volume in sheep. Urinary volume did not appear to be different between thyroidectomized and sham operated sheep when they were injected with vehicle. Urinary volume tended to be decreased by calcitonin injection in thyroidectomized sheep. However the decreased urinary volume by calcitonin administration was not dose related.

Barlet (2) reported that calcitonin infusion (20 mi.u./kg body weight/hour) increased urinary volume in intact and thyroparathyroidectomized sheep. On the other hand, Kimura and Ogata (9) indicated that a large dose of calcitonin administration (1-1.3 i.u./rat) increased urinary volume but a smaller dose of injection (2m i.u./rat) did not affect urine volume. The results suggested that a small amount of calcitonin injection decreased urinary volume. But it was not clear that a large amount of calcitonin injection affected urinary volume in
Calcitonin injection (i.u/kg)

Fig. 9-1: Effect of calcitonin injection on serum parathyroid hormone concentrations.
Values were indicated means ± SD for 3 sheep.
Thyroidectomized sheep (O) were injected with calcitonin.
Sham operated sheep (●) were injected with vehicle.
*; Significantly (P<0.05) different from the value before operation.
Fig. 9-2 Effect of calcitonin injection on urinary volume.

Values were indicated means ± SD for 3 sheep. Thyroidectomized sheep (open columns) were injected with calcitonin. Sham operated sheep (hatched columns) were injected with vehicle.
As shown in Fig. 9-3(a), urinary calcium excretion was significantly \((P<0.05)\) more in thyroidectomized sheep than in sham operated ones when they were injected with vehicle. The large amount of urinary calcium excretion in thyroidectomized sheep was also found in the previous experiment as discussed in chapter 4. The smallest dose of calcitonin injection significantly \((P<0.05)\) reduced calcium excretion in urine in thyroidectomized sheep. Talmage (30) indicated that one of the roles of calcitonin was calcium storage in the bone. He suggested that the less storage of calcium induced a large amount of urinary calcium excretion in thyroidectomized sheep and that a small dose of calcitonin injection increased the retention of calcium which decreased urinary calcium excretion.

However, urinary calcium excretion were recovered by 0.5 i.u./kg body weight of calcitonin injection and the largest dose of calcitonin administration increased urinary calcium excretion in thyroidectomized sheep. And urinary calcium excretion were significantly \((P<0.01)\) more in thyroidectomized sheep injected with 1 i.u./kg body weight than in sham operated ones injected with vehicle.

Serum parathyroid hormone concentrations were increased by a large dose of calcitonin injection. It is well known that parathyroid hormone increases calcium reabsorption in the kidney. However, the large dose of calcitonin injection increased calcium excretion. It was indicated that calcitonin increased urinary calcium excretion and this action overcame the inhibitory effect of parathyroid hormone on calcium excretion.
Fig.9-3 (a) Effect of calcitonin injection on urinary calcium excretion. Values were indicated means ± SD for 3 sheep. Thyroidectomized sheep (open columns) were injected with calcitonin. Sham operated sheep (hatched columns) were injected with vehicle.

*; P<0.05 compared with the value of 0 i.u./kg (vehicle) injection in thyroidectomized sheep. ++; P<0.01 compared with the value from sham operated sheep.

Fig.9-3 (b) Effect of calcitonin injection on urinary phosphorus excretion. Values were indicated means ± SD for 3 sheep. Thyroidectomized sheep (open columns) were injected with calcitonin. Sham operated sheep (hatched columns) were injected with vehicle.

*; P<0.05 compared with the value of 0 i.u./kg (vehicle) injection in thyroidectomized sheep. ++; P<0.01 compared with the value from sham operated sheep.
Kimura and Ogata (9) found that a small dose of calcitonin administration decreased calcium excretion in urine but a large dose of administration increased calcium excretion which was consistent with the present experiment. From these results, it is suggested that calcitonin plays 2 kinds of role related to urinary calcium excretion, i.e., 1) calcitonin usually stimulates storage of calcium which reduces urinary calcium excretion and 2) the excessive calcitonin secretion induces hypocalcemia by means of the increase in urinary calcium excretion.

Fig. 9-3(b) shows the effect of calcitonin injection on urinary phosphorus excretion. Phosphorus excretion in urine was much less (P<0.01) in thyroidectomized sheep than in sham operated ones when they were injected with vehicle. The smallest dose of calcitonin injection significantly (P<0.05) increased urinary phosphorus excretion in thyroidectomized sheep, however there was no difference of urinary phosphorus excretion among any dose of calcitonin administration. Phosphorus excretion was significantly (P<0.01) more in sham operated sheep than in thyroidectomized ones even if thyroidectomized sheep were injected with calcitonin throughout the experiment. Some investigators reported the hyperphosphaturic effect of calcitonin (2, 9, 58). However, it was obscure that calcitonin injection could not recover perfectly urinary excretion of phosphorus in thyroidectomized sheep. There is a possibility that chronic calcitonin deficiency reduces the responsibility of urinary phosphorus excretion to calcitonin administration.
Summary

This experiment was designed to investigate a role of calcitonin on calcium and phosphorus excretion in urine. Three thyroidectomized sheep were injected with 0, 0.25, 0.5 and 1 i.u./kg body weight of porcine calcitonin and 3 sham operated sheep were administrated vehicle.

Serum calcium concentrations were significantly decreased and serum phosphorus concentrations tended to be reduced by calcitonin injection. Serum parathyroid hormone concentrations were elevated by calcitonin injection in terms of the reduction of serum calcium. The urinary volume tended to be decreased by a small dose of calcitonin administration but the reduction was not dose related.

Urinary calcium excretion was more in thyroidectomized sheep than in sham operated ones which were injected with vehicle. The smallest dose of calcitonin injection reduced urinary calcium excretion while a larger dose of calcitonin increased calcium excretion in thyroidectomized sheep.

Urinary phosphorus excretion was much less in thyroidectomized sheep than in sham operated ones which were injected with vehicle. Any dose of calcitonin injection increased urinary phosphorus excretion, however there was no difference in phosphorus excretion among 0.25, 0.5 and 1 i.u./kg body weight of calcitonin injection in thyroidectomized sheep.

These results suggest that calcitonin usually stimulates conservation of calcium which reduces urinary calcium excretion and that the excessive calcitonin secretion induces the increase.
in urinary calcium excretion. And it was confirmed that calcitonin had a hyperphosphaturemia action in sheep.
CHAPTER 10 Effect of Calcitonin on Bone Metabolism

It is well known that calcitonin reduces bone resorption. Freidman et al. (67) found that stimulatory effect of parathyroid hormone on bone resorption were inhibited by calcitonin administration. Rasmussen et al. (58) found that calcitonin reduced urinary hydroxyproline excretion in rats and suggested that calcitonin inhibited bone resorption. On the other hand, as shown in chapter 4, bone resorption was not influenced by the deficiency of calcitonin in sheep because serum hydroxyproline concentrations were not changed by thyroidectomy. Furthermore Payne and Sanson (119) found that calcium release from the bone was not affected by thyroidectomy using kinetics study.

The effect of calcitonin on bone formation are still controversial. Some workers indicated that calcitonin administration increased the enzymic activities which were thought to stimulate bone calcification (78, 79). However a kinetics studies showed that calcitonin reduced calcium deposition (81, 119). And calcitonin might not affect bone formation because urinary polypeptide hydroxyproline excretion, which was thought to be an index of bone collagen synthesis, was not changed by calcitonin infusion (156). On the other hand, as described in chapter 5, shows that urinary polypeptide hydroxyproline excretion was increased by calcium load in sham operated sheep but was not changed in thyroidectomized one. And calcitonin injection increased calcium conservation in sheep as
presented in chapter 9.

This experiment was to study the effect of calcitonin injection on bone metabolism using indices in urine and serum.

Materials and Methods

Experiment 1

Six adult sheep, weighing about 40 kg, were used. Three wethers were thyroidectomized 3 months before the experiment which were injected intramuscularly with 2.5 mg of L-thyroxine (Nakarai Chemicals Ltd., Kyoto) dissolved in corn oil once a week. A siliconized catheter was inserted into the jugular vein in all wethers. Every animal was kept in a metabolism cage and given the diet shown in Table 7-1 at a level of 2% of body weight. Water was available at all times. Thyroidectomized animals were injected intramuscularly with 1 i.u./kg body weight of porcine calcitonin (Armor Pharmaceutical Company Ltd., Eastbourne England; 80 i.u./mg protein) dissolved in 16% gelatin twice 5 day-intervals during feeding.

Blood was collected on the days before calcitonin injection and the days of calcitonin injection before feeding, and 1, 2, 4 and 8 hours after feeding in thyroidectomized sheep. Blood was collected from intact sheep at the same time.

Serum calcium concentrations were measured by an atomic absorption spectrophotometry. Serum phosphorus concentrations were measured by the method of Gomori (121). Serum free hydroxyproline concentrations were analysed by the method of Bergmann and Loxley (122) and serum alkaline phosphatase activities were determined by the method of Bessey and Lowry.
Experiment 2

Six young sheep, weighing about 25 kg, were used. Three wethers were thyroidectomized and the other 3 were performed sham operation 2 weeks before the experiment. All sheep were given a diet shown in Table 7-1 at a level of 2% of body weight daily. Water was available at all times. Thyroidectomized animals were injected intramuscularly with 0.25 mg of L-thyroxine (Nakarai Chemicals Ltd., Kyoto) dissolved in corn oil daily. And thyroidectomized sheep were injected intramuscularly with 0, 0.25, 0.5 and 1 i.u./kg body weight of porcine calcitonin (Armor Pharmaceutical Company, Eastbourne, England; 80 i.u./mg protein) dissolved in 16% gelatin at 6 day intervals at feeding time. At the same time, sham operated sheep were injected with vehicle.

Blood samples were collected from the jugular vein 8 hours after injection on the day of calcitonin and vehicle administration. And urine collection were made over 24 hours after injection. Serum calcium concentrations were measured by an atomic absorption spectrophotometry, phosphorus concentrations were determined by the method of Gomori (121) and serum hydroxyproline concentrations were measured by the method of Bergmann and Loxley (122). Urinary total hydroxyproline excretion was determined by the method of Koevoet (136) and polypeptide hydroxyproline excretion was measured by the method of Krane et al. (137). Statistical differences were evaluated by student's t test.
Results

Experiment 1

There are no difference in serum calcium, phosphorus and hydroxyproline concentrations and alkaline phosphatase activities between the 1st and the 2nd trial.

Serum calcium, phosphorus and bone metabolism indices before feeding are shown in Table 10-1. There was no difference in serum calcium, phosphorus and free hydroxyproline concentrations between thyroidectomized and intact sheep. On the other hand, serum alkaline phosphatase activities in thyroidectomized sheep were significantly (P<0.05) lower than in intact ones.

Postprandial changes of serum calcium and phosphorus in arterial blood are shown in Fig. 10-1. There was little change in serum calcium concentrations in either thyroidectomized sheep or intact ones injected with vehicle. On the other hand, in thyroidectomized sheep, calcitonin injection began to decrease serum calcium concentrations 2 hour after calcitonin injection and serum calcium reached 76% of the initial calcium levels at the end of the experiment.

Though serum phosphorus concentrations increased significantly (P<0.05) in intact sheep at 2 hours after feeding, they tended to decrease at 4 hours after feeding. In all thyroidectomized sheep, injected with calcitonin and not, serum phosphorus concentrations did not increase and gradually reduced after feeding. As a result, serum phosphorus concentrations reduced significantly at 8 hours after feeding in all sheep. The reduction of serum phosphorus concentrations in thyroidectomized animals injected with calcitonin was more striking (P<0.05) than in
<table>
<thead>
<tr>
<th></th>
<th>Intact</th>
<th>TX</th>
<th>Sham operated sheep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mg/100ml)</td>
<td>8.14 ± 0.17</td>
<td>8.24 ± 0.33</td>
<td>8.24 ± 0.33</td>
</tr>
<tr>
<td>Phosphorus (mg/100ml)</td>
<td>6.89 ± 0.24</td>
<td>5.88 ± 0.18</td>
<td>2.24 ± 0.21</td>
</tr>
<tr>
<td>HYP (B.L.U.)</td>
<td>2.44 ± 0.15</td>
<td>2.74 ± 0.21</td>
<td>0.77 ± 0.09</td>
</tr>
<tr>
<td>ALP (B.L.U.)</td>
<td>0.93 ± 0.15</td>
<td>0.93 ± 0.15</td>
<td>0.77 ± 0.09</td>
</tr>
</tbody>
</table>

Values were indicated means ± SE, for 3 sheep. TX means thyroidec-tomized sheep. Sham means sham operated sheep. HYP means hydroxyproline. ALP means alkaline phosphatase activities. *P<0.05 compared with the value from sham operated sheep.
Fig. 10-1 Percentage changes of serum calcium and phosphorus concentrations after feeding in intact sheep (O), thyroidectomized ones (▲) and thyroidectomized ones injected with calcitonin (■). Values were indicated means ± SD for 3 sheep. *: P<0.05, **: P<0.01 compared with the value before feeding. +: P<0.05 compared with the value from sham operated sheep. Arrows indicated feeding and injection.
thyroidectomized and intact ones injected vehicle.

Postprandial change of serum bone metabolism indices in arterial blood are shown in Fig. 10-2. In all sheep, serum alkaline phosphatase activities were slightly reduced 1 hour after and significantly (P<0.05) decreased 8 hours after feeding. Serum hydroxyproline concentrations also decreased (P<0.05) in all sheep as time pass. At 8 hours after feeding, serum hydroxyproline concentrations in thyroidectomized sheep injected with calcitonin were significantly lower (P<0.05) than in the other groups.

Experiment 2

As shown in Fig. 10-3, serum calcium concentrations tended to be decreased by 0.25 i.u./kg body weight calcitonin injection in thyroidectomized sheep and were significantly (P<0.01) decreased by 1 i.u./kg body weight of calcitonin injection. Serum phosphorus concentrations tended to be decreased by calcitonin injection though a significant reduction was not found in the present experiment.

Calcitonin injection at the level of 0.25 i.u./kg body weight tended to increase serum parathyroid hormone concentrations in thyroidectomized wethers. And the increment in serum parathyroid hormone was accompanied by the reduction of serum calcium concentrations.

As shown in Table 10-2, there was no difference in serum free hydroxyproline concentrations between thyroidectomized and sham operated sheep when they were injected with vehicle. Serum free hydroxyproline concentrations were significantly (P<0.05 by 0.5
Fig. 10-2 Percentage changes of bone metabolism indices in serum after feeding in intact sheep (○), thyroidectomized ones (▲) and thyroidectomized ones injected with calcitonin (■). Values were indicated means ± SD for 3 sheep. *; P<0.05, **; P<0.01 compared with the value before feeding. +; P<0.05, ++; P<0.01 compared with the value from sham operated sheep. Arrows indicated feeding and injection.
Fig. 10-3 Effect of calcitonin injection on serum calcium and phosphorus concentrations. Values were indicated means ± SD for 3 sheep. Thyroidectomized sheep (O) were injected with calcitonin. Sham operated sheep (●) were injected with vehicle.

*; P<0.05 compared with the value of 0 i.u./kg (vehicle) injection in thyroidectomized sheep. ++; P<0.01 compared with the value from sham operated sheep.
Table 10-2 Effect of calcitonin injection on bone metabolism indices in serum and in urine

<table>
<thead>
<tr>
<th></th>
<th>Amount of Calcitonin Injection (i.u./kg)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Serum Hyp (mg/l)</td>
<td></td>
</tr>
<tr>
<td>TX</td>
<td>2.36 ± 0.05</td>
</tr>
<tr>
<td>Sham</td>
<td>2.36 ± 0.13</td>
</tr>
<tr>
<td>Urinary Total Hyp (mg/day)</td>
<td></td>
</tr>
<tr>
<td>TX</td>
<td>143 ± 32</td>
</tr>
<tr>
<td>Sham</td>
<td>148 ± 31</td>
</tr>
<tr>
<td>Urinary Polypeptide Hyp (mg/day)</td>
<td></td>
</tr>
<tr>
<td>TX</td>
<td>5.8 ± 1.7</td>
</tr>
<tr>
<td>Sham</td>
<td>6.1 ± 2.3</td>
</tr>
</tbody>
</table>

Values were indicated means ± SD for 3 sheep.
TX means thyroidectomized sheep which were injected with calcitonin.
Sham means sham operated sheep which were injected with vehicle.
Hyp means hydroxyproline.
**; P<0.01 compared with the value when sheep were injected with 0 i.u./kg of calcitonin (vehicle).
a; P<0.05, b; P<0.01 compared with the value from sham operated sheep.
i.u./kg body weight injection; P<0.01 by 1 i.u./kg body weight injection) decreased in thyroidectomized sheep injected with calcitonin in comparison with sham operated ones injected with vehicle.

Urinary total hydroxyproline excretion also tended to be decreased by calcitonin injection while there was no significant difference between vehicle injection and calcitonin injection (Table 10-2).

Urinary polypeptide hydroxyproline excretion were not different between thyroidectomized and sham operated sheep injected with vehicle. However, urinary polypeptide hydroxyproline excretion tended to be increased by the smallest dose of calcitonin injection in thyroidectomized sheep and 0.5 and 1 i.u./kg body weight of calcitonin injection significantly (P<0.01) increased urinary polypeptide hydroxyproline excretion in thyroidectomized sheep. There was a significant (P<0.01) difference between thyroidectomized animals injected with 0.5 and 1 i.u./kg body weight and sham operated ones injected with vehicle.

Discussion

The results from experiment 1 that serum hydroxyproline concentrations decreased after feeding in intact wethers was agreed with a report of Evance et al. (58) on cattle. The decrease in serum hydroxyproline concentrations after feeding in all animals suggested that bone resorption was reduced in order to prevent an elevation of serum calcium concentrations which was induced by calcium absorption. Some factors other than calcitonin
might contribute to the decrease of serum hydroxyproline concentrations after feeding because the lowering of serum hydroxyproline concentrations was also found in thyroidectomized wethers.

As shown in experiment 1, the lowering of serum hydroxyproline concentrations was more remarkable in thyroidectomized sheep injected with calcitonin than in the other animals. Furthermore the reduction of serum hydroxyproline concentrations by calcitonin was also found in experiment 2 and it was dose related response. This agreed with the indication by Johnston and Deiss (159) that calcitonin decreased bone resorption in rats. Rasmussen et al. (58) found that calcitonin decreased urinary total hydroxyproline excretion in rats and suggested that calcitonin inhibited bone resorption. However, in the present experiment, a significant reduction in urinary total hydroxyproline excretion was not found although calcitonin injection tended to decrease urinary total hydroxyproline excretion. It was suggested that serum hydroxyproline concentration reflected bone resorption more clearly than urinary total hydroxyproline excretion.

It is known that parathyroid hormone increases bone resorption. Though serum parathyroid hormone concentrations were increased by calcitonin injection, bone resorption was reduced with dose related manner. It was considerable that the inhibitory effect of calcitonin on bone resorption was stronger than the action of parathyroid hormone.

Krane et al. (137) found that disorders with stimulation of
bone formation increased urinary polypeptide hydroxyproline excretion and suggested that procollagen was cleaved and divided into the polypeptide contained hydroxyproline and collagen molecule. Haddad et al. (156) showed that calcitonin infusion decreased urinary polypeptide hydroxyproline excretion in a Pagetic subject. And they suggested that calcitonin inhibited bone formation. However, in this experiment, urinary polypeptide hydroxyproline excretion was increased by calcitonin injection in thyroidectomized sheep. Furthermore the increase was dose related response. It was not clear why the differences between the report by Haddad et al. and the present experiment occurred. The results in the present study, on the contrary, indicated that calcitonin stimulated bone formation (collagen synthesis) in calcium deficient sheep.

Serum alkaline phosphatase activities were reduced in all animals, however, the reduction was inagreement with the findings of Longmann et al. (160). They indicated that serum alkaline phosphatase activities increased after diet in man.

Usually serum alkaline phosphatase activities were used as an index of bone formation because bone alkaline phosphatase was thought to stimulate bone calcification. And it was reported that calcitonin increased bone alkaline phosphatase activities (78). The changes of serum alkaline phosphatase activities were not accompanied with the other index of bone formation (urinary polypeptide hydroxyproline excretion) but were similar to the changes of the index of bone resorption (serum free hydroxyproline concentrations). Furthermore Hurwitz and Griminger (101) found that serum alkaline phosphatase activities indicated
the statue of bone resorption rather than bone formation. The reduction of alkaline phosphatase activities might be induced by the decrease in released bone alkaline phosphatase that was resulted from the depression of bone resorption.

Serum phosphorus concentrations increased 2 hours after feeding in intact sheep. As shown in chapter 6, phosphorus absorption was much less in both of thyroidectomized sheep injected with calcitonin and not than in intact ones injected with vehicle from 2 to 4 hours after feeding. The absence of the increment in serum phosphorus concentrations may be due to the depression of phosphorus absorption in thyroidectomized sheep.

Summary

In experiment 1, three thyroidectomized sheep were injected with 1 i.u./kg body weight of calcitonin or vehicle at feeding time and 3 intact animals were injected with vehicle. Serum alkaline phosphatase activities were decreased in all groups at 8 hours after feeding. Serum hydroxyproline concentrations were decreased in all sheep, however the reduction was most remarkable in thyroidectomized sheep injected with calcitonin.

Experiment 2 were designed to study a dose related response of calcitonin injection to bone metabolism. Three thyroidectomized sheep were injected with 0, 0.25, 0.5 and 1 i.u./kg body weight of calcitonin and sham operated sheep were injected with vehicle. As same as experiment 1, serum hydroxyproline concentrations were decreased by calcitonin injection and the reduction was dose related. And urinary total hydroxyproline excretion tended to be
decreased by calcitonin administration. Urinary polypeptide hydroxyproline excretion were significantly increased by calcitonin injection and the response was also dose related.

From these results, it was suggested that calcitonin decreased bone resorption and increased bone formation in sheep and these effects were dose related manner.
CHAPTER 11 Conclusion

It was found by a radioimmunoassay using porcine calcitonin that serum calcitonin concentrations were 50-100 pg/ml in sheep and were increased about 10 fold by intravenous calcium load. On the other hand, serum calcitonin concentrations were not detected in thyroidectomized sheep even after the calcium load. It might be concluded that thyroidectomy induced calcitonin deficiency in sheep.

The portal vein and artery blood difference of serum calcium concentrations were more in thyroidectomized sheep than in intact ones at 8 hours after feeding. And calcitonin administration decreased the veno-arterial blood difference of serum calcium concentrations in thyroidectomized sheep after 1 hour of administration. It was clear that calcitonin directly inhibited calcium absorption in the gut.

Calcitonin injection decreased serum hydroxyproline concentrations and urinary total hydroxyproline excretion in thyroidectomized sheep. On the other hand, urinary polypeptide hydroxyproline excretion was increased by calcitonin injection. Calcitonin might inhibit bone resorption and stimulated bone formation in sheep.

Urinary calcium excretion was more and urinary phosphorus excretion was less in thyroidectomized sheep than in intact ones. The small dose of calcitonin injection decreased calcium excretion in urine while the large dose of calcitonin injection
Table 11-1. Effect of calcitonin administration on calcium and phosphorus metabolism in sheep.

*: Large dose of calcitonin injection increased calcium loss but small dose of calcitonin injection decreased calcium loss.
increased calcium excretion. On the other hand, phosphorus excretion was reduced by any dose of calcitonin injection. Calcium excretion via bile was decreased in sham animals infused with vehicle but was not changed in thyroidectomized ones infused with a physiological level of calcitonin. And the calcitonin infusion increased phosphorus excretion via bile.

Salivary phosphorus excretion was less in thyroidectomized sheep than in sham operated ones. Furthermore, calcitonin infusion reduced phosphorus excretion via urine, bile and saliva in thyroidectomized sheep.

These action of calcitonin were summarized in Fig. 11-1.

The results that thyroidectomy did not affect serum calcium concentrations in sheep was in agreement with several reports using ruminants. And postprandial increment of serum calcium shown in thyroidectomized rats did not occur in thyroidectomized sheep. However, urinary calcium excretion was increased by thyroidectomy in sheep. And the small dose of calcitonin administration decreased urinary calcium excretion. In addition, urinary calcium excretion was less in sham operated sheep than in thyroidectomized ones during oral calcium load. These results indicated that calcitonin stimulated calcium conservation in sheep.

Postprandial hyperphosphatemia occurred in intact sheep at 2 hours after feeding but was not found in thyroidectomized sheep whether calcitonin was injected or not. The absence of increment in serum phosphorus concentrations in thyroidectomized sheep were partly due to the decrease in phosphorus absorption before and just after feeding which might be induced by the lower endogenous
phosphorus excretion into the gastrointestinal tract. It was possible that the increment of serum phosphorus might promote calcium conservation after feeding because phosphorus was necessary for bone calcification.

When a high calcium diet was given, serum calcium concentrations were increased in thyroidectomized sheep more than in sham-operated ones. It was shown that calcitonin inhibited the increment in serum calcium concentrations which was brought about by the reduction of bone resorption and the stimulation of bone formation.

From these results, it is suggested that calcitonin has 3 roles as following in ruminants at least, 1) antihypercalcemic action in animals given a large amount of calcium, 2) the stimulation of phosphorus excretion via urine, bile and saliva, 3) calcium conservation in the skeletal tissue.
Acknowledgement

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