EFFECT OF VITAMIN D_3 ON CALCIUM HOMEOSTASIS OF BEEF STEERS

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ABSTRACT

Supplementation of high levels of vitamin D_3 (D_3) to cattle has enhanced beef tenderness. However, high levels of D_3 possess a food safety concern. Therefore, the objective of this thesis was to evaluate feeding strategies that would reduce the amount of D_3 required to increase serum calcium (Ca) to levels where tenderness has been improved. In experiment 1, 15 steers (452 ± 30kg) were fed with 75% barley grain-base concentrate and 25% barley silage (as fed) and 1.25, 2.5 or 5 MIU D_3/hd/d for 7 d. Feed intake was depressed ($P<0.05$) while total (T) and ionized (I) Ca were increased ($P<0.05$) in relationship to the amount of D_3 fed. Feeding 5 MIU D_3 increased TCa and ICa by 2 and 1 mg/dl, respectively. Peak values were reached 3-5 d post-supplementation. Serum Ca levels were similar to those related with meat tenderization. In experiment 2, 15 steers (607 ± 12 kg) were supplemented with 0, 2.5 or 5 MIU D_3/hd/d for 7 d. Feed intake, TCa, and ICa showed similar trends as in experiment 1. Plasma D_3 and $25(OH)D_3$ were increased ($P<0.05$) quadratically and linearly, respectively. Parathyroid hormone (PTH) was depressed ($P<0.05$). In experiment 3, 19 steers (522 ± 33 kg) were fed with a low Ca diet for 10 d prior to supplementation with 0, 1.25, 2.5 or 5 MIU D_3/hd/d for 7 d. Blood TCa, ICa, and D_3 decreased ($P<0.05$) during the first 2-4 d of the low Ca period. All variables responded as in experiment 2. Plasma $1,25(OH)_2D_3$ was increased ($P<0.05$). Calcium response was no higher. In experiment 4, 20 steers (448 ± 26 kg) were fed a low Ca for 14 d and then switched to a high Ca diet and anionic salts (NH_4Cl and $MgSO_4$) at –1500 and –3000 mEq/hd/d for 3 and 7 d, respectively, and fed with 0, 0.6, 1.2 or 2.4 MIU D_3/hd/d (10 d). All variables responded as in experiment 3. Similar

total and ionized serum Ca levels were achieved with 30 to 80 % less D_3, indicating that such a feeding strategy may be a successful approach to improving beef tenderness.

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DEDICATION

I would like to dedicate this thesis

To

My Parents

Vicente Aranda Nicolas and *Veda Osorio Osorio*

For having sown in me the principles of discipline,
respect and admiration for life.
For having taught me to believe in my dreams...
"dream and truly believe in them, just be sure that
you put enough hope and work, and
sooner or later they will come true".
For their love, care and support
throughout my life

To

My Brothers and Sisters and their Families

To

My Relatives and Friends in Mexico

To

My Latin American-Canadian Friends

To

Carlos Gilberto

Hoping he could learn to believe in his dreams as well

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LIST OF ABBREVIATIONS

$1,25(OH)_2D_3$	1,25-Dihydroxyvitamin D_3 or calcitriol
$25(OH)D_3$	25-hydroxyvitamin D_3 or calcidiol
AS	Anionic salts
BW	Body weight
Ca	Calcium
Ca^{2+}	Ionized calcium
CT	Calcitonin
d	Day
D_3	Vitamin D_3 or cholecalciferol
dl	Deciliter
DM	Dry matter
g	Gram
HCa	High Ca diet
hd	Head
ICa	Ionized calcium
kg	Kilogram
LCa	Low Ca diet
M	Moles / Molar
mg	Milli gram
MIU	Million international units
mEq	Milli equivalents
ml	Milliliter
NCa	Normal Ca diet
ng	Nanograms
P	Probability
pg	Picograms
PTH	Parathyroid hormone
SEM	Standard error of the mean
TCa	Total serum calcium

CHAPTER I

INTRODUCTION

Since domestication beef cattle have been a source of highly nutritious food for people around the world. Intense and continuous research in genetics, nutrition, reproduction, and management has resulted in the production of highly specialized animals that grow faster and produce beef of excellent quality. Beef quality is determined by its color (both meat and fat), intramuscular fat content, firmness, lean content and tenderness (Greaser 1986; Hedrick *et al.* 1994; Canadian Beef Grading System). These factors are used to predict eating quality. Of all the attributes of eating quality, tenderness is considered by consumers to be the most important factor (Morgan *et al.* 1991a; Smith *et al.* 1995). Generally, consumers are willing to pay a premium for meat known to be tender (Boleman *et al.* 1997). However, there is concern within the industry with respect to the tenderness of today's beef, in particular, the lack of consistency and uniformity (Morgan *et al.* 1991a; Smith *et al.* 1995). Inconsistency in beef tenderness can have a negative effect on the consumer's preference for beef relative to other meats and/or more importantly affect international markets (reducing beef exports). In Canada, beef has lost market share in comparison to other meats over the past decade. At this moment the consumption of beef *per capita* (23 kg) is similar to pork (22 kg), but less than chicken (29 kg) (Canfax 2002). If this trend continues, the contribution of the beef industry to the national economy will be seriously affected. On the other hand, Canada is the third largest exporter of beef in the world (Canfax 2002). To maintain or increase the volume of exports, it is important to assure a product of consistent quality. Morgan *et al.* (1991a) found that current beef production practices result in considerable variation in beef tenderness and an unacceptable percentage (> 20 %) of tough meat. Therefore, methods that can be used to improve beef tenderness and lead to the production of uniform and consistent products will be of particular importance to the beef industry (Pringle *et al.* 1999).

Tenderness is influenced by several pre- and post-slaughter factors. Once the animal has been slaughtered, the rate and extent of rigor development and subsequent

loss of rigor within muscle tissues appears to be the most important determinant influencing the final degree of tenderness (Dayton *et al.* 1981; Huff-Lonergan *et al.* 1996; Goll *et al.* 1998). This process is how meat becomes tender following slaughter of the animal and is referred to in industry as "aging" of the meat. Tenderization refers to the process by which meat losses its postmortem rigidity as a result of the degradation of muscle proteins. Under normal conditions this process usually takes between 14 to 21 days to complete (Lawrie 1991). It has been found that it is possible to modify the rate and extent of proteolysis by stimulating higher activity of the proteolytic enzymes (Geesink and Koohmaraie 1999). This reduces the time to reach maximum tenderness to 7 days or less and results in more tender (lower shear force values) beef (Koohmaraie *et al.* 1988a; Goll *et al.* 1998; Lonergan *et al.* 1998). Reducing the time to reach full relaxation of rigor in muscles could lead to the reduction in processing costs (days in cold room), and improve meat quality, benefits that will impact the whole beef industry.

The proteolytic calpain enzyme system (μ-calpain, m-calpain, and calpastatin) is considered to be the principal group of proteases involved in postmortem myofibril degradation (Etherington 1984; O'Halloran *et al.* 1997; Pringle et al. 1999). The calpains are activated by calcium (Ca), and when intracellular Ca in muscle is increased, the proteolytic activity of these enzymes is increased (Koohmaraie *et al.* 1990; Ilian *et al.* 2001), resulting in improved beef tenderness (Swanek *et al.* 1999; Montgomery *et al.* 2000; Karges *et al.* 2001).

Research has focused on methods to increase the concentration of Ca in muscle. Two approaches have proved to be effective, although substantially different. The first deals with injection, infusion or marination of calcium chloride ($CaCl_2$) to carcasses or meat cuts (Koohmaraie *et al.* 1987, 1988a,b; Wheeler *et al.* 1992; Whipple and Koohmaraie 1992). The main disadvantages of this method in commercial applications is it involves a significant increase in labor and facilities to treat the meat, and has a high likelihood of producing bitter and off-flavors in meat (St. Angelo *et al.* 1991; Miller *et al.* 1995).

The second application is the supplementation of high levels of vitamin D_3 to feedlot animals for a short period of time prior to slaughter. It has been found that feeding 5 or more million IU (MIU) of vitamin D_3 to cattle for 5 to 10 days prior to

slaughter has significantly increased (around 20 %) plasma Ca concentrations, which led to higher levels of Ca in muscle (20-40 %) and improvements in the eating quality of the meat (Owens *et al.* 1998). The higher intracellular Ca in muscle is thought to activate and enhanced the proteolytic activity of the calpain enzymes, resulting in an improvement in tenderness, as measured by mechanical (reduction in shear force values) and gustative (sensory panelists) means (Swanek *et al.* 1997, 1999; Montgomery *et al.* 1998; 2000; 2002; Karges *et al.* 2001).

However, supplementation of high levels of vitamin D_3 induces higher concentrations of it and its metabolites in plasma, muscle and other tissues (Montgomery *et al.* 2000). This situation raises two main concerns with the use of supplemental vitamin D_3. The first is related to the animal, to its well-being and performance; and the second, with food safety and human health. Feeding high levels of vitamin D_3 for prolonged periods to finishing animals could lead to vitamin D_3 toxicity (Karges *et al.* 1999b; Berry *et al.* 2000). The second and most important is in relation to the risk of excessive amounts of vitamin D_3 (and its metabolites) in meat and other edible tissues (Owens *et al.* 1998; Montgomery *et al.* 2000). Residual vitamin D_3 (and its metabolites) in meat can induce vitamin D_3 toxicity in consumers, which will cause disturbances in Ca metabolism, resulting in formation of renal stones and calcification of soft tissues (Glerup and Eriksen 2000).

As a result of these concerns, it would be of interest to reduce the level of vitamin D_3 required to raise plasma Ca to levels that stimulate proteolytic enzyme activity in post-rigor muscle tissue.

The objective of this thesis was to characterize the effects of supplemental vitamin D_3 on Ca homeostasis of finishing steers under western Canadian feedlot conditions, and to determine if specific feeding management strategies can be devised that will allow a reduced amount of vitamin D_3 to be fed yet still achieve the desired increase in serum Ca concentration.

The literature review will cover muscle fiber structure and function, rigor development, Ca metabolism in ruminants, and methods to alter plasma Ca concentrations.

CHAPTER II

LITERATURE REVIEW

2.1 Beef Tenderness

A goal of the Canadian beef industry is to produce beef of excellent quality. In recent years the beef cattle industry has contributed over $25 billion annually to the national economy (Canfax 2002). Approximately 55 % of the beef from Canadian cattle is exported. This makes Canada the third larger exporter in the world (Canfax 2002). This success in the international beef trade has been based on quality products and safety. The domestic beef market, in contrast, has been losing meat market share (i.e. from 35 to 29 % in 1990 and 2000, respectively; Canfax 2002). In this sense, beef tenderness has been identified as the second most important factor affecting meat quality and subsequently consumer preference. The main problem related to beef tenderness is the lack of consistency and uniformity (Morgan *et al.* 1991a; Smith *et al.* 1995).

Generally, consumers are willing to pay a premium for meat known to be tender (Boleman *et al.* 1997). This situation is clearly observed when one looks at the relationship that exists between the expected tenderness of a specific meat cut and its price. Morgan *et al.* (1991a) found that current beef production practices result in considerable variation in beef tenderness and an unacceptable percentage of tough meat (> 20 %). Therefore, methods that can be used to improve beef tenderness and lead to the production of uniform and consistent product will be of particular importance to the beef industry (Davis *et al.* 1979; Pringle *et al.* 1999).

Numerous factors influence the tenderness of meat. Some variables are related to the animal such as breed, age, and sex; others are related to the muscle itself such as the amount of intramuscular fat, sarcomere length, collagen content, size and type of muscle fibers; while others are related to postmortem aging such as pH, temperature, and enzyme proteolytic activity (Davis *et al.* 1979; Dayton *et al.* 1981; Wheeler *et al.* 1990; Whipple *et al.* 1990; Geesink and Koohmaraie 1999). Once the animal has been slaughtered, the most important factors influencing meat quality are those associated with postmortem aging.

2.1.1 Muscle Structure and Function

There are more than 300 muscles in the animal body (Lawrie 1991). These can be classified into three types: skeletal, smooth and cardiac muscle. Skeletal muscle constitutes the greater proportion of the carcass weight. Smooth muscle is associated primarily with the gastrointestinal tract and blood vessels, while cardiac muscle is confined solely to the heart. Skeletal and cardiac muscle are also known as striated muscle because of the transverse banding pattern. Muscle tissue can also be classified as voluntary (skeletal) and involuntary (smooth and cardiac) muscle (Bhagavan 1992; Hedrick *et al.* 1994).

In terms of structure, muscle tissue is surrounded and separated from other external and internal structures by a sheath of connective tissue. The connective tissue surrounding the muscle, the bundles, and each individual muscle fiber are known as epimysium, perimysium (which contains the larger blood vessels and nerves) and endomysium (Figure 2.1). The relative proportions of connective tissue and muscle fibers vary between muscles and, in part, account for the relative toughness of meat (Bechtel 1986).

The structural unit of skeletal muscle tissue is the muscle fiber cell. Muscle fibers constitute 75 to 92 % of the total muscle volume. Connective tissues, blood vessels, nerve fibers, and extracellular fluid make up the remaining volume, with extracelluar fluid comprising the major proportion of this volume. Skeletal muscle fibers are long, unbranched, threadlike cells that taper slightly at both ends. Although fibers may attain a length of many centimeters, they generally do not extend the length of the entire muscle. They vary considerable in diameter, ranging from 10 μm to more than 100 μm (West 1985; Hedrick *et al.* 1994; Guyton and Hall 1996).

The main components of the muscle fiber are the sarcolemma, sarcoplasmic reticulum, nuclei, myofibrils, myofilaments, mitochondria, lysosomes, and Golgi complex. The sarcolemma is the plasma membrane of the muscle cell, which is composed of proteins and lipids, and is relatively elastic, a property that enables it to endure great distortion during contraction, relaxation, and stretching. It surrounds the contractile units and regulates the uptake and release of specific molecules by the muscle cell. The sarcolemma also depolarizes in response to a nerve impulse. The transverse

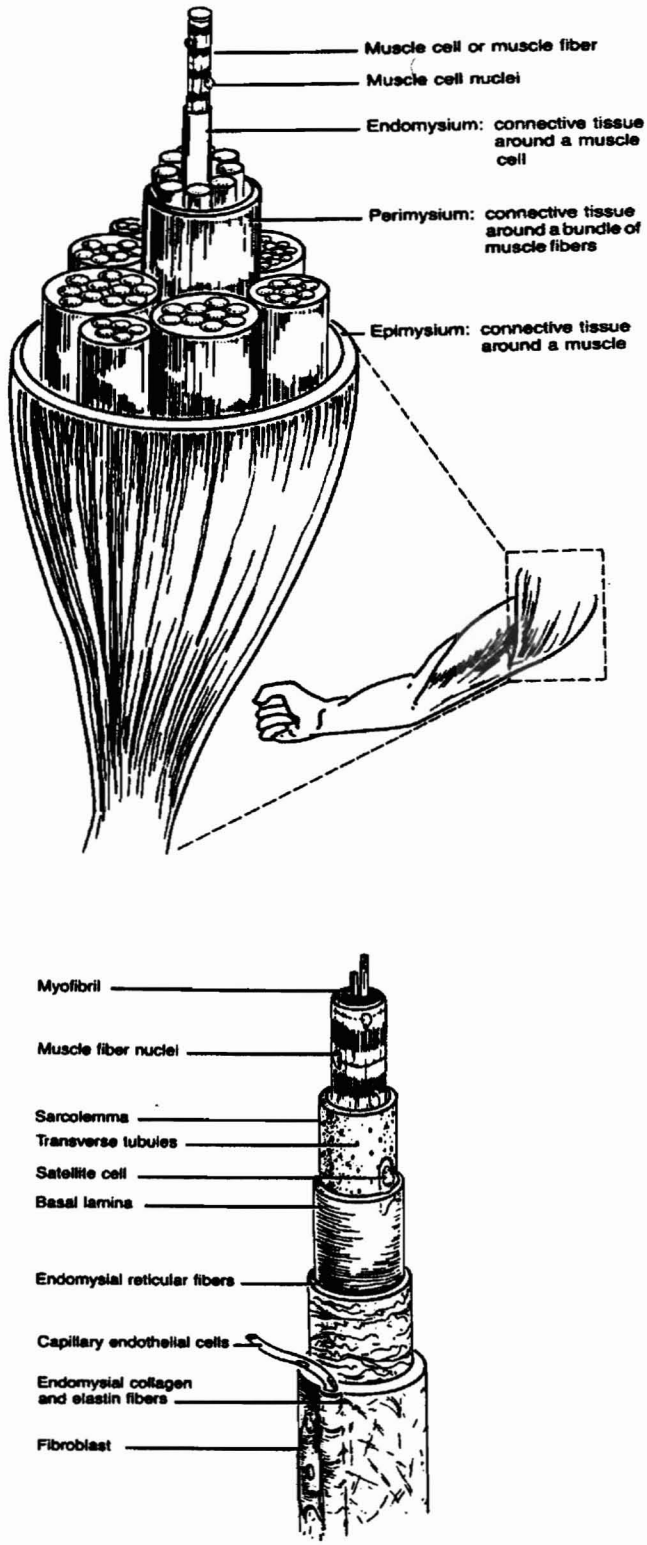

Figure 2.1. Diagrammatic representation of muscle and connective tissue and organization of the muscle cell (Adapted from Bechtel 1986).

tubule (T-tubule) system is a series of small invaginations of the sarcolemma. Its function is to bring the membrane depolarization event from the sarcolemma to the inner regions of the muscle cells. The sarcoplasmic reticulum forms a matrix around the myofibrils between the T-tubules; and has the function to release and sequester Ca in the muscle cell. Calcium is sequestered by the sarcoplasmic reticulum against a concentration gradient, a process that requires energy. Muscles with faster contraction speeds have a more developed sarcoplasmic reticular system. A more developed sarcoplasmic reticulum may allow for faster, more uniform release and uptake of Ca (Bechtel 1986). The sarcoplasm is the cytoplasm of the muscle fiber. Water constitutes about 75 to 80 % of the sarcoplasm, but in addition, sarcoplasm of skeletal muscle contains lipid droplets, glycogen granules, ribosomes, numerous proteins, nonprotein nitrogenous compounds, and a number of inorganic constituents. Skeletal muscle fibers are multinucleated, however, the number of nuclei per fiber is not constant. Nuclei are located at the periphery of the fiber, just beneath the sarcolemma (Lawrie 1991; Hedrick *et al.* 1994).

Each individual muscle fiber is composed of a number of smaller units, the myofibrils (Figure 2.2). The myofibrils are composed of numerous parallel filaments, the myofilaments. Myofilaments are referred as thick and thin filaments of the myofibril. Thick filaments are approximately 14 to 16 nm in diameter and 1.5 μm long. Thin filaments are about 6 to 8 nm in diameter and they extend approximately 1 μm on either side of the Z disk. Thick filaments are aligned parallel to each other and arranged in exact alignment across the entire myofibril. Thin filaments are aligned exactly across the myofibril, parallel to each other and to the thick filaments. This arrangement of myofilaments, and the fact that thick and thin filaments overlap in certain regions along their longitudinal axis, gives the appearance of alternating light and dark areas and accounts for the characteristic banding or striated appearance of the myofibrils (Smith *et al.* 1983; Hedrick *et al.* 1994).

Thick filaments constitute the A band of the sarcomere. Since the predominant protein in thick filaments is myosin, they are referred to as myosin filaments. Thin filaments constitute the I band of the sarcomere and also extend beyond the I band into

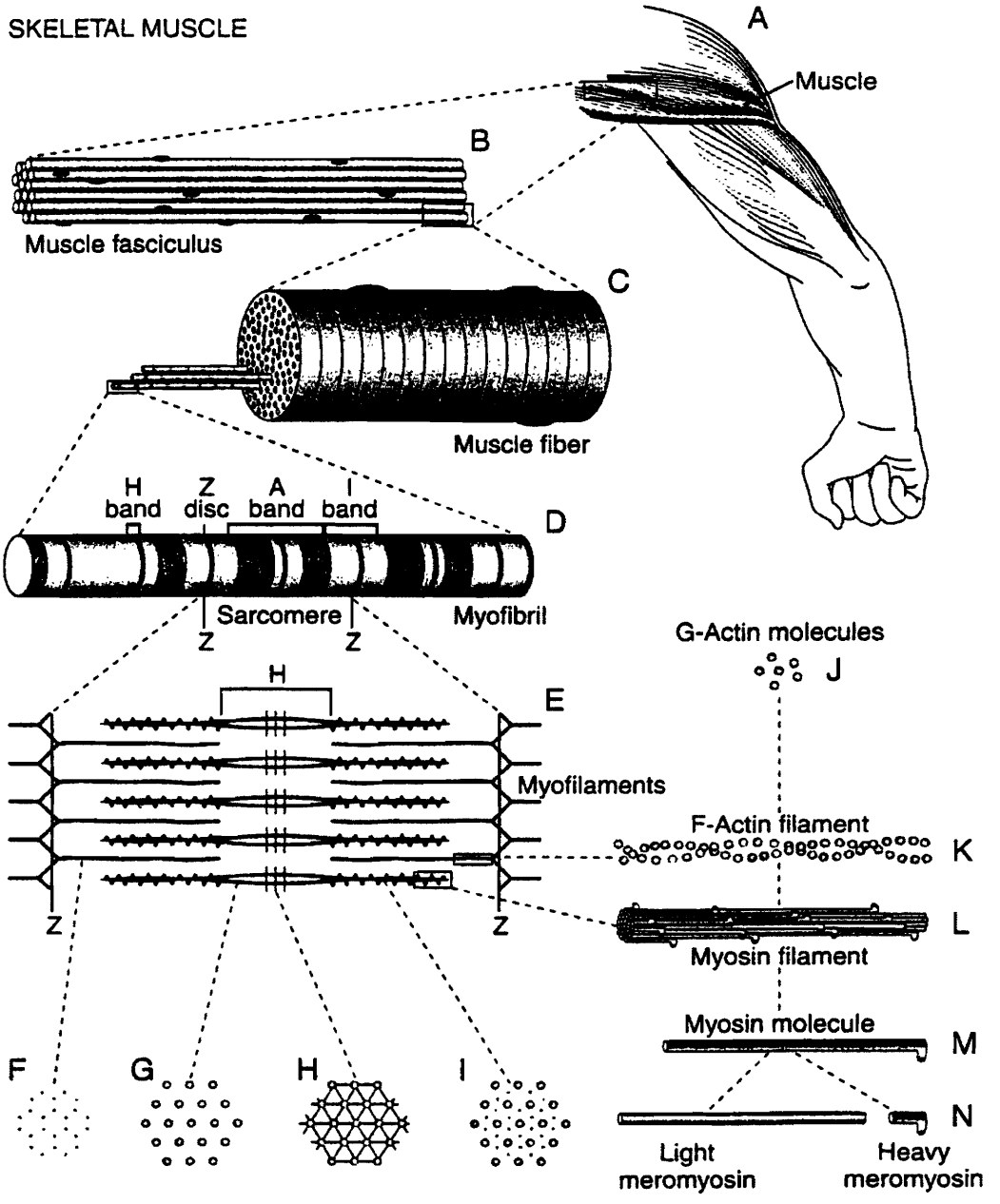

Figure 2.2. Diagrammatic representation of macroscopic and microscopy muscle structure (Adapted from Guyton and Hall 1996, 1997).

the A band, lying alongside the thick myosin filaments. Since actin is the most abundant protein in the thin filament, they are called actin filaments (Guyton and Hall 1997).

The sarcomere is the repeating structural unit of the myofibril, and it is also the basic unit involved in muscle contraction (Figure 2.2). The sarcomere is encompassed between two adjacent Z disks. The sarcomere includes both an A band and the two half I bands located on either side of the A band (Guyton and Hall 1996).

The Z disk is a dense fibrous structure in which one end of each thin (actin) filament is anchored. An actin filament on one side of the Z disk lies between two actin filaments on the opposite side of the Z disk. This arrangement indicates that the actin filaments do not pass through the Z disk. Ultrathin filaments, called Z filaments, constitute the material of the Z disk, and they connect with actin filaments on either side of the Z disk. Each actin filament connects to four Z filaments that pass through the Z disk (Murphy 1998; Cunningham 2002).

There are more than 20 different proteins associated with the myofibril. However, six of these comprise approximately 90 % of the total myofibrillar protein content. These six proteins in order of abundance are myosin, actin, titin, tropomyosin, troponin, and nebulin. Myofibrillar proteins are classified by their function as contractile, regulatory, or cytoskeletal. Actin and myosin constitute the major contractile proteins, and they participate directly in the processes of muscle contraction through the formation of the actomyosin complex. The major regulatory proteins include tropomyosin, troponin, α-actinin, and β-actinin. They are called regulatory proteins because of their role in regulating the actin-myosin interaction during muscle contraction and in maintaining myofibril integrity. The cytoskeletal proteins serve as the template and/or provide the scaffold for the alignment of myofilaments during myofibril and sarcomere formation. In mature muscle, they maintain its overall longitudinal and lateral alignment as well as its structural integrity (Lawrie 1991; Hedrick *et al.* 1994).

Myosin is a fibrous protein that constitutes approximately 45 % of total myofibrillar protein content. The structure of the myosin molecule is an elongated rod shape, with a thickened portion at one end. The thickened end of the myosin molecule is usually referred to as the head region, and the long, thin portion that forms the backbone of the thick filaments is called the rod or tail. When myosin is subjected to proteolytic

enzyme degradation, it is split into two fractions that differ in molecular weight; light meromyosin and heavy meromyosin (Guyton and Hall 2000).

Actin constitutes approximately 20 % of the myofibrillar protein. It is a globular shaped molecule approximately 5.5 nm in diameter. This molecule is called G-actin (for globular actin) and as such constitutes the monomeric form of actin. The fibrous nature of the actin filament is due to the G-actin monomers linking to form F-actin (fibrous actin). Two strands of F-actin are spirally coiled around one another, to form a super helix that is characteristic of the actin filament (Hedrick *et al.* 1994).

Tropomyosin constitutes approximately 5 % of myofibrillar protein and lies in close contact with the actin filament. A strand of tropomyosin lies alongside each groove of the actin super helix, and a single molecule extends the length of seven G-actin molecules in the actin filament (Greaser 1986).

Troponin constitutes about 5 % of myofibrillar proteins and is also present in the grooves of the actin filament, where it lies astride the tropomyosin strands. Troponin units show a periodicity along the length of the actin filament. There is one molecule of troponin for every seven or eight G-actin molecules along the actin filament (Bechtel 1986).

Among the cytoskeletal proteins, titin is the most abundant, compromising about 10 % of the myofibrillar protein. It extends longitudinally in each half of the sarcomere. The portion of titin in the A band is inelastic, while that in the I band is elastic. It is bound to the outside shaft of the thick filament. Titin provides the scaffold for alignment of the filaments during myofibril and sarcomere formation. In mature myofibrils, it maintains the ordered structure and integrity of myofibrils within the sarcomere. Nebulin comprises approximately 4 % of the myofibrillar protein. It is located close and parallel to the thin filament. Nebulin extends longitudinally along the entire length of the thin filament from the A band to the Z disk. In developing muscle, it plays a role in the organization of thin filaments during myofibril formation. In mature muscle, it serves as a template for assembly and/or a scaffold for stability of thin filaments. It also may function in anchoring thin filaments to the Z disk (Hedrick *et al.* 1994; Murphy 1998).

2.1.2 The Mechanism of Muscle Contraction

Muscle contraction involves four of the myofibrillar proteins: actin, myosin, tropomyosin, and troponin. Actin and myosin are the contractile proteins, which form the cross-bridges, and at this point this protein complex is called actomyosin. Tropomyosin and troponin regulate the contraction process (Cunningham 2002). Muscle contraction is initiated by a stimulus that arrives at the surface of the muscle fiber (the sarcolemma). Contraction is initiated by a nerve action potential, which results from the release of acetylcholine. Contraction begins with formation of cross-bridges between the thick and thin fibers, causing shortening of the sarcomere. Tension then develops in these cross-bridges and pull the two arrays of thin filaments and their attached Z disks toward each other. As a result the thin and thick filaments slide past each other with changes in the degree of interlocking between these filaments. The maximum force developed by a muscle is related to the number of cross-bridges that can be formed and to the degree of interlocking between the filaments (Figure 2.3)(Bhagavan 1992; Guyton and Hall 1996).

Relaxed muscle has very low levels of calcium ions (Ca^{2+}) in the sarcoplasmic fluid that bathes the myofibrils. Typical concentrations are less than 10^{-7} M/L of free Ca^{2+}. However, total concentration of Ca^{2+} in skeletal muscle is more than 1000 times this level (greater than 10^{-4} M/L), nearly all of which is bound in the sarcoplasmic reticulum. For muscle to remain in the relaxed state, it must also have a relatively high concentration of adenosine triphosphate (ATP). Most of the ATP is found in the form of a magnesium ion (Mg^{2+}) complex. The Mg^{2+}-ATP complex must be present in order to prevent formation of cross-bridges between actin and myosin. When the sarcoplasmic Ca^{2+} concentration is low (less than 10^{-7} M), and the Mg^{2+}-ATP concentration is high, troponin and tropomyosin inhibit cross-bridge formation between the actin and myosin filaments (Figure 2.3) (Smith *et al.* 1983; West 1991; Hedrick *et al.* 1994).

When the action potential is transmitted from the sarcolemma to the interior of the fiber along the T tubules, it causes bound Ca^{2+} to be released from the sarcoplasmic reticulum into the sarcoplasm. Increased free Ca^{2+} concentration in the sarcoplasm is the trigger that initiates the contractile mechanism. It order to do so; it is necessary to increase the concentration of free Ca^{2+} to about 10^{-6} or 10^{-5} M/L (a 10- to 100-fold increase). When free Ca^{2+} is released into the sarcoplasm, it is bound by troponin. This

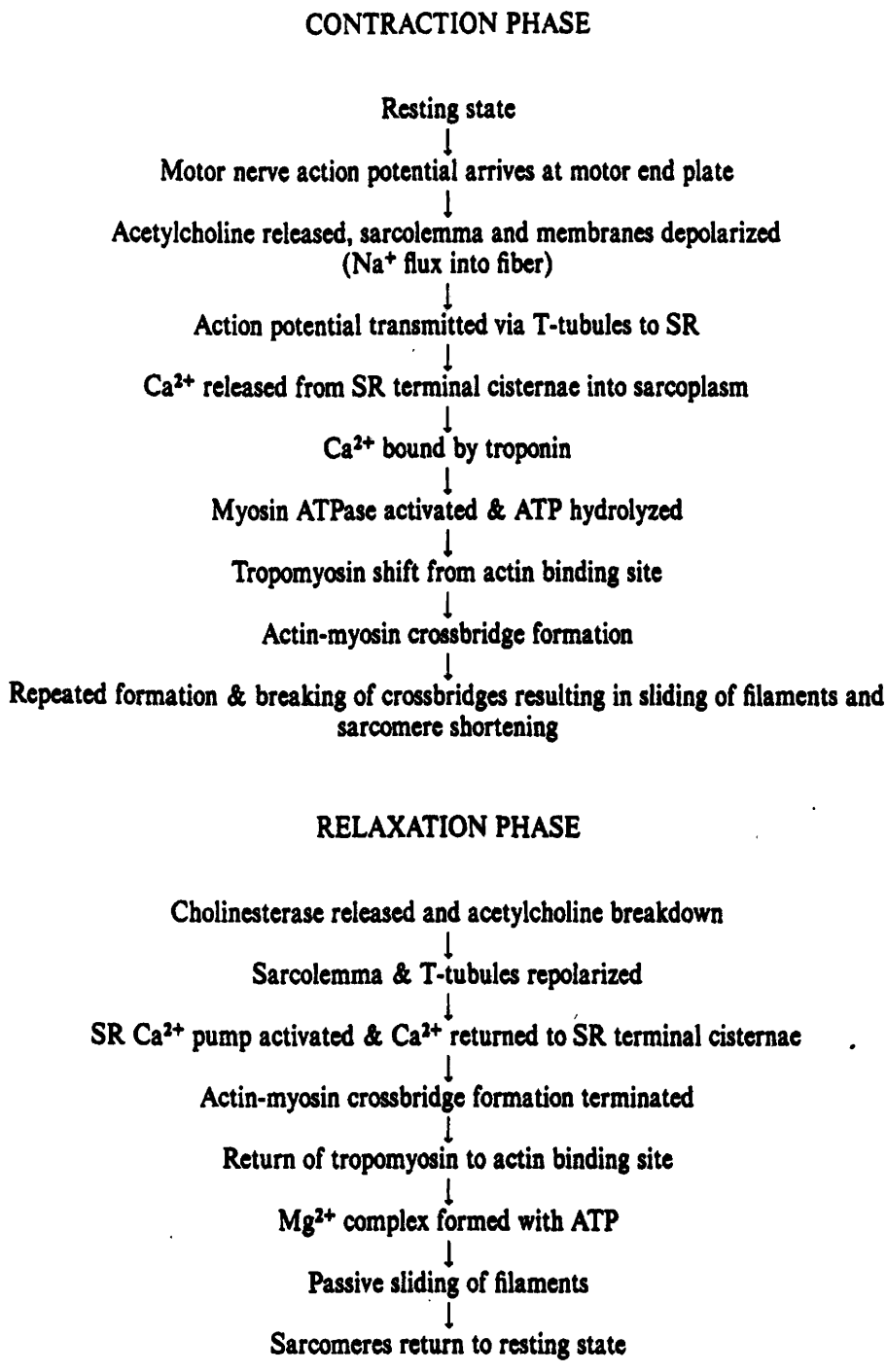

Figure 2.3. Flow diagram of muscle contraction-relaxation cycle (adapted from Hedrick *et al.* 1994).

relieves the inhibition that tropomyosin exerts on cross-bridge formation between actin and myosin because the calcium-activated troponin interacts with tropomyosin, causing it to shift its position along the actin filament. The shift by tropomyosin allows the myosin heads to form cross-bridges between the myosin and actin filaments. These cross-bridges develop a contractile force, and the actin filaments in each half of the sarcomere are pulled toward the center of the sarcomere. During contraction, the length of individual actin and myosin filaments does not change. Rather, the filaments slide along each other, pulling the Z disk closer to the myosin filaments, thereby decreasing sarcomere length (Figure 2.3) (Hedrick *et al.* 1994; Guyton and Hall 1997).

Muscle contraction requires energy in addition to that normally consumed by the resting muscle. This energy is derived from ATP by a reaction catalyzed by the enzyme myosin ATPase, in which ATP is hydrolyzed to adenosine diphosphate (ADP) and inorganic phosphate (Pi). The enzyme system responsible for this reaction is located in the head of the myosin molecule. The activity of the myosin ATPase is greatly enhanced by Ca^{2+} released into the sarcoplasm. Thus, increased free Ca^{2+} concentration in the sarcoplasm promotes cross-bridge formation between actin and myosin filaments, and simultaneously increases ATP splitting, yielding the required chemical energy. Cross-bridges between actin and myosin convert chemical energy into mechanical energy and initiate filament sliding, thus generating a contractile force (Figure 2.3) (Hedrick *et al.* 1994; Murphy 1998).

Relaxation of skeletal muscle is defined as the reestablishment of the resting state. This state is reestablished when the intracellular free Ca^{2+} concentration in the sarcoplasm is 10^{-7} M (or less), and a relatively high concentration of ATP. Intracellular free Ca^{2+} concentration in the sarcoplasm is returned to a low level by the action of the sarcoplasmic reticulum, which removes the excess Ca^{2+} from the sarcoplasmic fluid and binds it in an inactive form. Bound Ca^{2+} is then transferred to the terminal cisternae for storage and subsequent release when the next stimulus arrives. When free Ca^{2+} concentration in the sarcoplasm decreases, the actin-myosin cross-bridges are broken and troponin releases the Ca^{2+} it had bound during the initiation of contraction. As troponin releases Ca^{2+}, it is again able to inhibit the formation of cross-bridges by allowing tropomyosin to regain its original protective position over the cross-bridge site. In the

resulting absence of cross-bridges, tension is not generated and the stretching imposed by elastic components in the muscle causes the filaments to slide passively over one another (West 1991; Cunningham 2002).

2.1.3 Post-Mortem Changes and Meat Quality

When the animal is slaughtered, muscle tissue undergoes a series of chemical and biochemical changes. These changes are associated with the stoppage of circulation and the switch from aerobic to anaerobic metabolism. Some of the most notable changes include a decline in temperature, depletion of available energy, decline in pH, accumulation of metabolites such as lactic acid, a rise in the relative ionic strength, and a decrease in the ability of cellular systems to maintain control of the redox conditions within the cell (Hedrick *et al.* 1994; Huff-Lonergan *et al.* 1995). These biochemical changes have a profound effect on the conversion of muscle to meat, with tenderness being one aspect of meat quality that can be affected either positively or negatively (Lawrie 1991).

2.1.3.1 Rigor Mortis

Several hours after death, all the muscles of the body go into a state of contracture called rigor mortis. Rigor mortis is due to formation of permanent cross-bridges in muscle between the actin and myosin filaments. This is the same chemical reaction that forms actomyosin during muscle contraction. This rigidity is caused by loss of all the ATP, which is required in the living animal to cause separation of the cross-bridges from actin and myosin filaments during the relaxation process. The muscles remain in rigor until the muscle proteins are destroyed by autolysis caused by enzymes released from the lysosomes (Guyton and Hall 1996) and/or by proteolytic enzymes (Goll *et al.* 1998). The difference between living and rigor mortis is that relaxation is impossible in the latter state, as no energy is available for breaking the actomyosin bonds (Hedrick *et al.* 1994).

Rigor mortis is comprised of three phases. These include: a) the delay phase, that corresponds to the period in which muscle is relatively extensible and elastic; b) the onset phase, where muscle begins to lose extensibility, and c) the completion phase, which corresponds to depletion of creatine phosphate and as a result ATP can no longer be

regenerated from ADP, and the muscle becomes relatively inextensible (Dayton *et al.* 1981; Greaser 1986; Lawrie 1991).

Two main enzyme systems present in skeletal muscle are implicated as being responsible for the postmortem proteolytic degradation of myofibrillar proteins. The first is the calpain-calpastatin (neutral proteases) system (Etherington 1984; Goll *et al.* 1998; Geesink and Koohmaraie 1999). The second is the cathepsin-cystatins (acidic proteases) system (Penny and Ferguson-Pryce 1979; Johnson *et al.* 1990) present only in the lysosomes of muscle fibers. It has been observed that cathepsins are not released from lysosomes even after electrical stimulation and extended post-mortem storage (Hedrick *et al.* 1994). Without their release, these enzymes cannot degrade myofibrillar proteins (Calkis and Siderman 1998). Muscle proteins would have to be endocytosed into lysosomes for proteolysis. This is almost impossible in post-mortem muscle because endocytosis is an active process requiring energy (Lawrie 1991). Thus, the calpain enzyme system is considered the most likely system responsible for the proteolytic changes in myofibrillar proteins during postmortem storage and consequently the most important system involved in meat tenderness (Koohmaraie *et al.* 1987; Kendall *et al.* 1993; Huff-Lonergan *et al.* 1996; Goll *et al.* 1998).

2.1.3.2 The Proteolytic Calpain Enzyme System

The calpain system is comprised, principally, of three proteins: 1) μ-calpain, a proteolytic enzyme that requires micromolar concentrations (3 to 50 μM) of ionized Ca (Ca^{2+}) for a half-maximal rate of proteolytic activity; 2) m-calpain, a proteolytic enzyme that requires nearly millimolar concentrations (400 to 800 μM) of ionized Ca for a half-maximal rate of proteolytic activity; and 3) calpastatin, an enzyme responsible for inhibition of the two calpains (Dayton *et al.* 1981; Hedrick *et al.* 1984; Geesink and Koohmaraie 1999).

The two calpains are both cysteine proteases (i.e. they have a sulfhydryl group at their active site) with a pH optima of 7.2 to 8.2. These enzymes are intracellular in nature. Within the muscle cell, these enzymes are found in the cytoplasm and associated with subcellular organelles, primarily the cytoskeleton (myofibrils in skeletal muscle), but also with the plasma membrane, mitochondria, and nuclei. The calpains are

expressed in a single isoform. Calpastatin is a multiheaded inhibitor that can be expressed in several different isoforms having one, three, or four inhibitory domains (Lawrie 1991; Huff-Lonergan *et al.* 1996; Goll *et al.* 1998).

Normally calpains degrade myosin, the major muscle protein, very slowly causing few cleavages in the light and heavy chain. Undernatured actin, the second most abundant protein in skeletal muscle myofibrils, is not cleaved. However, the calpains rapidly cleave troponin T, desmin, vinculin, talin, spectrin, nebulin, and titin. They act more slowly on troponin I, filamin, C-protein, dystrophin, and tropomyosin, and cleave α-actinin and M protein very slowly (Hedrick *et al.* 1984; Goll *et al.* 1998).

Ultrastructurally, incubation with the calpains results first in loss of the N_2 line, and then in complete loss of Z-disks leaving a gap in the middle of the sarcomere as well as loss of periodicity in the I-band area, likely due to troponin and tropomyosin degradation. Alpha-Actinin is a major Z-disk protein, but is degraded slowly by the calpains. The loss of the Z-disk structure is caused by release of α-actinin from the Z-disk in an almost intact form. Calpains cleave relatively few peptide bonds in each protein and leave large polypeptide fragments rather than reducing the protein to small peptides and amino acids. Nevertheless, these cleavages to the structural myofibrils are of fundamental importance to the break down of the sarcomere structural integrity, which will result in a significant reduction in the postmortem rigidity, and eventually in a more tender meat (Goll *et al.* 1991, 1998).

It has been observed that the calpain enzymes not only need Ca to be activated but that their rate of activity is closely regulated by the amount of available Ca. That means that higher concentrations of Ca will induce higher proteolytic activity and consequently improvements in meat tenderness (Koohmaraie *et al.* 1988a,b; Swanek *et al.* 1997).

2.2 Calcium

Calcium (Ca) is a white silvery, alkaline earth metal, which does not occur in free state. However, Ca is associated with other minerals widely distributed in nature. Calcium is the fifth most abundant element on the earth and the most abundant cation in the animal body, comprising 1 to 2 % of the total weight. Approximately 99% of the Ca in the body is found in the bones and teeth, with the remaining 1 % widely distributed in

various soft tissues. Calcium has a very close interrelationship with phosphorus, parathyroid hormone, calcitonin and vitamin D_3 and its metabolites (Underwood 1981; West 1991; Bhagavan 1992).

2.2.1 Importance of Calcium

Calcium is essential for nerve impulse transmission, muscle contraction, blood coagulation, the secretion of hormones and neurotransmitters, cell growth, motility, replication, as an activator of a number of enzymes, and for cell membrane integrity (Rodan 1973; Smith *et al.* 1983; Wasserman 1989). Calcium levels both intra- and extra-cellular are tightly regulated. Abnormally low or high levels either in the cell or in the plasma can induce metabolic disorders (i.e. hypocalcemia or hypercalcemia, respectively) that can seriously affect not only animal performance but the well-being of the animal (Guyton and Hall 1997).

2.2.2 Forms of Calcium

Total Ca in serum and extracellular fluid is divided into three forms: a) ionized (Ca^{2+}), which comprises approximately 50% of the total, b) protein-bound Ca, which represents approximately 45% of the total, and c) Ca that is complexed to anions such as citrate, bicarbonate, phosphate or lactate. This latter form of Ca comprises approximately 5% of the total. The protein-bound Ca^{2+} is bound principally to negatively-charged sites of albumin with smaller amounts bound to globulins. The protein-bound form of Ca^{2+} is dependent on serum parathyroid hormone (PTH). Alterations in blood pH will change the concentration of the ionized Ca. As the pH of serum becomes more acidic, the concentration of the ionized Ca will increase. This is caused by the competition of H^+ for binding to the negatively-charged sites on serum proteins. The ionized and complexed Ca^{2+} comprise the ultrafilterable fraction of Ca^{2+} and represents the fraction that is present in the glomerular filtrate (Rosol *et al.* 1995; Rosol and Capen 1997; Guyton and Hall 2000).

The concentration of total Ca in the serum in most mammalian species is approximately 2.5 mM or 10 mg/dl, with a range between 2.2 to 2.6 mM or 8.8 to 10.4 mg/dl. The concentration of ionized Ca in serum is approximately 1.2 mM or 4.8 mg/dl,

with a range between 1.2 to 1.6 mM or 4.8 to 6.4 mg/dl (Copp 1973; Bell *et al.* 1976; Ganong 1987; West 1991; Rosol *et al.* 1995; Rosol and Capen 1997).

2.2.3 Calcium Homeostasis

The concentration of Ca in the extracellular fluid is regulated by the hormones parathyroid hormone (PTH), calcitonin, vitamin D_3 and its metabolites. These hormones are highly sensitive to variations in plasma Ca. They can act alone or in concert to promote intestinal Ca absorption, bone Ca resorption, and renal Ca reabsorption to achieve plasma Ca homeostasis (Goff 1989).

2.2.3.1 Intestinal Calcium Absorption

The absorption of Ca occurs in all segments of the small intestine although the efficiency of absorption follows the order: duodenun > jejunum > ileum. It also occurs in the colon and cecum. However, it seems that most of the Ca is absorbed in the ileum. This is due to the long resident time of the ingesta in this segment as compared to the duodenum and jejunum (Wasserman 1989).

Two transport processes are responsible for the absorption of Ca by the intestinal cells. The first is an active process, transcellular, energy-requiring, and regulated by vitamin D_3 ($1,25(OH)_2D_3$). This process saturates at an intracellular Ca concentration of about 2 to 5 mM. The second absorption mechanism is a passive diffusion-like process that is non-saturable. This method appears to be paracellular (i.e. absorbed between cells rather than through them). The rate of Ca absorption by the passive process is directly related to the intraluminal concentration of Ca (Wasserman 1989; Hunt and Groff 1990).

The absorption of Ca across the intestine occurs in three phases. The first is the transport of Ca from the intestine throughout the brush border into the epithelial cell. Then the intracellular Ca is transported to the basal-lateral surface, and finally Ca is transported across the basal-lateral membrane into the blood stream (Spirichev and Sergeev 1988).

The first step is the transfer of Ca across the brush border membrane. Much of the Ca accumulated by the brush border vesicles is bound to components on the inner surface of the membrane. The rate of entry of Ca across the brush border membrane

allows a concentration gradient and is positively affected by $1,25(OH)_2D_3$ (Figure2.4). The Ca-binding proteins (CaBP) located in the cytosol of the epithelial cells are translocated to the brush border region. These proteins are responsible for the uptake of Ca in the cell. The sequestering of Ca by the CaBP and other organelles such as the endoplasmic reticulum, mitochondria and Golgi apparatus in the cytosol is very important in order to maintain epithelial cell integrity (Spirichev and Sergeev 1988; Wasserman 1989).

The second step is the transfer of Ca through the cytosol. The translocation of Ca from the brush border membrane to the basolateral membrane is largely regulated by the action of $1,25(OH)_2D_3$ through the formation of the CaBP, which is responsible for binding Ca and forming the complex Ca-CaBP. It is this complex which transverses the cell cytoplasm to the basolateral membrane, where the Ca is released (Figure 2.4) (DeLuca 1980; Wasserman 1989; Rhoades and Tanner 1995).

The third step is the transfer across the basolateral membrane. Extrusion of Ca from the cell is the function of an ATP-dependent active Ca transport system, and also occurs by Na-Ca exchange (Smith *et al.* 1983; Wasserman 1989).

2.2.3.2 Bone Calcium Resorption

Bone, besides its structural function serves as a source of readily available Ca and phosphate. This Ca pool is important for maintenance of Ca homeostasis. The deposition of Ca in bone and the mobilization of it to the extracellular fluid is under the control of several hormones (PTH, vitamin D, calcitonin, growth hormone, insulin, thyroxine, estrogen and testosterone, and the glucocorticoids) and hormone like factors (somatomedins, prostaglandins). A process that is very complex (Smith *et al.* 1983; Strand 1983).

Bone is composed of bone cells called osteoblasts, osteocytes and osteclasts and extracellular matrix. Osteoblasts are located on the bone surface and are responsible for osteoid synthesis. Osteoids are secreted into the space adjacent to the bone. Eventually, new osteoid becomes mineralized, and in the process, osteoblasts are surrounded by mineralized bone, losing their ability to form bone and as such become quiescent. At this point they are called oesteocytes. Osteoclasts are cells responsible for bone resorption.

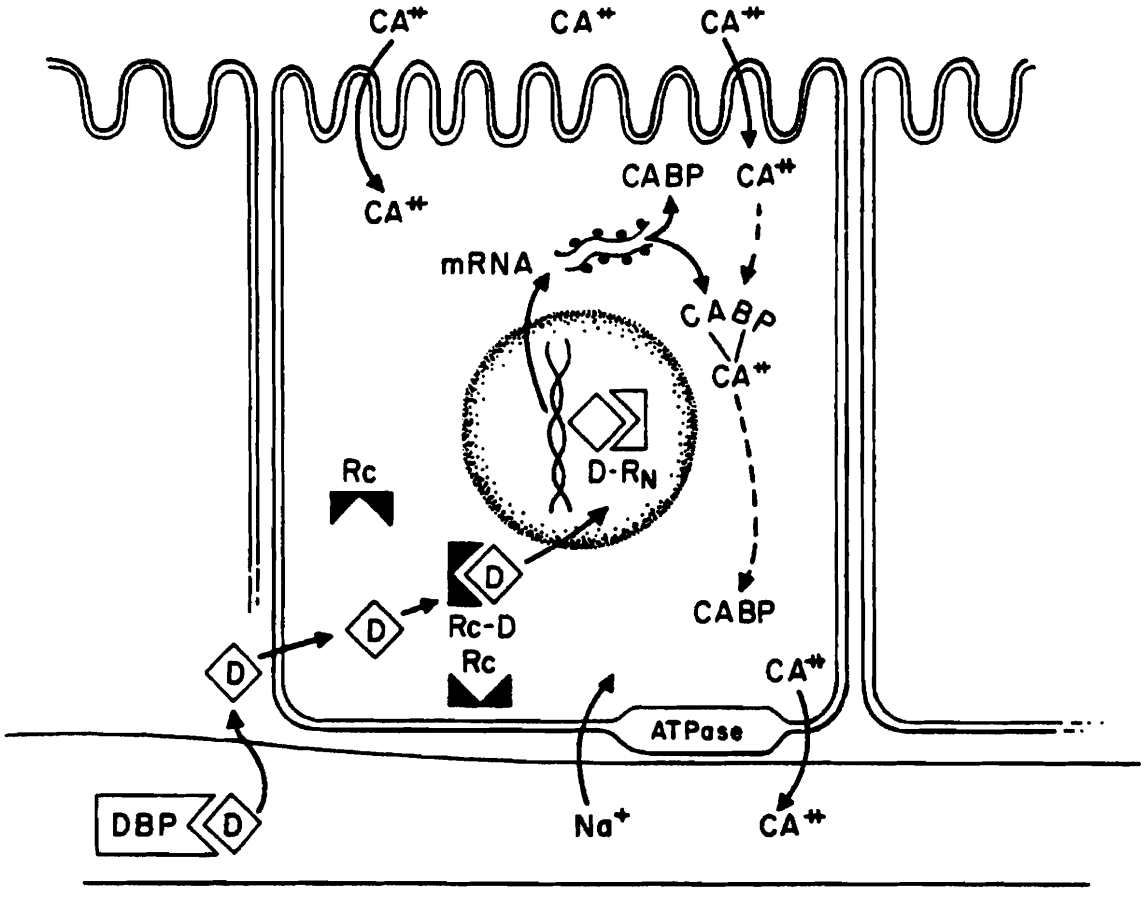

Figure 2.4. Molecular mechanisms of action of the hormonal form of vitamin D ($1,25(OH)_2D$) on the active transport of calcium across the intestinal epithelial cells.

DBP = Vitamin D Binding Protein

CABP = Calcium Binding Protein

(Adapted from Rosol and Capen 1997).

They are large, multinucleated cells located on bone surfaces. Osteoclasts promote bone resorption by secreting acid and proteolytic enzymes into the space adjacent to the bone surface. Bone resorption is two-step event. First, osteoclasts create a local acidic environment that increases solubility of surface bone mineral. Second, proteolytic enzymes secreted by osteoclasts degrade the organic matrix of bone. The result is a flux of Ca and phosphate into and out of bone, which reflects a turnover of bone mineral and changes in bone structure. This phenomenon called remodeling is a beneficial adaptive process that allows bone to be reshaped to meet changing mechanical demands placed on the skeleton. It also allows the body to store or mobilize Ca rapidly in order to maintain Ca homeostasis (Strand 1983; West 1985; Bhagavan 1992; Rhoades and Tanner 1995).

There are two independent but interacting homeostatic systems affecting bone Ca. One is the system that regulates ionized plasma Ca, through the readily exchangeable bone Ca pool. In the operation of this system, about 500 mM of ionized Ca per day moves into and out of the readily exchangeable pool in bone. The other system is the one concerned with bone remodeling by the constant interplay of bone resorption and deposition. This latter system takes more time to respond but constitutes a larger source of Ca than the readily exchangeable Ca pool (Ganong 1987; Endres *et al.* 2001).

2.2.3.3 Renal Calcium Reabsorption

Reabsorption occurs in both the proximal and distal tubules and in the loop of Henle of the kidneys. Reabsorbable or filterable Ca comprises 60% of the total Ca in plasma and consists of ionized Ca and Ca bound to filterable anions such as bicarbonate or citrate. The remaining 40% of the total Ca circulates bound to proteins and thus is not filterable by the glomerular tubules. Ordinarily, only about 1% of filtered Ca is eventually excreted in the urine, with the remaining 99% reabsorbed. Active transport mechanisms are involved in the uptake from the proximal and distal tubules. Approximately 60% of filtered Ca is reabsorbed in the proximal tubule, 30% from the loop of Henle, and 9% from the distal tubule. The remaining 1% is excreted in the urine. Calcium excretion is regulated by the kidney primarily at the distal tubule. PTH stimulates Ca absorption in the distal tubule, thus promoting Ca retention and lowering excretion (Bhagavan 1992; Rhoades and Tanner 1995; Guyton and Hall 1997).

2.3 Hormonal Regulation of Calcium

2.3.1 Vitamin D

Vitamin D is a group of fat-soluble compounds collectively known as the calciferols. There are two main forms of vitamin D. These are vitamin D_2 and D_3. The metabolism of the two forms of vitamin D in the body is identical (Figure 2.5) (DeLuca and Schnoes 1983; Solvstan 2000).

Vitamin D_3 is also called cholecalciferol or activated dehydroxycholesterol. This type of vitamin D is naturally synthesized by the body, and consequently is the main source for the animal and also the main form from animal food (Rhoades and Tanner 1995). Vitamin D_2 or ergocalciferol is produced in plants (primarily yeast) by ultraviolet irradiation of the sterol ergosterol (DeLuca et al. 1977). Vitamin D_2 differs from D_3 only in a double bound between carbons 21 and 22 and a methyl group on carbon 24 (Figure 2.5) (Smith *et al.* 1983). Vitamin D_2 is used to supplement milk, cereals, and bread in the United States, Canada and Mexico (Holick 2000).

Vitamin D functions in Ca and bone metabolism in amphibians, reptiles, birds and mammals through the active metabolite 1,25-dihydroxyvitamin D ($1,25(OH)_2D_2$ and $1,25(OH)_2D_3$) (Solvesten 2000). The $1,25(OH)_2D$ is considered a hormone (secosteroid hormone with a disrupted B ring) because it modifies the function of target tissues or organs (Figure 2.5) (Glerup and Eriksen 2000).

Vitamin D is stored in adipose tissue from which it can be released into the circulation. The main function of vitamin D is to maintain serum Ca levels in the normal range to support metabolic functions involving Ca (Holick 2000). Specifically, vitamin D regulates calcification and mobilization of Ca from bone, Ca absorption from the intestine, and Ca reabsorption from kidneys (Figure 2.6) (DeLuca 1979; Glerup and Eriksen 2000).

2.3.1.1 Synthesis of Vitamin D_3

During exposure to sunlight, the high-energy ultraviolet (UV) radiation penetrates the skin, where they are absorbed by epidermal and dermal stores of 7-dehydro-cholesterol (provitamin D_3). Provitamin D_3 then, undergoes bond cleavage between

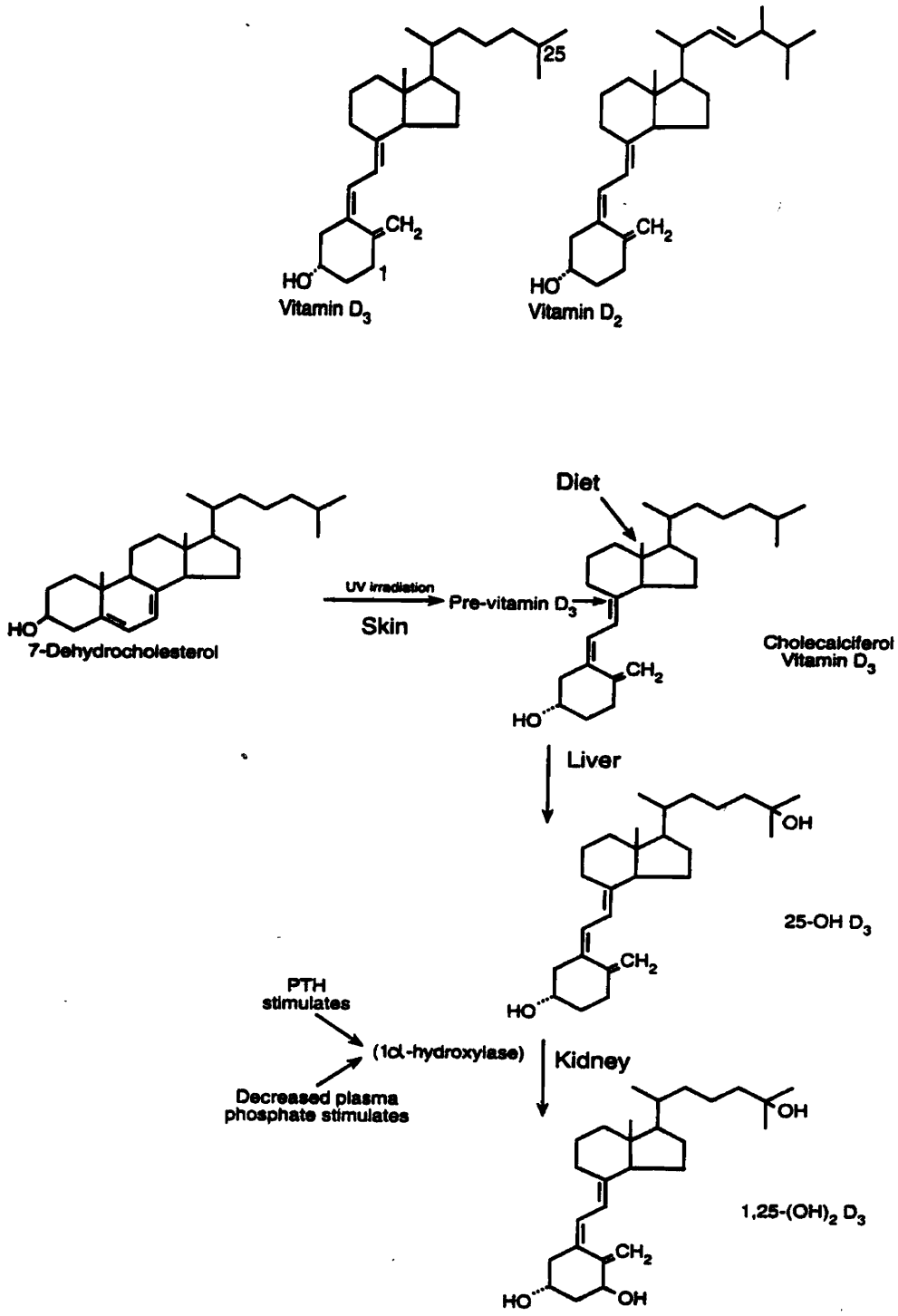

Figure 2.5. Structures for vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocholecalciferol). Pathways for conversion of vitamin D_3 into its hormonal form: $1,25(OH)_2D_3$ (Adapted from Rhoades and Tanner 1995).

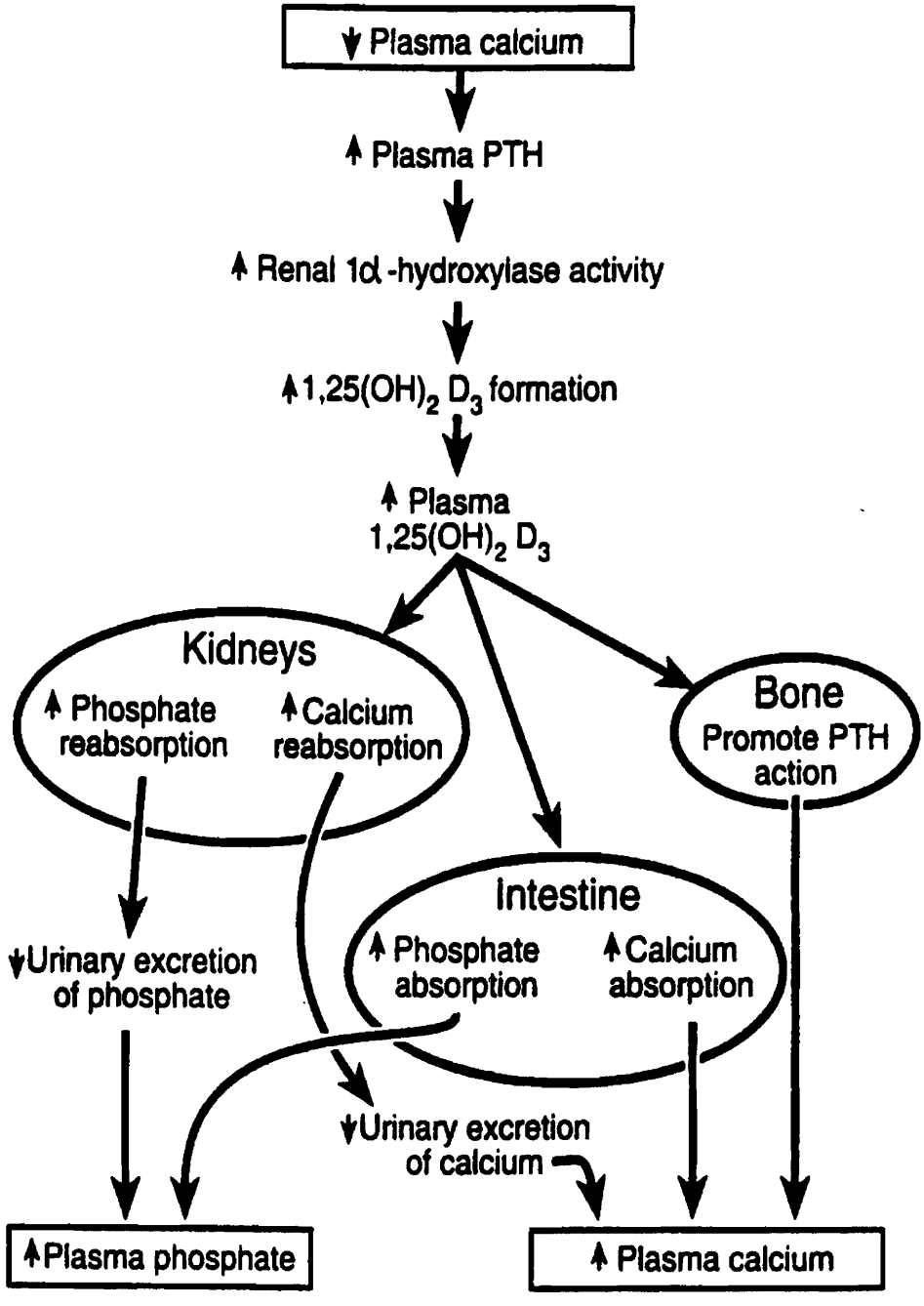

Figure 2.6. The effect of vitamin D_3 ($1,25(OH)_2D_3$) on calcium metabolism (Adapted from Rhoades and Tanner 1995).

carbons 9 and 10 to form a 9-10-secosteroid known as previtamin D_3. This compound is biologically inert and is thermodynamically unstable and spontaneously rearranges to form vitamin D_3 (Figure 2.5). At a physiological temperature of 37^0C, this process takes approximately 24 h to reach completion, although, it has been found that it could be completed in only 4 h (DeLuca 1979, 1980; Holick 2000).

Vitamin D_3 from the diet is absorbed in the ileum of the small intestine passively as it is dependent on bile salts for solubilization (West 1991). It is transported by the small intestine in free form, predominantly in association with chylomicrons. During the metabolism of chylomicrons, vitamin D_3 is transferred to a binding protein in plasma called vitamin D binding protein (DBP). Vitamin D_3 is not stored in the liver but is distributed between the various organs, depending on their lipid content (Rhoades and Tanner 1995). The normal circulating concentration of vitamin D_3 for humans and cattle ranges from approximately less than 0.2 to 20 ng/ml, with a half-life of 22 h (DeLuca 1979; Rhoades and Tanner 1995).

After entering the circulation, vitamin D_3 is activated by undergoing two different hydroxylation reactions, each in a different tissue. In the liver, vitamin D_3 is hydroxylated at carbon 25 to yield the major circulating form of vitamin D_3, 25-hydroxyvitamin D_3 ($25(OH)D_3$), also called calcidiol (Wasserman 1989). This hydroxylation occurs in the endoplasmic reticulum, catalyzed by the hepatic enzyme 25-hydroxylase. This first hydroxylation does not appear to be tightly regulated (Smith *et al.* 1983; Ganong 1987). Circulating concentrations of $25(OH)D_3$ in plasma are normally between 10 to 50 ng/ml, with a half-life of 15 days (DeLuca 1979; Rhoades and Tanner 1995) . This metabolite appears to be approximately five times more potent in regulating Ca homeostasis than vitamin D_3 (DeLuca 1979; Smith *et al.* 1983; Rhoades and Tanner 1995).

In the kidneys, $25(OH)D_3$ is hydroxylated at carbon 1 to form the hormonal form of vitamin D_3, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), also called calcitriol (Wasserman 1989). This hydroxylation is catalyzed by the mitochondrial enzyme 1α-hydroxylase located in the proximal tubules. This step is considered to be the most significant point of vitamin D metabolism. The main factors involved in the regulation of its activity are PTH (positive feedback), low levels of Ca and P (positive feedback), and

levels of $1,25(OH)_2D_3$ (negative feedback) (DeLuca 1979; Weiser 1984; Spirichev and Sergeev 1988; Wasserman 1989; Guyton and Hall 1997). Specific hormones such as growth hormone, estrogens, androgens, prolactin, and insulin may also influence the activity of this enzyme (Bhagavan 1992). Normal plasma concentration of $1,25(OH)_2D_3$ is approximately 10 to 60 pg/ml, with a half-life of 15 h (Rhoades and Tanner 1995). This metabolite is considered to be 500 to 1000 times more biologically active than vitamin D_3 (Rhoades and Tanner 1995; Glerup and Eriksen 2000).

The major excretory route for vitamin D and its metabolites is the bile, with little excreted in the urine. Vitamin D metabolites may undergo conjugation in the liver prior to excretion in feces (Smith *et al.* 1983; Bhagavan 1992).

2.3.1.2 Role of Vitamin D_3 in Intestinal Calcium Absorption

The small intestine in the absence of vitamin D_3 will absorb about 10-15% of the dietary Ca. In the presence of $1,25(OH)_2D_3$, this amount will increase to approximately 30% (Holick 2000). During periods of rapid growth, pregnancy and lactation, the increased Ca demand results in increased $1,25(OH)_2D_3$ plasma concentrations, which in turn will increase the efficiency of Ca absorption from intestine (West 1991; Holick 2000).

The $1,25(OH)_2D_3$ stimulates absorption of Ca^{2+} and phosphorus (Pi) across intestinal epithelial cells by active transport mechanisms. The $1,25(OH)_2D_3$, enters the circulation and is bound to a vitamin D_3 plasma binding protein (DBP; $G_c$protein, Transcalciferin; MW of 56,000) and α-globulin (Weiser 1984; Bhagavan 1992). When the complex reaches the small intestine, the $1,25(OH)_2D_3$ dissociates from the DBP and enters the intestinal epithelial cell by facilitated diffusion. It then binds to a highly specific cytosolic receptor. Binding is followed by activation of the receptor and translocation of the $1,25(OH)_2D_3$-receptor complex to the nucleus, where it binds to chromatin. This results in increased mRNA synthesis and increased activity of RNA polymerase II. A subsequent increase in the synthesis of two specific intestinal proteins occurs. The first is a Ca ATPase, and the second is calbandin, the specific Ca-binding protein (CaBP), both of which are essential for the active absorption of Ca (Smith *et al.* 1983; Weiser 1984; Rhoades and Tanner1995). Mammalian intestinal mucosa contains a

CaBP (MW 9,000) that binds 2 moles of Ca^{2+} per mole of protein (Bhagavan 1992). Typically, there is a lag of several hours in Ca^{2+} absorption and is associated with increased synthesis and concentrations of a soluble CaBP in the cytoplasm of the intestinal mucosal cells as a result of $1,25(OH)_2D_3$ action in intestine (Smith *et al.* 1983; Weiser 1984).

2.3.1.3 Vitamin D_3 Toxicity

Toxic effects of vitamin D_3 are mediated through its hypercalcemic and hypercalciuric actions. The symptoms are the same independently of the type of vitamin D that causes the intoxication. They include anorexia, nausea, vomiting, polyuria, polidipsia, constipation, weakness, muscle pain, changes in mental status, fatigue, confusion, difficulty in concentration, drowsiness, apathy, and eventually coma. Metastatic calcification of the soft tissues may develop. One of the most apparent symptoms is nephrocalcinosis, causing a gradual decline in kidney function (Pennington 1976; Hunt and Groff 1990; Guyton and Hall 1996; Glerup and Eriksen 2000).

2.3.2 Parathyroid Hormone (PTH)

Parathyroid hormone (PTH or parathormone) is an 84-amino acid peptide produced by the parathyroid glands. There are two pairs of parathyroid glands, located in the dorsal surface of the left and right lobes of the thyroid gland. The parathyroid glands are highly sensitive to changes in the concentration of circulating Ca, specifically plasma concentrations of ionized Ca (Ganong 1987; Goff 1989; Bhagavan 1992).

The parathyroid glands respond within minutes to slight changes in blood Ca levels. A small decline in blood Ca stimulates the release of large amounts of PTH (Figure 2.7). If the plasma Ca concentration decreases below normal, PTH acts principally on the kidneys, in which it stimulates Ca reabsorption in the thick ascending limb and distal tubules. This reduces the loss of Ca in the urine and increases plasma Ca concentration. Only a few grams of Ca are conserved, but this is often sufficient to return blood Ca to normal. PTH also inhibits phosphate reabsorption in the proximal tubule, leading to increased urinary phosphate excretion and a decrease in plasma phosphate. This decrease in phosphate is important with regard to Ca homeostasis. At normal

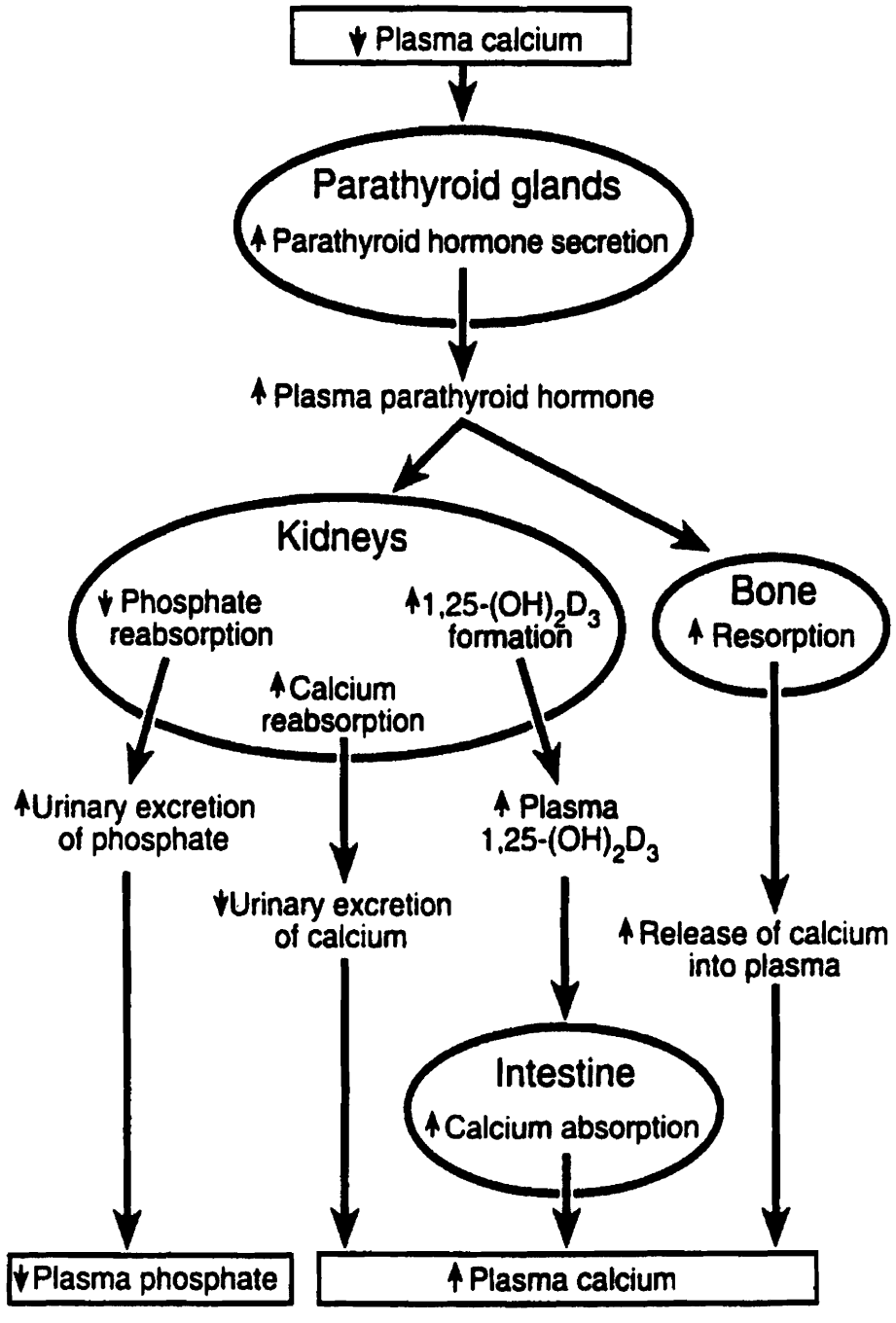

Figure 2.7. The effect of parathyroid hormone (PTH) on calcium metabolism (Adapted from Rhoades and Tanner 1995).

plasma concentrations, Ca and phosphate are at or near chemical saturation levels. If PTH was to increase both Ca and phosphate levels, they would simple crystallize in bone or soft tissues as calcium phosphate, and the necessary increase in plasma Ca concentration would not occur. Thus, the effect of PTH to lower plasma phosphate is an important part of its role in regulating plasma Ca. In the event of a larger Ca drain, continued secretion of PTH stimulates the activity of the 1α-hydroxylase enzyme, which is essential in the formation of the $1,25(OH)_2D_3$, which functions to promote the absorption of Ca from the intestine. In bone, PTH stimulates the development and activity of bone osteoclasts, cells that reabsorb bone matrix, and release Ca into the blood. The action of PTH to promote bone resorption is augmented by $1,25(OH)_2D_3$ (Figure 2.7) (Rhoades and Tanner 1995; Cunningham 2002).

2.3.3 Calcitonin

Calcitonin or thyrocalcitonin is a 32-amino acid peptide produced by the parafollicular cells (C-cells) of the thyroid gland. Hormones of the gastrointestinal tract, especially gastrin, promote calcitonin secretion. Since the net effect of calcitonin is to promote Ca deposition in bone, stimulation of calcitonin secretion by gastrointestinal hormones provides an additional mechanism facilitating Ca uptake into bone after ingestion of a meal (Rhoades and Tanner 1995; Guyton and Hall 1996).

When plasma Ca concentration increases above normal levels, the parfollicular cells increase the synthesis and secretion of calcitonin (Fig. 2.8). The main target organs of calcitonin are the kidneys and bone. In the kidneys, calcitonin decreases tubular reabsorption of Ca and phosphate. This leads to an increase in urinary excretion of both Ca and phosphate and ultimately to decreased levels of both ions in the plasma. In bone, calcitonin opposes the action of PTH on osteoclasts by inhibiting their activity. This leads to decreased bone resorption and an overall net transfer of Ca from plasma into bone, thus decreasing Ca levels in plasma and returning it to normal. Calcitonin has little or no effect on the gastrointestinal tract (Figure 2.8) (Rosol and Capen 1997; Cunningham 2002).

2.4 Acid-Base Balance

The ability of the animal to maintain a constant composition of the extracellular fluid is essential for the maintenance of life. Normal metabolic activities require a very close regulation of the volume, ionic composition, and pH of the extracellular fluid (Smith *et al.* 1983). The pH of the body fluids is maintained within narrow limits (7.35 to 7.45). This is necessary for the maintenance of protein structure and function, which is an essential condition for normal metabolism. Hydrogen ions (H^+) have a profound effect on metabolic events largely through interaction with cellular proteins. These interactions result in altered protein configuration and thus altered protein function. Most enzymatic reactions need to be carried out under optimum pH, and changes in H^+ concentration can have a negative effect on the rate and extent of the reaction, thus influencing homeostasis. Without adequate control systems in place the metabolic functions of the cells would either cease or become so active within a few minutes that the cells would die. Such activity can lead to acidosis in which coma and death are the final results, or to alkalosis in which tetany or convulsions result (Guyton 1956; Carlson 1997).

Metabolic processes, however, result in the production of relatively large amounts of carbonic, sulfuric, phosphoric, and other acids. These products of metabolism are transported to the excretory organs (lungs and kidneys) via the extracellular fluid without producing any appreciable change in pH. This transportation and subsequent excretion is accomplished by the combined functions of the buffer system of the blood and by respiratory and renal regulatory mechanisms (Heusel *et al.* 2001).

Acid-base balance refers to the homeostatic maintenance of acids and bases within the body to achieve a physiological constant pH of approximately 7.40 ± 0.1. Acid-base balance involves an accounting of the carbonic (H_2CO_3, HCO_3^-, CO_3^{2-}, and CO_2) and noncarbonic acids and conjugate bases in terms of input (intake plus metabolic production) and output (excretion plus metabolic conversion) over a given period of time. The acid-base status of the body fluids typically is assessed by the measurements of plasma pH and pCO_2 as the bicarbonate/carbonic acid system is the most important buffering system of the plasma (Strand 1983; West 1991; Heusel *et al.* 2001).

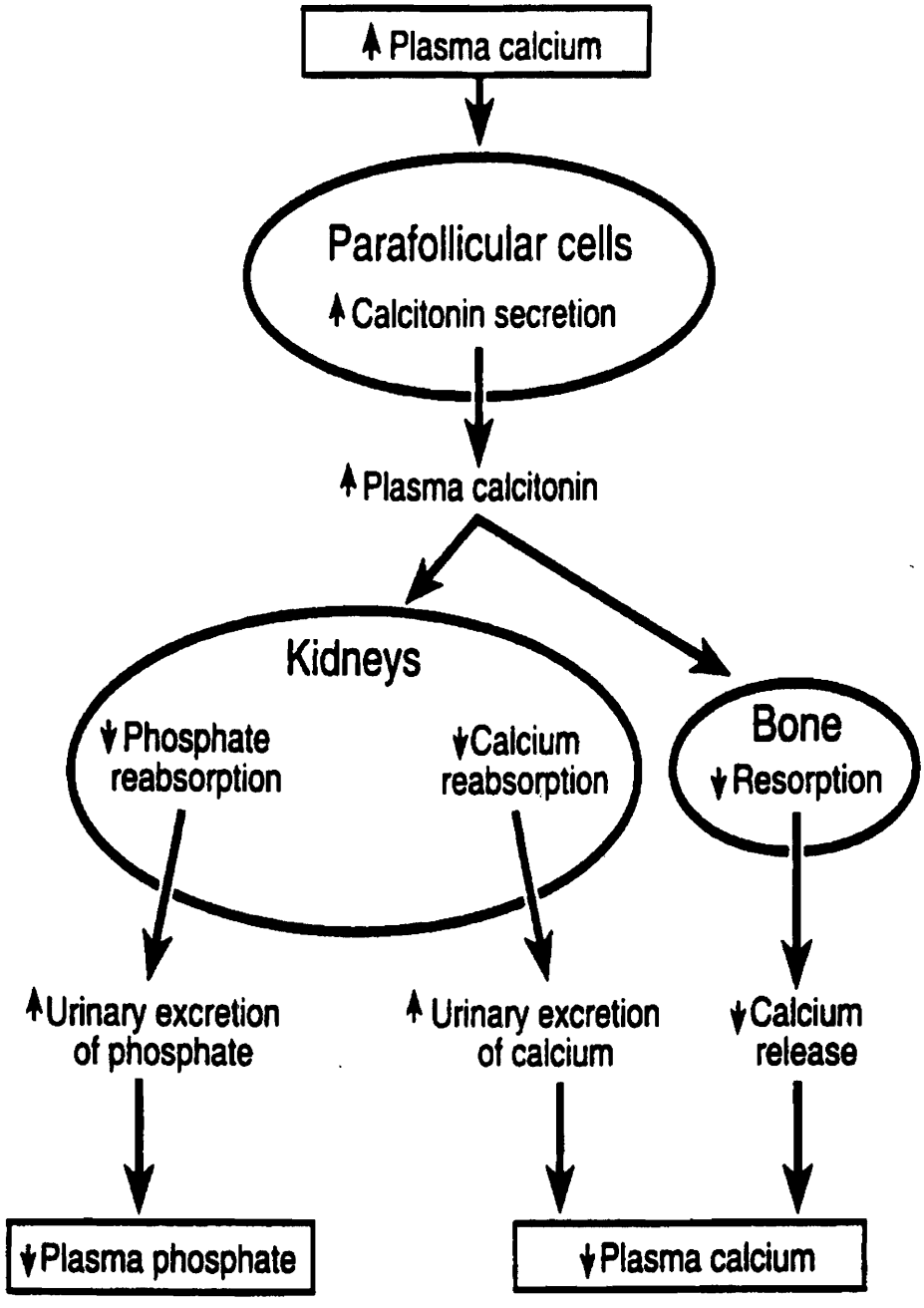

Figure 2.8. The effect of calcitonin on calcium metabolism (Adapted from Rhoades and Tanner 1995).

2.4.1 Regulation of the Acid-Base Homeostasis

The three most important systems involved in pH regulation are the chemical buffers of the blood and tissues, the respiratory system, and the kidneys. Removal of the acids formed by active tissues is crucial because the excess of acid or alkali can contribute to metabolic disorders such as acidosis or alkalosis (Smith *et al.* 1983; Bhagavan 1992; Guyton and Hall 2000):

a) ***The buffer systems***: A buffer pair is the combination of a weak acid and its conjugate base that resists a change in H^+ concentration. Examples of conjugate buffer pairs include H_2CO_3 and HCO_3^- and HbO_2 (oxyhemoglobin) and Hb- (deoxygenated hemoglobin). Proteins can buffer as Zwitter ions because of the presence of NH_3^+ (amino) groups, which can act as an acid (donate H^+) and the presence of a carboxylic group (COO^-), which can act as a base (accept H^+). These systems react within a fraction of a second, preventing sudden changes in pH (Example: $HA \leftrightarrow H^+ + A^-$).

b) ***The respiratory control***: An increase in H^+ concentration in the body fluids directly affects the respiratory center of the medulla to increase the rate of pulmonary ventilation. A decrease in H^+ concentration decreases the rate of pulmonary ventilation. Pulmonary ventilation is stimulated as much as by increased H^+ concentration in the body fluids as by increased CO_2 concentration. They both can act independently or in concert to affect the rate and depth of respiration. However, they can neutralize each other as well. In general, H^+ concentration can stimulate the ventilatory rate to 5 times normal, while CO_2 can stimulate the ventilatory rate to 10 times normal. The major purpose of the respiratory control is to prevent excessive accumulation of CO_2 in the body and subsequent acidosis. Large quantities of CO_2 are removed from the body fluids and as a result the H^+ concentration returns toward normal. In alkalosis the opposite effect occurs. The respiratory system takes approximately 1 to 3 min to respond after sudden changes in H^+ concentration have occurred.

c) ***The kidneys control***: The kidneys regulate the extracellular fluid H^+ concentration through three basic mechanisms: 1) secretion of H^+, 2)

reabsorption of filtered bicarbonate ions, and 3) production of new bicarbonate. An increase in the H^+ concentration to above normal (acidosis) causes increased reabsorption of basic compounds from the tubular fluid; a decrease in the H^+ concentration to below normal (alkalosis) causes increased reabsorption of acidic compounds from the tubules. Thus, the kidneys eliminate the excess of acid or base through their ability to change the composition of the urine, by excreting either acidic (larger amounts of H^+) or basic (larger amount of bicarbonate) urine. This system usually takes hours or days to respond, and is considered the most powerful acid-base balance regulatory system.

2.4.2 Disturbances of the Acid-Base Balance:

Normal blood pH ranged from 7.35 to 7.45 (corresponding to 35 to 45 nM of H^+/L). Values below 6.80 (160 nM of H^+/L) or above 7.70 (20 nM of H^+/L) are seldom compatible with life. A large amount of acid is produced everyday, and most of the organic acids are produced by incomplete oxidation of proteins, carbohydrates and fats, and in some conditions (e.g. intoxication, diseases, etc.), which should be eliminated from the body. Failure to eliminate or neutralize, leads to disorders in the acid-base balance (Kaneko 1989; Bhagavan 1992).

Disorders in the acid-base balance are classified according to their cause and the direction of the pH change. Acidemia is defined as an arterial blood pH of less than 7.35. Alkalemia is when the arterial blood pH is greater than 7.45. Acidosis and alkalosis refers to pathological states that can lead to academia or alkalemia (Autran de Morais and DiBartola 1993, 1994).

Acid-base disorders are divided in two types: respiratory and metabolic. These can be further classified as respiratory acidosis, respiratory alkalosis, metabolic acidosis, or metabolic alkalosis. However, more than one type of pathological process can occur simultaneously, given rise to a mixed acid-base disturbance, in which the blood pH may be low, high, or within the normal interval (Bhagavan 1992; DiBartola 2000; Guyton and Hall 2000; Heusel *et al.* 2001):

a) ***Respiratory acidosis***: If breathing is slow and shallow, the alveoli is not able to rid itself of the CO_2 that diffuses from the blood. Therefore, the concentration of CO_2 rises in the alveoli, causing the CO_2 to build up in the body fluids. As the quantity of dissolved CO_2 in the body fluids increases, the concentration of carbonic acid, bicarbonate ion, and H^+ also increases ($CO_2 + H_2O \leftrightarrows H_2CO_3 \leftrightarrows H^+ + HCO_3^-$). Therefore, the body fluids become more acid and the pH falls, causing acidosis. In respiratory acidosis pCO_2 is increased.

b) ***Respiratory alkalosis***: If breathing is rapid and deep, the CO_2 concentration in the alveoli falls very low and there is increased diffusion of CO_2 from the blood through the pulmonary membrane into the alveoli. Therefore, the concentrations of dissolved carbon dioxide, carbonic acid, bicarbonate ion, and H^+ are depressed. The pH of the body fluids rises resulting in alkalosis. In respiratory alkalosis pCO_2 is increased.

c) ***Metabolic acidosis***: Normal metabolic processes produce considerable amounts of nonvolatile acids. Some acids are ingested and absorbed from the gastrointestinal tract into the blood. Generally, the acidic salts of organic compounds are absorbed, and the organic portions of the compounds are then metabolized into water and CO_2, leaving the acid radical behind causing an increase in H^+ concentration, thus leading to acidosis. Acidosis due to retained CO_2 is also a type of metabolic acidosis, because CO_2 is derived from metabolism. Thus, the term metabolic acidosis usually refers to the acidosis causes by acid that cannot be excreted by the respiratory system but instead must be excreted by the kidneys. Metabolic acidosis can result from: 1) failure of the kidneys to excrete metabolic acids normally formed in the body, 2) formation of excess quantities of metabolic acids in the body, 3) addition of metabolic acids to the body by ingestion or infusion of acids, and 4) loss of base from the body fluids. In metabolic acidosis, pCO_2 and plasma bicarbonate are decreased while the anion gap (difference between unmeasured anions and unmeasured cations) can be increased.

d) ***Metabolic alkalosis***: When there is an excess retention of bicarbonate or loss of H^+ from the body, the result is alkalosis. When large quantities of alkaline

drugs are ingested or alkaline salts of organic compounds an excess quantity of alkali can occurs in the body fluids, or when excessive quantities of hydrochloric acid are lost from the stomach. Metabolic alkalosis is not nearly as common as metabolic acidosis.

There is a close association between blood gases and acid-base metabolism. Appropriate electrodes allow the determination of partial pressure of oxygen (pO_2), partial pressure of carbon dioxide (pCO_2), and pH in a single blood sample. From these, the bicarbonate concentration [HCO_3^-], CO_2 content, base excess, and oxygen saturation can be calculated and the general acid-base status evaluated (Lasker 1981).

2.5 Importance of Normal Plasma Calcium Levels

Concentration of Ca in plasma under normal conditions is maintained in a very close range by the interaction of several factors, among them are the amount and form of Ca and phosphorus (P) in the diet, the Ca:P ratio, the physiological state of the animal and the functioning of the Ca-regulating hormones (PTH, vitamin D_3 and calcitonin). If any of these factors is altered it can affect the Ca homeostasis in the animal. The most apparent effect is in decreasing (hypocalcemia = < 8 mg/dl) or increasing (hypercalcemia = > 10 mg.dl)) plasma Ca levels, which can cause adjustments in the Ca homeostatic mechanisms or, depending on severity, specific metabolic disorders (Kaneko *et al.* 1997). In hypocalcemia, the nervous system becomes progressively more excitable because of increased neuronal membrane permeability. The peripheral nerve fibers become so excitable that they begin to discharge spontaneously, initiating nerve impulses that pass to the peripheral skeletal muscles, where they elicit tetanic contraction. In severe hypocalcemia, the spasm of the skeletal muscle increase, particularly the muscles of the extremities and the larynx. Laryngospasms can become so severe that the airway is obstructed, and death by asphyxia results (Guyton 1956; Underwood 1981; Ganong 1987). In hypercalcemia, the nervous system is depressed, and reflex activity of the central nervous system becomes sluggish. This induces depression in the contractility of the muscular walls of the gastrointestinal tract causing constipation and lack of appetite. In severe cases, calcium phosphate crystals can precipitate forming renal stones, damaging renal function, and inducing calcification of blood and soft tissues (West 1991;

Guyton and Hall 1997). The most common of these two metabolic dysfunctions in animal production is hypocalcemia, which induces the metabolic disease known as parturient hypocalcemia or milk fever. Hypocalcemia (< 8 mg of total Ca/dl) occurs because Ca leaves the extracellular fluid pool to enter the mammary gland faster than it can be replaced by intestinal Ca absorption or bone Ca resorption. The disease is characterized by recumbence, which can damage nerves and muscles, and also by paralysis of the smooth muscle, which causes loss of the eructation reflex, resulting directly or indirectly in bloat or secondary pneumonia caused by inhalation of rumen contents, then comma and eventually death (Allen and Sansom 1985).

2.6 Approaches Used to Increase Plasma Calcium Levels

The most common approaches used to increase plasma Ca and reduce the likelihood of milk fever have been the administration of massive doses of vitamin D, manipulation of dietary Ca, and acidification of prepartal diets.

2.6.1 Administration of Vitamin D

Vitamin D has been used to prevent parturient hypocalcemia in dairy cows for many years (Hibbs and Pounden 1955). For example, vitamin D_2 has been supplemented at levels of 20 to 30 million IU (MIU) daily for a maximum of 7 days starting 3 to 8 days prepartum with positive results in reducing the incidence of milk fever (Hibbs and Conrad 1976). Feeding vitamin D_3 at levels of 10,000, 50,000, or 250,000 IU daily approximately two weeks prepartum tended to increase plasma Ca concentration reducing the incidence of milk fever (McDermott *et al.* 1985).

Reinhardt and Conrad (1980) conducted an experiment in which vitamin D_3 at the level of 10 MIU via intramuscular injection was administrated to pregnant non-lactating Holstein and Jersey cows 5 to 7 days prior to parturition. The injection was combined with a high Ca diet (130 g Ca and 35 g P/d) or with a high phosphorus diet (45 g Ca and 70 g P/d). Administration of vitamin D_3 resulted in a 150 % rise (in both treatments) in circulating $1,25(OH)_2D_3$ 24 h post-injection. By day 1 prepartum the $1,25(OH)_2D_3$ levels returned to pre-injection levels. The levels of $1,25(OH)_2D_3$ in the plasma of control cows did not change until parturition at which time the hormone increased from 100-200 pg/ml

to 600 pg/ml. This was followed by a decrease to 200 pg/ml by day 2 post-partum. These researchers concluded that injection of 10 MIU of vitamin D_3 overrode the tightly controlled kidney 1α-hydroxylase enzyme resulting in overproduction of $1,25(OH)_2D_3$.

Littledike and Horst (1982) studied the effect of large parenteral doses (15 to 17.5 MIU) of vitamin D_3 on plasma Ca, vitamin D_3 and $25(OH)D_3$, and $1,25(OH)_2D_3$ concentrations in Jersey cows. The results showed a significant increase in all the compounds. None of the vitamin D_3 treated cows showed signals of milk fever. However, 22 % of the control cows developed clinical signs of milk fever. Pregnant cows injected with vitamin D_3 developed signs of toxicity. Ten out of 17 cows died, showing evidence of metastic calcification. Those researches concluded that due to the extreme toxicity of vitamin D_3 and the low margin of safety in doses that effectively prevent milk fever, they did not recommend the use of massive doses of vitamin D_3 as a means to prevent milk fever when injected several weeks prepartum.

2.6.2 Manipulation of Dietary Calcium

2.6.2.1 Low Calcium Diets

One of the most effective means to prevent milk fever is to avoid excessive dietary Ca in the prepartum diet (Allen and Sansom 1985; Horst 1986; Horst *et al.* 1994). This approach is based on stimulation of the Ca homeostatic mechanisms of the animal. Low Ca in the diet will induce lower Ca plasma levels, this lower plasma Ca level will be sensed by the parathyroid glands and synthesis and secretion of PTH will be increased. This increase in circulating PTH will stimulate higher activity of the renal enzyme 1α-hydroxylase and thus greater synthesis of $1,25(OH)_2D_3$, which in turn will stimulate higher Ca absorption from the intestine, Ca reabsorption from kidneys and Ca resorption from bone (Goff 1989).

It has been found that low Ca diets effectively increase the concentration of circulating $1,25(OH)_2D_3$, and the efficiency of intestinal Ca absorption. Ramberg *et al.* (1984) found that when dietary Ca in dairy cows dropped below 50 to 60 g/d, the efficiency of intestinal Ca absorption increased. When dietary Ca was 10 to 15 g/d, the

animals depended almost solely on bone Ca resorption to maintain normal plasma Ca levels.

Goings *et al.* (1974) prevented parturient paresis in dairy cows by feeding a low Ca diet (8 g of Ca daily per 450 kg body weight) for about 14 days prepartum. Changes in plasma Ca and PTH occurred within 4 days of initiation of the feeding of the low Ca diet. Calcium concentration reached a minimum (8.3 mg/dl) approximately 36 h after initiation of feeding the low Ca diet. Calcium concentration then slowly rose (9 mg/dl) until approximately 50 h after the minimum. These workers concluded that Ca homeostatic mechanisms were effectively sensitized by the low Ca diet.

Yarrington *et al.* (1977) investigated the effect of feeding a low (9.5 g Ca/d and 25 g P/d) or normal (25 g Ca/d and 25 g P/d) Ca diet on Ca homeostatic mechanisms. Feeding started 70 days prior to the calculated date of parturition. The specific activity of intestinal and renal CaBP, percentage of bone surfaces undergoing resorption, urinary hydroxyproline, and plasma PTH concentrations were higher 1 day postpartum for cows fed the low Ca compared to the control diet. Urinary hydroxyproline excretion 1 day postpartum was increased 553 % above prepartum values for cows fed the low Ca diet compared to 345 % for the control cows. Serum Ca remained within normal limits in the cows fed the low Ca diet. Plasma PTH concentrations increased with the approach of parturition in both groups of cows. Calcitonin activity in the thyroid gland was greater in the cows fed the low Ca diet and ultrastructurally, secretory granules were more numerous in thyroid C-cells. These workers concluded that Ca homeostasis in cows fed the low Ca diet prepartum was more under the fine control of PTH-mediated bone resorption with the approach of parturition. Thus, greater reliance on bone Ca mobilization than on intestinal Ca absorption may be a significant factor in the prevention of parturient hypocalcemia from low Ca diets.

Green *et al.* (1981) characterized the changes in vitamin D metabolites of plasma in dairy cows fed a high (80 g/d) or low (8 g/d) Ca diet for approximately 14 days before parturition. Plasma Ca concentration decreased after initiation of either diet, but cows fed the low Ca diet tended to have lower prepartum but greater peripartal plasma Ca concentrations. Magnesium concentrations in plasma were not affected. Prepartum $1,25(OH)_2D_3$ in plasma tended to be greater in cows fed the low Ca diet. It was

concluded that the changes observed in plasma of the cows fed the low Ca diet were associated with mobilization of the Ca homeostatic mechanisms several days before the Ca demand of initiation of lactation.

Kichura *et al.* (1982) investigated the effect of feeding diets with different combinations of Ca and phosphorus (P) to cows for about 4 weeks prepartum. Low Ca diets had greater concentrations of plasma $1,25(OH)_2D_3$ and hydroxyproline prepartum, greater plasma Ca concentration at parturition, and less incidence of parturient paresis. These workers concluded that low Ca diets, regardless of dietary P intake, seemed to activate Ca homeostatic mechanisms before parturition by stimulating both intestinal Ca absorption and bone Ca resorption.

2.6.2.2 Calcium Supplements

Oral administration of large amounts of Ca salts to force Ca into the blood by passive diffusion can also be used to increase blood Ca concentration during the periparturient period. The Ca salts used has traditionally been calcium chloride ($CaCl_2$) and calcium propionate (Goff and Horst 1993). The $CaCl_2$ solutions and gel preparations are a soluble concentrated (36 %) and rapidly absorbed source of Ca (54 g of Ca). However, $CaCl_2$ solutions and gels have several disadvantages. Aqueous solutions of $CaCl_2$ and some gel products are very caustic and cause ulceration of the mouth and digestive mucosa of some cows. All $CaCl_2$ products reduce blood pH, and excessive oral $CaCl_2$ can induce metabolic acidosis (Horst *et al.* 1994, 1997).

Calcium propionate can be formed into a thick paste. It effectively raises plasma Ca concentration, although its effect is not as rapid as with $CaCl_2$. Its activity however, is more sustained (Goff and Horst 1993). In contrast to $CaCl_2$, calcium propionate does not affect blood pH. Furthermore, the propionate could serve as a gluconeogeneic precursor at a time when the animal is in a negative energy balance. Calcium propionate has the disadvantage of being only 21.5 % Ca, thus requiring larger volumes of the preparation to be given orally (Horst *et al.* 1997).

Goff *et al.* (1996) conducted an experiment to test the efficiency of a calcium propionate paste as an aid to prevent milk fever. Two or three calcium propionate tubes (37 g of Ca) were given at calving and again at 12 h after calving to Jersey and Holstein

cows. For the Jersey herd, calcium propionate treatment (2 tubes) reduced the incidence of milk fever from 50 % in control animals to 29 % in treated cows. Plasma obtained 24 h after calving from treated cows had higher Ca (7.23 mg/dl) than plasma from control (6.87 mg/dl) cows. No effect was observed on the Holstein herd. However, calcium propionate reduced the number of cows with subclinical hypocalcemia (< 7.5 mg/dl of plasma Ca) in both herds. The conclusion was that calcium propionate was beneficial in reducing subclinal hypocalcemia and in reducing the incidence of milk fever.

Dhiman and Sasidharan (1999) carried out a study to determine the effectiveness of $CaCl_2$ supplements such as gels and drench in increasing blood serum Ca concentrations in periparturient dairy cows. Treatments were inert gel, gel containing $CaCl_2$ and vitamins, gel containing $CaCl_2$ and minerals, and drench containing $CaCl_2$ and vitamins. The results showed that oral supplements of $CaCl_2$ as gel or drench increased the blood Ca levels in periparturient dairy cows. These researchers concluded than an increased supply of Ca through oral supplements of $CaCl_2$ may prevent milk fever in cows that are marginally hypocalcemic.

2.6.3 Supplementation of Anionic Salts

Manipulation of the acid-base balance has successfully been used as a nutritional tool to increase plasma Ca levels in periparturient dairy cows and so reduce the incidence of milk fever (Block 1984, 1994; Block and LeClerc 1989; Tucker *et al.* 1992; Horst *et al.* 1997; Goff and Horst 1998). It is recognized that acidification of the diets improves Ca homeostasis by increasing the proportion of dietary Ca absorbed from the intestines, as well as the rate of mobilization of Ca from the skeleton. This effect seems to be mediated by increases in the rate of production of 1,25-dihydroxyvitamin D_3 and the responsiveness of tissues to parathyroid hormone (Abu Damir *et al.* 1994; Vagnoni and Oetzel 1998). Anionic salts, including magnesium sulfate, ammonium chloride, and calcium chloride, generally induce a mild compensated metabolic acidosis which is characterized by extensive Ca excretion in urine (hypercalciuria), which in turn may be responsible for stimulation of the vitamin D/Ca homeostatic mechanisms. It has been observed that plasma hydroxyproline concentrations increase suggesting that bone resorption may be stimulated because of the role of bone in buffering systemic acidosis.

In these studies, Ca absorption from intestine has been reported to be increased, decreased or unchanged (Block 1984; Goff *et al.* 1991; Oetzel 1993; Vagnoni and Oetzel 1998).

Alterations in dietary cation-anion balance will influence acid- base status of the animal. The degree depends on the quantity of the ion entering the system. Since anions are considered acidogenic and cations alkalogenic, their content in a diet is commonly used as a measure of its alkalinity or acidity. The dietary cation-anion balance (DCAB) is defined as the sum in milliequivalents (mEq) of the cations, sodium (Na) and potassium (K), minus the sum of the anions, chloride (Cl) and sulfur (S), and expressed in the formula: [Na + K) – (Cl + S]. Thus, a positive DCAB means an alkaline diet while a negative DCAB means an acidic diet. Calculation of the DCAB of a diet requires using the equivalent weights of the electrolytes because acid-base balance is affected by electrical charge rather than mass. One equivalent weight is equal to the molecular weight divided by the valence (Block 1984; LeClerc and Block 1989; Oetzel 1993; Perhson *et al.* 1999).

2.7 Manipulation of Plasma Calcium to Improve Beef Tenderness

It is generally accepted that proteolysis of myofibrillar proteins is a major contributor to tenderization of beef during the postmortem period. Among the proteolytic enzymes found in muscle tissue, the neutral proteases known as the calpain enzyme system appears to be, at least partially, responsible for these changes. Calpains depend on Ca for activity, and their activity is directly related to the concentration of Ca (the μ- and m-calpains require 3-50 and 400-800 μM of Ca to be activated, respectively). These enzymes degrade principally the proteins of the Z disk, resulting in changes in structural and physical characteristics of muscle tissue, resulting in more tender meat. It has been hypothesized if intracellular Ca levels of muscle fibers can be increased, the activity of these enzymes postmortem will be increased. Two main approaches have been used to increase Ca concentration in muscle: a) postmortem treatment, in which calcium chloride is injected to meat cuts, and b) premortem treatment, in which vitamin D_3 is supplemented to live animals in order to increase Ca concentration in plasma and subsequently in muscle.

2.7.1 Calcium Chloride Injection

It was found that incubation of bovine *longissimus* muscle slices obtained 12 h postmortem, in a buffer solution containing calcium chloride ($CaCl_2$), accelerated myofibrillar proteolysis. These changes occurred within the first 24 h of incubation. No beneficial effect was observed with the same buffers containing Ca chelators (10 mM EGTA or 10 mM EDTA)(Koohmaraie *et al.* 1988a). Because none of the treatments affected lysosomal enzyme activities (cathepsin B, H and L) and because there was a decrease in calpains activity, these researchers concluded that a) postmortem tenderization was mediated by Ca, and b) that the calcium-dependant proteases (calpains) were responsible for postmortem tenderization. In a subsequent study, Koohmaraie *et al.* (1988b) infused lamb carcasses (through carotid artery) with 0.3 M CaCl2 immediately after death. Infusion of $CaCl_2$ resulted in acceleration of the postmortem tenderization process. These workers concluded that Ca is important to increase the activity of the calpain enzymes, which are responsible for myofibrillar proteolysis. In another study, Koohmaraie *et al.* (1989) examined the effect of infusing different concentrations and ionic strengths of $CaCl_2$ to ovine carcasses. It was observed that 0.3 M $CaCl_2$ gave the best response in increasing the activity of the calpains. Enzyme activity was maximum during the first 24 h after infusion. Shear force values were reduced 50 % in carcasses treated with $CaCl_2$ compared with untreated carcasses on day 1.

Subsequently, $CaCl_2$ infusions were extended to beef carcasses (Koohmaraie and Whipple 1990; Morgan *et al.* 1991b), and to specific muscle cuts (Wheeler *et al.* 1991; Wheeler and Koohmaraie 1992), or by marination of subprimal meat cuts (Whipple and Koohmaraie 1992).

It was concluded from these studies that: 1) injection of $CaCl_2$ represents an effective way to increase intracellular Ca levels of muscle, 2) the external Ca stimulates higher activity of the calpain enzyme system, 3) which results in greater myofibril protein degradation, and 4) increased myofibrillar degradation is associated with reduced shear force values, an indication of more tender meat.

There are some disadvantages in the use of this technique in commercial practice. These include: 1) $CaCl_2$ at the concentrations of 0.3 M has proved to be the most adequate infusion dose (Koohmaraie *et al.* 1989), however, at this concentration $CaCl_2$

tends to produce bitter and off-flavors (Morgan *et al.* 1991b); 2) manipulation of carcasses or meat cuts for $CaCl_2$ infusion represents a significant increase in labor and facilities as well as a safety issue with respect to preparation and administration of solution. The risks and expenses associated with this method outweigh its advantages.

Methods to increase the concentration of Ca in muscle while the animal is still alive, could potentially be of interest to the beef industry. In this case, Ca will be distributed throughout the muscles of carcass such that the improvement in meat tenderness will be on the whole carcass (systemic distribution) and not just in some meat cuts. Moreover, the need for increased labor and/or facilities will be eliminated.

2.7.2 Vitamin D_3 Supplementation on Beef Tenderness

Owens *et al.* (1998) stated that the supplementation of high levels of vitamin D_3 (5 million IU or more per head per day) for approximately 5 to 10 days to finishing steers prior to slaughter could result in improved beef tenderness. These authors suggested that the improved beef quality was a result of higher activation of the Ca-activated enzymes, the calpains. The rationale is that higher levels of vitamin D_3 in plasma will induce the synthesis of the hormonal form of vitamin D_3 ($1,25(OH)_2D_3$), which will act upon the epithelial cells to improve the efficiency of Ca absorption, as well as on kidneys and bone to increase the rate of Ca reabsorption and resorption, respectively. All these actions will induce greater plasma Ca levels, however, as plasma Ca is maintained in a very close range, the excess of Ca in plasma is mobilized to other tissues, among them muscle. It is hypothesized that this excess of Ca in muscle will stimulate higher activity of the proteolytic calpain enzymes, which dissolve myofibrillar proteins in the Z-disk of the sarcomere. The result is an improvement in beef tenderness.

The first research in this area was carried out by Swanek *et al.* (1997), in which two experiments were reported. Beef steers were supplemented in trial 1 with 5 million IU (MIU) of vitamin D_3 (D_3) per day for 5 days immediately prior to slaughter. In trial 2 the cattle were supplemented with 7.5 MIU D_3 per day for 10 days prior to slaughter. In trial 1, total Ca in plasma Ca increased from 9.23 to 10.39, a 12.6 % increase. Shear force values of steaks from treated animals were reduced by 6.6 % and the number of unacceptable tough steaks by 21.8 % at 7 days postmortem. In trial 2, shear force values

were reduced by 18 % at 7 days postmortem aging, and the number of tough steaks was reduced by 23.3 and 22.5 % at 14 and 21 days of postmortem aging. These workers concluded that an increase in activity of the proteases associated with the calpain system could be responsible for the improvement in beef tenderness. In subsequent work, supplementation of steers was carried out at levels of 0, 2.5, 5, and 7.5 MIU D_3 per day for 10 days (Swanek *et al.* 1999). Blood ionized Ca increased linearly between days 6 and 13. The 7.5 MIU D_3 treatment had blood Ca levels that were 40 to 50% greater than those from control animals. The 5 MIU D_3 treatment resulted in greater muscle Ca concentration than from control muscle tissue. Sensory panelists rated samples from vitamin D_3 supplemented steers as more tender than the unsupplemented steers (Swanek *et al.* 1999).

Montgomery *et al.* (1998) administered to beef steers 0, 5 or 7.5 MIU of vitamin D_3 (via boluses) per day for 9 days. Cattle were slaughtered 2 days later. Vitamin D_3 treatment increased plasma (30 and 40 % for the 5 and 7.5 MIU D_3, respectively) and muscle Ca (30 % for the 5 MIU D3) concentration. The two levels of vitamin D_3 were equally effective in reducing shear force values (approximately 15 % reduction at 14 days postmortem). In a subsequent report, Montgomery *et al.* (2000) using the same levels of supplemental vitamin D_3 than in previous work, found that concentrations of vitamin D_3 and $25(OH)_2D_3$ increased in plasma, liver and muscle in direct relationship with the amount of vitamin D_3 supplemented. In contrast, $1,25(OH)_2D_3$ was not affected. These workers also found higher proteolytic activity (greater activity of the calpain enzyme system, in particular μ-calpain) in muscle tissue from vitamin D_3 treated steers than from controls. However, this proteolytic activity was higher from the 5 MIU D_3 than for the 7.5 MIU D_3 treatment. This suggests that 5 MIU D_3 per day for at least 5 days represents a potential method to increase beef tenderness and consumer acceptance.

Karges *et al.* (1999a) supplemented beef yearling steers with 0, 5, 7.5, 15 and 75 MIU D per day. It was observed that feed intake was significantly depressed in an inverse relationship with the amount of vitamin D supplemented. In a subsequent work Karges *et al.* (1999b) supplemented finishing steers with 0 or 6 MIU of vitamin D (in pellets) for either 4 or 6 days. It was found that tenderness was numerically increased as a result of vitamin D supplementation. In a subsequent study, it was found that plasma

Ca concentration increased by vitamin D supplementation and was increased to a greater degree by feeding vitamin D for 6 than for 4 days (Karges *et al.* 1999c). It was observed that calpastatin activity at 0 or 24 h was not affected by vitamin D supplementation. Karges *et al.* (2001) conducted an experiment to determine the effect of vitamin D_3 supplementation on plasma Ca concentration, carcass traits, and tenderness. Vitamin D_3 was fed to beef steers at 0 or 6 MIU daily for 4 or 6 days. The results showed a significant increase on plasma Ca concentration, a decrease in shear force values, no difference in palatability traits, a delayed pH decline, and an increase in water holding capacity in meat from the supplemented steers. These researchers concluded that supplementation of vitamin D at levels of 6 MIU appears to represent a reliable method to increase plasma Ca concentration, improve tenderness and the water holding capacity of beef.

Scanga *et al.* (2001) carried out an experiment to develop a recommendation for the optimum dose and duration of administration of vitamin D_3 for purposes of improving beef tenderness. Beef heifers were supplemented with vitamin D_3 (via boluses) at 1, 2 (2 plus 75 g of $CaCO_3$), 3, 4 (4 plus 75 g of $CaCO_3$), or 5 MIU daily for 2, 4, 6, or 8 days prior to slaughter. The results showed that hot carcass weight, dressing percentage, USDA Yield Grade, or USDA marbling score were not affected. Total serum Ca concentrations were increased as a result of vitamin D_3 supplementation. Surprisingly, the 1 MIU D_3 treatment had the highest serum Ca concentration (13.3 vs 12.7 mg/dl for the 5 MIU D_3 treatments). There were no differences in shear force values or in tenderness of cooked steaks (at 75 or 85 °C) from supplemented cattle. These results do not agree with previous research. These workers concluded that more work needs to be carried out to determine the impact of vitamin D_3 on beef tenderness.

From this review, is clear that supplementation of vitamin D_3 to beef cattle can result in increased plasma Ca concentration, and subsequently in higher muscle Ca concentrations. This intracellular Ca can stimulate greater activity of the calpains, enhancing beef tenderness. However, as pointed out by Owens *et al.* (1998), high levels of vitamin D_3 supplementation present two main disadvantages, the first has to do with animal welfare and animal performance; and the second with food safety and public health. Feeding high levels of vitamin D_3 depresses feed intake (Berry *et al.* 2000). This

affects animal performance, and prolonged high dietary doses can cause vitamin D_3 toxicity characterized by muscle weakness, renal and cardiovascular failure and death (DeLuca 1980; Spirichev and Sergeev 1988). Montgomery *et al.* (2000) found that supplementation of high levels (5 or 7.5 MIU/hd/d) of vitamin D_3 induced significant increases in vitamin D_3 and 25(OH)D_3 concentrations in plasma, muscle, liver and kidney. Thus, meat containing high levels of vitamin D_3 can potentially induce vitamin D_3 toxicity in consumers, characterized by anorexia, nausea, formation of renal stones, and calcification of soft tissues (Glerup and Eriksen 2000). It is clear that supplementation of high levels (5 MIU/hd/d or more) of vitamin D_3 represents a viable method to increase plasma Ca levels to a point where improvements in beef tenderness has been observed (Swanek *et al.* 1997, 1999; Montgomery *et al.* 1998, 2000; Karges *et al.* 1999a,b,c, 2001). However, high levels of vitamin D_3 also represent high risks in animal and human health. It would be of value to minimize the risks by feeding lower levels of vitamin D_3 or to augment the effects of vitamin D_3 feeding via other methods of dietary manipulation of plasma Ca levels. Such practices might include a designated period prior to slaughter where dietary Ca levels are reduced as common with dairy cows prior to calving or manipulation of the dietary cation-anion balance combined with lower levels of vitamin D_3 feeding. This approach may increase plasma Ca levels to a point where beef tenderness is ultimately improved, without using levels of vitamin D_3 that risk toxicity.

2.8 The Objectives of the Research Project

The general objectives of the present thesis were to evaluate:

a) The effects of feeding graded levels of vitamin D_3 on total and ionized serum Ca concentrations and on feed intake of cattle fed diets common to Western Canada.
b) How graded levels of vitamin D_3 affect circulating concentrations of vitamin D_3 and its metabolites and subsequently Ca homeostatic mechanisms.
c) The effects of manipulation of dietary Ca levels on the response of serum Ca levels to vitamin D_3 supplementation.

d) If dietary Ca manipulation coupled with anionic salt supplementation could be a suitable method to increase serum Ca concentrations as a result of vitamin D_3 supplementation.

CHAPTER III

Effect of Supplemental Vitamin D_3 on Feed Intake and on Serum Calcium Concentrations of Steers fed Barley Grain-Based Finishing Diets

3.1 Introduction

Beef is greatly appreciated and consumed in many countries in the world. However, its consumption can be limited by several factors, among them, its perceived quality. In particular, tenderness and consistency constitute one of the most important issues in the beef industry (Boleman *et al.* 1997).

Tenderness is considered to be the single most important component in meat quality, as evidenced in the relationship between the price of a cut and its estimated tenderness (Karges *et al.* 1999b). Furthermore, inadequate tenderness can cause valuable losses to the beef industry (Smith *et al.* 1995).

Tenderness is related to postmortem proteolysis (Boehm *et. al.* 1998), and it has been widely hypothesized to be the result of degradation of the myofibrillar proteins near the Z-disks (Kendall *et al.* 1993). The calpain proteolytic system plays a key role in the postmortem proteolysis that leads to increased tenderness (Geesink and Koohmaraie 1999). This proteolytic system consists of two isoenzymes that are activated by Ca with differing sensitivities. The μ-calpain isoenzyme requires a micromolar order of Ca^{2+} and m-calpain a millimolar order of Ca^{2+} for activation. The third enzyme is called calpastatin, which has the function to inhibit the activity of the calpains (Kendall *et al.* 1993; Geesink and Koohmaraie 1999).

It has been shown that calcium chloride infusion to pre-rigor beef can improve tenderness (Koohmaraie *et al.* 1989). This work led to the theory that manipulation of plasma Ca levels in the living animal, prior to slaughter, would also lead to an improvement in tenderness. Increases in muscle Ca concentration could be achieved by increasing Ca concentration in plasma (Montgomery *et al.* 1998) because Ca concentration in the extracellular fluid is maintained within a narrow range (8 to 10 mg/dl), and any excess is immediately diverted to other tissues or excreted (Rosol *et al.* 1995). This homeostatic mechanism is controlled by the calciotrophic hormones:

parathyroid hormone (PTH), calcitonin and vitamin D_3 in its hormonal form (1,25-dihydroxyvitamin D_3) (DeLuca 1979; Cunningham 2002).

Vitamin D_3 has been used to regulate Ca homeostasis in the event of metabolic diseases such as rickets and osteomalacia (Guyton 1956), or to prevent milk fever (Higgins 1955). In both cases, vitamin D_3 has effectively increased plasma Ca concentration. Recently, high levels of vitamin D_3 have been supplemented to beef cattle at the end of the finishing period with the purpose of increasing plasma Ca, and subsequently in muscle, with the expectation that excess Ca in muscle can increase calpain activity and so, enhance meat tenderness.

Swanek *et al.* (1997, 1999) conducted several trials to evaluate the effect of supplemental vitamin D_3 on beef tenderness. In one of the trials the steers were supplemented with 5 million IU (MIU) of vitamin D_3 (D_3) per day for 5 days immediately prior to slaughter, which resulted in an increase of 12.6 % in plasma Ca, leading to a reduction of 6.6% in shear force values and a 21.8% decrease in the number of unacceptably tough steaks. In a second trial, steers were supplemented with 7.5 MIU D_3 per day for 10 days prior to slaughter. Plasma Ca was increased approximately 34 %, which resulted in a reduction of 18.0 % in shear force values and 23.3 % in the number of unacceptably tough steaks. These authors concluded that the observed improvement in tenderness could be due to increased activity of the calpain system.

Montgomery *et al.* (1998, 2000) studied the effects of high levels of vitamin D_3 supplementation on plasma and muscle Ca concentration and subsequently on beef tenderness. Vitamin D_3 treatments were 5 and 7.5 MIU D_3 per day for 9 days, with the cattle slaughtered 2 days later. Plasma and muscle Ca concentrations were significantly increased in treated steers, although, the 5 MIU level produced a higher muscle Ca concentration than the 7.5 MIU treatment. As a result, muscle shear force values were lowered. These workers concluded that 5 MIU D_3 per day for 10 days before slaughter may be an effective way to improve beef tenderness.

Karges *et al.* (1999b) supplemented finishing steers with 6 MIU D_3 per day for 4 or 6 days prior to slaughter. They found that vitamin D_3 supplementation reduced the number of unacceptable steaks. However, supplementation tended to decrease feed intake and hot carcasses weights.

It can be concluded from the results of these studies, that the supplementation of high levels of vitamin D_3 (5 MIU or more per day) for 5 to 10 days prior to slaughter can increase Ca concentration in plasma and muscle, resulting in an improved meat tenderness. However, there are two concerns with the use of high levels of vitamin D_3. The first has to do with food safety (Owens *et al.* 1998). High levels of vitamin D_3 can result in high residual levels in meat and other edible tissues (Montgomery *et al.* 2000 and in press). The second has to do with animal performance, as high supplemental levels tend to depress feed intake (Owens *et al.* 1998; Karges *et al.* 1999a,b; Scanga *et al.* 2001), a situation that can affect carcass characteristics (Berry *et al.* 2000).

In the face of these potential problems, it is of interest to determine the response of plasma Ca to lower levels of supplemental vitamin D_3. It is also of interest to examine the relationship between supplemental vitamin D_3 levels and serum Ca using diets common to Western Canada.

Therefore, the objective of this study was to investigate the effects of feeding graded levels (1.25, 2.5 and 5 MIU/hd/d for seven days) of vitamin D_3 on serum total and ionized Ca levels and on feed intake of cattle fed diets common to Western Canada.

3.2 Material and Methods

3.2.1 Experimental Design

3.2.1.1 Adaptation Period (days A1 to A33)

Fifteen Angus steers (452 ± 28 kg) were housed in individual pens (3.6 x 3.6 m) with automatic water bowls in the Livestock Research Barn of the Department of Animal and Poultry Science at the University of Saskatchewan. Animals were cared for according to the guidelines of the Canadian Council of Animal Care. The cattle were adapted to the experimental diet for 33 days. The ration consisted of 75 % concentrate (barley grain base) and 25 % barley silage (as fed) meeting the nutritional requirements for this type of cattle according to the National Research Council (NRC 1996) (Table 3.1).

Table 3.1 Ingredient composition and formulated nutrient levels of the experimental diet.

Item	DM (%)
Total mixed diet:	
Concentrate	87.2
Barley silage	12.8
Concentrate:	
Barley grain	87.8
Canola meal	3.9
Tallow	5.5
Limestone	1.2
Rumensin [z]	0.6
Vitamin [y]	0.4
Salt	0.6
Formulated composition [x]:	
Digestible energy, Mcal/kg [w]	3.60
Crude protein, %	13.15
Calcium, %	0.52
Phosphorus, %	0.36
Ca:P	1.48

[z] Premix: Monensin (20% active) mixed with barley grain.

[y] Premix: Vitamin A (416,650 IU/kg) and vitamin D (83,333 IU/kg).

[x] Nutrient Requirements of Beef Cattle. National Research Council (1996).

[w] Predicted TDN x 4.4 Mcal/kg (Weiss *et al.* 1992).

Feed was offered twice a day (0700 and 1600 h), with orts collected and weighed everyday before the morning feeding. Individual maximum voluntary intake were estimated from day A20 to A26. Then, voluntary intake was then restricted to 85% from days A27 to A33 to allow for cattle to even their intake and consume all feed offered.

Initial blood samples were obtained by jugular venipunture on days A17, A24 and A31 at approximately 1300 h. These samples served as the pre-treatment control samples. Blood samples were collected into 7ml-draw evacuated (Vacutainer®) tubes without anticoagulant, blood was then allowed to clot at room temperature for approximately 2 h. They were centrifuged at 3500 rpm for 15 min. The serum was used for the determination of total and ionized Ca concentrations.

3.2.1.2 Supplemental Period (days S1 to S7)

During this period, the diet and the feeding pattern were the same as in the last week of the adaptation period (feed restriction to 85%, two feedings a day). Feed intake was monitored daily, with orts collected and weighed everyday before the morning feeding.

The steers were randomly assigned to one of the following vitamin D_3 supplemental treatments: 1.25, 2.5, and 5 MIU of vitamin D_3 per steer per day, for seven consecutive days, with five steers per treatment.

The vitamin D_3 supplement was prepared from the vitamin D_3 premix containing 500,000 IU per gram (Rhone-Poulenc Canada Inc.), using ground barley as a carrier. The vitamin D_3 supplement was formulated to have a final concentration of 13.0 MIU D_3 per kg of supplement. This supplement was analyzed (New Jersey Feed Laboratory, Inc.) and the actual concentration was 13.13 ± 0.15 MIU D_3 per kg. The total daily amount of vitamin D_3 supplement assigned per steer per day was equally divided and supplied at each feeding. The supplement was top-dressed onto the ration and thoroughly mixed by hand before delivering into the feed bunk.

Daily blood samples (from day S1 to S7) were obtained and processed from each steer, under the same protocol as in the previous period.

3.2.1.3 Withdrawal Period (days W1 to W8)

Once vitamin D_3 supplementation ended, the steers were maintained for five days under the same experimental conditions to evaluate post-treatment effects. Immediately after the bleeding on day W5, the cattle were shipped back to the feedlot.

During this period, feed was offered without restriction according to their individual daily consumption. Similarly, feed intake was monitored daily with orts collected and weighed before every morning feeding.

Blood samples were obtained from day W1 to W5 in the metabolism barn, and on day W8 at the feedlot. Blood sampling was carried out under the same protocol as in previous periods.

3.2.2. Laboratory Analyses

3.2.2.1 Total Calcium Determination

Total Ca in serum was determined by indirect potentiometry utilizing a Ca ion selective electrode in conjunction with a sodium reference electrode under the SYNCHRON LX System (Beckman Coulter, Inc., Fullerton, CA. USA). Total Ca was calculated from free Ca once the molar ratio between free and total Ca concentrations was constant. To achieve a constant molar ratio between total and free Ca, a buffered solution (which contains strong Ca complexing agents) was used. The ratio was one part serum to 21 parts buffered solution (LX ISE Electrolyte Buffer Reagent: Tris). The high molar strength buffer served to establish a constant activity coefficient for Ca ions. The electrode was then calibrated to standard concentration values, and unknowns calculated.

3.2.2.2 Ionized Calcium Determination

Ionized Ca in serum was determined by the use of an ion selective electrode (Radiometer ABL 700 Series Analyzer. Copenhagen). The analyzer measures the differential in voltage between a reference electrode and a Ca ion-selective electrode in a solution. The potential established by the unknown solution is compared with the potential developed by a standard solution of known ionic strength and ion composition. Three electrodes are seated in a temperature-controlled module (37°C). The serum

sample moves along the sample path by peristaltic pumps. The pH of the serum was measured by a pH glass and reference electrode, adjusting ionized Ca values to pH 7.4 (Gases Ionized Calcium. Operator's Manual. Ciba-Corning 634 Ionized Ca/pH system).

3.2.3 Statistical Analysis

The data was analyzed using the Analysis of Variance Technique including Repeated Measures Analysis using the General Linear Model procedure of SAS (SAS 1989) to compare treatments within days and over time (across the experimental periods). The following model for the repeated measures analysis was used:

Model: $Y_{ijk} = \mu + \alpha_i + \tau_{ij} + \delta_k + (\alpha\delta)_{ij} + \varepsilon_{ijk}$

where:

Y_{ijk} = is the response at time k on animal j in treatment i.

μ = is the overall mean.

α_i = is a fixed effect of treatment i.

τ_{ij} = is a random effect of animal j in treatment i.

δ_k = is a fixed effect of time k.

$(\alpha\delta)_{ij}$ = is a fixed interaction effect of treatment i with time k.

ε_{ijk} = is the random error at time k on animal j in treatment i.

Single degree of freedom contrasts:

A = Pre-treatment control value vs the 1.25 MIU treatment.

B = Pre-treatment control value vs the 2.5 MIU treatment.

C = Pre-treatment control value vs the 5.0 MIU treatment.

3.3 Results and Discussion

3.3.1 Feed Intake (DMI)

Voluntary individual intake calculated from day A18 to A24 of the adaptation period averaged 10.89 ± 1.42 kg of DM (Table 3.2 and Figure 3.1). Feed intake was then restricted to 85% of *ad libitum* for days A25 to A31. The aim was to eliminate as much

as possible any daily fluctuation. Variation in intake during the supplemental period could then be considered a direct effect of treatment.

Vitamin D_3 supplementation depressed ($P<0.05$) feed consumption from day 6 of supplementation (S6) to approximately day 3 of the withdrawal period (W3) for all the treatments. Minimum intake values were attained on day W1, and they represented an 18.7, 27.7, and 36.7 % reduction ($P<0.05$) in feed consumption with respect to the average pre-treatment value for the 1.25, 2.5 and 5.0 MIU treatments, respectively. No differences ($P>0.05$) among treatments were detected. Feed intake tended to recover after supplementation, however, intakes were still lower than pre-treatment values, 4 days after supplementation (Table 3.2 and Figure 3.1).

Repeated measures analysis indicated that feed intake was depressed ($P<0.0001$) over time as a result of vitamin D_3 supplementation with no differences between treatments (Table 3.2). This depression in intake as a result of vitamin D_3 supplementation has been acknowledged in other studies. Karges *et al.* (1999a) conducted an experiment to determine the effect of high levels of vitamin D_3 supplementation on feed intake. Yearling steers were supplemented with 5, 7.5, 15 and 75 MIU D_3, per day for 12 days. Feed consumption began to be depressed on days 2, 4, 5, and 6 for the 75, 15, 7.5 and 5 MIU treatments, respectively. These workers concluded that depression in intake as result of high levels of vitamin D_3 was a dose-response effect. In the present experiment this tendency was also observed, even with lower doses of supplementation.

In another study, Karges *et al.* (1999b) restricted intake to 90% of *ad libitum* intake when supplementing 6 MIU D_3 to finishing steers for 4 or 6 days. Intake decreased 11.7 and 14 % on days 4 and 6 of supplementation, respectively. In the current study, restriction was slightly higher (85%) but the depression observed was much higher in the 5 MIU treatment (36.7 %).

Montgomery *et al.* (in press) supplemented feedlot steers with 0.5, 1, 2.5, 5 and 7.5 MIU D_3 for 9 days before slaughter. Feed intake was linearly decreased during the last 3 days of supplementation, with only the 2.5, 5 and 7.5 MIU doses negatively affecting ADG and feed intake. In the present study, even the 1.25 MIU D_3 treatment depressed intake. Similar results were observed by Berry *et al.* (2000).

Table 3.2. Effect of supplementing high levels of vitamin D_3 for seven consecutive days on feed intake (DMI) of finishing Angus steers (452 ± 30 kg BW).

	DMI (kg)						
	Vitamin D_3 Level (MIU/hd/d)				Contrast [z]		
Days	1.25	2.5	5.0	*SEM*	A	B	C
Group	1	2	3				
Adaptation Period [y]:							
A	10.26	11.78	10.63	0.69			
85%	8.72	10.01	9.03				
Supplemental Period [x]:							
S1	8.69	9.86	9.00	0.61	0.4552	0.2067	0.8251
S2	8.69	9.78	9.00	0.60	0.4552	0.2742	0.8251
S3	8.43	8.80	8.97	0.81	0.2556	0.4446	0.7980
S4	8.50	9.28	8.79	0.63	0.3032	0.8784	0.6494
S5	8.39	9.12	7.53	0.63	0.2333	0.8750	0.0715
S6	7.84	7.10	6.70	0.89	0.0479	0.0002	0.0080
S7	7.75	7.74	6.34	0.74	0.0362	0.0066	0.0026
Withdrawal Period [y]:							
W1	7.07	7.13	5.70	0.82	0.0026	0.0002	0.0003
W2	7.80	8.28	6.63	0.84	0.0428	0.0807	0.0065
W3	8.34	7.43	7.13	0.87	0.2083	0.0012	0.0269
W4	8.87	8.30	8.08	0.69	0.6229	0.0887	0.2220
Repeated Measures Analysis:							
	P values						
Treatment	0.5787						
Time	<0.0001						
Time x treatment	0.0926						

[z] Contrasts:

A = Control restricted vs the next experimental day in 1.25 MIU treatment.

B = Control restricted vs the next experimental day in 2.5 MIU treatment.

C = Control restricted vs the next experimental day in 5 MIU treatment.

[y] Feed intake *ad libitum*.

[x] Feed intake restricted to 85% of *ad libitum*.

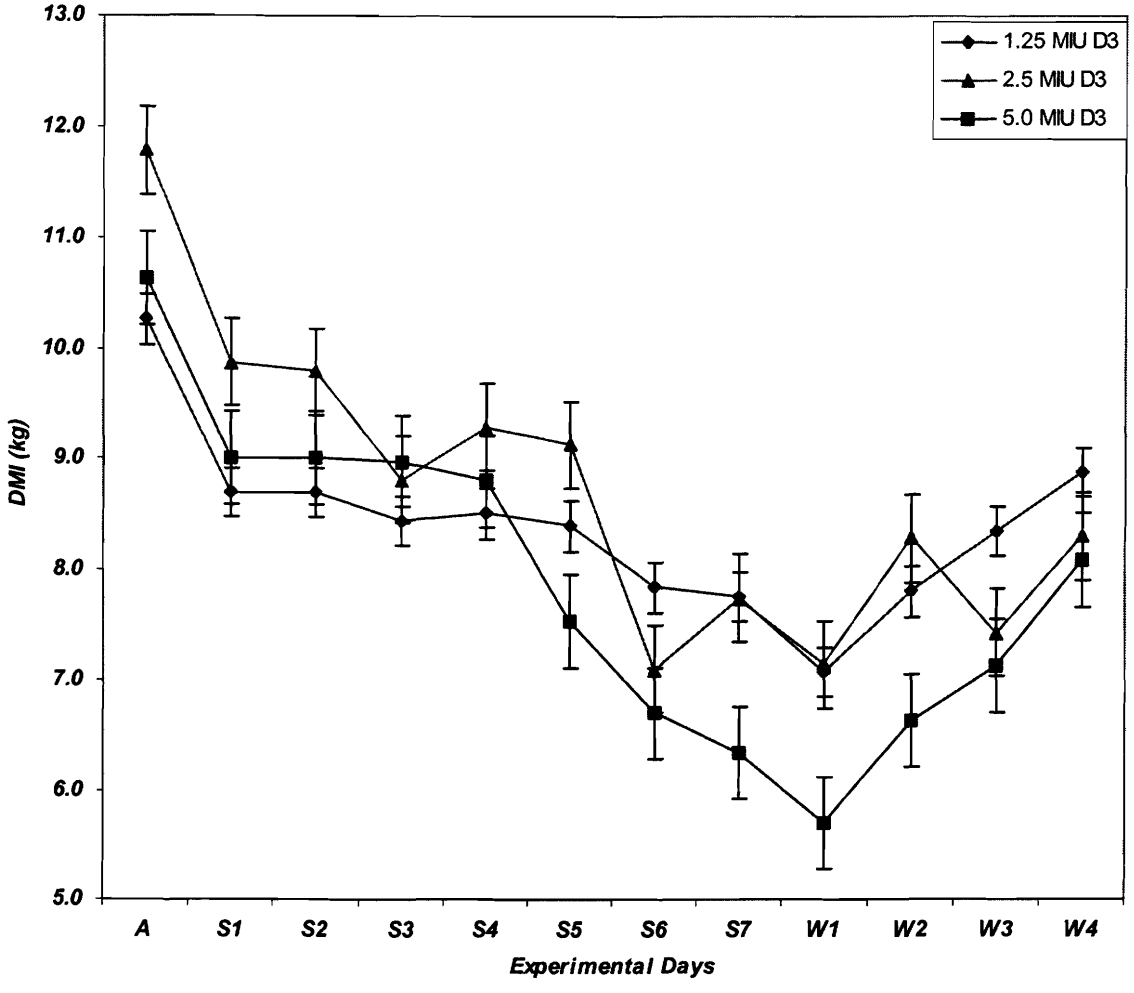

Figure 3.1. Effect of supplementing high levels of vitamin D_3 on feed intake (DMI) of finishing Angus steers (452 ± 30 kg BW), during pre-treatment (A), treatment (S1 to S7) and post-treatment (W1 to W4) periods.

Depression of feed intake of feedlot animals as a result of supplementing high levels of vitamin D_3 appears not to be related to palatability factors. This conclusion is based on the observation that vitamin D_3 has been administered by top-dressing the diet (Karges *et al.* 1999a; Montgomery *et al.* in press), as pellets (Karges *et al.* 1999b,c), and by intraruminal boluses (Montgomery *et al.* 1998, 2000). In all cases, a significant depression in feed consumption was observed. This decline in consumption has been directly related to the amount of vitamin D_3 supplemented. That is, higher levels of supplementation induced higher levels of depression. It is likely that vitamin D_3 supplementation causes metabolic (alteration of the normal acid-base balance) or physiological changes (Owens *et al.* 1998), which could be responsible for the reduced intake of the supplemented cattle.

The depression in feed intake observed in the present study influenced the total amount of vitamin D_3 consumed. Total vitamin D_3 consumption averaged 96.1 % (1.20 MIU/d), 90.3 % (2.26 MIU/d) and 89.4 % (4.48 MIU/d) for the 1.25, 2.5 and 5 MIU treatments, respectively.

3.3.2 Total and Ionized Serum Ca

Serum levels of the three pre-treatment control samples for total and ionized Ca concentrations were 10.61 ± 0.35 and 5.12 ± 0.15 mg/dl, respectively (Tables 3.3 and 3.4 and Figures 3.2 and 3.3). Normal Ca concentration in blood ranges from 9.0 to 11.0 mg/dl and from 4.8 to 6.4 mg/dl, for total and ionized Ca, respectively (Guyton 1956; Copp 1973; Smith *et al.* 1983; Rosol and Capen 1997). Thus, the total and ionized serum Ca concentrations found in the present experiment appeared to be in good agreement with the normal ranges reported in the literature.

In this study, it was found that supplementation of vitamin D_3 did not influence ($P>0.05$) total Ca concentrations relative to the control period during the first three days of supplementation. For the 2.5 and 5 MIU treatments, total Ca concentration was increased ($P<0.0001$) from day 4 of supplementation (S4) until day 5 of the withdrawal (W5) period. Even the concentration on day W8, while decreased was still higher ($P<0.05$) than pre-treatment control values. In contrast, total Ca concentrations for the 1.25 MIU treatment were not different ($P>0.05$) from pretreatment values throughout the

Table 3.3. Effect of supplementing high levels of vitamin D_3 on total serum Ca concentrations of finishing Angus steers (452 ± 30 kg BW).

	Total Ca (mg/dl)						
	Vitamin D_3 Level (MIU/hd/d)				Contrasts [z]		
Days	1.25	2.5	5.0	*SEM*	A	B	C
Group	1	2	3				
Adaptation Period:							
A10	10.59	10.90	10.66	0.12			
A24	10.34	10.68	10.60	0.19			
A31	10.26	10.80	10.68	0.13			
Average:		10.61 ± 0.35					
Supplemental Period:							
S1	10.38	10.66	10.61	0.08	0.2666	0.7986	1.0000
S2	10.40	10.80	10.89	0.10	0.3021	0.3085	0.1946
S3	10.60	10.98	11.02	0.16	0.9682	0.0526	0.0600
S4	11.04	11.58	11.47	0.18	0.0337	<0.0001	0.0001
S5	10.77	11.45	11.25	0.21	0.4267	<0.0001	0.0036
S6	10.93	11.33	11.30	0.21	0.1137	0.0002	0.0016
S7	10.99	11.70	11.98	0.21	0.0584	<0.0001	<0.0001
Withdrawal Period:							
W1	10.96	11.63	11.94	0.26	0.0823	<0.0001	<0.0001
W2	10.78	11.55	12.21	0.29	0.4041	<0.0001	<0.0001
W3	10.85	11.67	12.29	0.23	0.2342	<0.0001	<0.0001
W4	11.03	11.42	12.25	0.22	0.0370	<0.0001	<0.0001
W5	10.98	11.52	12.43	0.27	0.0695	<0.0001	<0.0001
W8	10.52	11.15	11.35	0.24	0.6907	0.0048	0.0008

Repeated Measures Analysis

	P values
Treatment	0.0041
Time	<0.0001
Time x treatment	0.0003

[z] Contrasts:

A = Control restricted vs the next experimental day in 1.25 MIU treatment.

B = Control restricted vs the next experimental day in 2.5 MIU treatment.

C = Control restricted vs the next experimental day in 5 MIU treatment.

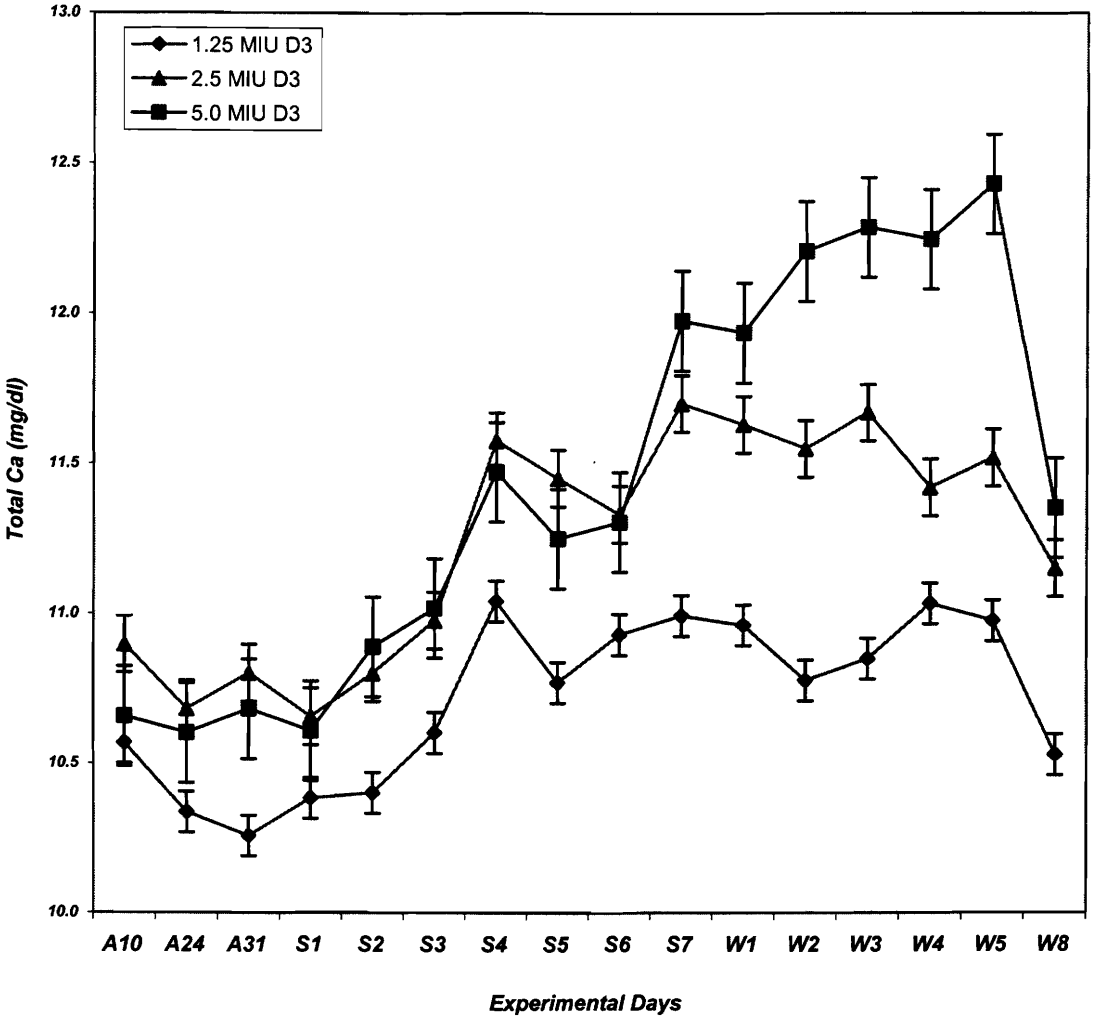

Figure 3.2. Effect of supplementing high levels of vitamin D_3 on total serum Ca concentrations of finishing Angus steers (452 ± 30 kg BW), during adaptation (A1 to A31), supplemental (S1 to S7), and withdrawal (W1 to W8) periods.

Table 3.4. Effect of supplementing high levels of vitamin D_3 on ionized serum Ca concentrations of finishing Angus steers (452 ± 30 kg BW).

Ionized Ca (mg/dl)							
	Vitamin D_3 Level (MIU/hd/d)				Contrasts [z]		
Days	1.25	2.5	5.0	*SEM*	A	B	C
Group	1	2	3				
Adaptation Period:							
A10	5.08	5.18	5.16	0.05			
A24	5.05	5.25	5.22	0.08			
A31	4.91	5.16	5.05	0.05			
Average:		5.12 ± 0.15					
Supplementation Period:							
S1	5.01	5.21	5.17	0.05	0.2359	0.3518	0.6912
S2	4.91	5.17	5.19	0.07	0.0280	0.6051	0.5562
S3	5.13	5.39	5.34	0.07	0.9160	0.0053	0.0845
S4	5.15	5.38	5.37	0.07	0.7155	0.0067	0.0484
S5	5.08	5.36	5.39	0.06	0.6801	0.0134	0.0308
S6	5.26	5.46	5.55	0.08	0.1392	0.0006	0.0008
S7	5.14	5.66	5.58	0.09	0.7809	<0.0001	0.0004
Withdrawal Period:							
W1	5.19	5.61	5.74	0.16	0.4266	<0.0001	<0.0001
W2	5.21	5.73	5.90	0.14	0.3337	<0.0001	<0.0001
W3	5.22	5.70	5.89	0.14	0.2552	<0.0001	<0.0001
W4	5.39	5.63	6.03	0.14	0.0038	<0.0001	<0.0001
W5	5.26	5.59	6.00	0.16	0.1392	<0.0001	<0.0001
W8	4.82	5.25	5.30	0.18	0.0015	0.1729	0.1579
Repeated Measures Analysis							
	P values						
Treatment	0.0040						
Time	<0.0001						
Time x treatment	0.0070						

[z] Contrasts:

A = Control restricted vs the next experimental day in 1.25 MIU treatment.

B = Control restricted vs the next experimental day in 2.5 MIU treatment.

C = Control restricted vs the next experimental day in 5 MIU treatment.

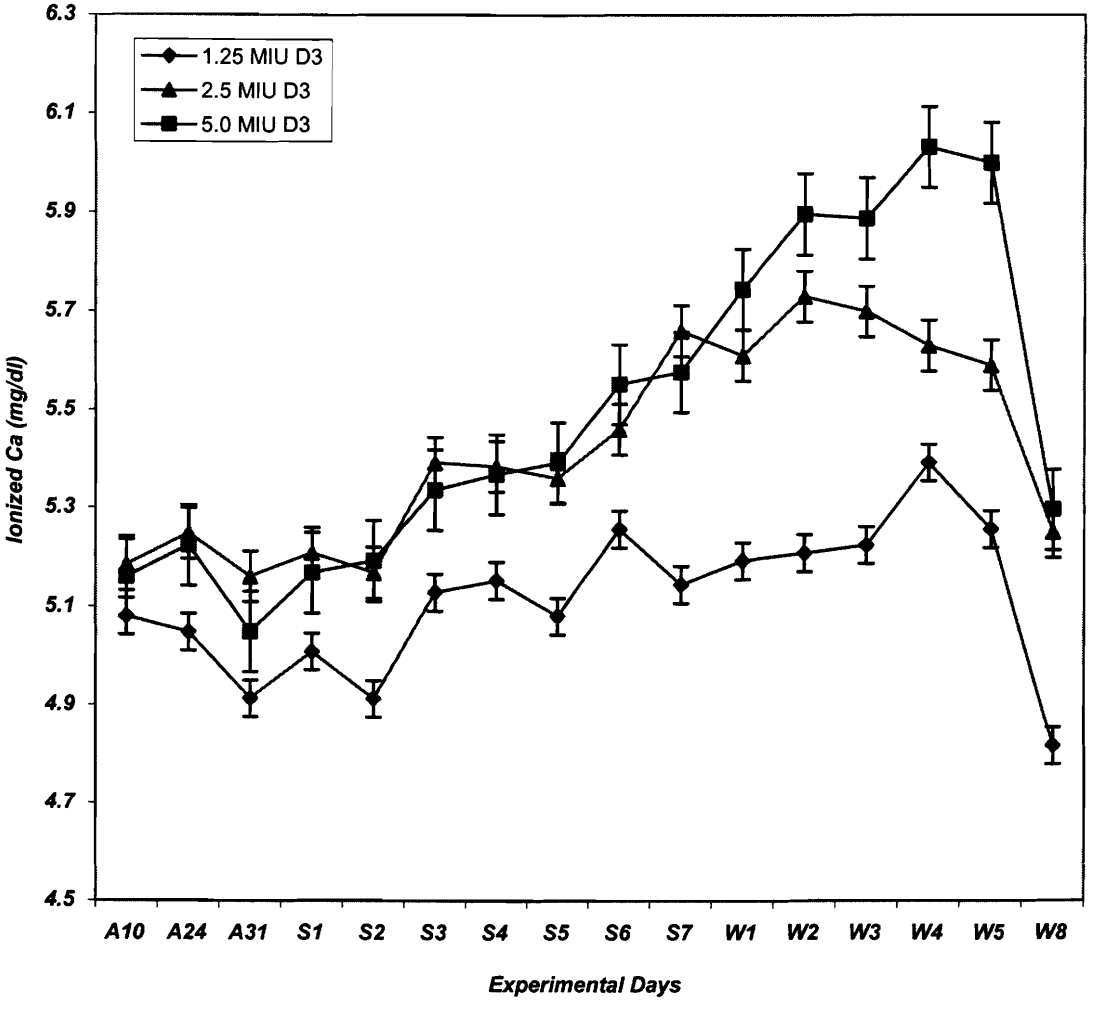

Figure 3.3. Effect of supplementing high levels of vitamin D_3 on ionized serum Ca concentrations of finishing Angus steers (452 ± 30 kg BW), during adaptation (A1 to A31), supplemental (S1 to S7), and withdrawal (W1 to W8) periods.

the experiment (Table 3.3 and Figures 3.2).

Parallel changes were observed in regard to ionized Ca. However, increments were significant ($P<0.05$) from day S4 to W5, for the 2.5 and 5 MIU treatments. On day W8, ionized Ca concentrations returned to normal levels for all treatments. The 1.25 MIU treatment had similar ($P>0.05$) serum ionized Ca levels as the pre-treatment control value throughout the experiment (Table 3.4 and Figure 3.3).

The repeated measures analysis indicated a significant effect of time, treatment and time by treatment interaction for total and ionized Ca, which is illustrated in Figures 3.2 and 3.3. While total and ionized serum Ca concentrations increased over time for all the treatments, they did so to a greater extent for the 5 MIU treatment.
Maximum total Ca concentrations were 11.03, 11.67 and 12.43 mg/dl, which represented an increase in 4.0, 10.0 and 17.2 % with respect to the adaptation period, for the 1.25 (on day W4), 2.5 (on day W3) and 5.0 (on day W5) MIU treatments, respectively (Table 3.3 and Fig. 3.2). Maximum ionized Ca concentrations were 5.39, 5.73 and 6.03 mg/dl, which represented an increase of 5.3, 11.9 and 17.8 % with respect to the pre-treatment control value for the 1.25 (on day W4), 2.5 (on day W2) and 5.0 (on day W4) MIU treatments, respectively (Table 3.4 and Figure 3.3).

Comparing these results with the literature, the total Ca concentration found with the 5 MIU treatment was higher than the 10.39 mg/dl reported by Swanek *et al.* (1997) who also supplemented 5 MIU D_3 to finishing steers for 5 days. This difference can be explained firstly, by the number of days of supplementation (7 vs 5) and, secondly, because the steers in their study were sacrificed immediately after supplementation without a withdrawal period. In the present study, maximum values were attained during the withdrawal period. In a subsequent study, Swanek *et al.* (1999) reported a plasma Ca value of 12.39 mg/dl, when 5 MIU D_3 was supplemented for 7 days. Results that are in agreement with this study. Increments of approximately 2 mg/dl in total Ca concentration as a result of 5 MIU D_3 supplementation have also been reported by Montgomery *et al.* (1998 and 2000) and Karges *et al.* (1999a,c).

The ratio of ionized to total Ca is given in Table 3.5. This ratio is very important as it indicates the proportion of total Ca available to tissues for metabolic purposes

Table 3.5. Effect of supplementing high levels of vitamin D_3 on the ionized to total serum Ca ratio of finishing Angus steers (452 ± 30 kg BW).

Ionized to Total Ca Ratio (%)							
	Vitamin D_3 Level (MIU/hd/d)				Contrasts [z]		
Days	1.25	2.5	5.0	*SEM*	A	B	C
Group	1	2	3				
Adaptation Period:							
A10	48.07	47.59	48.42	0.36			
A24	48.86	49.14	49.29	0.41			
A31	47.90	47.80	47.28	0.41			
Average:		48.26 ± 0.86					
Supplementation Period:							
S1	48.23	48.87	48.72	0.37	0.9595	0.3016	0.4798
S2	47.22	47.86	47.69	0.47	0.0594	0.4979	0.3803
S3	48.39	49.15	48.44	0.44	0.8088	0.1321	0.7865
S4	46.68	46.53	46.79	0.30	0.0046	0.0040	0.0253
S5	47.18	46.93	47.95	0.66	0.0496	0.0259	0.6297
S6	48.10	48.20	49.19	0.66	0.7728	0.9192	0.1558
S7	46.85	48.38	46.57	0.60	0.0113	0.8383	0.0104
Withdrawal Period:							
W1	47.37	48.19	48.09	0.48	0.1066	0.9084	0.7894
W2	48.40	49.57	48.30	0.54	0.7947	0.0276	0.9531
W3	48.18	48.82	47.90	0.60	0.8782	0.3427	0.5763
W4	48.89	49.30	49.22	0.58	0.2489	0.0798	0.1438
W5	47.88	48.51	48.21	0.37	0.4830	0.6786	0.9390
W8	45.75	47.16	46.63	1.43	<0.0001	0.0638	0.0138

Repeated Measures Analysis

	P values
Treatment	0.5366
Time	<0.0001
Time x treatment	0.8810

[z] Contrasts:

A = Control restricted vs the next experimental day in 1.25 MIU treatment.

B = Control restricted vs the next experimental day in 2.5 MIU treatment.

C = Control restricted vs the next experimental day in 5 MIU treatment.

(Guyton 1956; Rosol *et al.* 1995; Guyton and Hall 1997). It can be seen that by day 4 of supplementation, the ratio for all treatments was reduced and was also lower on day S7 for the 1.25 and 5 MIU treatments. During the withdrawal period there were no differences in this ratio relative to pre-treatment values. This indicates that during the supplementation period, total Ca increased at a relatively greater rate than ionized Ca thus reducing the ratio, with the situation reversed during the withdrawal period.

The rise in total and ionized Ca levels observed in this study has important implications for the beef industry. Other workers have observed that high levels of vitamin D_3 supplementation induced higher plasma Ca concentrations, which in turn produced higher muscle Ca concentrations (Montgomery *et al.* 1998, 2000 and in press; Karges and Morgan 1999; Swanek *et al.* 1999). This excess of Ca in muscle is though to stimulate higher activity of the calpain proteolytic enzyme system (m-calpain, μ-calpain), which leads to more tender beef (Koohmaraie 1992, 1996; Zamora *et al.* 1998).

Swanek *et al.* (1997) supplemented 5 MIU D_3 for 5 days and observed a rise in plasma Ca of 12.6 %. This increase in Ca was enough to reduce shear force values by 6.6% and the number of tough steaks by 21.8 % (seven days postmortem aging). In this study, supplementation of 5 MIU D_3/hd/d increased total Ca to levels where improvements in shear force values and tenderness have been observed by Swanek *et al.* (1999) and Montgomery *et al.* (2000).

The results of this study showed that the rise in serum Ca is related to the level of vitamin D_3 supplementation. Minimal increases were seen with the 1.25 MIU treatment, in contrast the greatest increases were seen with the 5 MIU treatment. To more fully understand the response of serum Ca to vitamin D_3 supplementation and to possibly manipulate the response, it is necessary to more fully understand the biological mechanism that leads to this rise including the role of the calciotrophic hormones such as parathyroid hormone, calcitonin and vitamin D_3 and its major metabolites.

3.4 Conclusions

The results of this study showed that feed intake was depressed as a result of vitamin D_3 supplementation, and the depression followed an inverse relationship with the amount of vitamin D_3 supplemented. Intake recovery was observed only after supplementation ended, with the rate of recovery being related to the degree of depression. From these observations and from other studies in which vitamin D_3 was intraruminally administered, it was concluded that the depression in feed intake when high levels of vitamin D_3 are supplemented is possibly due to metabolic or physiological alterations and not to palatability factors.

The levels of supplemental vitamin D_3 used in this study have been shown to be an effective way to increase serum Ca concentrations in beef steers fed with a common barley grain-based feedlot finishing diet. The observed increments in serum Ca were in direct relationship with the amount of vitamin D_3 supplemented, that is, higher levels produced larger increments.

Interestingly, the maximum increments in total and ionized serum Ca concentrations from supplemented steers were found not during the supplemental but during the withdrawal period. This post-treatment rise may result from alterations in the hormonal control of Ca metabolism, a phenomenon that deserves further investigation.

The results of this study where barley grain-based finishing rations were fed have important implications for the beef industry in western Canada. The rise in serum Ca obtained in this study with the 5 MIU treatment is similar to that noted by other workers who with corn-based diets have noted a considerable improvement in beef quality, notably a reduction in shear force values. If this can be achieved for Canadian beef, particularly at lower levels of vitamin D_3 supplementation, then it is possible that such dietary manipulation of Ca homeostasis may become a common management tool.

CHAPTER IV

Effect of Supplementing High Levels of Vitamin D_3 on the Homeostatic Control of Serum Calcium in Finishing Steers

4.1 Introduction

Calcium (Ca) plays a key role in many physiological processes including structural (bones and teeth); in contraction of skeletal, cardiac, and smooth muscles; in transmission of nerve impulses; blood clotting; hormone secretion and enzyme activity (Guyton and Hall 1996). Approximately 99 % of the total Ca in the body is deposited in bone and teeth; 0.9 % is sequestered in the plasma membrane and endoplasmic reticulum of cells and the remaining 0.1 % in the extracellular fluid. Total serum Ca concentration is about 10 mg/dl, with approximately 50 % of it in the ionized form (Ca^{2+}), which is the biologically active form of Ca (Rhoades and Tanner 1995; Rosol and Capen 1997).

Fluctuations in the normal Ca concentration can cause important metabolic alterations. For example, slight increases in Ca concentration in the extracellular fluid (hypercalcemia) can cause progressive depression of the nervous system; conversely, slight decreases in Ca concentration (hypocalcemia) cause the nervous system to become more excited (Guyton and Hall 1996). Therefore, maintenance of a constant Ca concentration, despite variations in intake and excretion, is one of the primary homeostatic functions of the body. The metabolism of Ca is regulated, fundamentally, by the interaction of three hormones: parathyroid hormone (PTH) which is secreted by the parathyroid glands; calcitonin (CT), secreted by the C-cells of the thyroid gland; and vitamin D_3 in its hormonal form, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), produced in the kidneys (Hunt and Groff 1990; Horst *et al.* 1997).

PTH is highly sensitive to declines in plasma Ca concentration, even a small decline will stimulate the release of large amounts of this hormone. Calcitonin, on the other hand, is secreted when Ca concentration is higher than normal, although, it seems to be less sensitive than PTH to changes in Ca concentration (Goff 1989; Bhagavan 1992).

Vitamin D_3, from skin and that from the diet which is absorbed in the small intestine (duodenum) enters the lymphatic circulation. It is rapidly taken up by the liver,

where it is hydroxylated to 25-hydroxyvitamin D_3 ($25(OH)D_3$), which constitutes the major circulating form of vitamin D_3. Subsequently, it undergoes a second hydroxylation in the kidney to form the steroid hormone $1,25(OH)_2D_3$, whose main function is to increase the efficiency of Ca absorption from the intestine. The $1,25(OH)_2D_3$ stimulates the synthesis of Ca-binding protein in the intestinal epithelial cells, which results in the active transport of Ca from the intestine. However, the metabolic conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ is regulated by PTH, and is dependent upon dietary Ca levels, plasma Ca and P concentrations, and circulating $1,25(OH)_2D_3$ levels (DeLuca 1980; Spirichev and Sergeev 1988; Wasserman 1989; Host *et al.* 1994).

It has been observed that large amounts of vitamin D_3 (more than 10 million IU in a single dose) effectively increased plasma Ca concentrations in dairy cattle (Reinhardt and Conrad 1980; Littledike and Horst 1982). This effect has been observed in beef cattle with quantities around or higher than 5 million IU per head per day (Owens *et al.* 1998). Indeed, supplementation of feedlot animals with 5 to 7.5 MIU D_3 per day for 5 to 10 days has resulted in increases in plasma Ca concentration (Swanek *et al* 1999; Montgomery *et al.* 2000; Karges *et al.* 2001). Similar results were noted in the first trial of this study. Increases in plasma Ca levels of approximately 2 mg/dl as noted in the above studies have been shown to increase intracellular Ca concentration in myofibrils. For instance, supplementation of 5 MIU D_3 to finishing steers for 7 or 9 days increased muscle Ca concentration by 43 % (Swanek *et al.* 1999) and 34 % (Montgomery *et al.* in press), respectively. In both studies, improvements in beef tenderness were observed.

In the previous experiment (Chapter III), it was found that serum Ca concentration was effectively increased as a result of high levels (2.5 and 5 MIU/d) of vitamin D_3 supplementation when fed to steers on a common western Canadian feedlot diet. Total and ionized serum Ca concentrations exhibited very similar patterns, that is, increasing simultaneously as a result of vitamin D_3 supplementation. This rise in total and ionized Ca is hard to explain in light of the tight regulatory control mechanisms that govern Ca levels in blood (Guyton 1956; DeLuca and Shnoes 1983; West 1991; Kaneko *et al.* 1997). To this point none of the previous studies have attempted to understand from a hormonal point of view what is the underlying reason for the observed increases in serum Ca when high levels of vitamin D_3 are fed.

Understanding the hormonal control of serum Ca levels is of interest, as it will help to explain how supplementation of vitamin D_3 influences this parameter and ultimately beef tenderness. As such, the objective of this study was to evaluate the effect of supplementing high levels of vitamin D_3 (0, 2.5 and 5 MIU per day for seven consecutive days) on the following parameters (during supplemental and post-treatment periods):

1) Total and ionized serum Ca concentrations, and the ionized to total Ca ratio;
2) Vitamin D_3 and 25-hydroxyvitamin D_3 plasma concentrations;
3) Parathyroid hormone and calcitonin serum concentrations;
4) Feed intake.

4.2 Material and Methods

4.2.1 Experimental Design

4.2.1.1 Adaptation Period (days A1 to A18)

Fifteen Hereford steers (607 ±12 kg BW) were housed in individual pens (3.6 x 3.6 m) with automatic water bowls in the Livestock Research Barn of the Department of Animal and Poultry Science at the University of Saskatchewan. Animals were cared for according to the guidelines of the Canadian Council of Animal Care. The cattle were adapted to the experimental diet for 18 days. The diet consisted of 75% concentrate (barley grain base) and 25% barley silage (as fed), meeting the requirements for this type of cattle according to the National Research Council (1996) (Table 4.1).

The steers were fed twice a day (0800 and 1600 h), with orts collected and weighed every day before the morning feeding. Individual maximum voluntary feed intake was measured from days A5 to A11. Subsequently, feed was offered at this level from days A12 to A18, with the objective to level out intake before vitamin D_3 supplementation and so be able to measure its effect on feed intake.

Table 4.1. Ingredient composition and formulated nutrient levels of the experimental diet.

Item	DM (%)
Total mixed diet:	
Concentrate	87.2
Barley silage	12.8
Concentrate:	
Barley grain	89.4
Canola meal	3.9
Tallow	3.9
Limestone	1.2
Rumensin [z]	0.6
Vitamin [y]	0.4
Salt	0.6
Formulated composition [x]:	
Digestible energy, Mcal/kg [w]	3.60
Crude protein, %	13.30
Calcium, %	0.52
Phosphorus, %	0.36
Ca:P	1.49

[z] Premix: Monensin (20% active) mixed with barley grain.

[y] Premix: Vitamin A (416,650 IU/kg) and vitamin D (83,333 IU/kg).

[x] Nutrient Requirements of Beef Cattle. National Research Council (1996).

[w] Predicted TDN x 4.4 Mcal/kg (Weiss *et al.* 1992).

4.2.1.2 Supplemental Period (days S1 to S7)

The steers were randomly assigned to one of the following supplemental treatments: 0, 2.5, or 5.0 million IU (MIU) of vitamin D_3 per steer per day for seven consecutive days, with five steers per treatment.

The vitamin D_3 supplement was prepared from a vitamin D_3 concentrate containing 500,000 IU per gram (Rhone-Poulec Canada Inc.), using ground barley as a carrier. This supplement was formulated to have a final concentration of 13.0 MIU D_3 per kg of premix.

The total daily amount of supplement per steer per treatment was equally divided and supplied at each feeding. The supplement was top-dressed to the ration and thoroughly mixed by hand before delivering it into the feed bunk.

During this period, daily blood samples (S1 to S7) were obtained by jugular venipunture, from all the steers, at approximately 1300 h. Three sets of samples were taken. For the first and second sets, blood was collected into 7ml-draw evacuated (Vacutainer®) tubes without anticoagulant. The blood obtained was allowed to clot at room temperature for approximately 2 h. Then, one set was centrifuged at 3500 rpm for 15 min, and the serum obtained was immediately used to determine total and ionized Ca concentrations. The second set was centrifuged at 3000 rpm for 10 min at –4°C, then the serum was collected and transferred to 1.7 ml centrifuge clear vials and stored at –70°C until analysis for PTH and calcitonin concentrations. For the third set, blood was collected into 7 ml-draw vacutainer tubes with anticoagulant (100 USP units of lithium heparin), which were placed in crushed ice immediately after withdrawal, and approximately 2 h later centrifuged at 3000 rpm for 10 min at –4°C, then the plasma was collected and transferred to 1.7 ml centrifuge clear vials and stored at –70°C until analysis for vitamin D_3 and 25(OH)D_3 concentrations.

4.2.1.3. Withdrawal Period (W1 to W9)

Once the vitamin D_3 supplementation period ended, the steers were maintained for a further five days under the experimental conditions to evaluate post-treatment effects, then the steers were shipped back to the feedlot. Feed was offered without restriction and was monitored daily with orts collected and weighed before every

morning feeding. Daily blood samples were obtained from day W1 to W5, and a final sample on day W9, under the same protocol as described previously.

4.2.2 Laboratory Analyses

4.2.2.1 Total and Ionized Calcium Determinations

Serum samples were analyzed for total and ionized Ca as described in Chapter III. Briefly, total Ca was determined by indirect potentiometry utilizing a Ca ion selective electrode (Radiometer ABL 700 Series Analyzer. Copenhagen, Denmark).

4.2.2.2 Parathyroid Hormone (PTH) Determination

Serum concentrations of PTH were analyzed by a two-site immunoradiometric assay (IRMA)(Intact PTH- Parathyroid Hormone. Nichols Institute Diagnostics). Two different goat polyclonal antibodies to human PTH, purified to be specific to two different regions of the PTH molecule were used. One antibody bound the mid-region and C-terminal PTH 38-84, this antibody was immobilized onto plastic beads. The other antibody bound the N-terminal PTH 1-34, this was radiolabeled for detection. Further details on the assay are provided in the Appendix A.

4.2.2.3 Calcitonin Hormone Determination

Calcitonin (thyrocalcitonin) concentrations in serum were determined by a double –antibody radioimmunoassay (Double Antibody Calcitonin. Diagnostic Products Corporation DPC®, Los Angeles, CA. USA). This is a sequential competitive radioimmunoassay procedure. The samples were first pre-incubated with anti-calcitonin antiserum. ^{125}I-labeled calcitonin competed with calcitonin in the sample for antibody sites. After incubation for a fixed period of time (18 h), separation of bound from free was achieved by the PEG-accelerated double-antibody method. Finally, the antibody-bound fraction was precipitated and counted by a gamma counter. Calcitonin concentration in the samples was read from a calibration curve. Further details of the assay are provided in Appendix B.

4.2.2.4 Vitamin D_3 and 25-Hydroxyvitamin D_3 Determinations

Plasma concentrations of vitamin D_3 and 25-hydroxyvitamin D_3 25(OH)D_3 were determined using reverse-phase high-performance liquid chromatography (HPLC). These two compounds were extracted from plasma samples by using acetonitrile (1:2 ratio). Solutions were thoroughly mixed (vortexed) for a fixed period of time (30 sec), then centrifuged. The supernatant from control animals underwent a second extraction with chloroform and hexane and evaporation under nitrogen and then reconstitution with acetonitrile. The supernatant from treated steers was subjected to only the first extraction. Supernatants were filtered and transferred to injection vials. Samples were introduced to the HPLC using an autosampler (Waters™. Millipore Co. Milford, MA. USA). Samples were analyzed with a reverse-phase column (Phenomenex C18 3µ 15 x 4.6 mm) and acetonitrile as the mobile phase under isocratic conditions. The metabolite 25(OH)D_3 was eluted first and vitamin D_3 minutes later. The absorbance of these analytes (peak area) was computed and integrated by the software Millennium 2010 (Millennium™. Millipore Co. Milford, MA. USA). Calibration curves were used to calculate concentrations from the experimental samples. Further details of the assay are provided in Appendix C.

4.2.3 Statistical Analysis

The feed intake and blood sample data were analyzed using the Analysis of Variance Technique including Repeated Measures Analysis using the General Linear Model procedure of SAS (SAS 1989) to compare treatments within days and over time.

The following model for the repeated measures analysis was used:

Model: $Y_{ijk} = \mu + \alpha_i + \tau_{ij} + \delta_k + (\alpha\delta)_{ij} + \varepsilon_{ijk}$

where:

Y_{ijk} = is the response at time k on animal j in treatment i.

μ = is the overall mean.

α_i = is a fixed effect of treatment i.

τ_{ij} = is a random effect of animal j in treatment i.

δ_k = is a fixed effect of time k.

$(\alpha\delta)_{ij}$ = is a fixed interaction effect of treatment i with time k.

ε_{ijk} = is the random error at time k on animal j in treatment i.

Single degree of freedom contrasts:

1) Control versus the average response of the two vitamin D_3 treatments.
2) Comparison between the 2.5 and 5 MIU D_3 treatments.

4.3 Results and Discussion

4.3.1 Feed Intake (DMI)

Feed intake during the adaptation period averaged 8.99 ± 1.21 kg of DM. Intake from control animals was maintained without change throughout the supplemental and withdrawal periods. During the first days of the supplementation period, feed intake was not different ($P>0.05$) between control and supplemented steers, with the exception on the second day of supplementation (S2) where it was highly depressed ($P<0.05$) in the 5 MIU treatment, but it recovered the following day. The trend over time was to reduce ($P<0.05$) feed intake as supplementation advanced with no difference ($P>0.05$) between levels of supplementation. Feed intake tended to recover after supplementation ended, following an inverse relationship with the level of vitamin D_3 supplemented, although it had not fully recovered four days post-treatment. Minimum intakes were attained towards the end of the supplemental period, which represented a decrease of 18.2 and 37.2 % relative to control animals for the 2.5 and 5 MIU treatments, respectively (Table 4.2).

Repeated measures analysis indicated a highly significant effect of time ($P<0.0001$) and the interaction of time by treatment ($P<0.0001$), but no treatment effect ($P=0.0992$). The interaction indicates that over time the depression in feed intake was greater with the 5 MIU treatment (Table 4.2).

Depression of feed intake as a result of high levels of vitamin D_3 supplementation was evidenced in the previous study (Chapter III) and this response has also been observed in other studies (Owens *et al.* 1998). The degree of depression seems to be directly related to the amount of vitamin D_3 supplemented (Karges *et al.* 1999a). As discussed in the

Table 4.2 Effect of high levels of vitamin D_3 supplementation on dry matter intake (DMI) of finishing Hereford steers (607 ± 12 kg BW).

DMI (kg)						
	Vitamin D_3 Level (MIU/hd/d)				Contrasts [z]	
Days	0	2.5	5.0	*SEM*	C vs D	2.5 vs 5.0
Adaptation Period:						
A	9.20	8.66	9.11	0.57		
Supplemental Period:						
S1	8.57	7.46	8.94	0.65	0.6482	0.1348
S2	8.80	8.92	4.42	0.76	0.0405	0.0012
S3	8.36	8.70	7.75	0.76	0.8853	0.3969
S4	8.31	8.01	6.95	0.81	0.4172	0.3751
S5	7.98	6.53	5.98	0.77	0.0939	0.6251
S6	8.52	6.88	5.35	0.77	0.0259	0.1889
S7	8.72	7.43	5.72	0.59	0.0120	0.0649
Withdrawal Period:						
W1	8.49	7.82	6.65	0.70	0.1656	0.2559
W2	8.83	8.80	6.62	0.57	0.0665	0.0744
W3	9.06	8.31	6.42	0.58	0.0358	0.0411
W4	8.98	8.54	6.94	0.60	0.1181	0.0849
Repeated Measures Analysis:						
	P value					
Treatment	0.0992					
Time	<0.0001					
Time x treatment	<0.0001					

[z] Contrast:

C vs D = Control vs the average of the two vitamin D_3 treatments.

2.5 vs 5.0 = 2.5 vs 5.0 MIU D_3 treatments.

previous experiment, the depression in intake appears to be not related to palatability factors, because decreases in feed consumption has been observed independently of the vitamin D_3 route of administration. That is, with top-dressing (Karges *et al.* 1999a; Montgomery *et al.* in press), with pellets (Karges *et al.* 1999a,b,c; 2001), or by oral boluses (Montgomery *et al.* 1998, 2000).

The vitamin D_3 supplement was formulated to have a final concentration of 13.0 MIU per kg. Analysis of the supplement indicated an actual concentration of 12.90 ± 0.46 MIU D_3 per kg (New Jersey Feed Laboratory Inc. Trenton, New Jersey). As the supplement was added to the daily ration, vitamin D_3 consumption was directly related to daily feed intake. As such, the average vitamin D_3 consumption for the 2.5 and 5 MIU treatments were 85.4 % (2.14 MIU/d) and 68.38 % (3.41 MIU/d) of the targeted intakes, respectively.

4.3.2 Total and Ionized Ca Concentrations

Total and ionized serum Ca concentrations on the first day of supplementation were 10.11, 10.18 and 10.26 mg/dl and 4.92, 5.08 and 5.06 mg/dl for the 0, 2.5 and 5 MIU treatments, respectively, with no difference ($P>0.05$) between treatments (Tables 4.3 and 4.4 and Figures 4.1 and 4.2).

These initial concentrations, as well as the range from 9.90 to 10.19 mg/dl for total and 4.78 to 5.11 mg/dl for ionized Ca from control animals during the whole experiment were in the normal range for cattle of 9.0 to 11.0 and 4.8 to 6.4 mg/dl for total and ionized plasma Ca concentrations, respectively (Guyton 1956; Copp 1973; Rosol and Capen 1997).

Total and ionized serum Ca concentrations were increased ($P<0.0001$) as a result of vitamin D_3 supplementation. Contrast analysis indicated that Ca serum concentrations in supplemented animals were higher ($P<0.05$) than from controls, from day S3 to W5 and from S4 to W9 for total and ionized Ca, respectively. Conversely, differences due to treatments (2.5 vs 5 MIU) were only evident ($P<0.05$) during the withdrawal period (from day W2 to W9), with highest levels achieved in the 5 MIU treatment (Tables 4.3 and 4.4 and Figures 4.1 and 4.2).

Table 4.3. Effect of high levels of vitamin D_3 supplementation on total serum Ca concentrations of finishing Hereford steers (607 ± 12 kg BW).

Total Ca (mg/dl)						
	Vitamin D_3 Level (MIU/hd/d)				Contrasts [z]	
Days	0	2.5	5.0	*SEM*	C vs D	2.5 vs 5.0
Supplemental Period:						
S1	10.11	10.18	10.26	0.16	0.5819	0.7324
S2	10.17	10.40	10.46	0.14	0.1503	0.7526
S3	10.02	10.34	10.53	0.14	0.0273	0.3595
S4	10.10	10.42	10.74	0.15	0.0274	0.1601
S5	10.19	10.68	10.91	0.09	0.0002	0.1507
S6	10.06	10.73	10.98	0.09	<0.0001	0.0694
S7	9.96	10.66	10.90	0.11	<0.0001	0.1408
Withdrawal Period:						
W1	9.98	10.84	11.28	0.14	<0.0001	0.0503
W2	10.09	10.77	11.61	0.26	0.0046	0.0402
W3	10.02	10.79	11.90	0.28	0.0021	0.0150
W4	10.18	11.02	12.12	0.27	0.0013	0.0148
W5	9.90	10.70	12.11	0.19	<0.0001	0.0002
W9	10.04	9.82	10.34	0.12	0.7563	0.0090

Repeated Measures Analysis:

	P value
Treatment	0.0002
Time	<0.0001
Time x treatment	<0.0001

[z] Contrast:

C vs D = Control vs the average of the two vitamin D_3 treatments.

2.5 vs 5.0 = 2.5 vs 5.0 MIU D_3 treatments.

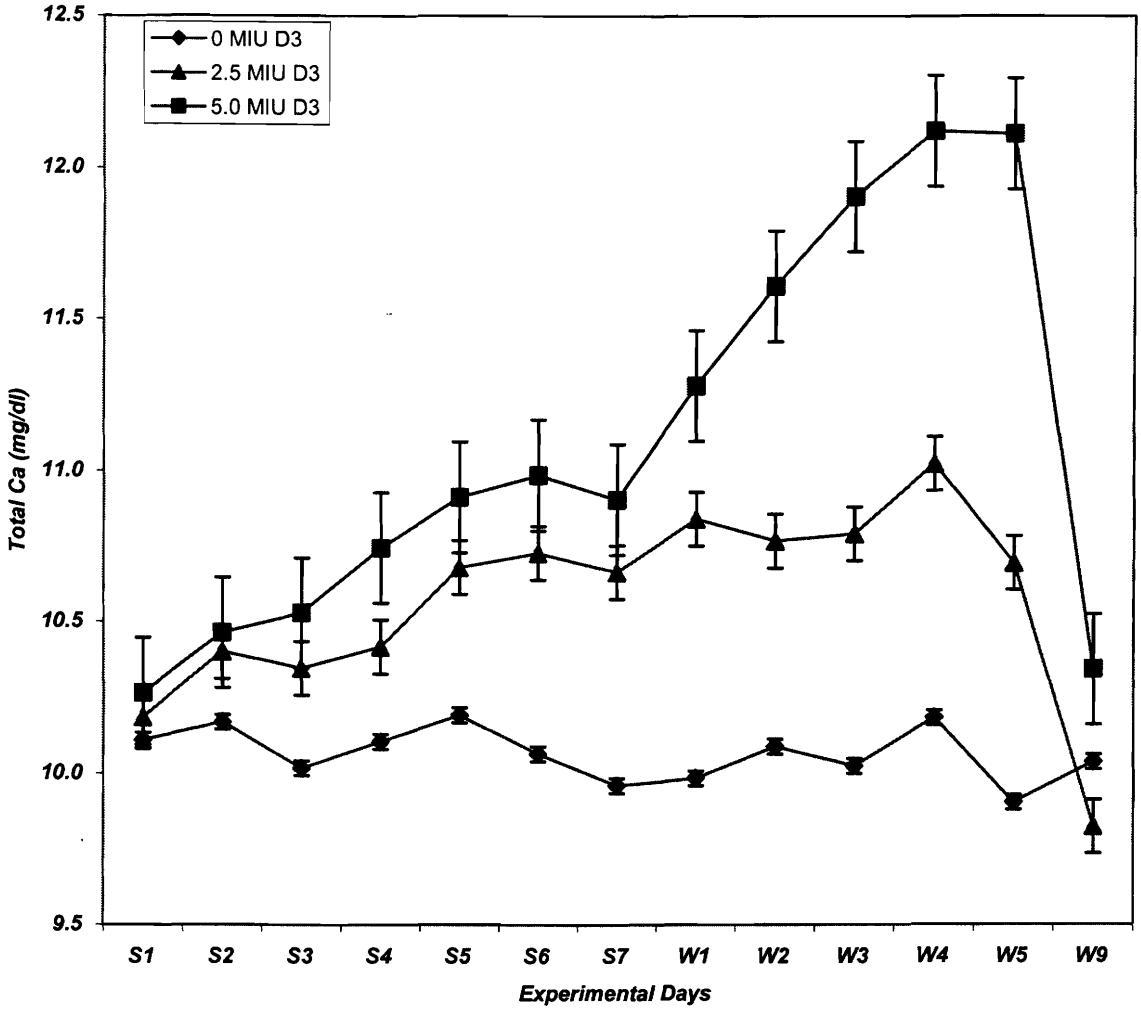

Figure 4.1. Effect of high levels of vitamin D_3 supplementation (0, 2.5 and 5 MIU per day for seven consecutive days) on total serum Ca concentrations of finishing Hereford steers (607 ± 12 kg BW), during supplemental (S1 to S7) and withdrawal (W1 to W9) periods.

Table 4.4. Effect of high levels of vitamin D_3 supplementation on ionized serum Ca concentrations of finishing Hereford steers (607 ± 12 kg BW).

Ionized Ca (mg/dl)						
	Vitamin D_3 Level (MIU/hd/d)				Contrasts [z]	
Days	0	2.5	5.0	*SEM*	C vs D	2.5 vs 5.0
Supplemental Period:						
S1	4.92	5.08	5.06	0.07	0.1011	0.8741
S2	4.78	4.94	4.95	0.08	0.1102	0.8892
S3	4.92	5.06	5.14	0.07	0.0662	0.4973
S4	4.87	5.02	5.11	0.05	0.0124	0.2275
S5	4.95	5.12	5.19	0.04	0.0025	0.2666
S6	5.03	5.27	5.34	0.07	0.0062	0.5128
S7	5.04	5.28	5.42	0.06	0.0019	0.1558
Withdrawal Period:						
W1	5.07	5.34	6.38	0.52	0.2345	0.1800
W2	5.06	5.26	5.78	0.15	0.0321	0.0326
W3	5.11	5.34	5.97	0.15	0.0115	0.0107
W4	5.06	5.36	5.98	0.14	0.0045	0.0095
W5	5.03	5.24	5.97	0.12	0.0016	0.0008
W9	5.07	4.67	4.99	0.08	0.0369	0.0174

Repeated Measures Analysis:

	P value
Treatment	0.0007
Time	<0.0001
Time x treatment	0.0094

[z] Contrast:

C vs D = Control vs the average of the two vitamin D_3 treatments.

2.5 vs 5.0 = 2.5 vs 5.0 MIU D_3 treatments.

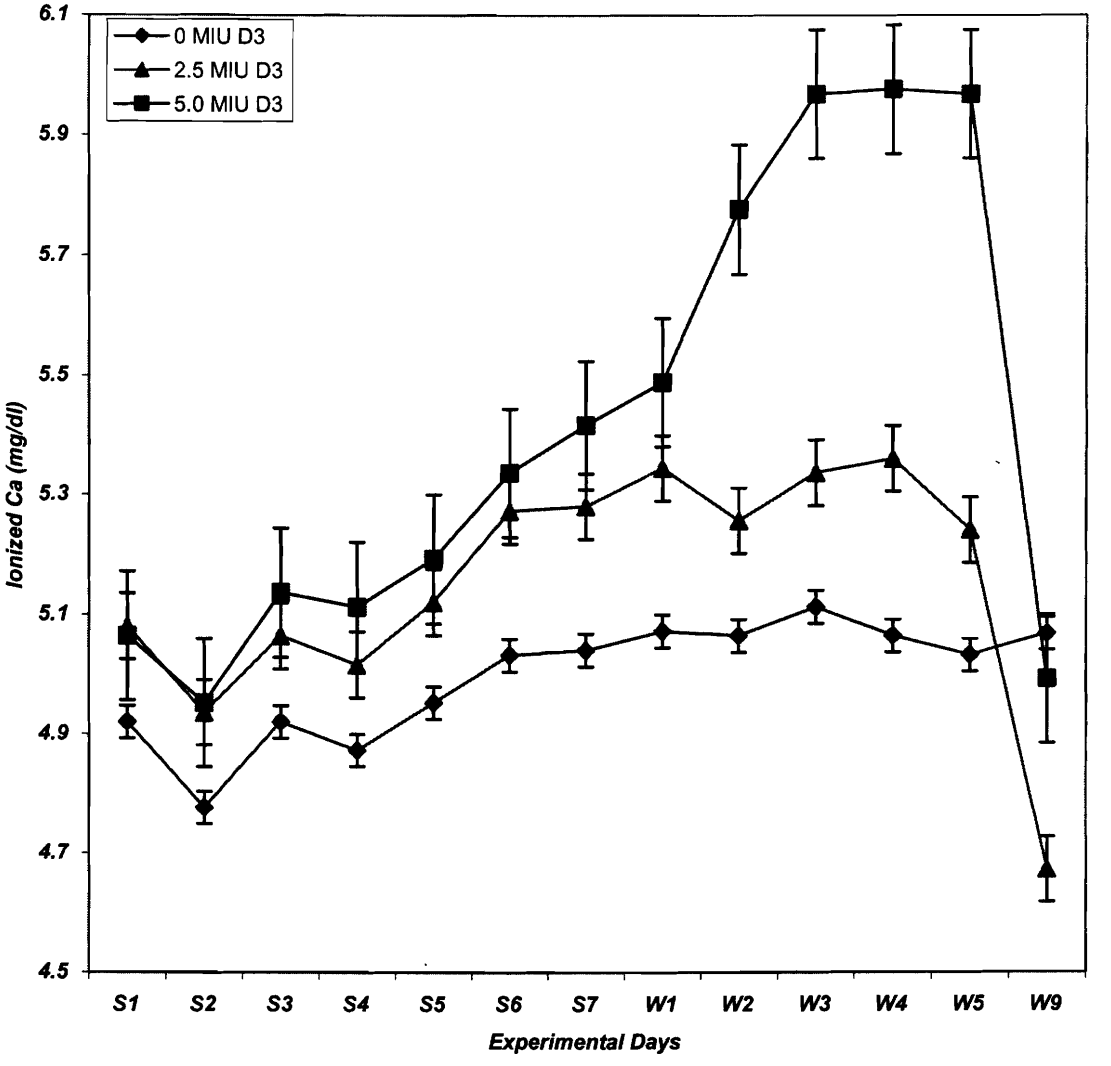

Figure 4.2. Effect of high levels of vitamin D_3 supplementation (0, 2.5 and 5 MIU per day for seven consecutive days) on ionized serum Ca concentrations of finishing Hereford steers (607 ± 12 kg BW), during supplemental (S1 to S7) and withdrawal (W1 to W4) periods.

It is important to note that maximum Ca concentrations were achieved four days after the end of the vitamin D_3 supplemental period (W4), and were reached the same day for total and ionized Ca for the 2.5 and 5 MIU treatments (Tables 4.3 and 4.4 and Figures 4.1 and 4.2).

The maximum rise in total and ionized serum Ca in response to vitamin D_3 supplementation was similar (11.67 vs 11.02 and 12.43 vs 12.12 mg/dl for total and 5.73 vs 5.36 and 6.03 vs 5.98 mg/dl for ionized Ca) for the 2.5 and 5 MIU treatments in the previous and present study of this series, respectively. Maximum total Ca concentration of 12.12 mg/dl achieved in the present study with the 5 MIU D_3 treatment for seven consecutive days, was higher than the reported by Swanek *et al.* (1997) (10.39 mg/dl), at the same level of supplementation. The difference in values may be due to a lower period of supplementation (5 days), and the fact that the steers in their study were slaughtered immediately after supplementation. In a subsequent study, Swanek *et al.* (1999) supplemented 5 MIU D_3 for seven days, and found a final plasma Ca concentration of 12.39 mg/dl, which was very close to the value found in the present experiment.

Montgomery *et al.* (1998) administered 5 MIU D_3 to finishing steers for eight days and reported that plasma Ca concentrations were increased from 9 to 11 mg/dl. In absolute terms, their final plasma Ca concentration was lower than that found in the current experiment, even though vitamin D_3 was supplemented by intraruminal boluses. However, the magnitude of the increase was similar as Ca concentration was increased by approximately 2 mg/dl in both studies (from 9 to 11 and from 10.11 to 12.12 mg/dl, respectively). Karges *et al.* (1999a,b) reported a similar rise in plasma Ca when 5 or 6 MIU D_3 were supplemented to steers for twelve (13 mg/dl) or six days (12.12 mg/dl), respectively. It is important to note that the rise in total and ionized Ca levels in serum in this study were similar to that of the studies where this increase has been associated with improved meat quality (Swanek *et al.* 1997, 1999; Karges *et al.* 1999a,b, 2001; Montgomery *et al.* 1998, 2000).

Ionized to total Ca ratios are presented in Table 4.5. There were no differences in the ratio between the two vitamin D_3 treatments. There was, however, a decrease in the ratio due to supplementation with vitamin D_3 from day S5. A reduction in this ratio

Table 4.5 Effect of high levels of vitamin D_3 supplementation on serum ionized to total Ca ratio of finishing Hereford steers (607 ± 12 kg BW).

Ionized to Total Ca Ratio (%)						
	Vitamin D_3 Level (MIU/hd/d)				Contrasts [z]	
Days	0	2.5	5.0	*SEM*	C vs D	2.5 vs 5.0
Supplemental Period:						
S1	48.65	49.92	49.34	0.36	0.0460	0.2746
S2	46.98	47.46	47.33	0.49	0.5050	0.8527
S3	49.13	48.95	48.79	0.36	0.5709	0.7595
S4	48.23	48.17	47.60	0.39	0.4787	0.3155
S5	48.59	47.94	47.60	0.34	0.0769	0.4934
S6	50.00	49.14	48.57	0.36	0.0224	0.2866
S7	50.60	49.52	49.67	0.36	0.0395	0.7717
Withdrawal Period:						
W1	50.81	49.31	48.65	0.37	0.0016	0.2279
W2	50.20	48.80	49.73	0.41	0.0871	0.1383
W3	51.00	49.42	50.15	0.44	0.0454	0.2666
W4	49.73	48.62	49.31	0.43	0.1746	0.2747
W5	50.81	48.99	49.25	0.49	0.0150	0.7084
W9	50.49	47.55	48.24	0.38	0.0001	0.2242
Repeated Measures Analysis:						
	P value					
Treatment	0.0081					
Time	<0.0001					
Time x treatment	0.0001					

[z] Contrast:

C vs D = Control vs the average of the two vitamin D_3 treatments.

2.5 vs 5.0 = 2.5 vs 5.0 MIU D_3 treatments.

indicates that total Ca was increasing at a relative faster rate than ionized Ca. This phenomenon was also observed in the previous experiment of this series with the exception that the ratio returned to normal values by the end of the withdrawal period. Total content of Ca in the extracellular fluid is found in the following forms: approximately 50 % is free or ionized Ca, 45 % is bound to proteins, and the remaining 5 % is complexed (Rosol *et al.* 1995; Rosol and Cappen 1997).

4.3.3 Parathyroid Hormone (PTH) Concentrations

Serum PTH concentrations of the experimental steers on day S1 were: 147.07, 149.84 and 199.60 pg/ml for the 0, 2.5 and 5 MIU treatments, respectively. Values that were not statistically different ($P>0.05$). Over the course of the supplemental period, PTH values fell in all (supplemented and unsupplemented) steers (Table 4.6 and Figure 4.3).

Values for the control steers reached a plateau on day S5, however, for the treated steers they continued to decrease through the supplemental and withdrawal periods. The lowest PTH values attained were 80.75, 19.35 and 8.31 pg/ml on day W5, W5 and W4 for the 0, 2.5 and 5 MIU treatments, respectively, which represented a decrease of 45.0, 87.0 and 96.0 % with respect to their initial values (day S1) for each group (Table 4.6 and Figure 4.3).

Contrast analysis indicated that supplemented animals had lower ($P<0.05$) PTH values than control animals from day S6 to W9. However, no differences ($P>0.05$) between levels of supplementation during supplemental or withdrawal periods were identified, with the exception of day S3 and S4, where the 5 MIU treatment had the lowest levels of PTH (Table 4.6 and Figure 4.3).

PTH hormone appears to be the principal hormone involved in the minute-to-minute regulation of Ca concentration in blood. It is produce by the chief cells of the parathyroid glands (Rosol and Capen 1997). The parathyroid glands respond to minor fluctuations in Ca concentration by rapidly altering the rate of hormonal secretion and more slowly by altering the rate of hormonal synthesis (Rosol *et al.* 1995). PTH controls plasma Ca concentrations by acting at the glomerular level to increase the reabsorption of Ca by stimulating the development and activity of bone osteoclasts, and by stimulating

Table 4.6. Effect of high levels of vitamin D_3 supplementation on serum parathyroid hormone (PTH) concentrations of finishing Hereford steers (607 ± 12 kg BW).

PTH (pg/dl)						
	Vitamin D_3 Level (MIU/hd/d)				Contrasts [z]	
Days	0	2.5	5.0	*SEM*	C vs D	2.5 vs 5.0
Supplemental Period:						
S1	147.07	149.84	199.60	22.21	0.3295	0.1391
S2	216.35	224.56	139.38	51.64	0.5967	0.2661
S3	181.60	216.84	66.03	32.14	0.3277	0.0061
S4	129.93	123.87	36.88	22.49	0.0971	0.0181
S5	109.65	108.11	57.00	24.67	0.3875	0.1687
S6	120.59	42.89	20.82	16.14	0.0007	0.3529
S7	117.39	33.01	14.54	15.99	0.0004	0.4300
Withdrawal Period:						
W1	94.65	19.21	16.99	9.65	<0.0001	0.8734
W2	141.71	46.35	18.71	26.44	0.0056	0.4741
W3	118.57	24.87	23.31	16.69	0.0006	0.9483
W4	116.06	29.41	8.24	20.19	0.0020	0.4767
W5	80.75	19.35	10.86	8.24	<0.0001	0.4807
W9	131.19	60.32	43.76	13.64	0.0005	0.4074

Repeated Measures Analysis:

	P value
Treatment	0.0013
Time	<0.0001
Time x treatment	0.0014

[z] Contrast:

C vs D = Control vs the average of the two vitamin D_3 treatments.

2.5 vs 5.0 = 2.5 vs 5.0 MIU D_3 treatments.

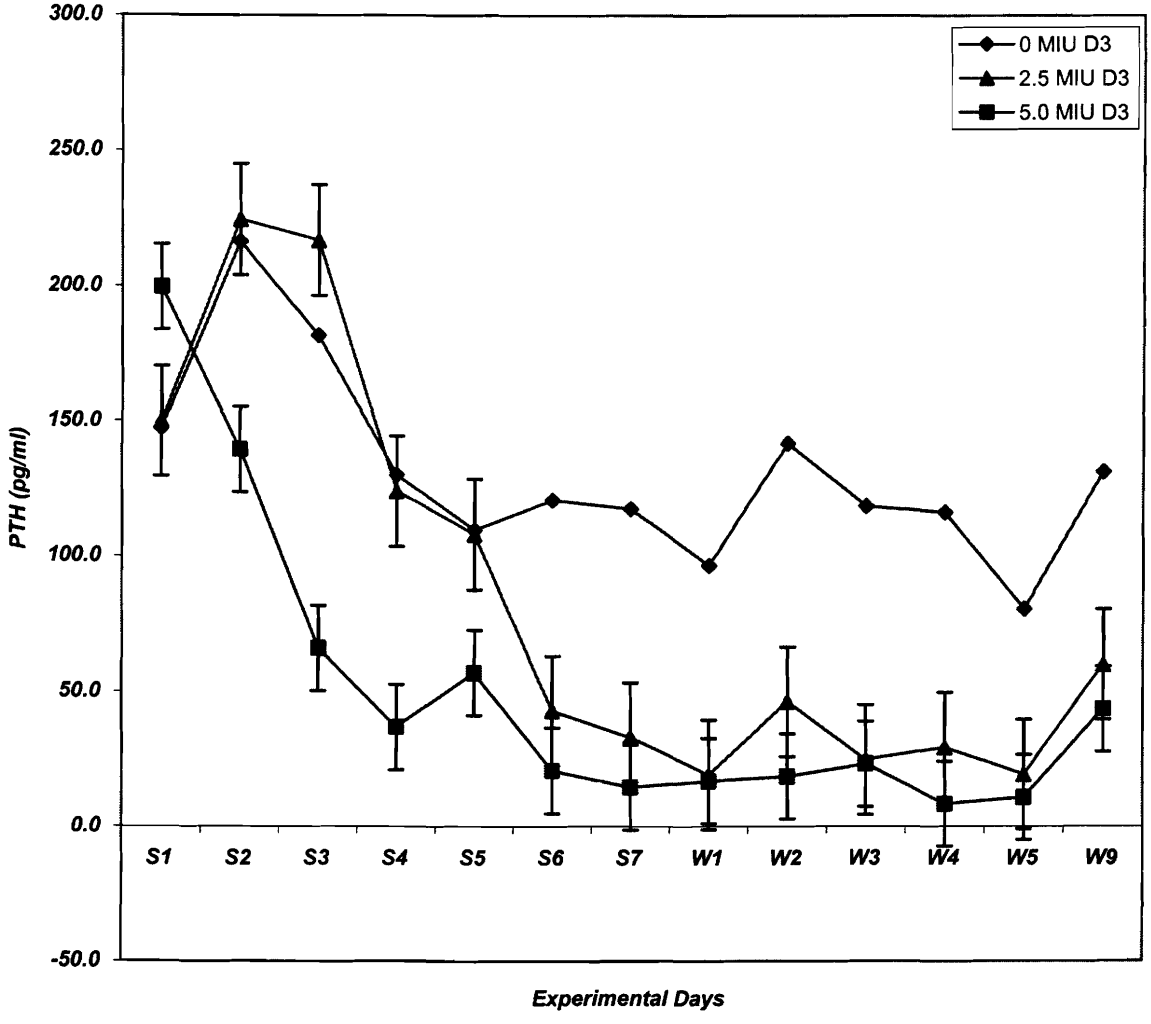

Figure 4.3. Effect of high levels of vitamin D_3 supplementation (0, 2.5 and 5 MIU for seven days) on serum parathyroid hormone (PTH) concentrations of finishing Hereford steers (607 ± 12 kg BW), during supplemental (S1 to S7) and withdrawal (W1 to W4) periods.

the synthesis of the hormonal form of vitamin D_3, which acts on intestinal tissue to increase the efficiency of Ca absorption (Goff 1989; Guyton and Hall 1996). When Ca plasma reaches levels above normal, PTH secretion is reduced, and in acute hypercalcemia the parathyroid glands can suffer hypoplasia (Guyton and Hall 1997). It would appear, therefore, that the reduced levels of PTH in serum were a metabolic adjustment to reduce the high levels of Ca in serum caused by vitamin D_3 supplementation.

4.3.4 Calcitonin Concentrations

Serum calcitonin concentrations of the experimental steers were determined on days S1, S3, S6, W1, W3 and W5 (Table 4.7). On the first day of the supplemental period there was no difference ($P>0.05$) in calcitonin concentrations between control and supplemented steers, however, there was ($P<0.05$) between the 2.5 and 5 MIU treatment (Table 4.7). On the third day of supplementation the situation was inversed, that is, there was a difference ($P<0.05$) between control and supplemented steers, but not between vitamin D_3 treatments. From the end of the supplementation period (S6) and throughout withdrawal period, there were no differences ($P>0.05$) between control and vitamin D_3 treatments.

Calcitonin secreted by the parafollicular cells, or C cells of the thyroid gland has the function to reduce blood Ca concentration (Rosol *et al.* 1995). This is achieved by increasing urinary Ca excretion, by decreasing absorption from bone osteoclasts (immediate effect), and by preventing the formation of new osteoclasts (prolonged effect) (Goff 1989; Guyton and Hall 1997). However, even though serum Ca concentrations were significantly increased by vitamin D_3 supplementation, and PTH decreased, calcitonin concentrations were not significantly altered throughout the whole experiment. Rosol *et al.* (1995) pointed out that the function of calcitonin in inhibiting osteoclastic bone resorption is transitory, because the osteclasts rapidly become refractory to its effect. For this reason, the calcitonin regulatory mechanism is considered weaker than the PTH system because its effect only lasts a few hours to a few days (Rhoades and Tanner 1995). This may explain why serum Ca concentrations from supplemented steers were increased and reached high levels for a prolonged period of time, because calcitonin

Table 4.7. Effect of high levels of vitamin D_3 supplementation on serum calcitonin hormone concentrations of finishing Hereford steers (607 ± 12 kg BW).

Calcitonin (pg/ml)						
	Vitamin D_3 Level (MIU/hd/d)				Contrasts [z]	
Days	0	2.5	5.0	*SEM*	C vs D	2.5 vs 5.0
Supplemental Period:						
S1	354.02	381.94	297.64	15.94	0.4799	0.0028
S3	278.16	381.52	339.68	22.48	0.0112	0.2128
S6	296.36	323.34	352.68	23.27	0.1697	0.3902
Withdrawal Period:						
W1	318.62	309.08	281.72	30.86	0.5505	0.5425
W3	289.16	312.46	347.60	19.54	0.1133	0.2275
W5	332.04	310.42	328.08	22.99	0.6578	0.5970
Repeated Measures Analysis:						
	P value					
Treatment	0.4222					
Time	0.2246					
Time x treatment	0.0071					

[z] Contrast:

C vs D = Control vs the average of the two vitamin D_3 treatments.

2.5 vs 5.0 = 2.5 vs 5.0 MIU D_3 treatments.

appears not to play a key role in controlling plasma Ca levels as the PTH and $1,25(OH)_2D_3$ system does in ruminants.

4.3.5 Vitamin D_3 and 25-Hydroxyvitamin D_3 Concentrations

Plasma vitamin D_3 concentrations from the experimental steers on the first day of supplementation (S1) were: 4.92, 6.70 and 11.51 ng/ml for the 0, 2.5 and 5 MIU treatments, respectively. Vitamin D_3 concentrations from control animals ranged from 2.71 to 8.20 ng/ml throughout the experiment, with an average of 4.23 ± 1.47 ng/ml (Table 4.8 and Figure 4.4). This average was close to the normal values of 1 to 3 ng/ml reported for dairy cattle (McDermott *et al.* 1985; Horst 1986); or the 3.1 ng/ml for finishing beef cattle (Montgomery *et al.* 2000).

Plasma $25(OH)D_3$ concentrations from the experimental steers on day S1 were: 50.13, 50.11, 54.03 and 54.03 ng/ml for the 0, 2.5 and 5 MIU treatments, respectively. The $25(OH)D_3$ concentrations for the control steers ranged from 43.49 to 60.65 ng/ml throughout the trial, with an average of 48.47 ± 4.33 ng/ml (Table 4.9 and Figure 4.5). This average was in good agreement with the normal range of 20 to 60 ng/ml for dairy cattle (Horst and Reinhardt 1982; Littledike and Horst 1982; Horst *et al.* 1994); or almost identical to the 48.1 ng/ml reported for finishing steers (Montgomery *et al.* 2000). Vitamin D_3 and $25(OH)D_3$ were increased ($P<0.0001$) as a result of vitamin D_3 supplementation, and this response was directly related ($P<0.0001$) to the level of supplementation, but their responses were different (Tables 4.8 and 4.9 and Figures 4.4 and 4.5). The increase in vitamin D_3 in plasma fit a bell shaped curve. Values increased almost immediately once supplementation started, with a maximum located between days S5 and S7, and declining and plateauning on day W3. At this point, vitamin D_3 concentration remained elevated relative to control values through the end of the trial (W9) (Figure 4.4). The increase in $25(OH)D_3$ concentration was linear from almost the beginning through the end of the trial with the highest value on day W9 (Figure 4.5). In contrast, the levels of these compounds in control animals remained constant throughout the trial (Figures 4.4 and 4.5).

Maximum vitamin D_3 concentrations were 132.4 and 149.8 ng/ml, which represented a 32.6- and 36.9-fold increase relative to the control animals for the 2.5 and 5

Table 4.8. Effect of high levels of vitamin D_3 supplementation on plasma vitamin D_3 concentrations of finishing Hereford steers (607 ± 12 kg BW).

Vitamin D_3 (ng/ml)						
	Vitamin D_3 Level (MIU/hd/d)				Contrasts [z]	
Days	0	2.5	5.0	*SEM*	C vs D	2.5 vs 5.0
Supplemental Period:						
S1	4.92	6.70	11.51	1.86	0.0914	0.0934
S2	4.64	44.27	79.07	12.14	0.0024	0.0656
S3	3.03	44.01	107.81	7.11	<0.0001	<0.0001
S4	3.28	114.60	132.23	10.60	<0.0001	0.2623
S5	4.06	132.43	149.75	10.12	<0.0001	0.2495
S6	2.71	90.19	139.99	7.89	<0.0001	0.0008
S7	3.99	107.05	148.48	9.32	<0.0001	0.0085
Withdrawal Period:						
W1	4.10	103.55	135.22	9.62	<0.0001	0.0383
W2	4.05	61.46	102.24	6.49	<0.0001	0.0008
W3	5.40	33.62	56.40	4.29	<0.0001	0.0027
W4	8.20	60.39	43.80	6.32	0.0001	0.0881
W5	2.44	58.44	51.52	7.49	<0.0001	0.5265
W9	4.23	40.76	51.23	3.10	<0.0001	0.0342

Repeated Measures Analysis:

	P value
Treatment	<0.0001
Time	<0.0001
Time x treatment	<0.0001

[z] Contrast:

C vs D = Control vs the average of the two vitamin D_3 treatments.

2.5 vs 5.0 = 2.5 vs 5.0 MIU D_3 treatments.

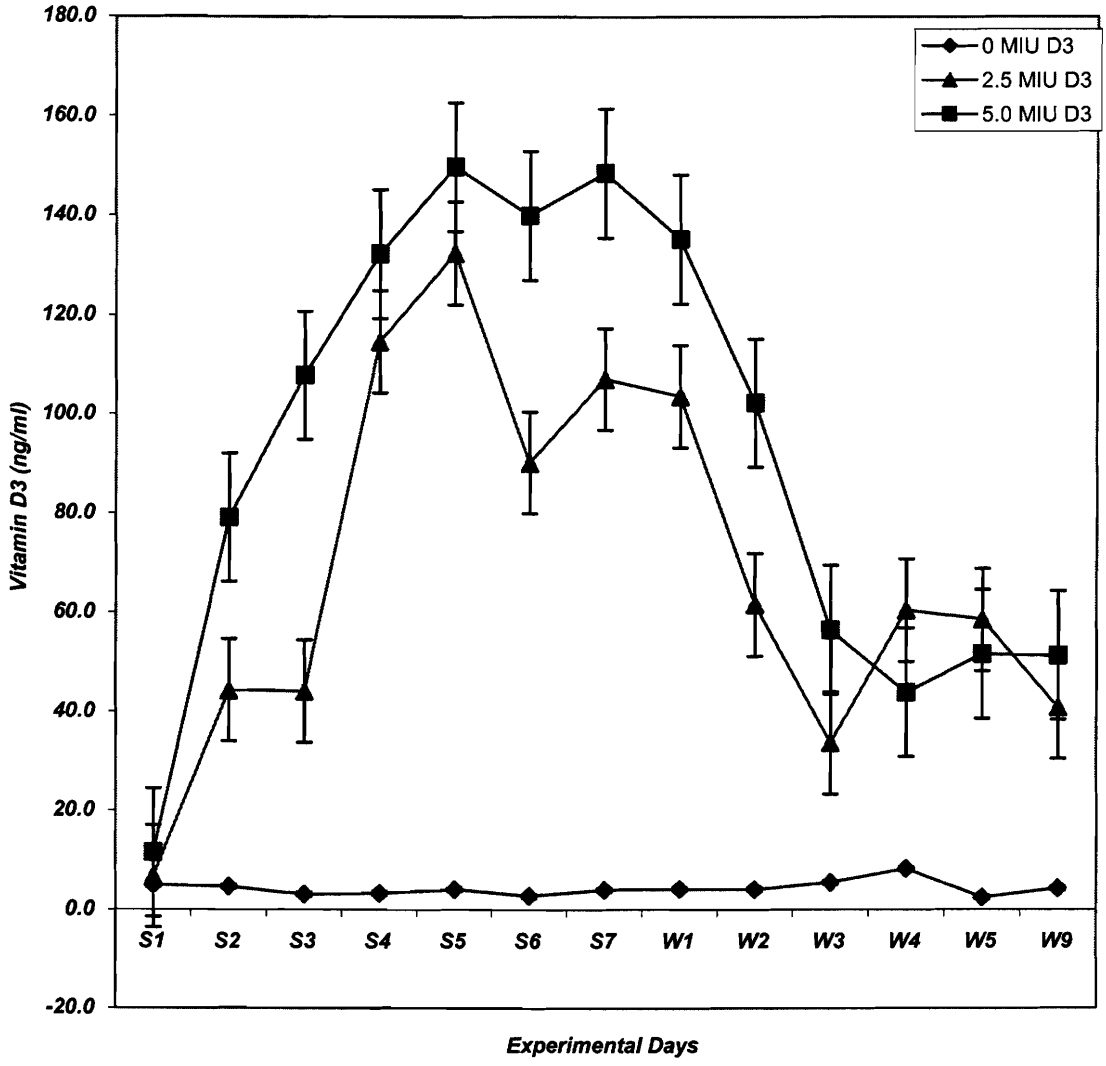

Figure 4.4. Effect of high levels of vitamin D_3 supplementation (0, 2.5 and 5 MIU for seven days) on plasma vitamin D_3 concentrations of finishing Hereford steers (607 ± 12 kg BW), during supplemental (S1 to S7) and withdrawal (W1 to W4) periods.

Table 4.9. Effect of high levels of vitamin D_3 supplementation on plasma 25-hydroxy vitamin D_3 ($25(OH)D_3$) concentrations of finishing Hereford steers (607 ± 12 kg BW).

	$25(OH)D_3$ (ng/ml)					
	Vitamin D_3 Level (MIU/hd/d)				Contrasts [z]	
Days	0	2.5	5.0	*SEM*	C vs D	2.5 vs 5.0
Supplemental Period:						
S1	50.13	50.11	54.03	3.84	0.6870	0.4838
S2	48.58	68.56	84.03	7.56	0.0112	0.1738
S3	49.84	60.54	120.39	6.95	0.0005	<0.0001
S4	46.90	93.33	166.60	8.31	<0.0001	<0.0001
S5	46.69	130.65	204.92	7.69	<0.0001	<0.0001
S6	43.82	139.34	249.53	9.82	<0.0001	<0.0001
S7	48.01	162.78	265.41	10.49	<0.0001	<0.0001
Withdrawal Period:						
W1	43.49	178.40	288.77	11.50	<0.0001	<0.0001
W2	44.29	180.41	285.46	15.50	<0.0001	0.0004
W3	49.85	160.92	308.67	11.59	<0.0001	<0.0001
W4	60.65	237.30	325.48	20.98	<0.0001	0.0117
W5	49.34	242.81	297.54	16.82	<0.0001	0.0401
W9	48.47	226.92	335.89	23.69	<0.0001	0.0069

Repeated Measures Analysis:

	P value
Treatment	<0.0001
Time	<0.0001
Time x Treatment	<0.0001

[z] Contrast:

C vs D = Control vs the average of the two vitamin D_3 treatments.

2.5 vs 5.0 = 2.5 vs 5.0 MIU D_3 treatments.

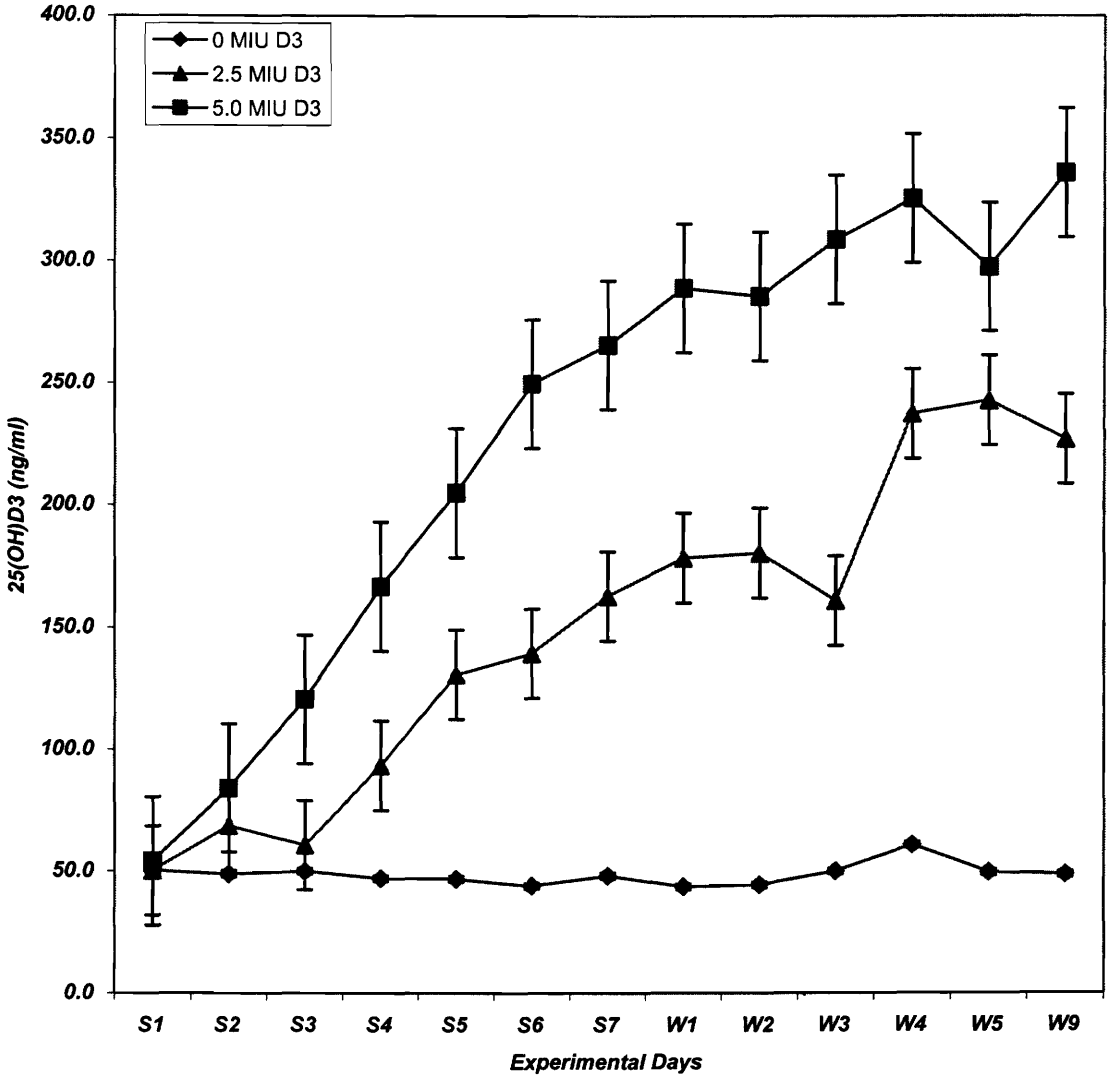

Figure 4.5. Effect of high levels of vitamin D_3 supplementation (0, 2.5 and 5 MIU for seven days) on plasma circulating 25-hydroxyvitamin D_3 ($25(OH)D_3$) concentrations of finishing Hereford steers (607 ± 12 kg BW), during supplemental (S1 to S7) and withdrawal (W1 to W4) periods.

MIU treatments, respectively (Table 4.8). Maximum $25(OH)D_3$ concentrations were 242.8 and 335.9 ng/ml, which represented a 4.9- and 6.9-fold increase relative to the control animals for the 2.5 and 5 MIU treatments, respectively (Table 4.9).

Horst and Reinhardt (1983) conducted an experiment in which nonlactating non-pregnant dairy cows were injected (single dose) with 15 MIU D_3. These workers found an immediate increase in plasma vitamin D_3, $25(OH)D_3$ and $1,25(OH)_2D_3$ levels. Vitamin D_3 concentration peaked approximately 10 days after treatment, and then began to fall through day 35, at which time a plateau was attained, which lasted until the end of the trial (day 90). The $25(OH)D_3$ concentration was also immediately increased, peaking approximately 30 days after treatment, and then began to fall slowly until the end of the trial. Similarly, $1,25(OH)_2D_3$ had a sharp increase after 12 to 15 days after vitamin D_3 injection, and then began to decline. Interestingly, the plasma concentration patterns of vitamin D_3 and $25(OH)D_3$ found in the current experiment were remarkably similar to this study, despite the difference in the route of vitamin D_3 administration and in the amount used.

Montgomery *et al.* (2000) supplemented finishing steers with 5 MIU D_3 for 9 days and found plasma vitamin D_3 and $25(OH)D_3$ concentrations of 3.1 and 464.3 ng/ml and 48.1 and 578.4 ng/ml for control and supplemented steers, respectively. The control values found in the present trial were in close agreement with this report. However, differences in maximum plasma vitamin D_3 (150- vs 37-fold) and $25(OH)D_3$ (12- vs 7-fold) values of treated animals were identified. These differences may be due to actual vitamin D_3 consumption. These researchers administered vitamin D_3 by intraruminal boluses, while in the present trial vitamin D_3 was top-dressed to the daily ration, with depression in feed intake affecting total consumption. Vitamin D_3 intake for the 5 MIU treatment in the present trial was only 68.4 % of the targeted value. However, despite the differences in vitamin D_3 intake, the relationship between vitamin D_3 supplementation and plasma vitamin D_3 and $25(OH)D_3$ concentrations were very similar in both studies.

When one considers the metabolic response to supplemental vitamin D_3 it is clear that supplementation caused significant increases in plasma vitamin D_3 and $25(OH)D_3$, and in total and ionized serum Ca, a reduction in PTH and no effect on calcitonin levels. In addition feed intake was drastically depressed. This rise in total and ionized serum Ca

levels is likely a result of several metabolic signals that ultimately influence Ca absorption and resorption. It is clear, first, that PTH levels declined rapidly due to a rise in serum Ca with no detectable affect on calcitonin levels. As discussed, this drop in PTH is fully consistent with its metabolic function (Guyton and Hall 1996). Mega levels of dietary vitamin D_3 were found to dramatically increase plasma vitamin D_3 levels. It is obvious that as a result of increased of $25(OH)D_3$ levels, the first hydroxylation, which is carried out in the liver by the 25-hydroxylase enzyme was carried out relatively unregulated. Again this is consistent with known functions of this enzyme. The rise in $25(OH)D_3$ levels could increase serum Ca levels in one or two fashions. First, increased levels of $25(OH)D_3$ could stimulate conversion in the kidney to $1,25(OH)_2D_3$ (hormonal form of vitamin D_3), which acts to stimulate Ca binding protein synthesis in intestinal epithelial cells which then increase active Ca transport across the epithelium. In humans and many other species, this step is normally a tightly regulated step with $1,25(OH)_2D_3$ levels held in close check (DeLuca *et al.* 1977; Guyton and Hall 1997). Supporting this reasoning, Montgomery *et al.* (2000), did not find significant changes in $1,25(OH)_2D_3$ plasma concentrations in steers supplemented with 5 MIU D_3 for nine days. The second possible mechanism for increased serum Ca levels due to vitamin D_3 supplementation is that $25(OH)D_3$ may override the normal process, by performing the functions of $1,25(OH)_2D_3$ in intestine and bone by virtue of excess of substrate, that is, although the affinity of the receptors in the target tissues is much higher for $1,25(OH)_2D_3$ than for $25(OH)D_3$, the excess of $25(OH)D_3$, as a result of vitamin D_3 supplementation, could bind the receptors and simulate the functions of $1,25(OH)_2D_3$ in kidney, bone and intestine (Smith *et al.* 1983; Bhagavan 1992; Rhoades and Tanner 1995).

Finally, it has also been shown in other studies that supplementation with high levels of vitamin D_3 (2.5 and 5 MIU) to finishing steers results in significant increases in plasma vitamin D_3 and $25(OH)D_3$ concentrations (Montgomery *et al.* 2000). These higher levels of vitamin D_3 in plasma will lead to higher concentrations in muscle and in other organs (liver) and tissues (adipose) as has been demonstrated by Montgomery *et al.* (2000 and in press). High levels of vitamin D_3 in edible tissues represent the main concern with regard to human health (Owens *et al.* 1998). High residual amounts of vitamin D_3 have been found in meat, from supplemented animals (5 MIU/d), even after

the meat has been cooked (Montgomery *et al.* in press). The residual concern is based on the fact that vitamin D_3 regulates Ca metabolism and elevated consumption (via meat) may lead to alteration in the normal metabolism of Ca in humans, causing, principally, calcification of the soft tissues and formation of kidney stones (Hunt and Groff 1990; Guyton and Hall 1996, 1997). Acute intoxication from vitamin D_3 includes lost of appetite, nausea, weakness, hypertension, renal failure, cardiovascular dysfunction and death, which is the result of nephrocalcinosis and heart and aortic calcification (Smith *et al.* 1983; Hunt and Groff, 1990; Glerup and Eriksen 2001).

In light of the documented potential for residues of vitamin D_3 in organ and tissues, it is doubtful if this dietary treatment to improve beef tenderness would ever be approved by Canadian regulatory agencies. It is therefore of interest to explore alternative mechanisms to increase serum Ca levels that may work either alone or in combination with vitamin D_3 to improve beef tenderness.

4.4 Conclusions

Vitamin D_3 supplementation at levels used in this experiment (2.5 and 5 MIU/hd/d for 7 days) caused a significant depression in feed intake, with the degree of depression and the rate of recovery inversely related to the amount of vitamin D_3 supplemented. Both levels of supplemental vitamin D_3 induced significant increases in total and ionized serum Ca concentrations. The increments were directly related to the level of supplementation. Maximum serum Ca concentrations were attained several days after supplementation ended. This suggests that the cascade of events leading to synthesis of Ca binding protein and subsequently increased Ca absorption were activated, however the mechanisms require time to fully develop. Vitamin D_3 supplementation at both levels, induced a significant increase in plasma vitamin D_3 and 25(OH)D_3 concentrations. These two metabolites had different response curves to supplementation. Plasma vitamin D_3 was increased only during supplementation, whereas 25(OH)D_3 was linearly increased throughout the supplemental and withdrawal periods. This suggests that the activity of the 25-hydroxylase enzyme was not tightly regulated, allowing the conversion of vitamin D_3 to 25(OH)D_3.

The increase in plasma vitamin D_3 and $25(OH)D_3$ and subsequently in the total and ionized serum Ca caused a depression in the normal PTH values, although calcitonin was not significantly affected. This suggests that the calciotrophic hormone responsible for the increased Ca levels was $1,25(OH)_2D_3$, either acting by itself or by the $25(OH)D_3$ by virtue of excess substrate.

CHAPTER V

Effect of Feeding a Low Calcium Diet and Vitamin D_3 on Calcium Metabolism in Finishing Steers

5.1 Introduction

It has been observed that manipulation of dietary calcium (Ca) can directly affect plasma Ca levels. The efficiency of Ca absorption in the intestine is related to the amount of Ca in the diet, high levels of Ca reduce the efficiency of absorption and low levels tend to improve it (Verdaris and Evans 1975). This phenomenon is a response to maintain Ca homeostasis in the organism. Reduced levels of Ca in the diet results in lower Ca in the blood, thus the animals' response is to secrete greater amounts of PTH, which in turn stimulates the kidneys to synthesize $1,25(OH)_2D_3$, whose main function is to accelerate the active transport of Ca from the intestine, increasing plasma Ca levels and consequently its availability. Calcium can be absorbed from the lumen of the intestine by passive diffusion between the intestinal epithelial cells (parecellular transport) and by active transport across the epithelial cells. Passive diffusion is directly related to the concentration of Ca ions in the lumen of the gut. The active transport of Ca across the intestinal epithelial cells takes place when dietary Ca is low or when demand is very high. This process requires $1,25(OH)_2D_3$, which facilitates the active process through the synthesis of the Ca binding protein (CaBP) which has the function to transport Ca across the epithelial cell. It appears that the rate of transcellular Ca transport is directly correlated to the amount of CaBP in the cells. In other words, low Ca diets stimulate Ca homeostatic mechanisms and the contrary is true for high Ca diets (DeLuca 1980; Goff 1989; Horst *et al.* 1994).

A common use of this concept is with dairy cows approaching parturition. Such cows are prone to milk fever. Low Ca diets have been used during the latter stages of the dry period to increase or at least maintain plasma Ca concentration at parturition and so reduce the likelihood of parturient paresis or milk fever (Beitz *et al.* 1973; Goings *et al.* 1974; Kichura *et al.* 1982; Allen and Sansom 1985).

Goings *et al.* (1974) fed a low Ca diet (8 g of Ca/d) to dairy cows for 14 (7 to 17) days prepartum. Plasma Ca concentrations of the cows decreased within four days after the initiation of feeding the low Ca diet, although minimum values were reached approximately 36 h after the initiation of feeding and then slowly rose to pre-treatment values. An increase in PTH concentration also occurred during the same 4 days. This feed management was sufficient to prevent parturient paresis in all the treated cows. These authors made the observation that feeding cows a low Ca diet for at least 7 days prepartum is likely to stimulate Ca homeostatic mechanisms to the degree that milk fever can be prevented.

Kichura *et al.* (1982) conducted an experiment in which dairy cows were fed with different proportions of Ca and P from four weeks prepartum. They found that cows fed the low Ca diets regardless of the dietary phosphorus intake, had greater $1,25(OH)_2D_3$ and hydroxyproline concentrations prepartum, and greater plasma Ca concentrations at parturition, and less incidence of parturient paresis than cows fed high Ca diets. These workers concluded that low Ca diets effectively activated the Ca homeostatic mechanisms before parturition by stimulating both bone resorption and intestinal absorption of Ca.

In the previous experiments of this study, the basis for high levels of vitamin D_3 supplementation under western Canadian conditions (Chapter III) and the relationship between supplemental vitamin D_3 and Ca homeostatic mechanisms (Chapter IV) were studied. It was found that the levels of serum Ca concentrations achieved from the 5 MIU D_3 level were, in both experiments, in the range in which significant improvements in beef tenderness were reported (Swanek *et al.* 1997, 1999; Montgomery *et al.* 1998, 2000; Karges *et al.* 2001). However, high levels of supplemental vitamin D_3 also induce high levels of residual vitamin D_3 in liver and other edible tissues (Montgomery *et al.* 2000), as well as negatively influencing animal performance (Karges *et al.* 1999a,b; Berry *et al.* 2000). These conditions are the main concerns that limit the utilization of this practice as a common tool for feedlot producers to improve beef tenderness.

It is possible, however, that feeding a low Ca diet for a set period of time may induce Ca absorption, reabsorption and resorption mechanisms and thus prime the response to vitamin D_3 supplementation. In such situations it may be possible to feed

lower levels of vitamin D_3 yet obtain a significant rise in plasma Ca. Therefore, the objective of this study was to evaluate the feasibility of using a low Ca diet prior to vitamin D_3 supplementation at one of the three levels: 1.25, 2.5 and 5 MIU/hd/d for 7 days, with the aim to increase serum Ca to higher levels than supplementation of vitamin D_3 without dietary Ca manipulation. It was also of interest to determine the effect of dietary treatment on circulating concentrations of parathyroid hormone, calcitonin, vitamin D_3, $25(OH)D_3$ and $1,25(OH)_2D_3$.

5.2 Material and Methods

5.2.1 Experimental Design

5.2.1.1 Adaptation Period (days A1 to A36)

Nineteen Angus steers (522 ± 34 kg BW) were housed in individual pens (3.6 x 3.6 m) with automatic water bowls in the Livestock Research Barn of the Department of Animal and Poultry Science at the University of Saskatchewan. Animals were cared for according to the guidelines of the Canadian Council of Animal Care. The cattle were adapted to the experimental diet for 36 days. The diet consisted of 75% concentrate (barley grain base) and 25% barley silage (as fed), meeting the requirements for this type of cattle according to the National Research Council (1996) (Table 5.1).

The steers were fed twice a day (0800 and 1600 h), with orts collected and weighed every day before the morning feeding. Individual maximum voluntary feed intake was measured from day A30 to A36, constituting the pre-treatment feed intake value.

The experimental steers were bled by jugular venipuncture starting at 1230 h on the last day of this period (A36). Two sets of samples were taken. For the first, blood was collected into 5ml-draw evacuated (Vacutainer®) tubes without anticoagulant. The blood was allowed to clot at room temperature for approximately 2 h, then centrifuged at 3500 rpm for 15 min, the serum obtained was immediately used to determine total and ionized Ca concentrations. For the second set, blood was collected into 10 ml-draw

Table 5.1. Ingredient composition and formulated nutrient levels of the experimental diets.

Item	Normal Ca diet	Low Ca diet
	DM (%)	DM (%)
Total mixed diet:		
Concentrate	89.6	89.6
Barley silage	10.4	10.5
Concentrate:		
Barley grain	88.1	89.5
Canola meal	7.3	7.3
Tallow	1.7	1.7
Limestone	1.6	0.0
Rumensin [z]	0.4	0.5
Vitamin [y]	0.4	0.5
Salt	0.6	0.6
Formulated composition [x]:		
Digestible energy Mcal/kg [w]	3.50	3.55
Crude protein, %	12.09	12.24
Calcium, %	0.62	0.14
Phosphorus, %	0.43	0.44
Ca:P	1.43	0.32

[z] Premix: Monensin (20% active) mixed with barley grain.

[y] Premix: Vitamin A (416,650 IU/kg) and vitamin D (83,333 IU/kg).

[x] Nutrient Requirements of Beef Cattle. National Research Council (1996).

[w] Predicted TDN x 4.4 Mcal/kg (Weiss *et al.* 1992).

evacuated (BD Vacutainer™) tubes with anticoagulant (sodium heparin), which were placed on crushed ice immediately after withdrawal, and approximately 2 h later centrifuged at 3000 rpm for 10 min at –4°C, then the plasma was collected and transferred to 1.7 ml centrifuge clear vials and stored at –20°C. These plasma samples were used to determine vitamin D_3 and 25-hydroxyvitamin D_3 concentrations.

5.2.1.2 Low Ca Diet Period (days L1 to L10)

The steers were randomly dived into four groups of five steers each. Groups 1, 2 and 3 were fed with a low Ca diet (Table 5.1) for ten days. This low Ca diet was essentially the same as the normal Ca diet but with the main source of Ca (limestone) withdrawn, resulting in a total Ca content of 0.14 % and a Ca to P ratio of 0.32, in contrast to 0.62 % and 1.43 for the normal Ca diet, respectively. Group 4 was fed the normal Ca (control) diet during this period.

Feed was offered and intake monitored as in the previous period. Blood samples were obtained on days L2, L4, L6, L8 and L10 starting at 1230 h. These blood samples were collected, processed, stored and analyzed as described for the adaptation period.

5.2.1.3 Supplemental Period (days S1 to S7)

All the steers were shifted to the normal Ca diet on day 11 (S1). The steers that received the low Ca diet (groups 1, 2 and 3) were then randomly assigned to one of the following levels of vitamin D_3 supplementation: 0, 1.25 and 2.5 million IU (MIU) D_3 per steer per day for seven consecutive days. The steers from group 4 (with no low Ca period) were supplemented with 5 MIU D_3 per steer per day for seven consecutive days, the purpose of this group was to serve as a general control (to compare the effect of dietary Ca manipulation and vitamin D_3 supplementation).

The vitamin D_3 supplement was prepared from a vitamin D_3 concentrate containing 500,000 IU per gram (Rhone-Poulec Canada Inc.), using ground barley as a carrier. This supplement was formulated to have a final concentration of 13 MIU D_3 per kg of premix. Analysis of the supplement indicated an actual concentration of 14.06 ±0.53 MIU D_3 per kg (Aventis Animal Nutrition, Inc. Alpharetta, GA. USA). The corresponding daily total amount of the vitamin D_3 supplement was equally divided and

supplied at each feeding. The supplement was top-dressed onto the ration and thoroughly mixed by hand into the feed bunk. Feed intake was restricted to 85 % of the average voluntary intake during the last 7 days of the low Ca period with the exception of the control group (0 MIU) to ensure maximum vitamin D_3 consumption, with additional daily adjustments in order to avoid excessive feed refusals. Feed was supplied and feed intake monitored daily as described in previous periods.

Blood samples were obtained on day S2, S4 and S6, and these blood samples were collected, processed, stored and analyzed as described for the adaptation period.

5.2.1.4 Withdrawal Period (days W1 to W11)

Once vitamin D_3 supplementation ended, the steers were maintained for a further six days under the experimental conditions to evaluate post-treatment effects, then the steers were shipped back to the feedlot. Feed was offered without restriction. The ration and intake monitoring were the same as in previous period.

The steers were bled by jugular venipunture on days W1, W3, W5 and W11 (at the feedlot), these blood samples were collected, processed, stored and analyzed as described for the adaptation period. In addition, on the first day of the withdrawal period (W1) blood was collected using 10 ml-draw evacuated (BD Vacutainer™) tubes with anticoagulant (sodium heparin). Samples were placed in crushed ice immediately after withdrawal and approximately 2 h later centrifuged at 3000 rpm for 10 min at –4°C. Plasma was then transferred to 50 ml screw conical centrifuge tubes and stored at –20°C. These plasma samples were used to determine parathyroid hormone (PTH), calcitonin, and $1,25(OH)_2D_3$ concentrations.

5.2.2 Laboratory Analyses

5.2.2.1 Total and Ionized Ca Determinations

Serum concentrations of total and ionized Ca were determined by indirect potentiometry utilizing a Ca ion selective electrode, as described in Chapter III.

5.2.2.2 Parathyroid Hormone (PTH) Determination

PTH concentrations in plasma were determined by immunoradiometric assay (Coat-A-Count® Intact PTH IRMA, Diagnostic Products Corporation®, Los Angeles, CA. USA). Coat-A-Count Intact PTH IRMA (I-34) is a solid-phase immunoradiometric assay employing ^{125}I-labeled affinity-purified polyclonal anti-PTH (I-34) antibody in liquid phase, in conjunction with affinity-purified polyclonal anti-PTH (44-84) antibodies immobilized to the wall of a polystyrene tube. Intact PTH was captured between the anti-PTH immobilized on the tube and the radio-labeled anti-PTH. Unbound ^{125}I-labeled anti-PTH antibody was removed by decanting the reaction mixture and washing the tube, which reduced the nonspecific binding to a very low level. The PTH concentration was directly proportional to the radioactivity present in the tube after the wash step. The radioactivity was counted using a gamma counter, after which the concentration of PTH in the sample was obtained by comparing the sample counts-per-minute with those obtained from standards (calibration curves). Further details of the assay are provided in Appendix A.

5.2.2.3 Calcitonin Hormone Determination

Calcitonin (thyrocalcitonin) concentrations in plasma were determined by a double –antibody radioimmunoassay as described in Chapter IV. Further details of the assay are provided in Appendix B.

5.2.2.4 Vitamin D_3 and 25-Hydroxyvitamin D_3 Determinations

Concentrations in plasma of vitamin D_3 and 25-hydroxyvitamin D_3 ($25(OH)D_3$) were carried out using a reverse-phase high-performance liquid chromatography (HPLC) assay. These two compounds were extracted from plasma samples by using acetonitrile (1:2 ratio). Solutions were thoroughly mixed (vortexed) for a fixed period of time, then centrifuged. The supernatant was then filtered and transferred to injection vials. Samples were introduced into the HPLC by an autosampler (Agilent Series1100, Waldbronn, Germany). The samples were analyzed with a reverse-phase column (Prodigy ODS3 5μ 250 x 4.6 mm) and acetonitrile as the mobile phase under isocratic conditions. The metabolite $25(OH)D_3$ was eluted first and vitamin D_3 second. The absorbance of these

analytes (peak area) was computed and integrated by the software ChemStation (Hewlett-Packard. Agilent Technologies Waldbornn, Germany). Calibration curves were used to calculate concentrations from the experimental samples. Further details of the assay are provided in Appendix C.

5.2.2.5 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) Determination

Concentrations in plasma of $1,25(OH)_2D_3$ were determined by radioimmunoassay (RIA)(1,25-Dihydroxyvitamin D ^{125}I RIA kit. DiaSorin, Stillwater, MN. USA). This assay consisted of a two-step procedure involving a preliminary extraction and subsequent purification of vitamin D (D_2 and D_3) metabolites from serum using $C_{18}OH$ "Extra Clean" cartridges. Following extraction, the treated samples were then assayed using a competitive RIA procedure. The RIA method is based on a polyclonal antibody that is specific for both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$. The sample, antibody and tracer were incubated for 2 h at 20-25°C. Phase separation was accomplished after a 20 min incubation at 20-25°C with a second antibody precipitating complex. After centrifugation and decantation, the bound fraction remaining in the pellet was counted in a gamma counter. Hormone concentration in the samples was calculated directly from a standard curve of known concentrations. Further details of the assay are provided in Appendix D.

5.2.3 Statistical Analysis

The data was analyzed using the Analysis of Variance Technique including Repeated Measures Analysis using the General Lineal Model procedure of SAS (SAS 1989) to compare treatments within days and over time.

The following model for repeated measures was used:

Model: $Y_{ijk} = \mu + \alpha_i + \tau_{ij} + \delta_k + (\alpha\delta)_{ij} + \varepsilon_{ijk}$

where:

Y_{ijk} = is the response at time k on animal j in treatment i.

μ = is the overall mean.

α_i = is a fixed effect of treatment i.

τ_{ij} = is a random effect of animal j in treatment i.

δ_k = is a fixed effect of time k.

$(\alpha\delta)_{ij}$ = is a fixed interaction effect of treatment i with time k.

ε_{ijk} = is the random error at time k on animal j in treatment i.

Single degree of freedom contrasts:

A = Normal Ca diet versus low Ca diet.

B = Control animals versus the average of vitamin D_3 treatments.

C = Between 1.25 and 2.5 MIU D_3 treatments.

D = Between 2.5 and 5 MIU D_3 treatments.

5.3 Results and Discussion

5.3.1 Feed Intake (DMI)

Feed intake during the adaptation period averaged 9.67 ± 1.42 kg of DM, in which group 4 had a lower ($P<0.05$) intake than the other three groups, an effect attributed to differences in live weight, as this group was lighter than the other three. During the low Ca period (ten days), groups 1, 2 and 3 were fed the low Ca diet (0.14 % Ca and 0.32 Ca to P ratio) and group 4 with the normal Ca diet (0.62 % Ca and 1.43 Ca to P ratio). Feeding the low Ca diet did not alter ($P>0.05$) feed intake, with the exception of days L5 and L6 where intake was higher ($P<0.05$) than the controls (group 4)(Table 5.2). These results are in accordance with Verdaris and Evans (1975), who did not find significant difference in intake of dairy cows when they were fed with a low (0.2 %) or high (2.1 %) Ca diet.

During the supplemental period (seven days), all the groups were shifted to the normal Ca diet. Groups 1, 2, 3 and 4 received 0, 1.25, 2.5 and 5 MIU D_3 per steer per day, respectively. Control animals (group 1) were fed *ad libitum*, while the vitamin D_3 supplemented steers were fed at 85% of *ad libitum* relative to the average intake from the previous period, and additionally daily adjustments were performed to avoid excessive refusals. There was no difference ($P>0.05$) in feed intake among levels of vitamin D_3 supplemented (Table 5.2).

Table 5.2. Effect of feeding a low Ca diet for ten days prior to vitamin D_3 supplementation on feed intake (DMI) of finishing Angus steers (522 ± 33 kg BW).

DMI (kg)									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum)*:									
A	9.84	10.07	10.21	8.27	0.66				
Low Ca Diet Period (*ad libitum)*:									
	LCa	LCa	LCa	NCa					
L1	8.63	9.76	9.11	7.80	0.83	0.1633			
L2	7.17	7.65	6.79	7.58	1.34	0.8039			
L3	8.49	8.15	8.48	8.10	1.16	0.8378			
L4	7.93	8.70	8.29	7.33	0.79	0.2872			
L5	9.01	9.95	8.52	6.63	1.02	0.0433			
L6	10.10	10.21	8.46	7.62	0.68	0.0208			
L7	9.85	9.85	9.45	8.14	0.71	0.0693			
L8	8.83	9.70	9.06	7.65	0.76	0.1914			
L9	8.29	9.39	8.11	8.44	0.73	0.8524			
L10	8.45	9.23	7.01	6.98	1.17	0.3548			
Supplemental Period (normal Ca diet; D_3 treatments fed at 85% of *ad libitum*):									
	Vitamin D_3 Level (MIU/hd/d)								
	0	1.25	2.5	5.0					
S1	8.64	6.97	6.58	6.36	1.04			0.7381	0.8535
S2	7.69	7.25	6.52	6.48	0.98			0.5103	0.9732
S3	8.09	7.06	6.50	6.42	1.01			0.6251	0.9423
S4	7.65	6.15	6.24	5.64	1.53			0.9599	0.7395
S5	7.57	6.52	6.39	5.52	1.22			0.9215	0.5500
S6	6.73	6.28	5.51	5.44	1.32			0.6041	0.9648
S7	7.34	6.22	5.58	5.25	1.32			0.6656	0.8359
Withdrawal Period (normal Ca diet *ad libitum)*:									
W1	6.37	7.60	5.37	5.48	1.67		0.9055	0.2448	0.9558
W2	7.64	7.99	5.71	4.93	1.51		0.4083	0.1928	0.6647
W3	8.64	7.83	5.23	3.90	1.68		0.1304	0.1813	0.5060
W4	8.76	7.87	6.07	3.98	1.23		0.0603	0.2043	0.1677
W5	11.23	9.93	6.90	5.38	1.09		0.0070	0.0243	0.2490

[z] Contrasts: **A** = Normal Ca (NCa) diet (group 4) vs low Ca (LCa) diet (groups 1, 2 and 3)./ **B** = Control animals vs vitamin D_3 treated steers / **C** = 1.25 vs 2.5 MIU D_3 / **D** = 2.5 vs 5 MIU D_3.

During the withdrawal period (five days), the normal Ca diet was fed to all steers *ad libitum*. No differences ($P>0.05$) among supplemented and control steers were found, except on the last day (W5), where consumption from control animals was higher than from vitamin D_3 supplemented steers. Similarly, there was a significant difference ($P<0.05$) between the 1.25 and 2.5 MIU, but not between the 2.5 and 5 MIU treatments (Table 5.3).

Minimum intakes were 6.37 (W1), 6.15 (S4), 5.23 (W3) and 3.90 (W3) kg, which represented a decrease in 10.8, 13.9, 26.8 and 45.4 % relative to the average intake on day 1 of supplementation, for the 0, 1.25, 2.5 and 5 MIU treatments, respectively (Table 5.2). The decline in intake from control animals (group 1) could be due to the handling of the steers for bleeding. Additional to this effect, supplementation of vitamin D_3 appeared to decrease feed intake, in a direct relationship to the amount supplemented. This depression in intake has also been noted in other studies (Owens *et al.* 1998; Karges *et al.* 1999a; Berry *et al.* 2000; Scanga *et al.* 2001), and in the previous experiments of this series.

The vitamin D_3 concentration of the supplement was 14.06 ± 0.53 MIU per kg (Aventis Animal Nutrition, Inc. Alpharetta, GA. USA). The supplement consumption averaged 1.27, 2.56 and 5.14 MIU D_3 per day for the 1.25, 2.5 and 5 MIU treatments, respectively. The higher concentration and better adjustment of the daily feed intakes helped to achieve the targeted consumption.

5.3.2 Total and Ionized Ca Concentrations

Serum total and ionized Ca concentrations in the adaptation period averaged 9.97 ± 0.26 and 4.90 ± 0.19 mg/dl, respectively. These concentrations and the ranges of 9.96 to 10.18 and 4.89 to 5.01 mg/dl for total and ionized Ca, respectively, for the control group during the low Ca period, were within the normal ranges reported for cattle (Wasserman 1989; Rosol *et al.* 1995; Rosol and Capen 1997) (Tables 5.3 and 5.4 and Figures 5.1 and 5.2).

Total Ca concentrations from the low Ca groups (1, 2 and 3) were lower ($P<0.05$) than the control group (4), with the exception on days L4 and L10 (Table 5.3). Ionized Ca concentrations were also lower, with the exception on day L6 (Table 5.4). The

Table 5.3. Effect of feeding a low Ca diet for ten days prior to supplementation of vitamin D_3 on total serum Ca concentrations of finishing Angus steers (522 ± 33 kg BW).

Total Ca (mg/dl)									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum)*:									
A	9.77	9.89	9.97	10.18	0.13				
Low Ca Diet Period (*ad libitum)* [y]:									
	LCa	LCa	LCa	NCa					
L2	9.49	9.75	9.75	10.11	0.15	0.0269			
L4	9.51	9.66	9.63	9.96	0.17	0.0952			
L6	9.74	9.70	9.83	10.15	0.15	0.0443			
L8	9.71	9.86	9.85	10.18	0.12	0.0179			
L10	9.79	9.70	9.86	10.05	0.14	0.1462			
Supplemental Period (normal Ca diet; D_3 treatments fed at 85% of *ad libitum*):									
	Vitamin D_3 Level (MIU/hd/d)								
	0	1.25	2.5	5.0					
S2	9.59	9.78	9.87	9.93	0.14		0.1240	0.6088	0.7699
S4	9.91	10.02	10.27	10.60	0.16		0.0593	0.2421	0.1622
S6	9.94	10.50	10.90	11.54	0.15		<0.0001	0.0690	0.0086
Withdrawal Period (normal Ca diet *ad libitum)*:									
W1	9.85	10.46	11.25	12.02	0.20		<0.0001	0.0108	0.0163
W3	9.90	10.65	11.50	12.41	0.30		0.0005	0.0514	0.0519
W5	9.78	10.11	11.47	12.10	0.25		0.0003	0.0012	0.0982
W11	9.60	9.50	10.06	10.62	0.24		0.1286	0.0952	0.1208

Repeated Measures Analysis:

	P value
Treatment	0.0003
Time	<0.0001
Time x treatment	<0.0001

[z] Contrasts: A = Normal Ca diet (group 4) vs low Ca diet (groups 1, 2 and 3).

B = Control animals vs vitamin D_3 treated steers

C = 1.25 vs 2.5 MIU D_3

D = 2.5 vs 5.0 MIU D_3

[y] LCa = low Ca diet; NCa = normal Ca diet

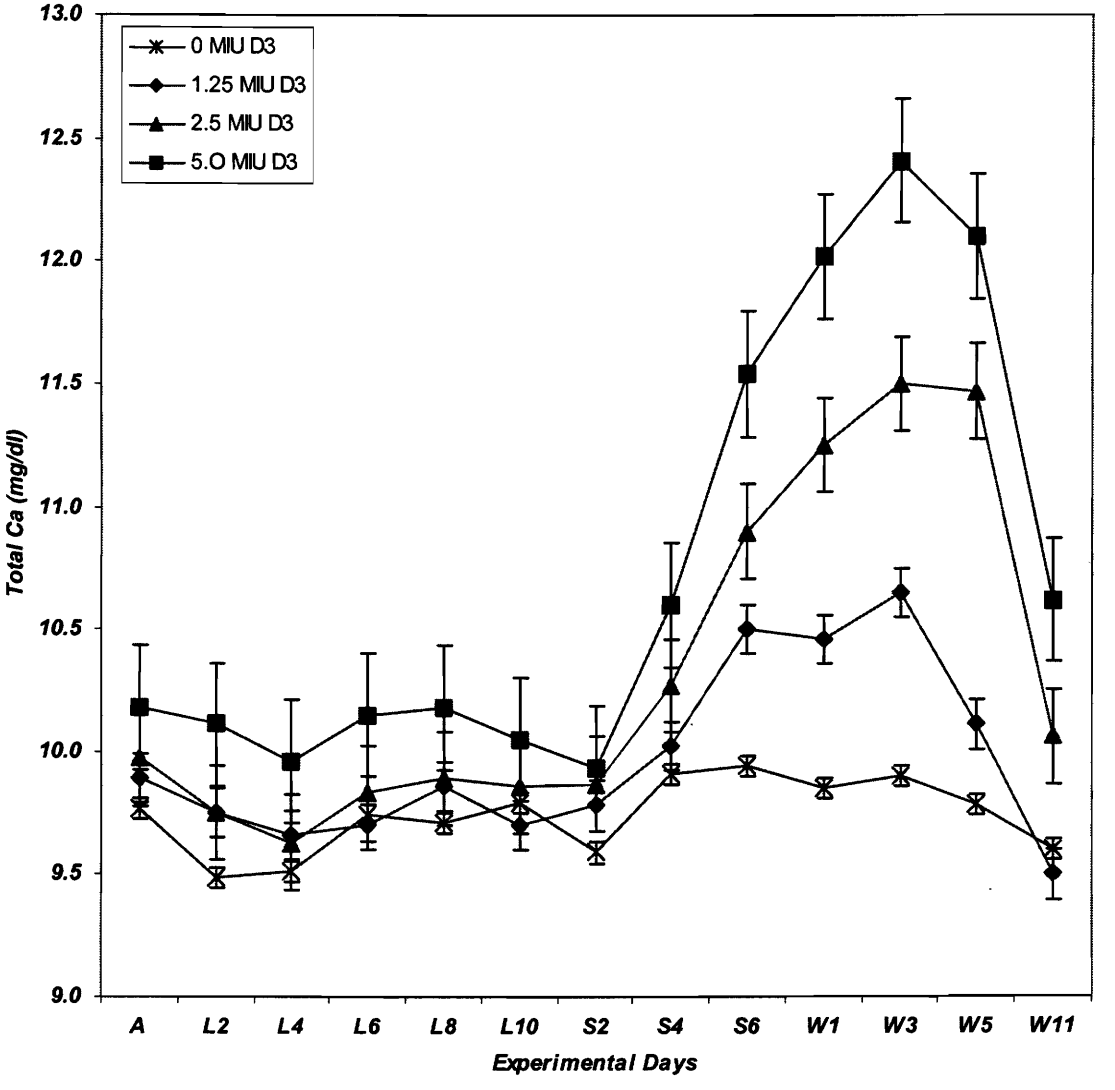

Figure 5.1. Effect of feeding a low Ca diet for ten days prior to supplementation of vitamin D_3 (0, 1.25, 2.5 and 5 MIU per day for seven consecutive days) on total serum Ca concentrations of finishing Angus steers (522 ± 33 kg BW), during adaptation (A), low Ca (L1 to L10), supplemental (S1 to S7) and withdrawal (W1 to W11) periods.

Table 5.4. Effect of feeding a low Ca diet for ten days prior to supplementation of vitamin D_3 on ionized serum Ca concentrations of finishing Angus steers (522 ± 33 kg).

Ionized Ca (mg/dl)									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum)*:									
A	4.79	4.99	4.91	4.94	0.10				
Low Ca Diet Period (*ad libitum)* [y]:									
	LCa	LCa	LCa	NCa					
L2	4.66	4.78	4.75	5.01	0.08	0.0111			
L4	4.64	4.69	4.72	4.89	0.09	0.0563			
L6	4.75	4.74	4.82	4.89	0.08	0.1998			
L8	4.78	4.82	4.87	4.97	0.06	0.0592			
L10	4.75	4.82	4.84	4.96	0.05	0.0144			
Supplemental Period (normal Ca diet; D_3 treatments fed at 85% of *ad libitum*):									
	Vitamin D_3 Level (MIU/hd/d)								
	0	1.25	2.5	5.0					
S2	4.72	4.74	4.73	4.63	0.06		0.7401	0.9136	0.2223
S4	4.78	4.82	4.86	4.96	0.08		0.2704	0.6943	0.3793
S6	4.67	4.88	4.98	5.34	0.07		0.0002	0.2904	0.0015
Withdrawal Period (normal Ca diet *ad libitum)*:									
W1	4.87	5.00	5.29	5.64	0.13		0.0120	0.1117	0.0707
W3	4.75	4.90	5.37	5.90	0.22		0.0217	0.1152	0.0909
W5	4.78	4.98	5.57	6.03	0.18		0.0022	0.0182	0.0694
W11	4.76	4.84	5.04	5.23	0.14		0.1006	0.2732	0.3244

Repeated Measures Analysis:

	P value
Treatment	0.0024
Time	<0.0001
Time x treatment	<0.0001

[z] Contrasts: A = Normal Ca diet (group 4) vs low Ca diet (groups 1, 2 and 3).

B = Control animals vs vitamin D_3 treated steers

C = 1.25 vs 2.5 MIU D_3

D = 2.5 vs 5.0 MIU D_3

[y] LCa = low Ca diet; NCa = normal Ca diet

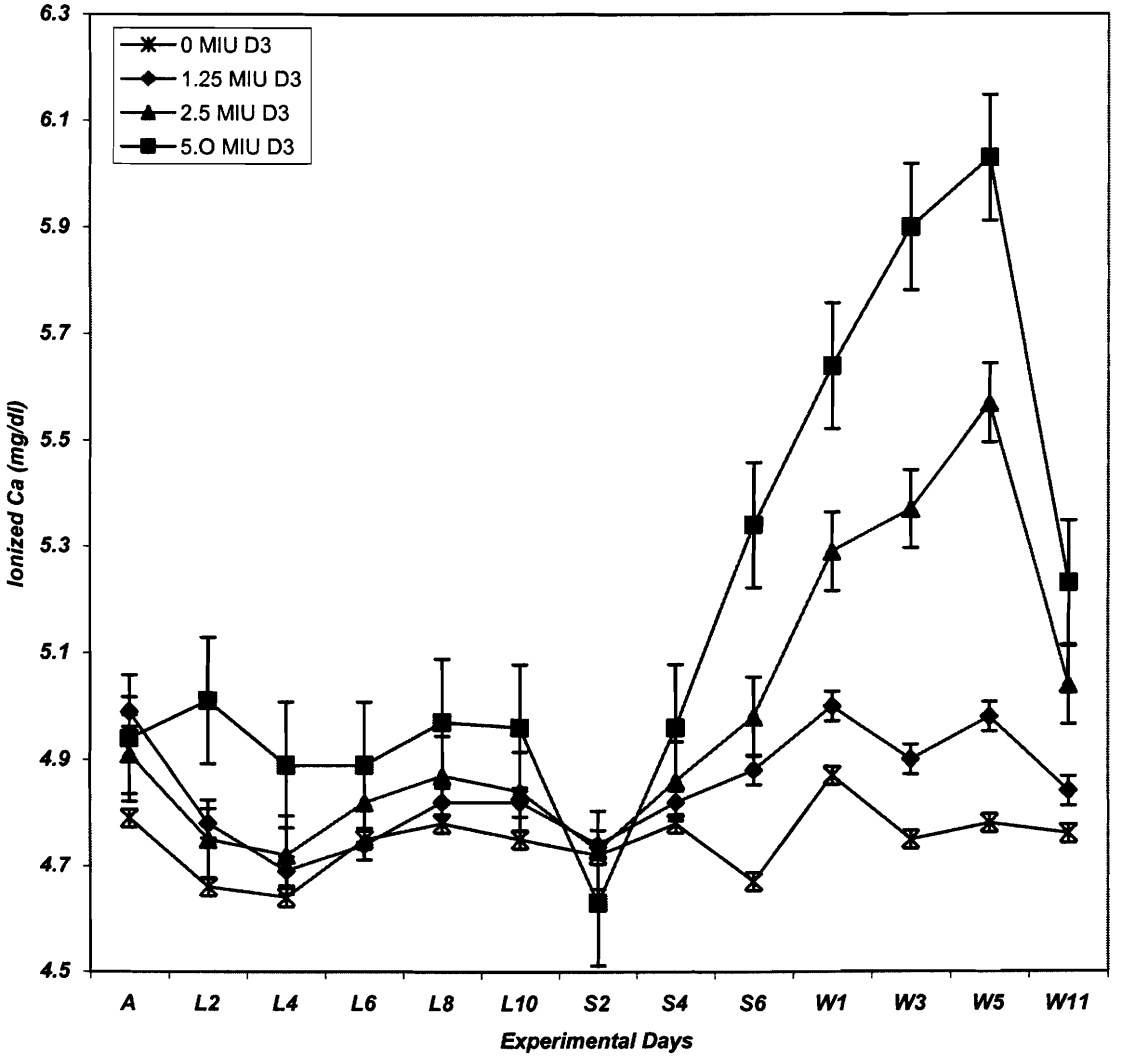

Figure 5.2. Effect of feeding a low Ca diet for ten days prior to supplementation of vitamin D_3 (0, 1.25, 2.5 and 5 MIU per day for seven consecutive days) on ionized serum Ca concentrations of finishing Angus steers (522 ± 33 kg BW), during adaptation (A), low Ca (L1 to L10), supplemental (S1 to S7) and withdrawal (W1 to W11) periods.

ionized to total Ca ratio was not altered ($P>0.05$) during the period of low Ca feeding (Table 5.5).

During the first four days of the supplemental period, total and ionized Ca concentrations were not different ($P>0.05$) between the control and supplemented steers or within levels of supplementation. However, at the end of this period (S6) total and ionized Ca from treated steers were higher ($P<0.0001$ and $P=0.0002$, respectively) than controls, and there were also differences ($P<0.05$) within levels of supplementation. A response that was in direct relationship to the amount of vitamin D_3 supplemented. The ionized to total Ca ratio was not different between treated and control steers (Tables 5.3, 5.4 and 5.5 and Figures 5.1 and 5.2).

During the withdrawal period, total and ionized Ca concentrations continued rising, and were elevated ($P<0.05$) relative to control animals, increases that followed the same pattern as in the previous period. However, these differences tended to disappear at the end of the withdrawal period (W11). The ratio of ionized to total Ca was not affected ($P>0.05$) with the exception of day W1 where it was higher ($P<0.05$) for supplemented steers (Table 5.5).

Maximum serum Ca values were achieved during the withdrawal period. Total Ca maximal concentrations were 10.65, 11.50 and 12.41 mg/dl, which represented a 6.8, 15.3 and 24.5 % increase relative to control animals for the 1.25, 2.5 and 5 MIU treatments, respectively, on day W3 (Table 5.3 and Figure 5.1). Maximal concentrations for ionized Ca were 5.00, 5.57 and 6.03 mg/dl, which represented a 2.0, 13.7 and 23.1 % increase relative to control animals for the 1.25, 2.5 and 5 MIU treatments, respectively, on day W5 (Table 5.4 and Figure 5.2).

Repeated measures analysis indicated a significant effect of treatment ($P<0.0001$ and $P=0.0024$), time ($P<0.0001$, both) and time by treatment interaction ($P<0.0001$, both) for total and ionized serum Ca levels, respectively. The nature of these interactions is illustrated in Figures 5.1 and 5.2.

The maximum serum Ca concentration of 12.41 mg/dl from the 5 MIU D_3 treatment was in good agreement with the range of results reported by other workers using the same level of supplementation and where improvements in beef tenderness

were observed (Swanek *et al.* 1997, 1999; Montgomery *et al.* 1998, 2000; Karges *et al.* 1999b, 2001).

Comparing total and ionized serum Ca concentrations obtained in this experiment with previous experiments of this study, there was found a remarkable similarity among levels of supplementation. For example, comparing study 1 with this study, total Ca: 11.67 vs 11.50 mg/dl and 12.43 vs 12.41 mg/dl for the 2.5 and 5 MIU treatments, respectively. For ionized Ca: 5.73 vs 5.57 mg/dl and 6.03 vs 6.03 mg/dl for the 2.5 and 5 MIU treatments, respectively. Additionally, the ionized to total Ca ratios were very similar among treatments and experiments, which ranged from 47.7 to 49.2 %.

The ionized to total Ca ratio during the adaptation period averaged 49.40 ± 1.81 %. This ratio is in accordance with the normal physiological relationship between ionized and total Ca of approximately 50:50 (Copp 1973; Coles 1986; Rosol *et al.* 1995). This Ca ratio was not affected ($P>0.05$) either by the low Ca diet or by vitamin D_3 supplementation, with the exception on day W1, where supplemented steers had a lower ($P<0.05$) ratio than controls, with no difference ($P<0.05$) amongst treatments (Table 5.6). No effect ($P>0.05$) was observed during the withdrawal period (Table 5.6). This indicates that any change in total serum Ca induced by the low Ca diet or by vitamin D_3 supplementation was paralleled, almost immediately by changes in ionized Ca levels.

5.3.3 Vitamin D_3 and 25-Hydroxyvitamin D_3 Concentrations

Vitamin D_3 and 25(OH)D_3 plasma concentrations from the experimental steers during the adaptation period averaged 20.76 ± 7.00 and 23.22 ± 5.74 ng/ml, respectively. The vitamin D_3 pre-treatment average value and the range (13.43 to 30.68 ng/ml) from control animals during the low Ca period were slightly higher than the normal range (1 to 6 ng/ml) reported by DeLuca (1979) and Montgomery *et al.* (2000), however, this range was in good agreement with the values (<0.2 to 20 ng/ml) reported by Endres and Rude (2001). The 25(OH)D_3 pre-treatment value and the range (15.05 to 20.24 ng/ml) from control animals were in agreement with the normal range (25 to 35 or 10 to 50 ng/ml) reported in the literature (DeLuca 1979; Endres and Rude 2001, respectively).

During the low Ca diet period, vitamin D_3 concentration was decreased ($P<0.05$) in steers fed the low Ca diet relative to those fed the control diet during the first two days

Table 5.5. Effect of feeding a low Ca diet for ten days prior to supplementation of vitamin D_3 on ionized to total serum Ca ratio of finishing Angus steers (522 ± 33 kg).

Ionized to Total Ca Ratio (%)									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum)*:									
A	49.05	50.48	49.30	48.53	0.91				
Low Ca Diet Period (*ad libitum)* [y]:									
	LCa	LCa	LCa	NCa					
L2	49.10	49.07	48.72	49.56	0.38	0.1860			
L4	48.78	48.53	49.00	49.11	0.50	0.5596			
L6	48.79	48.87	49.05	48.18	0.52	0.2348			
L8	49.23	48.89	49.49	48.83	0.66	0.6246			
L10	48.53	49.81	49.11	49.36	0.84	0.8251			
Supplemental Period (normal Ca diet; D_3 treatments fed at 85% of *ad libitum*):									
	Vitamin D_3 Level (MIU/hd/d)								
	0	1.25	2.5	5.0					
S2	49.24	48.46	47.91	46.64	0.66		0.0557	0.5202	0.1725
S4	48.24	48.19	47.36	46.79	0.53		0.2059	0.2341	0.4368
S6	47.01	46.48	45.69	46.28	0.70		0.2946	0.3899	0.5452
Withdrawal Period (normal Ca diet *ad libitum)*:									
W1	49.44	47.81	47.03	46.87	0.73		0.0194	0.4186	0.8717
W3	47.93	46.07	46.76	47.42	1.28		0.4274	0.6744	0.7054
W5	48.90	49.21	48.50	49.80	0.73		0.7488	0.4545	0.2081
W11	49.58	51.01	50.06	49.22	0.56		0.4342	0.2070	0.2849

Repeated Measures Analysis:

	P value
Treatment	0.6064
Time	<0.0001
Time x treatment	0.3259

[z] Contrasts: A = Normal Ca diet (group 4) vs low Ca diet (groups 1, 2 and 3).

B = Control animals vs vitamin D_3 treated steers

C = 1.25 vs 2.5 MIU D_3

D = 2.5 vs 5.0 MIU D_3

[y] LCa = low Ca diet; NCa = normal Ca diet

of this period. However, at the end of this period (L10), the situation was reversed (Table 5.6 and Figure 5.3). The 25(OH)D_3 concentrations were similar between treated and untreated steers, with the exception of the last day of the period (L10), where the normal Ca cattle had lower ($P<0.05$) plasma 25(OH)D_3 concentrations than the low Ca diet groups (Table 5.7 and Figure 5.4). These differences while significant were relatively small.

During the supplemental period, the supplemented animals showed an increase ($P<0.0001$) in vitamin D_3 and 25(OH)D_3 concentrations. The response was closely related to the amount of vitamin D_3 supplemented ($P<0.0001$). However, these two compounds had different response patterns. That is, the vitamin D_3 increase resembled a bell shaped curve, with the midpoint towards the end of the supplemental period. The 25(OH)D_3 levels exhibited a sustained linear increase until the beginning of the withdrawal period where plateaus were reached (Tables 5.6 and 5.7 and Figures 5.3 and 5.4).

Repeated measures analysis indicated a significant effect of treatment ($P<0.0001$), time ($P<0.0001$) and time by treatment interaction ($P<0.0001$) for plasma vitamin D_3 and 25(OH)D_3 concentrations. The nature of these effects is illustrated in Figures 5.3 and 5.4.

Maximum plasma concentrations of vitamin D_3 were 103.25, 171.59 and 352.98 ng/ml, which represented a 5-, 8.2- and 13.1-fold increment relative to control animals for the 1.25, 2.5 and 5 MIU treatments, respectively. Maximum concentrations for 25(OH)D_3, were 91.40, 160.87 and 271.62 ng/ml, which represented a 5.6-, 10.2- and 17.2-fold increase relative to control animals for the 1.25, 2.5 and 5 MIU treatments, respectively (Tables 5.6 and 5.7 and Figures 5.3 and 5.4).

Montgomery *et al.* (2000) supplemented finishing steers with 5 MIU D_3 for 9 days and found at slaughter that plasma vitamin D_3 and 25(OH)D_3 concentrations reached 464.3 and 578.4 ng/ml, respectively. In the present study the increases in plasma of these compounds for the 5 MIU treatment were lower than the values reported by Montgomery *et al.* (2000). These workers, however, used a different method of vitamin D_3 administration (intraruminal boluses). Nevertheless, the response trends were similar between the two studies.

Table 5.6. Effect of feeding a low Ca diet for ten days prior to supplementation of vitamin D_3 on plasma vitamin D_3 concentrations of finishing Angus steers (522 ± 33 kg).

Vitamin D_3 (ng/ml)									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A	10.22	22.90	22.47	26.46	1.98				
Low Ca Diet Period (*ad libitum*) [y]:									
	LCa	LCa	LCa	NCa					
L2	15.15	21.18	10.68	30.68	2.08	<0.0001			
L4	21.01	20.03	10.28	13.89	1.53	0.0848			
L6	20.88	17.92	10.27	13.43	3.22	0.4363			
L8	28.36	26.26	13.06	19.98	3.22	0.4911			
L10	29.68	22.96	23.95	13.56	2.43	0.0007			
Supplemental Period (normal Ca diet; D_3 treatments fed at 85% of *ad libitum*):									
	Vitamin D_3 Level (MIU/hd/d)								
	0	1.25	2.5	5.0					
S2	28.17	45.38	53.68	131.97	3.78	<0.0001	0.1226	<0.0001	
S4	28.79	73.77	141.44	336.17	15.14	<0.0001	0.0033	<0.0001	
S6	26.98	80.17	144.63	352.98	10.96	<0.0001	0.0004	<0.0001	
Withdrawal Period (normal Ca diet *ad libitum*):									
W1	20.79	103.25	171.59	301.25	13.77		<0.0001	0.0015	<0.0001
W3	24.25	86.61	108.20	157.11	6.85		<0.0001	0.0259	<0.0001
W5	27.62	55.87	64.67	104.38	4.56		<0.0001	0.1491	<0.0001
W11	15.22	33.75	36.57	36.52	4.47		0.0013	0.6251	0.9935
Repeated Measures Analysis:									
				P value					
Treatment				<0.0001					
Time				<0.0001					
Time x treatment				<0.0001					

[z] Contrasts: A = Normal Ca diet (group 4) vs low Ca diet (groups 1, 2 and 3).

B = Control animals vs vitamin D_3 treated steers

C = 1.25 vs 2.5 MIU D_3

D = 2.5 vs 5.0 MIU D_3

[y] LCa = low Ca diet; NCa = normal Ca diet

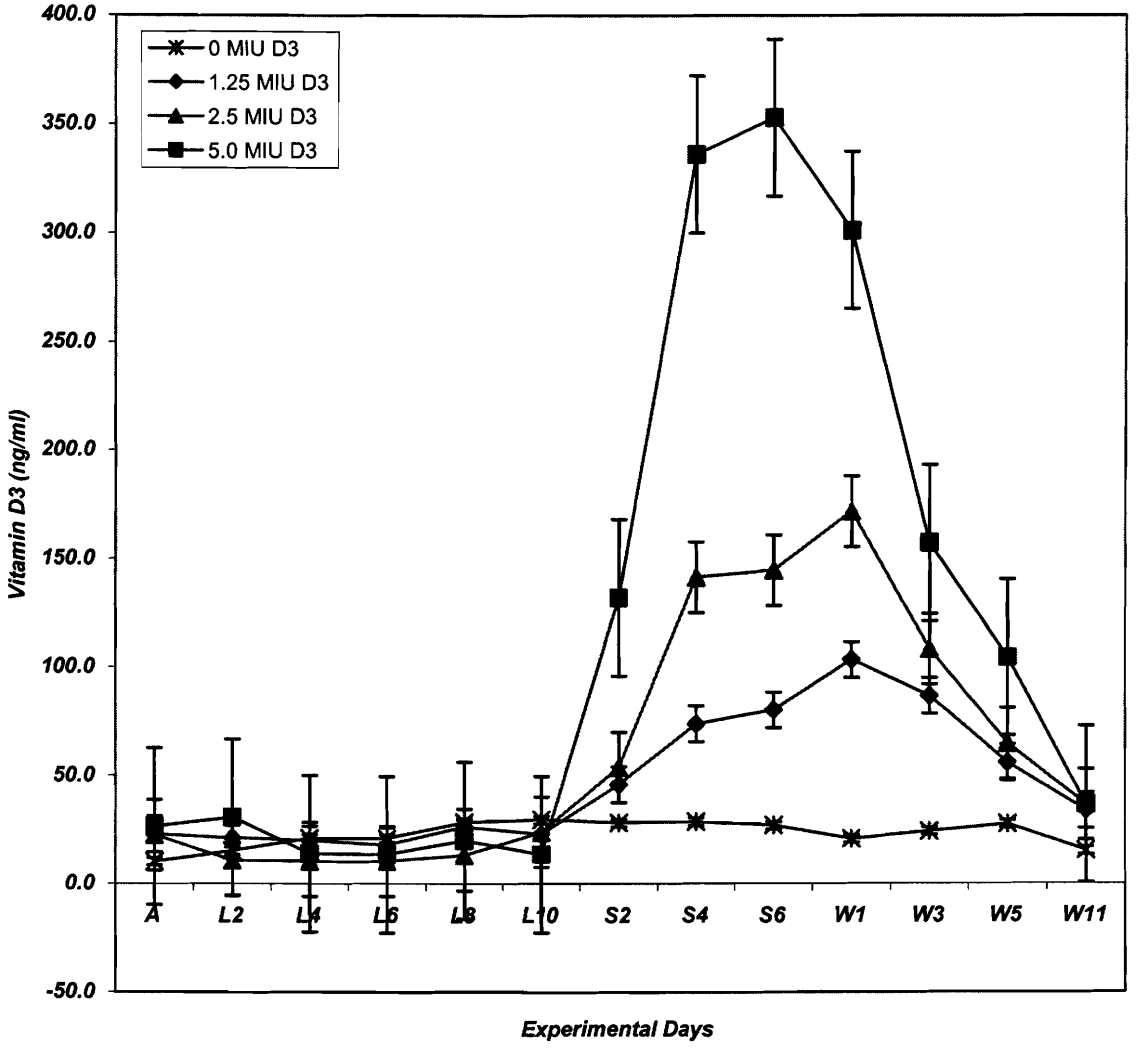

Figure 5.3. Effect of feeding a low Ca diet for ten days prior to supplementation of vitamin D_3 (0, 1.25, 2.5 and 5 MIU per day for seven consecutive days) on plasma vitamin D_3 concentrations of finishing Angus steers (522 ± 33 kg BW), during adaptation (A), low Ca (L1 to L10), supplemental (S1 to S7) and withdrawal (W1 to W11) periods.

Table 5.7. Effect of feeding a low Ca diet for ten days prior to supplementation of vitamin D_3 on circulating 25-hydroxyvitamin D_3 ($25(OH)D_3$) concentrations of finishing Angus steers (522 ± 33 kg BW).

$25(OH)D_3$ (ng/ml)									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A	18.94	19.16	30.94	22.91	1.38				
Low Ca Diet Period (*ad libitum*) [y]:									
	LCa	LCa	LCa	NCa					
L2	18.64	19.55	21.29	20.24	1.45	0.8065			
L4	18.12	18.49	21.02	17.97	1.34	0.4264			
L6	18.60	18.54	19.71	17.77	1.24	0.4191			
L8	18.16	19.01	20.45	20.22	1.69	0.6052			
L10	19.18	18.98	25.20	15.05	1.22	0.0006			
Supplemental Period (normal Ca diet; D_3 treatments fed at 85% of *ad libitum*):									
	Vitamin D_3 Level (MIU/hd/d)								
	0	1.25	2.5	5.0					
S2	18.05	30.42	36.97	53.04	2.50		<0.0001	0.0570	0.0003
S4	17.34	48.70	81.40	149.78	3.34		<0.0001	<0.0001	<0.0001
S6	16.72	75.42	117.97	218.42	3.99		<0.0001	<0.0001	<0.0001
Withdrawal Period (normal Ca diet *ad libitum*):									
W1	15.74	84.59	160.86	271.62	7.67		<0.0001	<0.0001	<0.0001
W2	16.26	91.40	148.32	262.13	10.55		<0.0001	0.0008	<0.0001
W3	15.66	81.60	151.83	235.45	11.45		<0.0001	0.0003	<0.0001
W4	17.38	75.28	128.19	171.08	6.95		<0.0001	<0.0001	0.0004
Repeated Measures Analysis:									
				P value					
Treatment				<0.0001					
Time				<0.0001					
Time x treatment				<0.0001					

[z] Contrasts: A = Normal Ca diet (group 4) vs low Ca diet (groups 1, 2 and 3).

B = Control animals vs vitamin D_3 treated steers

C = 1.25 vs 2.5 MIU D_3

D = 2.5 vs 5.0 MIU D_3

[y] LCa = low Ca diet; NCa = normal Ca diet

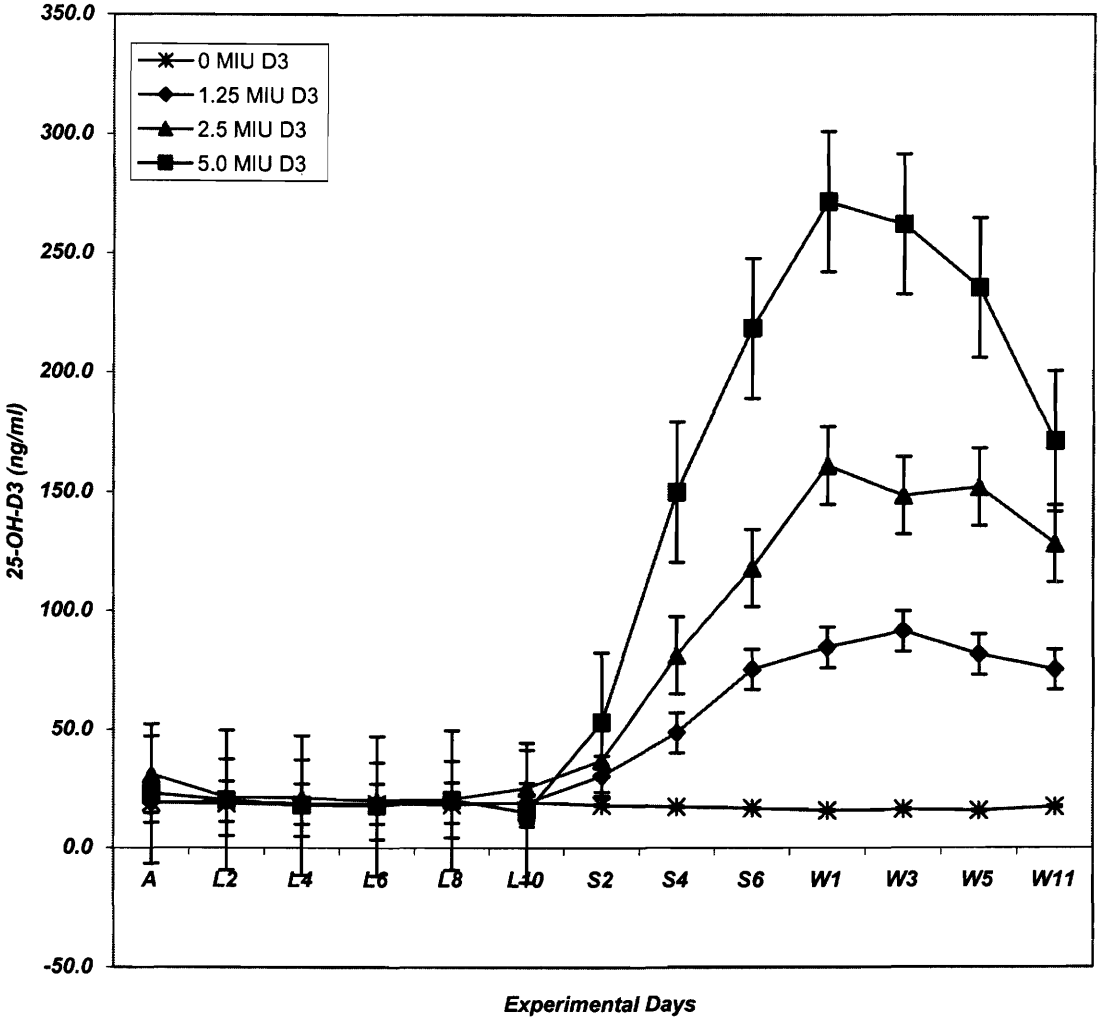

Figure 5.4. Effect of feeding a low Ca diet for ten days prior to supplementation of vitamin D_3 (0, 1.25, 2.5 and 5 MIU per day for seven consecutive days) on 25-hydroxyvitamin D_3 ($25(OH)D_3$) concentrations of finishing Angus steers (522 ± 33 kg BW), during adaptation (A), low Ca (L1 to L10), supplemental (S1 to S7) and withdrawal (W1 to W11) periods.

5.3.4 1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) Concentrations

Plasma $1,25(OH)_2D_3$ concentration was determined immediately after supplementation period ended (W1). The concentration was 26.83, 62.05, 113.99 and 148.71 pg/ml for the 0, 1.25, 2.5 and 5 MIU treatments, respectively, indicating a significant ($P<0.0001$) increase in $1,25(OH)_2D_3$ concentration with the level of vitamin D_3 supplementation (Table 5.8).

Increases in $1,25(OH)_2D_3$ concentration as a result of vitamin D_3 supplementation was to some extent unexpected. It has been pointed out that the second hydroxylation of vitamin D_3 in the kidney is a tightly regulated step, as it is controlled principally by Ca levels in plasma, by PTH, and the level of $1,25(OH)_2D_3$ itself. This control is accomplished by modulating the activity of the 1α-hydroxylase enzyme. When Ca in plasma reaches concentrations above normal, PTH is decreased and consequently the activity of this enzyme is reduced, shutting down the production of $1,25(OH)_2D_3$ (DeLuca and Schnoes 1983; Horst and Littledike 1979; Guyton and Hall 1996). In this respect, Montgomery *et al.* (2000) found no increase (20.8 vs 26.83 pg/ml) in plasma $1,25(OH)_2D_3$ as a result of vitamin D_3 supplementation (5 MIU D_3 for 9 days).

A possible explanation for the increase in $1,25(OH)_2D_3$ levels observed in this study is that high levels of vitamin D_3 supplementation induced high levels of vitamin D_3 and $25(OH)D_3$ in plasma. It is possible that high substrate concentrations, mainly $25(OH)D_3$, stimulated higher activity of the 1α-hydroxylase enzyme, by overwhelming the negative feedback. This would in turn result in higher levels of $1,25(OH)_2D_3$ which will stimulate the Ca homeostatic mechanisms. It has been observed that ruminants respond differently to administration of vitamin D_3. Intramuscular injection of 10 or more MIU of vitamin D_3 to dairy cows has resulted in elevated levels of $1,25(OH)_2D_3$ in plasma, this higher levels of $1,25(OH)_2D_3$ increase the influx of Ca to the extracellular fluid through the stimulation of Ca reabsorption, resorption and absorption from kidneys, bone and intestine, respectively, preventing the incidence of milk fever (Reinhardt and Conrad 1980; Littledike and Horst 1982; Goff *et al.* 1991). Although, no clear explanation to this phenomenon has been provided in the literature. Nevertheless, Horst and Reinhardt (1983) pointed out that ruminants appear unique in their response to large doses of vitamin D_3, suggesting that ruminants lack the tightly controlled 1α-hydroxylase

Table 5.8. Effect of feeding a low Ca diet for ten days prior to supplementation of vitamin D_3 on circulating calciotrophic hormones concentration of finishing Angus steers (522 ± 33 kg BW) on day 1 of the withdrawal period.

Calciotrophic Hormones (pg/ml)								
	Vitamin D_3 Level (MIU/hd/d)					Contrast [z]		
Day	0	1.25	2.5	5.0	*SEM*	A	B	C
Parathyroid Hormone (PTH):								
W1	74.65	29.44	12.51	5.00	6.40	<0.0001	0.0552	0.3964
1,25-Dyhydroxyvitamin D_3:								
W1	26.83	62.05	113.99	148.71	12.25	<0.0001	0.0047	0.0531
Calcitonin:								
W1	330.86	384.72	360.69	395.73	29.93	0.1671	0.5358	0.3976

[z] Contrasts:

A = Control animals (0 MIU) versus supplemented vitamin D_3 steers.

B = Treatment 1.25 versus 2.5 MIU D_3.

C = Treatment 2.5 vs 5 MIU D_3.

enzyme activity that has been observed in other species (monogastrics, including humans).

5.3.5 Parathyroid Hormone (PTH) Concentration

Plasma PTH concentrations were determined immediately after supplementation period ended (W1). PTH concentrations were 74.65, 29.44, 12.51 and 5.00 pg/ml for the 0, 1.25, 2.5 and 5 MIU treatments, respectively. There was a significant ($P<0.0001$) effect of treatment and between the lower and higher level of vitamin D_3 supplementation ($P<0.05$), although the 2.5 MIU treatment was similar ($P>0.05$) to both levels of supplementation (Table 5.8). PTH concentrations were in agreement with its theoretical response to high levels of plasma Ca. PTH is essential in protecting against hypocalcemia and in the maintenance of skeletal integrity (Rosol and Capen 1997). When Ca concentration in plasma decreases below normal values then the parathyroid glands secrete larger amounts of PTH, which in turn stimulates the hydroxylation of $25(OH)D_3$ to form the $1,25(OH)_2D_3$. PTH also acts concurrently on Ca reabsorption from kidneys, resorption from bone and Ca absorption from the intestine (Smith *et al.* 1983; Rhoades and Tanner 1995), in consequence Ca levels in plasma return normal levels (West 1991). When Ca concentrations are above normal, the opposite occurs, PTH concentration is decreased (Bhagavan 1992; Rhoades and Tanner 1995), effect that was observed in the present experiment.

5.3.6 Calcitonin Hormone Concentration

Calcitonin plasma concentrations were determined immediately after supplementation period ended (W1). The calcitonin concentrations were 330.85, 384.72, 360.69 and 395.73 pg/ml for the 0, 1.25, 2.5 and 5 MIU treatments, respectively, with no effect ($P<0.05$) of supplementation (Table 5.8).

Calcitonin concentrations in this experiment were different to the theoretical trends expected. Calcitonin functions together with the other calciotrophic hormones to maintain normal plasma Ca levels. When Ca concentrations are above normal, calcitonin is secreted immediately in order to prevent hypercalcemia (Copp 1973; Rhoades and Tanner 1995; Guyton and Hall 1997). Even though, vitamin D_3 supplementation did

increase serum Ca concentrations, calcitonin levels were not altered. As discussed in Chapter IV, Rossol *et al.* (1995) indicates that calcitonin regulatory mechanism is considered weaker than the PTH system. This may explain why calcitonin levels were not significantly affected by vitamin D_3 supplementation.

In summary, feeding steers with the low Ca diet for ten days tended to decrease feed intake, plasma vitamin D_3, and total and ionized serum Ca concentrations but only for the first one to two days of feeding. Supplementing steers with vitamin D_3 for seven days resulted in a significant depression in intake, and in PTH levels, and in elevated levels of total and ionized serum Ca, as well as in plasma vitamin D_3, $25(OH)D_3$, and $1,25(OH)_2D_3$ levels, with no effect on calcitonin levels.

Total serum Ca concentrations were increased by almost 0.5 mg/dl more (11.50 vs 11.02 mg/dl) than in the previous experiment (Chapter IV) for the 2.5 MIU treatment. It is possible to hypothesize that the increased response in the present study for the same level of vitamin D_3 supplementation was a result of the low Ca diet, which may have primed the animal to respond to vitamin D_3 supplementation. However, it is also possible that the improved response is in relation to differences in the actual consumption of vitamin D_3. In the previous experiment, the steers fed the 2.5 MIU D_3 level consumed 84 % (2.1 MIU/d) of the total amount targeted, while in the present trial the consumption was slightly higher than that targeted (2.7 MIU/d). In the previous study steers fed the 5 MIU D_3 level consumed approximately 70 % (3.4 MIU/d) of the total amount targeted, while in this experiment consumption was almost exactly on target (5.1 MIU/d).

The apparent lack of response of the low Ca diet during the feeding period or in enhancing the effect of vitamin D_3 on serum Ca concentrations may be due to the following factors. First, dairy cattle are fed low Ca diets for long periods of time (30 to 80 days); and secondly, and most importantly, low Ca diets usually do not increase plasma Ca levels during the feeding period, but they prevent sharp drops of plasma Ca levels at parturition and/or initiation of lactation, by increasing Ca resorption from bone and the efficiency of Ca absorption from the intestine. Through stimulation of the synthesis of PTH and $1,25(OH)_2D_3$, these diets actually prime the Ca homeostatic mechanisms of the animal. In this trial, feeding the low Ca diet altered the Ca homeostatic mechanisms as initial levels of total Ca and vitamin D_3 were initially lower

than normal. However, the lack of any major stress, as parturition imposes to the high-producing cow seems to neglect any advantage from the low Ca diet prior to supplementation of vitamin D_3. It does not appear that feeding a low Ca diet to finishing steers enhances the serum Ca response of the animals to vitamin D_3 feeding.

5.4 Conclusions

Feeding a low Ca diet for ten days prior to vitamin D_3 supplementation induced a decrease in feed intake, total serum Ca and plasma vitamin D_3 concentrations during the first three to four days of feeding, but had no effect on other parameters.

High levels of vitamin D_3 (1.25, 2.5 and 5 MIU/hd/d for seven consecutive days) had a significant effect on depressing feed intake and plasma PTH levels. On the other hand, it significantly increased total and ionized serum Ca, and plasma vitamin D_3, $25(OH)D_3$ and $1,25(OH)_2D_3$ concentrations. The degree of response was related to the level of vitamin D_3 supplementation. However, no effect on the ionized to total Ca ratio or in calcitonin concentrations were observed.

An interesting finding from this experiment is that supplementation with high levels of vitamin D_3 actually increases the plasma concentration of $1,25(OH)_2D_3$ despite high concentrations of total and ionized Ca in serum. This phenomenon has been also observed in dairy cows when they have been injected with high amounts of vitamin D_3. These results suggest that in ruminants the activity of the enzyme 1α-hydroxylase is not as tightly regulated as is in humans and other animals (pigs).

It seemed that the low Ca diet affected the Ca homeostatic mechanisms at least to some degree, because total Ca and vitamin D_3 concentrations in blood were lower than normal during the first days of the low Ca period. However, the lack of any considerable Ca stress such as parturition appears to counteract the potential benefits of feeding a low Ca diet to finishing steers.

In conclusion, it does not appear that feeding a low Ca diet to finishing steers enhances the serum Ca response of the animals supplemented with vitamin D_3.

CHAPTER VI

Effect of Low and High Calcium Diets, Anionic Salts and Vitamin D_3 Supplementation to Finishing Beef Steers on Calcium and Acid-Base Homeostasis

6.1 Introduction

It has been observed that feeding low calcium (Ca) diets to dairy cattle induce higher plasma Ca concentrations at and around parturition. These increased levels have helped to reduce milk fever problems (Beitz *et al.* 1973; Green *et al.* 1981; Horst *et al.* 1997). However, this effect has only been identified during the periparturient period. It is postulated that this rise in plasma Ca as a result of low Ca feeding is a response to the stress induced by the onset of lactation. In the last experiment of this series, there were no beneficial effects on serum Ca when beef steers were fed a low Ca diet either with or without vitamin D_3 supplementation, likely due to the lack of any significant stress on Ca metabolism such as the initiation of lactation.

On the other hand, the feeding of acidified diets has increased the levels of Ca in plasma, which has resulted in considerable reduction in the incidence of milk fever (Block 1984, 1994; LeClerc and Block 1989; Wang and Beede 1992). The proposed mechanism of action is that a higher proportion of dietary anions in relation to cations alters the normal acid-base status of the animal reducing the pH of the blood and consequently causing a mild acidosis. This in turn induces Ca excretion in urine (hypercalciuria). Hypercalciuria may be due to a competition between Ca^{++} and H^+ for binding sites on serum proteins (Moore 1970). Reduced Ca levels in plasma in turn stimulate the Ca homeostatic mechanisms. Parathyroid hormone (PTH) synthesis is stimulated and the responsiveness of target tissues to PTH is increased. Similarly, plasma $1,25(OH)_2D_3$ concentrations increase. As a result, the proportion of dietary Ca absorbed from intestine and the rate of Ca mobilization from bone are increased, leading to a correction in plasma Ca concentration. For this reason high Ca diets are

recommended when feeding acidified diets (Gaynor *et al.* 1989; Abu Damir *et al.* 1994; Horst *et al.* 1997; Pehrson *et al.* 1999).

The balance of the bioavailable ions that are not metabolized in the diet influences the acid-base balance in the animal (Oetzel *et al.* 1991). The dietary cation-anion balance (DCAB) is defined as the summation in milliequivalents (mEq) of the cations, sodium (Na) and potassium (K), minus the sum of the anions, chloride (Cl) and sulfur (S) per kg of dry matter or per day ([Na+K] – [Cl+S]). Thus, a positive DCAB reflects a cationic diet conversely a negative DCAB reflects an anionic diet (Block 1984; LeClerc and Block 1989).

Acidified diets (negative DCAB) have proved to be an important tool to increase plasma Ca concentration to manage milk fever problems in high producing dairy cows at and around parturition. Unfortunately, most of the research reported in the literature express the degree of acidification (DCAB) in terms of mEq/100 g of DM or mEq/kg of DM or they do not use the above formula. In some cases a derivation of it is used where for example the researcher does not take into account S or includes absorption factors for each mineral (Block 1984; Oetzel 1993). For this experiment the supplementation of anionic salts was carried out in terms of mEq/d and not per kg of DM consumed.

Anionic salts such as magnesium sulfate, calcium sulfate, ammonium sulfate, magnesium chloride, ammonium chloride and calcium chloride have been used to formulate acidogenic diets (Oetzel *et al.* 1991) with positive results in preventing milk fever (LeClerc and Block 1989; Goff *et al.* 1991; Erdman 1993). In the previous experiment of this series a low Ca diet was used prior to supplementation of vitamin D_3 to finishing steers with the objective to stimulate Ca homeostasis (i.e. increase serum Ca) and so enhance the effect of vitamin D_3 supplementation. However, this effect was not apparent, although serum Ca and plasma vitamin D_3 concentrations were decreased at the beginning of the low Ca feeding period, indicating that Ca homeostasis was altered in some degree. For this reason, the same approach was followed in this experiment. Feeding of anionic salts appears to be a viable way to pose a Ca stress, because they increase the amount of Ca excreted via urine.

Moreover, such diets also influence plasma Ca concentrations through an effect on bone Ca mobilization and intestinal Ca absorption. It is possible that a combination of

dietary Ca manipulation in conjunction with the feeding of anionic salts and vitamin D_3 may increase serum Ca values to the point where beef tenderness has been improved. In the process, if lower levels of vitamin D_3 are required to increase serum Ca concentrations, then the risk of residual vitamin D_3 in edible tissues could be reduced.

The objective of this experiment was to evaluate the effect of dietary Ca manipulation (low and high Ca diets), anionic salts ($MgSO_4$ and NH_4Cl) and low levels of vitamin D_3 on circulating concentrations of serum Ca, vitamin D_3, 25(OH) D_3, calciotrophic hormones, and in the acid-base regulatory mechanisms of finishing steers.

6.2 Materials and Methods

6.2.1 Experimental Design

An overview of the experimental design is given in Table 6.1.

6.2.1.1 Adaptation Period (days A1 to A19)

Twenty Hereford steers (448 ± 26 kg BW) were housed in individual pens (3.6 x 3.6 m) with automatic water bowls in the Livestock Research Barn of the Department of Animal and Poultry Science at the University of Saskatchewan. Animals were cared for according to the guidelines of the Canadian Council of Animal Care. The cattle were adapted to the experimental diet for 19 days. The diet consisted of 75 % concentrate (barley grain based) and 25 % barley silage (as fed) (Table 6.1). This diet was considered as the normal Ca diet and was formulated according to the Nutrient Requirements of Beef Cattle (NRC 1996), which had a total Ca concentration of 0.64 % and a Ca to P ratio of 1.3 (Enviro-Test Laboratories. Saskatoon, SK. Canada and Norwest Labs. Lethbridge, AB. Canada). The dietary cation-anion balance (DCAB) ([Na+K] – [Cl+S]) for this diet was 2.07 mEq/kg of DM (Oetzel 1993).

The steers were fed twice a day (0800 and 1600 h), with orts collected and weighed before the morning feeding. Individual maximum voluntary feed intake was measured from day A13 to A19, constituting the pre-treatment feed intake value.

Table 6.1. Experimental design.

Period	No. of Days	Type of Diet	Supplemental Anionic Salts [z]	Supplemental Vitamin D_3
Adaptation	19 (A1 to A19)	Normal Ca Diet DCAB = 2.07 mEq/kg DM Ca = 0.64 % Ca:P = 1.26		
Low Ca	14 (L1 to L14)	Low Ca Diet DCAB = 1.77 mEq/kg DM Ca = 0.16 % Ca:P = 0.31		
Supplemental	10 (S1 to S10)	High Ca Diet DCAB = 2.19 mEq/kg DM Ca = 0.84 % Ca:P = 1.68	S1 to S3: -1500 (mEq/hd/d) S4 to S10: -3000 (mEq/hd/d)	S1 to S10: 0, 0.6, 1.2 or 2.4 (MIU D_3/hd/d)
Withdrawal	5 (W1 to W5)	High Ca Diet DCAB = 2.19 mEq/kg DM Ca = 0.84 % Ca:P = 1.68		

[z] -1500 mEq/hd/d = 56.6 g of $MgSO_4$ + 56.6 g of NH_4Cl

-3000 mEq/hd/d = 113.5 g of $MgSO_4$ + 113.5 g of NH_4Cl

Table 6.2. Ingredient composition and formulated nutrient levels of the experimental diets.

	Normal Ca Diet	Low Ca diet	High Ca Diet
Item		DM (%)	
Total mixed diet:			
Concentrate	89.6	89.6	89.6
Barley silage	10.4	10.4	10.4
Concentrate:			
Barley grain	88.1	89.5	87.5
Canola meal	7.3	7.3	7.3
Tallow	1.7	1.7	1.7
Limestone	1.6	0.0	2.26
Rumensin [z]	0.4	0.5	0.4
Vitamin [y]	0.4	0.5	0.4
Salt	0.6	0.6	0.6
Chemical composition:			
Digestible energy, Mcal/kg [x]	3.47	3.52	3.45
Crude protein, %	16.14	16.35	16.04
Calcium, %	0.64	0.16	0.84
Phosphorus, %	0.51	0.51	0.50
Ca:P	1.26	0.31	1.68
DCAB mEq/kg DM [w]	2.07	1.77	2.19

[z] Premix: Monensin (20% active) mixed with barley grain.

[y] Premix: Vitamin A (416,650 IU/kg) and vitamin D (83,333 IU/kg).

[x] Predicted TDN x 4.4 Mcal/kg (Weiss *et al.* 1992).

[w] DCAB: Dietary Cation-Anion Balance: ([Na + K] – [Cl + S])(Oetzel 1993).

The experimental steers were bled by jugular venipuncture starting at 1230 h on the last day of this period (A19). Four sets of samples were taken. The first set was collected into 5ml-draw evacuated (Vacutainer®) tubes without anticoagulant. Blood was allowed to clot at room temperature for approximately 2 h, then centrifuged at 3500 rpm for 15 min. The serum obtained was immediately used to determine total and ionized Ca concentrations. The second set was collected into 10 ml-draw evacuated (Vacutainer®) tubes without anticoagulant. Samples were allowed to clot at room temperature for approximately 2 h and then centrifuged at 3000 rpm for 15 min at –4°C. The serum was collected and transferred to 1.7 ml centrifuge clear vials and stored at –20°C until analysis of parathyroid hormone, calcitonin and 1,25-dihydroxyvitamin D_3. The third set was collected into 10 ml-draw evacuated (BD Vacutainer™) tubes with anticoagulant (sodium heparin), which were placed in crushed ice immediately after withdrawal, and approximately 2 h later centrifuged at 3000 rpm for 15 min at –4°C. The plasma was collected and transferred to 1.7 ml centrifuge clear vials and stored at –20°C until analysis of vitamin D_3 and 25-hydroxyvitamin D_3 concentrations. The fourth set was collected into 2 ml syringes (Pico™ 50; Radiometer, Copenhagen, Denmark), which were placed on crushed ice immediately after extraction and within an hour analyzed for pH, partial pressure of carbon dioxide (pCO_2), total carbon dioxide (tCO_2), partial pressure of oxygen (pO_2), bicarbonate (HCO_3^-), base excess (BE), hemoglobin (tHb), sodium (Na), potassium (K), chloride (Cl) and anion gap (AG) using a blood gas analyzer (Radiometer 288 System, Copenhagen, Denmark).

6.2.1.2 Low Ca Diet Period (days L1 to L14)

The steers were randomly divided into four groups (five steers per group) and fed with a low Ca diet for fourteen days. The low Ca diet was similar in composition to the basal experimental diet but the source of additional Ca (limestone) was withdrawn (Table 6.1). The chemical analysis (Enviro-Test Laboratories. Saskatoon, SK. Canada and Norwest Labs. Lethbridge, AB. Canada) indicated that the complete ration had a total Ca concentration of 0.16 % and a Ca to P ratio of 0.31. The DCAB for this diet was 1.77 mEq/kg of DM (Oetzel 1993). The feeding pattern and the monitoring of the feed intake were the same as in the previous period.

Blood samples from all the steers were obtained on days L7 and L14, which were collected, processed, stored and analyzed as described for the adaptation period.

6.2.1.3 Supplemental Period (days S1 to S10)

Immediately after the low Ca diet period ended, all four groups of steers were switched to a high Ca diet. The high Ca diet was similar in composition to the basal experimental diet but with additional amount of Ca (limestone) (Table 6.1). The chemical analysis (Enviro-Test Laboratories. Saskatoon, SK. Canada and Norwest Labs. Lethbridge, AB. Canada) indicated that the complete ration had a total Ca concentration of 0.84 % and a Ca to P ratio of 1.68.

The DCAB for this diet was 2.19 mEq/kg of DM (Oetzel 1993; Norwest Labs. Lethbridge, AB. Canada). The feeding pattern and the monitoring of feed intake were the same as in the previous period. During this period, all four groups of steers were supplemented with anionic salts, including magnesium sulfate ($MgSO_4$) and ammonium chloride (NH_4Cl). The addition of these salts was such that the DCAB was decreased to –1500 mEq/hd/d. This was accomplished by adding to the ration 56.57 g of $MgSO_4$ and 56.57 g of NH_4Cl per steer per day for three days (S1 to S3). For the remaining seven days of the supplemental period (S4 to S10) the DCAB was reduced to –3000 mEq/hd/d by adding to the ration 113.5 g of $MgSO_4$ and 113.5 g of NH_4Cl per steer per day. The total amount of these minerals were equally divided and offered at each feeding by top-dressing the ration and then mixing it by hand into the feed bunk. Additionally, thiamin (50 mg/hd/d) was added to the diet in the morning feeding to prevent any negative effect (e.g. polyencephalomalacia) as a consequence of sulfur excess.

Groups 1, 2, 3 and 4 were assigned to the following levels of supplemental vitamin D_3: 0, 0.6, 1.2 and 2.4 MIU per steer per day for 10 consecutive days, respectively.

The vitamin D_3 supplement was prepared from a vitamin D_3 concentrate containing 518,000 IU per gram (Aventis Animal Nutrition Canada Inc. Campbellsville, Ontario), using ground barley as a carrier. This supplement was formulated to have a final concentration of 13 MIU D_3 per kg of premix. Analysis of the supplement indicated an actual concentration of 12.66 ± 0.21 MIU D_3 per kg (CN Laboratories. Courtland,

MN. USA). The daily total amount of vitamin D_3 per steer was equally divided and supplied at each feeding.

Feed intake was restricted to 85% relative to the average consumption from the last 7 days of the low Ca period in all the steers, and additional daily adjustments were carried out to avoid excessive feed refusals and assure maximal consumption of anionic salts and vitamin D_3. Feeding times and feed intake monitoring were as described in previous periods.

Blood samples from all the steers were obtained on days S1, S3, S5, S7, and S9. These blood samples were collected, processed, stored and analyzed as described in the adaptation period.

6.2.1.4 Withdrawal Period (days W1-W5)

Once anionic salt and vitamin D_3 supplementation ended, the steers were maintained for a further 5 days under the same experimental conditions to evaluate post-treatment effects. The steers were fed with the high Ca diet *ad libitum*. Feeding pattern and feed intake monitoring were as described in previous periods.

Blood samples from all the steers were obtained on days W1 and W5. These blood samples were collected, processed, stored and analyzed as described in the adaptation period.

6.2.2 Laboratory Analyses

6.2.2.1 Total and Ionized Ca Determinations

Concentrations of total and ionized Ca in serum were determined by indirect potentiometry utilizing a calcium ion selective electrode as described in Chapter III.

6.2.2.2 Parathyroid Hormone (PTH) Determination

Concentrations of PTH in serum were determined by immunoradiometric assay as described in Chapter V. Further details of the assay are presented in Appendix A.

6.2.2.3 Calcitonin Hormone Determination

Concentrations of calcitonin in serum determined by a double–antibody radio-immunoassay as described in Chapter IV. Further details of the assay are presented in Appendix B.

6.2.2.4 Vitamin D_3 and 25-Hydroxyvitamin D_3 ($25(OH)D_3$) Determinations

Concentrations of vitamin D_3 and $25(OH)D_3$ in plasma were determined by reverse-phase high-performance liquid chromatography (HPLC) as described in Chapter V. Further details of the assay are presented in Appendix C.

6.2.2.5 1,25-Dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) Determination

Concentrations of $1,25(OH)_2D_3$ in serum were determined by radioimmunoassay as described in Chapter V. Further details of the assay are presented in Appendix D.

6.2.2.6 Blood Gas Analysis

Blood gas analysis was carried out by using the Radiometer 288 System (Radiometer 288 System, Copenhagen, Denmark). The analysis included pH, partial pressure of carbon dioxide (pCO_2), total carbon dioxide (tCO_2), partial pressure of oxygen (pO_2), bicarbonate (HCO_3^-), base excess (BE), hemoglobin (tHb), sodium (Na), potassium (K), chloride (Cl) and anion gap (AG). The description for each analysis is presented in Appendix E.

6.2.3 Statistical Analysis

The data from feed intake and blood sample analyses were analyzed using the Analysis of Variance Technique including Repeated Measures Analysis using the General Lineal Model procedure of SAS (SAS 1989) to compare treatments within days and over time.

The following model was used for the repeated measures analysis:

Model: $Y_{ijk} = \mu + \alpha_i + \tau_{ij} + \delta_k + (\alpha\delta)_{ij} + \varepsilon_{ijk}$

where:

Y_{ijk} = is the response at time k on animal j in treatment i.

μ = is the overall mean.

α_i = is a fixed effect of treatment i.

τ_{ij} = is a random effect of animal j in treatment i.

δ_k = is a fixed effect of time k.

$(\alpha\delta)_{ij}$ = is a fixed interaction effect of treatment i with time k.

ε_{ijk} = is the random error at time k on animal j in treatment i.

Single degree of freedom contrasts:

A = Pre-treatment control value versus low Ca groups.

B = Control animals versus vitamin D_3 supplemented steers.

C = 0.6 versus 1.2 MIU D_3 treatments.

D = 1.2 versus 2.4 MIU D_3 treatments.

6.3 Results

6.3.1 Feed Intake (DMI)

Feed intake during the adaptation period averaged 9.93 ± 1.16 kg DM. Feed intake was not affected ($P<0.05$) during the fourteen days of feeding the low Ca diet. During the supplemental period the high Ca diet (fed at 85 % of *ad libitum*) was supplemented with anionic salts at -1500 and -3000 mEq/hd/d for 3 (S1-S3) and 7 (S4-S10) days, respectively, and vitamin D_3 (0, 0.6, 1.2 or 2.4 MIU/hd/d). Anionic salts did not decrease ($P<0.05$) feed intake of control animals (group 1) during the first seven days of supplementation. However, feed intake was decreased ($P<0.05$) during the last 3 days of this period. Feed intake of the group 1 steers was depressed by 26.1 % on day S10 relative to pre-supplementation of anionic salts (Table 6.3).

Feed intake was not different ($P>0.05$) between anionic and vitamin D_3 treated steers or between levels of supplemental vitamin D_3 from days S1 to S3. However, it started to decrease from day S4, reaching minimum values toward the end of this period: 6.19, 5.57, 3.84 and 4.04 kg which represented a decrease, relative to their intakes on day S4 (initiation of -3000 mEq/d) of approximately 26, 34, 54 and 49 % for the 0, 0.6, 1.2 and 2.4 MIU treatments, respectively (Table 6.3). The pattern of decline was similar for

Table 6.3. The effects of anionic salts and vitamin D_3 supplementation on feed intake (DMI) of finishing Hereford steers (448 ± 26 kg BW).

DMI (kg)									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A19	9.82	10.10	10.17	9.63	0.56				
Low Ca Diet Period (low Ca diet *ad libitum*):									
L14	9.82	10.44	9.94	9.62	0.58				
Supplemental Period (high Ca diet; 85% of *ad libitum*) [y]:									
	Vitamin D_3 Level (MIU/hd/d)								
	0	0.6	1.2	2.4					
-1500 mEq/d:									
S1	8.36	8.79	8.58	7.99	0.42	0.8922	0.8496	0.7205	0.3348
S2	8.42	8.84	8.33	8.04	0.47	0.9583	0.9741	0.4525	0.6740
S3	8.37	8.55	8.23	8.05	0.49	0.9007	0.8723	0.6492	0.7988
-3000 mEq/d:									
S4	8.38	8.45	8.29	7.93	0.50	0.9113	0.7949	0.8218	0.6217
S5	8.06	7.79	7.60	6.89	0.55	0.5909	0.3355	0.8125	0.3729
S6	7.76	7.18	6.93	6.50	0.63	0.3473	0.2408	0.7783	0.6386
S7	7.37	6.74	6.65	5.71	0.68	0.1468	0.2237	0.9255	0.3484
S8	6.34	6.37	5.35	4.54	0.86	0.0059	0.3696	0.4162	0.5179
S9	6.31	6.20	4.06	4.10	0.96	0.0053	0.1896	0.1342	0.9756
S10	6.19	5.57	3.84	4.04	0.88	0.0033	0.1124	0.1844	0.8793
Withdrawal Period (high Ca diet *ad libitum*):									
W1	7.77	7.30	6.47	5.57	0.72		0.1289	0.4272	0.3857
W2	8.67	8.41	4.23	4.57	1.25		0.0601	0.0312	0.8469
W3	8.94	8.57	5.86	5.06	1.08		0.0694	0.0963	0.6082
W4	9.06	8.95	6.91	5.43	1.04		0.1216	0.1839	0.3306

[z] Contrasts: **A** = Average during the low Ca period (85% restriction= 8.46 kg) versus anionic salts (group 1) / **B** = Control vs vitamin D_3 treatments / **C** = 0.6 vs 1.2 MIU D_3 / **D** =1.2 vs 2.4 MIU D_3

[y] High Ca diet with a DCAB = 2.19 mEq/d.

all groups of steers. From days S7 to S10 there was a difference ($P<0.05$) between control steers (group 1) and vitamin D_3 supplemented steers, although, there were no differences ($P>0.05$) among vitamin D_3 groups. Feed intake tended to be decreased in an inverse relationship to the amount of vitamin D_3 supplemented. Once supplementation ended feed intake for all steers began to recover.

The DCAB of the ration consumed by the steers was a function of the DCAB content of the high Ca diet (2.19 mEq/kg DM), the level of anionic salts supplemented (-1500 or –3000 mEq/hd/d), and the actual DMI of the steers. For example, if a steer consumed 10 kg of DM per day of the high Ca diet, the DCAB would be 21.9 mEq/d. Anionic salt supplementation at –1500 mEq/hd/d (hand mixing), results in DCAB of –1478 mEq/hd/d with a DMI of 10 kg. Thus, the DCAB of the steers (all groups) during the first three days of the supplemental period averaged –1472 ± 46.1 mEq/hd/d. However, when the supplementation of anionic salts was increased to –3000 mEq/hd/d, the DCAB ranged from -2936 to -2478 mEq/hd/d from days S4 to S10, with no difference ($P>0.05$) among groups.

The average consumption of the vitamin D_3 supplement was 94.3 % (0.55 MIU/d), 87.8 % (1.0 MIU/d) and 88.9 % (2.1 MIU/d) of the targeted intake for the 0.6, 1.2 and 2.4 MIU/hd/d treatments, respectively.

6.3.2 Total and Ionized Ca Concentrations

Total and ionized serum Ca concentrations during the adaptation period averaged 9.81 ± 0.34 and 4.99 ± 0.19 mg/dl, respectively. These averages were not affected ($P>0.05$) by feeding the low Ca diet, with the exception of group 1 and 4 that had lower ($P<0.05$) ionized and total Ca concentrations on day L14, respectively. In order to know the effect of anionic salts on serum Ca the average concentrations from the low Ca period were contrasted with the measurements from the steers supplemented with anionic salts only. Anionic salts increased ($P<0.05$) total Ca and ionized Ca concentrations during the supplemental and withdrawal periods, with the exception of day S1 (total Ca) and W5 (ionized Ca) (Tables 6.4 and 6.5 and Figures 6.1 and 6.2).

Table 6.4. The effects of anionic salts and vitamin D_3 supplementation on total serum Ca concentrations of finishing Hereford steers (448 ± 26 kg BW).

Total Ca (mg/dl)									
	Group					Contrast [z]			
Days	1	2	3	4	SEM	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A19	9.78	9.83	9.78	9.86	0.17				
Low Ca Diet Period (low Ca diet *ad libitum*) [y]:									
L7	9.70	9.79	9.58	9.56	0.10				
L14	9.64	9.81	9.68	9.46	0.10				
Supplemental Period (high Ca diet; 85% of *ad libitum*) [x]:									
	Vitamin D_3 Level (MIU/hd/d)								
	0	0.6	1.2	2.4					
- 1500 mEq/d:									
S1	9.57	9.84	9.70	9.52	0.11	0.5293	0.3407	0.3770	0.2367
S3	9.93	10.12	10.21	10.07	0.14	0.0341	0.2061	0.6509	0.4863
- 3000 mEq/d:									
S5	10.04	10.52	10.79	10.80	0.22	0.0037	0.0177	0.3896	0.9796
S7	10.11	10.70	11.43	11.35	0.27	0.0007	0.0042	0.0743	0.8382
S9	9.98	10.83	11.25	11.73	0.38	0.0138	0.0098	0.4527	0.3876
Withdrawal Period (high Ca diet *ad libitum*):									
W1	9.89	10.97	11.07	12.24	0.41	0.0677	0.0054	0.8613	0.0635
W5	9.94	10.55	10.81	11.51	0.37	0.0254	0.0322	0.6350	0.2020
Repeated Measures Analysis:									
				P value					
Treatment				0.0167					
Time				<0.0001					
Time x treatment				<0.0001					

[z] Contrasts: **A** = Average low Ca period vs anionic salts (group1) / **B** = Control vs vitamin D_3 treatments / **C** = 0.6 vs 1.2 MIU D_3 / **D** =1.2 vs 2.4 MIU D_3

[y] Comparison: Average adaptation period vs low Ca period

(Only group 4 had lower ($P<0.05$) concentration on day L14).

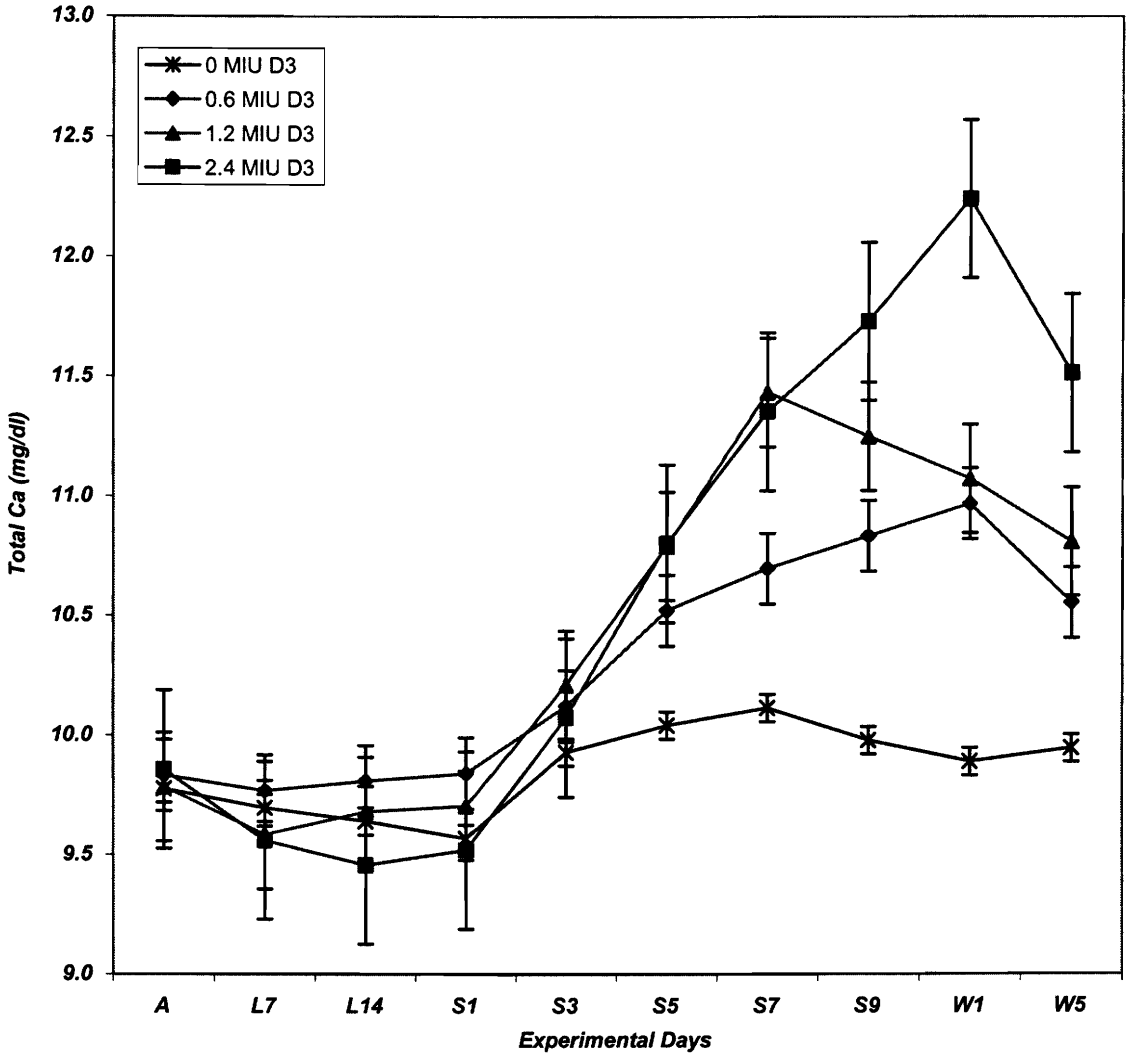

Figure 6.1. Effect of feeding a low Ca diet for 14 days prior to supplementation of anionic salts (-1500 mEq/hd/d from S1-S3 and -3000 mEq/hd/d from S4-S10) and vitamin D_3 (0, 0.6, 1.2 and 2.4 MIU/hd/d) to a high Ca diet for 10 days on total serum Ca concentrations of finishing Hereford steers (448 ± 26 kg)(*A=adaptation; L=low Ca; S=supplemental; and W=withdrawal periods*).

Table 6.5. The effect of anionic salts and vitamin D_3 supplementation on ionized serum Ca concentrations of finishing Hereford steers (448 ± 26 kg BW).

Ionized Ca (mg/dl)									
	Group					Contrast [z]			
Days	1	2	3	4	SEM	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A19	4.93	4.98	4.99	5.06	0.09				
Low Ca Diet Period (low Ca diet *ad libitum*) [y]:									
L7	4.94	4.93	4.83	4.94	0.05				
L14	4.81	4.95	4.88	4.82	0.05				
Supplemental Period (high Ca diet; 85% of *ad libitum)* [x]:									
	Vitamin D_3 Level (MIU/hd/d)								
	0	0.6	1.2	2.4					
- 1500 mEq/d:									
S1	4.85	4.91	4.86	4.88	0.04	0.5430	0.4775	0.4563	0.8023
S3	5.02	5.10	5.21	5.17	0.06	0.0494	0.0632	0.2305	0.6381
- 3000 mEq/d:									
S5	5.26	5.42	5.53	5.54	0.11	<0.0001	0.0672	0.4653	0.9162
S7	5.40	5.55	5.97	5.95	0.14	<0.0001	0.0179	0.0506	0.9362
S9	5.43	5.78	5.98	6.21	0.20	<0.0001	0.0265	0.4864	0.4366
Withdrawal Period (high Ca diet *ad libitum*):									
W1	5.36	5.90	5.91	6.52	0.21	<0.0001	0.0079	0.9793	0.0625
W5	4.95	5.14	5.39	5.76	0.21	0.3512	0.0673	0.4199	0.2370
Repeated Measures Analysis:									
				P value					
Treatment				0.0197					
Time				<0.0001					
Time x treatment				0.0037					

[z] Contrasts: **A** = Average low Ca period vs anionic salts (group1) / **B** = Control vs vitamin D_3 treatments / **C** = 0.6 vs 1.2 MIU D_3 / **D** =1.2 vs 2.4 MIU D_3

[y] Comparison: Average adaptation period vs low Ca period

(Only group 1 had lower ($P<0.05$) concentration on day L14).

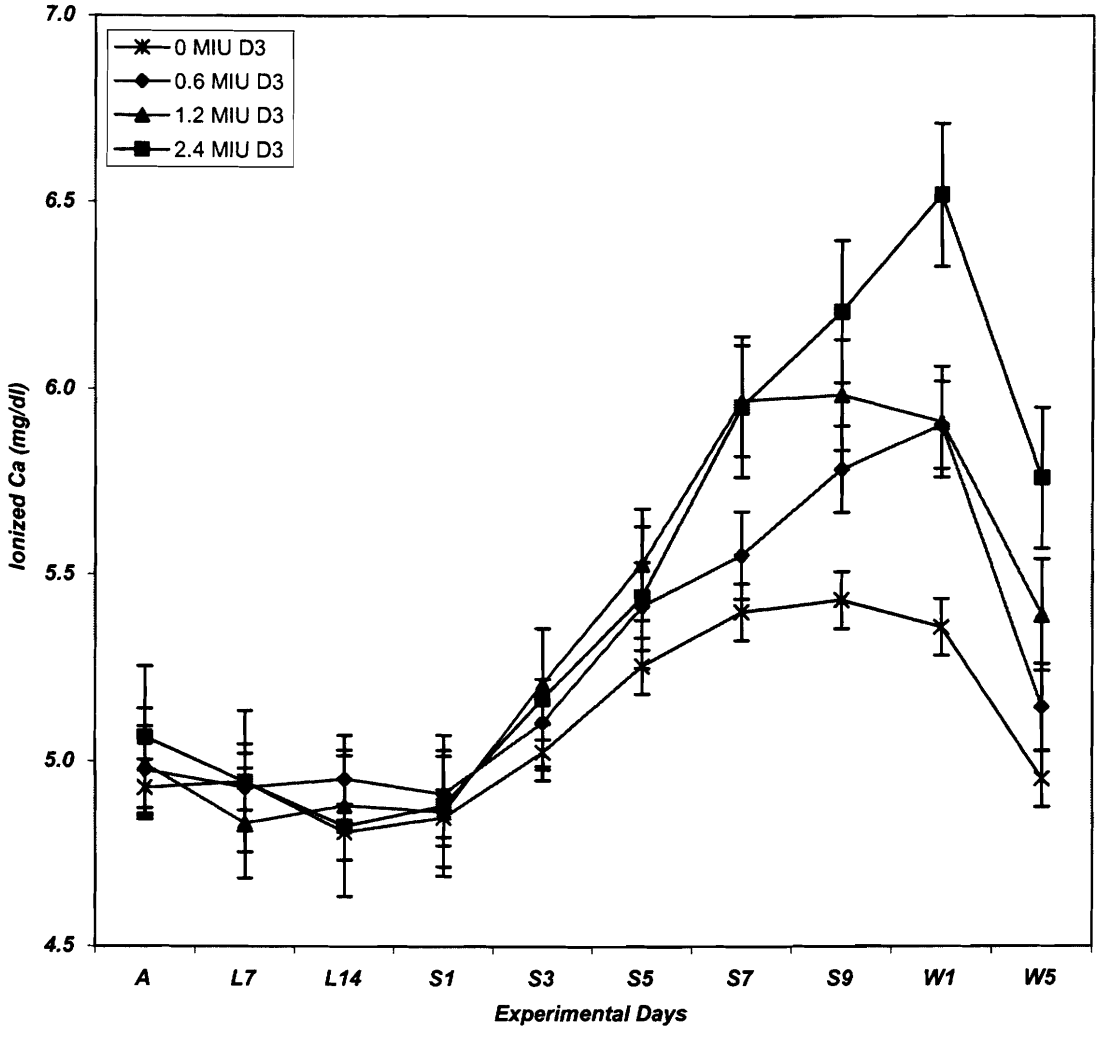

Figure 6.2. Effect of feeding a low Ca diet for 14 days prior to supplementation of anionic salts (-1500 mEq/hd/d from S1-S3 and -3000 mEq/hd/d from S4-S10) and vitamin D_3 (0, 0.6, 1.2 and 2.4 MIU/hd/d) to a high Ca diet for 10 days on ionized serum Ca concentrations of finishing Hereford steers (448 ± 26 kg)(*A=adaptation; L=low Ca; S=supplemental; and W=withdrawal periods*).

Total and ionized Ca upon supplementation (-1500 mEq/d and vitamin D_3) began to increase ($P<0.05$), with no difference ($P>0.05$) between the control and vitamin D_3 treated animals (Figures 6.1 and 6.2). From day S4 to S10 (-3000 mEq/d and vitamin D_3) total and ionized Ca continued rising ($P<0.0001$) and a clear differentiation ($P<0.05$) between control and the vitamin D_3 treatments was found, but not within levels of vitamin D_3 ($P>0.05$) (Tables 6.4 and 6.5 and Figures 6.1 and 6.2).

Maximum total Ca concentrations achieved were 10.11 (S7), 10.97 (W1), 11.43 (S7) and 12.24 (W1) mg/dl for the 0, 0.6, 1.2 and 2.4 MIU D_3 treatments, respectively. This represents a 3.1, 11.8, 16.5 and 24.8 % increase relative to the pre-treatment control value, respectively (Table 6.4 and Figure 6.1). Maximum ionized Ca concentrations were 5.43 (S7), 5.90 (W1), 5.98 (S7) and 6.25 (W1) mg/dl for the 0, 0.6, 1.2 and 2.4 MIU D_3 treatments, respectively. This represents an 8.8, 18.2 19.8 and 30.7 % increase relative to the pre-treatment control value, respectively (Table 6.5 and Figure 6.2).

Ionized to total Ca ratio during the adaptation period averaged 50.86 ± 1.06 %. This average was not altered ($P>0.05$) by the low Ca diet. Anionic salt supplementation at the level of -1500 mEq/d had no effect ($P>0.05$) on this ratio. However, at the level of -3000 mEq/d, the ionized to total Ca ratio was increased ($P<0.0001$), an effect that continued until the first day of the withdrawal period. There was no difference ($P>0.05$) between the anionic salt group and the vitamin D_3 treatments, with the exception ($P<0.05$) of days S5 and S7. There was no difference ($P>0.05$) among the vitamin D_3 treatments. After day W1, the Ca ratio dropped in all groups, attaining a minimum value that was lower ($P<0.05$) than in the adaptation period (Table 6.6).

6.3.3 Vitamin D_3 and 25-Hydroxyvitamin D_3 (25(OH)D_3)

Vitamin D_3 plasma concentrations during the adaptation period averaged 16.81 ± 5.04 ng/ml. The low Ca diet decreased ($P<0.05$) vitamin D_3 plasma concentration in all groups on day L7, however, concentrations returned to normal by day L14. Anionic salts at –1500 mEq/d decreased ($P<0.05$) and at –3000 mEq/d had no effect ($P>0.05$) on plasma vitamin D_3 concentrations. However, during the withdrawal period, concentrations of vitamin D_3 were higher ($P<0.0001$) in cattle that were fed the anionic salts relative to values for cattle fed the low Ca diet (Table 6.7 and Figure 6.3).

Table 6.6. The effect of anionic salts and vitamin D_3 supplementation on ionized to total Ca ratio of finishing Hereford steers (448 ± 26 kg BW).

Ionized to Total Ca Ratio (%)									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A19	50.42	50.62	51.02	51.39	0.48				
Low Ca Diet Period (low Ca diet *ad libitum*) [y]:									
L7	50.99	50.46	50.42	51.74	0.40				
L14	49.88	50.50	50.42	51.03	0.42				
Supplemental Period (high Ca diet; 85% of *ad libitum*) [x]:									
	Vitamin D_3 Level (MIU/hd/d)								
	0	0.6	1.2	2.4					
- 1500 mEq/d:									
S1	50.70	49.92	50.13	51.28	0.46	0.9616	0.6284	0.7487	0.0966
S3	50.61	50.43	51.03	51.33	0.32	0.8975	0.4081	0.2109	0.5358
- 3000 mEq/d:									
S5	52.35	51.49	51.24	51.35	0.40	0.0018	0.0489	0.6699	0.8470
S7	53.40	51.90	52.27	52.40	0.39	<0.0001	0.0167	0.5158	0.8229
S9	54.46	53.43	53.15	53.02	0.62	<0.0001	0.0998	0.7594	0.8826
Withdrawal Period (high Ca diet *ad libitum*):									
W1	54.23	53.86	53.37	53.34	0.74	<0.0001	0.4239	0.6453	0.9760
W5	49.79	48.76	49.92	49.93	0.55	0.0857	0.6961	0.1552	0.9859

Repeated Measures Analysis:

	P value
Treatment	0.1938
Time	<0.0001
Time x treatment	0.5696

[z] Contrasts: **A** = Average low Ca period vs anionic salts (group1) / **B** = Control vs vitamin D_3 treatments / **C** = 0.6 vs 1.2 MIU D_3 treatment / **D** =1.2 vs 2.4 MIU D_3 treatment.

[y] Comparison: Average adaptation period vs low Ca period

(No difference ($P<0.05$) among groups).

Table 6.7. The effect of anionic salts and vitamin D_3 supplementation on plasma vitamin D_3 concentrations of finishing Hereford steers (448 ± 26 kg BW).

Vitamin D_3 (ng/ml)									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A19	13.17	15.04	20.54	18.48	1.99				
Low Ca Diet Period (low Ca diet *ad libitum*) [y]:									
L7	9.60	12.08	10.87	8.96	1.49				
L14	15.24	17.64	16.45	19.49	2.15				
Supplemental Period (high Ca diet; 85% of *ad libitum*) [x]:									
	Vitamin D_3 Level (MIU/hd/d)								
	0	0.6	1.2	2.4					
- 1500 mEq/d:									
S1	11.03	12.29	15.38	14.74	1.74	0.0215	0.1424	0.2280	0.7976
S3	9.05	39.77	53.02	79.15	4.14	0.0029	<0.0001	0.0381	0.0004
- 3000 mEq/d:									
S5	19.21	46.61	78.24	122.55	6.85	0.4423	<0.0001	0.0049	0.0003
S7	11.99	38.47	70.91	118.83	5.72	0.0502	<0.0001	0.0010	<0.0001
S9	16.43	37.87	64.23	81.16	3.63	0.7658	<0.0001	0.0001	0.0046
Withdrawal Period (high Ca diet *ad libitum*):									
W1	24.56	50.13	44.59	82.81	4.44	0.0006	<0.0001	0.3913	<0.0001
W5	28.31	36.49	32.90	48.35	2.41	<0.0001	0.0012	0.3068	0.0003
Repeated Measures Analysis:									
				P value					
Treatment				<0.0001					
Time				<0.0001					
Time x treatment				<0.0001					

[z] Contrasts: **A** = Average low Ca period vs anionic salts (group1) / **B** = Control vs vitamin D_3 treatments / **C** = 0.6 vs 1.2 MIU D_3 treatment / **D** =1.2 vs 2.4 MIU D_3 treatment.

[y] Comparison: Average adaptation period vs low Ca period

(All the four groups had lower ($P<0.05$) concentration on day L7).

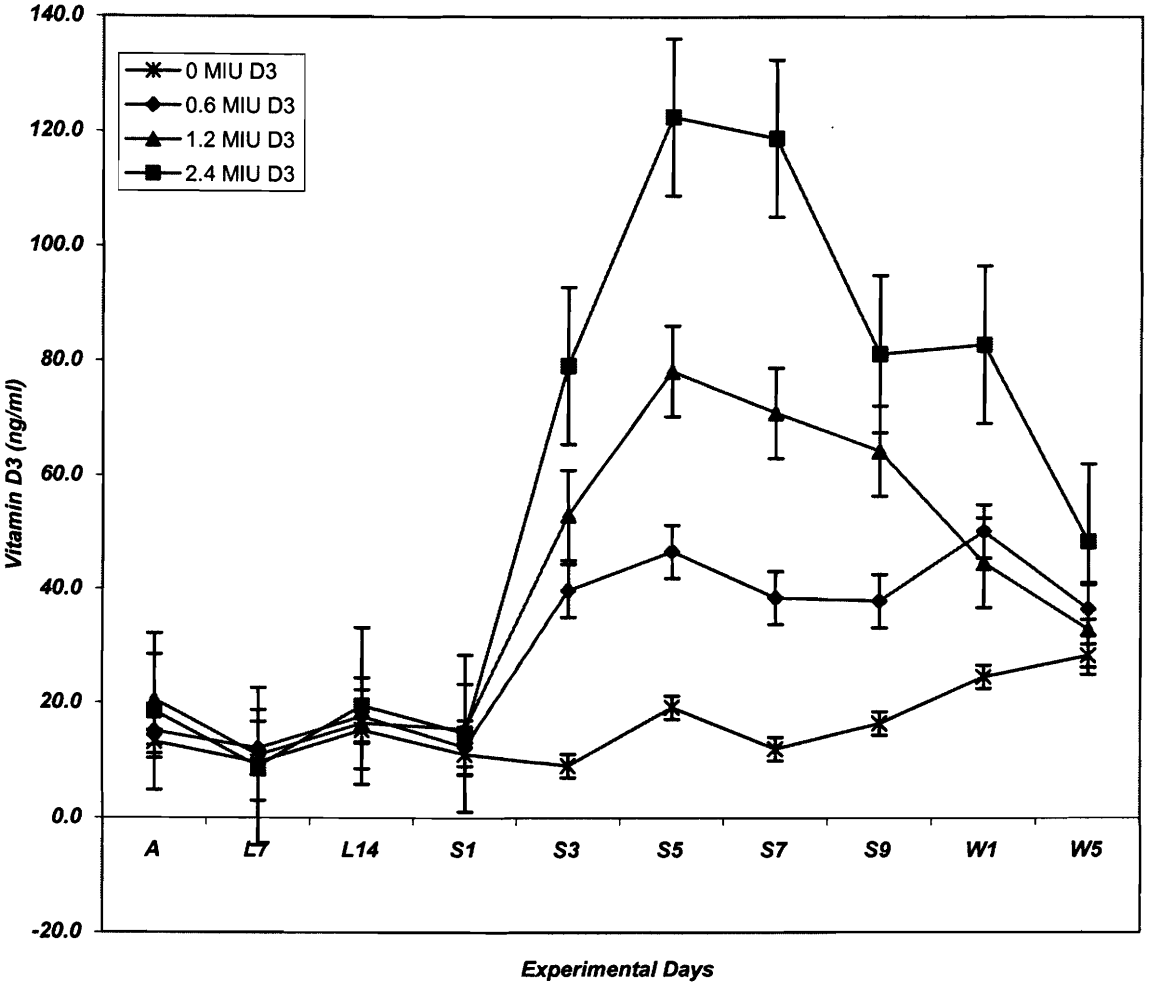

Figure 6.3. Effect of feeding a low Ca diet for 14 days prior to supplementation of anionic salts (-1500 mEq/hd/d from S1-S3 and -3000 mEq/hd/d from S4-S10) and vitamin D_3 (0, 0.6, 1.2 and 2.4 MIU/hd/d) to a high Ca diet for 10 days on plasma vitamin D_3 concentrations of finishing Hereford steers (448 ± 26 kg)(*A=adaptation; L=low Ca; S=supplemental; and W=withdrawal periods*).

Plasma concentrations of vitamin D_3 were not different ($P>0.05$) between steers supplemented with anionic salts or in combination with vitamin D_3, irrespective of the level of vitamin D_3 fed, on day S1. However, plasma vitamin D_3 concentrations increased ($P<0.0001$) in steers supplemented with vitamin D_3 from day S3 to W5, with clear differentiation ($P<0.05$) among vitamin D_3 treatments (Table 6.7 and Figure 6.3).

Plasma vitamin D_3 concentration response curves described a bell shaped curve, with the degree of response directly related to the level of vitamin D_3 supplemented. The response was observed almost immediately after supplementation initiated and returned almost to normal by the end of the withdrawal period. Maximum plasma vitamin D_3 concentrations were 28.31 (W5), 50.13 (W1), 78.24 (S5) and 122.55 (S5) ng/ml, which represented a 1.7-, 3.0-, 4.6- and 7.3-fold increase relative to pretreatment values for the 0, 0.6, 1.2 and 2.4 MIU treatments (Table 7.6 and Figure 6.3).

Plasma 25(OH)D_3 concentrations during the adaptation period averaged 20.77 ± 3.39 ng/ml. This average was not affected ($P>0.05$) by the low Ca diet. Anionic salt supplementation decreased ($P<0.05$) the concentration of 25(OH)D_3 during the first 6 days of supplementation, and on day W1. Combination of anionic salts and vitamin D_3 induced sustained ($P<0.0001$) increases in plasma 25(OH)D_3 that started to rise almost immediately after supplementation began, reaching maximum values toward the end of the withdrawal period. There were large differences ($P<0.0001$) in 25(OH)D_3 levels between anionic salt and vitamin D_3 supplemented steers. Differences ($P<0.05$) in plasma 25(OH)D_3 among vitamin D_3 treatments were evident in both supplemental and withdrawal periods. Maximum 25(OH)D_3 concentrations were 63.81 (W1), 97.34 (W1), and 154.54 (W5) ng/ml, which represented a 3.1-, 4.7- and 7.4-fold increase relative to adaptation period values for the 0.6, 1.2 and 2.4 MIU treatments, respectively (Table 6.8 and Figure 6.4).

6.3.4 Calciotrophic Hormones

Serum concentrations of the parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3 and calcitonin were determined immediately after supplementation of anionic salts and vitamin D_3 ended (W1). Supplementation of vitamin D_3 decreased ($P<0.0001$) PTH values, and there also was a difference ($P<0.05$) between the 0.6 and 1.2 MIU D_3

Table 6.8. The effect of anionic salts and vitamin D_3 supplementation on plasma 25-hydroxyvitamin D_3 ($25(OH)D_3$) levels of finishing Hereford steers (448 ± 26 kg).

$25(OH)D_3$ (ng/ml)									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A19	19.96	19.35	23.08	20.69	1.49				
Low Ca Diet Period (low Ca diet *ad libitum*) [y]:									
L7	18.26	17.95	16.87	18.14	1.23				
L14	19.79	19.07	20.49	17.46	1.17				
Supplemental Period (high Ca diet; 85% of *ad libitum*) [x]:									
	Vitamin D_3 Level (MIU/hd/d)								
	0	0.6	1.2	2.4					
- 1500 mEq/d:									
S1	15.24	19.86	17.29	18.61	1.27	0.0019	0.0370	0.1723	0.4730
S3	15.93	25.35	36.61	49.51	2.57	0.0127	<0.0001	0.0070	0.0027
- 3000 mEq/d:									
S5	16.27	34.79	56.67	90.91	3.24	0.0292	<0.0001	0.0002	<0.0001
S7	16.85	46.34	66.58	109.50	3.71	0.1027	<0.0001	0.0005	<0.0001
S9	19.66	51.81	85.80	132.35	5.56	0.2537	<0.0001	0.0005	<0.0001
Withdrawal Period (high Ca diet *ad libitum*):									
W1	16.36	63.81	97.34	146.14	4.42	0.0361	<0.0001	<0.0001	<0.0001
W5	16.71	60.53	95.30	156.54	7.39	0.0768	<0.0001	0.0043	<0.0001
Repeated Measures Analysis:									
				P value					
Treatment				<0.0001					
Time				<0.0001					
Time x treatment				<0.0001					

[z] Contrasts: A = Average low Ca period vs anionic salts (group1) / B = Control vs vitamin D_3 treatments / C = 0.6 vs 1.2 MIU D_3 treatment / D =1.2 vs 2.4 MIU D_3 treatment.

[y] Comparison: Average adaptation period vs low Ca period

(Only group 3 had lower ($P<0.05$) concentration on day L7).

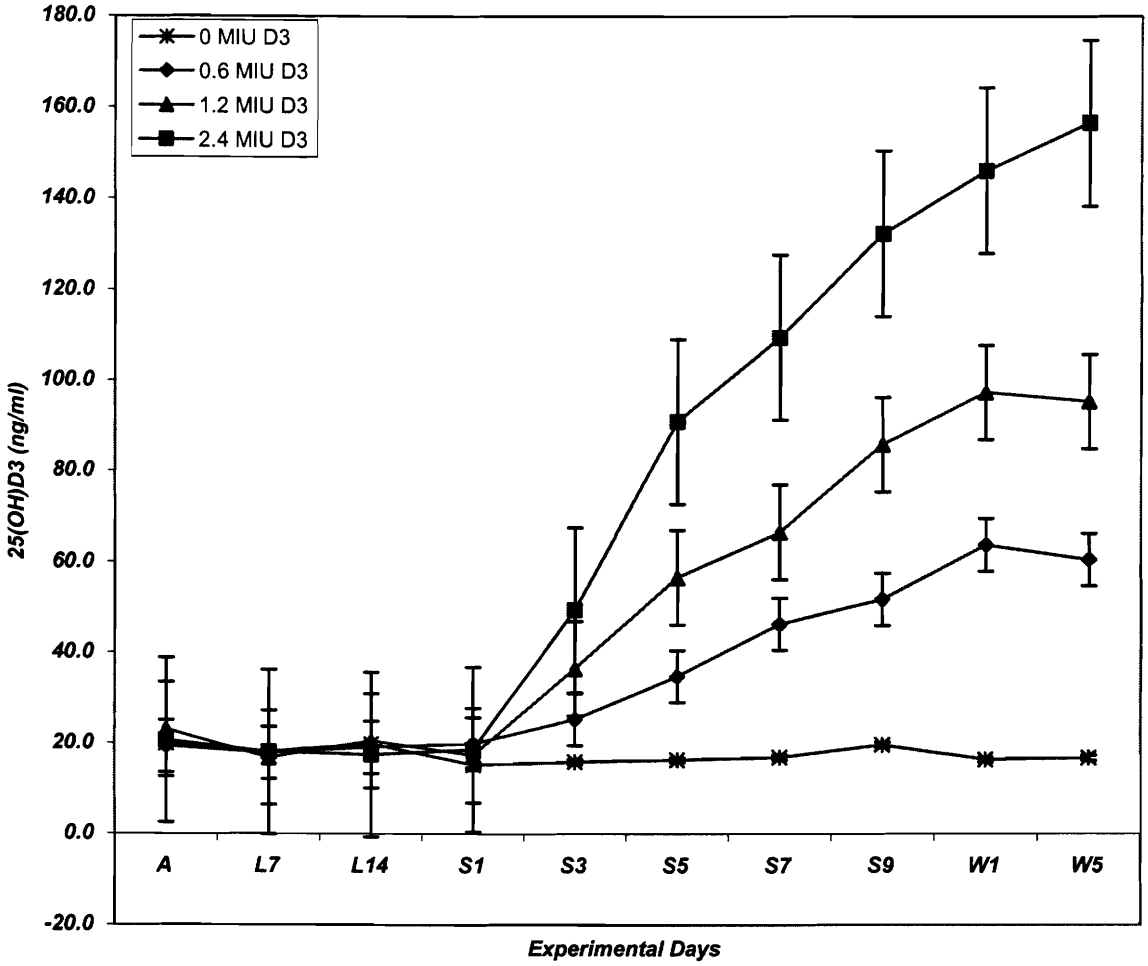

Figure 6.4. Effect of feeding a low Ca diet for 14 days prior to supplementation of anionic salts (-1500 mEq/hd/d from S1-S3 and -3000 mEq/hd/d from S4-S10) and vitamin D_3 (0, 0.6, 1.2 and 2.4 MIU/hd/day) to a high Ca diet for 10 days on plasma 25-hydroxyvitamin D_3 ($25(OH)D_3$) levels of finishing Hereford steers (448 ± 26 kg)(*A=adaptation; L=low Ca; S=supplemental; and W=withdrawal periods*).

Table 6.9. The effect of anionic salts and vitamin D_3 supplementation on circulating concentration of the calciotrophic hormones in finishing Hereford steers (448 ± 26 kg BW) at the first day of the withdrawal (W1) period.

Calciotrophic Hormones (pg/ml)								
	Vitamin D_3 Level (MIU/hd/d)					Contrast [z]		
Days	0	0.6	1.2	2.4	*SEM*	A	B	C
Parathyroid Hormone (PTH):								
W1	21.22	12.92	4.14	4.32	2.20	<0.0001	0.0124	0.9536
1,25-Dihydroxyvitamin D_3:								
W1	27.36	52.68	67.74	78.52	8.11	0.0007	0.2079	0.3616
Calcitonin:								
W1	304.85	300.55	291.63	313.80	16.02	0.8791	0.6990	0.3422

[z] Contrasts:

A = Control (anion salts only) vs Vitamin D_3 treatments.

B = 0.6 vs 1.2 MIU D_3 treatments

C = 1.2 vs 2.4 MIU D_3 treatments

treatments, but not between the 1.2 and 2.4 MIU D_3 treatments (Table 6.9). The $1,25(OH)_2D_3$ concentration was increased ($P<0.001$) as a result of vitamin D_3 supplementation, although there was no difference ($P>0.05$) between vitamin D_3 treatments (Table 6.9). Calcitonin concentrations were not significantly altered ($P>0.05$) as a consequence of vitamin D_3 supplementation (Table 6.9).

6.3.5 Acid-Base Status

Blood pH values during the adaptation period averaged 7.40 ± 0.02. During the low Ca period, blood pH was decreased ($P<0.05$) in all groups on day L14. Anionic salts at -1500 mEq/d did not affect ($P>0.05$) blood pH values. However, feeding the anionic salts at -3000 mEq/d reduced ($P<0.0001$) blood pH. This effect was still evident at the beginning of the withdrawal period. The depressing effect of anionic salts on pH was such that when ionic salts were withdrawn, pH values were slightly higher ($P<0.05$) than normal on day W5. There was no difference ($P>0.05$) in pH values between cattle fed anionic salts and those fed anionic salts plus vitamin D_3, no difference ($P>0.05$) was also found between vitamin D_3 treatments. The repeated measures analysis showed a highly significant effect of time ($P<0.0001$), but not treatment ($P=0.7014$) or time by treatment interaction ($P=0.6959$). Indicating that pH values were effectively altered from normal as a result of anionic salt supplementation (Table 6.10 and Figure 6.5).

This same pattern of response for blood pH values to anionic salt supplementation was observed for blood total carbon dioxide (tCO_2), bicarbonate (HCO_3^-), and base excess (BE) values. However, partial pressure of carbon dioxide (pCO_2), partial pressure of oxygen (pO_2) and anionic gap (AG) were not significantly altered ($P<0.05$) at any level of anionic salt or vitamin D_3 supplementation. The results tables for pCO_2, tCO_2, pO_2, HCO_3^-, BE and AG are presented in Appendix E.

Table 6.10. The effect of anionic salts and vitamin D_3 supplementation on blood pH values of finishing Hereford steers (448 ± 26 kg BW).

Blood pH									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A19	7.41	7.40	7.40	7.39	0.01				
Low Ca Diet Period (low Ca diet *ad libitum*) [y]:									
L7	7.39	7.40	7.40	7.40	0.01				
L14	7.40	7.38	7.39	7.37	0.01				
Supplemental Period (high Ca diet; 85% of *ad libitum*) [x]:									
	Vitamin D_3 Level (MIU/hd/d)								
	0	0.6	1.2	2.4					
- 1500 mEq/d:									
S1	7.39	7.38	7.39	7.37	0.01	0.8099	0.5976	0.7458	0.3372
S3	7.38	7.39	7.38	7.38	0.01	0.5049	0.7828	0.5020	0.6857
- 3000 mEq/d:									
S5	7.34	7.34	7.34	7.37	0.01	0.0122	0.6565	1.0000	0.0829
S7	7.30	7.30	7.27	7.32	0.02	<0.0001	0.8838	0.3322	0.744
S9	7.29	7.30	7.27	7.32	0.02	<0.0001	0.8600	0.2706	0.0713
Withdrawal Period (high Ca diet *ad libitum*):									
W1	7.30	7.29	7.29	7.30	0.02	<0.0001	0.9344	0.2706	0.6875
W5	7.43	7.42	7.40	7.41	0.02	0.0445	0.4426	0.8403	0.7705
Repeated Measures Analysis:									
				P value					
Treatment				0.7014					
Time				<0.0001					
Time x treatment				0.6959					

[z] Contrasts: **A** = Average low Ca period vs anionic salts (group1) / **B** = Control vs vitamin D_3 treatments / **C** = 0.6 vs 1.2 MIU D_3 / **D** =1.2 vs 2.4 MIU D_3

[y] Comparison: Average adaptation period vs low Ca period

(Only group 4 had lower ($P<0.05$) concentration on day L14).

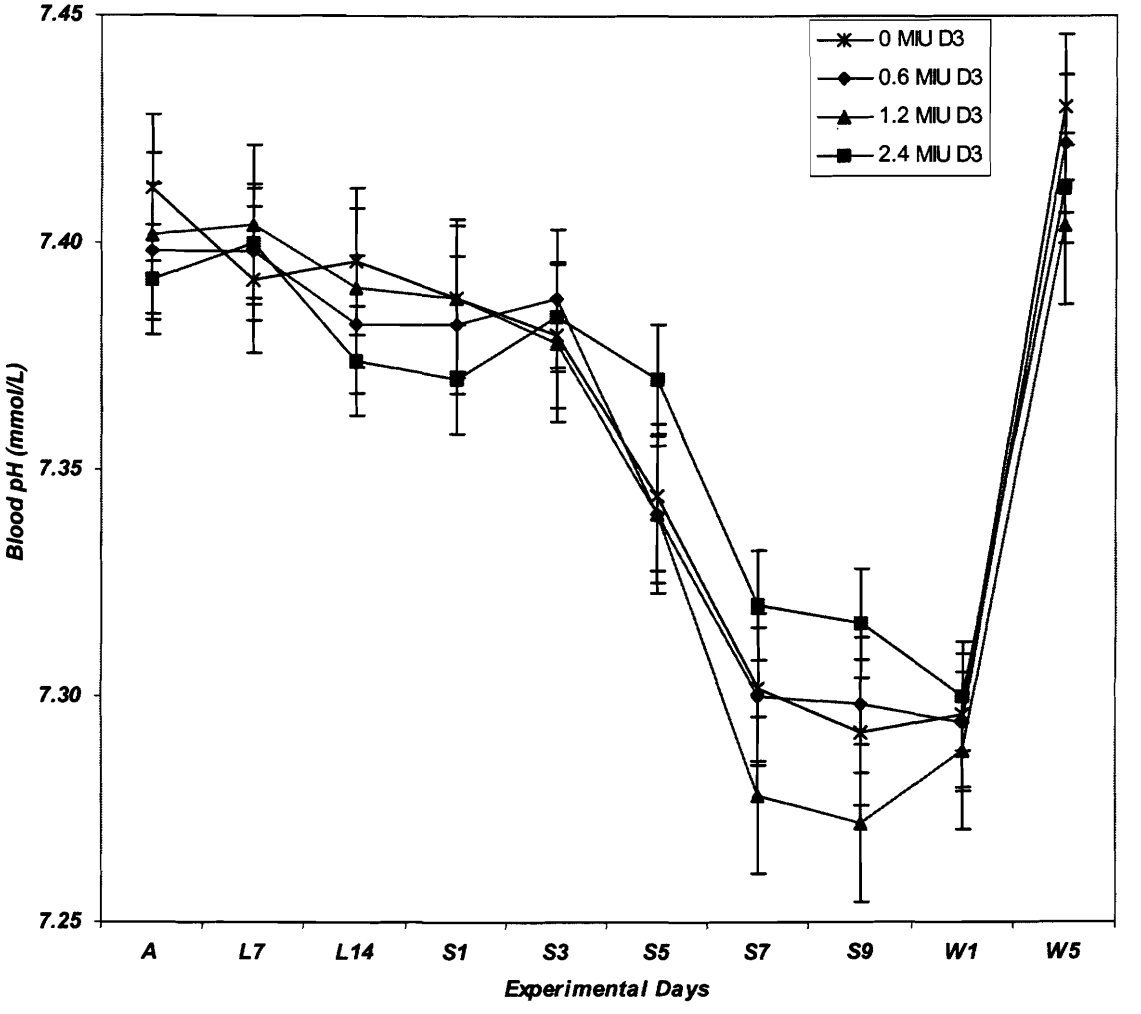

Figure 6.5. Effect of feeding a low Ca diet for 14 days prior to supplementation of anionic salts (-1500 mEq/hd/d from S1-S3 and -3000 mEq/hd/d from S4-S10) and vitamin D_3 (0, 0.6, 1.2 and 2.4 MIU/hd/day) to a high Ca diet for 10 days on blood pH concentrations of finishing Hereford steers (448 ± 26 kg)(*A=adaptation; L=low Ca; S=supplemental; and W=withdrawal periods*).

6.4 Discussion

The main objective of this experiment was determine if it is possible to enhance the effect of vitamin D_3 supplementation on increasing total and ionized serum Ca concentrations through the manipulation of dietary Ca and the feeding of anionic salts. A low Ca diet was fed initially for fourteen days prior to supplementation of vitamin D_3. In the previous experiment it was found that the feeding a low Ca diet decreased total and ionized serum Ca as well as plasma vitamin D_3 concentrations during the first days of feeding. It was concluded in this study that the low Ca diet might have primed the Ca homeostatic mechanism in the steers. For this reason, the same approach was used in this experiment. It has also been shown that the acidification of the diet through the addition of anionic salts causes hypercalciuria (Moore 1970; Horst *et al.* 1997). This phenomenon also activates the Ca homeostatic mechanisms, principally by increasing the efficiency of intestinal Ca absorption. For this reason, a ration high in Ca was fed while supplementation of anionic salts and vitamin D_3 was carried out. On the other hand, anionic salts appear to be unpalatable and can lead to a depression of intake (Block 1984). For this reason, such products should be added gradually to the ration. Thus, anionic salts were supplemented first at –1500 and then at –3000 mEq/hd/d, for 3 and 7 days, respectively.

Feed intake in this experiment was depressed as a result of anionic salt supplementation more than due to vitamin D_3. However, the effect was only significant at the highest level of anionic salts (–3000 mEq/d). The decline in intake followed a trend that was inversely related to the levels of vitamin D_3 supplemented (time by treatment interaction, $P = 0.0002$). The lack of significant ($P>0.05$) effect of vitamin D_3 on feed intake may be due to the fact that lower levels of vitamin D_3 were supplemented compared with previous experiments of this series and with other studies (Swanek *et al.* 1997, 1999; Montgomery *et al.* 2000; Karges *et al.* 2001).

Anionic salts are considered unpalatable (Oetzel *et al.* 1991), and their addition, depending on the amount used, have maintained (Jackson *et al.* 1992; Vagnoni and Oetzel 1998) or depressed (Tucker *et al.* 1988, 1992; Gaynor *et al.* 1989; Erdman 1993) feed intake in dairy cattle. The depression in intake appears to be related to alteration of the normal acid-base balance (Block 1984; LeClerc and Block 1989; Horst *et al.* 1997).

The normal acid-base balance of the steers was not altered ($P<0.05$) by feeding a high Ca diet and supplemented with anionic salts at –1500 mEq/d and vitamin D_3. However, when anionic salts were increased to –3000 mEq/d and vitamin D_3 levels maintained, the acid-base status deviated from normal (lower blood pH, tCO_2, HCO_3^-, and BE; $P<0.05$). It is possible that a metabolic acidosis was produced at this time in which the normal homeostatic mechanisms (renal excretion and respiratory rate) were able to buffer the excess of anions, such that the normal blood pH and the other parameters were maintained. No changes in blood pH have been observed in dry cows fed with acidified diets at –1200 mEq/hd/d (Pehrson *et al.* 1999) or –2000 mEq/d (Oetzel *et al.* 1991). The acid-base characteristic of the blood is described by pH, which refers to hydrogen ion (H^+) activity. Metabolism of proteins, carbohydrates, and fats yields high amounts of H^+ every day, and these ions (protons) are highly reactive. Thus, maintaining a constant blood pH is critical for normal body function, because changes in the normal $[H^+]$ result in alterations in charge and molecular configuration of proteins, which in turn affects enzyme function and cellular structure (DiBartola 2000).

Maintenance of pH in blood is in direct relationship with bicarbonate and pCO_2, as is shown by the Henderson-Hasselbach equation: blood pH = 6.1 x $\log_{10}$ $[HCO_3^-/(0.03 \times pCO_2)]$ (Guyton and Hall 1997). So, alterations in blood bicarbonate or blood pCO_2 will have an immediate effect on blood pH. In order to maintain a constant ratio between bicarbonate and pCO_2, the organism adjusts respiration rate to control pCO_2 and renal excretion to control bicarbonate concentrations (Kaneko 1989; DiBartola 2000). In this study, anionic salts decreased bicarbonate and pCO_2 concentrations, which resulted in a decreased pH. Bicarbonate was decreased either by loss or buffering of non-carbonic acid. Significant declines in blood pH and bicarbonate are indicative that the steers suffered metabolic acidosis (Autran de Morais 1993, 1994; Constable 1999). The decreased pCO_2 was a response to compensate for the acidosis. An explanation for how anionic salts induced acidosis is the fact that Cl is often absorbed from the gastrointestinal tract in exchange for the secretion of bicarbonate ion (Kaneko *et al.* 1997). Thus an excess of Cl and S ions in the gastrointestinal tract might cause a decrease in bicarbonate ions, altering the normal blood bicarbonate-pCO_2 ratio, and thus depressing blood pH.

It has been found that feeding acidified diets has resulted in higher plasma Ca concentrations in dairy cattle (Block 1984, 1994), in goats (Fredeen *et al.* 1988) and laying hens (Cohen *et al.* 1972). Total and ionized serum Ca levels were increased by anionic salt supplementation in this study by approximately 3 and 9 %, respectively. This response seems to be related to the induction of metabolic acidosis, which causes initially hypercalciuria (Tucker *et al.* 1992). This excess of Ca excretion is likely the result of hydrogen excretion interfering with Ca absorption (Gaynor *et al.* 1989), which might cause sufficient loss of Ca from the plasma pool to activate Ca homeostatic mechanisms (Oetzel *et al.* 1991). Thus, when plasma Ca decreased, stimulation of plasma PTH (Goff *et al.* 1991; Phillippo *et al.* 1994) and $1,25(OH)_2D_3$ (Gaynor *et al.* 1989; Goff *et al.* 1991; Abu Damir *et al.* 1994) occurs, which in turn activates bone and intestinal Ca resorption and absorption mechanisms (Block 1984; Oetzel *et al.* 1988; Gaynor *et al.* 1989; LeClerc and Block 1989). In this experiment, serum PTH, $1,25(OH)_2D_3$ and calcitonin were measured at the end of the supplemental period, and at this point, PTH was decreased ($P<0.05$) while $1,25(OH)_2D_3$ was increased ($P<0.05$), responses that were related to the amount of vitamin D_3 fed. As discussed in the previous chapter (Chapter V), vitamin D_3 supplementation likely induced higher synthesis of $1,25(OH)_2D_3$, either directly or via $25(OH)D_3$ which in turn led to elevated levels of Ca in serum. This effect was likely due to excess of substrate ($25(OH)D_3$) overwhelming normal homeostatic mechanisms (Horst *et al.* 1997).

With respect to the objectives of this study, the most important effect of anionic salt supplementation was that it helped to elicit the response in serum Ca to vitamin D_3 supplementation. In the previous experiment (Chapter V), maximum serum total Ca concentrations for the 2.5 and 5 MIU treatments were 11.50 and 12.41 mg/dl, respectively, while in the present study they were 11.43 and 12.24 mg/dl for the 1.2 and 2.4 MIU treatments, respectively. That is, serum Ca concentrations were similar between experiments but now using only half of the amount of vitamin D_3. On the other hand, ionized Ca concentrations in the previous experiment were 5.57 and 6.03 mg/dl for the 2.5 and 5 MIU treatment, respectively, and for this experiment they were 5.98 and 6.52 mg/dl for the 1.2 and 2.4 MIU treatments, respectively. In this case, the response was even higher, with ionized Ca levels exceeding the limits of 6.03 mg/dl from 5 MIU

treatment in the previous experiment. The increase was approximately 0.5 mg/dl of ionized Ca over the 5 MIU treatment without anionic salts. This is a very interesting finding because the total serum Ca concentrations found in previous experiments for the 5 MIU treatment were in very good agreement with the range in which significant improvements in beef tenderization were obtained (Swanek *et al.* 1997, 1999; Montgomery *et al.* 1998, 2000; and Karges *et al.* 1999a,b, 2001). Furthermore, the increase in ionized Ca is critical as ionized or free Ca is the fraction of the total Ca in the extracellular fluid that can be transferred to other tissues. In particular, muscle, where the high Ca levels can stimulate higher activity of the calpain proteolytic system and consequently enhance beef tenderness (Boehm *et al.* 1998; Geesink and Koohmaraie 1999; Montgomery *et al.* 2000).

These results suggest that by reducing the level of vitamin D_3 to 0.6, 1.2 or 2.4 MIU D_3/hd/d in combination with the feeding of anionic salts at -3000 mEq/hd/d may be an alternative method of stimulating high serum Ca levels and to improve beef tenderness. However, before this can be stated with certainty, further research is required to determine the actual effect on the eating quality of beef and the potential for carcass residues of vitamin D_3.

6.5 Conclusions

The low Ca diet appeared to be a feasible way to prime the Ca homeostatic mechanisms as feeding this diet resulted in lower total and ionized serum Ca concentrations, as well as plasma vitamin D_3 and 25(OH)D_3 concentrations. The acid-base status of the steers was not altered, however. Anionic salt supplementation effectively increased total and ionized Ca concentrations and helped to enhance the effect of vitamin D_3 supplementation, as serum Ca concentrations were similar or higher than the concentrations achieved with 5 MIU D_3/hd/d fed in previous experiments. This response of serum Ca was accomplished by the alteration of the normal acid-base status of the steers, which resulted in a mild metabolic acidosis that was not compensated for by respiratory means.

This response in serum Ca as a result of feeding anionic salts (-3000 mEq/hd/d) and vitamin D_3 (1.2 and 2.4 MIU/hd/d) is a possible alternative to feeding 5 MIU or greater amounts of vitamin D_3 to improve beef tenderness. Further research is required to define the benefits and/or limitations of such an approach.

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSIONS

7.1 General Discussion

Tenderness is considered to be one an important determinants of beef quality and it is very likely to be the most important organoleptic characteristic of meat (Greaser 1986; Smith *et al.* 1995). Tender meat is priced higher than less tender meat (Boleman *et al.* 1997). With respect to beef there are two main problems with tenderness, its lack of consistency and uniformity (Morgan *et al.* 1991a; Smith *et al.* 1995; Pringle *et al.* 1999). Several premortem (age, sex, nutrition, etc.) and postmortem (handling of the animal, processing, etc.) factors affect meat tenderness. Among them, the rate and extent of postmortem tenderization appears to be important for the final degree of tenderness (Dayton *et al.* 1981; Huff-Lonergan *et al.* 1996; Goll *et al.* 1998). The proteolytic enzyme system known as calpains (μ-calpain, m-calpain, and calpastatin) is considered to be one of the principal group of proteases involved in postmortem myofibril degradation (Koohmaraie *et al.* 1988a,b, 1989). The calpains are activated by calcium (Ca), and when intracellular Ca in muscle is increased, enzymes activity is increased (Koohmaraie *et al.* 1990; Ilian *et al.* 2001). Increased activity of the calpains has been hypothesized to result in more tender beef (Swanek *et al.* 1999; Montgomery *et al.* 2000; Karges *et al.* 2001). To increase the Ca concentration in muscle two procedures can be followed, injection of calcium chloride ($CaCl_2$) directly into meat (Miller *et al.* 1995), or by supplementing vitamin D_3 to feedlot animals for a short period of time prior to slaughter. Vitamin D_3 supplementation can potentially overcome the disadvantages of the $CaCl_2$ such as that of extra costs for handling of carcass and meat cuts, the potential for off-flavors associated with $CaCl_2$, and the fact that Ca is potentially more homogeneously distributed throughout the whole animal with vitamin D_3 feeding. High levels of vitamin D_3 (5 or more MIU/hd/d) have been used to increase plasma Ca concentrations in feedlot cattle and subsequently increase the concentration of Ca in muscle, and this excess of Ca in muscle has stimulated higher proteolytic activity, resulting in important reductions in shear force values and consequently in improvements in beef tenderness (Swanek *et al.*

1997, 1999; Montgomery *et al.* 1998; 2000; Karges *et al.* 2001). However, supplementation of high levels of vitamin D_3 imposes two main problems, the first is related to animal welfare and the second with food safety. Feeding high levels of vitamin D_3 tends to depress feed intake and affect feedlot performance (Karges *et al.* 1999b). The second and most important concern is in relation to the accumulation of vitamin D_3 and/or its metabolites in meat and other edible tissues (Owens *et al.* 1998; Montgomery *et al.* 2000). Excess of vitamin D_3 in meat can induce vitamin D_3 toxicity, which can cause formation of renal stones and calcification of edible tissues (Glerup and Eriksen 2001). Hence, it is important if this management practice is to gain widespread acceptance that strategies be devised to reduce the levels of supplemental vitamin D_3 required to increase plasma Ca levels such that the risk of residual vitamin D_3 and its metabolites in the carcass is reduced.

Thus, the objective of this research project was to characterize the effect of supplemental vitamin D_3 on Ca homeostasis of finishing steers under Western Canadian feedlot conditions, and with this knowledge, integrate alternatives aimed to reduce the levels of vitamin D_3 that can be used to increase serum Ca concentrations sufficiently to enhance beef tenderness. In the first experiment (Chapter III) the objective was to characterize the effect of titrated doses of vitamin D_3 on feed intake and serum Ca concentrations of finishing steers fed a common Western Canadian finishing diet. In the second experiment (Chapter IV) the objective was to evaluate the effect of vitamin D_3 on feed intake, and circulating Ca, calcium-regulating hormones, vitamin D_3 and 25(OH)D_3 concentrations, in order to understand how vitamin D_3 affects Ca homeostasis. In the third experiment (Chapter V) the objective was to know if dietary Ca manipulation (low Ca diet) could be a suitable method to sensitize Ca homeostatic mechanisms and so enhance the serum Ca response to vitamin D_3 supplementation, preferably at lower levels than reported in the literature. In the fourth experiment (Chapter VI), the objective was to know if dietary Ca manipulation (low and high Ca diets) coupled to anionic salt supplementation could be a suitable method to improve the serum Ca response to lower levels of supplemental vitamin D_3.

Results from experiments 1 to 4 showed that total and ionized serum Ca levels of control steers averaged 10.14 ± 0.35 and 5.03 ± 0.10 mg/dl, respectively (Table 7.1 and

7.2). Supplemental vitamin D_3 at levels of 1.25, 2.5 or 5 MIU/hd/d for 7 days (experiments 1 to 3) induced significant increases in these values, and the response was directly related with the amount of vitamin D_3 supplemented. This situation was confirmed by the high coefficients of correlation obtained between levels of vitamin D_3 supplementation and the resulting increase in total (R = 0.9909) and ionized (R = 0.9751) serum Ca concentrations from experiments 1 to 4 (Tables 7.1 and 7.2).

Maximum total and ionized serum Ca concentrations were reached 3 to 5 days after supplementation of vitamin D_3 ended, a situation that had not been reported before in the literature. This aspect is of particular importance because vitamin D_3 tends to depress feed intake, affecting subsequently animal feedlot performance (daily gain weight, gut fill, etc.) and/or carcass characteristics (Karges *et al.* 1999c; Berry *et al.* 2000). However, it was observed (experiments 1 to 4) that feed intake started to recover as soon as vitamin D_3 supplementation was withdrawn. Thus, if the supplemented animals are slaughtered not immediately after supplementation (as it has been done in all the published studies) but approximately 5 days later, the supplemented animals will have first, the opportunity to reestablish their feed intake and general condition, and secondly, and most importantly, around this time plasma and muscle Ca concentrations will be at their highest.

There seems to be agreement in the published literature that the administration of 5 MIU of vitamin D_3 per head per day constitutes the minimum amount required to raise plasma Ca levels to the point where improvements in beef tenderization are obtained (Swanek *et al.* 1997, 1999; Montgomery *et al.* 1998, 2000, in press; Karges *et al.* 2001). This research showed that 5 MIU D_3 daily for 5 to 10 days increased total plasma Ca, with respect to control animals by approximately 2.0 mg/dl. In experiment 1 to 3, supplementation of 5 MIU D_3 daily, increased total serum Ca concentration by approximately 2.0 mg/dl (from 10.25 to 12.32) (Table 7.1). Thus, the increase in serum Ca levels was in good agreement with the above studies. On the other hand, ionized serum Ca concentrations were increased by approximately 1.0 mg/dl (from 5.05 to 6.01) (Table 7.2). Ionized Ca is physiologically the most important form of Ca in the body. Few other studies have reported the effect of vitamin D_3 supplementation on ionized Ca.

Table 7.1. Effect of vitamin D_3 supplementation on total serum Ca concentrations of finishing steers (experiments 1 to 4).

Total Serum Ca					
Vitamin D_3 (MIU/hd/d)	Vitamin D_3 (MIU total period)	Maximum value (mg/dl)	Increment from control (mg/dl)	Increment from control (%)	Correlation Coefficient [z]
Experiment 1:					
0	0	10.61			
1.25	8.75	11.03	0.42	4.0	
2.50	17.50	11.67	1.06	10.0	
5.00	35.00	12.43	1.82	17.0	0.9947
Experiment 2:					
0	0	10.18			
2.50	17.50	11.02	0.84	8.0	
5.00	35.00	12.12	1.94	19.0	0.9970
Experiment 3:					
0	0	9.90			
1.25	8.75	10.65	0.75	7.6	
2.50	17.50	11.50	1.60	16.0	
5.00	35.00	12.41	2.51	25.4	0.9899
Average 1-3:					
0	0	10.23			
1.25	8.75	10.84	0.61	6.0	
2.50	17.50	11.40	1.35	13.0	
5.00	35.00	12.32	2.09	20.4	0.9975
Experiment 4:					
0	0	9.81			
0 [y]	0	10.11	0.30	3.0	
0.6	6.0	10.97	0.86	8.5	
1.2	12.0	11.43	1.32	13.0	
2.4	24.0	12.24	2.13	21.0	0.9822

[z] Correlation between levels of supplemental vitamin D_3 and response in total serum Ca concentrations.

[y] Anionic salt supplementation only

Table 7.2. Effect of vitamin D_3 supplementation on ionized serum Ca concentrations of finishing steers (experiments 1 to 4).

Ionized Serum Ca					
Vitamin D_3 (MIU/hd/d)	Vitamin D_3 (MIU, total period)	Maximum value (mg/dl)	Increment from control (mg/dl)	Increment from control (%)	Correlation Coefficient [z]
Experiment 1:					
0	0	5.12			
1.25	8.75	5.39	0.27	5.3	
2.50	17.50	5.73	0.61	12.0	
5.00	35.00	6.03	0.91	18.0	0.9828
Experiment 2:					
0	0	5.06			
2.50	17.50	5.36	0.30	6.0	
5.00	35.00	5.98	0.92	18.0	0.9649
Experiment 3:					
0	0	4.78			
1.25	8.75	5.00	0.22	4.6	
2.50	17.50	5.57	0.79	16.5	
5.00	35.00	6.03	1.25	26.0	0.9749
Average 1-3:					
0	0	4.99			
1.25	8.75	5.20	0.15	4.2	
2.50	17.50	5.55	0.60	11.2	
5.00	35.00	6.01	0.96	20.4	0.9964
Experiment 4:					
0	0	4.99			
0 [y]	0	5.43	0.44	9.0	
0.6	6.0	5.90	0.47	9.0	
1.2	12.0	5.98	0.55	10.0	
2.4	24.0	6.52	1.09	20.0	0.9778

[z] Correlation between levels of supplemental vitamin D_3 and response in ionized serum Ca concentrations.

[y] Anionic salt supplementation only

Manipulation of dietary Ca (low Ca diet) alone (experiment 3) did not improve the response of serum Ca to vitamin D_3 supplementation. This is despite the fact that Ca homeostatic mechanisms appeared to be stimulated by low Ca feeding, as serum Ca and plasma vitamin D_3 concentrations were lower than control values during the first 2 to 4 days of feeding the low Ca diet. This response is somewhat different than that observed for dairy cows that were fed low Ca diets prior to parturition to prevent milk fever. The likely explanation is that dairy cows experience a significant Ca stress with the onset of lactation. Low Ca feeding sensitizes the Ca homeostatic mechanisms to this need. In finishing steers no such stress is imposed, thus no response in plasma Ca was observed when dietary Ca levels were reduced

Acidification of prepartum diets has also been used to elevate plasma Ca concentrations in dairy cows and so reduce the incidence of milk fever. It was hypothesized that the addition of anionic salts to the diet of the finishing steers could function as a suitable Ca stressor to enhance the response to vitamin D_3 feeding. This is due to the fact that anionic salts cause hypercalciuria (Fredeen *et al.* 1988; Gaynor *et al.* 1989) and it is recommended to use diets high in Ca when adding anionic salts (Block 1984; LeClerc and Block 1989). In experiment 4, finishing steers were fed with a low Ca diet for 14 days, and then switched to a high Ca diet plus anionic salts and supplementation of vitamin D_3 for 10 days. Anionic salts were added at –1500 mEq/d during the first 3 days of supplementation, and at –3000 mEq/d for the remaining 7 days of this period. Vitamin D_3 was supplemented at levels of 0, 0.6, 1.2 and 2.4 MIU/hd/d. It should be noted that in this experiment, the 1.2 and 2.4 MIU D_3 treatments represented only 48 and 70 %, respectively, of the levels fed in experiments 1 to 3 (Table 7.1 and 7.2). Total and ionized serum Ca concentrations were significantly increased as a result of dietary Ca manipulation, anionic salt and vitamin D_3 supplementation. In experiments 1 to 3, supplementation of 1.25, 2.5 and 5 MIU D_3 elevated total serum Ca concentration to 10.84, 11.58, and 12.32 mg/dl, respectively (Table 7.1). Average ionized Ca levels were 5.20, 5.55, and 6.01 mg/dl, respectively (Table 7.2). Supplementation of 0.6, 1.2 and 2.4 MIU D_3 in experiment 4 elevated total Ca concentrations to 10.97, 11.43, and 12.24 mg/dl, respectively (Table 7.1), and ionized Ca levels to 5.90, 5.98, and 6.52 mg/dl, respectively (Table 7.2). These results indicate that the amount of supplemental vitamin

D_3 required to induce equivalent total Ca concentration to the 5 MIU D_3 treatment was effectively reduced by approximately 65 and 30 % (1.2 and 2.4 MIU D_3 treatments, respectively) (Table 7.1). This response was even higher with ionized Ca. The 1.2 MIU D_3 treatment induced approximately 13 and 7 % more ionized Ca than the 1.25 and 2.5 MIU D_3 treatments. The 2.4 MIU D_3 treatment, induced approximately 15 and 8 % more ionized Ca than the 2.5 and 5 MIU D_3 treatments, respectively (Table 7.2). That is, the nutritional management (low Ca diet and anionic salts) effectively improved the serum Ca response to vitamin D_3 supplementation. This aspect represents one of the most meaningful findings from this research. The amount of vitamin D_3 required to increase ionized Ca to levels sufficiently high to cause an impact on beef tenderness can be reduced by dietary manipulation with anionic salts. In experiments 1 to 3, the 5 MIU D_3 treatment increased ionized Ca to an average of 6.01 mg/dl, while in experiment 4 the 0.6 and 1.2 MIU D_3 treatments resulted in 5.90 and 5.98 mg/dl, respectively. This means that the amount of vitamin D_3 required to induce equivalent ionized Ca concentrations to 5 MIU D_3 treatment was effectively reduced by approximately 80 and 65 % (0.6 and 1.2 MIU D_3 treatments, respectively) (Table 7.2).

It is very likely that this reduction in the amount of vitamin D_3 fed will reduce carcass residues of vitamin D_3 and its metabolites appreciably. Further work should be carried out to determine if feeding this reduced level of vitamin D_3 in combination with anionic salts will not only raise serum Ca to levels where tenderness of beef is improved but also to determine the extent of any tissue residue problem.

The second objective of this research project was to know how vitamin D_3 supplementation affected Ca homeostasis in order to understand how high doses of vitamin D_3 increased the concentration of Ca in serum. It was found that supplementation of high levels of vitamin D_3 caused elevated levels of vitamin D_3 and 25(OH)D_3 in plasma, a response that was directly related to the amount of vitamin D_3 supplemented. However, the response patterns were different for vitamin D_3 and 25(OH)D_3. Plasma vitamin D_3 concentrations increased as supplementation proceeded and began to fall as supplementation ended. In contrast, plasma 25(OH)D_3 concentrations increased linearly throughout the supplemental and withdrawal periods, attaining maximal concentrations at the end of the latter period. This suggests that once

vitamin D_3 is absorbed from the small intestine, it is transferred to the liver and hydroxylated by the 25-hydroxylase enzyme. The rapid rise in plasma 25(OH)D_3 levels suggests that this step in ruminants, as in other species, is not tightly regulated. Parathyroid hormone (PTH) concentration in plasma was decreased by vitamin D_3 feeding. This finding was in agreement with the literature, because serum Ca levels were elevated causing a mild hypercalcemia, and plasma vitamin D_3 and 25(OH)D_3 concentrations were at levels much higher than normal. These factors affect the synthesis and release of PTH from the parathyroid glands (Rhoades and Tanner 1995; Guyton and Hall 2000). Furthermore, PTH regulates the activity of the 1α-hydroxylase enzyme, which is primarily found in the kidneys. This enzyme is responsible for the second hydroxylation of the vitamin D_3 to its hormonal form, 1,25$(OH)_2D_3$ (DeLuca 1979; Smith *et al.* 1983; West 1991). It is generally assumed that this is a tightly controlled step and that the plasma concentration of 1,25$(OH)_2D_3$ should be low as a consequence of high levels of Ca, vitamin D_3, and 25(OH)D_3, and low levels of PTH in plasma. However, when the concentration of 1,25$(OH)_2D_3$ in serum was analyzed in experiment 3 and 4, it was found that the concentrations of this metabolite were higher in the supplemented animals than in controls, with the increase being in direct relationship to the amount of vitamin D_3 supplemented.

The increase in 1,25$(OH)_2D_3$ could be due to two reasons associated with an excess of 25(OH)D_3 in plasma. An excess of 25(OH)D_3 could stimulate the activity of the 1α-hydroxylase enzyme and induce higher synthesis of 1,25$(OH)_2D_3$ as noted in experiments 3 and 4. Secondly, while cellular receptors are highly specific for 1,25$(OH)_2D_3$, it has been shown that an excess of 25(OH)D_3 in plasma can bind to the receptors and induce the effect of 1,25$(OH)_2D_3$ in bone, kidneys and intestine (Smith *et al.* 1983; West 19991; Bhagavan 1992). This statement is based on the fact that concentrations of plasma 25(OH)D_3 continued increasing during the withdrawal period (experiments 2 to 4), and serum Ca concentrations followed a similar response pattern (experiment 1 to 4). In conclusion, supplementation of vitamin D_3 appears to increase serum Ca concentration through increases in the action of 25(OH)D_3 and 1,25$(OH)_2D_3$ in kidneys, bone and intestine.

Serum calcitonin concentrations were determined in experiments 2 to 4, however no significant response from this hormone was found as a result of vitamin D_3 supplementation, even though elevated serum Ca levels were evident in all the experiments. This lack of response has been observed in some species, for this reason calcitonin is considered to be weaker than PTH and $1,25(OH)_2D_3$ in controlling plasma Ca concentrations (Smith *et al.* 1983; Bhagavan 1992; Guyton and Hall 1997). However, in marine species, where sea water contains elevated levels of Ca, calcitonin plays an active role in regulating plasma Ca concentrations (Rosol *et al.* 1995).

The results found in this thesis show that feeding as low as 0.6 MIU D_3/hd/d coupled to dietary Ca manipulation and anionic salt supplementation can be an effective method to raise serum Ca levels when cattle are fed feedlot diets common to Western Canada. However, before this technique can be used commercially, more research work has to be done in order to assure that vitamin D_3 supplementation maximizes biological (animal welfare and food safety) and economic (animal performance and beef quality) responses. This work should focus on evaluation of treatment on tenderness (shear force and palatability), and the effects on residues in muscle, liver, kidneys and adipose tissue relationship. Finally, there is a need to define the relationship between dietary Ca and anionic salts manipulation more closely in order to avoid the negative effects on feed intake and subsequently on animal performance.

7.2 General Conclusions

The results of this study showed that:

- Total and ionized serum Ca concentrations were increased by vitamin D_3 supplementation to levels similar to or higher than those reported in the literature using the same amounts of vitamin D_3 per day. Values were increased to threshold levels where improvements in beef tenderness have been reported by other workers. Total and ionized Ca concentrations rose in a parallel reaching their maximum concentrations toward the end of the withdrawal period. The 5 MIU D_3 treatment caused an average increase (experiments 1 to 3) of 2.0 and 1.0 mg/dl of total and ionized serum Ca concentrations, respectively.

- Plasma vitamin D_3 concentrations were increased in a quadratic fashion, attaining their maximum values towards the end of the supplemental period. In contrast, $25(OH)D_3$ concentrations were linearly increased, reaching their maximum values toward the end of the withdrawal period. The concentrations of these two metabolites started to rise immediately upon supplementation of vitamin D_3. This indicates that once vitamin D_3 entered the circulation it was rapidly hydroxylated in the liver to the $25(OH)D_3$. The 5 MIU D_3 treatment (experiments 2 and 3) increased plasma vitamin D_3 and $25(OH)_2D_3$ concentrations by approximately 15- to 30-fold and 7- to 15-fold, respectively, compared to unsupplemented steers. This indicates that supplementation of 5 MIU D_3 daily increases the concentrations of vitamin D_3 in plasma, and is very likely to result in significant residues of vitamin D_3 and its metabolites in muscle and other edible tissues.
- In relation to the calciotrophic hormones it was found that the concentration of PTH in serum was decreased, a response that corresponded to its physiological role on Ca metabolism. Somewhat unexpectly, the concentration of $1,25(OH)_2D_3$ in serum was increased, indicating failure of normal homeostatic control measures in the face of high levels of vitamin D_3 feeding.
- Feeding a low Ca diet prior to vitamin D_3 supplementation did not improve the response to vitamin D_3 supplementation in inducing higher serum Ca concentrations than normal Ca diets. However, low Ca diets sensitized Ca homeostatic mechanisms because total and ionized serum Ca, plasma vitamin D_3 and $25(OH)D_3$ concentrations were lower than normal during the first four days of feeding.
- The addition of 1.25, 2.5 or 5 MIU D_3 to common barley grain-base feedlot finishing diets caused a significant decline in feed intake. The depression in feed intake and its recovery followed an inverse relationship with the amount of vitamin D_3 supplemented. This observation led to the conclusion that the negative effect of vitamin D_3 on feed intake was not related to palatability factors, but most likely due to physiological and/or metabolic alterations.
- The fact that total and ionized Ca concentrations were highest during the withdrawal periods opens the possibility of reducing the negative effect on feed

intake if the supplemented animals are slaughtered following a 3 to 5 day withdrawal period. The animals will have the opportunity to recover feed intake and other feedlot performance characteristics, and thus reduce the likelihood of affecting carcass quality characteristics.

- Manipulation of dietary Ca plus supplementation of anionic salts (-3000 mEq/d) and low levels of vitamin D_3 (0.6, 1.2 and 2.4 MIU/hd/d) increased serum Ca concentration to levels consistent with improvements in beef tenderness found in other studies. This was accomplished with as low as 20 % of the vitamin D_3 that was fed in other studies. Further work is required to study the effect on beef tenderness, on residual effects on meat and other edible tissues, and on the levels of anionic salts that could be fed without affecting feed intake.
- The use of lower levels of vitamin D_3 to elevate serum Ca concentrations to levels high enough to improve beef tenderness will lead to an important reduction in residues of vitamin D_3 (and/or its metabolites) that are found in meat and other edible tissues, and consequently reduce the risk for vitamin D_3 toxicity in consumers.

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APPENDIX A

Determination of the Parathyroid Hormone (PTH)

Experiment 2 - Chapter IV

The native or intact human parathyroid hormone (iPTH) is an 84 amino acid polypeptide, which is biosynthesized in the parathyroid gland. The Intact PTH Immunoassay carries out the measurement of the biologically intact 84 amino acid chain of PTH, which is a two-site immunoradiometric assay (IRMA)(Intact PTH- Parathyroid Hormone 100T Kit-Catalog No. 40-2170. Nichols Institute Diagnostics. San Juan Capistrano, CA. USA).

Two different goat polyclonal antibodies to human PTH, purified to be specific to two different regions of the PTH molecule were used. One antibody bound the mid-region and C-terminal PTH 38-84, this antibody was immobilized onto plastic beads. The other antibody bound the N-terminal PTH 1-34, this was radiolabeled for detection.

The sample (bovine serum) was incubated simultaneously with the antibody-coated bead and 1251-labeled antibody. Thus, the intact PTH present in the sample was bound by both the immobilized and the labeled antibodies to form a complex:

Bead Anti-PTH (39-84) -------- Intact PTH (1-84) ------ ^{125}I Anti-PTH (1-34)

Although mid-region and C-terminal fragments are bound by the antibody coated bead, only the intact PTH 1-84 forms the complex necessary for detection. At the end of the assay incubation, the bead was washed to remove unbound components and the radioactivity bound to the solid phase was measured in a gamma counter. A dose response curve of radioactivity versus concentration was generated using results obtained from standards, which were assayed concurrently with the unknowns. Concentrations of intact PTH present in the controls and in the samples were determined directly from this curve (Radioisotopic Assay. Intact PTH – Parathyroid Hormone. Nichols Institute Diagnostics).

Materials:

- Reagent A = PTH Antibody Coated Beads.
- Reagent B = ^{125}I-PTH Antibody Solution.
- Reagent C = Intact PTH Zero Standard.
- Reagents D_2-H = Intact Standards.
- Reagents I = Wash Solution Concentrate.
- Reagents J-K = Intact PTH Controls 1 & 2.

Assay Procedure:

- 200 µL of standard (reagents C-H), control (Reagents J-K) and plasma samples were placed in individual test tubes.
- 100 µL of the ^{125}I-PTH Antibody Solution (Reagent B) were added to all tubes.
- Tubes were thoroughly mixed (vortexed).
- Using Bead Dispenser one bead (Reagent A) was added to each test tube.
- Then test tubes were covered with parafilm and incubated at room temperature for approximately 24 h.
- Beads were washed twice by dispensing 2.0 ml of working Wash Solution into each tube, and the reaction mixture was decanted from each tube.
- Each tube was counted in a gamma counter for one minute to measure concentration.

Performance Characteristics:

- Precision: the precision (intra-assay variance) of the assay was calculated from replicate determinations on each of two quality control sera in a single assay (CV = 5.35 %).
- Accuracy: correlation coefficient (r) = 0.98 when compared to radioimmunoassay (Nichols Institute Diagnostics INS-PTH).
- Sensitivity: 1 pg/ml. Defined as the smallest single value which can be distinguished from zero at the 95% confidence limit.

Experiments 3 (Chapters V) and 4 (Chapter VI)

Parathyroid hormone was determined in serum samples by the Coat-A-Count Intact PTH IRMA (Diagnostic Products Corporation. Los Angeles, CA. USA), which is an immunoradiometric assay designed for the quantitative measurement of intact parathyroid hormone (parathyrin, PTH). PTH is a single-chain polypeptide containing 84 amino acids, exerts significant influence in the maintenance of optimal Ca ion concentrations.

Principle of the Procedure:

Coat-A-Count Intact PTH IRMA is a solid-phase immunoradiometric assay employing ^{125}I-labeled affinity-purified polyclonal anti-PTH (1-34) antibody in liquid phase, in conjunction with affinity-purified polyclonal anti-PTH (44-84) antibodies immobilized to the wall of a polystyrene tube. Intact PTH is captured between the anti-PTH immobilized on the tube and the radio-labeled anti-PTH. Unbound 125I-labeled anti-PTH antibody is removed by decanting the reaction mixture and washing the tube; this reduces nonspecific binding to a very low level, and ensures excellent low-end precision. The PTH concentration is directly proportional to the radioactivity present in the tube after the wash step. The radioactivity is counted using a gamma counter, after which the concentration of PTH in the sample is obtained by comparing the counts-per-minute with those obtained for the standards.

Materials:

- PTH AB-Coated Tubes (IPH1).
- ^{125}I PTH Ab (IPH2).
- Intact PTH Calibrators (PHI3-9).
- Buffered Wash Solution Concentrate (1TSBW).
- Intact PTH Controls (PHCO1-2).

Immunoradiometric Assay Procedure:

- Calibrators, controls, serum samples and assay tubes were all kept on ice while setting up the assay, due to the highly labile nature of intact PTH.

- Fourteen PTH Ab-Coated Tubes A (nonspecific binding) and B through G ("maximum binding") were labeled in duplicate. Additional PTH Ab-Coated Tubes, also were labeled in duplicates, for control and samples.
- 200 μL of each calibrator, control and sample were pipeted into the prepared tubes.
- 100 μL of ^{125}I PTH Ab were added to every tube.
- All tubes were gently vortexed.
- Incubation was carried out for 24 hours at 4°C.
- All tubes were thoroughly decanted. 2 ml of Buffered Wash Solution were added to each tube. After a wait of 1-2 min, the tubes were again thoroughly decanted. The addition of the buffered solution and decantation was repeated one more time.
- The tubes were set in a gamma counter and count for one minute.
- Counts-per-minute (CPM) for all tubes was corrected by subtracting the average CPM of the non specific binding tubes (calibrator A).

Performance Data:

- Sensitivity: 1.0 pg/ml. The assay-s detection limit, defined as the concentration two standard deviations above the response at zero dose.
- Precision: intraassay (within-run) coefficient of variation = 6.46 %.
- Specificity: the Coat-A-Count Intact PTH IRMA antibodies are highly specific for intact PTH, with particularly low cross reactivity to the most PTH fragments, as well as to other naturally occurring compounds in the serum samples.

APPENDIX B

Determination of the Calcitonin Hormone

Experiments 2 (Chapters IV), 3 (Chapter V) and 4 (Chapter VI)

Calcitonin concentrations in serum were determined by a double-antibody ^{125}I-Radioimmunoassay (Diagnostic Products Corporation. Los Angeles, CA. USA). The assay is designated for quantitative measurement of calcitonin (thyrocalcitonin) in serum (Catalog No. KLCD). Human calcitonin is a single-chain peptide hormone produced primarily in the thyroid gland, where the parafollicular "C" cells secrete it. The physiological significance of calcitonin is uncertain, but it does have a modest Ca- and phosphorus-lowering effect. Rising and falling Ca levels normally modulate its secretion, with an increase in circulating Ca prompting an increase in the calcitonin level. A fasting basal range of 3-19 pg/ml for healthy adults, based on radioimmunoassays of extracted plasma. Elevated levels (> 100 pg/ml) are encountered in a variety of pathological conditions.

Principle of the Procedure:

DCP's Double Antibody Calcitonin procedure is a sequential competitive radioimmunoassay. The sample is first preincubated with anti-calcitonin antiserum. 125I-labeled calcitonin then competes with calcitonin in the sample for antibody sites. After incubation for a fixed time, separation of bound from free is achieved by the PEG-accelerated double-antibody method. Finally, the antibody-bound fraction is precipitated and counted. Sample concentrations are read from a calibration curve.

Materials:

- Calcitonin Antiserum (CLD1).
- ^{125}I Calcitonin (CLD2).
- Calcitonin Calibrators (CLD3-8).
- Precipitating Solution (CLGG).

- Calcitonin Controls (CLC1-2).

Assay Procedure:

- Sixteen tubes in duplicate were labeled: T (total counts), NSB (nonspecific binding), A (maximum binding) and B through F. And additional tubes for control and samples, also in duplicates, were labeled.
- 200 μL of the zero calibrator A were pipeted into the NSB and A tubes, and 200 μL of each of the remaining calibrators B through F into correspondingly labeled tubes. And 200 μL of each serum sample and control were pipeted into the prepared tubes.
- 100 μL of Calcitonin Antiserum were added to all tubes except the NSB and T tubes. All tubes were vortexed.
- All tubes (except T tubes) were incubated for 3 hours at room temperature.
- 100 μL of ^{125}I Calcitonin were added to all tubes and then vortexed.
- All tubes (except T tubes) were incubated for 18 hours at –4°C.
- Then, 1.0 ml of cold Precipitating Solution was added to all tubes and then vortexed.
- All tubes were incubated for 30 minutes at room temperature.
- All tubes were centrifuge for 15 minutes at 3000 x g.
- Using a foam decanting rack, the supernatants were decanted, and the precipitate was retained for counting.
- All tubes were placed in a gamma counter, and each tube was counted for 1 min.
- Calculation of results: calcitonin concentrations were calculated from a logit-log representation, in which count-per-minute (CPM) from serum samples were corrected by CPM of the nonspecific binding tubes.

Performance Data:

- Sensitivity: 16 pg/ml. As a minimal detectable dose of the assay.
- Precision: intra-assay (within-run) a CV of 5.32 %. Inter-assay (run-to-run) a CV of 0.44 % (n=2).

Specificity: the antiserum is highly specific for human calcitonin, with an extremely low cross reactivity to other peptides present in the sample.

APPENDIX C

Determination of Vitamin D_3 and 25-hydroxyvitamin D_3

Experiment 2 (Chapter IV)

Vitamin D_3 and its metabolite 25-hydroxivitamin D_3 ($25(OH)D_3$) were analyzed using a high-performance liquid chromatography (HPLC) technique. This separation technique is based on two main phases, the stationary (represented by the column) and the liquid (represented by the mobile phase). In this analysis, a silica-based carbon 18 packed HPLC column was used which represented the non-polar phase and the mobile phase, which was an organic solvent represented the polar phase. The column used was a reverse-phase Luna column C18 3μ 150 x 4.60 mm (Phenomenex, Torrance, CA. USA), and acetonitrile for the mobile phase, under isocratic conditions.

Assay Procedure:

1) Plasma Preparation from untreated animals:
    a. Acetonitrile extraction:
        i. Two ml of acetonitrile were placed in a glass test tube.
        ii. One 1 ml of plasma was added in a drop-wise fashion.
        iii. The solution was thoroughly vortexed for 30 sec and then centrifuged at 3000 rpm for 10 min.
        iv. The supernatant was collected and transferred to a separate tube.
    b. Chloroform extraction:
        i. To the supernatant, 1 ml of chloroform was added.
        ii. The solution was thoroughly vortexed for 30 sec and then centrifuged at 3000 rpm for 10 min.
        iii. The organic (bottom) layer was collected and transferred to a separate glass test tube.
        iv. The solution was then exposed to a steady stream of nitrogen until completely dry.

v. To this test tube, containing the dried residue, 0.5 ml of acetonitrile was added and then vortexed for 30 sec.
vi. The solution was filtered through a 0.45 μm filter attached to a glass syringe.
vii. The filtered solution was transferred to an amber vial, which was ready for injection onto the HPLC.

2) Plasma preparation from treated animals:
   a. Acetonitrile extraction:
      i. 800 μL of acetonitrile were placed in a glass test tube.
      ii. 200 μL of plasma were added in a drop-wise fashion.
      iii. The solution was thoroughly vortexed for 30 sec and then it was centrifuged at 3000 rpm for 10 min, resulting in two layers, a solid one at the bottom and a liquid (supernatant).
      iv. The supernatant was filtered through 0.45 μm filter attached to a glass syringe.
      v. The filtered solution was transferred to an amber vial, which was ready for injection to the HPLC.

The samples dissolved in acetonitrile were injected by an autosampling device (Sampler Processor, Water 715, Ultra Wisp. Waters™. Waters Limited. Mississauga, ON Canada) onto the head of the column. Each sample was carried through the column by a continuous flow (1.2 ml/min) of mobile phase (acetonitrile) from the pump. The elution times for $25(OH)D_3$ was 3.0 min while that for vitamin D_3 was 8.6 min. A typical chromatograph for this separation is illustrated in Fig. C.1.

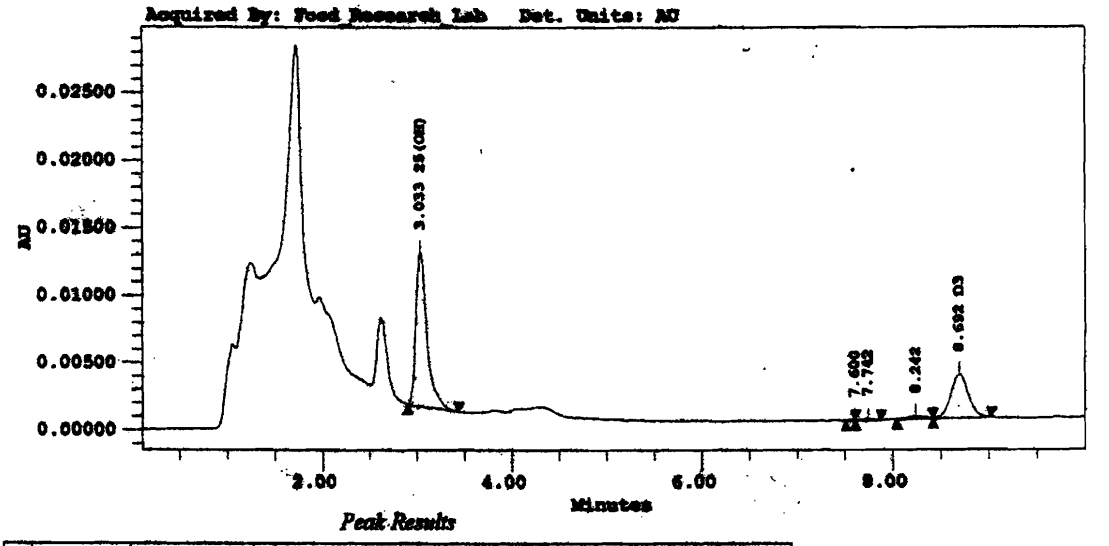
Identification and quantification of vitamin D_3 and $25(OH)D_3$ was carried out by using a photodiode array detector (Photodiode Array Detector, Waters 996. Waters™. Waters Limited. Mississauga, ON Canada) at 265 nm.

Analysis of 130 blood samples were carried out in duplicate, with coefficients of variation for 6.12 for vitamin D_3 and 3.97 for $25(OH)D_3$.

Millennium Results Report December 28, 2000 Page: 1 of 1
Report Method: Vit_D3 Version: 2.15
For Sample: Pl-70 Vial: 22 Injection: 1 Channel: 996
Proc Chan: PDA_265.0nm Processed: 28/12/00 07:03:54 PM
Channel Descr: PDA 265.0 nm

Millennium Sample Information

Project Name:	Vit_D3		
Sample Name:	Pl-70		
Vial:	22	Sample Type:	Unknown
Injection:	1	Volume:	150.00
Channel:	996	Run Time:	10.0 min
Date Acquired:	28/12/00 02:57:42 AM	Date Processed:	28/12/00 07:03:54 PM
SampleWeight:	1.00000	Dilution:	1.00000
Acq Meth Set:	Vit_D3		
Processing Method:	Vit_D3		



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1	25(OH)	3.033	92783	11419	454.259	BB
2		7.600	141	42		BV
3		7.742	799	80		VB
4		8.242	2113	192		BV
5	D3	8.692	40836	3245	204.673	VB

Figure C.1. Determination and separation of vitamin D_3 and 25(OH)D_3 from plasma. A typical chromatograph from the reverse-phase HPLC analysis.

Calibration Curves:

Calibration curves were created by using standards of both analytes, vitamin D_3 and 25(OH)D_3, diluted in acetonitrile at the concentrations of 2, 4, 8, 16, 32, 64 and 128 ng/ml. The coefficients of correlation and regression for both analytes were R = 0.9999 and R^2 = 0.9999, respectively, in the majority of the cases but never lower than 0.999 (Figures C.2 and C.3). Integration and calculation of calibration curves were automatically performed by the computer software (Millennium). The output from the computer was a chromatograph showing the absorbance peaks with information about their retention or elution time, peak area, peak high and concentration (in ng/ml of sample injected) for the vitamin D_3 and 25(OH)D_3 analytes for each sample (Figure C.1).

Calculation of the Final Concentrations:

To convert the output from the HPLC analysis (ng/ml of sample injected) to ng/ml of plasma, the results were adjusted according to the sample's extraction procedure. In the case of the acetonitrile-chloroform procedure, the results were corrected by two factors: a) by the volume of reconstitution in acetonitrile (0.5 ml); and b) by the volume of the bottom layer collected (in accordance with the maximum volume collected without disturbing the upper layer), which was calculated to be around 90%. Condensation and dilution factors considered were 2 (because 0.5 ml were used to reconstitute the sample) and 1.1 (because only 90% of the layer was collected). Therefore, the results from the HPLC output were multiplied by 1.1 and divided by 2 to obtain the concentration in ng/ml of plasma.

Similarly, two correction factors were use in the one-step acetonitrile extraction, the first had to do with the plasma-acetonitrile ratio (1:4); and the second, with the volume of the sediment that was formed after centrifugation and which was calculated to be 10%. Thus, the results were multiplied by 5 and then by 0.9 to obtain the final concentration in ng/ml of plasma.

Equipment:

- HPLC Waters (Waters™. Waters Limited. Mississauga, ON Canada):
    i. Water 715. Ultra Wisp. Sample Processor.

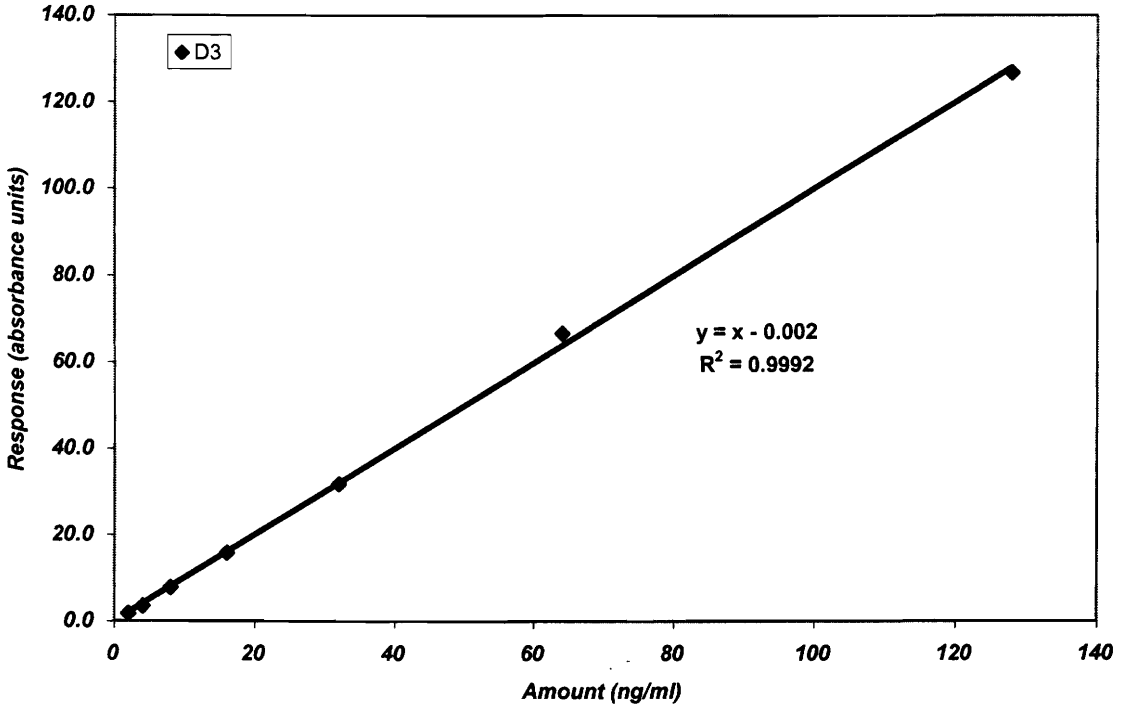


Figure C.2. Typical standard curve for estimation of vitamin D_3 using a HPLC assay.

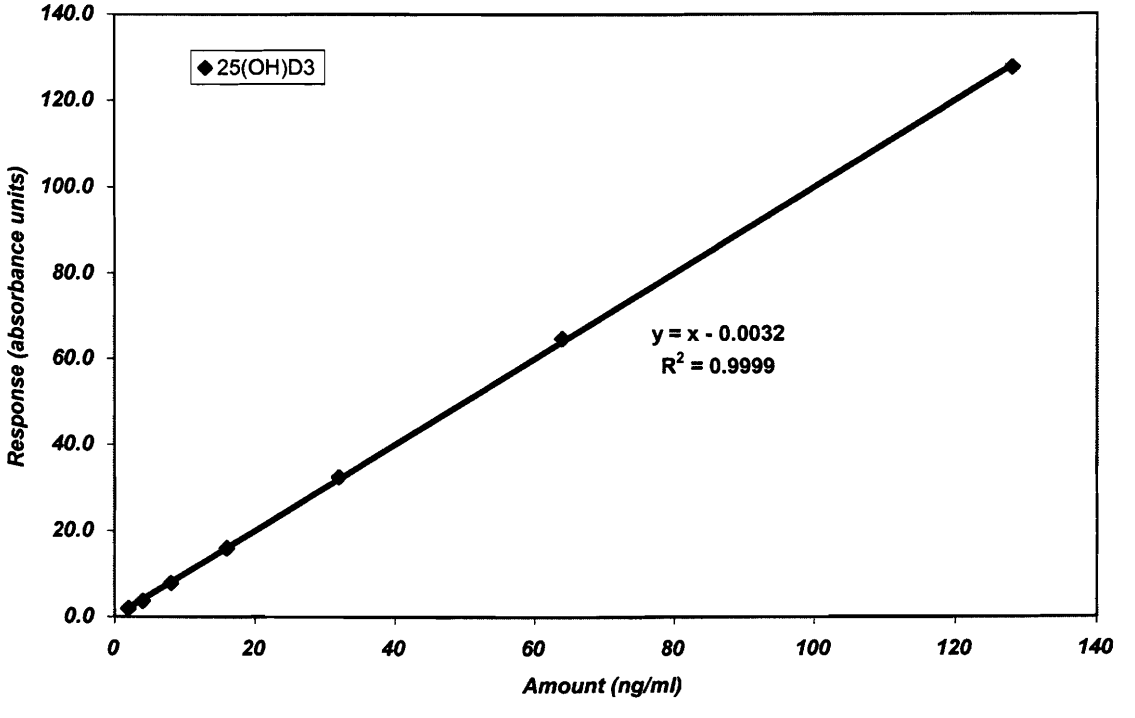


Figure C.3. Typical standard curve for estimation of 25(OH)D_3 using a HPLC assay.

ii. Water 600. Controller.

iii. Waters 996. Photodiode Array Detector.

iv. Software: Millennium (Waters™. Waters Limited. Mississauga, ON. Canada).

- Column:
  i. Type: silica-based reverse-phase.
  ii. Phenomenex: Luna (Torrance, CA. USA)
  iii. C18 3μ 150 x 4.60 mm.
- Assay conditions:
  i. Mobile phase: Acetonitrile
  ii. Flow rate: 1.2 ml/min.
  iii. Injection: 150 μL
  iv. Running time: 10 min.
- Centrifuge: Beckman G6.
- Vortex: Genie 2™. Fisherbrand®

Experiments 3 (Chapters V) and 4 (Chapter VI)

Vitamin D_3 and its metabolite 25(OH)D_3 were analyzed using a high-performance liquid chromatography (HPLC) assay.

Assay Procedure:

1) Analyte Extraction:
    a. 1 ml of acetonitrile was placed in a glass test tube (10 x 70 mm).
    b. 0.5 ml of plasma was added in a drop-wise fashion.
    c. The solution was thoroughly vortexed for about 60 sec.
    d. This mix was centrifuged at 3000 rpm for 15 min.
    e. The supernatant was filtered through 0.45 μm filter attached to a glass syringe and transferred to 1.7 ml amber vials.
    f. Samples were ready for injection onto the HPLC.

2) Injection Characteristics:
    a. Amount injected: 100 μL.
    b. Running time: 15 min.
    c. Mobile phase: Acetonitrile (isocratic).
    d. Flow rate: 2 ml/min.
    e. Column: Prodigy ODS3 100A 5μ 250 x 4.60 mm (Phenomenex).
    f. Wavelength: 266 nm.

3) Equipment:
    a. HPLC Agilent. 1100 Series (Agilent Technologies, Waldbronn, Germany):
        i. Vacuum Degasser.
        ii. Autosampler.
        iii. Quaternary Pump.
        iv. Thermostatted Column Compartment.
        v. Diode Array Detector and Multiple Wavelength Detectors.

vi. Software: ChemStation (Hewllet-Packard. Agilent Technologies, Waldbronn, Germany)

b. Centrifuge: Centrifuge: Beckman J6-MC.

c. Vortex: Genie 2™. Fisherbrand®.

4) Calibration Curves:

a. Calibration curves were created by using standards of both analytes, vitamin D_3 and 25(OH)D_3, diluted in acetonitrile at the concentrations of 3.125, 6.25, 12.5, 25.0, 50.0 and 100 ng/ml.

b. Correlations coefficients for both analytes were: R = 0.999 or higher (Figures C.4 and C.5).

c. The calibration curves were automatically calculated and integrated by the computer software ChemStation (Hewlett-Packard. Agilent Technologies, Waldbronn, Germany).

5) Results:

a. Identification of the peaks of interest was in relation to the standards of both compounds, vitamin D_3 and 25(OH)D_3. Figure C.6 illustrates a typical chromatograph from the HPLC analysis (identification and quantification) of vitamin D_3 and 25(OH)D_3 from plasma.

b. Quantification was based on peak area according to the calibration curves.

c. Two correction factors were applied to the sample's peak area to convert the absorbance units to concentration in ng/ml. The first was the dilution factor of 3, which corresponded to the extraction procedure (2 parts of acetonitrile and 1 part of plasma). The second had to do with the quantification of the total volume of the sample after centrifugation, in which the solid phase constituted approximately 10 % of the total volume. For this reason, a factor of 1.1 was applied to convert concentration in ng/ml of solution injected to ng/ml of plasma.

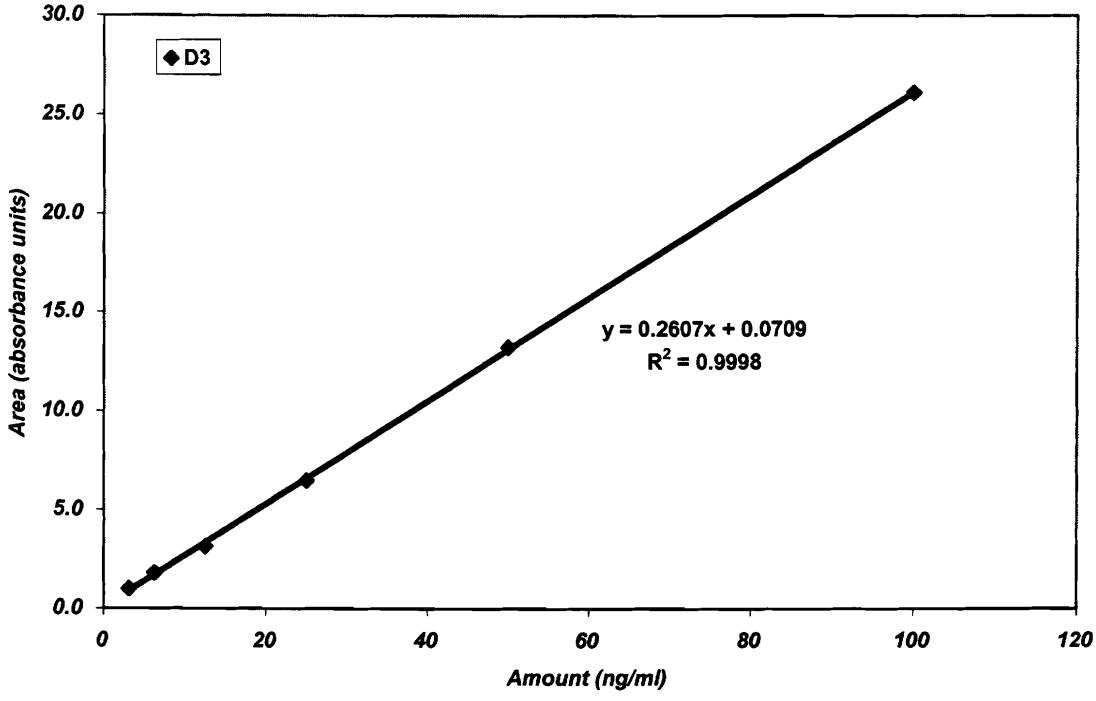


Figure C.4. Typical standard curve for estimation of vitamin D_3 using a HPLC assay.

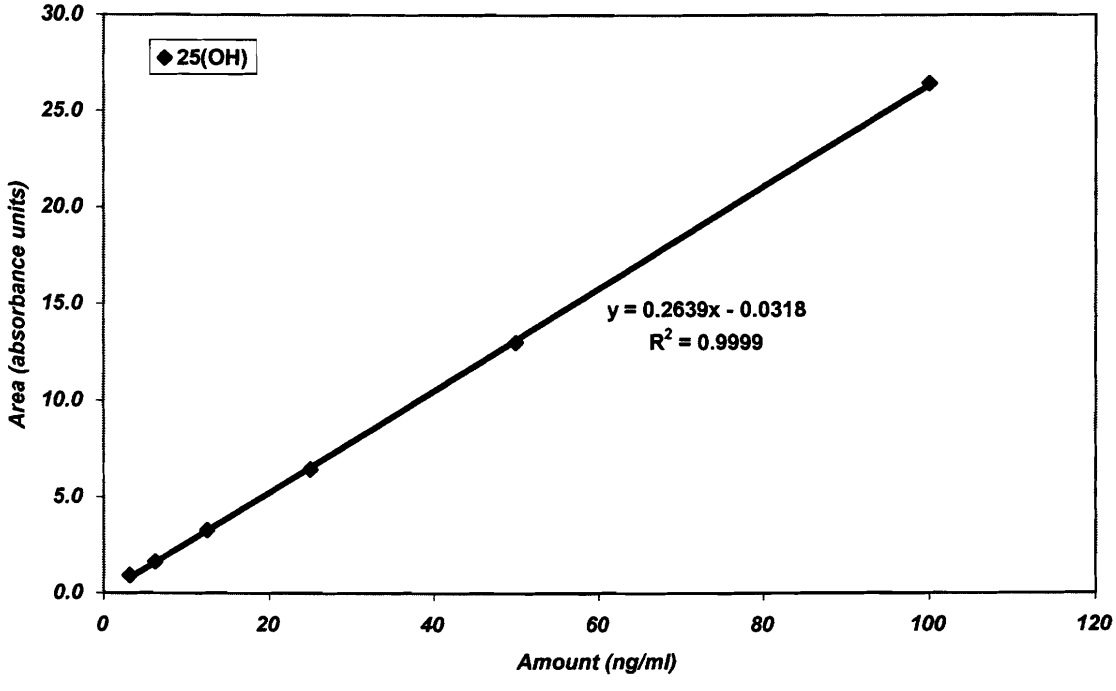


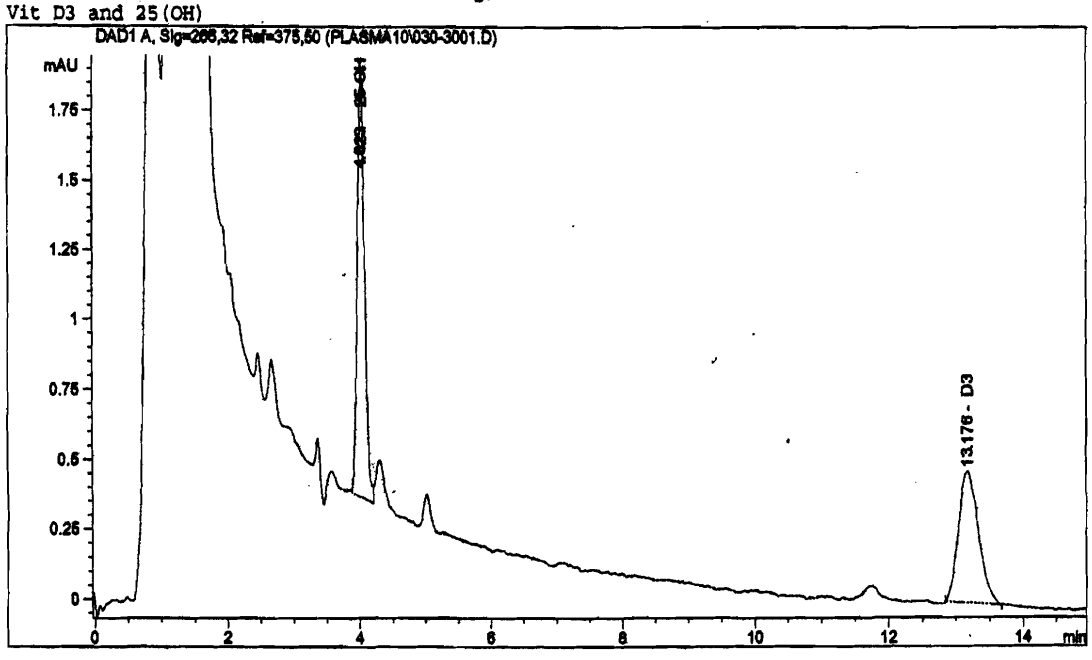
Figure C.5. Typical standard curve for estimation of 25(OH)D_3 using a HPLC assay.

Data File C:\HPCHEM\1\DATA\PLASMA10\030-3001.D Sample Name: 380

Injection Date : 10/15/01 8:00:59 PM Seq. Line : 30
Sample Name : 380 Location : Vial 30
Acq. Operator : Inj : 1
Inj Volume : 100 µl

Acq. Method : C:\HPCHEM\1\METHODS\VITD3A.M
Last changed : 10/15/01 7:58:54 PM
(modified after loading)
Analysis Method : C:\HPCHEM\1\METHODS\VITD3A.M
Last changed : 3/12/02 4:10:17 PM
(modified after loading)

Vit D3 and 25(OH)



External Standard Report

Sorted By : Signal
Calib. Data Modified : 3/12/02 4:10:15 PM
Multiplier : 1.0000
Dilution : 3.0000

Signal 1: DAD1 A, Sig=266,32 Ref=375,50

RetTime [min]	Type	Area [mAU*s]	Amt/Area	Amount [ng/mL]	Grp	Name
4.023	BV	11.71898	4.53433	159.41308		25-OH
13.176	PB	9.71783	4.36749	127.32758		D3

Totals : 286.74067

Results obtained with enhanced integrator!

*** End of Report ***

Figure C.6 Determination and separation of vitamin D_3 and 25(OH)D_3 from plasma. A typical chromatograph from the reverse-phase HPLC analysis.

Method Validation:

Plasma samples (1 ml) were extracted with 2, 3 or 4 ml of acetonitrile, which were equivalent to extraction ratios of 1:2, 1:3 and 1:4, respectively. The peak areas from these levels of extraction were highly correlated: R = 0.9318 for vitamin D_3 and R = 0.9673 for 25(OH)D_3. This indicates that any of the ratios of extraction involved could be used to process plasma samples in order to extract vitamin D_3 or 25(OH)D_3 with good accuracy. We chose the 1:2 ratio because a lower dilution represented more concentration of the analyte(s) and thus better probability of identification during the HPLC analysis. This was a concern principally from untreated steers. However, plasma samples were stored in a 1.7 ml centrifuge clear vials, which were filled to approximately 1.5 ml. Thus, in order to carry out the analysis in duplicate, it was necessary to reduce the aliquot to 0.5 ml of plasma and 1 ml of acetonitrile. The correlation coefficients for 0.5 ml of plasma and acetonitrile in a 1:2 ratio were: R = 0.8953 for vitamin D_3 and R = 0.9781 for 25(OH)D_3. In experiment 3 (Chapter V) 234 plasma samples were analyzed in duplicate, with a coefficient of variation of 17.0 for vitamin D_3 and 4.9 for 25(OH)D_3. In experiment 4 (Chapter VI) there were 200 plasma samples analyzed in duplicate with a coefficient of variation of 16.4 for vitamin D_3 and 11.4 for 25(OH)D_3. Even though, these coefficients of variation (CV) for these two analytes seem to be high, the actual amounts corresponded very well. For example, vitamin D_3 concentrations of 24.83 and 31.02 ng/ml for duplicates gave a CV of 15.7; and 25(OH)D_3 concentrations of 17.41 and 20.39 ng/ml for duplicates gave a CV of 11.2. Values that were considered acceptable under the objective of this research project, which was to identify the general trends in vitamin D_3 and 25(OH)D_3 concentrations in plasma as a result of vitamin D_3 supplementation.

Another test consisted in the evaluation of the recovery rate: to 4 ml of acetonitrile, 2 ml of plasma were added in a drop wise fashion, the solution was then thoroughly mixed (vortexed for approximately 30 sec), then centrifuge at 3000 rpm for 10 min, the supernatant collected and filtered through 0.45 μm filter attached to a glass syringe. Two ml of this solution was set apart and called native, another 2 ml were set in a different test tube and was spiked with 20 ng of both vitamin D_3 and 25(OH)D_3 and called spiked solution. One ml of native and spiked solution were directly injected into

the HPLC and the other ml of both solutions was used for chloroform extraction (method previously described that was used for vitamin D extraction from untreated animals). The difference between the chloroform extraction and original solution was designated as the recover rate, which was for both metabolites (vitamin D_3 and 25(OH)D_3) around 90 to 100 %. Concluding that this procedure was suitable for vitamin D_3 and 25(OH)D_3 extraction from bovine plasma at physiological levels.

The HPLC apparatus was tested using ten injections of the same standard under the same conditions of the analyses described above. A standard containing both vitamin D_3 and 25(OH)D_3, at the concentration of 3.125 ng/ml was injected ten consecutive times. The resultant coefficient of variation for vitamin D_3 was 12.9 and for 25(OH)D_3 was 6.45. Thereafter, a standard containing a 100 ng/ml of both analytes was injected ten times. The coefficient of variation for vitamin D_3 was 4.9 and for 25(OH)D_3 was 2.17. Plasma from an untreated steer was processed under the same conditions of the standards and the analysis of the experimental samples and injected ten consecutive times. The coefficient of variation for vitamin D_3 was 12.2 and for 25(OH)D_3 was 5.23. Furthermore, plasma from five different steers (external to the experimental ones) was assayed in quadruplicate. The mean values for vitamin D_3 and 25(OH)D_3 concentrations were 12.4 ± 2.6 and 13.6 ± 1.5, with coefficient of variations of 11.6 and 4.3, respectively. This condition confirmed the good reproducibility of the analytical procedure.

Additionally, it was identified that after 60 to 80 injections, the column started to lose sensitivity, as the chromatographs showed no clear definition of the peaks (shoulders, wrong integration, etc.), and/or additional peaks, and/or the baseline started to lose its parallelism. These alterations could be due to accumulation of proteins in the column. For this reason, the system had to be washed in a cycle of methanol-double distilled water-acetonitrile for approximately 2 h every 60 to 80 injections or in some cases earlier, according to the characteristics of the chromatographs.

APPENDIX D

Determination of 1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]

Experiments 3 (Chapter V) and 4 (Chapter VI)

Serum concentrations of 1,25$(OH)_2D_3$ were determined by the 1,25-Dihydroxyvitamin D ^{125}I RIA Kit Catalog No. 65100E (DiaSorin Inc. Stillwater, MN. USA). The DiaSorin 1,25$(OH)_2D$ assay consists of a two-step procedure. The assay involves a preliminary extraction and subsequent purification of the vitamin D metabolites from serum using C_{18}OH "Extra Clean" cartridges. Following extraction, the treated sample is then assayed using a competitive RIA procedure. The RIA method is based on a polyclonal antibody that is specific for both 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$. The sample, antibody and tracer are incubated for 2 hours at 20-25°C. Phase separation is accomplished after a 20 min incubation at 20-25°C with a second antibody precipitating complex. After centrifugation and decantation, the bound fraction remaining in the pellet is counted in a gamma counter. Values are calculated directly from a standard curve of known concentrations. The final concentration of the 1,25$(OH)_2D$ in serum is expressed as pg/ml.

Material and Reagents:

- C_{18}OH "Extra Clean" cartridges.
- 1,25$(OH)_2D$ NSB Buffer.
- 1,25$(OH)_2D$ 0 Standard.
- 1,25$(OH)_2D_3$ Standards (A-E).
- 1,25$(OH)_2D$ Antiserum.
- ^{125}I 1,25$(OH)_2D_3$.
- 1,25$(OH)_2D_3$ Pretreatment Solution.
- 1,25$(OH)_2D$ Controls: Level 1 (normal), Level 2 (elevated).
- Goat Anti-Rabbit (GAR) Precipitating Complex.
- 95% Ethanol: 95% ethanol and 5% water.

Assay Procedure:

- Reagents were allowed to equilibrate to room temperature.
- Disposable borosilicate tubes, in duplicates, were labeled for each standard (zero, A-E), control and samples. 75 µL of the reconstituted standard, control and sample extracts were added into the duplicate assay tubes.
- 75 µL from the TC (Total Count) tube were added into duplicate assay tubes. This step was repeated for the NSB duplicate assay tubes.
- 300 µL of NSB buffer were added into the NSB tubes.
- 300 µL of the primary antibody were added into all tubes except the TC and NSB tubes.
- All tubes were thoroughly vortexed and then incubated for 2 hours (± 15 min) at 20-25^{0}C.
- All the tubes were centrifuged for 20 min at 20-25°C at 1800 x g, except the TC tubes.
- The GAR Precipitating Complex was reconstituted and mixed 5-10 min before use to ensure homogeneous suspension.
- 500 µL of the well-mixed GAR Precipitating Complex were added into all tubes except the TC tubes, which were incubated for 20 min (± 5 min) at 20-25°C.
- All the tubes were centrifuged for 20 min at 20-25°C at 1800 x g, except the TC tubes.
- The supernatants were decanted, except the TC tubes, using a foam rack tube holder by inverting the rack into a waste container. The inverted rack was place onto absorbent paper for 2-3 min, and the tubes were gently blotted to ensure that all liquid was removed.
- Radioactivity was measured by counting all tubes for 1 min on a gamma counter.
- Maximum binding: average counts per minute (CPM) of 0 standard tubes/average CPM of total count tubes.
- Nonspecific binding: average CPM of the NSB tubes/average CPM of total count tubes.

Performance Data:

- Sensitivity: ≤ 4.0 pg/ml. Defined as the lowest quantity differentiated from zero at 2 standard deviations below the means cpm of the zero standard.
- Accuracy: correlation coefficients of 0.86 and 0.96, when compared to the original and modified DiaSorin Radio-Receptor (RRA) kits, respectively.
- Specificity: assay highly specific to $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$ with cross-reactivity around 100%.
- Intraassay coefficient of variation of 2.0 %.

APPENDIX E

Determination of Acid-Base Balance Status

The acid-base balance status of the finishing steers was determined by analysis of whole blood samples using the Blood Gases Analysis (Radiometer 288 System, Copenhagen, Denmark):

5.1 pH Electrode System:

The acid-base characteristics of the blood is described by pH, which refers to hydrogen ion activity, that is, the product of hydrogen ion activity coefficient and the hydrogen ion concentration, which in turns determines the acidity of blood. For definition, the pH value increases with decreasing $[H^+]$ and vice versa. The pH measurement system consists of a measuring electrode, capable of detecting hydrogen ions, as well as a reference electrode. The pH electrode is a glass electrode, which develops a potential difference when the pH of the sample differs from the pH of the electrode fill solution.

5.2 Partial pressure of Carbon Dioxide (pCO_2) Electrode System:

Carbon dioxide (CO_2) is a natural product of cellular metabolism. Disturbance in the partial pressure of CO_2 in the blood indicate disorders in acid-base balance. The pCO_2 measurement system consists of a glass pH electrode separated from a sample by a membrane permeable to gaseous CO_2. The internal Ag/AgCl reference electrode provides a stable reference potential. A bicarbonate fill solution is utilized in the pCO_2 electrode. As CO_2 from the sample diffuses through the membrane and reaches equilibrium, the pH of the bicarbonate fill solution changes, and is detected by the glass pH electrode. The change in pH is related to the log of the partial pressure of CO_2.

5.3 Total Carbon Dioxide (tCO_2):

Total carbon dioxide (tCO_2), in combination with pH and pCO_2, is useful in distinguishing between metabolic and respiratory acid-base disorders. The following equation is used:

$$tCO_2 = (0.031\ pCO_2) + [HCO_3^-]$$

5.4 Partial pressure of oxygen (pO_2) Electrode System:

The extent of oxygen (O_2) exchange in the lungs and the ability of the blood to adequately perfuse the body tissues with O_2 may be assessed in part by determining the partial pressure of O_2 (pO_2) in whole blood. The pO_2 measurement system is based upon a O_2 electrode. This electrodes is amperometric, measuring the current produced by an electrolytic process which taker place due to the presence of O_2. The pO_2 electrode consists of a Pt (platinum) cathode, an Ag (silver) anode, an electrolyte fill solution and an O_2 permeable membrane. A constant voltage, called the polarizing voltage, is maintained between the anode and the cathode. When dissolved O_2 from the sample diffuses across the membrane into the fill solution, it is reduced at the cathode due to the applied voltage. The circuit is completed at the anode, when the Ag is oxidized. The magnitude of the resulting current is proportional to the pO_2 in the sample.

5.5 Bicarbonate (HCO_3^-):

Most of the CO_2 in the blood is present in the form of bicarbonate ions (HCO_3^-). The rate of HCO_3^- ions to pCO_2 in the blood depends upon the acid-base balance, and in conjunction with other data, is useful in assessing the extent to which metabolic renal control contribute to the acid-base disturbance that exists. The relationship of pCO_2 to HCO_3^- is described by the equation:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

5.6 Base Excess (BE):

The term BE refers to the expression which approximates the amount of acid or base which would be needed to titrate 1 L of blood back to a pH of 7.4. The BE in blood with a 7.4, pCO_2 of 40 mm Hg, tHb of 15 g/dL and a temperature of 37°C is zero. BE is

useful in the management of patients with acid-base disorders as it permits the estimation of the number of equivalents of $NaHCO_3$ or NH_4Cl required to correct pH to normal.

5.7 Anion Gap (AG):

The AG is an approximation of the difference between unmeasured cations and anions:

$$\text{Anion Gap} = (Na^+ + K^+) - (Cl^- + HCO_3^-)$$

Table E.1. The effect of anionic salts and vitamin D_3 supplementation on partial pressure of carbon dioxide (pCO_2) in blood of finishing Hereford steers (448 ± 26 kg).

pCO_2 (mm/Hg)									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A19	44.24	45.34	41.38	45.10	1.02				
Low Ca Diet Period (low Ca diet *ad libitum*) [y]:									
L7	44.14	43.72	42.96	42.84	0.95				
L14	43.56	42.70	43.22	44.12	1.10				
Supplemental Period (high Ca diet; D_3 treatments fed at 85% of *ad libitum*):									
	Vitamin D_3 Level (MIU/hd/d)								
	0	0.6	1.2	2.4					
- 1500 mEq/d:									
S1	43.82	43.42	43.68	45.50	0.99	0.7223	0.7431	0.8546	0.2107
S3	42.40	40.50	42.02	42.68	0.73	0.3869	0.4386	0.1586	0.5299
- 3000 mEq/d:									
S5	42.20	42.50	41.38	42.96	1.32	0.3006	0.9588	0.5574	0.4104
S7	40.50	40.88	41.78	42.22	1.37	0.1552	0.4868	0.6487	0.8233
S9	40.44	38.10	41.64	40.74	1.32	0.0133	0.8566	0.0763	0.6364
Withdrawal Period (high Ca diet *ad libitum*):									
W1	41.80	39.50	41.18	41.90	1.50	0.1700	0.5959	0.4413	0.7394
W5	45.16	44.46	44.90	44.88	1.24	0.1354	0.7768	0.8053	0.9911
Repeated Measures Analysis:									
				P value					
Treatment				0.5905					
Time				<0.0001					
Time x treatment				0.9145					

[z] Contrasts: **A** = Average low Ca period vs anionic salts (group1) / **B** = Control vs vitamin D_3 treatments / **C** = 0.6 vs 1.2 MIU D_3 / **D** =1.2 vs 2.4 MIU D_3

[y] Comparison: Average adaptation period vs low Ca period

(No difference (P>0.05) between groups at any day).

Table E.2. The effect of anionic salts and vitamin D_3 supplementation on total carbon dioxide (tCO_2) concentration in blood of finishing Hereford steers (448 ± 26 kg).

	tCO_2 (mmol/L)								
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A19	29.48	29.26	26.96	29.76	0.57				
Low Ca Diet Period (low Ca diet *ad libitum*) [y]:									
L7	28.10	28.28	28.10	27.92	0.50				
L14	28.08	26.58	27.40	27.10	0.78				
Supplemental Period (high Ca diet; D_3 treatments fed at 85% of *ad libitum*):									
	Vitamin D_3 Level (MIU/hd/d)								
	0	0.6	1.2	2.4					
- 1500 mEq/d:									
S1	27.72	27.16	27.26	27.54	0.62	0.9860	0.5846	0.9108	0.7540
S3	26.56	25.56	25.92	26.72	0.78	0.4270	0.5907	0.7479	0.4779
- 3000 mEq/d:									
S5	24.18	24.22	23.64	25.96	0.78	0.0167	0.6435	0.6078	0.0525
S7	21.24	21.34	20.64	23.04	0.82	<0.0001	0.6536	0.5549	0.0552
S9	21.00	19.90	20.46	22.22	1.26	<0.0001	0.9246	0.7576	0.3385
Withdrawal Period (high Ca diet *ad libitum*):									
W1	21.98	20.52	20.92	22.18	1.60	0.0002	0.6818	0.8622	0.5862
W5	31.60	30.60	29.38	30.08	1.80	0.0083	0.4584	0.6384	0.7869
Repeated Measures Analysis:									
				P value					
Treatment				0.6815					
Time				<0.0001					
Time x treatment				0.9460					

[z] Contrasts: **A** = Average low Ca period vs anionic salts (group1) / **B** = Control vs vitamin D_3 treatments / **C** = 0.6 vs 1.2 MIU D_3 / **D** =1.2 vs 2.4 MIU D_3

[y] Comparison: Average adaptation period vs low Ca period

(Only group 2 had lower ($P<0.05$) concentration on day L14).

Table E.3. The effect of anionic salts and vitamin D_3 supplementation on partial pressure of oxygen (pO_2) in blood of finishing Hereford steers (448 ± 26 kg).

	pO_2 (mm/Hg)								
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A19	38.48	36.30	35.88	35.60	2.90				
Low Ca Diet Period (low Ca diet *ad libitum*) [y]:									
L7	34.36	34.80	34.88	35.06	1.24				
L14	32.92	34.08	36.22	33.92	1.18				
Supplemental Period (high Ca diet; D_3 treatments fed at 85% of *ad libitum*):									
	Vitamin D_3 Level (MIU/hd/d)								
	0	0.6	1.2	2.4					
- 1500 mEq/d:									
S1	43.06	36.86	43.88	41.16	3.31	<0.0001	0.5345	0.1532	0.5694
S3	32.96	35.72	35.14	33.34	0.98	0.3226	0.1368	0.6813	0.2126
- 3000 mEq/d:									
S5	37.32	35.36	37.70	36.40	1.75	0.0821	0.6862	0.3595	0.6074
S7	37.24	36.66	36.84	37.74	1.57	0.0910	0.9309	0.9365	0.6912
S9	35.26	36.78	33.88	37.34	1.56	0.6442	0.6860	0.2062	0.1355
Withdrawal Period (high Ca diet *ad libitum*):									
W1	36.50	38.22	36.34	36.38	1.93	0.2159	0.8204	0.4778	0.9885
W5	35.60	35.84	36.02	38.30	1.36	0.4990	0.4864	0.9267	0.2535
Repeated Measures Analysis:									
				P value					
Treatment				0.9655					
Time				<0.0001					
Time x treatment				0.4659					

[z] Contrasts: **A** = Average low Ca period vs anionic salts (group1) / **B** = Control vs vitamin D_3 treatments / **C** = 0.6 vs 1.2 MIU D_3 / **D** =1.2 vs 2.4 MIU D_3

[y] Comparison: Average adaptation period vs low Ca period

(Only group 4 had lower ($P<0.05$) concentration on day L14)

Table E.4. The effect of anionic salts and vitamin D_3 supplementation on blood bicarbonate (HCO_3^-) concentrations of finishing Hereford steers (448 ± 26 kg).

HCO_3^- (mmol/L)									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A19	28.14	27.82	25.70	27.34	0.56				
Low Ca Diet Period (low Ca diet *ad libitum*) [y]:									
L7	26.74	26.96	26.76	26.60	0.49				
L14	26.72	25.32	26.04	25.74	0.75				
Supplemental Period (high Ca diet; D_3 treatments fed at 85% of *ad libitum*):									
	Vitamin D_3 Level (MIU/hd/d)								
	0	0.6	1.2	2.4					
- 1500 mEq/d:									
S1	26.38	25.86	26.30	26.14	0.65	0.9885	0.7126	0.6369	0.8633
S3	25.22	24.30	24.42	25.40	0.77	0.4133	0.5700	0.9132	0.3793
- 3000 mEq/d:									
S5	22.86	22.90	22.36	24.62	0.76	0.0147	0.6277	0.6218	0.0514
S7	20.00	20.00	19.32	21.70	0.79	<0.0001	0.7134	0.5501	0.0484
S9	09.72	18.70	19.14	20.94	1.22	<0.0001	0.9294	0.8019	0.3121
Withdrawal Period (high Ca diet *ad libitum*):									
W1	20.70	19.30	19.62	20.90	1.56	0.0002	0.6789	0.8866	0.5702
W5	30.20	29.22	27.98	28.72	1.77	0.0078	0.4557	0.6266	0.7710
Repeated Measures Analysis:									
					P value				
Treatment					0.6804				
Time					<0.0001				
Time x treatment					0.9375				

[z] Contrasts: **A** = Average low Ca period vs anionic salts (group1) / **B** = Control vs vitamin D_3 treatments / **C** = 0.6 vs 1.2 MIU D_3 / **D** =1.2 vs 2.4 MIU D_3

[y] Comparison: Average adaptation period vs low Ca period

(Only group 2 had lower ($P<0.05$) concentration on day L14).

Table E.5. The effect of anionic salts and vitamin D_3 supplementation on blood base excess (BE) concentrations of finishing Hereford steers (448 ± 26 kg).

Base Excess (mmol/L)									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A19	3.76	3.14	1.48	2.58	0.62				
Low Ca Diet Period (low Ca diet *ad libitum*) [y]:									
L7	2.08	2.46	2.36	2.22	0.62				
L14	2.20	0.60	1.44	0.80	0.81				
Supplemental Period (high Ca diet; D_3 treatments fed at 85% of *ad libitum*):									
	Vitamin D_3 Level (MIU/hd/d)								
	0	0.6	1.2	2.4					
- 1500 mEq/d:									
S1	1.68	1.08	1.64	1.00	0.80	0.9545	0.6401	0.6272	0.5793
S3	0.58	-0.06	-0.24	0.78	0.87	0.4518	0.6810	0.8853	0.4186
- 3000 mEq/d:									
S5	-2.38	-2.38	-2.80	-0.24	0.79	0.0110	0.5372	0.7110	0.0353
S7	-5.76	-5.74	-6.96	-3.82	0.94	<0.0001	0.8186	0.3730	0.0314
S9	-6.16	-6.88	-7.22	-4.56	1.37	<0.0001	0.9703	0.8631	0.1893
Withdrawal Period (high Ca diet *ad libitum*):									
W1	-5.52	-6.52	-6.42	-5.04	1.78	<0.0001	0.7480	0.9689	0.5919
W5	5.82	4.84	3.34	4.16	1.91	0.0130	0.4496	0.5857	0.7650
Repeated Measures Analysis:									
				P value					
Treatment				0.6862					
Time				<0.0001					
Time x treatment				0.8692					

[z] Contrasts: **A** = Average low Ca period vs anionic salts (group1) / **B** = Control vs vitamin D_3 treatments / **C** = 0.6 vs 1.2 MIU D_3 / **D** =1.2 vs 2.4 MIU D_3

[y] Comparison: Average adaptation period vs low Ca period

(Only group 2 and 4 had lower ($P<0.05$) concentration on day L14).

Table E.6. The effect of anionic salts and vitamin D_3 supplementation on anion gap (AG) concentration in blood of finishing Hereford steers (448 ± 26 kg).

Anion Gap (mmol/L)									
	Group					Contrast [z]			
Days	1	2	3	4	*SEM*	A	B	C	D
Adaptation Period (normal Ca diet *ad libitum*):									
A19	12.46	12.50	13.40	11.84	1.04				
Low Ca Diet Period (low Ca diet *ad libitum*) [y]:									
L7	9.60	9.52	10.06	9.18	9.18				
L14	10.96	11.06	9.94	9.64	9.64				
Supplemental Period (high Ca diet; D_3 treatments fed at 85% of *ad libitum*):									
	Vitamin D_3 Level (MIU/hd/d)								
	0	0.6	1.2	2.4					
- 1500 mEq/d:									
S1	11.90	12.02	12.18	11.46	1.28	0.1203	0.9929	0.9305	0.6955
S3	14.50	15.58	16.42	1308	1.28	0.0005	0.7264	0.6491	0.0838
- 3000 mEq/d:									
S5	16.88	17.48	16.98	15.72	1.00	<0.0001	0.8955	0.7271	0.3840
S7	12.86	14.74	15.38	13.14	0.95	0.0215	0.2361	0.6343	0.0406
S9	11.02	12.50	12.52	9.72	0.86	0.3990	0.5807	0.9871	0.0351
Withdrawal Period (high Ca diet *ad libitum*):									
W1	12.24	14.02	14.84	12.30	1.01	0.0685	0.2209	0.5725	0.0932
W5	10.28	11.68	11.94	10.84	1.10	0.8139	0.3550	0.8690	0.4885
Repeated Measures Analysis:									
				P value					
Treatment				0.3705					
Time				<0.0001					
Time x treatment				0.4644					

[z] Contrasts: **A** = Average low Ca period vs anionic salts (group1) / **B** = Control vs vitamin D_3 treatments / **C** = 0.6 vs 1.2 MIU D_3 / **D** =1.2 vs 2.4 MIU D_3

[y] Comparison: Average adaptation period vs low Ca period

(Only group 1 and 2 had no lower (P>0.05) concentrations on day L14).