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PREVENTION OF PARTURIENT PARESIS (MILK FEVER) IN DAIRY COWS

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Prevention of parturient paresis (milk fever) in dairy cows

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GENERAL INTRODUCTION

Calcium homeostasis is a complex endocrinological process involving phosphorus, magnesium, parathyroid hormone, calcitonin, and the vitamin D metabolites. This homeostatic mechanism is capable of maintaining normal blood calcium concentrations under most conditions. However, there are certain pathological conditions in which the homeostatic mechanism is insufficient. One such state is exhibited in the periparturient dairy cow - parturient paresis (milk fever). The outstanding clinical features of this syndrome are severe hypocalcemia and recumbency. Left untreated, the cow becomes comatose and would die.

The incidence of parturient paresis is greatly reduced by feeding cows a low calcium diet the 2 weeks before parturition (Goings et al., 1974). Green et al. (1981) found that low calcium diets act to increase blood concentrations of immunoreactive parathyroid hormone and 1,25-dihydroxyvitamin D. By stimulating production of these homeostatic hormones prior to parturition, the calcium demands associated with the onset of lactation could be adequately met. However, using low calcium diets (< 20 g calcium/day) to prevent parturient paresis has not been universally feasible. Therefore, other methods for preventing parturient paresis have been sought.

Since endogenously-produced parathyroid hormone and 1,25-dihydroxyvitamin D are capable of preventing parturient paresis, perhaps by exogenously supplying the cow with these hormones prior to parturition

the physiologic and clinical benefits of a low calcium diet can be achieved. In these experiments, the ability of vitamin D metabolites and parathyroid hormone to prevent parturient paresis were assessed.

Dissertation Format

This dissertation is presented in the alternate thesis format, which includes three manuscripts. The paper in Section I is coauthored by R. L. Horst, E. T. Littledike, A. Boris, and M. R. Uskokovic, and has been accepted for publication in the Journal of Nutrition. The papers in Sections II and III are coauthored by R. L. Horst and E. T. Littledike. Both are being submitted to the Journal of Dairy Science for publication. A review of the literature precedes the first manuscript. It is primarily concerned with parathyroid hormone. At the end of each section, a separate Literature Cited section has been included to facilitate publication. A general Summary and Discussion follows the final manuscript.

The doctoral candidate, Jesse Paul Goff, was the principal investigator in each of these studies.

LITERATURE REVIEW

Calcium Homeostasis

Intracellular calcium concentration

Free calcium ion concentration within the cytosol of cells is estimated to be between 10^{-7} and 10^{-8} M (Kretsinger, 1979). Under normal conditions, extracellular fluid calcium ion concentration is about 1.2 mM. This means that the extracellular free calcium ion concentration is 10,000 to 100,000 times the intracellular free calcium concentration. Internal calcium ion concentration is precisely controlled since even minor changes can have wide-ranging physiologic, and even pathologic, effects (Carafoli and Penniston, 1985). Calcium ions act as a second messenger, relaying electrical and chemical messages that arrive at the cell surface to the biochemical machinery within the cell. Before the implications of changes in intracellular calcium concentration can be understood, it is necessary to first discuss how very low calcium ion concentrations within the cell can be so precisely maintained against a tremendous concentration gradient. Extracellular calcium ions continually leak into the cytosolic compartment of the cell through calcium channels located within the plasma membrane. There are three mechanisms available for removal of calcium ions from the cytosolic compartment of the cell. Initially, the free calcium ions can become bound to proteins within the cytosol. There are a great number of proteins that are specialized for binding calcium within the cytosol; some of them, e.g., calmodulin, are important as physiologic messengers in addition to

their role in regulating calcium ion concentration (Rasmussen, 1980). The capacity of this mechanism for removal of cytosolic calcium is very low. The second mechanism is capable of moving many calcium ions out of the cytosol. Within the cell membrane there is a protein structure that allows sodium ions outside the cell to be exchanged for calcium ions inside the cell. Three sodium ions are brought into the cell for every calcium ion extruded. The sodium gradient is the driving force for this process. Ultimately, the sodium ions must be actively pumped out of the cell by a membrane-bound Na^{+}-K^{+}-ATPase (Carafoli and Penniston, 1985). Third, calcium ions can be pumped outside the cell or into cell organelles -- in particular, the cisternae of the endoplasmic reticulum (sarcoplasmic reticulum of muscle) and the mitochondria. This is accomplished by a membrane-bound Ca^{++}-Mg^{++}-ATPase (Borle, 1967). As calcium concentrations within the cytosol increase, binding sites on calmodulin become fully occupied and calmodulin changes its configuration. Calcium-activated calmodulin then becomes associated with the Ca^{++}-Mg^{++}ATPase embedded within the membranes, initiating active pumping of calcium from the cytosol (Carafoli and Penniston, 1985).

The mechanisms that remove calcium ions from the cytosol are able to compensate for the normal rate of influx of calcium ions into the cell from the extracellular fluid so that intracellular calcium ion concentration remains unchanged. However, if the rate of entry of calcium into the cell changes, the intracellular calcium ion concentration would be expected to change. In some excitable cells,

chemical or electrical excitation of the plasma membrane induces an increase in the size of the calcium channels, allowing more into the cell. In the case of a muscle cell, this triggers contraction. However, in most of the cells of the body the calcium channels do not appear to be so flexible. Therefore, changes in extracellular calcium ion concentrations play a major role in determining the rate of influx of calcium into a cell (Carafoli and Penniston, 1985). Cytosolic calcium ion concentration in parathyroid gland cells seem to be especially sensitive to changes in extracellular calcium ion concentration.

Parathyroid glands

The parathyroid glands of mammals, birds, and amphibians arise from differentiation of the third and fourth branchial pouches of the developing embryo. In mammals, the pair of parathyroid glands arising from the third branchial pouch tend to migrate with the developing thymus gland. The pair of parathyroid glands arising from the fourth branchial pouch migrate with the thyroid primordium (Roth and Schiller, 1976). In some species, one pair of parathyroid glands fails to develop while in others additional accessory parathyroid tissue can be found (Table 1). Fish apparently do not have a parathyroid gland (Pang, 1973).

The histology of the parathyroid gland is similar in all species. There is species variation in the amount of stromal fat present, vascularity, and amount of fibrous connective tissue, but parathyroid hormone (PTH)-producing chief cells of all species are similar, even

Table 1. Parathyroid gland location in several species

Chicken	- III and IV (often coalesced together) - lie near origin of common carotid arteries - caudal or within thyroid - occasionally have accessory glands
Rat, mouse	- III only - embedded in dorsolateral border of cranial pole of thyroid - occasionally accessory tissue within the thymus
Rabbit	- III-within carotid sheath anywhere from base of tongue to thymus - IV-within thyroid gland
Horse	- III-within fat cranial, dorsal, and lateral to thyroid - IV-near or within thymus at bifurcation of common carotid artery
Cow	- III-within medial surface of thyroid near trachea - IV-near common carotid artery near caudal pole of thyroid along superior thyroid artery. (Calf may surround internal carotid near its origin)
Sheep and goat	- III-within medial surface of thyroid near trachea - IV-near carotid bifurcation, often along submaxillary artery
Pig	- III only - within thymus or attached to common carotid artery
Dog	- III-dorsolateral aspect of thyroid - IV-caudal portion of cranial pole of thyroid - Accessory glands within thymus are common
Man	- III-within inferior pole of thyroid - IV-slightly superior to thyroid where middle thyroid artery crosses recurrent laryngeal nerve

at the electron microscope level (Altenähr, 1972; Capen, 1971). Oxyphil cells are common in some species and are most common in the elderly. Oxyphils are believed to be a degenerated form of the chief cell. They are not capable of synthesizing PTH (Munger and Roth, 1963).

PTH biosynthesis

The mRNA that directs the synthesis of parathyroid hormone codes the production of a 115 amino acid peptide (PRE-PRO-PTH) consisting of an initiator peptide (2 amino acids), a signal sequence (23 amino acids), a pro-component (6 amino acids), and the parathyroid hormone (84 amino acids). The entire 115 amino acid sequence never exists as such within the parathyroid cell because the peptide is cleaved before translation is concluded. The initiator peptide is removed when the nascent chain is only 20-30 amino acids long (Habener, 1981a).

A signal sequence is common to all proteins that are produced by cells for export out of the cell. It facilitates transport of the growing peptide from the ribosome surface into the cisternae of the rough endoplasmic reticulum (Lingappa and Blobel, 1980). The signal sequence is cleaved off the peptide just after translation is completed.

The 90 amino acid peptide remaining (PRO-PTH) is transported to the Golgi apparatus for further processing. There an enzyme cleaves the first six amino acids from the amino terminal end of the peptide, converting it to PTH. PTH-(1-84) then is incorporated into membrane-bound granules where it is stored until secreted. No PRO-PTH is

thought to be incorporated into secretory granules (Habener et al., 1976). Electron microscopists describe two types of secretory granules within parathyroid cells (Roth and Capen, 1974). Type I granules have a large core relative to vesicle diameter. Type II granules are believed to be the storage form of nascent PTH. Type II granules have a smaller core. They are the predominant form seen in animals under hypercalcemic conditions. They contain some acid phosphatase activity indicating the presence of hydrolytic enzymes. Type II granules arise from Type I granules (probably after fusion with lysosomes) and appear to represent an intracellular method for degradation of PTH-(1-84), especially under hypercalcemic conditions (Setoguti et al., 1985a).

Up to 70% of nascent PTH-(1-84) is not incorporated into secretory granules within the Golgi apparatus, but is released into the cytoplasm (Morrissey et al., 1980) where it is vulnerable to proteolysis. Most commonly, cleavage occurs between the 33-34 position, but cleavage can occur at many sites (Hanley et al., 1978). Parathyroid glands also possess cathepsin B, which cleaves PTH-(1-84) between amino acids 36 and 37 and cathepsin D, which cleaves PTH-(1-84) between amino acids 34 and 35 (Cohn and Elting, 1983).

Amino terminal fragments PTH-(1-29) and PTH-(1-34) are produced within the parathyroid cells (Morrissey et al., 1980). However, they apparently undergo rapid intracellular degradation as they cannot be detected in venous effluent from parathyroid glands in vivo (Mayer et

al., 1979) nor can they be detected in media in which parathyroid cells have been cultured in vitro (Morrissey et al., 1980).

A major regulatory site for the production of PTH is DNA synthesis leading to multiplication of parathyroid cells. Chronic hypocalcemia stimulates hyperplasia of the parathyroid glands with concomitant increases in PTH production (Habener, 1981a).

Synthesis of new hormone by the parathyroid cells is apparently continuous and not a site of regulation. Neither transcription nor translation of PTH mRNA is greatly affected by the calcium status of the animal (Habener, 1981b) or by the concentration of cyclic AMP within the parathyroid cell (Morrissey and Cohn, 1979a).

Post-translational intracellular degradation of PTH-(1-84) may be an important means of regulating the production of biologically active PTH within the glands. Intracellular degradation of PTH-(1-84) is stimulated by high extracellular concentrations of calcium, whereas low extracellular calcium concentration inhibits intracellular destruction of PTH (Habener et al., 1975; Chu et al., 1973).

Parathyroid glands also produce and release a glycoprotein with a molecular weight of around 150,000 known as parathyroid secretory protein (Morrissey and Cohn, 1980). Its release from parathyroid cells is governed by the same factors that govern PTH secretion. Its function is unknown. It has been suggested that parathyroid secretory protein is a component of the Golgi vesicle whose extrusion from the parathyroid cells is coincidental to PTH secretion (Habener and Potts, 1979).

Peripheral metabolism of PTH

Circulating immunoreactive PTH consists of a mixture of intact PTH-(1-84) and both carboxy- and amino-terminal fragments of PTH-(1-84) (Berson and Yalow, 1968). Metabolism of intact hormone by peripheral organs contributes greatly to the heterogeneity of the circulating PTH (Canterbury et al., 1975). Intravenously injected radioiodine-labeled intact PTH-(1-84) is rapidly cleaved by peripheral organs, primarily between amino acids 33-34 (Segre et al., 1974) and 36-37 (Segre et al., 1976), of the PTH molecule. Since the biological activity of PTH resides within the first 27 amino acids (Marcus and Aurbach, 1969; Parsons et al., 1973; Treagear et al., 1973), it is likely that cleavage of PTH-(1-84) at these sites can give rise to fragments of PTH that are biologically active. The major organs involved in the peripheral catabolism of PTH are liver, kidney, and bone.

Fang and Tashjian (1972) observed that the rate of disappearance of intact PTH from the plasma was dramatically decreased following partial hepatectomy of rats. Canterbury et al. (1975) found that isolated perfused rat liver was capable of cleaving PTH-(1-84) to two fragments, the amino (NH_2)-terminal fragment, retaining full biological activity (based on rat renal cAMP production), while the carboxy (COOH)-terminal fragment was devoid of activity. They also observed that production of the NH_2-terminal fragment was inversely proportional to the concentration of calcium in the perfusion media. More recent evidence suggests that only the intact hormone is taken up

by the liver, for there is no arterial-venous difference in concentrations of NH_2-terminal or COOH-terminal fragments across the liver in dogs (Martin et al., 1976).

An interesting observation is that the hepatic uptake and catabolism of PTH-(1-84) is markedly reduced in chronic renal failure (Hruska et al., 1981).

The Kupffer cells of the liver seem to be the cells responsible for cleavage of intact PTH (Segre et al., 1981). Kupffer cells are the macrophages of the liver tissue.

The kidney also plays a major role in PTH metabolism. Several laboratories have reported that intact PTH-(1-84) is degraded by kidney tissue in vitro (Chu et al., 1975; Martin et al., 1977). In studies of isolated perfused kidney of dogs, it was found that the rate of degradation of PTH was accelerated in the presence of hypocalcemia and retarded in the presence of elevated calcium concentration in the perfusate. Both COOH-terminal and NH_2-terminal PTH fragments are produced and released into the circulation by the kidney.

Nephrectomy leads to greatly diminished metabolic clearance rate of PTH and PTH fragments (Hruska et al., 1981; Martin et al., 1977). The studies of Freitag et al. (1978), Kau and Maack (1977), and Martin et al. (1977) were instrumental in the development of the following model for renal clearance of PTH as described in a review article by Slatopolsky et al. (1981). All forms of PTH undergo glomerular filtration and tubular reabsorption. The biologically active forms of PTH (intact PTH-(1-84) and NH_2-terminal fragments) are removed by both

glomerular filtration and by uptake from peritubular capillaries followed by degradation within renal tubular epithelium. Biologically inactive COOH-terminal fragments can only be cleared by glomerular filtration. When glomerular filtration rate is decreased, as it is in patients with chronic renal failure, the clearance rate of all PTH fragments is greatly decreased. Because NH_2-terminal PTH can still be removed by tubular cell uptake from peritubular capillaries, the relative amount of COOH-terminal PTH in plasma rises faster than does NH_2-terminal PTH. These COOH-terminal fragments are biologically inactive in terms of calcium homeostasis, but recent reports indicate these COOH-terminal fragments, along with the NH_2-terminal fragments, are potentially toxic in patients with renal failure. Slatopolsky et al. (1980) have reviewed some of these effects that include osteitis fibrosa, encephalopathy, neuropathy due to brain tissue calcification, glucose intolerance, acidosis, hyperlipidemia, and anemia.

The metabolism of PTH in bone is currently a subject of considerable controversy. Many laboratories report that intact PTH-(1-84) is removed from the circulation by bone and stimulates resorption of bone calcium (Calvo et al., 1985; Goltzman, 1978; Peck et al., 1973). However, there is a great deal of evidence that indicates that intact PTH must be cleaved to an NH_2-terminal fragment for complete activity in bone. Parsons and Robinson (1968) found that perfusion of bPTH-(1-84) into the isolated feline tibia nutrient artery had little effect on release of calcium from the bone. However, if the bPTH-(1-84) was first circulated through the whole body prior to infusion,

it greatly stimulated calcium release from the bone. Martin et al. (1978) reported that isolated perfused canine tibia selectively takes up bPTH-(1-34) but not bPTH-(1-84). They also observed that only bPTH-(1-34) would markedly stimulate release of cAMP from the perfused tibia. Recently, Sugimoto et al. (1985) developed a system for perfusion of isolated rat femora. They conclude that hPTH-(1-34) is capable of stimulating cAMP release from bone and that hPTH-(1-84) is not.

Most of the work that ascribes PTH-(1-84) full biologic activity in bone has been done using fetal rat calvaria. There is little doubt that PTH-(1-84) is capable of stimulating adenylate cyclase in these cells in culture. However, Freitag et al. (1979) suggest that fetal rat calvaria first catabolizes PTH-(1-84) to NH_2-terminal fragments which then activate adenylate cyclase of the cultured cells. Adult bone tissue does not seem to be capable of catabolizing intact PTH-(1-84) (Martin et al., 1978). Therefore, a tissue-age effect may account for some of the differences in the activity ascribed to PTH-(1-84). Calvo et al. (1985) reported that PTH-(1-84) was fully capable of stimulating skeletal release of cAMP when administered to perfused mature rat hindquarters. The model excluded the possibility of PTH catabolism by visceral organs but maintained the bone in situ. These results conflict with those of Sugimoto et al. (1985). The only difference between the two experiments was the presence of muscle and skin in the preparation of Calvo et al. (1985). Perhaps the PTH-(1-84) was catabolized by this extra-osseous tissue.

The relative contribution of bone, liver, and kidney to the metabolic clearance of PTH-(1-84) has been calculated in adult dogs by determining arteriovenous differences of immunoreactive bPTH-(1-84) across these organs. Slatopolsky et al. (1981) found that 56% of PTH-(1-84) was "cleared" from the blood by the liver, 33% was cleared by the kidneys, and bone cleared less than 5% of the PTH-(1-84), leaving 6% unaccounted for.

If PTH-(1-84) requires extraskeletal cleavage to achieve complete activity in bone, the peripheral catabolism of PTH-(1-84) secreted by the parathyroid glands may represent an activating step rather than simply a degradative step. Regulation of liver and kidney metabolism of PTH may significantly affect the circulating levels of NH_2-terminal PTH fragments and, hence, the effects of PTH at the level of bone.

PTH Secretion

The cells of the parathyroid gland are continuously engaged in the biosynthesis, partial degradation, and secretion of PTH. Little active product is stored within the cells, in contrast to other peptide-secreting endocrine organs. For instance, pancreatic cells contain enough insulin to satisfy normal needs for several days, whereas PTH reserves do not support normal requirements for more than 6-7 hours (Martin, 1985).

The parathyroid chief cells never totally shut down secretion of PTH. Individual chief cells seem to maintain a basal level of PTH secretion even if exposed to the suppressive effect of a sustained

hypercalcemia (Mayer et al., 1979). In the event of sustained hypercalcemia, the only way to decrease PTH concentration in the blood is to decrease the amount of parathyroid tissue (Habener, 1981a; Mayer et al., 1976).

Studies on the effects of different secretagogues on parathyroid cells indicate two intracellular pools of PTH are available for secretion (Morrissey and Cohn, 1979b). One pool contains newly synthesized hormone, whereas the other contains older stored PTH (Morrissey and Cohn, 1979b) which may very well correspond to the two types of secretory granules that have been observed within parathyroid cells (Setoguti et al., 1985a).

There are at least two major pathways by which PTH secretion is stimulated. The first pathway is mediated by cytosolic calcium ion concentration. Early reports indicated that a decrease in cytosolic calcium ion concentration stimulates release of PTH from both the newly synthesized and the older pool of PTH (Morrissey and Cohn, 1979b). However, it now appears that low calcium concentration has little or no effect on release of the older storage granule pool of PTH (Hanley and Wellings, 1985). The second pathway is via changes in intracellular cAMP concentrations. Increased cAMP concentrations result in secretion of PTH from the older pool of PTH only (Morrissey and Cohn, 1979b). Two cAMP-dependent protein kinases have been identified within the parathyroid cells which are believed to play a role in PTH secretion (Lasker and Spiegel, 1982), but their exact function is unknown.

Parathyroid hormone is released from the cell by exocytosis. Shortly after secretion of PTH, the secretory granule membranes are retrieved by endocytosis, presumably for recycling (Wild et al., 1985).

Factors Affecting PTH Secretion

Calcium

Extracellular fluid calcium ion concentration is the major regulator of PTH secretion. Hypercalcemia inhibits, while hypocalcemia rapidly stimulates PTH release. Initially, it was felt that there was a linear relationship between PTH secretion and extracellular calcium concentration (Sherwood et al., 1968). Subsequently, a sigmoidal relationship was shown to exist between PTH release and extracellular calcium concentration (Brown, 1983; Mayer and Hurst, 1978). This relationship results from the fact that a certain degree of PTH secretion is nonsuppressible; i.e., no matter how high the extracellular calcium concentration, the chief cells continue to secrete a low basal level of PTH. Also, at some low calcium concentration, the chief cells are maximally stimulated. Further reduction in calcium does not result in increased secretion of PTH. There also seem to be individual differences in the calcium set-point to which the glands respond. The set-point for a parathyroid gland is defined as the calcium concentration at which PTH secretion is 50% of maximal secretion. Often humans suffering from hyperparathyroidism do not have a neoplastic parathyroid gland. Instead, recent research

indicates that these patients simply have parathyroid tissue with a higher set-point for calcium than normal. At a blood calcium concentration of 10 mg/dl, a normal parathyroid gland would only be secreting PTH at a rate slightly greater than the basal secretion rate. However, glands of a patient diagnosed with hyperparathyroidism will often secrete PTH at a rate well above the basal rate at this same calcium concentration (Brown, 1983; Insogna et al., 1985). Conversely, hypoparathyroidism may in some cases be due to parathyroid glands with a low set-point for calcium.

Neonatal calf parathyroid tissues have a higher set-point for calcium than do parathyroids of adult cows (Brown, 1981; Keaton et al., 1978). Calves also maintain higher plasma calcium concentrations than do adult cows (Goff et al., 1982). Heifers almost never develop severe hypocalcemia at calving despite the fact that some heifers produce more milk than older cows that do develop hypocalcemia and paresis. These facts raise the following questions concerning the pathogenesis of parturient paresis. Does the calcium set-point for PTH secretion decline continuously throughout the life of a cow? If so, does the decreased calcium set-point leave the older cow in a somewhat hypoparathyroid state? While the heifer may begin secreting PTH when her blood calcium concentration falls below 9 mg/dl, perhaps the older cow does not begin to secrete PTH until her plasma calcium concentration is below 8 mg/dl. This could cause a delay in activation of those calcium homeostatic mechanisms necessary to prevent development of severe hypocalcemia.

Magnesium

Magnesium and calcium are equipotent inhibitors of PTH secretion in vitro (Targovik et al., 1971). Although high levels of magnesium inhibit PTH secretion, a certain level of magnesium must be present to allow PTH secretion (Anast et al., 1972; Buckle et al., 1968). Although the effects of hypermagnesemia on PTH secretion are rarely of clinical importance, the effects of hypomagnesemia have been implicated in the development of several syndromes, including grass tetany of cattle. Grass tetany is a disorder in which clinical signs are associated with the rapid development of severe hypocalcemia following a prolonged period of hypomagnesemia. Littledike et al. (1983) observed inappropriately low PTH concentrations in the blood of cattle with grass tetany. They concluded that magnesium deficiency prevents PTH secretion which then leads to the development of hypocalcemia and clinical signs.

Sodium and potassium

Parathyroid cells utilize Na^+/Ca^{++} exchange mechanisms to remove excessive numbers of calcium ions (Rothstein et al., 1982). Low extracellular Na^+ and Na^+/K^+-ATPase inhibitors (such as ouabain and monensin) impair operation of this system. As a result, calcium ions accumulate in the cytosol-inhibiting PTH secretion. High extracellular levels of K^+ may enhance PTH secretion by stimulating the Na^+-K^+-ATPase pump.

Phosphate

Phosphate was once thought to stimulate PTH secretion. Acute intravenous phosphate infusion results in a gradual increase in PTH secretion. However, phosphate infusion also causes a decrease in plasma calcium concentration, which is the true stimulus for PTH secretion since phosphorus infusion does not stimulate PTH secretion if normocalcemia is maintained by simultaneous infusion of calcium (Sherwood et al., 1968). Chronic hyperphosphatemia (as in renal failure) is also associated with high plasma PTH concentrations but, again, release of PTH is believed to be secondary to the hypocalcemia associated with hyperphosphatemia.

The mechanism by which acute intravenous phosphate infusion causes hypocalcemia remains a subject of debate. Hebert et al. (1966) felt that the degree of hypocalcemia was proportional to the magnitude by which the solubility product for $CaHPO_4$ was exceeded. Markedly elevated levels of phosphorus undoubtedly do cause precipitation of $CaHPO_4$ salts on body surfaces (Carey et al., 1968; Craig, 1959). There are other observations that do not support the solubility product hypothesis to explain the hypocalcemia associated with phosphate infusion. Goldsmith and Ingbar (1966) infused phosphate into a patient with hypercalcemia due to malignancy (mammary carcinoma). Plasma calcium concentration decreased from 18 mg/dl to 8 mg/dl within 4 days when infusion was ended. Plasma phosphate then rapidly returned to normal. However, calcium concentration did not begin to increase for another 11 days. If calcium had simply been deposited

extracellularly on body tissues, it should have returned into the plasma compartment once phosphate concentration had returned to normal. There are probably several mechanisms involved in the effect of phosphate on plasma calcium. First, the phosphate ion ties up some of the free calcium in the blood. The reduction in ionized calcium then stimulates PTH secretion. Reiss et al. (1970) found that total calcium, as well as ionized calcium, decreased during phosphate infusion. Other work indicates that phosphate infusion stimulates bone formation by causing alterations in the concentration of calcium ion within bone-forming cells (Rasmussen, 1971) or through its reported regulatory role in mineralization (Glimcher and Krane, 1968). There has been little recent work on this subject - despite the fact that phosphate is commonly employed to treat hypercalcemia of malignancy and hyperparathyroidism (Scolding, 1985).

Chronic oral phosphate administration or hyperphosphatemia also results in hypocalcemia. The major mechanism is inhibition of renal 25-hydroxycholecalciferol-1α-hydroxylase (Gray et al., 1977). The product of this enzyme, 1,25-dihydroxyvitamin D, is vital to maintenance of normal plasma calcium concentration, primarily through its effects on intestinal calcium absorption.

Factors that act through cAMP

Epinephrine, dopamine, isoproterenol, and other β-2-adrenergic agonists increase PTH secretion through their activation of membrane-bound adenylate cyclase (Blum et al., 1980). Norepinephrine and α-adrenergic agonists depress cAMP levels and PTH release (Brown et

al., 1978). Prostaglandin E_2 and secretin also stimulate PTH release by activating adenylate cyclase, but this action is independent of adrenergic receptors (Habener, 1981a). Prostaglandin $F_2\alpha$ lowers adenylate cyclase activity and also activates cyclic nucleotide phosphodiesterase to decrease cAMP concentration and reduce PTH secretion (Gardner, 1980). Theophylline and other phosphodiesterase inhibitors retard cAMP degradation and therefore enhance PTH secretion (Habener, 1981a).

Elevated cAMP concentrations stimulate release of PTH from the older stored pool of PTH. Because the size of this pool is small, cAMP-mediated release of PTH is transient and of short duration (Hanley and Wellings, 1985). High plasma calcium concentration can prevent activation of adenylate cyclase, but low calcium concentrations do not stimulate adenylate cyclase in the chief cells (Hanley and Wellings, 1985). High cytosolic calcium concentration leads to formation of calcium-calmodulin complexes that bind to and inhibit adenylate cyclase and also stimulate activity of phosphodiesterase (Peck and Klahr, 1975).

The parathyroid glands are innervated by vagal and sympathetic fibers; however, their physiologic significance is unknown (Metz et al., 1978). Some species exhibit diurnal PTH secretory rhythms that may be controlled by these nerves (Fischer et al., 1982). Setoguti et al. (1985b) have shown that vagal stimulation (parasympathetic) leads to increased conversion of Type I secretory granules to Type II secretory granules.

Miscellaneous factors

In vitro studies of cultured parathyroid gland cells have shown that 1,25-dihydroxyvitamin D directly inhibits PTH secretion (Chertow et al., 1975). Cantley et al. (1985) have indicated that 1,25-dihydroxyvitamin D suppresses transcription of pre-pro-PTH mRNA in chief cells. Chan et al. (1986) have shown this effect is independent of an increase in extracellular calcium concentration.

The inhibitory effects of 1,25-dihydroxyvitamin D on PTH secretion are not seen in hypocalcemic animals (Seshadri et al., 1985). Dietel et al. (1979) found that 1,25-dihydroxyvitamin D also decreased cAMP production within chief cells, which also depresses PTH secretion. Since one of the functions of PTH is to stimulate renal production of 1,25-dihydroxyvitamin D, inhibition of PTH secretion by 1,25-dihydroxyvitamin D may represent a negative feedback mechanism to control 1,25-dihydroxyvitamin D synthesis. Vitamin D_3 and 24,25-dihydroxyvitamin D_3 had no inhibitory effects on PTH secretion (Dietel et al., 1979).

Vitamin A (retinol) is capable of stimulating PTH secretion in vitro and in vivo (Chertow et al., 1977). Retinoic acid has no effect on PTH secretion. Chertow et al. (1977) felt that vitamin A acts by altering the structure of the PTH secretory granule membrane facilitating fusion of the secretory granule with the cell membrane. The membrane stabilizers, such as hydrocortisone and vitamin E, antagonize the effects of vitamin A on chief cells, supporting the theory that vitamin A acts on secretory granule membranes. Although the

hypercalcemia seen in vitamin A intoxicosis is primarily due to direct effects of vitamin A on bone resorption, it is possible that increased PTH secretion may also contribute.

Physiologic Actions of PTH

Effects on the kidney

Parathyroid hormone has major physiologic effects on renal excretion of minerals and on renal conversion of vitamin D to a hormone.

Phosphate excretion When PTH is administered to a parathyroidectomized rat, it results in rapid urinary excretion of phosphorus. Normally, about 70-80% of filtered phosphate is reabsorbed in the proximal convoluted tubule, a small amount (< 5%) is absorbed in the loop of Henle, and the rest is excreted. During phosphorus deprivation, almost all the phosphate is reabsorbed (Martin, 1985). Parathyroidectomized animals excrete less phosphate than normal animals. Parathyroid hormone acts tonically on the kidney to maintain a basal level of phosphate excretion. Parathyroid hormone primarily inhibits proximal tubule reabsorption of phosphorus (Talmage and Kraintz, 1954). This process is mediated by cAMP generated as a result of PTH stimulation of adenylate cyclase (Chase and Aurbach, 1968). The cAMP activates cytosolic and brush border protein kinase (Sacktor et al., 1977). Kinne and Schwartz (1978) postulate that stimulation of the protein kinase results in phosphorylation of a specific phosphate carrier protein which regulates the rate of uptake

of inorganic phosphate from the urinary space into the cell. Phosphorylation results in inhibition of phosphate reabsorption.

Kempson et al. (1981) found that NAD^+ inhibits sodium-dependent uptake of phosphorus by tubule brush border membranes. They propose that PTH, acting via cAMP, stimulates gluconeogenesis which increases the NAD^+/NADH ratio. The change in the ratio of NAD^+/NADH inhibits phosphate reabsorption. The activity of alkaline phosphatase, a renal tubular brush border enzyme, is highly correlated with the rate of tubule phosphate reabsorption (Kempson et al., 1979). However, substances which block the activity of alkaline phosphatase do not impair renal reabsorption of phosphorus (Shirazi et al., 1981). Therefore, alkaline phosphatase probably plays no role in phosphate transport.

The phosphaturic response to exogenous PTH is impaired in several conditions, including vitamin D deficiency (Forte et al., 1976), metabolic acidosis (Beck et al., 1975), and phosphate deprivation (Steele et al., 1976). The mechanism is unknown. It is known that, at least in phosphate deprivation, PTH continues to stimulate cAMP production (Bonjour and Fleisch, 1980). Bellorin-Font et al. (1985) present evidence that in metabolic acidosis the coupling of the components of adenylate cyclase is impaired.

Calcium excretion About 56% of blood calcium is not bound to plasma proteins. This fraction is able to cross the glomerulus and enter the urinary space. Under normal conditions, 50-55% of the filtered load is reabsorbed within the proximal tubule, 30% in the

loop of Henle, and most of the remainder is reabsorbed in the more distal segments of the nephron. Usually less than 1% of the filtered calcium actually ends up in the urine (Sutton and Dirks, 1978). For a human this amounts to about 100 mg calcium per day. Parathyroidectomy leads to increased loss of calcium in the urine, even if plasma calcium is subnormal. Parathyroid hormone decreases urinary calcium loss primarily by increasing calcium reabsorption within the ascending sections of the cortical loops of Henle with lesser effects on the distal convoluted tubules. Parathyroid hormone has no effect on calcium reabsorption in the medullary ascending loops of Henle. In the proximal convoluted tubules, PTH actually decreases calcium reabsorption slightly (Bengele et al., 1980; Bourdeau and Burg, 1980). Calcium reabsorption from the proximal tubule is dependent on co-transport of sodium. Na^+-K^+-ATPase inhibitors block the reabsorption of both ions. More distally, the calcium reabsorptive mechanism is independent and separate from sodium transport (Goldberg et al., 1976). The effects of PTH on calcium reabsorption are primarily mediated by cAMP. When dibutyryl cAMP, an analog of cAMP, is administered to dogs there is an increase in calcium reabsorption in the ascending limb of the loop of Henle and a decrease in calcium reabsorption in the proximal tubule (Sutton et al., 1976).

Parathyroid hormone stimulates synthesis of acidic phospholipids within the renal cortex (Farese et al., 1980). These acid phospholipids (phosphatidic acid and polyphosphoinositides) increase divalent cation partitioning into lipid phases (Green et al., 1980). An

increase in the amount of these acid phospholipids in brush border membrane vesicles results in an increase in calcium uptake (Humes et al., 1981), probably through their action as calcium ionophores within the renal tubule membrane.

Magnesium excretion Administration of exogenous PTH extract to man results in renal conservation of magnesium. Parathyroid hormone has no effect on proximal tubule handling of magnesium. It does increase magnesium transport within the ascending limb of the loop of Henle (Shareghi and Agus, 1979). It is unclear whether the mechanisms for reabsorption of magnesium and calcium are separate or identical.

Excretion of other substances Parathyroid hormone causes a decrease in the amount of bicarbonate ion reabsorbed within the proximal tubule (Arruda et al., 1977). This effect is not mediated by inhibition of carbonic anhydrase (Garg, 1975). Loss of bicarbonate contributes to the metabolic acidosis that is a common finding in hyperparathyroid people (Slatopolsky et al., 1981).

Parathyroid hormone decreases proximal tubule reabsorption of sodium. However, little natriuresis is seen because most of the sodium is reabsorbed by more distal segments of the nephron (Wen, 1974). In man, PTH increases the tubular maximum for glucose transport and increases amino acid excretion (Slatopolsky et al., 1981).

Renal production of 1,25-dihydroxyvitamin D Renal conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D is accomplished by renal 25-hydroxycholecalciferol-1α-hydroxylase. The activity of this

enzyme is closely regulated by PTH and/or plasma calcium concentration. Parathyroidectomy results in decreased production of 1,25-dihydroxyvitamin D (Garabedian et al., 1972). Hyperparathyroidism generally results in excessive production of 1,25-dihydroxyvitamin D (Broadus et al., 1983). Exogenous PTH stimulates 1,25-dihydroxyvitamin D production in vitro (Garabedian et al., 1972; Kremer and Goltzman, 1982) and in vivo (Aksnes and Aarskog, 1980).

Formation and resorption of bone

This section of the Literature Review is intended to present a brief overview of bone dynamics. Reviews by Jaworski (1984), Parfitt (1984), Raisz and Kream (1981), Recker (1983), and Rodan and Martin (1981) present current concepts on bone formation and remodeling in greater depth.

In the young animal and in small mammals of short life span, bone is in a state of growth. Growth occurs through a process of modeling (sometimes referred to as external remodeling) in three areas of the bone: the physis, the periosteum, and the endosteum. When bone from a young animal is observed under the microscope, one finds that nearly all free bone surfaces are actively engaged in either bone formation or bone resorption (Parfitt, 1984). Growth in length of a bone is achieved through growth of a cartilagenous model within the physis. First, chondrocytes proliferate and differentiate, then begin to secrete cartilage matrix (Type II collagen). The cartilage cells undergo hypertrophy and the cartilage matrix becomes mineralized.

Blood vessels from the metaphysis invade the calcified cartilage, bringing in osteogenic cells which produce woven bone. The new woven bone is resorbed by osteoclasts and the woven bone is replaced by lamellar bone (Type I collagen) formed by osteoblasts. Increase in width of a long bone occurs by the removal of bone by osteoclasts on endosteal surfaces and formation of bone on periosteal surfaces by osteoblasts.

In the adult stage of large long-lived animals, bone ceases growth in length and width. However, the bone tissue continues to be slowly resorbed and replaced by a process known as remodeling (internal remodeling). This process is distinct from modeling. In remodeling, old bone is replaced by new bone; in modeling, new bone is added to the old bone in the growth process or as a reaction to mechanical stress, such as the increase in bone width that occurs with exercise. Modeling can lead to loss of bone from old surfaces in response to changes in mechanical stress also (such as disuse).

As bone tissue ages, it becomes more mineral dense. It is more brittle than young bone and the calcium within the bone is bound more tightly to the collagen, making it less available to meet calcium homeostasis needs. Remodeling is a bone replacement mechanism which functions to prevent the accumulation of fatigue damage in the skeleton and to maintain an adequate supply of young bone of relatively low mineral density to subserve mineral homeostasis. When adult bones are observed histologically, only about 20% of the trabecular (cancellous) bone surface and about 5% of the intracortical bone surface are active

with respect to bone remodeling (Parfitt, 1984). The next sections of this review will focus on the mechanisms of bone resorption and formation during the remodeling processes in trabecular and cortical bone.

Cancellous bone remodeling The normal sequence of events during remodeling begins with activation of a quiescent site on the trabecular surface of cancellous bone. In the quiescent stage, trabeculae are lined by endosteal lining cells. They probably are deactivated osteoblasts (Parfitt, 1984; Rodan and Martin, 1981). They differ from active osteoblasts in that they are flattened and appear to have lost their ability to synthesize collagen (Parfitt, 1984). Initiating remodeling requires the recruitment of osteoclasts, a means for them to gain access to the bone, and a mechanism for their attachment to the bone surface. The site chosen for bone remodeling seems to be random, although focal structural damage is also a likely initiator of remodeling. The events involved in activation of remodeling are not well understood. They will be discussed later in the review.

Once the osteoclasts have made contact with the bone, they begin to erode a cavity within the bone. This cavity is known as a Howship's lacuna. Generally, the mean depth of the lacuna is 60 μm from the surface of the trabecula. The area covered by the lacuna seems to be dependent on the number of nuclei incorporated into the osteoclasts. In trabecular bone, osteoclasts rapidly erode the first two-thirds of the lacuna cavity. They then disperse (or die) and the

remainder of the cavity is eroded slowly by extracellular enzyme products left by the osteoclasts and by mononuclear cells that have followed the osteoclasts into the cavity (Ericksen et al., 1983). The mononuclear cells could be bone-resorbing monocytes or, more likely, they could be mononuclear osteoclast precursors that have failed to fuse with the osteoclast.

How the depth of the lacuna is determined is unknown. Several possible controls have been postulated. The simplest explanation is that osteoclastic activity ceases when the nuclei within the osteoclast live out their life span and no new nuclei are recruited. However, recently prostacyclin has been shown to inhibit osteoclast motility and bone resorption (Parfitt, 1984). Osteoblasts have been implicated as cells that might produce prostacyclin to modulate osteoclast activity.

While the osteoclasts are burrowing into the trabecular surface, the process of bone formation is beginning to fill in the space created by the osteoclasts. First, mononuclear cells (which may be pre-osteoclasts or pre-osteoblasts) clean up cellular debris left by the osteoclasts. They smooth the lacuna surface and then secrete a thin layer of highly mineralized, but collagen-poor, bone matrix (cement substance) which prepares the surface for bone formation. Osteoblast precursors then begin to multiply along the cement line. The stimulus for this division is unknown. Local bone growth factors, such as skeletal growth factor, are believed to play a role (Farley et al., 1982). Plump, round osteoblasts eventually line the surface of

the cement substance and begin to secrete collagen and the other components of bone matrix. If there is an adequate supply of calcium and phosphate present, mineralization of the matrix occurs once the matrix has matured. Details of the mineralization process are controversial and will not be discussed here.

During active formation of bone, the osteoblasts are separated from the mineralized matrix by unmineralized matrix (osteoid). This osteoid seam eventually disappears as mineralization catches up with the osteoblast once the lacuna has been filled with osteoid. [In osteomalacia (adult rickets), much of the osteoid never is mineralized due to lack of calcium, phosphate, and/or vitamin D.] As the matrix surrounding the nonmotile osteoblast becomes mineralized, the osteoblast is converted to an osteocyte. As bone formation proceeds, eventually the lacuna is entirely filled with bone. Once level with the original trabecular surface, the osteoblasts revert to a quiescent flattened form that lines the trabecular surface. Some authors refer to them as "lining cells" at this stage. Once again, they are inactive until bone remodeling is activated in that site. In the human, this occurs about every 7 years (Martin, 1985). The extent to which the lacunae are refilled is probably more highly dependent on the number of osteoblasts recruited into the area than on the biosynthetic activity of an individual osteoblast (Parfitt, 1984). Hormonal agents that regulate bone formation probably act more on osteoblast recruitment than on osteoblast function (Jaworski, 1984). This has

especially important implications for bone disease states such as osteoporosis.

Cortical bone remodeling Lamellar cortical bone is found under the periosteum and articular surfaces of all bones. It is very dense and compact. In many respects, remodeling of lamellar bone is similar to remodeling of trabecular bone. Both require some activation factor that causes quiescent bone surface to become active. In both cases, old bone is attacked by osteoclasts and replaced by osteoblasts. The net result is replacement of old bone with new, more structurally sound bone in both areas.

However, there are important differences. In trabecular bone, the endosteum is adjacent to the hematopoietic system which serves as a source of pre-osteoclasts and monocytes. To begin remodeling of periosteal or articular surfaces of lamellar bone, the pre-osteoclasts and monocytes must move to the activation site via the capillaries. Once at the site of remodeling, the osteoclastic precursors fuse into giant multinucleated osteoclasts and begin resorbing bone. This resorbing process is similar to that in trabecular bone except that on average the depth of the lacunae "drilled out" by the osteoclasts is deeper (100 μm) and more cylindrical in shape. In lamellar bone, these lacunae outline the Haversian system that is easily recognized in histologic sections of bone. As the osteoclasts drill into the bone, bone-lining cells and mononuclear cells migrate into the lacuna along the bone just behind the osteoclasts, just as in trabecular bone. The mononuclear cells may play an important role in regulating

the osteoclastic activity as well. Mundy (1983) lists possible mechanisms by which the mononuclear cells may affect bone resorption. These include production of prostaglandin E_2, osteoclast-activating factor (OAF), and interleukin I which act to stimulate bone resorption. In addition, the mononuclear cells may be precursors for the osteoclasts.

As the bone-lining cells follow the osteoclasts, they lay down cement substance along the surface of the lacunae. Then some of them differentiate into osteoblasts which begin to secrete collagen and form bone at a right angle to the direction in which the osteoclasts have "drilled." The lacunae then is filled in from the outer wall towards the center (Jaworski, 1984). An analogy is to think of drilling a hole into a piece of wood, then having the wood grow back into the hole from the new surface created by the drill toward the center of the hole.

Hormonal control of bone resorption

A number of hormones are known to stimulate calcium release from bone both *in vitro* and *in vivo*. These bone-resorbing hormones include PTH, 1,25-dihydroxyvitamin D, and prostaglandins. One might expect that these substances would act directly on osteoclasts to stimulate bone-resorbing activity. However, no one has been able to demonstrate receptors for PTH or 1,25-dihydroxyvitamin D in osteoclasts. Chambers *et al.* (1985) have found that the direct effect of prostaglandin E_1 is to actually decrease bone resorption by osteoclasts.

Parathyroid hormone and 1,25-dihydroxyvitamin D receptors have been demonstrated in osteoblast cells of bone (Rodan and Martin, 1981). Activation of PTH receptors on osteoblasts results in stimulation of adenylate cyclase activity, resulting in a cAMP surge, rapid activation of cAMP-dependent protein kinase, inhibition of collagen synthesis, inhibition of alkaline phosphatase activity, stimulation of calcium uptake, and production of changes in cell shape of the osteoblast. The osteoblastic response to 1,25-dihydroxyvitamin D is transcriptional inhibition of collagen synthesis and decreased alkaline phosphatase activity. In addition, 1,25-dihydroxyvitamin D can inhibit the replication of bone cells under some conditions (Raisz, 1984).

It is difficult to understand how these hormones act. In the case of 1,25-dihydroxyvitamin D, it is well documented that it is necessary for normal bone growth, yet it inhibits bone collagen synthesis in vitro. Raisz (1984) attempts to reconcile these incongruities. He feels that 1,25-dihydroxyvitamin D acts as a bone growth factor only at low concentrations, when calcium and phosphorus are abundant. Under these circumstances, it permits growth. When calcium and phosphate are in short supply, the synthesis of 1,25-dihydroxyvitamin D increases greatly, blocking osteoblast synthesis of bone collagen - which probably could not calcify properly until blood calcium and phosphate had returned to normal anyway.

Rodan and Martin (1981) proposed the following hypothesis to explain how hormones that act directly on osteoblasts can stimulate

bone resorption by osteoclasts. According to this hypothesis, in quiescent bone inactive osteoclasts are separated from the bone by a contiguous layer of inactive osteoblasts. These osteoblasts are flattened and completely cover the surface of the bone. Bone-resorbing hormonal agents, such as PTH and some prostaglandins, induce (via cAMP or calcium flux) a shape change in the osteoblasts. As the osteoblasts retract, the matrix beneath them is uncovered, exposing it to osteoclasts or osteoclast projections. The idle osteoclast is stimulated to begin resorbing bone simply by exposure to bone matrix. The resulting matrix digestion further enhances resorption by releasing collagen and osteocalcin locally which attracts monocytic osteoclast precursors and idle osteoclasts to the site. A second aspect is the direct activation of osteoclasts by products of hormone action on osteoblasts. Osteoblasts may produce osteocalcin or prostaglandins that contribute to the activation of the osteoclasts. Activity of the osteoclasts ceases when the osteoblasts are no longer stimulated by the bone-resorbing hormones or by the direct action of calcitonin on the osteoclasts. Calcitonin receptors have been demonstrated on osteoclasts and their activation increases adenylate cyclase activity within the osteoclasts and decreases bone resorption mechanisms. Although many aspects of this hypothesis remain to be tested, it does seem to be the best hypothesis based on the information at hand.

SECTION I.

BONE RESORPTION, RENAL FUNCTION, AND MINERAL STATUS IN COWS TREATED WITH 1,25-DIHYDROXYCHOLECALCIFEROL AND ITS 24-FLUORO ANALOGUES

INTRODUCTION

Parturient paresis is a syndrome seen in dairy cows that develop severe hypocalcemia at the onset of lactation. Nonparetic cows meet the periparturient calcium demand by adequately increasing the amount of calcium entering the blood from the diet and from bone. Paretic cows do not. Both groups of cows respond to the calcium drain with increased parathyroid hormone (PTH) (Mayer et al., 1969) and 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] (Horst et al., 1978). However, in the paretic cows this homeostatic response does not activate bone resorptive and intestinal calcium absorption mechanisms in time to meet the lactational drain of calcium, possibly because of delayed target organ responsiveness. To effectively prevent parturient paresis, bone resorption and intestinal absorption of calcium and inorganic phosphorus should be stimulated before "paresis-prone" cows become hypocalcemic. This can be done by feeding low calcium diets prior to parturition (Goings et al., 1974; Green et al., 1981), but the management required to maintain cows on these diets has proven impractical for many producers.

The use of the vitamin D family of compounds in the prophylaxis of parturient paresis has been of interest for some time. The prophylactic regimes using the parent compound, vitamin D, require the use of near-toxic doses of vitamin D ($10-20 \times 10^6$ IU), and are only effective when given within a precise time period prior to parturition (Hibbs and Pounden, 1955; Julien et al., 1977; Littledike and Horst,

1982; Manston and Payne, 1964). When relatively nontoxic doses of vitamin D are used (2.5-5 x 10^6 IU), they are found to be ineffective and actually increase the incidence and severity of milk fever (Littledike and Horst, 1979). The elucidation of the vitamin D endocrine system and the availability of potent vitamin D metabolites renewed the search for an agent capable of preventing parturient paresis. 25-Hydroxycholecalciferol (Jorgensen et al., 1978; Olson et al., 1972), 1α-OHD_3 (Barlet, 1977; Sachs et al., 1977; Sansom et al., 1976; Wittwer and Ford, 1978), and 1,25-$(OH)_2D_3$ (Gast et al., 1979; Hoffsis et al., 1978) have been used with some success. These compounds have the advantage of a shorter biological life than vitamin D; therefore, toxicity problems are reduced. However, the shorter biological life also requires a more accurate prediction of the time of parturition for full effectiveness. A synthetic vitamin D metabolite with a longer biologic activity than 1,25-$(OH)_2D_3$ could better accommodate inaccuracies in predicting the time of parturition, while compromising only slightly on possible toxicities.

Several workers have shown that 24,24-F_2-1,25-$(OH)_2D_3$ has greater biologic activity than 1,25-$(OH)_2D_3$ in rats (Okamoto et al., 1983) and chicks (Corradino et al., 1980). The biologic activity of 24-F-1,25-$(OH)_2D_3$ has not been determined for any species, but, based on its similarity to 24,24-F_2-1,25-$(OH)_2D_3$ it might also be expected to be more potent than 1,25-$(OH)_2D_3$. The present report demonstrates the relative potencies of 1,25-$(OH)_2D_3$, 24-F-1,25-$(OH)_2D_3$, and 24,24-F_2-1,25-$(OH)_2D_3$ in nonlactating, nonpregnant adult dairy cows by

comparing the effects on plasma mineral concentrations, bone resorption, and renal excretion of calcium and phosphorus. In addition, we present evidence that renal function is impaired following administration of these compounds.

MATERIALS AND METHODS

Vitamin D Compounds

The 1,25-$(OH)_2D_3$, 24-F-1,25-$(OH)_2D_3$, and 24,24-F_2-1,25-$(OH)_2D_3$ were supplied from Hoffmann-La Roche, Inc., Nutley, NJ.

Animals

Mature (≥ 3 years of age) nonlactating, nonpregnant Jersey cows that had been fed the experimental diet for at least 1 month were used in these experiments. Animals were housed indoors in individual stanchions during the trials.

Diet

Each cow was fed 10 kg of a diet composed of 50% alfalfa hay cubes and 50% beet pulp-based commercial supplement (Sweet and Bulky, Wayne Feeds Division, Continental Grain, Chicago, IL) per day. The calculated daily intake of minerals (calcium, 75 g; phosphorus, 40 g; magnesium, 25 g) was well above the NRC requirement (National Research Council, 1978).

Design

Treatment trials were run for each of the 3 compounds [24-F-1,25-$(OH)_2D_3$, 24,24-F_2-1,25-$(OH)_2D_3$, and 1,25-$(OH)_2D_3$] at the following three doses: 25 µg, 100 µg, 400 µg, thus resulting in 9 treatment groups. In addition, a tenth group of cows received drug vehicle only (ethanol). Each treatment group consisted of 3 cows.

Protocol

A Foley catheter was placed into the bladder of each cow, and urine collections were begun 24 hours later. Urine was collected into vessels containing 100 ml glacial acetic acid which brought the pH between 4 and 5. Daily blood and total urine collections were made for the next 4 days to obtain baseline physiologic data for each individual cow during this pretreatment period. Plasma and urine samples were kept frozen at -19°C until analyzed.

At the start of day 5, a specific dose (calibrated by ultraviolet absorption, λ_{max} = 264 nm, Σ = 18,200) of one of the three compounds dissolved in 1 ml of ethanol was administered intramuscularly. Daily blood and total urine collections were then continued over a 9-day posttreatment period to determine the influence of drug treatment on various physiologic parameters.

Physiologic Parameters Monitored

Daily concentrations of calcium and magnesium in plasma and urine were determined for each cow by atomic absorption spectrophotometry (Perkin-Elmer Corp., 1965). Plasma and urine inorganic phosphorus concentrations were determined colorimetrically (Fiske and Subbarrow, 1925).

Concentrations of hydroxyproline in plasma and urine were monitored daily as an estimate of bone resorption activity (Dull and Henneman, 1963). Free plasma hydroxyproline and total urinary

hydroxyproline were determined colorimetrically (Bannister and Burns, 1970; Kiviriko et al., 1967). Renal function was assessed by monitoring urine specific gravity and renal glomerular filtration rate (GFR). Glomerular filtration rate was estimated by determining endogenous creatinine clearance rates. Plasma and urine creatinine concentrations were determined colorimetrically on an AutoAnalyzer (Chasson et al., 1961).

The hydration state of the animals was estimated by measuring total plasma protein using a refractometer (American Optical Company, Buffalo, NY). In addition, plasma glucose and β-hydroxybutyrate concentrations were measured by colorimetric assays (Trinder, 1969; Zivin and Snarr, 1973) to give some indication of the metabolic state of the animals throughout the experiment.

Analysis

Pretreatment (baseline) and posttreatment samples were obtained. The 9 days after treatment were divided into four 2-day periods and a single 1-day period to more accurately assess the duration and intensity of the drugs' effects. For each physiologic parameter, the effect of the drug was expressed as a percentage of the baseline value obtained during the pretreatment period, allowing each cow to act as its own control. The means of each group of treated cows were subjected to t-tests (LSD) (Barr et al., 1976), comparing them to the means obtained from the ethanol-treated cows.

RESULTS

All three compounds effectively elevated plasma calcium and inorganic phosphorus concentrations at the 100 and 400 μg doses (Tables 1 and 2). The 24,24-F_2-1,25-$(OH)_2D_3$ compound was approximately 4 times as effective as 1,25-$(OH)_2D_3$ in elevating plasma calcium and inorganic phosphorus concentrations based on the observation that 100 μg of 24,24-F_2-1,25-$(OH)_2D_3$ and 400 μg of 1,25-$(OH)_2D_3$ resulted in similar plasma calcium and inorganic phosphorus concentrations. The 24-F-1,25-$(OH)_2D_3$ compound appears to be only slightly more effective than 1,25-$(OH)_2D_3$ in elevating plasma calcium and inorganic phosphorus concentrations. At the 400 μg dose, both the fluorinated compounds induced significantly elevated plasma calcium concentrations for a longer period of time than did 1,25-$(OH)_2D_3$ (Fig. 1). Administration of any one of the three compounds resulted in similar decreases in plasma magnesium concentration.

Plasma hydroxyproline concentrations were not significantly affected by 25 μg or 100 μg of any of the compounds. At the 400 μg dose, the 24-F-1,25-$(OH)_2D_3$ compound significantly depressed plasma hydroxyproline concentration when compared to the effects of ethanol. The 400 μg dose of 1,25-$(OH)_2D_3$ and 24,24-F_2-1,25-$(OH)_2D_3$ had no significant effect on plasma hydroxyproline concentration. Urinary hydroxyproline excretion rate exhibited the same pattern as plasma hydroxyproline concentration (Table 3), except that hydroxyproline

excretion via the urine was depressed significantly following the 400 µg dose of all three compounds.

Total plasma protein, plasma glucose, and plasma β-hydroxybutyrate were not significantly affected by treatment with any of the compounds.

Urine specific gravity was reduced following administration of any of the compounds. Isosthenuria (Sp. gr. ≤ 1.012 and ≥ 1.008) was seen in most cases, although several of the cows that received 400 µg of 24,24-F_2-1,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ were hyposthenuric (Sp. gr. ≤ 1.008) for several days.

The GFR was significantly reduced by all three compounds at the 400 µg dose and in cows that received 100 µg of 24,24-F_2-1,25-$(OH)_2D_3$ (Table 4). The depression of GFR induced by 400 µg of the fluorinated compounds persisted longer than the depression in GFR induced by 400 µg of 1,25-$(OH)_2D_3$. The GFR of the cows was determined again 1 month after the end of the experimental period and had returned to pretreatment levels in all cases.

Urinary calcium excretion rates (Table 5) exhibited a considerable degree of variation, as shown in the control cows. There was generally an increase in urinary calcium excretion after treatment with 25 or 100 µg of the drugs. However, urinary calcium excretion rates at the 400 µg dose were less than those seen at the 100 µg dose for all three drugs. In addition, the 400 µg doses of 1,25-$(OH)_2D_3$ and 24,24-F_2-1,25-$(OH)_2D_3$ were associated with lower urinary calcium excretion rate than those determined in the pretreatment periods.

Urinary phosphorus excretion rates were also generally increased by treatment with any of the compounds. There was considerable variation among the cows, which made statistical inferences difficult.

Assuming that 56% of plasma calcium and all plasma phosphorus is filterable across the renal glomeruli, the resting renal tubular reabsorption rates (TR) for calcium and phosphorus were estimated using the following formulae:

TR (Ca) = (GFR x [plasma Ca x 0.56]) - (urine vol. x [urine Ca])

TR (P) = (GFR x [plasma P]) - (urine vol. x [urine P]).

Reabsorption of calcium by the renal tubules ranged between 95 and 98% efficient. No significant difference was found in renal tubular reabsorption rate for calcium or phosphorus after administration of any of the compounds studied.

DISCUSSION

The addition of fluorine to the 24-carbon of the side-chain increases the duration and intensity of the hypercalcemic and hyperphosphatemic activity of 1,25-$(OH)_2D_3$. We found the 24,24-F_2-1,25-$(OH)_2D_3$ compound to be approximately 4 times as active as 1,25-$(OH)_2D_3$, while the 24-F-1,25-$(OH)_2D_3$ compound was intermediate in its ability to raise plasma calcium concentrations. Okamoto et al. (1983) and Corradino et al. (1980) reported similar findings in experiments with 24,24-F_2-1,25-$(OH)_2D_3$ in rats and chicks.

Urinary hydroxyproline excretion has been utilized as a sensitive index of bone resorptive activity in cattle (Black and Capen, 1971). Hoffsis et al. (1978) reported that 600-µg injections of 1,25-$(OH)_2D_3$ to dairy cows caused significant increases in urinary hydroxyproline excretion when expressed as a ratio to urinary creatinine. However, we could not demonstrate any increase in total urinary hydroxyproline excretion with 1,25-$(OH)_2D_3$ or the fluorinated analogues. We found that the higher doses of the compounds tested actually inhibited urinary hydroxyproline excretion.

The discrepancy in results may reflect the manner in which the data was presented. However, we have demonstrated that high doses of any of the compounds tested are capable of dramatically decreasing GFR, so urinary hydroxyproline excretion may not be the best indicator of bone matrix catabolism in animals with compromised renal function.

Plasma hydroxyproline concentration is also a useful indicator of bone resorption activity and is less likely to be affected by reduced renal function than urinary hydroxyproline excretion rates. Although reduced GFR might result in higher plasma hydroxyproline, we measured plasma hydroxyproline concentrations and found no significant change with any of the compounds tested. Likewise, Hove et al. (1983) and Bar et al. (1985) found that administration of 1α-OHD_3, 1,25-$(OH)_2D_3$, 1,24,25-$(OH)_3D_3$, and 1,25,26-$(OH)_3D_3$ to mature cows either had no effect or caused a slight reduction in plasma hydroxyproline concentration. Therefore, bone calcium resorption probably has little or no role in the hypercalcemic activity observed when pharmacologic doses of cholecalciferol compounds are administered to normocalcemic cows.

The compounds tested induced acute transient changes in renal function manifested by an inability to concentrate the urine and a reduction in GFR. The hypercalcemic activity of the three compounds may be responsible for the observed renal insufficiencies. Hypercalcemia has long been known to alter the ability of the kidneys to concentrate the urine (Lee et al., 1978; Osborne and Stevens, 1977; Schwartz and Relman, 1967). Mechanisms proposed include a blockade of the activity of anti-diuretic hormone at the level of the collecting ducts (Manitins et al., 1960) or a defect in sodium transport in the loop of Henle (Epstein et al., 1959).

Hypercalcemia has also been observed to result in a reduction of GFR. There are believed to be two mechanisms at work. Hypercalcemia can result in calcification of renal tubules and loss of functional

nephron mass (Capen et al., 1966; Ganote et al., 1975). The loss of these nephrons is presumably permanent. Hypercalcemia has also been shown to cause significant alterations in renal hemodynamics. Calcium ions potentiate the vasoconstrictive activity of angiotensin II on renal afferent arterioles, thus reducing renal blood flow and GFR (Levi et al., 1983). This effect is reversible once normocalcemia is established.

Vitamin D sterols have also been incriminated as the direct cause of reduced renal function. Dinkel (1966) showed that nephrocalcinosis could be caused by dihydrotachysterol administration despite the presence of hypocalcemia. Morrissey et al. (1977) found renal calcification occurred in chicks fed varying amounts of 25-OHD at dose levels lower than those required to induce hypercalcemia. Hartenbower et al. (1977) suggested that 1,25-$(OH)_2D_3$ has a specific toxic effect on the kidney since the pattern of calcification of the kidney in rats following 1,25-$(OH)_2D_3$ intoxication differs from that occurring in vitamin D toxicity.

We could not discern the extent of any direct contribution that the compounds tested had on reduction of GFR. However, we have shown that the fluorinated compounds produced greater changes in GFR than did 1,25-$(OH)_2D_3$ at the same doses.

Initially, the compounds induced hypercalcemia and hypercalciuria. However, as the degree of renal insufficiency increased the amount of calcium excreted in the urine decreased, despite greater plasma loads of calcium. Therefore, the inability to excrete calcium

in the urine appropriate to the plasma load of calcium actually adds to the hypercalcemia. Hoffsis et al. (1978) reported that urinary calcium excretion rates of cows were increased by administration of 1,25-$(OH)_2D_3$, but that the changes in urine calcium did not correlate directly with the intravenous dose of steroid. Cows in their experiment may have had compromised renal function at the high doses of 1,25-$(OH)_2D_3$, accounting for the lack of a direct correlation between urinary calcium excretion and steroid dose. Kopple and Coburn (1973) noted that even mild to moderate renal insufficiency can be associated with lower than normal urinary calcium excretion.

Vitamin D compounds can induce hypercalcemia and hyperphosphatemia by increasing intestinal calcium and phosphorus absorption, increasing bone resorption, or by decreasing the loss of calcium and phosphorus via the urine. In this experimental model, the compounds tested did not act by stimulating bone resorption or by inducing renal conservation of calcium and phosphorus over control animals. The hypercalcemia and hyperphosphatemia caused by the compounds tested are probably the result of stimulation of active transport mechanisms for calcium and phosphorus within the intestinal tract (Braithwaite, 1980; Hove, 1984).

The 24-F-1,25-$(OH)_2D_3$ and 24,24-F_2-1,25-$(OH)_2D_3$ compounds have equal or greater biopotency than 1,25-$(OH)_2D_3$ in all physiologic parameters we measured. Okamoto et al. (1983) suggest that fluorine atoms on the 24-carbon block hydroxylation which may be necessary for inactivation of the compound, thus prolonging the biological activity.

In this study, we have found that the prophylactic activity of the compounds tested would reside mainly in their ability to markedly increase intestinal calcium and phosphorus absorption. Further studies must capitalize on this beneficial effect while minimizing the potentially harmful effects of these compounds which include reduced renal function and inhibition of bone resorption activity.

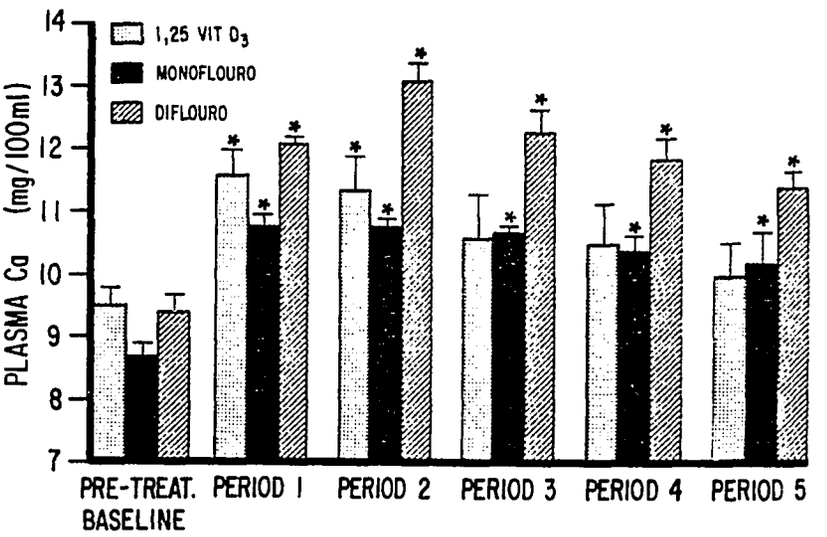

Figure 1. Plasma calcium concentrations following a 400-μg dose of either 1,25-$(OH)_2D_3$, 24-F-1,25-$(OH)_2D_3$ or 24,24-F_2-1,25-$(OH)_2D_3$. Mean ± SEM. *Denotes significantly increased plasma calcium concentration above baseline. Period 1 = days 1 and 2; Period 2 = days 3 and 4; Period 3 = days 5 and 6; Period 4 = days 7 and 8; Period 5 = day 9

Table 1. Plasma calcium concentration during the 5 posttreatment periods expressed as a percentage of pretreatment calcium concentration

	% of Pretreatment Level[a]				
Treatment	Period 1 (Days 1&2)	Period 2 (Days 3&4)	Period 3 (Days 5&6)	Period 4 (Days 7&8)	Period 5 (Day 9)
<u>Ethanol</u>					
Control	101.1	102.4	99.6	101.1	98.8
(9.55 ± 0.13)	± 2.0	± 0.8	± 0.6	± 1.1	± 1.4
<u>24-F-1,25-$(OH)_2D_3$</u>					
25 µg	103.4	109.1*	101.4	106.2	ND
(9.24 ± 0.23)	± 2.6	± 0.8	± 1.5	± 1.1	
100 µg	107.4	113.2*	110.2*	109.7*	ND
(8.36 ± 0.13)	± 1.4	± 2.5	± 1.6	± 1.0	
400 µg	124.3*	124.0*	123.0*	120.4*	117.8*

400 µg (8.68 ± 0.23)	124.3* ± 5.9	124.0* ± 4.4	123.0* ± 3.6	120.4 ± 4.5	117.8 ± 4.6
$24,24\text{-}F_2\text{-}1,25\text{-}(OH)_2D_3$					
25 µg (9.29 ± 0.14)	111.3* ± 3.6	110.9* ± 4.4	106.5* ± 5.4	104.4 ± 3.4	ND
100 µg (8.91 ± 0.24)	119.9*† ± 1.9	117.1* ± 2.5	115.1* ± 4.7	112.8* ± 3.2	111.9 ± 6.6
400 µg (9.38 ± 0.25)	129.3* ± 4.7	139.9*† ± 3.7	131.1*† ± 5.2	126.8*† ± 7.3	122.0*† ± 6.2
$1,25\text{-}(OH)_2D_3$					
25 µg (8.58 ± 0.13)	106.0 ± 2.4	109.3* ± 2.0	105.8 ± 2.2	104.5 ± 1.1	103.3 ± 1.1
100 µg (9.01 ± 0.03)	111.9* ± 1.0	115.8* ± 1.4	111.4* ± 0.9	109.2* ± 0.9	107.7 ± 1.0
400 µg (9.50 ± 0.26)	122.3* ± 3.1	119.4* ± 4.2	111.4 ± 7.0	110.3 ± 4.5	105.5 ± 3.1

[a]Values in parentheses are the pretreatment baseline concentration of calcium in mg/100 ml. Pretreatment data are reported as the mean ± SEM. ND = Not determined. *Significantly different ($P \leq 0.05$) from control (ethanol) values. †Significantly different ($P \leq 0.05$) from $1,25\text{-}(OH)_2D_3$ values at same dose.

Table 2. Plasma phosphorus concentration during the 5 posttreatment periods expressed as a percentage of pretreatment phosphorus concentration

Treatment	% of Pretreatment Level[a]				
	Period 1 (Days 1&2)	Period 2 (Days 3&4)	Period 3 (Days 5&6)	Period 4 (Days 7&8)	Period 5 (Day 9)
Ethanol					
Control (5.83 ± 0.14)	109.3 ± 5.2	100.4 ± 9.2	88.9 ± 9.1	100.0 ± 5.5	105.1 ± 1.5
24-F-1,25-$(OH)_2D_3$					
25 µg (5.65 ± 0.21)	115.7 ± 8.7	117.4 ± 5.2	111.6 ± 7.5	123.1 ± 9.8	ND
100 µg (5.62 ± 0.45)	110.2 ± 9.7	134.0* ±11.4	123.8* ± 4.9	116.0 ±10.7	ND
400 µg (5.82 ± 0.69)	140.2 ± 6.8	157.5* ±14.3	155.1* ±16.8	160.4* ±16.4	150.0 ±29.9
24,24-F_2-1,25-$(OH)_2D_3$					

$24,24\text{-}F_2\text{-}1,25\text{-}(OH)_2D_3$					
25 µg (7.12 ± 0.26)	117.2 ± 2.2	116.3 ± 5.8	116.4* ± 8.0	126.2* ± 2.3	ND
100 µg (6.42 ± 0.33)	136.5*† ± 2.1	131.6* ±10.6	135.2 ±15.3	133.8* ± 9.5	136.7 ±10.2
400 µg (5.37 ± 0.68)	144.9 ±11.7	153.5* ±23.6	145.6* ± 9.7	154.7* ±21.8	150.1 ±22.7
$1,25\text{-}(OH)_2D_3$					
25 µg (6.24 ± 0.52)	105.3 ± 3.4	107.9 ± 9.2	112.0 ± 5.1	116.3 ± 9.3	109.7 ± 6.4
100 µg (6.45 ± 0.23)	116.0 ± 4.8	128.2 ± 6.7	133.7* ± 4.0	139.1* ± 6.1	129.3 ±13.8
400 µg (6.21 ± 0.58)	142.7 ±17.5	129.2 ±11.7	126.7 ±12.4	118.9 ±10.3	131.4 ± 7.4

[a]Values in parentheses are the pretreatment baseline phosphorus concentrations in mg/100 ml. Pretreatment data are reported as the mean ± SEM. ND = Not determined. *Significantly different ($P \leq 0.05$) from control (ethanol) values. †Significantly different ($P \leq 0.05$) from $1,25\text{-}(OH)_2D_3$ values at same dose.

Table 3. Urinary hydroxyproline excretion rate during the 5 posttreatment period expressed as a percentage of pretreatment urinary hydroxyproline excretion rate

Treatment	% of Pretreatment Level[a]				
	Period 1 (Days 1&2)	Period 2 (Days 3&4)	Period 3 (Days 5&6)	Period 4 (Days 7&8)	Period 5 (Day 9)
Ethanol					
Control (11.3 ± 1.2)	90.7 ±13.5	97.3 ±14.1	119.7 ± 9.8	113.1 ± 4.3	132.7 ±14.8
24-F-1,25-$(OH)_2D_3$					
25 µg (13.3 ± 2.8)	136.2* ± 7.4	109.3 ±16.2	113.5 ± 1.8	94.1 ±19.8	ND
100 µg (13.2 ± 3.1)	154.8 ±38.5	136.0 ±20.3	132.5 ±11.3	138.0 ±16.6	ND
400 µg (17.6 ± 2.3)	104.2 ± 4.5	91.7 ± 4.7	68.3* ± 6.5	60.8*† ± 3.4	45.5* ±24.9
24,24-F_2-1,25-$(OH)_2D_3$					

24,24-F_2-1,25-$(OH)_2D_3$					
25µg (15.8 ± 1.3)	113.6 ± 8.7	88.1 ± 9.3	84.5* ±11.7	79.4 ± 8.0	ND
100 µg (12.9 ± 0.6)	114.9 ±17.2	87.5 ±16.1	78.2 ±21.5	68.6* ± 4.0	55.7 ± 7.5
400 µg (16.0 ± 0.2)	110.0 ± 8.5	78.5 ± 8.8	70.6* ± 7.6	67.1*† ± 6.1	73.1 ± 8.9
1,25-$(OH)_2D_3$					
25 µg (19.3 ± 3.0)	106.3 ± 5.7	107.5 ± 4.6	95.2 ± 1.7	99.9 ±11.5	98.5 ± 9.9
100 µg (14.6 ± 1.2)	138.2 ± 9.1	102.0 ± 1.9	99.3 ± 5.7	89.6 ±13.0	100.9 ± 9.3
400 µg (15.4 ± 3.4)	105.0 ±18.9	87.1 ±18.5	75.6* ±12.0	91.6* ±10.3	ND

[a]Values in parentheses are the pretreatment baseline urinary hydroxyproline excretion rates in mg/min. Pretreatment data are reported as the mean ± SEM. ND = Not determined. *Significantly different ($P \leq 0.05$) from control (ethanol) values. †Significantly different ($P \leq 0.05$) from 1,25-$(OH)_2D_3$ values at same dose.

Table 4. Glomerular filtration rate during the 5 posttreatment periods expressed as a percentage of pretreatment glomerular filtration rate

Treatment	% of Pretreatment Level[a]				
	Period 1 (Days 1&2)	Period 2 (Days 3&4)	Period 3 (Days 5&6)	Period 4 (Days 7&8)	Period 5 (Day 9)
Ethanol					
Control (922 ± 108)	91.7 ± 9.1	89.5 ±10.8	106.8 ± 6.1	103.5 ± 4.1	101.4 ± 7.0
24-F-1,25-$(OH)_2D_3$					
25 µg (651 ± 136)	113.9 ± 7.5	86.4 ± 0.6	93.5 ± 1.7	84.6 ± 4.8	ND
100 µg (693 ± 96)	127.2 ±14.2	109.1 ±23.6	114.2 ±20.6	123.4 ±24.6	ND
400 µg (955 ± 125)	87.4 ± 7.5	81.9 ± 7.6	68.4* ± 2.6	64.2* ± 2.3	61.5* ± 1.3

400 µg (955 ± 125)	87.4 ± 7.5	81.9 ± 7.6	66.4 ± 2.6	[illegible] ± 2.3	[illegible] ± 1.3
<u>24,24-F_2-1,25-$(OH)_2D_3$</u>					
25 µg (1091 ± 91)	89.6 ± 4.1	94.8 ± 4.0	90.4 ± 7.1	88.9 ± 8.6	ND
100 µg (794 ± 129)	107.5 ±14.9	92.4 ± 3.1	88.6 ± 5.1	67.8* ± 2.7	60.5* ± 7.1
400 µg (899 ± 83)	84.2 ± 3.5	69.3 ± 4.8	62.6* ± 4.6	63.8* ±10.3	57.9* ± 9.3
<u>1,25-$(OH)_2D_3$</u>					
25 µg (819 ± 58)	105.6 ± 3.9	102.9 ± 1.2	105.7 ±12.7	97.2 ± 3.4	101.0 ± 2.6
100 µg (758 ± 13)	110.9 ± 6.3	99.1 ± 0.8	94.4 ± 4.1	89.4 ± 6.6	97.7 ± 5.8
400 µg (916 ± 65)	104.6 ±28.3	83.9 ± 9.7	69.7* ±12.5	96.4 ±12.9	ND

[a]Values in parentheses are the pretreatment baseline glomerular filtration rate in ml/min. Pretreatment data are reported as the mean ± SEM. ND = Not determined. *Significantly different ($P \leq 0.05$) from control (ethanol) values.

Table 5. Urinary calcium excretion rate during the 5 posttreatment periods expressed as a percentage of pretreatment urinary calcium excretion rate

Treatment	% of Pretreatment Level[a]				
	Period 1 (Days 1&2)	Period 2 (Days 3&4)	Period 3 (Days 5&6)	Period 4 (Days 7&8)	Period 5 (Day 9)
Ethanol					
Control (0.87 ± 0.43)	111.0 ±23.0	132.2 ±44.6	168.0 ±26.6	129.5 ± 5.5	84.0 ±19.7
24-F-1,25-$(OH)_2D_3$					
25 µg (0.52 ± 0.22)	137.6 ±76.7	182.3 ±72.3	315.9 ±42.8	260.4* ±38.7	ND
100 µg (0.99 ± 0.52)	977.0 ±800.6	546.3 ±291.8	817.8 ±509.2	685.4 ±389.5	ND
400 µg (1.96 ± 1.40)	232.7 ±137.0	214.2 ±118.3	250.0 ±146.2	279.7 ±157.8	106.0 ±34.5

400 µg (1.96 ± 1.40)	232.7 ±137.0	217.2 ±118.3	±146.2	±157.8	±34.5
24,24-F_2-1,25-$(OH)_2D_3$					
25 µg (0.26 ± 0.03)	281.0 ±47.3	360.8 ±91.2	259.0 ±37.0	190.5 ±20.4	ND
100 µg (0.92 ± 0.29)	310.0 ±208.6	194.6 ±155.5	166.7 ±79.2	163.0 ±22.5	86.0 ±30.6
400 µg (1.22 ± 0.17)	180.1 ±38.7	68.5 ±22.9	64.6 ± 4.6	148.0 ±63.6	65.0 ±14.5
1,25-$(OH)_2D_3$					
25 µg (0.37 ± 0.28)	237.7 ±82.1	735.8 ±429.5	353.0 ±98.6	166.0 ±33.5	198.0* ±30.6
100 µg (0.30 ± 0.08)	582.0 ±193.0	872.6* ±280.6	811.5 ±350.3	602.0 ±260.7	690.0 ±292.5
400 µg (0.71 ± 0.28)	123.1 ±52.9	97.7 ±32.6	61.0 ±10.5	67.6 ±13.2	ND

[a]Values in parentheses are the pretreatment baseline urinary calcium excretion rates in g/day. Data are reported as the mean ± SEM. ND = Not determined. *Significantly different ($P \leq 0.05$) from control (ethanol) values.

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SECTION II.

USE OF 24-F-1,25-DIHYDROXYVITAMIN D_3 TO PREVENT PARTURIENT PARESIS (MILK FEVER) IN DAIRY COWS

INTRODUCTION

Parturient paresis (milk fever) continues to be a major source of loss in terms of productivity, increased predisposition to secondary disease problems, treatment costs, and cow deaths. Many measures to prevent parturient paresis have been suggested. Among these are adjustment of calcium content of prepartal diets (Hove, 1986; Jorgensen, 1974) and the use of vitamin D and its derivatives (Barlet, 1977; Gast et al., 1979; Hibbs and Pounden, 1955; Hove and Kristiansen, 1982; Sachs et al., 1977).

The major problem with the use of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] and 1α-hydroxyvitamin D_3 (1α-OHD_3) is in the timing of the administration of these sterols relative to parturition. When 1,25-$(OH)_2D_3$ or 1α-OHD_3 are administered between 1 and 3 days before calving, they are effective in preventing parturient paresis (Hoffsis et al., 1978; Sachs et al., 1977). Outside this time period, they are less effective. Prevention of parturient paresis would be more practical if the period of efficacy of the vitamin D sterols could be extended so as to circumvent difficulties in predicting the actual time of parturition.

The duration of action of 1,25-$(OH)_2D_3$ can be extended by fluoridation of the 24th carbon which blocks 24-hydroxylation of the side-chain of 1,25-$(OH)_2D_3$, a major degradative pathway (Okamoto et al., 1983). We have found that 24-F-1,25-$(OH)_2D_3$ is about 1.5 times as potent and acts over a longer period than does 1,25-$(OH)_2D_3$ in

nonpregnant cows (Goff et al., 1986). Our objective was to develop a protocol for the prevention of parturient paresis in dairy cows using 24-F-1,25-$(OH)_2D_3$ administration.

MATERIALS AND METHODS

Twenty-nine periparturient Jersey cows, weighing between 400 and 450 kg, were selected from the National Animal Disease Center research herd. All cows had at least three previous lactations and all had histories of parturient paresis at a previous calving. Six weeks prepartum, cows were fed alfalfa hay ad libitum and 4.5 kg concentrate feed, which supplied approximately 110 g calcium and 33 g phosphorus daily. Approximately 2 weeks prepartum, the cows received an additional 7.3 kg of concentrate daily so that their diet supplied about 150 g calcium and 76.8 g phosphorus per day in the last days of gestation and for the first 2 weeks of lactation.

Cows were randomly allocated to either the control group or one of the two treatment groups. Five days before the expected day of parturition, experimental cows were treated with either 100 μg (n = 7) or 150 μg (n = 10) of 24-F-1,25-$(OH)_2D_3$ intramuscularly (IM). Cows that did not calve within 7 days received a second dose of 24-F-1,25-$(OH)_2D_3$. One cow in each experimental group did not calf within 7 days of the second injection and was given a third injection of the experimental drug. Twelve control cows were left untreated.

The 24-F-1,25-$(OH)_2D_3$ used in these trials was the gift of M. R. Uskokovic (Hoffmann-La Roche, Inc., Nutley, NJ). The concentration of 24-F-1,25- $(OH)_2D_3$ was determined using UV spectroscopy (Σ_{264} = 18,200 m^{-1}) and then dissolved in Neobee oil at a concentration of either 100 or 150 μg/ml.

Daily plasma samples were obtained from all cows between the 10th day prior to parturition and the 14th day postpartum. Around the time of parturition, sampling was more frequent.

Concentrations of calcium and magnesium in plasma were determined by atomic absorption spectrophotometry (Perkin-Elmer Corp., 1965). Plasma inorganic phosphorus (Fiske and Subbarrow, 1925), hydroxyproline (Bannister and Burns, 1970), and creatinine (Chasson et al., 1961) were determined by colorimetric methods adapted to an Auto-Technicon Analyzer. Plasma parathyroid hormone (PTH) concentrations were determined by radioimmunoassay using an antibody directed against middle and N-terminal portions of the PTH molecule (Arnaud et al., 1971). The method of Reinhardt et al. (1984) was modified to allow determination of both 1,25-$(OH)_2D_3$ and 24-F-1,25-$(OH)_2D_3$ in plasma. 24-F-1,25-Dihydroxyvitamin D_3 and 1,25-$(OH)_2D_3$ comigrated on C18 Sep-Pak Silica columns. Separation was achieved by subjecting the dihydroxyvitamin D metabolite fraction from the initial C18 Sep-Pak column to high-performance liquid chromatography (HPLC) on a Zorbax Sil column (0.45 x 25 cm, Dupont) developed in 3:97 isopropanol/methylene chloride. Once separated, concentrations of both sterols were determined by using a radioreceptor assay (Reinhardt et al., 1984). Recovery estimates for 1,25-$(OH)_2D_3$ were used to estimate recovery of 24-F-1,25-$(OH)_2D_3$.

RESULTS

A summary of animal allocation and clinical results within each experimental group is presented in Table 1. Ninety-two percent of the control cows developed clinical parturient paresis. Most of these cases occurred between parturition (day 0) and 2 days postpartum. One cow became paretic 12 days after calving. Three of the 11 paretic control cows suffered relapses of parturient paresis requiring further treatment with intravenous calcium solution.

Treatment of cows with either 100 µg or 150 µg of 24-F-1,25-$(OH)_2D_3$ prior to parturition reduced the incidence of parturient paresis to 43% and 20%, respectively. No relapses were seen in the 24-F-1,25-$(OH)_2D_3$-treated cows that developed parturient paresis.

Plasma calcium concentrations of control and 24-F-1,25-$(OH)_2D_3$-treated cows are presented in Figure 1. Data derived from the control cow that did not develop parturient paresis are included in the data for the control cows. The degree of hypocalcemia exhibited by paretic 24-F-1,25-$(OH)_2D_3$-treated cows is similar to that of control cows with paresis. Cows treated with 24-F-1,25-$(OH)_2D_3$ that did not develop paresis exhibited mild hypocalcemia when compared to control or treated cows that developed paresis.

Control cows exhibited a fivefold increase in plasma 1,25-$(OH)_2D_3$ concentration around the time of parturition. Plasma concentrations of 1,25-$(OH)_2D_3$ were only slightly elevated in both paretic and

nonparetic 24-F-1,25-$(OH)_2D_3$-treated cows (Figure 2). Plasma concentrations of 1,25-$(OH)_2D_3$ was inversely proportional to plasma calcium concentration in control cows, but not in paretic 24-F-1,25-$(OH)_2D_3$-treated cows.

Plasma concentrations of iPTH were similar in control and paretic 24-F-1,25-$(OH)_2D_3$-treated cows (Figure 3), exhibiting a threefold increase around the time of parturition. Plasma iPTH was increased only slightly in nonparetic 24-F-1,25-$(OH)_2D_3$-treated cows. Plasma concentration of iPTH was inversely proportional to plasma calcium concentration in all cows.

Plasma hydroxyproline concentrations were low in all cows prior to parturition and rose significantly in all cows after parturition. Plasma hydroxyproline and creatinine concentrations in control cows and 24-F-1,25-$(OH)_2D_3$-treated cows were not significantly different before or after parturition.

DISCUSSION

These studies demonstrate that 24-F-1,25-$(OH)_2D_3$ can prevent parturient paresis in susceptible dairy cows. The greatest protection against parturient paresis was afforded by 150-µg doses of 24-F-1,25-$(OH)_2D_3$. Cows that calved between one day and 7 days after the first injection were protected from parturient paresis. A second injection, 7 days after the first, prevented parturient paresis for an additional 7 days in all but one cow. When 24-F-1,25-$(OH)_2D_3$ was administered in 100-µg doses, the period of protection was less than 7 days (Table 1). We did not detect any undesirable effects of repeated 150-µg doses of 24-F-1,25-$(OH)_2D_3$ in this study. Plasma creatinine concentrations were normal and did not change significantly in treated cows, indicating that renal function was not affected. A larger dose of 24-F-1,25-$(OH)_2D_3$ might be more effective in preventing parturient paresis; however, the possibility of toxicity would be greatly increased as well. This conclusion is based on studies in nonpregnant cows in which 400-µg doses of 24-F-1,25-$(OH)_2D_3$ caused significant reductions in renal glomerular filtration rate (Goff et al., 1986). Hydroxyproline is an amino acid unique to collagen. Plasma levels of hydroxyproline can be useful indices of bone resorption activity (Dull and Henneman, 1963). However, because hydroxyproline of uterine origin also enters the blood after calving, the usefulness of hydroxyproline determinations is limited to the prepartal period. We did not observe any significant differences in prepartal plasma

hydroxyproline concentrations between control cows and those 24-F-1,25-$(OH)_2D_3$-treated cows that had been injected more than 6 days prior to calving (data not shown). In controlled studies on nonpregnant cows, the administration of 1,25-$(OH)_2D_3$, 24-F-1,25-$(OH)_2D_3$, 24,24-F_2-1,25-$(OH)_2D_3$, and 1α-OHD_3 either had no effect on or inhibited bone resorption (Bar et al., 1985; Goff et al., 1986). Our results confirm that vitamin D sterols do not stimulate bone resorption in dairy cows prior to parturition. The hypercalcemic activity of vitamin D sterols in ruminants resides primarily in their ability to enhance intestinal calcium absorption (Braithwaite, 1978; Hove, 1984).

Despite nearly identical degrees of hypocalcemia, the 24-F-1,25-$(OH)_2D_3$-treated cows that developed parturient paresis had much lower plasma 1,25-$(OH)_2D_3$ concentrations around the time of parturition than did control cows. The use of 24-F-1,25-$(OH)_2D_3$ inhibited the expected increase in plasma 1,25-$(OH)_2D_3$ as a homeostatic response to hypocalcemia. Other vitamin D sterols are known to inhibit endogenous production of 1,25-$(OH)_2D_3$ in cows (Littledike et al., 1986), and 24-F-1,25-$(OH)_2D_3$ probably acts similarly. While vitamin D sterols stimulate one calcium homeostatic mechanism (intestinal calcium absorption), they may inhibit the cow's natural homeostatic mechanisms that respond to hypocalcemia.

We feel that a 150-μg dose of 24-F-1,25-$(OH)_2D_3$ enhances intestinal calcium absorption over a 7-day period to the extent that the calcium demands of initiating lactation can be met solely from dietary calcium. When 24-F-1,25-$(OH)_2D_3$ is administered in 100-μg doses,

intestinal calcium absorption is adequately enhanced to meet calcium demands for only about 5 days.

Several groups have reported that vitamin D sterols reduced the incidence of parturient paresis occurring around the time of calving, but increased the incidence of parturient paresis occurring more than 4 days postpartum (Hove, 1986; Littledike and Horst, 1982). Prolonged inhibition of the cow's natural homeostatic mechanisms were believed to have contributed to the development of hypocalcemia in these cases. In this study, only one 24-F-1,25-$(OH)_2D_3$-treated cow developed parturient paresis more than 4 days postpartum. However, climatic conditions were a major factor in this case. Ten days after this cow calved, Iowa received a severe snowstorm which prevented normal feeding and milking procedures that afternoon and the following morning. Shortly after being milked on the 11th day postpartum, the cow developed clinical parturient paresis. A possible significant difference between this study and earlier studies in which delayed cases of parturient paresis were prominent was the level of calcium supplied in the postpartum diet. The high calcium diet used in this study may have allowed the cows to better utilize the remaining calcium transport mechanisms as exogenous 24-F-1,25-$(OH)_2D_3$ action on the intestine declined. The vitamin D sterol-treated cow may have a greater requirement for dietary calcium after parturition than does the untreated cow. Endogenous 1,25-$(OH)_2D$ production is inhibited by these compounds, and it seems likely that hypercalcemia induced by these compounds during the peripartal period might leave bone calcium

resorption mechanisms inactivated. Thus, plasma calcium concentration in the vitamin D-treated cow may be dependent on just one branch of the calcium homeostatic mechanism-intestinal calcium absorption. We are currently investigating the efficacy of 24-F-1,25-$(OH)_2D_3$ in cows fed diets containing less calcium.

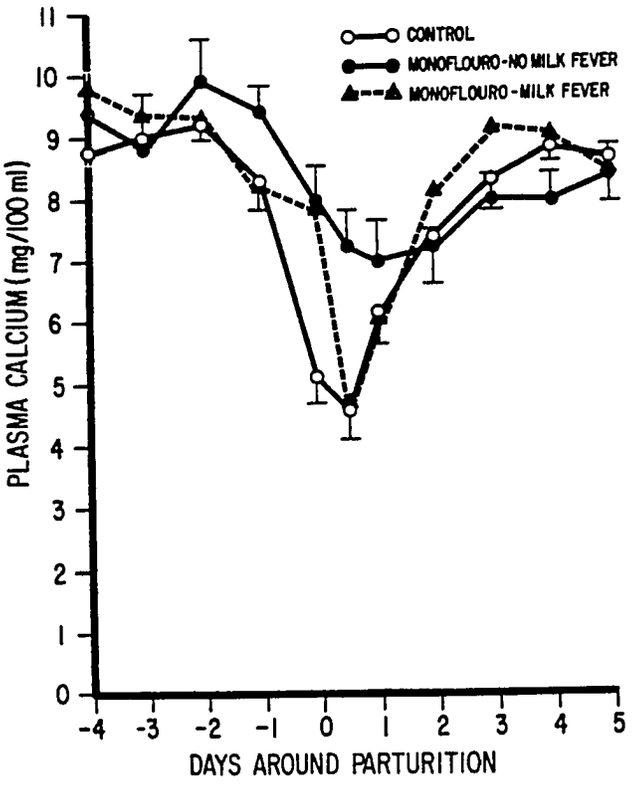

Figure 1. Plasma calcium concentration around the time of parturition in control cows and cows treated with 24-F-1,25-$(OH)_2D_3$. Eleven of the 12 control cows and those 24-F-1,25-$(OH)_2D_3$-treated cows that developed paresis were treated with intravenous calcium at their calcium nadir

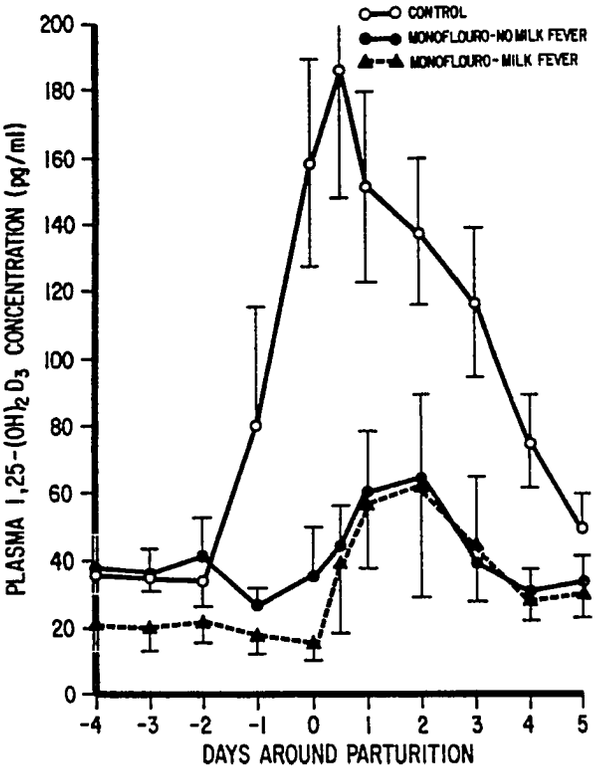

Figure 2. Plasma 1,25-$(OH)_2D$ concentration around the time of parturition in control cows and cows treated with 24-F-1,25-$(OH)_2D_3$

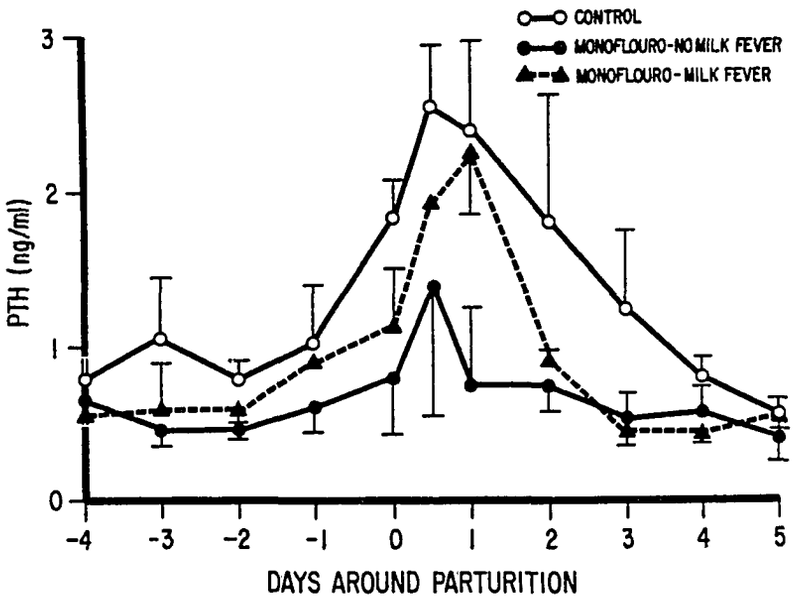

Figure 3. Plasma immunoreactive PTH concentration around the time of parturition in control cows and cows treated with 24-F-1,25-$(OH)_2D_3$

Table 1. Summary of animal allocation and clinical results within each experimental group[a]

Control cow	Calcium minimum observed	Day of occurrence	No. of relapses	Interval from treatment to parturition
		Control		
1	4.9	+2	No-PP	N/A
2	3.8	+1	0	N/A
3	4.5	+1	0	N/A
4	3.7	+1.5	0	N/A
5	3.7	+2	1	N/A
6	3.3	+0.5	0	N/A
7	4.7	0	0	N/A
8	4.3	0	2	N/A
9	3.5	+0.5	0	N/A
10	4.0	0	0	N/A
11	3.5	+1	1	N/A
12	3.8	+12	0	N/A
		100 µg 24-F-1,25-$(OH)_2D_3$		
1	6.0	+0.5	No-PP	3
2	5.5	+1	No-PP	7
3	6.0	+2	No-PP	3
4	7.6	+2	No-PP	3**
5	5.0	-0.5	0	1*
6	4.3	+0.5	0	7
7	2.7	+1	0	7
		150 µg 24-F-1,25-$(OH)_2D_3$		
1	7.7	+4	No-PP	6*
2	6.2	+9	No-PP	1
3	5.6	+3	No-PP	7
4	6.1	+2	No-PP	6
5	7.2	0	No-PP	0.5*
6	5.8	0	No-PP	0.5**
7	6.1	+0.5	No-PP	0***
8	6.2	+7	No-PP	2
9	4.5	+0.5	0	1
10	4.1	+11	0	0.5*

[a]No-PP = Did not develop clinical parturient paresis. N/A = Not applicable. *After 2nd dose of 24-F-1,25-$(OH)_2D_3$. **After 3rd dose of 24-F-1,25-$(OH)_2D_3$. ***Calved within 4 hours after 2nd dose.

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SECTION III.

EFFECT OF SYNTHETIC BOVINE PARATHYROID HORMONE IN DAIRY COWS: PREVENTION OF PARTURIENT PARESIS (MILK FEVER)

INTRODUCTION

Parturient paresis (milk fever) is primarily a hypocalcemic disorder of dairy cows associated with the onset of lactation. Though the predisposing factors, clinical signs, and clinical pathology of the syndrome have been well characterized, the basic cellular and biochemical mechanisms that cause parturient paresis are not well understood. In 1925, Dryerre and Greig suggested that the hypocalcemia might be the result of insufficient parathyroid hormone (PTH) secretion. However, subsequent workers using radioimmunological and histologic techniques concluded that PTH secretion in response to hypocalcemia in cows developing parturient paresis is equal to or greater than that of nonparetic cows (Capen and Young, 1967; Horst et al., 1978; Mayer et al., 1969). Several research groups (Hibbs et al., 1947; Jackson et al., 1962; Little and Mattick, 1933) injected cows with crude extracts of PTH and found that older prepartal cows were less responsive to PTH than young cows and that PTH administration did not prevent parturient paresis. Martig and Mayer (1972) observed that the prepartum cow responded to exogenous PTH, although this response was blunted when compared with the postpartal response. These findings lead to the current theory that the responsiveness of the target tissues to PTH stimulation may be deficient or delayed in the periparturient cow.

Since these earlier reports, the physiologic function of PTH and its interactions with the vitamin D endocrine system have been

discovered. PTH is secreted in response to hypocalcemia and in turn stimulates renal conservation of calcium and release of calcium from bone stores. Also, by activating renal enzymes, PTH plays an intimate role in the production of 1,25-dihydroxyvitamin D [1,25-$(OH)_2D$], which increases intestinal calcium absorption (DeLuca, 1979).

Parturient paresis can be prevented by feeding cows very low calcium diets (< 20 g/d) for 2 wk prior to parturition (Goings et al., 1974). This stimulates endogenous PTH secretion which in turn increases renal production of 1,25-$(OH)_2D$. Together, PTH and 1,25-$(OH)_2D$ stimulate mechanisms that resorb bone calcium and increase the active transport of calcium across the intestinal epithelium (Green et al., 1981; Kichura et al., 1982). The effectiveness of the low calcium diet in preventing parturient paresis suggested that calcium homeostatic mechanisms had to be primed during the prepartal period if they were to be fully responsive to the calcium demand associated with the onset of lactation.

The purpose of the present study was to determine if exogenous PTH administration could also prime the calcium homeostatic mechanisms and thus prevent parturient paresis.

MATERIALS AND METHODS

Pregnant Cow Experiment

Nine Jersey cows in the last trimester of pregnancy, weighing between 380 and 440 kg, were kept in individual stanchions and fed a complete pelleted diet that supplied 10.1 MCal Net Energy, 0.83 kg protein, 68 g calcium, and 35 g phosphorus daily.

Teflon catheters were placed in one external jugular vein under local anesthesia 12 to 20 h before the start of the experiment. At the same time, a noninvasive urine collection device was sutured to the cow's perineal region under standing epidural anesthesia. Urine was collected in polyurethane bottles to which 100 ml glacial acetic acid had been added as a preservative before the start of each collection period.

All cows were infused intravenously with 0.9% saline containing 0.2% bovine serum albumin (BSA) without PTH for 32 to 48 h and then infused with 0.9% saline containing BSA and PTH in accord with one of three treatment regimens. Bovine serum albumin was added to infusion solutions to minimize loss of PTH activity as a result of nonspecific binding of protein to glass and tubing. Highly-purified BSA (essentially fatty acid and globulin-free, Sigma Chemical Co., St. Louis, MO) was used to minimize introduction of endotoxins into infusion solutions. Crude synthetic N-terminal 1-34 fragment of bovine PTH (lot #007242, Peninsula Laboratories, Inc., San Carlos, CA) containing 767 units/mg (Nissenson et al., 1981) was given to four

cows at a rate of 146 µg/h for 48 h (3.5 mg/d), to three cows at a rate of 146 µg/h for 96 h (3.5 mg/d), and to two cows at a rate of 73 µg/h for 96 h (1.7 mg/d). The PTH solution was prepared fresh every 24 h and contained 1 to 2 ml of a stock solution of PTH in 0.01 M acetic acid diluted in sterile 0.9% saline containing 0.2% BSA. Infusate solutions were maintained in ice water and delivered at a rate of 0.5 ml/min by means of an external infusion pump (Rainin Instrument Co., Inc., Emeryville, CA).

Blood samples were taken by subcutaneous abdominal (milk vein) venipuncture at regular intervals throughout the experiment. Plasma was separated immediately by centrifugation and stored at -15°C until analyzed. Urine was collected over 8- or 12-h periods throughout the experiment. Volumes were recorded, and a representative sample was frozen for analysis.

Periparturient Cow Experiment

Eight periparturient Jersey cows, weighing between 400 and 450 kg, were selected from the National Animal Disease Center research herd. All cows had at least three previous lactations and all had histories of parturient paresis at a previous calving. Six-wk prepartal cows were fed alfalfa hay ad libitum and 4.5 kg concentrate feed that supplied approximately 110 g calcium and 33 g phosphorus daily. Approximately 2 wk prepartum, the cows received an additional 7.3 kg of concentrate daily so that their diet contained approximately

150 g calcium and 78 g phosphorus per d in the last days of gestation and for the first 2 wk of lactation.

Approximately 3 wk prepartum, a small programmable titanium pump (Medtronics Inc., Minneapolis, MN) was implanted under the skin of four of the cows. In two of the cows, the pump was located in the paralumbar fossa and was connected to a teflon catheter placed in the circumflex iliac vein. The pump was implanted in the neck in two other cows and connected to a catheter in the jugular vein. All surgery was performed under local anesthesia (lidocaine). Ace-promazine (20 mg, Fort Dodge Laboratories, Fort Dodge, IA) was used as a sedative. High epidural spinal block was used in the case of the cows with the catheterized circumflex iliac vein. The pump had a 20-ml reservoir that could be refilled by injection through the skin into a silicone port on the pump. Four other cows (that did not undergo surgery) served as controls.

All four treated cows received saline at a rate of 8.7 ml/d from the time of surgery until the PTH solution was administered. PTH solutions were made up every 48 h to refill the pump by dilution of stock PTH (in 0.01 M acetic acid) with 0.1% bovine serum albumin saline.

Cow #1 received PTH at a rate of 217 µg/h from 60 h prepartum to 12 h prepartum, at which time the infusion rate was decreased to 104 µg/h and continued until 55 h postpartum.

Cow #2 received PTH at a rate of 104 µg/h from 116 h prepartum until 55 h postpartum.

Cow #3 received PTH at a rate of 58 µg/h from 16 h prepartum to 5 h postpartum, at which time the rate was increased to 146 µg/h and continued until 48 h postpartum. At that time, the infusion rate was returned to 58 µg/h for another 48 h.

Cow #4 received PTH at a rate of 104 µg/h beginning 10 h prepartum and continuing until 96 h postpartum.

Plasma samples were obtained by jugular venipuncture on the side opposite the pump daily prior to parturition and for 14 d postpartum. Around the time of parturition sampling was more frequent.

Parameters Monitored

Concentrations of calcium and magnesium in plasma and urine were determined by atomic absorption spectrophotometry (Perkin-Elmer Corporation, 1965). Plasma and urine inorganic phosphorus concentrations were determined colorimetrically (Fiske and Subbarrow, 1925).

Concentrations of hydroxyproline in plasma and urine were monitored as an estimate of bone resorption activity (Dull and Henneman, 1963). Free plasma hydroxyproline and total urinary hydroxyproline were determined colorimetrically (Bannister and Burns, 1970; Hosley et al., 1970). Renal function was assessed by monitoring urine specific gravity and renal glomerular filtration rate. Glomerular filtration rate was estimated by determining endogenous creatinine clearance rate. Plasma and urine creatinine concentrations were determined colorimetrically on an AutoTechnicon Analyzer (Chasson et al., 1961). Plasma concentrations of 1,25-$(OH)_2D$ were determined

as described by Reinhardt et al. (1984). Plasma PTH concentrations were determined by radioimmunoassay using an antibody directed against middle and N-terminal portions of the PTH molecule (Arnaud et al., 1971).

The differences between the observed values at each time point during the infusion and the values observed during the control period prior to infusion were determined for each parameter measured. Student's paired t test was used to test the hypothesis that the mean difference was equal to zero.

RESULTS

Pregnant Cow Experiment

Parathyroid hormone infusion at 146 μg/h for 48 h resulted in significant changes in plasma calcium, 1,25-$(OH)_2D$, and magnesium concentrations (Table 1, Figure 1). Plasma calcium was increased 32% ($P < 0.05$), while plasma phosphorus was decreased 30% ($P < 0.05$) after 48 h of infusion. Plasma concentration of magnesium increased more rapidly than did calcium concentration and also decreased more rapidly once PTH infusion ended. No significant changes were seen in plasma hydroxyproline or plasma PTH concentrations.

Plasma 1,25-$(OH)_2D$ concentrations increased within 8 h (Figure 2) and were maximal between 24 and 32 h after the start of infusion, at which time they were 4 to 5 times pretreatment levels. Although plasma 1,25-$(OH)_2D$ concentrations decreased as infusion of PTH continued, they remained above preinfusion levels. Within 48 h after PTH infusion ended, plasma 1,25-$(OH)_2D$ concentrations were equivalent to pretreatment concentrations.

Parathyroid hormone caused a rapid decrease in urinary excretion of calcium and magnesium within 24 h of the start of infusion. Once PTH infusion was stopped, urinary excretion of calcium increased to nearly four times the rate during the control period. In contrast, there was no excessive loss of magnesium via the urine when PTH was withdrawn. Urinary phosphorus excretion was significantly increased ($P < 0.05$) by PTH infusion and remained high for 48 h after PTH was

withdrawn. Urinary excretion of hydroxyproline was not increased by 48 h of infusion of 146 μg PTH/h.

The effects of infusion of PTH for 96 h were similar to those seen when PTH was administered for only 48 h (Table 2, Figure 1). Plasma calcium concentration increased linearly as long as PTH infusion was maintained (Figure 1). Plasma concentration of $1,25\text{-}(OH)_2D$ was greatly elevated by PTH infusion initially. However, as PTH infusion continued, the mean plasma concentration of $1,25\text{-}(OH)_2D$ decreased so that by the end of the infusion period plasma $1,25\text{-}(OH)_2D$ concentration was only about two times the pretreatment level (Figure 2). Plasma concentration of hydroxyproline was significantly increased after 72 h of PTH infusion ($P < 0.05$) and continued to increase until PTH infusion ceased. Plasma hydroxyproline concentration returned to pretreatment level within 24 h after PTH withdrawal.

Urinary losses of calcium, phosphorus, and magnesium paralleled those changes seen during the 48-h infusion experiment (data not shown). An important difference was that urinary excretion of hydroxyproline was significantly increased ($P < 0.05$) between 48 and 96 h of PTH infusion (Figure 3). Urinary hydroxyproline excretion rapidly returned to pretreatment levels when infusion ceased.

Between 72 and 96 h of the infusion period, feed intake was greatly reduced. This corresponds to the time of maximal hypercalcemia. Appetite returned to normal about 48 h after PTH infusion ceased.

When 73 µg PTH/h was administered for 96 h, there were no significant changes in plasma concentrations of calcium, hydroxyproline, 1,25-$(OH)_2D$, magnesium, phosphorus, or PTH (Table 2). There were no significant changes in the urinary excretion rates for calcium, magnesium, or hydroxyproline. However, 73 µg PTH/h did increase urinary phosphorus excretion ($P < 0.05$) during the infusion period (data not shown).

Plasma concentrations of immunoreactive PTH (iPTH) were not significantly increased during any of the PTH infusions, even at the 146 µg PTH/h dose (Table 1). Plasma PTH levels did not decrease significantly once PTH infusion ceased.

Parathyroid hormone treatment did not result in any significant changes in renal glomerular filtration rate during the course of these trials (data not shown). Urine specific gravity was lower at the end of the 146 µg/h infusion periods than before infusion, but the cows did not exhibit any other indications that renal function was impaired.

Periparturient Cow Experiment

All four of the cows that did not receive PTH infusions developed severe hypocalcemia, became recumbent with parturient paresis (Figure 4), and required treatment with an intravenous solution containing calcium, magnesium, and phosphorus (Norcalciphos, Norden Labs., Lincoln, NE). One control cow suffered a relapse, requiring a second treatment.

The two cows that received PTH intravenously for at least 60 h before parturition (Nos. 1 and 2, Figure 5) did not become hypocalcemic at any time during the experiment. Both cows were actually hypercalcemic at parturition in contrast to the control cows. The two cows that began receiving PTH intravenously less than 24 h prior to parturition (Nos. 3 and 4, Figure 6) were hypocalcemic at parturition and shortly after. Cow #3 never exhibited any symptoms of parturient paresis, though her plasma calcium was 5.8 mg/100 ml at 18 h postpartum. Between 18 and 36 h postpartum, her plasma calcium rose rapidly and she became slightly hypercalcemic. Cow #4 exhibited several signs of parturient paresis, though she never became recumbent. She was dull, inappetant, and exhibited muscle tremors. She remained severely hypocalcemic (approximately 4.0 mg/100 ml) during the 48 h following parturition. Her plasma calcium then increased rapidly until by 96 h postpartum she was slightly hypercalcemic, at which time the PTH infusion was ended. None of the PTH-treated cows developed any significant hypocalcemia from the time PTH infusion was ended until the time sampling ceased (day 14 postpartum).

The control cows exhibited great increases in plasma concentrations of PTH and 1,25-$(OH)_2D$ which were inversely proportional to the degree of hypocalcemia present (Figure 4). The two PTH-treated cows that became hypocalcemic also responded by greatly increasing plasma PTH and 1,25-$(OH)_2D$ concentrations (Figure 6). The two cows that received PTH and did not develop hypocalcemia did not have greatly

elevated levels of PTH or 1,25-$(OH)_2D$ during the infusion period (Figure 5).

DISCUSSION

Parathyroid hormone is an important regulator of renal 1α-hydroxylase and, therefore, synthesis of 1,25-$(OH)_2D$, which is necessary for active transport of calcium across the intestinal epithelium. While it is clear that PTH can stimulate 1α-hydroxylase activity under hypocalcemic conditions (Aksnes and Aarskog, 1980; Treschel et al., 1980; Kremer and Goltzman, 1982), the effects of PTH under normocalcemic or even slightly hypercalcemic conditions are not clearly defined.

Hove et al. (1984) reported that PTH infusion increased plasma 1,25-$(OH)_2D$ concentrations when administered to thyroparathyroidectomized lactating goats that were hypocalcemic, but not if the goats were hypercalcemic. This suggested that hypercalcemia may block PTH stimulation of renal 1α-hydroxylase.

When we administered 146 μg PTH/h to pregnant dairy cows in late gestation, a fivefold increase in plasma concentrations of 1,25-$(OH)_2D$ developed after 32 h of infusion. The concentration of 1,25-$(OH)_2D$ decreased as the PTH infusion continued. However, even after 2 d of hypercalcemic conditions, plasma concentrations of 1,25-$(OH)_2D$ remained above pretreatment levels. We conclude that hypercalcemia blunts the responsiveness of plasma 1,25-$(OH)_2D$ to exogenous PTH, but does not eliminate it. In support of this conclusion is the observation that plasma 1,25-$(OH)_2D$ concentrations in humans with primary

hyperparathyroidism are often elevated despite hypercalcemia (Broadus et al., 1980; Gray et al., 1977; Haussler et al., 1975).

Parathyroid hormone administration to pregnant cows dramatically increased urinary excretion of phosphorus while decreasing urinary calcium losses as seen in earlier experiments (Mayer et al., 1966). In contrast to PTH effects in humans (Aksnes and Aarskog, 1980), phosphaturia is not an immediate response to PTH administration in cows. We did not detect significant phosphaturia in the cows until after 38 to 48 h of PTH infusion. This also coincides with the time of development of hypercalcemia in the cows. Possibly, calcitonin secretion in response to the hypercalcemia may be responsible for much of the phosphaturia seen (Munson, 1976). PTH decreased urinary calcium loss, but the total amount of calcium conserved was actually less than 500 mg/d for most cows.

Plasma hydroxyproline concentration and urinary hydroxyproline excretion were increased at 72 and 96 h of PTH infusion at 146 µg/h. The 3-day lag time between the start of PTH infusion and the stimulation of significant bone resorption may explain the failure of PTH infusion to prevent hypocalcemia in those pregnant cows that calved less than 24 h after the start of PTH infusion. If maintained for at least 3 d, exogenous PTH can stimulate bone resorption in the pregnant cow.

Infusion of bovine N-terminal 1-34 PTH had obvious physiologic effects on the cows; however, we could not detect any increase in plasma PTH concentration. Depending on the antisera used,

radioimmunoassay of PTH detects varying amounts of both active and inactive fragments of PTH. Bioassays of plasma PTH concentrations in man indicate that only about 1% of the radioimmunoassayable PTH is biologically active (Nissenson et al., 1981). Therefore, even if PTH infusion into cows greatly increased the amount of biologically active PTH circulating in plasma, it is unlikely it could be detected by radioimmunoassay. Our data suggest that the ability of cows to secrete or produce biologically active PTH in the prepartal period may not be adequate. Whether this is a defect in the form or amount of PTH secreted or in peripheral metabolism necessary for activation of PTH is not known. Characterization of the forms of PTH secreted and its peripheral metabolism in the periparturient cow will be crucial to establish the fundamental pathogenesis of parturient paresis.

The management of our experimental herd of Jersey cows results in an extremely high incidence of parturient paresis. Of the 28 cows that have been subjected to our high-calcium feeding regime, 26 developed parturient paresis. Prepartal PTH administration to the four periparturient cows in this study prevented parturient paresis in all four cows and the development of hypocalcemia in two of the cows. Parathyroid hormone may be able to prevent parturient paresis because it stimulates bone resorption of calcium and enhances intestinal absorption of calcium. Although PTH can cause a significant reduction in urinary calcium losses, the amount of calcium conserved is not likely to contribute significantly to the maintenance of plasma calcium in the paresis-prone cow.

Vitamin D metabolites have been used to prevent parturient paresis (Bar et al., 1985; Barlet, 1977; Gast et al., 1979; Hibbs and Pounden, 1955; Hoffsis et al., 1978; Hove and Kristiansen, 1982; Jorgenson et al., 1978; Julien et al., 1977; Littledike and Horst, 1979; Manston and Payne, 1964; Olsen et al., 1972), but they act only to enhance intestinal calcium absorption (Braithwaite, 1978; Hove, 1984). They do not increase bone resorption in the cow (Goff et al., 1986), probably because the hypercalcemia resulting from enhanced intestinal calcium absorption inhibits endogenous secretion of PTH necessary for bone resorption. In cows pretreated with vitamin D metabolites that develop parturient paresis, the endogenous $1,25\text{-}(OH)_2D$ response to hypocalcemia is inhibited (Littledike et al., 1981). When vitamin D metabolites fail to prevent hypocalcemia, the resulting parturient paresis is often clinically more severe than normal parturient paresis (Littledike and Horst, 1979), suggesting that vitamin D compounds replace the cow's calcium homeostatic mechanisms rather than augment them. In the two cows (Nos. 3 and 4) in the present study that became hypocalcemic despite PTH infusion, plasma concentrations of PTH and $1,25\text{-}(OH)_2D$ indicate that there is no suppression of endogenous PTH or $1,25\text{-}(OH)_2D$ production in response to hypocalcemia. In contrast to the vitamin D compounds, it appears that exogenous PTH may augment the cow's homeostatic mechanisms rather than replace them.

There was no evidence of renal toxicity during the administration of PTH to cows in these experiments. Because PTH lowers plasma

phosphorus as it increases plasma calcium concentrations, the danger of metastatic calcification due to PTH administration should be less than that seen with the vitamin D compounds. However, the hypercalcemic activity of PTH dictates that the nephrotoxic potential of PTH needs to be carefully characterized.

Parathyroid hormone may prove superior to vitamin D administration to prevent parturient paresis for several reasons. It is capable of stimulating bone resorption as well as intestinal calcium absorption at the time of parturition. Because it lowers plasma phosphorus, any hypercalcemia it induces should be less toxic than the hypercalcemia and hyperphosphatemia induced by vitamin D compounds. Parathyroid hormone administration should present little risk to the consumer of milk or meat from treated animals as it is inactivated when ingested. These considerations and the results of this study lead us to conclude that PTH has potential for therapeutic intervention in dairy management.

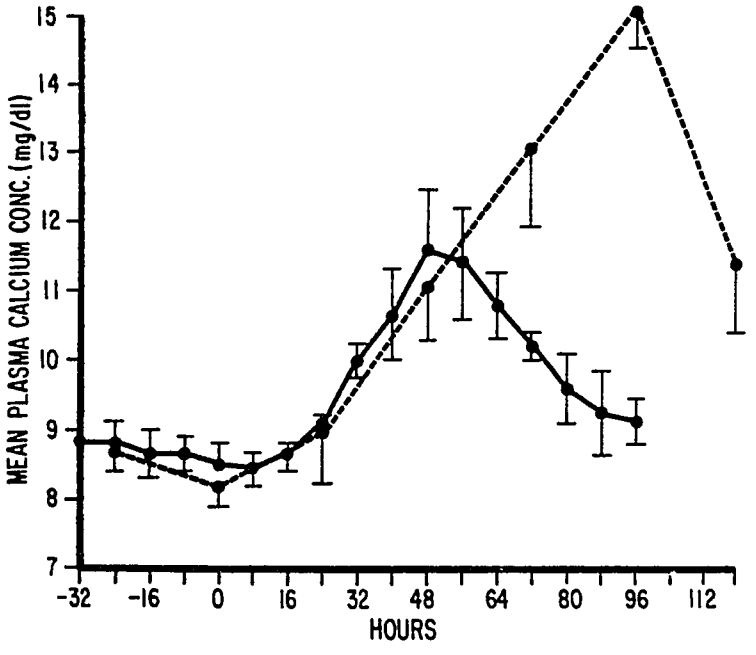


Figure 1. Plasma calcium concentration of pregnant cows that received intravenous infusion of synthetic bovine PTH-(1-34) at a rate of 146 µg/h. Mean ± SE of four cows that received PTH between h 0-48 (0——0). Mean ± SE of three cows that received PTH between h 0-96 (0----0)

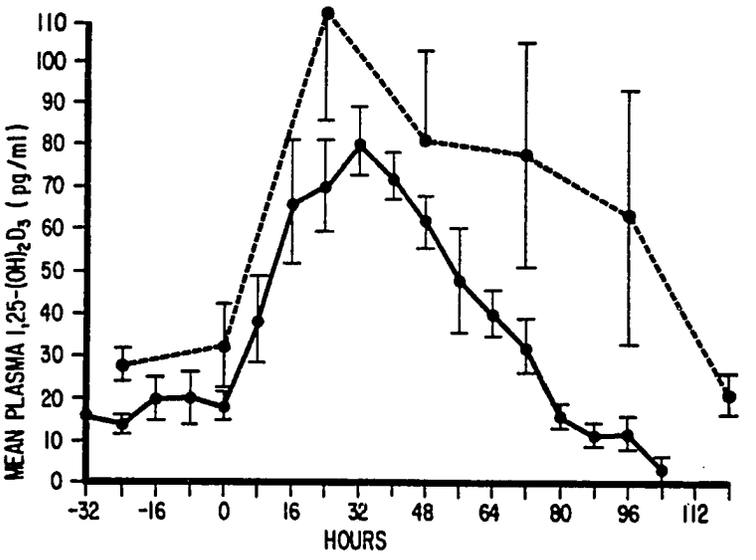


Figure 2. Plasma 1,25-$(OH)_2D$ concentration of pregnant cows that received intravenous infusion of synthetic bovine PTH-(1-34) at a rate of 146 µg/h. Mean ± SE of four cows that received PTH between h 0-48 (O——O). Mean ± SE of three cows that received PTH between h 0-96 (O----O)

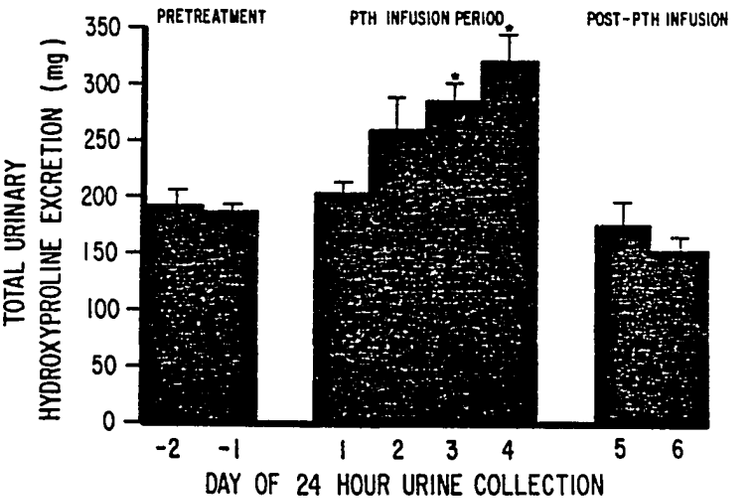
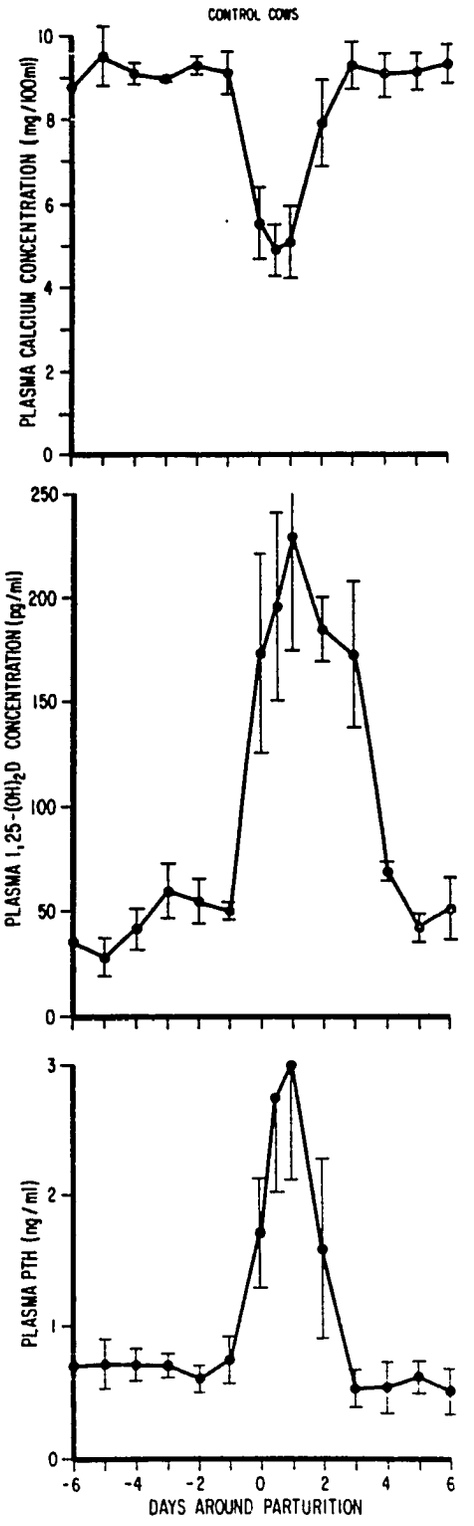
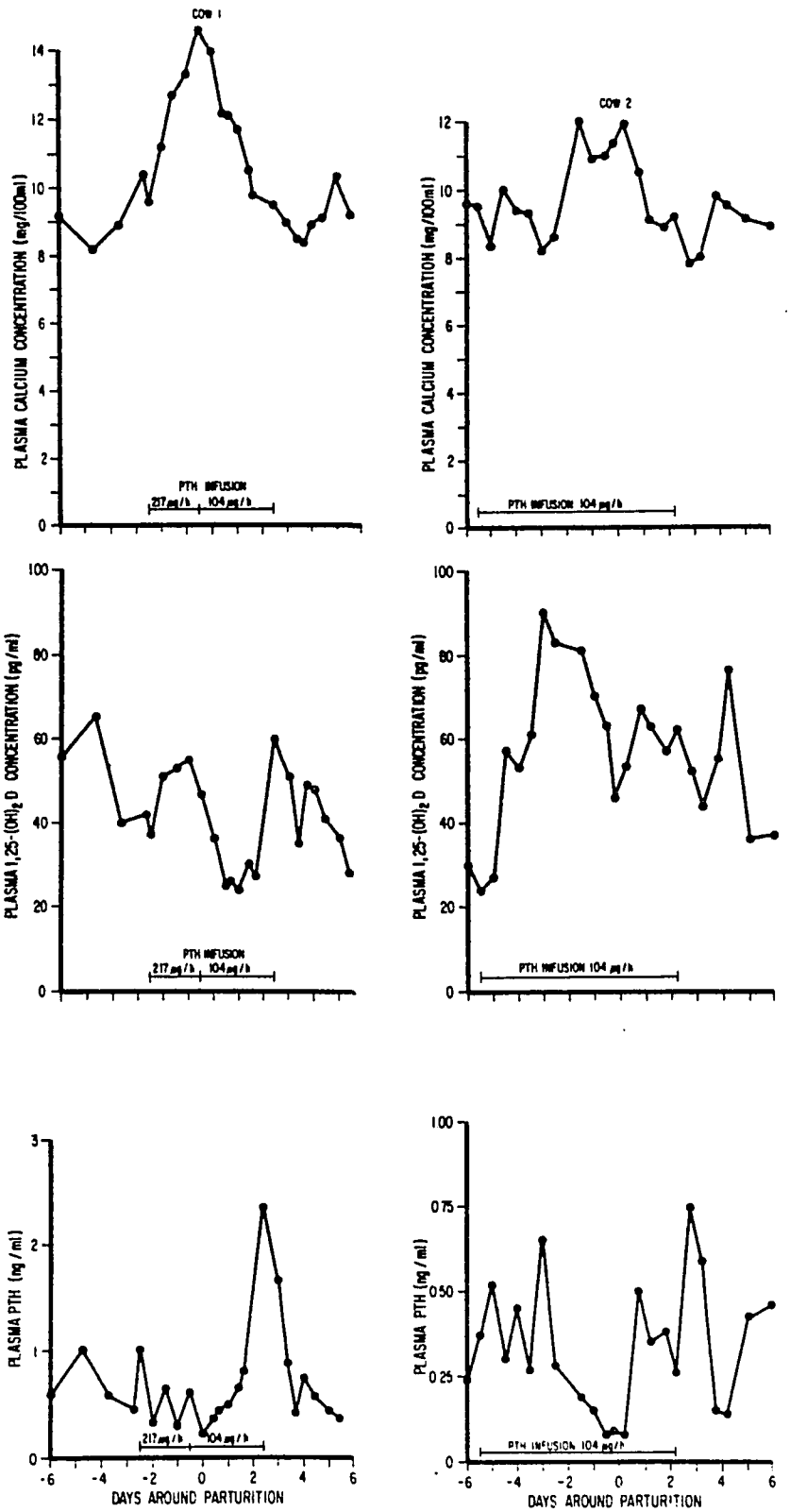


Figure 3. Daily total urinary hydroxyproline excretion before, during, and after treatment of three pregnant cows with synthetic bovine PTH-(1-34) at a rate of 146 µg/h for 96 h. Mean ± SE. Astrisk denotes significantly different from pretreatment ($P < 0.05$)


CONTROL COWS
PLASMA CALCIUM CONCENTRATION (mg/100ml)
10
8
6
4
2
0
PLASMA 1,25-(OH)2D CONCENTRATION (pg/ml)
250
200
150
100
50
0
PLASMA PTH (ng/ml)
3
2
1
0
-6
-4
-2
0
2
4
6
DAYS AROUND PARTURITION


Cow 1
Cow 2
PLASMA CALCIUM CONCENTRATION (mg/100ml)
PTH INFUSION
217 μg/h
104 μg/h
PTH INFUSION 104 μg/h
PLASMA 1,25-(OH)2 D CONCENTRATION (pg/ml)
PLASMA PTH (ng/ml)
DAYS AROUND PARTURITION

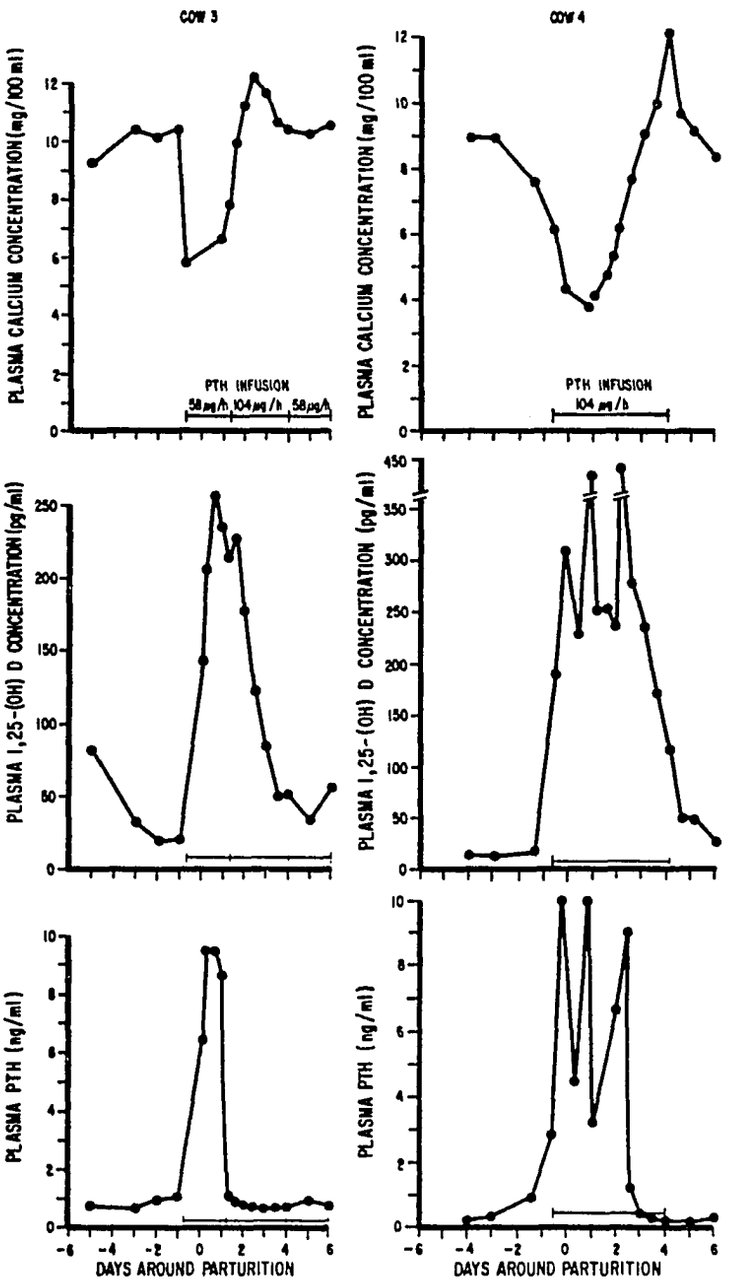

COW 3
COW 4
PLASMA CALCIUM CONCENTRATION (mg/100 ml)
PTH INFUSION
58 µg/h 104 µg/h 58 µg/h
104 µg/h
PLASMA 1,25-(OH) D CONCENTRATION (pg/ml)
PLASMA PTH (ng/ml)
DAYS AROUND PARTURITION

Table 1. Plasma concentrations of minerals, 1,25-$(OH)_2D$, PTH and OH
OH-proline 24-h excretion rates in cows in late gestation
146 µg PTH/h for 48 h (mean ± SE, n = 4)

	Control	PTH infusion	
		Day 1	Day 2
Plasma concentrations			
Calcium (mg/dl)	8.8 ± 0.2	9.8 ± 0.3*	11.6 ± 0
Phosphorus (mg/dl)	5.7 ± 0.4	4.7 ± 0.6*	4.0 ± 0
Magnesium (mg/dl)	2.10 ± 0.09	2.78 ± 0.11**	2.57 ± 0
1,25-$(OH)_2D$ (pg/ml)	16.7 ± 2.3	78.0 ± 10.6***	57.5 ± 1
OH-Proline (µg/ml)	1.23 ± 0.14	1.41 ± 0.25	1.54 ± 0
PTH (ng/ml)	0.52 ± 0.12	0.67 ± 0.17	0.58 ± 0
Urinary excretion rates			
Calcium (mg/h)	31.3 ± 7.9	6.5 ± 1.3**	7.7 ± 4.
Phosphorus (mg/h)	274 ± 91	310 ± 48	789 ± 61
Magnesium (mg/h)	21.6 ± 2.5	15.9 ± 3.4**	14.8 ± 2.
OH-Proline	5.45 ± 2.17	6.70 ± 3.49	7.43 ± 4.

*$P < 0.10$. **$P < 0.05$. ***$P < 0.025$

OH-proline and urinary mineral and
on before, during and after they received

	Recovery	
y 2	Day 1	Day 2
± 0.7**	9.7 ± 0.4	8.8 ± 0.3
± 0.2**	6.4 ± 0.9	5.9 ± 0.4
± 0.10*	1.70 ± 0.06***	1.79 ± 0.01**
± 13.2**	21.0 ± 2.1	15.0 ± 1.0
± 0.36	1.28 ± 0.19	1.48 ± 0.03
± 0.07	0.46 ± 0.17	0.63 ± 0.01
± 4.6*	140.7 ± 51.0*	78.0 ± 18.6
± 61**	1059 ± 220***	1222 ± 194***
± 2.3***	17.8 ± 4.2	10.9 ± 3.3*
± 4.20	5.81 ± 2.30	5.85 ± 2.14

Table 2. Plasma calcium, OH-proline, and 1,25-$(OH)_2D$ concentrations dur in cows in late gestation (mean ± SE)

	Control	PTH infusion		
		Day 1	Day 2	Day 3
73 ug PTH/h for 96 h (n = 2)				
Calcium (mg/ml)	9.7 ± 0.1	9.4 ± 0.4	9.6 ± 0.4	9.3 ± 0.0
1,25-$(OH)_2D$ (pg/ml)	6.1 ± 1.6	20.5 ± 6.5	20.5 ± 1.5	18.0 ± 10.0
OH-Proline (µg/ml)	0.86 ± 0.20	1.00 ± 0.30	0.89 ± 0.26	0.95 ± 0.25
146 ug PTH/h for 96 h (n = 3)				
Calcium (mg/dl)	8.2 ± 0.3	9.0 ± 0.9	11.1 ± 0.8*	13.1 ± 0.1*
1,25-$(OH)_2D$ (pg/ml)	32.1 ± 10.1	113.3 ± 26.8**	81.0 ± 21.5	78.3 ± 26.8
OH-Proline (µg/ml)	0.98 ± 0.11	1.10 ± 0.19	1.20 ± 0.19	1.37 ± 0.16

*$P < 0.10$. **$P < 0.05$. ***$P < 0.025$

uring the infusion of PTH for 96 h

	Day 4	Recovery Day 1	Day 2
	10.3 ± 0.4	9.7 ± 0.2	9.5 ± 0.8
0	20.0 ± 5.0	11.0 ± 8.0	20.5 ± 9.8
5	0.95 ± 0.35	1.15 ± 0.35	
L*	15.1 ± 1.5**	11.4 ± 1.0*	9.3 ± 0.4
.8	63.7 ± 28.6	21.3 ± 5.1	8.7 ± 4.9
16**	1.54 ± 0.11***	1.01 ± 0.01	0.94 ± 0.06

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SUMMARY AND DISCUSSION

Feeding cows a low calcium diet prior to parturition is an effective, but not universally practical, means of preventing parturient paresis. The effectiveness of this method is probably due to the stimulation of PTH and 1,25-dihydroxyvitamin D production prior to parturition which primes the bone and gut so that the calcium demands of lactation can be met. In Section I of this thesis, the effects of administration of 1,25-dihydroxyvitamin D and its analogues were examined. We found that vitamin D action on the intestine was the only positive prophylactic effect one could expect in the cow. These studies also determined that renal function can be impaired by higher doses of these compounds. In Section II of this thesis, we have demonstrated that 24-F-1,25-dihydroxyvitamin D can be successfully used to prevent parturient paresis in dairy cows and that it may be superior to 1,25-dihydroxyvitamin D for this purpose. However, the study also points out that the vitamin D compounds are only effective when given within a certain time frame prior to parturition.

In Section III of this thesis, we have demonstrated that PTH can prevent parturient paresis in dairy cows. We have demonstrated that it acts by stimulating bone resorption, renal conservation of calcium and production of 1,25-dihydroxyvitamin D which, in turn, stimulates intestinal calcium absorption. We conclude that PTH has tremendous potential for prevention of parturient paresis. The obstacle to its use will be cost of the hormone and a method of delivery of the PTH.

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