Chapter 1. Introduction

Calcium and phosphorus are the two most abundant minerals in mammals. They are the primary constituents of the skeleton and are involved in many biological activities. The metabolic control of Ca, and to a lesser extent P, is finely tuned and controlled by various hormones. Vitamin D, and its metabolites, is one of the molecules that are critical for effective control of digestion and metabolism of both Ca and P.

Vitamin D is generated in mammals in the skin when they have access to direct sun light. Animals confined in doors, and also in winter, may have lower concentrations of plasma 25-vitD than ideal. However, increased levels of productivity in ruminants may have developed situations for supraphysiological (Greater than 100 ng/ml) plasma concentrations of 25-vitD may be beneficial.

In intensive pasture systems Ca and P are usually readily available in pasture and generally do not limit productivity. However, in the modern dairy cow milk fever, or hypocalcaemia, at parturition has become the cause of a major reduction in productivity and a significant animal health concern. Hypocalcaemia is estimated to cost $334 per incident (Guard, 1996) and shortens the productive life of the dairy cow by 3.4 years (Payne, 1968, Chan *et al.*, 2006).

The cause of the acute deficit of Ca experienced during hypocalcaemia is complex, however, it is basically a product of the cows inability to generate available Ca fast enough to meet the initial requirements of Ca. It is not due to a deficiency of Ca in the diet. As Vitamin D is highly involved with Ca metabolism, and more practical metabolites of Vitamin D have been developed, potential benefits of pharmacological treatments with Vitamin D are worthy of further investigation (Horst 1986).

Furthermore, in beef cattle under extensive grazing systems P deficiency is the cause of a major loss of productivity, which may halve the gross profit margin of a grazing enterprise (Holmes, 1990). The primary cause is a result of a reduction in feed intake that leads to a reduction in weight gain and subsequently a drop in fertility of females. For example, pastures deficient in P have been shown to reduce live weight gains in heifers by 60-80 kg/annum (Coates, 1994).

As vitamin D has been demonstrated to increase the rate of P absorption it is logical that investigation into the potential benefits be conducted. An increase in the utilisation of dietary P by bovine would have major economic and environmental benefits.

1.1 Objectives of the study

Calcium and P are both important minerals in bovine and play important roles in animal health and production. Both minerals are predominately under the control of the thyro-parathyroid glands and the subsequent control of Vitamin D metabolism.

With the advent of stable 25-vitD products, further investigation of therapeutic supplementation is warranted. Furthermore, with the knowledge that supraphysiological concentrations of 25-vitD in plasma can activate Vitamin D mediated responses without the required PTH secretion it is now logical that treatment with 25-vitD may improve both active P absorption and the activation of Ca availability in ruminants.

The primary hypotheses tested in this study include:

a) Dietary 25-vitD and dietary anionic salts increase urinary Ca excretion greater than anionic salts alone.
b) Dietary 25-vitD increased dietary Ca and P absorption and retention.
c) Intra-ruminal supplementation of 25-vitD increases the concentration of 25-vitD in plasma and increased urinary Ca in steers.
d) Different sources of 25-vitD have similar efficacy of conversion from diet to plasma.

Chapter 2. Literature Review

2.1 Vitamin D and its role in the ruminant

2.1.1 Overview of Vitamin D

Vitamin D is produced within the body from exposure of skin to ultraviolet light. The epithelial cells of the skin synthesize the immediate precursor of Vitamin D, 7-dehydrocholesterol from acetate (Fraser, 1980). Exposure of the skin to ultraviolet light results in cleavage of the C-9 to C-10 bond of 7-dehydrocholesterol, which results in the formation of Vitamin D (Fraser, 1980). Vitamin D can be supplemented in the diet by commercially available crystalline forms (Horst, 1986), which are essential for many modern day production systems that involve housed animals without natural light. Once adequate production of Vitamin D is obtained further production of Vitamin D from prolonged exposure to ultraviolet light is controlled by continued exposure to ultraviolet light (Webb *et al.*, 1989).

Vitamin D is absorbed from the intestinal tract in association with dietary lipids and the presence of bile salts, like the other fat soluble vitamins (NRC, 2000). While there is evidence of metabolism of Vitamin D within the rumen, the mechanisms are not fully understood. Sommerfeldt *et al.* (1979) demonstrated that radioactive Vitamin D was degraded within rumen fluid. However, recent research by Hymoller and Jensen (2010) identified that a single large dose (250 mg) of Vitamin D is not degraded by rumen fluid over a 24h period and is readily absorbed. There have been very few published studies investigating the degradability of 25-vitD within the rumen. In humans lower gastro intestinal tract health problems have been shown to inhibit the absorption of Vitamin D (Davies *et al.*, 1980).

In the blood Vitamin D circulates at relatively low concentrations (1 to 3 ng/ml) in cows (Horst *et al.*, 1981). Vitamin D is transported in blood the liver and is associated with a specific α globulin called *transcalciferin* (also known as the Vitamin D binding protein (VDP)), a molecule synthesised by the liver (Ponchon and DeLuca, 1969).

The liver first hydroxylates the Vitamin D at C-25 by microsomal enzymes which converts the Vitamin D to 25-vitD (Ponchon and DeLuca, 1969). This process does not appear to be under hormonal control and is substrate driven (Goff *et al.*, 1991a).

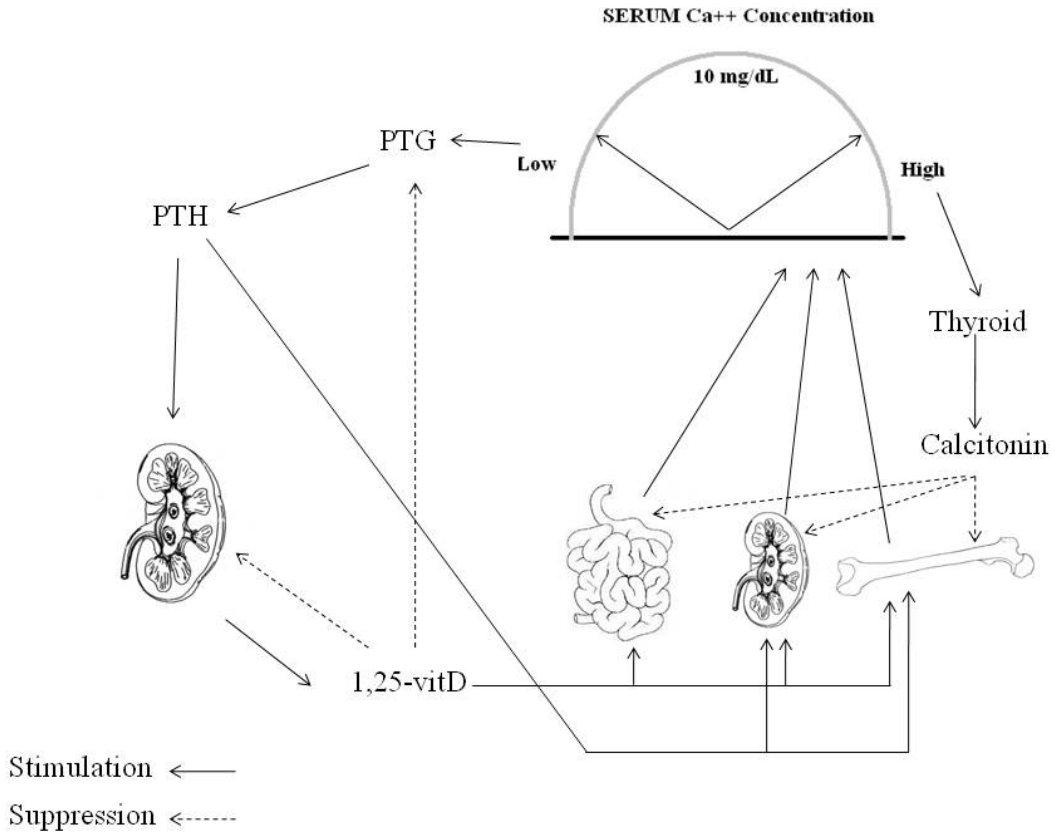

Figure 2.1 The vitamin D endocrine system. Low serum Ca concentrations are detected by a Ca sensor in the parathyroid gland (PTG), which initiates synthesis and secretion of parathyroid hormone (PTH). The Ca sensor for high concentrations in the thyroid gland initiates secretion of calcitonin (adapted from DeLuca, 2004).

Under normal conditions the majority of Vitamin D in circulation is present as 25-vitD until it is required for activation (Hove *et al.*, 1984). 25-vitD may be metabolised into a vast range of metabolites in the kidney, usually the only product of interest is the active form of Vitamin D, 1,25-vitD (Fraser, 1980, Horst, 1986). Another important metabolite is 24,25 dihydroxy vitamin D (24,25-vitD), which is the main pathway for degradation of Vitamin D and is quickly excreted through bile. When 25-vitD is metabolised into 24,25-vitD it is due to a relatively high level of 1,25-vitD circulation providing negative feedback (Shinki *et al.*,

1992). It appears that this mechanism aims to eliminate excessive levels of 1,25-vitD and 25-vitD from circulation.

1, 25 dihydroxy vitamin D circulates within plasma in extremely low concentrations (pg/ml). 1,25-vitD initiated immediate healing in patients and therefore proved to be the most active of the metabolites in a study comparing treatments for vitamin D dependent rickets with other Vitamin D metabolites (Fraser *et al.*, 1973).

2.1.2 25-vitD as an indicator of Vitamin D status

1,25-vitD is the Vitamin D metabolite of most activity and therefore most physiological importance. However, the measurement of 25-vitD is often preferred over 1,25-vitD for assessment of Vitamin D status, as the concentration of 25-vitD in plasma is much more consistent and circulating levels of 1,25-vitD are low and may be bound in tissues due to the affinity of Vitamin D receptors (VDR) (Christakos *et al.*, 2010). 25-vitD will usually have plasma concentrations of approximately 10-50 ng/ml and a 15-30d half-life in humans (Fraser, 1980) and approximately 34d in bovine (Hollis *et al.*, 1977). In comparison, 1,25-vitD concentrations are very low (eg. 10-100 pg/ml) and a very short half-life of approximately 5-8h in humans (Fraser, 1980) and sheep (Wilkens *et al.*, 2010).

The hydroxylation of Vitamin D into 25-vitD does not seem to be a controlled step and appears to reflect the vitamin D status of the animal (Goff *et al.*, 1991a, DeLuca, 2008), therefore the plasma concentration of 25-vitD is an excellent indicator of Vitamin D status and, unlike 1,25-vitD, it is not impacted by secretion of PTH or cacitonin. In humans the normal level is considered to be 25-35 ng/ml, but for protection against degenerative diseases higher levels (75-90 ng/ml) of 25-vitD appear to be desirable (DeLuca, 2008).

2.1.3 Metabolism of 25-vitD to 1,25-vitD

The vitamin D molecule 25-vitD is relatively inactive and must be hydroxylysed by the kidney before the molecule is biologically active. Hydroxylation in the kidney occurs at C-1 to produce the active compound, 1,25-vitD (Fraser, 1980). Control of the mitochondrial C-1 hydroxylase within the kidney by PTH is the most important control mechanism for the synthesis of 1,25-vitD (Horst, 1986, Goff *et al.*, 1991a). Decreases in the plasma Ca concentration stimulate PTH secretion, which in turn favours the synthesis of 1,25-vitD and increased intestinal absorption of Ca. In the proximal tubule cells, PTH stimulates the

CYP27B1 gene, producing the 1α-hydroxylase that causes synthesis of 1,25-vitD (Guo *et al.*, 1993). Although complete control of 1α-hydroxylase is not PTH dependent, inadequate secretion of PTH does result in lower levels of 1α-hydroxylase and subsequently lower 1,25-vitD (Horst, 1986) and as the primary driver of Ca homeostasis (Liesegang and Risteli, 2005) it will cause hypocalcaemia. Goff and Horst (1989) showed that 1,25-vitD concentration was steadily reduced in PTH infused cows, identifying the negative feedback of 1,25-vitD on PTH secretion. This demonstrates that PTH is only stimulatory to 1α-hydroxylase during hypocalcaemic events and that 1,25-vitD has an inherent negative feedback method that limits its production.

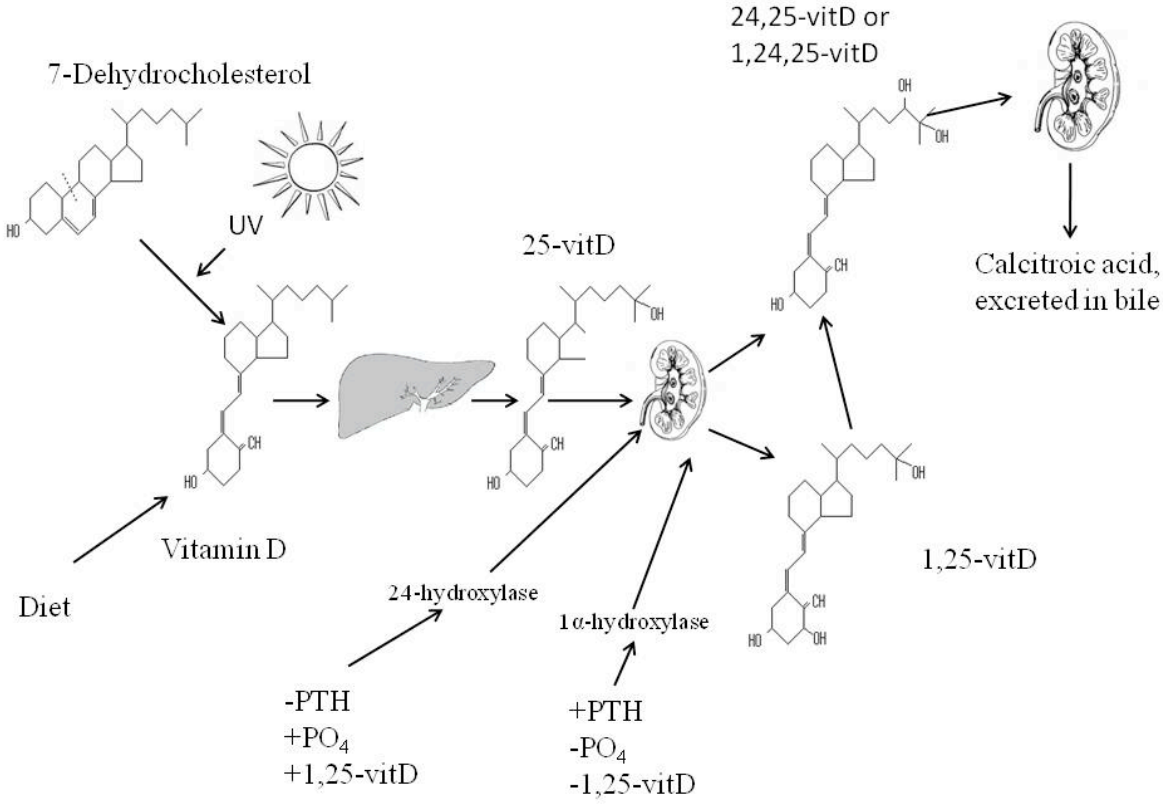

Figure 2.2 Synthesis and metabolism of vitamin D. Vitamin D is hydroxylated to 25-vitD in the liver. Plasma concentrations of PTH, PO_4 and 1,25-vitD determine the hydroxylation position of 25-vitD in the kidney. Hydroxylation of 25-vitD to 1,25-vitD utilises the enzyme 1α-hydroxylase, whereas hydroxylation to 24,25-vitD requires 24-hydroxylase (adapted from a combination of Cunningham, 1997 and Christakos *et al.*, 2010).

Other factors that control the rate of conversion of 25-vitD to 1,25-vitD may include plasma P concentration and various hormonal factors. Plasma phosphate regulates vitamin D metabolism in monogastrics via stimulation of the enzyme 1α-hydroxylase in kidneys (Horst, 1986) but not in ruminants (Breves *et al.*, 1985) However high concentrations of P may inhibit the production of 1α-hydroxylase in ruminants (Goff, 2000) as it does in rats (Tanaka and Deluca, 1973). Other sources of hydroxylation control include hormones involved with pregnancy such as growth hormone and prolactin increasing 1,25-vitD production by stimulation of C-1 hydroxylation (Christakos *et al.*, 2010). However their level of control over C-1 hydroxylation is minimal.

Monocytes and macrophages express 1α-hydroxylase and produce 1,25-vitD, however the regulation is controlled differently than renal hydroxylation (Stoffels *et al.*, 2007). Unlike renal, macrophage hydroxylation is not inhibited by elevated plasma Ca or positive feedback from 1,25-vitD and is upregulated by immune stimuli such as interferon-y and lipopolysaccharide (Christakos *et al.*, 2010).

Down regulation and elimination of Vitamin D

When plasma Ca concentration is adequate the parathyroid sensor is shut down stopping the secretion of PTH (**Error! Reference source not found.**). One of the important features of 1,25-vitD is to provide negative feedback, shutting down the preproparathyroid gene and preventing proliferation of parathyroid cells in response to hypocalcaemia (DeLuca, 2008). This negative feedback prevents over stimulation of Ca generating mechanisms and will usually prevent over correction of plasma Ca concentration, which would result in hypercalcaemia. However, if elevated concentrations of plasma Ca are maintained, the secretion of calcitonin from the "C" cells of the thyroid is promoted (DeLuca, 2008). Calcitonin completes the feedback loop by maintaining/returning Ca at/to the appropriate level by stopping the mobilisation of Ca from bone and the resorption of Ca in the kidney (DeLuca, 2004).

1,25-vitD is self-regulating with high concentrations of 1,25-vitD in plasma providing negative feedback and inhibiting further production. High concentrations of plasma 1,25-vitD result in hydroxylation of 25-vitD at the C-24 position instead of the C-1 position creating 24,25-dihydroxycholecalciferol (24,25-vitD) instead of the 1,25-vitD (Christakos *et al.*, 2010). The enzyme responsible for this, 24-hydroxylase, is only active in the presence of

Vitamin D and is activated by 1,25-vitD and its analogs working through the VDR (Ohyama *et al.*, 1991). The 24-hydroxylase preferred substrate is 1,25-vitD, meaning that high concentrations of 1,25-vitD result in greater production of the enzyme (Christakos *et al.*, 2010), hence the negative feedback effect. The enzyme causes metabolism of 1,25-vitD into calcitroic acid (1,24,25-vitD), which is eliminated through bile into the digestive tract (Figure 2.1). This process accounts for nearly all of the 1,25-vitD metabolism (Shinki *et al.*, 1992).

There does not appear to be any physiological role for 24,25-vitD besides elimination of Vitamin D from the body, although Horst (1986) suggested that 24,25-vitD may have had a role in bone accretion and other physiological processes. However, this has more recently shown not to be the case (DeLuca, 2008). There is also a negative feedback loop between 1,25-vitD and Fibroblast Growth Factor (FGF23) (Christakos *et al.*, 2010), as elevated concentrations of FGF23 (>4000 U/ml) decreased the concentration of 1,25-vitD (from ~120 to ~70 pg/ml) in mice (Bai *et al.*, 2004).

Vitamin D Receptor (VDR) response

The active form of Vitamin D is primarily involved with Ca and P homeostasis, which involves the small intestine, skeletal and renal system. For 1,25-vitD to be effective it requires receptor sites. In the rat VDR concentrations are the highest in the duodenum followed by the colon and then kidney and skin (DeLuca, 2008). The concentration of VDR at different sites in the body is highly variable and the concentration changes in response to circulating levels of 1,25-vitD and potentially other unknown products (Horst *et al.*, 1990, Goff *et al.*, 1995).

VDR concentration appears to rely on the presence of Ca and 1,25-vitD for maximum response (Figure 2.3). While it is generally accepted that the presence of 1,25-vitD increases the concentration of VDR in target organs (eg. small intestine and kidney), the presence of Ca is also required (Figure 2.3). However, the concentration of VDR also appears to be dependent on demand for Ca. In moderately Ca deficient animals (dietary Ca 0.25%), the concentration of VDR was much higher than in animals with access to diets with more typical Ca concentrations (eg. 1.2%) (DeLuca, 2008). This is an important consideration for understanding vitamin D mediated Ca absorption in ruminants as it demonstrates that while elevated concentrations of 1,25-vitD increase VDR concentration in target organs it is dependent on other factors such as dietary Ca concentration and potentially Ca demand.

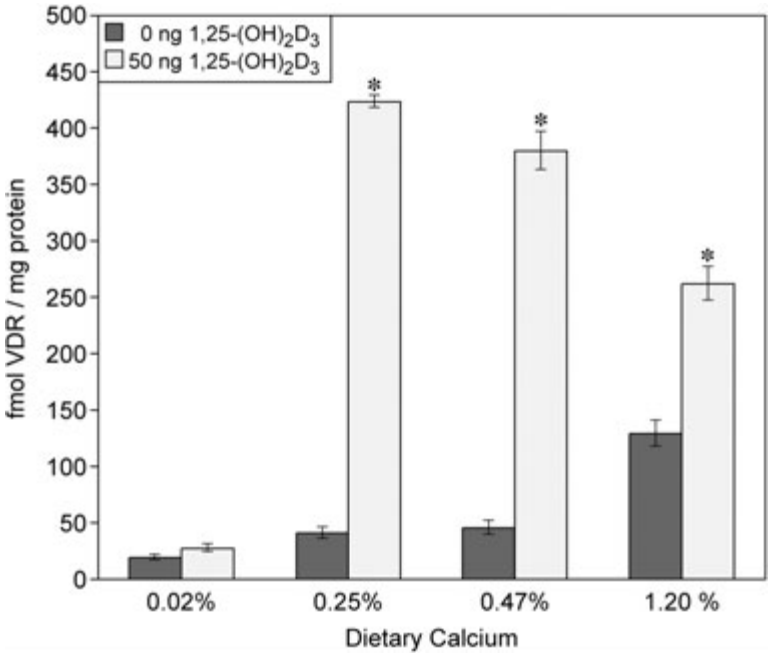

Figure 2.3 The effect of dietary calcium and 1,25-vitD on VDR in rat kidney. In the case of a 0.02% Ca diet and lack of 1,25-vitD, severe hypocalcaemia results with serum Ca levels of between 4 and 5 mg/dL. This represents regulation of the renal receptor by serum Ca levels and 1,25-vitD (DeLuca, 2008).

VDR concentrations decrease with age in humans, rats and cows (Horst *et al.*, 1990, Ebeling *et al.*, 1992, Goff *et al.*, 1995). The concentrations of VDR follow this pattern in both bone and the duodenum. Studies show that younger rats (4 to 5 weeks old) are able to increase their concentration of VDR by a greater proportion than adult rats when stimulated by 1,25-vitD (Horst *et al.*, 1990).

Goff (1995) monitored the concentration of VDR in colon mucosa of Jersey dairy cows during late gestation, parturition and early lactation and compared these values with non-pregnant non-lactating values. VDR concentrations were significantly greater than non-pregnant, non-lactating values on all occasions, regardless of incidence of clinical hypocalcaemia. A significant drop in VDR concentration was seen at parturition but that quickly recovered during early lactation. Neither the incidence of clinical hypocalcaemia nor the addition of anionic salts had an effect on VDR concentration at any stage (Goff *et al.*, 1995). The colon is not a major site of absorption but it may indicate the pattern of VDR in

duodenum and in rats evidence exists that the concentration is usually 60 to 70% of duodenal concentration (Goff *et al.*, 1995).

2.1.4 Vitamin D and mineral absorption from the alimentary tract

The most important effects of vitamin D are concerned with increased absorption of both Ca and P from the alimentary tract. When an animal is fed a diet containing less Ca than it requires, the proportion of dietary Ca absorbed is increased. Conversely when an animal consumes more Ca than needed, the proportion of Ca absorbed decreases (Horst, 1986, Liesegang and Risteli, 2005). Vitamin D is essential for increasing the proportion of absorbed Ca when dietary Ca concentration is low. 1,25-vitD stimulates the synthesis of the proteins that control active intestinal Ca absorption (DeLuca, 2004). Increased 1,25-vitD leads to the correction of hypocalcaemia by increasing the absorption of Ca from the gut (via 1,25-vitD) and resorption of Ca from bone when initiated as functions of PTH secretion (Fig 1.1) (Block, 1984, Gaynor *et al.*, 1989).

Active absorption of Ca from the small intestine is controlled via 1,25-vitD (Cai *et al.*, 1993). Transcellular transport of Ca involves a three step process, which includes entry of Ca from the gastro-intestinal lumen through the Ca channels TRPV5 and TRPV6 (van Abel *et al.*, 2003), translocation of Ca from the brush border to the basolateral membrane by the Ca-binding protein calbindin D9k (Bronner, 1987) and finally extrusion of Ca through the basolateral membrane by the Ca ATPase PMCA1b (Pannabecker *et al.*, 1995). However, much of the Ca digestion in ruminants, both active and passive, is prior to the small intestine (Khorasani and Armstrong, 1992, Schröder *et al.*, 1997) and there has been no clear description of the cellular process in the reticulo-rumen. In sheep Ca absorption was only increased by supplemented 1,25-vitD when consuming an adequate Ca diet (Wilkens *et al.*, 2010), suggesting that active mechanisms may not have adequate volume to meet total Ca requirements and that some passive diffusion is required.

The rate of P absorption in ruminants is negatively correlated with dietary concentration (Horst, 1986). However, the reduction in the rate of P absorption does not outweigh total P absorption, hence dietary absorption of P is still highest on high P concentration diets (i.e. >0.4% P). A decline in plasma phosphate concentrations results in decreased inhibition of the C-1 hydroxylation, which indirectly results in increased 1,25-vitD production and increased absorption of phosphate. This process is only effective in monogastrics. However, in

ruminants high plasma P concentration will inhibit 1,25-vitD production via this mechanism, but low P concentrations will not promote the production of 1,25-vitD via this method (Breves *et al.*, 1985). This appears to be one of the reasons why ruminants cannot increase the rate of active absorption of P when consuming low P diets.

Like Ca, P transport is conducted actively and passively. Passive absorption is via paracellular transport, which is associated with passive ion movement through tight junctions between adjacent epithelial cells in response to electrochemical gradients (Sabbagh *et al.*, 2009). The active process is stimulated by 1,25-vitD in the small intestine (Wasserman and Taylor, 1973), and is effective on both low and adequate P concentrations in ruminants (Wilkens *et al.*, 2010). In rats, rabbits and humans it is suggested that active absorption, linked to sodium dependent mechanisms, may contribute as much as 33 to 75% of total intestinal phosphate absorption (McHardy and Parsons, 1956, Walton and Gray, 1979, Danisi and Straub, 1980). There are several mechanisms responsible for the control of sodium dependent phosphate transport. One of the main mechanisms is the sodium-dependent phosphate co-transporter NPT2b (Sabbagh *et al.*, 2009). NPT2b has been shown to be primarily under the control of 1,25-vitD (Hildmann *et al.*, 1982, Katai *et al.*, 1999), although both estrogen and epidermal growth factor have an influence (Xu *et al.*, 2001, Xu *et al.*, 2003). Sabbagh *et al.* (2009) suggests that NPT2b dependent phosphate transport may contribute as much as 45% of total phosphate transport within the first hour of a meal and as much as 90% of active absorption in mice. Hence, if dietary P concentration is adequate or in excess then the rate of P absorption is drastically reduced in the absence of 1,25-vitD and therefore NPT2b.

The cellular activity of P transport in the alimentary tract of bovine is poorly described and most research is confined to humans and rats. However, Foote *et al*. (2011) have described the relative concentrations of NPT2b in bovine small intestine as being almost solely located in the distal jejunum and the ileum, and unlike other highly studied animals, not present in the proximal small intestine.

2.1.5 Ca and P resorption from bone

Hormonal control of bone breakdown

Vitamin D is an active component bone remodeling. Ca is obtained from bone by osteoclastic osteolysis (Christenson, 1997). Many researchers have tried to link bone

formative or degradative markers with the level of 25-vitD, 1,25-vitD, plasma Ca and P or even time relative to parturition, without consensus.

Osteoblasts contain receptors for PTH and 1,25-vitD unlike osteoclasts, which only have receptors for calcitonin (Goff *et al.*, 1991a). Osteoclasts only perform their bone degrading activity (acid release and lysosomal enzymes) under osteoblast direction. Continuous secretion of PTH will increase 1,25-vitD production after several hours (Goff *et al.*, 1986), which further enhances osteoclast activity. Calcitonin decreases the activity of osteoclasts (Goff *et al.*, 1991a).

Table 2.1 Dose-dependent effects of orally administered 1α,25-dihydroxyvitmain D3 on serum Ca and RANKL mRNA expression in bone (Suda *et al.*, 2003).

		Normal rats	Thyro-parathyroidectomised rats				
PTH Continuously infused	50 ng/h	-	-	+	+	+	+
1,25-vitD orally administered	ug/kg/d	-	-	-	0.01	0.1	0.5
Serum Ca	mg/dl	9.6	5.1	10.4	11.1	11.2	14.6
Bone RANKL mRNA expression		-	-	+++	+	+	+++

In a review by Horst (1986) it was stated that 1,25-vitD and PTH were required for bone resorption. More recent research has defined the homeostatic control of bone resorption and accretion. PTH is critical to bone resorption when extracellular Ca levels are deficient (Suda *et al.*, 2003) but unlike Horst's (1986) review, 1,25-vitD is now widely considered critical to bone accretion when dietary Ca is sufficient and influential on resorption when dietary Ca is deficient and PTH present (DeLuca, 2008). The role of 1,25-vitD is not direct as it is more a result of providing ample plasma Ca and P supplies to allow bone accretion (DeLuca, 2008).

From work with rats 1,25-vitD has been shown to have both resorption and accretive effects on bone (Suda *et al.*, 2003). Low dose rates of 1,25-vitD appear to reduce the effect (in thyro-parathyroidectomised (TPTX) rats that were continuously infused with PTH) of PTH as bone resorption is decreased and serum Ca is maintained in the normal range (Table

2.3) (Suda *et al.*, 2003). However, normal physiological doses inhibit PTH and increase serum Ca which leads to bone accretion (Suda *et al.*, 2003). Although, large supraphysiological doses of 1,25-vitD combined with regular administration of PTH will increase bone resorption (Suda *et al.*, 2003). This was confirmed by Wilkens *et al.* (2010) who found that in sheep 1,25-vitD increased serum cross laps, a marker for bone resorption, when combined with a low Ca diet. This suggests that PTH was secreted in response to low dietary Ca.

Table 2.2 Description of bone accretion and degradation markers.

	Name	**By-product of**	**Reference**
Bone Degradation	CTx	A eight amino-acid sequence in the C-terminal region of the collagen Type I chain	Christenson, 1997 Allen, 2003 Liesegang *et al.*, 2003
	ICTP	Type I collagen but via a different enzymatic process to CTx.	Christenson, 1997 Allen, 2003 Liesegang *et al.*, 2003
	Deoxypyridinoline or pyridinoline	A peptide bound molecule that results from the degradation of type I collagen	Moreira *et al.*, 2009
	Hydroxyproline	Originates from collagen breakdown. Several sources within the body may result in error.	Gaynor *et al.*, 1989 Schonewille *et al.*, 1994 Joyce *et al.*, 1997
Bone Accretion	Alkaline phosphatase (bALP)	A membrane bound protein that is synthesised by cells in a variety of tissues. Effective marker of osteoblastic activity.	Allen, 2003
	Osteocalcin	Released during osteoblastic activity	Ristelli and Ristelli, 1993
	Propeptides	Late stage bone accretion results in release of propeptides from the creation of amino and carboxy termini of collagen type I	Allen, 2003

From recent research and re-evaluation of past studies it appears that when dietary Ca is limiting and continual secretion of PTH and 1,25-vitD secretion does not adequately increase the concentration of plasma Ca the two hormones then progress to degrade the skeleton to adequately maintain plasma Ca. The implications of this are that treatment of animals with moderate amounts of Vitamin D, and a diet adequate in Ca, should not result in degradation of bone stores and may increase Ca deposition.

Bone degradation and formation markers

Research involving Ca and P metabolism often requires knowledge of the impact of experimental regimes on the status of Ca and P in skeletal storage. Direct assessment of bone densitometry is invasive, expensive and very stressful for animals (Cohen, 1973) and radiography is impractical for animals and only provides information on net results (Allen, 2003). Markers that are easily determined via serum and urinary analysis are essential for the effective diagnosis and regulation of osteoporotic diseases in humans (Allen, 2003) as well as providing information on cell activity. However, the transfer of these techniques to the assessment of bone mineral status in animals under research conditions has been conducted with varying levels of success. A summary of studies utilizing bone markers and their concentrations in plasma is presented in Table 2.3.

C-Terminal telopeptides of type-I collagen, ICTP and Cross Laps (CTx)

Type-I collagen represents more than 90% of the organic matrix of bone (Allen, 2003). The carboxyterminal telopeptides of type-I collagen are divided into two categories: CTx and ICTP. These refer to different domains of the c-terminus of type-I collagen (Christenson, 1997, Allen, 2003, Liesegang *et al.*, 2003). The concentrations of CTx in serum have been shown to correlate with bone age, bone density and predict fracture risk. CTx and ICTP are derived from different enzymatic processes. So in principle they both reflect bone resorption, but ICTP is hydrolysed by cathepsin K, an osteoclastic proteinase, whereas CTx is not. CTx in goats and sheep were measured as approximately 7000-60000 pmol/l of plasma using a one-step enzyme linked immunosorbant assay (ELISA) (Osteometer, Biotech, Copenhagen, Denmark), which demonstrated quite considerable variability within and between experiments on similar animals (Liesegang *et al.*, 2003, Liesegang and Risteli, 2005). Liesegang (2000) reported ICTP concentrations of typically 10 ug/L but up to 30 at calving in dairy cows and similar concentrations in goats and sheep (Liesegang and Risteli, 2005).

Liesegang (2000) showed that ICTP serum concentrations were higher in high yielding cows than low yielding cows post parturition. Staric (2010) recorded levels of 0.2 to 0.4 ng/L of CTx in plasma of parturient cows.

Osteocalcin

Osteocalcin is a small protein produced by osteoblasts during the matrix mineralization phase of their development (Ducy *et al.,* 1996). Expression of osteocalcin is strongly controlled by 1,25-vitD (Risteli and Risteli, 1993). It has been measured as a determinant of osteoblastic activity in corticosteroid-induced low bone formation in sheep (Liesegang *et al.*, 2003) (measured in ng/ml, 20-40 in goats and 10-20 in sheep). Osteocalcin (also known as serum bone-GLA protein), is a small (45-60 amino acid), vitamin K dependent protein synthesised only in osteoblasts and megakaryocytes (Allen, 2003). The release of osteocalcin is increased during bone resorption, so serum levels of osteocalcin accurately reflect the synthetic activity of osteoblast.

Bovine Ca flows demonstrated by marker studies

Holtenius and Ekelund (2005) examined CTx and osteocalcin in dairy cows covering both the lactation and dry period. Osteocalcin varied from 2 to 20 ng/ml and peaked during mid lactation and the nadir was immediately post calving, demonstrating that bone accretion immediately post parturition is virtually non-existent. Liesegang *et al.* (2000) demonstrated that CTx peaked at 40 nmol/L immediately post calving and became insignificant mid way through lactation, also demonstrating that bone degradation is greatly increased post parturition. The combination of both studies demonstrates that net skeletal Ca flow is highly negative (ie. bone degradation) past parturition and highly positive (ie. bone accretion) during late lactation and the dry period. Furthermore, Taylor *et al.* (2008) found that osteocalcin was highest post calving in cows during their first lactation and the post parturition peak decreased in magnitude as parity number increased suggesting that as cows age their ability to store skeletal Ca for use in subsequent parturitions is reduced.

Effectiveness in bovine

Liesegang (1998) compared markers of bone breakdown in the first few weeks of lactation. The study compared hydroxyproline (urine – colorimetric and RIA), deoxypyridinoline (urine – colorimetric and RIA) and carboxyterminal telopeptide (ICTP) (developed RIA on serum). Serum concentrations of type I collagen and amino-terminal

Table 2.3 Bone marker tests conducted with various metabolite concentrations in plasma or serum of bovine.

Metabolite	**Concentration**	**Physiological State**	**Reference**
Bone Accretion			
CTx	~40 nmol/L	Parturition	Holtenius *et al.*, 2005
	~3 nmol/L	35-40 weeks after parturition	Holtenius *et al.*, 2005
	0.417 ng/L	Parturition	Staric and Zadnik, 2010
ICTP	~25 ug/L	14d post parturition	Liesegang *et al.*, 2000
	~10 ug/L	Normal	Liesegang *et al.*, 2000
Hydroxyproline	1-1.25 ug/ml	Preparturition	Goff *et al.* 1998
	>1.75 ug/ml	3d postparturition	Goff *et al.* 1998
	1.5-1.8 ug/ml	Normal	Goff *et al.*, 1991
	1.8-2.5 ug/ml	Preparturition	Goff *et al.*, 1989
	1.2-1.5 ug/ml	Pre and post PTH infusion	Goff *et al.*, 1986
Pyridinoline	7.2-8.5 n*M*	Pre and post parturition	Moreira *et al.*, 2009
Bone Degradation			
Osteocalcin	15-20 (ng/ml)	12-15 weeks post parturition	Holtenius *et al.*, 2005
	~3 (ng/ml)	Parturition	Holtenius *et al.*, 2005
	0.7-0.9 ng/ml	Normal	Liesegang *et al.*, 2000
	0.3 ng/ml	14d post parturition	Liesegang *et al.*, 2000
	19.7-21.4 ng/ml	Pre and post parturition	Moreira *et al.*, 2009
ALP	55.9 U/L	Parturition	Staric and Zadnik, 2010
	150 U/L	Low parity	Van Mosel *et al.*, 2004
	65 U/L	High parity	Van Mosel *et al.*, 2004
bALP	20.5 U/L	Parturition	Staric and Zadnik, 2010
	~10 U/L	Preparturition	Kurosaki *et al.*, 2007
	≥15 U/L	Parturition	Kurosaki *et al.*, 2007
	20-35 U/L	Heifer, all the time	Kurosaki *et al.*, 2007

propeptide of type III procollagen (commercial RIA) were also analysed to study the effect of parturition on type III collagen, which is a component of soft connective tissue. Bone breakdown markers peaked approximately 1 week after parturition with slight differences in patterns, likely due to the timing of bone breakdown. Deoxypyridinoline and ICTP were useful tools for following the course of bone breakdown. However, it was suggested that

hydroxyproline in recumbent cows was different to normal cows due to hydroxyproline in urine being derived from other sources such as soft connective tissue. Hydroxyproline was not found to be an effective description of bone degradation.

Diurnal Variation

Liesegang (2003) monitored the diurnal variation in concentrations of some of the important bone markers in goats and sheep. There was evidence of circadian rhythms for OC and CTx but not for ICTP or bALP in both sheep and goats. However, in sheep there was much less diurnal variation for OC than goats (Figure 2.4). Goats appeared to have greater bone turn over as they consistently had higher bone ICTP concentrations (19 ug/L) than sheep (12 ug/L).

An extensive review of literature failed to find similar examples of comparisons of diurnal variations in bone markers of bovine.

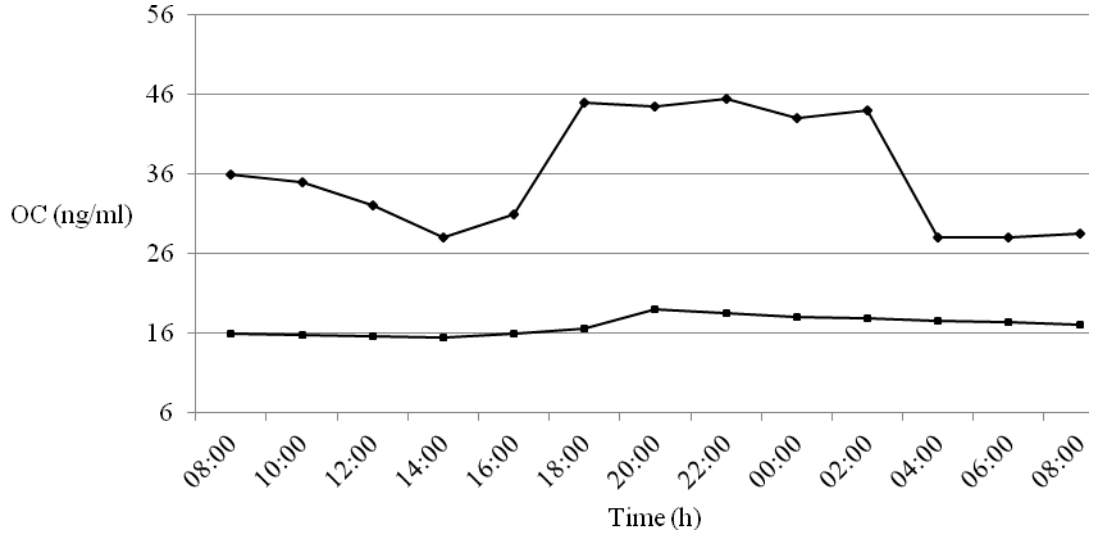

Figure 2.4 Diurnal variation in plasma of osteocalcin (OC) in sheep (■) and goats (♦) (adapted from Liesegang *et al.*, 2003).

2.1.6 Methods of 25-vitD supplementation

Treatment with 25-vitD in various studies has involved either supplementation of the metabolite in the diet (in feed, force fed bolus or through rumen fistula) or direct injection of the metabolite (daily, weekly or single dose injections). There is no published evidence

comparing different plasma or serum 25-vitD concentrations resulting from different application methods of 25-vitD, although several articles compare different vitamin D metabolites.

Taylor (2007) treated Jersey cows prior to parturition with a bolus containing 15 mg of 25-vitD or a similar bolus containing 15 mg of Vitamin D, 6d prior to parturition. The 25-vitD increases plasma 25-vitD from approximately 70 ng/ml to 120 ng/ml, there was no increase in plasma 25-vitD from the Vitamin D treatment. Bringe *et al.* (1971) found that dosing high milk fever incidence herds with massive amounts of 25-vitD (200 mg to 1000 mg) orally or injected, prior to parturition, reduced the incidence of milk fever from ca. 50% to ca. 15%, similar results were presented by Olson *et al.* (1974).

Carnagey *et al.* (2008) delivered 25-vitD into the rumen via a single bolus containing either 250 mg or 500 mg 7d before slaughter. Both supplementation levels increased plasma 25-vitD to between a range of 250 and 300 ng/ml over the following 7d, supplementation also increased levels of plasma 1,25-vitD to between 150 and 250 pg/ml. Interestingly, whether administering 500 mg or 250 mg the plasma level of 25-vitD was similar and only slightly less 1,25-vitD was seen in the 250 mg treatment suggesting that an upper limit of plasma 25-vitD may potentially be reached. Cho *et al.* (2006) presented similar results with dietary supplementation of 25-vitD with and without anionic salts increasing serum 25-vitD and 1,25-vitD and the affect was more pronounced with anionic salts. Unlike Carnagey *et al.* (2008) who found an increase in plasma Ca after application of 25-vitD, Cho *et al.* (2006) only found an increase in plasma Ca in the presence of anionic salts. However, Cho *et al.* (2006) only provided 125 mg of 25-vitD in bolus form as opposed to Carnagey's *et al.* (2008) much higher rate. However, serum levels of 25-vitD were increased from a base of 88 ng/ml in the control group to 383 and 488 in the 25-vitD and 25-vitD + Anionic salt treatments respectively, which was similar to the increases reported by Carnagey *et al.* (2008). Concentrations of 1,25-vitD reported by Cho *et al.* (2006) were much lower with an increase from 10 to 25 and 34 ng/ml in respective control, 25-vitD and 25-vitD + anionic salt groups.

It has been demonstrated that 25-vitD can successfully be applied to ruminants by either injection or through the gastrointestinal tract. Suitable supraphysiological levels of plasma 25-vitD have been attained from different methods of application; such as one time oral boluses (Cho *et al.*, 2006, Carnagey *et al.*, 2008, Taylor *et al.*, 2008), injections (Hollis *et al.*,

1977, Hidiroglou, 1987), in the diet (Montgomery *et al.*, 2000) and even buccal dosing (Rivera *et al.*, 2005). The application of 25-vitD appears to increase the plasma concentration of the active 1,25-vitD although the mechanisms for this are unclear and may not be a result of an increase in production of 1,25-vitD in the kidney but by a reduction in usage of 1,25-vitD at Vitamin D receptor sites due to excessive competition from 25-vitD (D. Fraser, pers comms 2011). The relationship between dose of 25-vitD and subsequent concentration of plasma 25-vitD is not consistent and would appear to be influenced by other factors. Such factors may consist of animal physiology (eg. Live weight, productivity or sex), season, access to direct sunlight and diet.

2.1.7 25-vitD as an active metabolite

As detailed above, the physiological process for obtaining Vitamin D is via contact with ultra-violet light (or to a lesser extent consumption of Vitamin D), then Vitamin D is metabolised to 25-vitD and further metabolised to the active 1,25-vitD. However, there are two independent aims of supplementing Vitamin D; the first is to provide adequate Vitamin D as an effective replacement for dietary or lack of contact with direct sunlight, this permits normal physiological processes to take place. The second supplementation purpose is to provide 25-vitD in pharmacological doses to attempt to activate additional health or productive benefits. If supraphysiological doses of Vitamin D are required, it is much more efficient and less toxic, to supplement with 25-vitD instead of the original Vitamin D as less vitamin is required. Hidiroglou and Knipfel (1983) injected 25-vitD at 10% of the rate of the Vitamin D, yet the concentration of 25-vitD in the plasma was a multiple of 6. It would appear that the efficiency of conversion of Vitamin D to 25-vitD is very limited and residual Vitamin D is transformed and excreted (Figure 2.2).

The mode of action of 25-vitD in pharmacological doses remains unclear. However, research suggests that extremely elevated concentrations of plasma 25-vitD may replicate the functions of 1,25-vitD (Rowling *et al.*, 2007). Studies measuring the influence of treatments of 25-vitD on the subsequent concentration of 1,25-vitD have demonstrated inconsistent results. Several studies have shown an increase in plasma concentration (Reinhardt and Conrad, 1980, Rivera *et al.*, 2005, Cho *et al.*, 2006, Carnagey *et al.*, 2008), some with no affect (Montgomery *et al.*, 2000, Wertz *et al.*, 2004) and others have demonstrated a decrease in 1,25-vitD concentration (Shepard and Deluca, 1980). However, the change in

concentration of 1,25-vitD is important to note. Whether the concentration is increased or decreased the change is likely to be inconsequential. Furthermore, the concentration of 1,25-vitD residing within VDR is also unknown, creating further variability in the estimation of the affect of large doses of 25-vitD on 1,25-vitD concentration. It appears that physiological effects generated from treatments with large doses of 25-vitD are not a result of an increase in 1,25-vitD concentration.

Moreover, pharmacological doses of 25-vitD cause hypercalcaemia, but maybe not directly as a function of increasing 1,25-vitD. Increases in concentration of 1,25-vitD may in fact be a consequence of sparing of the compound as the VDR may be overwhelmed with 25-vitD. Trummel *et al.* (1969) demonstrated that *in vitro* 25-vitD actually increased bone resorption and Foote *et al.* (2004) showed that hypercalcaemia would develop similar to 1,25-vitD, being more than double if 25-vitD was sufficient (ie. greater than 300 ng/ml, with no increase in 1,25-vitD). Furthermore, Shephard and Deluca (1980) summarized that physiological concentrations of 1,25-vitD and 1000 fold concentrations of 25-vitD caused equivalent displacement of radioactive 1,25-vitD at receptor sites. Thus, an increase in 1,25-vitD concentration may be an indication that an effective plasma concentration of 25-vitD has been achieved. However, this hypothesis has not been confirmed in bovine.

In bovine the majority of experiments demonstrating increases in 1,25-vitD as a response to pharmacological concentrations of 25-vitD are short duration experiments, usually less than 7d. The short duration of these studies may result in the recording of the initial sparing activity of 25-vitD on 1,25-vitD and the concomitant increase in plasma concentration. Studies of longer duration may demonstrate a reduction in the concentration of 1,25-vitD, this would be a logical progression as the high levels of circulating 1,25-vitD would provide negative feedback by inhibiting 1α–hydroxylase, and furthermore, hypercalcaemia would inhibit secretion of PTH and hence further deactivation of 1α–hydroxylase. This reasoning leads to the hypothesis that it is the displacement of 1,25-vitD by 25-vitD that is the mechanism of action of an increase in plasma 1,25-vitD not the increase in 1,25-vitD production.

Furthermore, recent research by Rowling *et al.* (2007) has demonstrated that in 1α-hydroxylase knockout mice supraphysiological doses of 25-vitD can almost completely replace physiological levels of 1,25-vitD. These mice have been genetically engineered to not

produce renal 1α-hydroxylase, which makes them perfect models for comparing 25-vitD and 1,25-vitD. In bovine, results of treatments with supraphysiological doses of 25-vitD are confounded by the potential activity of 1,25-vitD. Rowling's *et al.* (2007) research demonstrated that in normal white mice massive plasma concentrations of 25-vitD reduced the endogenous concentrations of 1,25-vitD. This suggests that in normal mice the 25-vitD had largely replaced 1,25-vitD and as a consequence adequate Ca concentrations inhibited the secretion of PTH, subsequently reducing the production of 1,25-vitD.

The research by Rowling *et al.* (2007) confirms the hypothesis presented in this section that when in supraphysiological concentrations 25-vitD can replace the activity of 1,25-vitD without the requirement of PTH. This has important implications as supraphysiological doses of 25-vitD can:

1. Increase Ca absorption from the gastrointestinal tract regardless of the requirement of the animal. This is important for pre-empting known Ca deficiencies, such as parturition.
2. Activate P absorption from the gastro intestinal tract, even when Ca concentration of plasma does not permit secretion of PTH, thus permitting improved P absorption when cattle are consuming low P concentration diets.

2.2 Calcium

Calcium is required for intracellular reactions including muscle contractions, nerve cell activity, the release of hormones through exocytosis and the activation of enzymes (Horst *et al.*, 2005). Extracellular functions include blood coagulation, the maintenance and stability of cell membranes and the maintenance of structural integrity of bone and teeth (Horst *et al.*, 2005). Approximate quantities required for daily function of a mature Holstein cow are shown in Figure 2.5.

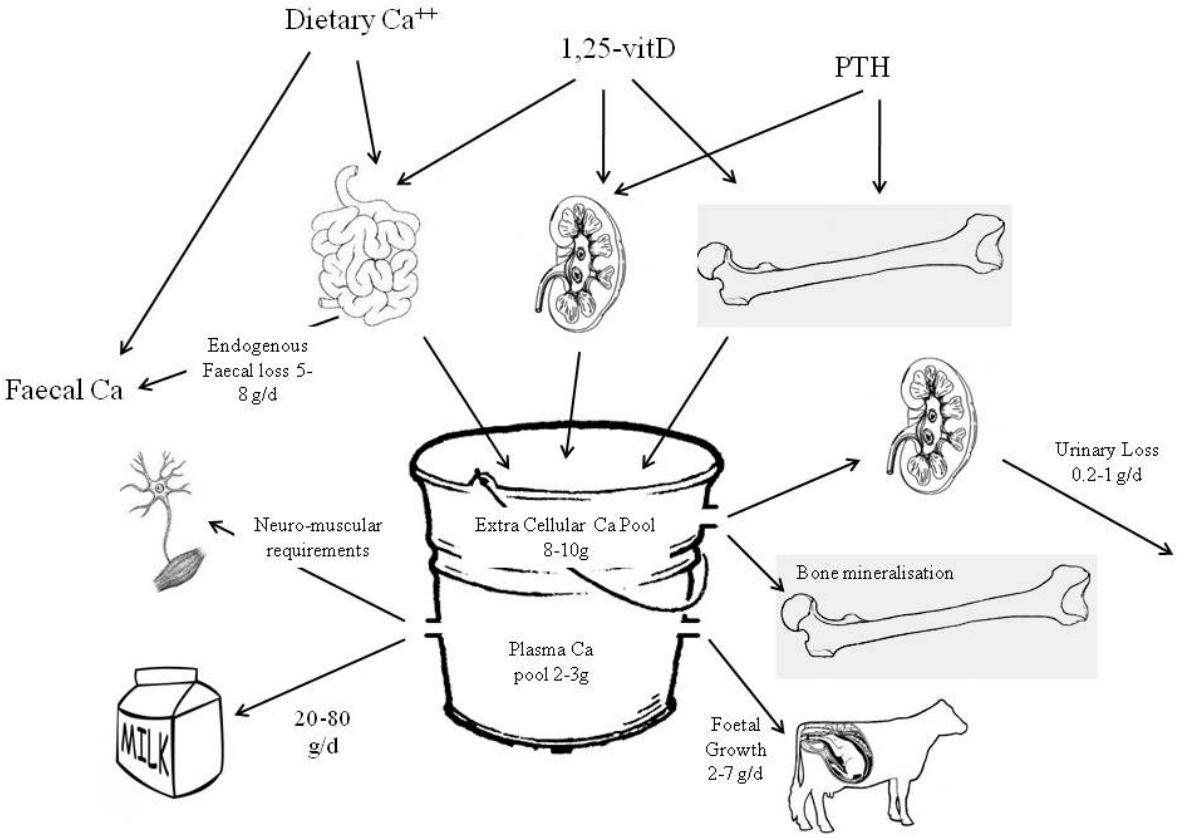

Figure 2.5 Representation of the role of active vitamin D and parathyroid hormone (PTH) for providing plasma Ca for various roles. Ca requirements (g/d) for each component are shown with order of importance governed by exit point on bucket, i.e. the most important functions come out of the bottom of the bucket and if Ca is in excess it is mineralized or excreted (developed from information presented by DeLuca, 2004 and Horst *et al.,* 1994a).

Control of Ca metabolism is important as the ions play an important role in many physiological processes. Ca homeostasis is tightly controlled and adjustments are made within 5% of the normal range. Typically, 9-10 mg/dl in plasma in most mammals (Goff, 2000, Horst *et al.*, 2005) which equates to approximately 3 g of Ca in the plasma pool of a 600 kg cow.

2.2.1 Ca Homeostasis

The regulation of Ca involves control of the movement of Ca between extracellular fluid and three body organs: bone, GI tract and the kidneys (Goff, 2000). Ca inputs into the extracellular pool originate from the diet, bone resorption and renal resorption. The homeostatic mechanisms are required to be dynamic and fast acting to avoid hyper- or hypocalcaemia as both irregularities can cause sudden death. In some mammals the initiation of lactation may cause hypocalcaemia. This is a consequence of a rapid increase in Ca demand. As milk production potential of the modern day dairy cow has increased so has the Ca requirement. For example, one Kg of colostrum contains approximately 2.5 g of Ca and a cow may produce 20 kg in the first 24h of lactation. Just one kilogram of colostrum is approximately equal to the extracellular Ca pool of a mature cow (Horst, 1986) (**Error! Reference source not found.**).

Most Ca in the body is in bone in the form of hydroxyapatite crystals, which contain Ca, phosphate and water (Goff *et al.*, 1991a). The next largest pool of Ca in the body is intracellular Ca. The smallest pool of Ca is in the extracellular fluid (Horst *et al.*, 1994a). Extracellular Ca is the most important pool for physiological control of Ca concentrations in blood. This component is composed of interstitial Ca, blood Ca and a small but important part of the bone Ca pool, which exists as amorphous crystals or in solution (Cunningham, 1997). The soluble bone Ca pool allows access to the large reserves of Ca that reside in bone and it is more available than Ca held in hydroxyapatite form.

Calcium is present in plasma in three major forms: ionised, protein bound, and complexed. The ionised form of Ca (iCa) is biologically active, representing about 50% of the total plasma Ca pool (Wang and Beede, 1992a). Potentially changes in iCa may not be significant if total Ca is recorded as the differences may be insignificant, this was the case in Wang and Beede's (1992a) study. However, measurement of iCa, while possible, is difficult when compared to measurement of total Ca.

Parathyroid Hormone (PTH)

The main organ involved in the control of Ca and phosphate metabolism is the parathyroid gland. In the ruminant, the gland lies anterior to the thyroid (Cunningham, 1997). PTH is secreted by the process of exocytosis in response to a reduction in plasma Ca concentration. It is primarily responsible for the overall control of Ca homeostasis, including changes to the labile pool of bone Ca as well as renal activity. However, it is rapidly metabolised by the kidney with a half life of 5-10 minutes in blood making it suitable for controlling minute changes in extracellular Ca concentration (Bringhurst *et al.,* 1988).

The effect of PTH is to increase Ca and decrease phosphate concentrations in extracellular fluids. PTH has direct effects on bone and kidney metabolism of Ca and indirect effects on GI metabolism of Ca (Goff *et al.*, 1989, DeLuca, 2004) via the production of 1,25-vitD.

Low dietary Mg has been shown to significantly increase incidence of hypocalcaemia (Lean *et al.*, 2006). One of the known effects of Mg on Ca homeostasis is the requirement of Mg for PTH function (Goff and Horst, 2003b). When PTH binds to its receptor this normally initiates activation of adenylate cyclase, resulting in production of the secondary messenger cyclic AMP, or phospholipase C, resulting in production of diacylglycerol and inositol 1,4,5-triphosphate. Both adenylate cyclase and phospholiase C require Mg for full activity. The level of Mg is only of concern when deficient and there is no benefit, or detriment, of excess Mg in diet (Wang and Beede, 1992).

Bone

The initial effect of PTH on bone is to promote the transfer of labile Ca across the osteblast-osteocyte membrane. This level of action occurs without the movement of phosphate and therefore has no effect on phosphate concentrations in blood. The soluble portion of bone is important for Ca homeostasis as it is able to be transferred rapidly into the extracellular pool of Ca.

The amorphous crystals and soluble Ca are located between the osteoblasts and the osteocytes (Termine and Posner, 1967). Osteoblasts line the blood vessels and osteocytes are deeper within the bone. These two cell types have cytoplasmic projections that interact intimately through the presence of tight cell junctions. For Ca to enter the blood, from the

labile bone Ca, it must cross the membrane barrier created by the osteoblasts and osteocytes (Cunningham, 1997).

PTH has additional resorptive effects on stable bone. This bone resorption is initiated by increased osteoclast activity and an inhibition of osteoblast activity. When stable bone is resorbed as a consequence of an increase in PTH, both Ca and phosphate are released (Goff *et al.*, 1989). Rapid bone resorption requires the presence of 1,25-vitD and PTH (DeLuca, 2004), however, 1,25-vitD without PTH does not result in bone degradation. After approximately 9 days, the concentration of bone degradation markers ICTP and DPD start to decrease (Liesegang *et al.*, 1998). This suggests that cows only actively degrade bone for approximately one week after parturition, which means that Ca absorption from the diet has been increased sufficiently to match the extra Ca secreted in lactation.

Renal Ca control

PTH acts on the distal convoluted tubules of the kidneys to increase absorption of Ca and decrease renal phosphate reabsorption. It achieves this by increasing resorption of Ca in the proximal tubules. Like bone degradation the process is further enhanced with the presence of 1,25-vitD (DeLuca, 2004).

Calcitonin

Calcitonin is a hormone produced by cells in the thyroid gland. The secretion of calcitonin is by exocytosis from granules. Increased levels of extracellular Ca results in increased levels of calcitonin excretion and concomitantly reduced levels of PTH production. Calcitonin balances the action of PTH by causing hypocalcaemia and hypophosphataemia. This balancing effect is achieved by activities primarily associated with bone (DeLuca, 2004). The hormone decreases the movement of Ca from the labile bone Ca pool into the extracellular fluid and decreases bone resorption through an inhibitory effect on osteoclasts.

Calcitonin also has non-skeletal effects. It increases renal secretion of Ca and P as well reducing GI activity, directly by inhibiting gastric acid secretion and indirectly by inhibiting gastrin secretion (Cunningham, 1997).

2.2.2 Ca absorption and metabolism in the ruminant

The absorption of Ca from the GI tract is by passive diffusion and active transport. The passive diffusion of Ca across the intestinal mucosa occurs in the presence of high

concentrations and is relative to Ca absorption when adequate levels of Ca are fed or requirement is very low.

Calcium intake does not affect the level of Ca absorption. For instance modifying Ca intake from 0.99% to 1.5% of dietary intake did not increase urine Ca content (Chan *et al.*, 2006). Both levels are considered to be well above requirement (NRC, 1989). This indicates that there was no increase in absorption of Ca as a result of the higher intake. NRC (1989) suggested that feeding diets with Ca higher than approximately 1% may inhibit feed intake, although this was not found by Chan (2006). However, if dietary Ca is below requirements then the rate of absorption will increase as active Ca absorption will be initiated.

Calcium secretion into the gastrointestinal tract is not affected by uptake and is relatively standard per unit of dry matter intake (Braithwaite, 1982, Takagi and Block, 1991c). Along with lactation, the kidney is the other route of variable Ca excretion. Most of the Ca that passes into the kidney is reabsorbed with a net loss of approximately 2% (Cunningham, 1997). Most of the Ca filtered by the kidneys is reabsorbed in the proximal tubules, with the next largest amount absorbed by the distal tubules and a lesser amount by the ascending loop of Henle. The distal tubules are the site of Ca regulation in the kidney as they are under hormonal control by PTH and 1,25-vitD (DeLuca, 2004).

2.2.3 Ca deficiency syndromes

Calcium deficiency in grazing animals, unlike most mineral deficiencies, tends to be acute and rarely result in long term losses of productivity. The most common type of acute disorder of Ca metabolism in domestic animals is that of hypocalcaemia in association with parturition in the dog and cow (Cunningham, 1997). Animals usually affected are recumbent with severe neuromuscular dysfunction. The main manifestation in cows is paralysis. The condition arrives from the heavy demand that is placed on Ca reserves by the sudden onset of lactation with the inability of the animal to maintain Ca homeostasis (Goff, 2003)

The most common type of chronic disorder of Ca metabolism in domestic animals is associated with secondary hyperparathyroidism, in which there is excess secretion of PTH because of chronically low plasma Ca concentrations. Causes include improper diets that involve inadequate intake of either Ca or vitamin D or inappropriate diets such as all meat

diets for carnivores that are inherently low in calcium (Cunningham, 1997). Horses may suffer from similar circumstances if the level of P in the diet is too high relative to Ca.

The Ca content of pastures is usually adequate (NRC, 2000). However, on rare occasions, usually when P has been identified as deficient, Ca has also been found to be deficient. In these situations the pasture is usually deemed to be of extremely low quality due to digestibility and invariably low in crude protein as well (Cohen, 1980). Mineral supplementation under these conditions does not usually result in an improvement in animal production as production is first limited by a lack of energy and protein (Cohen, 1980).

Kidney disease, also a cause of secondary hyperparathyroidism, results in increased Ca diuresis with retention of phosphates. The resultant hyperphosphatemia further depresses Ca concentrations through increased formation of hydroxyapatite crystals. This process removes excess phosphate, but also removes Ca from an already marginal extracellular fluid concentration (Brown and Slatopolsky, 2007).

2.2.4 Ca requirements of dairy and beef grazing cattle

The requirement of Ca for beef cattle is dependent on the animal's level of production and physiological status. In steers 100 g of protein gain requires approximately 7.1 g of retained Ca. Maintenance requirements have been estimated at 15.4 mg/kg of live weight (NRC, 2000). Ca usually has a digestibility of approximately 50% meaning that a 320 kg steer growing at 1 kg per day requires approximately 30 g of Ca per day in the diet (NRC, 2000). Lactating cows have a much higher requirement for Ca as the concentration of Ca in milk can be very large. Lactating dairy cows can secrete approximately 20-30 g of Ca every day in the milk alone (Goff, 2000). Furthermore, the foetal requirement for Ca of 13.7 g/kg of foetal weight is required for deposition during the last 3 months of gestation. Generally the Ca content of pasture is sufficient to meet the requirements of grazing animals and it is only specific short-term deficiencies as a result of inept homeostatic mechanisms that result in hypocalcaemia (McNeill *et al.*, 2002).

2.3 Phosphorus

The majority of phosphate requirements are, like Ca, primarily required for structural reasons with the compound being the second largest component of bone and teeth. However, inorganic phosphate also functions as a hydrogen ion buffering system in the blood and P is a component of cell plasma membrane, phospholipids, phosphoproteins, nucleic acids and is critical in energy transferring molecules such as ATP (Goff, 2000).

2.3.1 Homeostasis for P

Phosphorus homeostasis is controlled by homeostatic systems similar to Ca homeostasis, although P absorption may have some Vitamin D influenced absorption in the small intestine, independent of Ca absorption (Breves and Schröder, 1991).

Plasma P concentration, while important, is not nearly as tightly controlled as plasma Ca concentration. The concentration of P in plasma will often vary between between 4 and 8 mg/dl (Goff, 2000), with approximately 1-2 g of iP present in plasma and 4-7 g of P normally present in the extracellular pool of a 500 kg cow.

Extracellular P levels are maintained via absorption of P from the small intestine or resorption of P from bone. Potential losses of P from the extracellular pool include urinary, saliva, bone and muscle growth as well as lactation and fetal growth in females (Goff, 2000).

Hormonal regulation of P is mainly achieved by three hormones; PTH, calcitonin and 1,25-vitD (Horst, 1986, Breves and Schröder, 1991). The vitamin D metabolite, 1,25-vitD, is primarily involved with small intestine absorption and has less of an influence on bone and renal function. As opposed to PTH and calcitonin which are much more involved with bone and renal function and have little direct influence on small intestine function (Breves and Schröder, 1991).

As previously mentioned, 1,25-vitD is responsible for active mineral transport from the digestive tract and is up-regulated by parathyroid hormone (PTH) and 1α-hydroxylase when plasma Ca levels are low (Goff, 2000). However, unlike Ca, low plasma P concentrations cannot up-regulate 1,25-vitD in ruminants (Abdel-Hafeez *et al.*, 1982, Breves *et al.*, 1985). In this situation, plasma Ca levels will remain in an acceptable range and there will be no PTH

induced up-regulation of 1,25-vitD production, and consequently no improvement in P absorption.

Bone accretion cannot function without adequate amounts of P. As plasma Ca concentration is likely to remain within the acceptable range there will be no up-regulation of 1,25-vitD, thus not allowing this mechanism to improve P absorption. However, while a low concentration of plasma P cannot up-regulate 1,25-vitD it can increase receptor affinity (Breves and Schröder, 1991).

Since the 1950's it has been shown that supraphysiological doses of Vitamin D can increase P and Ca absorption and increase retention of both minerals (Conrad *et al.*, 1956). This was confirmed by Hollis (1977) who found that levels of 25-vitD over 200 ng/ml in plasma increased plasma P concentration, and that there was a linear relationship between plasma 25-vitD and plasma Ca. Furthermore, large doses of Vitamin D can potentially have negative health effects such as metastatic calcification and hypocalcaemia (Shepard and Deluca, 1980). However, the mechanisms for the findings are hard to elucidate. From more recent studies, it may be suggested that the large doses of Vitamin D given in these studies was transferred into 25-vitD (as it is not under regulated control), but not 1,25-vitD (which is), and the supraphysiological levels of 25-vitD increased Ca absorption from the digestive tract as previously mentioned.

Saliva P

The major area of P loss or excretion from the body in ruminants is salivary secretion, in a mature cow salivary secretion may be as high as 90 g of P per day (Goff, 2000). Other endogenous secretions of P in the gastro intestinal tract are considered to be minor compared to saliva and unregulated, as such they play a minor role in homeostasis (Scott and Buchan, 1988). Salivary P is both a recycling and excretion mechanism. When an animal is consuming excessive P, salivary P will be more concentrated than when an animal is consuming a low P diet. However, even when a diet is deficient in P, saliva will still contain some P (Mañas-Almendros *et al.*, 1982) demonstrating the key P recycling feature of saliva.

Salivary secretion of P is an important recycling mechanism in ruminants as P is a requirement for microbial growth in ruminants (Breves and Schröder, 1991). To maintain microbial growth during periods of low P intake, ruminants are able to resorb P from bone.

This allows maintenance of P in plasma and therefore saliva (Goff, 2000). Much of the iP from saliva is reabsorbed in the small intestine but a small amount is lost, ruminants are able to maintain adequate rumen fermentation and therefore productivity on marginal to low P diets for up to several months due to the mobilisation of bone stores (Gartner *et al.*, 1982).

Salivary P secretion is variable due to several factors. PTH stimulates parotid salivary P secretion and may double salivary phosphate concentrations (Wright *et al.*, 1984, Horst, 1986). However, in a separate experiment both PTH and 1,25-vitD reduced the concentration of salivary P when it was compared to plasma P concentrations. Furthermore, it was postulated both endogenous and exogenous PTH reduced salivary to plasma P concentration ratio by increasing the plasma concentration of 1,25-vitD (Mañas-Almendros *et al.*, 1982).

In most situations excretion of salivary P is the predominant P excretion point in ruminants. However, reducing total salivary output will decrease total salivary P excretion, even though saliva P concentration may have increased. Concentrate diets low in roughage and high in digestibility tend to have high P levels (NRC, 2000). These concentrate diets do not promote rumination and as a consequence urine plays an important role in plasma P removal (Tomas, 1974).

P Absorption

The small intestine is the major site of P absorption in both ruminants and single stomached animals (Breves and Schröder, 1991). Absorption of P in the small intestine is both active and passive (Breves and Schröder, 1991).

Active absorption of P in the small intestine is mediated by 1,25-vitD but only extremely low levels of plasma P will initiate the excretion of PTH which in turn up-regulates the production of 1,25-vitD in the kidney (Goff, 2000). Low plasma P may also increase the efficacy of 1,25-vitD production by not inhibiting the production of 1α-hydroxylase (Horst, 1986) but generally the active absorption of P via the release of 1,25-vitD is a consequence of low plasma Ca.

The presence of 1,25-vitD in plasma stimulates active Ca and P transport independently and it involves the classical steroid receptor mediated slow genomic effects and fast non genomic effects. The long term effects involve the induction of Ca binding proteins where as the short term effects appear to involve changes in membrane fluidity (Rasmussen *et al.*,

1979). Dietary P absorption can also be affected by other dietary constituents, low dietary protein and low energy reduce P digestibility in sheep (Liebholz, 1974). Supplemental P is absorbed as similar rates to the original low P diet (Coates and Ternouth, 1992).

On the other hand, passive absorption of P in bovine is not clearly defined (Breves and Schröder, 1991). However, there is a general understanding that there are primarily two forms of passive absorption, transcellular and paracellular, within the small intestine. Transcellular relies on an electrochemical gradient for the movement of ions into cells in the lumen and then further into capillaries. The function is independent of ATP (Cunningham, 1997). The second form of passive absorption is through tight junctions within the luminal wall. Small ions will move through the junctions in response to electrochemical gradients or osmotic pressure (Cunningham, 1997). Clearly, passive P absorption is dependent on adequate concentrations of P in the diet. Whilst this function is important in highly productive animals consuming diets high in P it is of much less importance in extensive grazing situations.

Urine P

Urinary P in ruminants is usually low and does not contribute to homeostasis (Breves and Schröder, 1991). The kidney is responsible for high levels of P resorption from urine (Mayer *et al.*, 1966). However, on low roughage diets that do not promote salivation P may be excreted through urine (NRC, 2000). Furthermore, urinary excretion of P may be stimulated by high levels of PTH (Goff, 2000). Fibroblast growth factor 23 is also responsible for increasing renal phosphate excretion by decreasing its reabsorption in the proximal tubule (Bai *et al.*, 2004). However, generally speaking in grazing ruminants renal P excretion is not a major component of P homeostasis.

Excretion of excess plasma P usually takes place via saliva (Horst, 1986, Breves and Schröder, 1991), however it remains uncertain whether the primary control of excess P is in the small intestine (absorption) or in saliva (excretion) (Breves and Schröder, 1991).

The high level of P in urine of lambs on concentrate diets compared to roughage diets has been demonstrated to be a result of reduced salivation due to a lack of rumination (Scott *et al.*, 1971). However, the same authors went on to demonstrate that sheep and calves fed concentrate diets with higher concentrations of P excreted more P in urine per unit of P intake than the roughage fed animals (Scott, 1972). This study demonstrated that more P was

absorbed in the higher concentrate diets, and it was disposed of in the urine. It is unclear what the mechanism for this increased urinary P is. However, it appears that more acidic diets with higher concentration of P promote greater P absorption and increased excretion via urine, potentially as saliva may be limited via volume. It is important to note that under the right circumstances ruminants can still excrete large volumes of urinary P if required.

Bone accretion and resorption

The skeleton is the major location of P in the body with up to 4 kg of P being present in the skeleton of a 500 kg cow (Goff, 2000). A small amount of this is available for resorption when plasma P concentration is low. Skeletal absorption is mediated by PTH, which breaks down bone and promotes circulation of P in saliva thus supplementing the low P diet that originally caused the low plasma P situation (Goff, 2000). However, this maintenance mechanism is only activated by plasma Ca concentrations.

Chronically low dietary P in young growing animals may lead to rickets as a result of an insufficient amount of P present for the accretion of bone during remodeling and growth (Goff, 2000). P and Ca are the major components of bone and are required in a ratio of 10 ions of Ca to 6 of P (Goff, 2000).

2.3.2 Diagnosis of P deficiency

A decrease in dry matter intake is the most obvious effect of P deficiency (Gartner *et al.*, 1982, Breves *et al.*, 1985, Bortolussi *et al.*, 1996). However, a decline in feed intake is difficult and impractical to measure in a grazing situation. Bone thickness is an effective measure of P storage but does not reflect the current P status of the animal and is only useful in regards to net bone accretion of degradation over time.

However, plasma P is an effective measure of P status and the use of the technique has evolved. Cohen (1973) showed that blood plasma iP levels were an inaccurate reflection of pasture P. The work showed that bone was a very accurate description of pasture P content when yearling steers were grazing a range of moderate to very deficient P pastures. However, after 19 weeks of low and moderately deficient P diets Gartner (1982) was able to show a difference in iP levels in serum. Ternouth (1990) went on to present data that iP was an excellent method of determing or identifying when P levels in the diet are at moderate to low levels, this is supported by several authors (Coates and Ternouth, 1992, Ternouth and Coates,

1997, Geisert *et al.*, 2010). Goff (2000) reported that plasma P is generally well correlated with dietary P absorption. Plasma iP levels of 6-8 mg/dl are considered normal whereas levels of 2-4 mg/dl are considered deficient. However there is a considerable range in between which is inconclusive and the values must be taken in context with nutritional and productive environment of the animal.

2.3.4 P requirements of cattle

Analysis of P requirements is inherently difficult as excess metabolic P is excreted through saliva not urine and P can be stored in the skeleton (and to a lesser extent muscle) and then released to compensate for periods of deficiency. Studies into P requirements often need to take the above features into account and this often makes such studies prohibitive. However, recycling of P and storage improves the adaptability of bovine to environments of variable nutrient availability.

Cattle have an excellent ability to conserve and then recycle P. This allows very effective adaptation to seasonal P deficiencies. Such seasonal P deficiencies are likely to be caused by differences in production rather than fluctuations in supply of P, for example the majority of growth and peak lactation are both primarily season specific. The skeleton effectively allows cows on sub-optimal P intake to have similar levels of production as non-deficient P intake cows. Call *et al.* (1986) and Fishwick *et al.* (1977) found that dramatic deficiencies in P over a short period of time (2 months) resulted in a reduction in tail bone density followed by recovery of production characteristics once cows had been returned to a normal diet. Furthermore, cattle tended to not show improved daily growth rate during the early wet season but daily growth rate during the late wet season were consistently higher on pastures with higher P concentration (McLean *et al.*, 1990). This indicates that P effectively stored during the dry season may be released during the early wet season thus reducing the effect of P being the limiting nutrient in pastures.

The P requirements for a 300kg steer gaining 0.5 kg/d and a 500 kg cow with average milking ability (5 kg/d) are 14.3 and 21.4 g/d, respectively (Ternouth, 1990). Growth requires *ca.* 8 g/kg gained, conceptus requires 1 g/d at 5 months and 5 g/d at 9 months (Ternouth, 1990). However, NRC (2000) presented different methods for expressing P requirements; with conceptus at 7.6 g/kg of foetus and 3.9 g/100 g of protein gain in growing animals. However, several experiments using lower than published P requirements have shown that

recommendations are often excessive (Fishwick *et al.*, 1977, Call *et al.*, 1978, Ternouth *et al.*, 1996).

Data presented by Ternouth (1990) suggests that maiden heifers (yearling and to a lesser extent 2 yr old) would be particularly susceptible to P deficiency as they require P for growth and conceptus. In recent research conducted in extensive grazing conditions in northern Australia, the pregnancy rate for heifers during their first lactation was approximately 20%, but was increased to 40% with year round mineral supplements that contain P (Schatz and Hearnden, 2008). Thus research on P digestibility or deficiency should be studied in highly productive animals, and since breeding animals with offspring are almost impossible to work with in an animal house situation young animals (1 to 2.5 yrs of age) are the next best animal to work with due to their growth rate increasing the requirement for P.

2.3.5 P deficiency in grazing ruminants

Phosphorus deficiency causes a major reduction in the productivity of the beef industry in extensive grazing regions of Australia (Ternouth, 1990). This is especially the case in the more extensive grazing systems in the north but is also seen in some intensive systems in southern areas were P is often a limiting factor. P is deficient when it is the limiting factor, during the dry season nitrogen and digestibility are limiting factors but during the wet season P is the limiting factor and digestibility and nitrogen concentrations are increased (McLean *et al.*, 1990, Bortolussi *et al.*, 1996). Both Ca and P may be deficient in certain pastures, responses to P supplementation in these situations is often negligible (Cohen, 1980). Often low pasture digestibility and protein will result in apparently deficient P concentrations not being the limiting factor for animal production.

As a decline in feed intake is the most obvious sign of P deficiency in ruminants (Gartner *et al.*, 1982, Breves and Schröder, 1991, Goff, 2000), it also has the most negative productive affect. Live weight is the most highly correlated factor for fertility in first calf heifers (Schatz and Hearnden, 2008), any reduction in feed intake will result in a concomitant loss in energy and protein intake causing a reduction in growth rates and potentially a reduction in live weight (Figure 2.6).

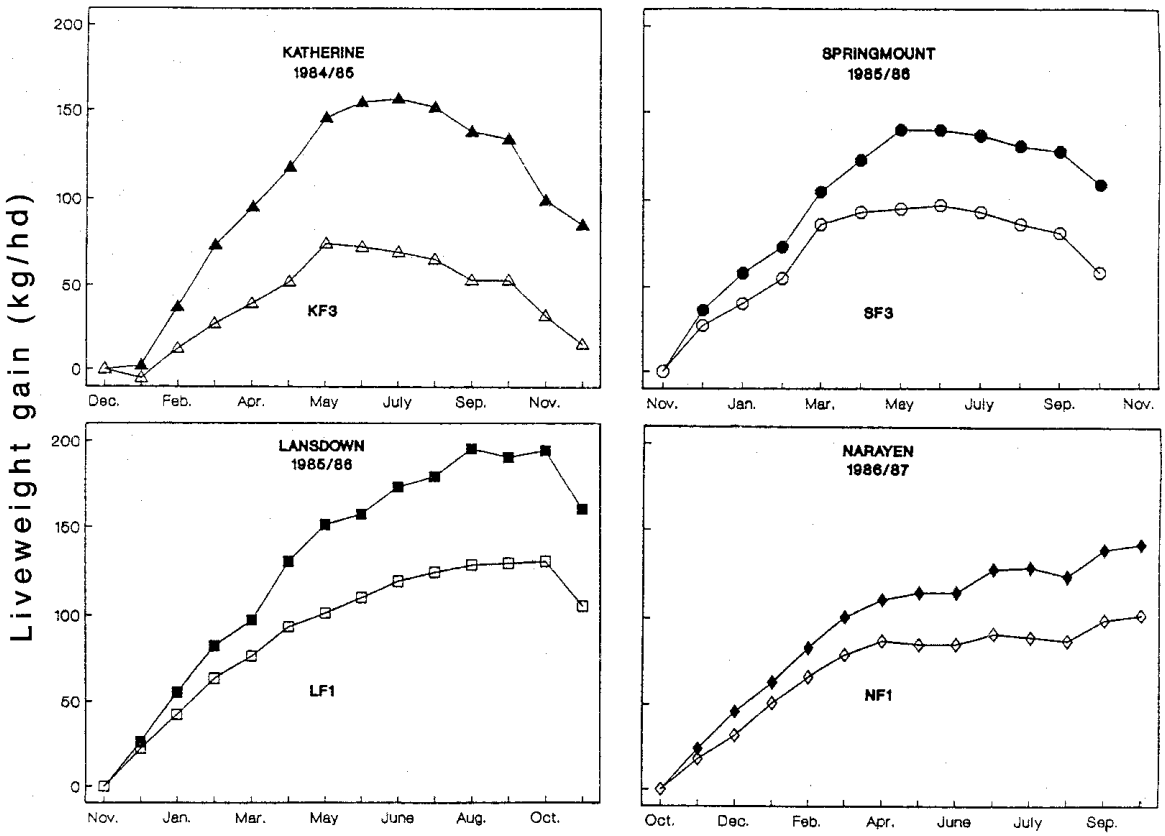

Figure 2.6 Cumulative liveweight gains for representative years at each site for unsupplemented (unfilled symbols) and P supplemented (filled symbols) treatments (Winter *et al.*, 1990).

P deficient diets should result in less microbial protein production and fermentation but it is inherently hard to cause complete P deficiency as a consequence of bone resorption, however Gartner *et al.* (1982) showed that heifers consuming diets that were restricted to the DM intake of the P deficient replicates but given supplemental P had higher rates of daily gain. This indicates that reduced content of feed was utilised more effectively, which was confirmed by *in vitro* research showing that VFA production, OM digestibility and ammonia utilisation were all increased at higher P concentrations (Komisarczuk *et al.*, 1987).

The time that it takes from the beginning of P deficient diets to a reduction in feed intake is dependent on the concentration of P in the diet; the greater the P deficiency in the diet the more quickly feed intake will drop. A reduction in feed intake has been shown to take from 3 weeks to 19 weeks depending on the P concentration of the diet (Gartner *et al.*, 1982). The

amount of P stored in the skeleton is limited, hence the greater the dietary deficiency the more quickly reserves are exhausted.

Gartner *et al.* (1982) demonstrated that P deficiency also has effects not related to feed intake. They pair fed P-deficient heifers with P supplemented heifers. The P supplemented heifers had a much greater feed efficiency than the P deficient heifers even though they had similar DM intakes, the theory behind this is that the P supplements improved rumen fermentation, however this was not overwhelmingly supported by digestibility data and individual animal variation was postulated as a cause of this.

2.3.6 P supplementation times and targets

Phosphorus supplementation is relatively inefficient (P absorption rates are often less than 50%) and is a costly strategy (Ternouth, 1990). The cost of P sources is continuously increasing, especially since the world supply has peaked or is rapidly approaching its peak supply of P (Cordell *et al.*, 2009). Increasing the ability of ruminants to retain P when supplementation is required would improve both the productivity and profitability of livestock enterprises.

"The class of cattle for which it is most important to assess their P requirements correctly is the breeding cow. In Australian extensive grazing situations, these animals appear to be particularly vulnerable to P deficiency as the probable negative P balance during lactation in the wet season cannot be restored during the dry season" (Ternouth, 1990). The most critical times for P supplementation in cattle in extensive grazing situations are in cattle with the highest demand for P. The most productive cattle are cattle with the highest demand for P. The two major products of cattle production are the deposition of body mass (and/or foetal mass) and milk production, both of these activities coincide with the wet season. The wet season is when other limiting nutritional factors, primarily dry matter digestibility, protein, available dry matter and sulphur, are no longer limiting. The promotion of greater animal productivity by the availability of these nutrients, combined with greater animal productivity associated with reproduction result in P becoming the limiting nutritional factor during the wet season. Hence supplementation of heifers during their initial joining period (ca. 2 yr old) and during the wet season of their first lactation are often referred to as being the most at risk for P deficiency and should be the primary target of supplementation programs (McIvor *et al.*, 2011).

Recommendations given for P requirements are often in excess of actual requirements under practical conditions (Coates and Ternouth, 1992). Diets offered to yearling heifers for two years at 0.66% of ARC recommended values did not decrease live weight or pregnancy status against P fed at 176% of recommendations. However, serum P levels were increased as a result of the higher P intake. Excess supplementation of P was not toxic and very large Ca:P ratios (approx 9:1) were not a problem in yearling heifers (Call *et al.*, 1978). It would appear that in this study that either P recommendations are too high or growth rates were not high enough to force P to become a limiting factor (growth approx 0.45 kg/day), it is likely that it is a combination of both as P requirements are a function of production and ARC recommendations have regularly been assessed as being excessive (Ternouth *et al.*, 1996).

Coates and Ternouth (1992) found that yearling heifers grazing P deficient and marginal P pastures (deficient with and without supplemental P) had adequate P for growth with only the lowest P intake group having slightly reduced live weight gain over the 12 month period. Plasma iP levels were considered to be low, < 60 mg/L, but weight gain was still adequate (ca. 0.5 kg/day). Plasma iP levels were increased on marginal P and supplemental P. Manure concentration of P showed difference between low and high P diets but supplemental P diets were similar to low with a low concentration of P and greater digestibility. Their research demonstrated that ARC recommendations over estimate P requirements in extensive situations and that P supplementation will increase live weight gain.

A strategy of classifying P deficiency based on soil P concentration has been developed to aid identification of P areas prone deficiency problems. The benefit of classifying the soil is that it avoids seasonal variations in plant P content and between species plant variability. P deficiency status has been classified into (reproduced from McIvor *et al.*, 2011):

- acute (< 4 mg/kg of bicarbonate extractable P)
- deficient (4-6 mg/kg)
- marginally deficient (7-8 mg/kg)

Within these classifications generalized recommendations can be made that suggest that all stock should be supplemented with P in acute situations; all stock during the productive season on deficient country with emphasis on reproductive stock during lactation, especially heifers; and lactating heifers on marginally deficient country.

Generalized recommendations for P supplementation need to be modified for individual circumstances. To increase voluntary feed intake and therefore productivity all dietary requirements need to be satisfied. Supplementary P usually only results in increases in weight gain when dietary nitrogen is in excess of 1.5% (Winks, 1990) and the remaining dietary requirements, predominately availability, composition, digestibility, Ca, Na and S composition, are also required to be satisfactory and not limiting.

While P is predominately a limiting factor during the wet season its availability is still low during the dry season. When NPN supplementation programs are conducted during the dry season it is critical that P is also included in the supplementation program, as the introduction of a protein source will cause it to become a limiting factor for microbial production in the rumen (Bortolussi *et al.*, 1996).

2.3.7 The potential role of Vitamin D for the improvement of dietary P absorption and/or status

Phosphorus deficiencies are a major cost to extensive grazing industries and the cost of P supplements will continue to increase in real terms. Many extensive grazing situations are only marginal deficient in P, and if the efficiency of P absorption was greater than the typical 50%, P requirements may be able to be met through the supply of P from pasture alone. This then raises the possibility that excessive "mining" of P reserves may occur (McIvor *et al.*, 2011). However, the extensive nature of grazing industries means that the actual P removed from the top soil is likely to be negligible, take many years to become critical and potentially be replaced by various mechanisms (McIvor *et al.*, 2011).

As 1,25-vitD can increase active absorption of P from the gastrointestinal tract, supraphysiological doses of 25-vitD can replace a large proportion of the functions of 1,25-vitD without the secretion of PTH (Rowling *et al.*, 2007). Thus adequate plasma Ca concentrations will not inhibit active absorption of P. If P absorption or retention can be increased by increasing Vitamin D status, i.e. 25-vitD plasma concentrations greater than 200 ng/ml, then P supplementation may not be required in many of the extensive grazing situations where P is the limiting factor. Delivery of 25-vitD, is the next challenge.

In extensive grazing situations that involve acute P deficiency an improvement in P absorption, regardless of how effective, will not meet P requirements. This is often the case on extremely P deficient soils or when an animal is highly productive due to other nutritional

factors such as protein, digestibility, Ca and S being in abundance (Cohen, 1980). Supplementation with P in these situations is likely to be of great benefit and may effectively be delivered by a lick block, loose supplement or even water medication (McIvor *et al.*, 2011). However, P is likely to be absorbed at a similar rate to the basal diet, which means that much of the P supplied by the supplement will not be digested and returned to the soil and largely wasted (Coates and Ternouth, 1992). If 25-vitD was included in supplementary diets the P digestibility of the diet may be increased. This would result in lower P requirements in supplementation programs, greater cost effectiveness or potentially greater retention and storage of P and greater productivity resulting from the supplement program.

2.4 Impacts on Ca homeostasis caused by metabolic acidosis or alkalosis on Ca homeostasis.

2.4.1 Dietary cation anion difference (DCAD)

Dietary cation anion difference (DCAD) is the difference in molecular equivalents of strong anions and strong cations in the diet (Ender *et al.*, 1962, Goff and Horst, 2003b). If an animal is consuming more anionic elements than cationic the diet is to be considered to have a negative DCAD and this will result in a degree of compensated metabolic acidosis (Block, 1994, Espino *et al.*, 2003). Conversely if an animal is consuming more cations than anions (typical pasture diet high in K) the animal will experience compensated metabolic alkalinity.

The theory is based on two beliefs. The first is that the number of moles of positively charged particles in any solution must equal the number of moles of negatively charged particles. The second is that the product of concentration of hydrogen ions and hydroxyl ions is always equal to the dissociation constant of water (Goff and Horst, 2003b). The pH of the solution is subject to the difference between the number of negatively and positively charged particles. If negatively charged particles (anions) are added to a solution the number of OH^- ions will decrease and the number of H^+ ions increase causing a reduction in pH (Goff and Horst, 2003b).

Salts of various cations and anions may be acidifying, alkalying or neutral – depending on the relative rate of absorption of each cation and anion within the salt. For example NaCl is a neutral salt as both cation and anion are efficiently absorbed. $CaCl_2$ on the other hand is an

acidifying salt as the Cl^- is absorbed almost completely while Ca^+ is generally absorbed at less than 40% (Goff *et al.*, 1991b).

The primary anions in the blood are bicarbonate anions. The relationship between the bicarbonate anion and CO_2 are responsible for minute-to-minute regulation of blood pH (Goff and Horst, 2003b). However, the concentration of non-metabolisable anions and cations has a much more long-term effect on metabolic pH. The strong ion difference theory refers to elements that enter through the digestive tract, hence the DCAD relationship with metabolic acid base balance (Goff and Horst, 2003b). Once absorbed the ions cannot be regulated by the respiratory tract and must be regulated, or removed, by the kidneys.

The major cations in the diet that have the most effect on metabolic acid base balance are Na, K, Ca and Mg. The major anions are S, Cl and phosphate (Goff and Horst, 2003b). The most common equation was originally proposed by Ender (1962)

$$DCAD\ (mEq/kg) = (\ Na^+ + \ K^+) - (\ Cl^- + \ S^{--})$$

Whilst this equation does not take into account several of the ions mentioned it does take into account the most influential and variable of the ions. Other equations may include some of the other ions mentioned but give them weightings that permit less influence on the equation. Goff and Horst (2003b) proposed the following equation

$$DCAD\ (mEq/kg) = (\ 0.15\ Ca^{++} + \ 0.15\ Mg^{++} + Na^+ + \ K^+) - (\ Cl^- + \ 0.6S^{--} + 0.5\ PO_4^{--})$$

For any of these ions to be effective they must be absorbed from the digestive tract (Goff and Horst, 2003b). The main ions represented in the equation are highly digestible. While some of the other ions mentioned may have varying degrees of digestibility, which are dependent on individual circumstances. Block (1984) used the original equation put forward by Ender (1962) but also referred to other more inclusive equations as potentially being more useful.

2.4.2 How is metabolic pH controlled?

The acid-base balance is finely tuned in most animals. There are three systems at work that maintain the acid-base homeostasis: intracellular and extracellular buffers, the respiratory system and the kidneys (Block, 1994). The normal blood pH is approximately 7.35 in bovine

(Goff and Horst, 2003b) and it is necessary for normal cellular function to maintain the balance in a very narrow range (Riond, 2001).

Intracellular and extracellular buffers and the respiratory system are responsible for rapid correction of blood pH to maintain a pH of approximately 7.35. While these two systems are effective short-term buffers they are unable to control long term variations in metabolic pH. The kidneys are responsible for long-term acid-base homeostasis and the excretion of excess hydrogen ions (Riond, 2001).

In monogastrics excess acid is typically the condition that is most prevalent in animals, although on occasions excessive basic ions must also be eliminated. Excess acid can be accumulated from a variety of sources such as exercise and other physiological processes, but the primary cause is diet (Riond, 2001). However, in grazing ruminants the predominant DCAD of the diet is highly positive, with grasses (especially heavily fertilized pastures) having a high, and often extremely high, K concentration. Furthermore, water consumed as part of the diet may also contain excessive Na, and often Ca may be excessive in ground water (NRC, 2000).

DCAD appears to exert its effects on blood pH by altering blood bicarbonate concentrations. Blood bicarbonate increases with increasing DCAD (Ross *et al.*, 1994), potentially in response either to increased systemic bicarbonate generation accompanying the absorption of Na and K from the gastrointestinal tract or to a reduction in systemic free proton generation as Cl absorption decreases. Of these three electrolytes (Na, K and Cl), serum Cl appears most profoundly affected in acid-base physiology (Tucker *et al.*, 1988a).

Vagnoni and Oetzel (1998) presented data that showed cows fed anionic diets had similar blood pH. However, they noted that blood $HCO3^-$ and base excess (i.e. a reduction in mEq/L) were reduced. This indicated that dietary anionic salts induced a mild metabolic acidosis that was adequately compensated by non-respiratory mechanisms (Vagnoni and Oetzel, 1998). Non-respiratory compensatory mechanisms include the degradation of bone for buffering, which results in an increase in urinary Ca excretion and an associated improvement in Ca homeostasis during parturition.

The kidneys respond to a decline in blood pH, which stimulates the Na^-/H^+ counter transport system in the renal tubules. Delaquis and Block (1995) found an increase in H^+

excretion in cows fed a diet that was more anionic. The acid stress caused by the dietary manipulation was apparent from the urinary analysis but was insufficient to overload the renal compensatory mechanisms resulting in no change in the acid-base parameters.

Buffers that are used to titrate excess H^+ to maintain pH within physiological limits include haemoglobin, carbonate from bone, phosphate and bicarbonate. The principal protein for buffering blood is potassium salt of oxyhaemoglobin in ethrocytes (Block, 1994). These buffers work rapidly and effectively until they reach the point where their buffering capacity is exceeded. In chronic metabolic acidosis conditions, bone provides a large supply of buffering capacity. In this state, excess H^+ in the extracellular fluid promotes physiochemical as well as osteoclast mediated dissolution of bone, releasing carbonate, which works as an effective buffer (Goff and Horst, 2003b).

The renal collecting duct can secrete protons and generate acidic urine if the animal is under metabolic acidotic stress (Goff *et al.*, 1991b). It is also capable of net HCO_3^- secretion in response to alkalosis (Cunningham, 1997). However, the proximal tubule is primarily responsible for the bulk of acid secretion, whereas the collecting duct is responsible for the control of net acid excretion and final urine pH. Therefore an animal under acidotic stress will have a urine pH concomitant with its level of metabolic acidosis and conversely the animal's urine pH will be basic when under alkalotic stress.

2.4.3 What are the primary effects of metabolic pH on Ca homeostasis?

Dietary cation anion difference has been found to have a very close relationship with the incidence of clinical hypocalcaemia (milk fever) in dairy cattle (Block, 1984, Goff *et al.*, 1991b). There are several affects that may cause this relationship. They include hormone sensitivity of tissue and bone resorption for buffering. Diets consumed by prepartum cows that cause compensated metabolic acidosis prevent a decline in plasma Ca at the initiation of lactation (Abu Damir *et al.*, 1994, Moore *et al.*, 2000, Chan *et al.*, 2006) and a reduction in incidence of milk fever (Gaynor *et al.*, 1989), although Ramos-Nieves *et al.* (2009) found no difference in plasma Ca concentration in cows at parturition. Low DCAD diets have been shown to increase an animal's ability to mobilise Ca into the extracellular Ca pool when plasma Ca content is reduced (Takagi and Block, 1991a).

PTH receptor sensitivity

Alkalosis affects PTH sensitivity of certain tissues (Goff *et al.*, 1991b, Goff and Horst, 2003b). Two PTH dependent functions, bone resorption and renal production of 1,25-vitD are enhanced in cows fed anionic diets (Block, 1984, Gaynor *et al.*, 1989). There is evidence to suggest that under neutral metabolic conditions when blood pH is approximately 7.35, PTH and its receptors interact much more effectively in target tissues located in critical areas such as kidney and bone (Beck and Webster, 1976, Goff and Horst, 2003b). Alkaline dietary conditions (positive DCAD) and hence metabolic alkalinity; may change the conformational structure of the PTH receptor so that PTH and its receptors do not interact as efficiently (Bushinsky, 2001, Espino *et al.*, 2003). Espino *et al.* (2003) found that in sheep, cationic diets resulted in increased levels of PTH at parturition but levels of osteocalcin were not increased, suggesting that PTH receptors were refractory due to the cationic state. This was further supported within the experiment as anionic treatment sheep had 're-organised' bone structure to allow for greater mobilisation of Ca.

Cationic diets have been shown to reduce renal synthesis of 1,25-vitD at the time of parturition in dairy cows (Gaynor *et al.*, 1989). Although, studies have also shown no difference in plasma concentrations of 1,25-vitD in cows offered diets with cationic or anionic DCAD (Kurosaki *et al.*, 2007). The evidence suggests metabolic alkalosis reduces the sensitivity of the renal tissue to PTH so that there is no increase in the hydroxylation of 25-vitD due to no increase in the production of 1α-hydroxylase (Goff and Horst, 2003b). Although in an EDTA challenge experiment with non-pregnant non-lactating cows Heron *et al.* (2009) found that during the challenge that PTH or 1,25-vitD were not influenced by DCAD even though the treatments with lower DCAD recovered their plasma Ca concentration faster than treatments on higher cationic diets, this has also been seen in other studies (Joyce *et al.*, 1997, Kurosaki *et al.*, 2007). The affect of metabolic alkalinity on the production of 1,25-vitD is not clear, it may well be that the refractory nature of PTH receptors results in a reduction of 1,25-vitD production in some cases or simply that there is less requirement for 1,25-vitD in cows supplemented with anionic salts as the PTH secreted is more effective and there is more Ca available as a consequence of the compensating mechanisms which control the metabolic acidosis.

Bone buffering

The blood buffering capacity of bone during metabolic acidosis may be beneficial for cows during hypocalcaemic events. As bone acts as a major buffer for blood it releases cations (primarily Ca) into the blood to buffer changes in pH (Vagnoni and Oetzel, 1998, Goff and Horst, 2003b). This has been conclusively shown in rats (Beck and Webster, 1976). The extra calcium is excreted by kidneys, resulting in hypercalciuria (Tucker *et al.*, 1988a, Tucker *et al.*, 1992, Vagnoni and Oetzel, 1998, Chan *et al.*, 2006, Kurosaki *et al.*, 2007). Liesegang (2008) used an aggressive anionic salt addition (approx DCAD -164 mEq/kg) and found that in goats both bone degradation markers were increased (CL and ICTP), urine Ca was increased and osteocalcin started to decrease 10d prepartum. The reduction in osteocalcin suggests that bone accretion was reduced but the markers for bone degradation clearly indicate that bone was being resorbed in response to the metabolic acidosis. The findings were also seen in dairy cows as Abu Damir *et al.* (1994) found that the addition of anionic salts increased the proportion of woven bone over lamellar cortical bone prior to calving. Woven bone is a temporary tissue that forms rapidly and has higher Ca content than compact bone, and may therefore act as a readily mobilised reserve of Ca during parturition (Abu Damir *et al.*, 1994). There is general acceptance in the literature (Goff and Horst 1997, 1998, Kurosaki *et al.*, 2007) that bone degradation increases during compensated metabolic acidosis.

Increase the flow of Ca through the exchangeable pool

Anionic diets increase the flow of Ca through the exchangeable Ca pool but do not increase the size of the pool (Fredeen *et al.*, 1988, Takagi and Block, 1991c, Chan *et al.*, 2006). Acidosis increases entry (bone and dietary) and clearance (urinary) rates and they are modified by PTH and calcitonin concentrations, which are determined by plasma Ca concentration. Plasma Ca concentration should remain constant regardless of anionic state except when influenced by other clearance factors, such as lactation. However, some experiments have reported a rise in extracellular Ca content as a result of low DCAD (Vagg and Payne, 1970) but usually only during periods of increased Ca removal, such as parturition or EDTA infusion (Block, 1984, Won *et al.*, 1996, Goff and Horst, 1998).

Urinary pH and Ca excretion

Urinary characteristics are influenced by DCAD. The contribution of the compensatory mechanisms involved in the control of metabolic acidosis or alkalosis are most easily measured in urine. Two of the primary measurements of compensatory acidosis are urine pH and Ca excretion.

Urine pH is a measure of the removal of excess H^+ ions, and Ca is a definitive by-product of bone degradation for buffering purposes (Goff and Horst, 2003b). Furthermore, Ca excretion from the kidney is also augmented by a depression of the renal tubular resorption of Ca induced by acidosis (Liesegang, 2008). However, increased urinary Ca excretion may also originate from higher dietary Ca intakes or even increased absorption from the alimentary tract. During experimental conditions there should not be confounding factors present that increase dietary intake of Ca and therefore the primary conclusion of elevated levels of urine Ca excretion is that extracellular (or available) Ca is in excess.

Diets supplemented with anionic salts, acid or generally negative DCAD in nature have been shown to reduce urinary pH in non-lactating dairy cows (Joyce *et al.*, 1997, Goff and Horst, 1998, Roche *et al.*, 2007), beef cows (Hersom *et al.*, 2010), sheep and goats (Liesegang, 2008). After the initial application of anionic salts, urinary pH will usually reach a nadir after 48h (Roche *et al.*, 2007), although Goff and Horst (1998) found that it took less than 16h to drop and would resume to normal, 24h after removal of anions from the diet. They also suggested that over time, the buffering system became more effective at maintaining metabolic pH as urinary pH tended to increase. This was also evident in both sheep and goats (Liesegang, 2008).

An increase in urinary Ca excretion almost always accompanies a reduction in urine pH (Schonewille *et al.*, 1994, Goff and Horst, 2003b, Kurosaki *et al.*, 2007, Roche *et al.*, 2007) although sheep appear to take longer to excrete urine Ca after consuming anionic diets than goats (Liesegang, 2008). Ca excretion may take up to 10d following addition of anions to diet (Roche *et al.*, 2007). Urine Ca excretion is a result of excessive extracellular Ca, excretion of Ca is critical for maintaining Ca homeostasis. Research has shown that this release is approximately 2-5 g/day (Kurosaki *et al.*, 2007). Animals in a state of hypercalciuria state are able to conserve Ca in the kidney extremely quickly once an alternative demand for the Ca is evident (Goff and Horst, 2003b, Chan *et al.*, 2006). Alternatively, cows that are in an

alkalotic state will only have this source of Ca available after they have activated hormonal control and the bone Ca has moved from the labile pool across the osteoblast-osteocyte membrane. This delay in Ca availability is potentially one of the critical factors behind clinical hypocalcaemia and is further impeded by PTH receptors being less sensitive due to cationic diets (Goff *et al.*, 1991b).

Liesegang (2008) suggested that anionic salts appear to increase urinary Ca due to increasing bone breakdown, decreasing accretion and up-regulating Ca absorption in the SI. However, excess Ca originating from bone during compensated metabolic acidosis is usually the primary origin of urinary Ca (Liesegang, 2008). However, phosphate is also a major constituent of bone but there is no increase in urinary phosphate excretion and it does not play a role in total acid excretion, unlike monogastrics (Vagnoni and Oetzel, 1998). In ruminants consuming a forage-based diet P is excreted via the saliva through the gastrointestinal tract (Breves *et al.*, 1985).

Feeding anionic salts has no effect on creatinine production and therefore urine spot samples adjusted for creatinine is an applicable assessment of total urinary Ca excretion (Wang and Beede, 1992a, Vagnoni and Oetzel, 1998). Roche (2007) found that anionic salts delivered twice a day resulted in no diurnal changes in urine pH. This enabled spot samples for urine pH analysis to be taken at any time.

Ca status

Compensated metabolic acidosis does not increase the plasma Ca concentration as Ca homeostatic mechanisms increase urinary Ca excretion to maintain homeostasis (Kurosaki *et al.*, 2007). Metabolic acidosis caused by DCAD levels below 0 have been shown to increase Ca release from bone (Beck and Webster, 1976, Takagi and Block, 1991c, Espino *et al.*, 2003, Charbonneau *et al.*, 2009) and to a lesser extent increase the rate of Ca digestibility, although most research indicates that negative DCAD diets reduce Ca retention as the loss of Ca from bone is greater than the improvement in Ca absorption from the small intestine (Takagi and Block, 1991b, Charbonneau *et al.*, 2009). However, in some situations the increase in small intestine Ca absorption is equal to the Ca lost in urine (Roche *et al.*, 2007). Schonewille *et al.* (1994) found that highly anionic diets resulted in 11.6% of Ca intake being absorbed and excreted through urine. Furthermore, using hydroxyproline as a marker there was no indication of increased bone degradation.

The conflicting evidence presented by Schonewille *et al.* (1994) may be a result of feeding the anionic salts for longer than most other studies, 19d as opposed to the usual adjustment period of 10 to 14d. Longer feeding periods may completely drain the labile Ca bone pool and allow increased absorption of Ca from the small intestine to compensate. However, results more commonly demonstrate continuous bone degradation and only marginally increased Ca absorption, which results in a net Ca loss (Braithwaite, 1975, Fredeen *et al.*, 1988, Takagi and Block, 1991c, Espino *et al.*, 2003).

2.4.4 Efficacy of various Anionic feeds?

Ender *et al.* (1962) discovered the DCAD principle with a series of experiments examining the addition of silages, made with mineralised acids, to dairy cow diets. This has been extended by many other researchers who have defined the strong cation anion theory and shown that anionic salts, of either Cl and S can effectively manipulate metabolic pH (Block, 1984, Gaynor *et al.*, 1989, Oetzel *et al.*, 1991, Goff and Horst, 1998, Chan *et al.*, 2006, Kurosaki *et al.*, 2007). Originally it was thought the DCAD equation was based on molecular Equivalents (Eq) and that each Eq of anion was equivalent, regardless of source. Goff *et al.*, (2004) reviewed the efficacy of several different sources of anions from salts and acids to compare acidifying efficacy. In general the findings were that the equivalent amount of acid had more of a metabolic acidifying effect than an anionic salt. This stands to reason as the anion in anionic salt is always coupled with a relevant cation, thus the relative strength of the cation varies from salt to salt. Goff *et al.* (2004) also found that Cl anionic salts were much more effective at inducing a metabolic acidosis than S based anionic salt (Figure 2.7).

However, other researchers found that sulphate anions had similar efficacy to chloride anions for reducing hypocalcaemia even though urine pH was usually not reduced to the same extent (Oetzel *et al.*, 1988, Oetzel *et al.*, 1991, Tucker *et al.*, 1991). Sulphur has potentially limited usage as the incidence of S toxicity is likely to increase when S is above 0.4% (NRC, 2000) where as Cl content may be increased much higher.

Anionic diets have been known to reduce dry matter intake of prepartum cows (Ender *et al.*, 1962, Vagnoni and Oetzel, 1998, Moore *et al.*, 2000) and reduce palatability (Wang and Beede, 1992a). Gaynor (1989) found that jersey cows fed additional anionic salts had a lower dry matter intake than cows that were not fed the anionic salts.

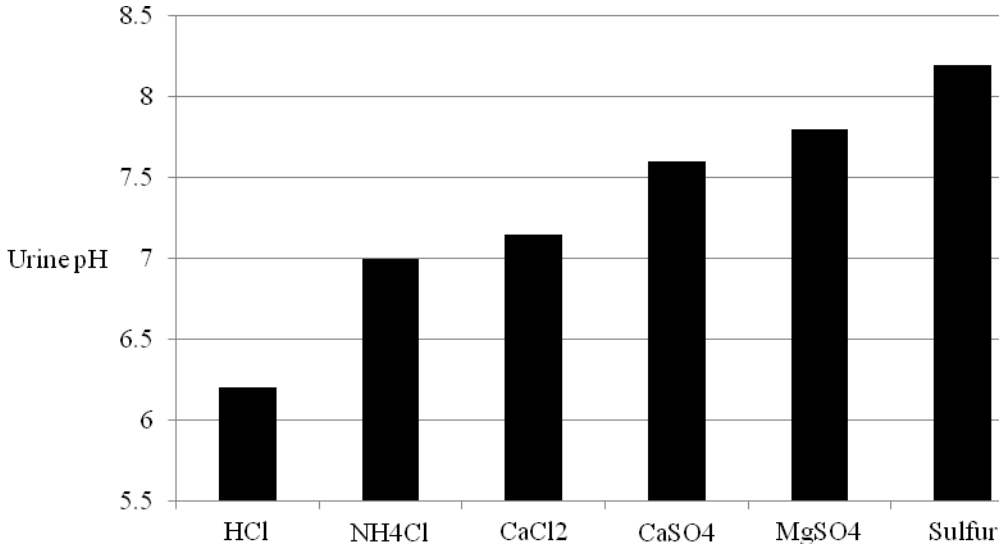

Figure 2.7 Mean urine pH of Jersey cows fed 2 Eq of anion using hydrochloric acid (HCl), ammonium chloride (NH4Cl), calcium chloride (CaCl2), calcium sulfate (CaSO4), magnesium sulfate (MgSO4), or elemental sulfur as sources of anions (Goff *et al.*, 2004).

The fertilisation of forage crops with chloride and sulphate-based fertilisers can reduce the DCAD without having to add unpalatable and corrosive salts to the diet (Pehrson *et al.*, 1999, Goff *et al.*, 2007, Heron *et al.*, 2009). Pehrson *et al.* (1999) found that this was more easily accomplished with Cl than S. Feeding low DCAD grass silage to cows resulted in a significant reduction in intake, indicating that the palatability of Cl even in biological form may be the major component of palatability issues (Charbonneau *et al.*, 2009). This suggests that the reduction in intake may be due to metabolic factors and not just palatability.

There are other dietary factors besides anions and cations that may have an impact on metabolic acid/base balance. Potentially high protein diets may result in ammonia becoming an excessive cation potentially causing an error in the calculation of DCAD, although Goff *et al.* (2004) could not prove this theory. Increasing the level of acid producing feed stuffs, such as grain, could theoretically alter metabolic pH (Scott *et al.*, 1971). However, the resulting level of metabolic acidosis is not as great as for those cows supplemented with anionic salts and it presents other potential health problems (Mellau *et al.*, 2004).

2.4.5 Metabolic acidosis and Vitamin D control

The effect of anionic diets on plasma 1,25-vitD concentration in published research is varied and far from consistent, although this is likely to be a consequence of inconsistent sampling periods. Some research has shown that anionic diets increase 1,25-vitD production (Gaynor *et al.*, 1989). Whilst others show that the total 1,25-vitD production is decreased or not affected (Goff *et al.*, 1991b, Abu Damir *et al.*, 1994, Kurosaki *et al.*, 2007). However, there are potential explanations for both cases. Cationic diets may cause PTH receptors to be refractory (Goff *et al.*, 1991b), this will initially result in lower concentrations of 1,25-vitD, but as low plasma Ca concentration continues to cause PTH secretion eventually 1,25-vitD production is increased to a greater level than animals consuming the equivalent anionic diet. Furthermore, greater increases in 1,25-vitD concentration in cows with anionic diets usually seen prior to parturition may be explained by increased receptiveness to PTH causing initially greater production of 1,25-vitD (Gaynor *et al.*, 1989, Abu Damir *et al.*, 1994, Liesegang, 2008). This early response to PTH secretion often results in lower total PTH secretion in cows consuming anionic diets during the parturition period as a consequence of plasma Ca returning to homeostasis more quickly than in cows that are more refractory to the initial secretions of PTH (Abu Damir *et al.*, 1994). Goff *et al.* (1991b) suggested that even more 1,25-vitD may need to be produced in cows with slight metabolic alkalinity as the rate of osteoclastic bone resorption was lower in the cows consuming the cationic diet suggesting that the PTH induced bone degradation may have been partly refractory as a consequence of metabolic alkalinity.

Until recently there has been a scarcity of research that combines both anionic salts and 25-vitD. However, one such study by Cho *et al.* (2006) found that anionic salts and 25-vitD treatments resulted in increased serum concentrations of both 1,25-vitD and 25-vitD over 25-vitD supplementation alone in beef cows destined for slaughter. Both treatments had greater 1,25-vitD and 25-vitD concentrations than the control diet, which did not contain anionic salts nor 25-vitD. The serum concentration of Ca was increased in the anionic salt and 25-vitD treatment but not in the control or 25-vitD treatment. In previous research Vitamin D supplementation without anionic salts has been more likely to increase serum Ca concentration than the use of anionic salts alone. When serum Ca is within homeostatic balance, the combination of both anionic salts and 25-vitD in this experiment may demonstrate a positive interaction when therapies are combined (Hollis *et al.*, 1977, Wang

and Beede, 1992a). However, it has been demonstrated that while total plasma Ca is not increased during metabolic acidosis there is often a small but significant increase in ionised plasma Ca. Unfortunately urine sample nor Ca intake or excretion were analysed so the effect on Ca status of the various treatments is unknown (Wang and Beede, 1992a).

A recently published study has examined the effect of the combination of supraphysiological doses of 25-vitD and anionic salts on the maintenance of plasma Ca during parturition in dairy cows (Wilkens *et al.*, 2012). The study successfully demonstrated several of the theories put forward in this literature review. Of most importance is that a combination of anionic salts and 25-vitD increase urinary Ca excretion prior to parturition, reduce the concentration of PTH in plasma, increase the PTH:1,25-vitD (which suggests, improves the level of PTH receptor sensitivity), decrease the rate of bone degradation post parturition and most importantly results in higher plasma Ca concentration at parturition.

2.5 The effect of Vitamin D and Anionic salts on the incidence of hypocalcaemia

2.5.1 Aetiology of hypocalcaemia

Cows that cannot maintain their Ca blood concentration between 8 and 10 mg/dl will suffer from hypocalcaemia (Ramberg *et al.*, 1984, Horst, 1986, Goff and Horst, 1997). When plasma Ca concentration becomes too low to support nerve and muscle function it results in parturient paresis (Goff and Horst, 1997).

A lack of 1,25-vitD during parturition is not considered to be the cause of hypocalcaemia at calving as production of 1,25-vitD is similar in cows both clinically and non-clinically affected with milk fever (Horst *et al.*, 1997, Goff and Horst, 2003b). However, a delay in the production of 1,25-vitD or inhibition of a response to PTH and 1,25-vitD may result in increased production of 1,25-vitD immediately post parturition (Abu Damir *et al.*, 1994).

Primiparous cows exhibit a much lower incidence of hypocalcaemia than multiparous cows because of lower milk yield and a greater ability to mobilise bone Ca deposits (Chan *et al.*, 2006, Kurosaki *et al.*, 2007). Due to the ongoing growth and development of young animals the bone structures are less stabile and there is probably greater bone turnover (Van Mosel *et al.*, 1994). Increased parity is linked to an increase in the incidence of hypocalcaemia, although milk yield is not (Fleischer *et al.*, 2001).

There does not appear to be any effect of season on the incidence of clinical hypocalcaemia when feeding and management are kept uniform through the year (Dohoo *et al.*, 1984). Therefore it is unlikely that inherently seasonally variable endogenous levels of 25-vitD have any impact on the incidence of hypocalcaemia.

2.5.2 Treatment methods and results

Prevention is the most desirable means of reducing the economic losses occurring from parturient paresis. The losses include but are not necessarily limited to milk production, veterinary costs, further animal health problems, labor costs, and possible loss of the cow (Block, 1994). There are several methods which can reduce the incidence of hypocalcaemia. They include offering a diet low in Ca, injections of various Vitamin D metabolites and reducing the DCAD prepartum (Delaquis and Block, 1995).

Low dietary Ca

When cows are fed less Ca in the diet than they require they secret PTH to increase the rate of osteoclastic bone resorption and renal production of 1,25-vitD (Goff *et al.*, 1989, Goff, 2000). At parturition the lactational drain of Ca is more easily replaced as the bone osteoclasts are already active and in high numbers. If there is an increase in dietary Ca immediately after calving the previous up regulation of 1,25-vitD will allow for more efficient utilisation of dietary Ca and an absence of the 24h delay that it takes for 1,25-vitD to increase active absorption of Ca (Goff, 2000). However, Chan (2006) found no difference in serum Ca concentration between cows with normal (0.99%) and high (1.5%) dietary Ca when fed a negative DCAD diet prepartum. Demonstrating that changes in dietary Ca may not have an influence when using the DCAD method to avoid hypocalcaemia.

However, it is difficult to maintain low Ca diets as most forages have more than adequate Ca concentrations for bovine requirements (NRC, 1989).

Anionic Diets

Anionic salts are the most effective and practical method of manipulating DCAD in forage diets. Many studies have analysed the use of various anionic salts and the effects of these on metabolic pH parameters and the incidence of hypocalcaemia. Studies investigating potential reductions in the incidence of hypocalcaemia are difficult as the incidence is low and often

extremely variable. Most successful studies involve either numerous replicates, highly susceptible animals and diets or both.

Pre-parturient forage diets that are supplemented with anionic salts and have a strongly negative DCAD have been shown to reduce the incidence of clinical hypocalcaemia in dairy cows (Block, 1984, Goff *et al.*, 1991b, Goff and Horst, 1998) and even reducing the DCAD from strongly cationic to approximately neutral has been demonstrated to be beneficial (Kurosaki *et al.*, 2007). Clinical evidence of a reduction in the incidence of hypocalcaemia has been demonstrated by plasma Ca concentrations being lower at parturition in cows consuming strongly positive DCAD diets (Block, 1984, Goff and Horst, 1998). However, some experimental work has shown no influence of anionic salt supplementation on the incidence of hypocalcaemia (Roche *et al.*, 2007), although these results are often influenced by low incidence of disease or insufficient replication and in some cases the control diets being close to neutral for DCAD (Gulay *et al.*, 2008).

For a variety of reasons, primarily timing, studies are very hard to conduct during parturition. Replication of hypocalcaemia is often undertaken by inducing a Ca deficiency by infusion of Na-EDTA. Non-lactating and non-pregnant cows fed highly anionic diets were able to withstand a larger volume of Na-EDTA than cows fed a cationic diet, their plasma Ca and iCa also recovered much quicker after the withdrawal of Na-EDTA application (Wang and Beede, 1992a). The study showed an increased input of iCa into plasma. However, the study was not able to show if the Ca originated from bone or small intestine but did successfully demonstrate the ability of cows that have a compensated metabolic acidosis to rectify Ca deficiency. Most authors consider that negative DCAD diets increase Ca availability from bone in the absence of PTH, and when plasma Ca is deficient and PTH is secreted, the increase in Ca availability is from the diet and bone (Braithwaite, 1975, Fredeen *et al.*, 1988, Takagi and Block, 1991c, Espino *et al.*, 2003). However, Roche (2007) presented data for prepartum cows on DCAD of -20 or +18 mEq/100g DM diets showing that low DCAD increased absorption of Ca from the alimentary tract, increased urinary Ca and actually increased retained Ca.

The suggested mode of action for negative DCAD diets reducing the incidence of hypocalcaemia in these studies is in general agreement with previously stated theorems. Bone resorption was found to be increased in parturient cows who were offered negative DCAD

(Block, 1984, Goff and Horst, 1998) and the concentration of 1,25-vitD appeared to increase more quickly in cows who were offered negative DCAD diets (Goff *et al.*, 1991b, Goff and Horst, 1998). Although, other experiments have found this not to be the case (Kurosaki *et al.*, 2007) and some experiments have found the opposite as PTH is continually secreted in hypocalcaemic cows and will eventually overcome the refractory nature of PTH receptors during metabolic alkalosis.

Greater incidences of associated transition diseases such as mastitis, retained placenta and metritis have been seen in cows on cationic diets when compared to similar cows consuming anionic diets (Goff and Horst, 1998) and during the subsequent lactation production was greater in cows who consumed negative DCAD diets (Block, 1994). This demonstrates the benefit of reducing the incidences of subclinical hypocalcaemia which enables a more healthy transition period for the cow.

Vitamin D metabolites and combinations

The use of Vitamin D and its metabolites for the prevention of hypocalcaemia in dairy cows has displayed varied results. The general trend is consistent with the role of Vitamin D in improving Ca availability. However, there are difficulties associated with practical application of the various Vitamin D metabolites, as well as negative feedback of elevated levels of Vitamin D on endogenous production of 1,25-vitD and animal health implications of the treatment regimes.

Treatment of animals (generally by injection) with 1,25-vitD or similar artificial metabolites within 7d of parturition is successful for reducing the incidence of clinical hypocalcaemia in dairy cows (Bar *et al.*, 1985, Goff *et al.*, 1988, Goff *et al.*, 1989, Hodnett *et al.*, 1992). It has been suggested by several of the authors that the beneficial mechanism is by an increase in Ca absorption from the diet (Bar *et al.*, 1985, Goff *et al.*, 1988). However, negative feedback from supraphysiological levels of 1,25-vitD has resulted in a requirement for continual treatment post-parturition and hypercalcaemia during initial treatment (Bar *et al.*, 1985, Horst, 1986). Continual treatment of pre-calving cows may have negative aspects in a practical environment due to added stress for the animal and increased work load for staff. The use of such toxic pharmaceuticals in an agricultural setting is neither practical nor safe.

The use of 25-vitD as a sole additive for bovine has been recently limited to investigations into meat quality (Montgomery *et al.*, 2000, Foote *et al.*, 2004, Cho *et al.*, 2006, Carnagey *et al.*, 2008) with the occasional investigation of hypocalcaemia (Taylor *et al.*, 2008). However, extensive research was conducted utilizing extremely large doses of Vitamin D from 1940's through until 1970's (Hibbs *et al.*, 1946b, Hibbs and Pounden, 1955, Conrad *et al.*, 1956, Julien *et al.*, 1977). Although, feeding Vitamin D in massive amounts did reduce the incidence of hypocalcaemia the technique was very delicate with the Vitamin D being required to be fed for 3d before calving, but not more than 7d due to toxicity. The injection of Vitamin D has had more success as a potential treatment for hypocalcaemia than supplementation through feeding but still retained problems with predicting calving date and metastatic calcification (Payne and Manston, 1967).

After the discovery of 25-vitD (Lund and DeLuca, 1966) the use of 25-vitD as a prophylactic either given in the diet or by injection was examined. The metabolite has been shown to decrease the incidence of hypocalcaemia in susceptible cows if calving is between 72h and 10d of treatment (Bringe *et al.*, 1971, Olson *et al.*, 1973). The use of 25-vitD was considered to be more successful than Vitamin D as feeding was not required to be as regular and less units of the metabolite required to be effective, making the product safer. However, these studies were conducted in herds of extremely high incidence and were still unable to prevent the occurrence of hypocalcaemia. These studies were also conducted without knowledge of the DCAD, which may have influenced the outcome of the results.

Hollis *et al.* (1977) injected large amounts of 25-vitD into dry cows and found that it increased plasma Ca, P and had no effect on Mg. This article was the first to record half life of 25-vitD at approximately 34d and demonstrated that plasma 25-vitD concentration was required to be above 200 ng/ml to increase plasma Ca concentration. Recently, Taylor *et al.* (2008) supplemented Jersey cows prior to parturition with 15 mg of 25-vitD 6 d prior to parturition. The supplementation level increased plasma 25-vitD to approximately 120 ng/ml. However, it is unlikely that this concentration of 25-vitD is sufficient to increase the concentration of plasma Ca or P, no effects were seen with pre or post parturition. As the DCAD was not given in the experimental data, the diet is likely to have been cationic, it is unlikely that the low level of 25-vitD would have positively influenced Ca homeostasis at calving as a consequence of the refractory nature of PTH receptors.

Wilkens *et al.* (2012) have recently published work that has investigated the effect of 25-vitD and anionic salts on improving plasma Ca concentration during parturition. The study compared a typical highly cationic diet with and without 25-vitD as well as a similar but anionic diet with and without 25-vitD. The study successfully demonstrated the changes in P and Ca homeostasis when 25-vitD was included in the diet and also the negative impact on plasma Ca concentration at parturition when 25-vitD is included in a cationic diet. The plasma concentration of 25-vitD in this experiment reached approximately 180 ng/ml prior to parturition. This concentration was not strong enough to change plasma concentrations of both Ca and P prior to parturition (Hollis, 2010). However, the concentration was strong enough to allow an interaction with anionic diets at parturition. Key findings from the experiment are analogous with summarised findings of this literature review. The combination of 25-vitD and anionic salts appeared to maintain plasma Ca concentration at parturition by increasing the sensitivity of PTH receptors, increase the excretion of urine Ca prior to parturition, maintain the labile bone Ca prior to parturition and increase the absorption of Ca from the diet.

2.5.3 DCAD and Vitamin D – a better treatment for hypocalcaemia

Based on this critical review of the literature, I believe that compensated metabolic acidosis and 25OHD may provide a better method for treating hypocalcaemia than anionic salt therapy alone. Both techniques improve the availability of Ca. However, complementary methods of improving Ca availability from both therapies may result in a synergistic relationship were the whole is greater than the individual affects of each therapy ().

It appears that supraphysiological doses of 25-vitD appear to usurp the PTH mediated control of 1,25-vitD and independently initiate Ca absorption from the small intestine, preemptively initiating the active absorption of Ca prior to parturition (Rowling *et al.*, 2007). As an increase in Ca availability from both the skeleton and diet may require up to 24h to activate, prior activation is critical for prevention of hypocalcaemia.

The combination of compensated metabolic acidosis and supraphysiological doses of 25-vitD should initiate all of the main aspects of Ca availability prior to parturition. This has recently been demonstrated in parturient dairy cows (Wilkens *et al.*, 2012). Prior activation of Ca mobilisation mechanisms prior to parturition is critical to the avoidance of hypocalcaemia (Horst *et al.*, 2005). The combination of both techniques may result in greater Ca availability

than just 25-vitD or metabolic acidosis as all homeostatic mechanisms that generate Ca availability are functioning. Furthermore, the improvement in PTH hormone receptor sensitivity should enable the animal to increase Ca availability further by initiating conversion of the available 25-vitD to 1,25-vitD if low serum Ca concentrations promote the secretion of PTH.

Table 2.4 Method of increase in Ca availability and period of affect generated by either metabolic acidity (strongly -ve DCAD) or metabolic neutrality (neutral DCAD) due to anionic salt supplementation or supraphysiological doses of 25-vitD.

Increase in Ca availability	**Cause**	**Period of Action**
Bone degradation	Metabolic acidity	24h after feeding of anions
Active absorption from alimentary tract	25-vitD	24-48h after feeding or injection of 25-vitD
Reduction in refractory nature of PTH receptors	Metabolic neutrality	24h after feeding of anions, but benefit is not seen until PTH secretion (i.e. parturition)
Excretion of large amounts of urinary Ca	Metabolic acidity or Metabolic neutrality and 25-vitD	24h after feeding of anions, but benefit is not seen until PTH secretion (i.e. parturition)
Availability of large amounts of substrate for metabolism to 1,25-vitD	25-vitD and to a lesser extent metabolic neutrality	When PTH is secreted (i.e. parturition)

In some practical situations, i.e. when pastures are extremely high in potassium or there is excessive Na in water, it is extremely difficult to promote metabolic acidosis to the advised levels. Furthermore, anionic salts are extremely unpalatable and cows required to consume supplemented feed may not do so. In such situations moderate reductions in DCAD may still be of benefit when combined with 25-vitD as the reduced alkalinity of the diet may not be sufficient enough to initiate Ca removal from bones but it may be adequate enough for reducing the refractory nature of highly alkalinic diets. This would result in the animal retaining four out of the five benefits given in Table 2.4.

Chapter 9. General Discussion

As the structure of this thesis incorporates published articles and stand alone studies many of the results have already been discussed. However, the discussion within the individual study chapters is generally specific to the individual chapter and does not seek to add to a general understanding of Vitamin D mediated Ca and P metabolism in cattle.

This general discussion aims to discuss the overarching theme of the thesis. It attempts to draw on the conclusions made within the individual chapters and present a concept of how 25-vitD influences mineral metabolism. The potential for the combination of 25-vitD and dietary anionic salts in alleviating hypocalcaemia experienced during parturition is also described.

25-vitD actively increases dietary absorption of Ca and P

This thesis study demonstrated the major physiological effects that supraphysiological plasma concentrations of 25-vitD can have on Ca and P homeostasis in cattle. Previous research has identified that large doses of 25-vitD can increase the plasma concentration of Ca and P in cattle but have been unable to identify the source of the increase in mineral concentration and have incorrectly concluded that the increase originated from bone (Hollis *et al.,* 1977. The results of the metabolism studies described in Chapter 3 and Chapter 6 identify that Ca and P homeostasis is manipulated by 25-vitD and the majority of the increase in availability of both Ca and P originates from the diet. Furthermore, the studies conducted on markers of bone degradation in Chapter 8 suggest that very high concentrations of plasma 25-vitD did not increase the rate of bone degradation. This finding is in agreement with the review of literature, which suggests that Vitamin D induced degradation of bone will only occur when low plasma Ca concentrations promotes the secretion of PTH. Rapid degradation of skeletal stores of Ca is an undesirable aspect of any Vitamin D supplementation program. While bone metabolism is normal, and some net bone degradation is normal immediately post parturition in dairy cows, bone degradation prior to parturition reduces the pool of labile Ca available for rapid release in response to severe hypocalcaemia.

Generating increased absorption of Ca and P from the small intestine is the most desirable solution for both hypocalcaemic and P deficient cattle. In all studies conducted within this thesis the high concentration of plasma 25-vitD occurred with the absence of PTH, thus enabling the Vitamin D mediated increase in Ca availability to be derived from the alimentary tract. 25-vitD can only improve both Ca and P absorption when PTH is absent, as in the presence of PTH bone degradation may also occur. However, in the case of hypocalcaemia an absence of bone degradation is not as important as in the case of P deficiency. The quantity of Ca required at parturition cannot be immediately met by diet alone and unlike P deficiency, hypocalcaemia is an acute disease and therefore bone degradation should only exist for one to two weeks maximum. However, it is beneficial if bone is only degraded at parturition and not prior.

Dietary absorption of minerals in absence of PTH

In the dairy industry, the current practice of dietary manipulation of the transition diet generates large amounts of Ca available from the skeleton. Negative DCADs, usually achieved by addition of anionic salts to positive DCADs, make large amounts of Ca available from skeleton by causing severe compensated metabolic acidosis. Part of the metabolic buffering system involves using Ca from bones as a cationic buffer, thus enabling the animal to compensate for the acidosis. In Chapter 5 strongly anionic diets resulted in daily urinary excretion of approximately 4 g of Ca per day. If this is based on live weight and extrapolated to dairy cows it equates to approximately 12 g of Ca per day, which is equivalent to approximately half the Ca excreted in an average daily lactation.

There is only a limited amount of labile bone Ca, which is easily exhausted. Further Ca release then must result from further osteoclastic degradation. Osteoclastic degradation is slow and less responsive and it also results in a release of P from bone stores, which may inhibit 1α-hydroxylase. The transition from labile Ca release to osteoclastic release may be the reason that cows consuming transition diets that are anionic (DCAD < 0 mEq/kg) for 30d instead of 20d increase their risk of clinical hypocalcaemia by 42% (Lean *et al.*, 2006). Therefore, improvements in transition feeding should aim to increase Ca availability from diets. If supraphysiological levels of 25-vitD can replace the actions of 1,25-vitD in cattle, as they can in mice, then the absence of PTH prior to parturition ensures that 25-vitD will increase Ca absorption from the small intestine. This will benefit the cow at parturition as the

very labile bone stores of Ca should still be available to be activated by PTH release during parturition.

While Ca absorption from the diet was not clearly shown in this thesis it was demonstrated that it was likely in Table 7.2, and this was explained in Chapter 6. Furthermore, in Chapter 7 bone degradation was shown to not be enhanced by 25-vitD. Thus, the inclusion of 25-vitD in the typical anionic transition diet will increase Ca absorption prior to parturition, enabling labile bone Ca stores to remain intact and available for immediate use at parturition.

On one hand, it is beneficial for cattle consuming P deficient diets to not have secretions of PTH, as PTH is known to cause excessive bone degradation and excretion of P in urine. These actions lead to the clinical signs of P deficiency such as 'peg-leg'. On the other hand, it is also the same low concentrations of PTH that cause P digestibility not to be increased. This may occur as PTH secretion is controlled by plasma Ca concentration and Ca is often satisfactory, or at least not the limiting factor, in P deficient situations. The supplementation of 25-vitD in P deficient situations enables the Vitamin D humoral control to effectively bypass regulation by PTH.

In Chapter 6 steers were supplemented daily with 3.25 mg of 25-vitD, after 10 days the plasma concentration of 25-vit D had increased to approximately 375 ng/ml, which was about 7 times the endogenous concentration. The steers with supplemented 25-vitD increased the absorption of both Ca and P, the concentration of both plasma Ca and P, and increased the level of Ca excretion in urine. Analysis of bone degradation markers in Chapter 7 confirmed that additional 25-vitD did not increase the rate of bone degradation. The result of Chapter 6 and Chapter 7 confirm the theory that active Vitamin D (whether it be 1,25-vitD or supraphysiological amounts of 25-vitD) in the absence of PTH increase both Ca and P absorption from the gastro-intestinal tract. Steers in Chapter 6 treated with 25-vitD retained P of 3g per day, this is equivalent to the amount of P found in typical supplement programs. In Chapter 5 the steers were consuming a diet with excessive Ca and as a consequence the extra Ca absorbed was expelled in the urine. Ruminants remove excess P by increasing P excretion through saliva, and while attempts were made to measure rumen turnover and salivary P concentration they were not successful.

25-vitD and Anionic salts synergy

Pharmacological concentrations of 25-vitD in dairy cows prior to parturition do not improve Ca availability and do not increase the cow's ability to maintain plasma Ca concentration during parturition (Taylor *et al.*, 2008). In Chapter 6 it was demonstrated that increased concentrations of plasma 25-vitD result in an increase in both Ca and P plasma concentration. Furthermore, the origins of these minerals are dietary. This should increase the availability of Ca. However, the demand for Ca at parturition in dairy cows is extreme and cannot be immediately met by dietary absorption. Furthermore, dietary intakes drop during parturition further reducing the intake of Ca. When plasma Ca concentrations inevitably dip during parturition PTH is released, activating the metabolism of 25-vitD to 1,25-vitD, enabling bone degradation and the conservation of urinary Ca. It may be possible that the high levels of plasma P as a consequence of 25-vitD supplementation, as seen in Chapter 7, inhibit the part of this process.

The combination of anionic salts and elevated concentrations of plasma 25-vitD allow an improvement in several factors that allow cows to adapt to rapid Ca demand at parturition (Table 2.4). Research has been published that demonstrated that a combination of plasma concentrations of ~180 ng/ml of 25-vitD and a negative DCAD result in an improved maintenance of plasma Ca at parturition (Wilkens *et al.*, 2012). In the studies presented within this thesis Chapter 4, 5 and 6 demonstrate that there is a positive interaction between anionic salt addition and the concentration of 25-vitD on Ca availability, which is demonstrated by urinary Ca excretion (Figure 5.1).

It is likely that the combination of 25-vitD and anionic salts (neutral to negative DCAD) enables the cow to improve Ca availability at parturition because:

- Compensated metabolic acidosis requires Ca as a buffer which may be partly or completely provided from the diet when elevated concentrations of 25-vitD increase the absorption of Ca. This prevents the labile pool of skeletal Ca from becoming exhausted prior to calving.
- Anionic salts permit PTH and its receptors in the bone and kidney to function effectively.
- Ample amounts of 25-vitD in plasma allow for optimum production of 1,25-vitD when PTH is secreted.

- Combination therapy results in a much greater increase urinary Ca excretion than 25-vitD alone.

The above points are in agreement with Wilkens *et al.* (2012), in fact the first three points are analogous with some of their discussion points. However, the article failed to present a compelling argument for the negative impact of 25-vitD therapy without anionic salts.

The effect of the combination of 25-vitD and anionic salts have of dramatically increasing urinary Ca excretion was demonstrated within this thesis and in Wilkens *et al.* (2012), in both cases 25-vitD without anionic salts has been shown to only marginally increase urinary Ca excretion (Table 7.2). However, the massive increase in urinary Ca excretion is a consequence of the reduced pH of the renal tubule fluid causing Ca to not be reabsorbed. This flow of Ca through the plasma pool would demand Ca be replaced, which enables the Ca generating mechanisms in the diet to up-regulate and increase the absorption rate of Ca to match that lost in urine.

In the case of treatment with 25-vitD only, urinary Ca excretion is inhibited and only marginally increased as the reabsorption of renal Ca is a function of 1,25-vitD and high plasma concentrations of 25-vitD are likely to replicate this affect (DeLuca, 2004). However, it is generally accepted that both 1,25-vitD and PTH are required to enable high rates of Ca renal reabsorption in the distal tubule. In this thesis, Chapter 6 demonstrated that extremely high levels (375 ng/ml, 8 x normal concentration) of plasma 25-vitD and no anionic salts resulted in hypercalcaemia and only a marginal increase in urinary Ca excretion, unlike other studies which contained both anionic salts and 25-vitD (Chapter 5: McGrath *et al.*, 2011a, Chapter 6: McGrath *et al.*, 2012b, Wilkens *et al.*, 2012). This contrast of results suggest that the action of 25-vitD without increased secretion of PTH still has some resorptive effects on renal Ca, which consequently results in mild hypercalcaemia.

However, this resorptive effect of 25-vitD is overcome by acidic urinary pH in steers that are consuming anionic diets. Consequently, urinary Ca excretion is increased (Chapter 4 and 5) and the high plasma concentrations of 25-vitD will continue to increase the absorption of Ca from the alimentary tract as this function is independent of PTH. However, when Ca demand is increased at parturition and PTH is secreted the combination of PTH and 25-vitD

increasing Ca reabsorption in the renal distil tubule is apparently greater than the low urine pH induced excretion of Ca, thus renal Ca is reabsorbed.

Treatment with 25-vitD in isolation reduces the ability of cows to maintain plasma Ca at parturition as it effectively mimics a high Ca diet by increasing the absorption of Ca from the diet in the days immediately pre parturition. The resulting state of hypercalcaemia would cause suppression of PTH secretion and potentially down regulation of alternative Ca generating pathways such as bone degradation and production of 1,25-vitD. When plasma Ca concentration is required for lactation at parturition the humoral system of the animal would be slower to respond (not unlike cows consuming high Ca diets), urinary Ca excretion has already been largely reabsorbed and it takes approximately 24h for small intestine absorption of Ca to be further increased (Horst *et al.*, 1994b). Thus, the use of 25-vitD in transition cows must not be conducted without a diet that enables high levels of urinary Ca excretion.

In conclusion, the combination of 25-vitD and anionic salts has physiological implications that allow sufficient generation of extracellular Ca at parturition. The combination treatment permits greater PTH receptor sensitivity, urinary Ca excretion and most importantly a dramatic increase in dietary Ca absorption, while maintaining skeletal Ca reserves. The thesis also demonstrates why 25-vitD therapy in isolation results in lower blood plasma Ca in cows during parturition.

Ideal 25-vitD concentration and toxicity

From the studies conducted in this thesis target plasma concentrations for 25-vitD have been developed. One for 25-vitD supplementation for an improvement in P absorption, and the other for the combination of 25-vitD and anionic salts. It would appear from Chapter 7 the required concentration of 25-vitD for increasing the absorption of P is in excess of 300 ng/ml. However, digestibility studies are difficult to conduct, if the concentration of P in plasma is related to an increase in absorption of P from the alimentary tract then other studies are able to be compared. Chapter 4 and 5 demonstrated treatments without anionic salts with 25-vitD concentrations in the range of 115 to 180 ng/ml, neither of these treatments demonstrated an increase in plasma P concentration. This is in agreement with other studies that have demonstrated that concentrations of 200 ng/ml are required to increase plasma P concentration (Hollis *et al.*, 1977).

The use of 25-vitD and anionic salts for the control of hypocalcaemia at parturition in dairy cattle appears to be an alternate methodology to current methods of prevention. From the studies conducted within this thesis it would appear that there is a synergy between Ca availability and the concentration of plasma 25-vitD when animals are consuming a negative DCAD (Figure 5.1). However, the plasma concentration of 25-vitD required to elicit the Ca homeostatic responses required to make Ca availability adequate at parturition is confounded by multiple factors. These factors are not able to be fully analysed within this proof-of-concept study. Nevertheless, the results generated from Chapter 4, 5 and 6 suggest that the concentration of 25-vitD required is negatively correlated with DCAD value of the diet. Highly anionic diets require a lower plasma concentrations of 25-vitD while neutral diets are more likely to require much higher concentrations of 25-vitD to elicit a response. In Chapter 4 plasma concentration of 25-vitD was estimated to be 200 ng/ml or greater, this estimate was derived from Chapter 6 and 7. Chapter 4 demonstrated that urinary Ca excretion was increased from positive DCAD diets with small amounts of anionic salts added to the diet. In Chapter 6, no significant increase in urinary Ca excretion occurred with similar DCAD concentrations. However the trend suggested a similar reaction to Chapter 4. However, in Chapter 5 much more Ca was excreted through urine when the diets were highly anionic (DCAD -140 mEq/kg) but plasma concentration of 25-vitD was much lower than the other experiments (~110 mEq/kg).

A target for plasma 25-vitD concentration of 200 – 350 ng/ml should permit an increase in P absorption without resulting in any short or long term toxicity. Further work is required to ascertain the ideal concentration of 25-vitD in the diet that permits a stable concentration of 25-vitD in plasma that allow for an increase in P absorption from the diet without potentially causing any long term physiological impairments.

Toxicity is of less importance in short term supplementation. The transition period in dairy cows is of short duration and thus does not permit continual accretion of 25-vitD, furthermore the target concentrations are likely to be much lower than for P deficient cattle and extremely large levels of 25-vitD have been fed to cattle for short periods without causing toxicity (Bringe *et al.*, 1971, Carnagey *et al.*, 2008). In dairy cows that can effectively be fed a highly negative DCAD, 25-vitD plasma concentrations of 100-150 ng/ml will greatly increase Ca

availability (Chapter 5). However, cows that are consuming neutral to positive DCAD may require plasma concentrations of 25-vitD greater than 200 ng/ml.

However, any benefit to Ca homeostasis that was attributed to 25-vitD when DCAD was positive was only evident when the diet contained additional anionic salts. For example, in Chapter 4 one of the treatments had a DCAD of 150 mEq/kg but the DCAD had been reduced from approximately 220 mEq/kg by anionic salts. However, in Chapter 5 and Chapter 6 the DCAD of the control diets was already approximately 150 mEq/kg and this diet did not show any response in Ca availability when the animals offered the diet were also supplied with 25-vitD. Further work is required to develop a guideline for the appropriate quantity of 25-vitD and anionic salts for control of hypocalcaemia during parturition. Of particular interest may be the interaction between additional anionic salts and 25-vitD, not specifically DCAD.

Intra-ruminal supplementation and type of 25-vitD

Chapter 5 of this thesis examined the prospect of supplementing cattle with 25-vitD via intra-rumen slow release boluses. There are several benefits for using this procedure. Intra-rumen supplementation guarantees the correct daily application rate per animal. This is very important in situations where the total ration is not being fed, or individual daily intake of feed supplements cannot be guaranteed. Intra-rumen supplementation enables animals in extensive grazing situations to be successfully supplemented when otherwise it would be logistically impractical.

In Chapter 5 intra-rumen boluses were used to deliver the 25-vitD. The release rate of the bolus was equivalent to the steers receiving approximately 4.1 mg/day for 11 days. This amount of 25-vitD should have resulted in 25-vitD plasma concentrations increasing above 300 ng/ml. However, plasma concentrations only averaged 120 ng/ml. The result led to the conclusion that the release pattern from the bolus may have resulted in the lower than expected plasma concentrations. As a consequence the study featured in Chapter 6 was designed to ascertain if the lower than expected plasma concentration was due to the release pattern from the bolus.

In Chapter 6 the contents of the bolus were removed from the bolus, ground and then added to the rumen at the equivalent rate as the typical 25-vitD beadlet powder. The results

clearly demonstrated that efficacy of absorption from the bolus content was much lower than from 25-vitD beadlet powder (Table 6.2). The study demonstrated that the physical attributes of the bolus are not a cause of poor efficacy of absorption of 25-vitD. The formulation of the 25-vitD crystal in the pellet which includes the combination of monensin and a lactose based carrier may affect absorption of 25-vitD, and therefore be a cause of low absorption rates reported in Chapter 4.

9.1 Implications and future research

Implications

The research conducted within this thesis has demonstrated aspects of the modification of Ca and P homeostasis by treatment with 25-vitD in cattle that influence productivity in both the beef and dairy industries. Calcium and P are major minerals in bovine; consequently any sub-clinical or clinical deficiency will result in dramatic reductions in productivity and economic loss.

Calcium and P nutritional management in both dairy and beef industries has been comprehensively researched. Nutritional programs for meeting the requirements of Ca and P in ruminant production systems have largely been reliant on increasing the amount of each mineral within the diet, either from supplementation or via fertilisation of the fodder prior to harvest. However, Ca and P are not completely available for absorption within ruminants and in many deficient situations there is adequate mineral content within the diet, but it is not available. Furthermore, acute Ca deficiency within a dairy cow at parturition is not a consequence of low Ca concentration in the diet but an inability of the animal to control Ca homeostasis.

In this thesis, the metabolite of Vitamin D, 25-vitD, has been proven to increase the retention of both Ca and P from normal forage diets. This finding has the potential for providing an alternative to supplementation method for meeting Ca and P deficiencies in ruminants. This technology has implications; which are environmental, welfare and economic in nature.

The animals in intensive ruminant industries, such as dairies, have a high requirement for P. Due to the low rate of digestibility (50-70%), much of the dietary P is lost in faeces. This loss can result in an excessive build up of P in the immediate environment.

Phosphorus deficiencies in extensive grazing industries in many countries are endemic. However, supplementation of P within this environment faces considerable logistical problems, which often results in ineffective supplementation and a continuation of P deficiency. P deficiency in beef cattle within this environment has serious economic implications. However, acute P deficiency also has animal welfare implications, which may lead to bone chewing, botulism and death.

The economic implications of replacing Ca and P supplements with 25-vitD are largely dependent on the price of Ca and P sources. While Ca is readily available and cost effective, the sources of economically available P are small and rapidly diminishing. Phosphorus is widely predicted to have a supply and cost pattern similar to other finite resources. Finding an alternative to P supplementation for alleviating P deficiencies may have definite economic benefits in the immediate future. Furthermore, the addition of 25-vitD to current Ca and P supplementations will increase the digestibility of supplemented minerals making the supply of the minerals more cost effective while also reducing the amount of the minerals supplied.

The studies conducted within this thesis confirm that supraphysiological plasma concentrations (~4 x normal) of 25-vitD effectively bypass the control of production of active Vitamin D by PTH. This has important implications for the control of hypocalcaemia at parturition, as it is largely a consequence of an inability of the cow to quickly adapt to the rapid increase in demand for extracellular Ca. The confirmation of very high concentrations of plasma 25-vitD largely replacing the functions of 1,25-vitD may have further implications in other aspects of human and animal health.

The use of a combination of dietary anionic salts and 25-vitD for the control of hypocalcaemia in dairy cows appears to be a novel and effective method for the control of the disease. Traditional approaches, while largely effective, have relied upon either a restriction of dietary Ca or by increasing Ca excretion and bone degradation by feeding anionic salts. Both techniques effectively ready the animal's homeostasis system for generating large

amounts of extracellular Ca. As previously mentioned there are considerable draw backs with either system.

The combination of both dietary anionic salts and 25-vitD aims to alleviate hypocalcaemia by stimulating the Ca homeostasis mechanisms without relying on increased production of PTH. This should allow for more effective control of the disease. Furthermore, it provides a multi-faceted control option which may be beneficial in production environments that are not suited the traditional methods of control for various reasons.

Future research

Work is required to analyse the ability of 25-vitD to be supplemented via intra-rumen techniques, specifically analysing the suitability of crystalline 25-vitD within the digestive tract. The benefit of individual animal application of the product is that it allows the exact application rate, if the product was included in fed supplements the actual intake of the animal would determine the application rate of 25-vitD. Furthermore, part of the attraction of the product for hypocalcaemia in the dairy industry is that it provides an alternative source of protection. For instance, if the animal, for various reasons, does not consume its allocated amount of supplement the intra-ruminal supply of 25-vitD may still provide some level of protection.

The use of 25-vitD for extended periods of time, for example the period of time that would be required for improving P digestibility during the wet season 3-6 months, requires investigation. There may be negative feedback mechanisms within the animal that result in rapid degradation of plasma 25-vitD, thus reducing the effectiveness of the supplementation program. Prolonged supplementation of 25-vitD may also cause negative health effects – a major concern would be metastatic calcification.

References

Abdel-Hafeez HM, Mañas-Almendros M, Ross R, Care AD and Marshall DH 1982. Effects of dietary phosphorus and calcium on the intestinal absorption of Ca in sheep. British Journal of Nutrition 47, 69-77.

Abu Damir H, Phillippo M, Thorp BH, Milne JS, Dick L and Nevison IM 1994. Effects of dietary acidity on calcium balance and mobilisation, bone morphology and 1,25 dihydroxyvitamin D in prepartal dairy cows. Research in Veterinary Science 56, 310-318.

Allen MJ 2003. Biochemical Markers of Bone Metabolism in Animals: Used and Limitations. American Society for Veterinary Clinical Pathology 32, 101-111.

Anderson DL and Henderson LJ 1986. Sealed chamber digestion for plant nutrient analysis. Agronomy Journal 78, 937-938.

Bai X, Miao D, Li J, Goltzman D and Karaplis AC 2004. Transgenic Mice Overexpressing Human Fibroblast Growth Factor 23 (R176Q) Delineate a Putative Role for Parathyroid Hormone in Renal Phosphate Wasting Disorders. Endocrinology 145, 5269-5279.

Bar A, Perlman R and Sachs M 1985. Observation on the use of 1[alpha]-hydroxyvitamin D3 in the prevention of bovine parturient paresis: The effect of a single injection on plasma 1[alpha]-hydroxyvitamin D3, 1,25-dihydroxyvitamin D3, calcium, and hydroxyproline. Journal of Dairy Science 68, 1952-1958.

Barton BA, Horst RL, Jorgensen NA and DeLuca HF 1981. Concentration of Calcium, Phosphorus, and 1,25-dihydroxyvitamin D in plasma of dairy cows during the lactation cycle. Journal of Dairy Science 64, 850-852.

Beck N and Webster S 1976. Effects of Acute Metabolic Acidosis on Parathyroid Hormone Action and Calcium Mobilization. American Journal of Physiology 230, 127-131.

Block E 1984. Manipulating dietary anions and cations for prepartum dairy cows to reduce incidence of milk fever. Journal of Dairy Science 67, 2939-2948.

Block E 1994. Manipulation of dietary cation-anion difference on nutritionally related production diseases, productivity, and metabolic responses of dairy-cows. Journal of Dairy Science 77, 1437-1450.

Blum JW, Wilson RB and Kronfeld DS 1973. Plasma insulin concentrations in parturient cows. Journal of Dairy Science 56, 459-464.

Bortolussi G, Ternouth JH and McMeniman NP 1996. Dietary nitrogen and phosphorus depletion in cattle and their effects on liveweight gain, blood metabolite concentrations and phosphorus kinetics. The Journal of Agricultural Science 126, 493-501.

Braithwaite GD 1975. Studies on the absorption and retention of calcium and phosphorus by young and mature Ca-deficient sheep. British Journal of Nutrition 34, 311-324.

Braithwaite GD 1982. Endogenous faecal loss of calcium by ruminants. Journal of Agricultural Science 99, 355-358.

Breves G and Schröder B 1991. Comparative aspects of gastrointestinal phosphorus metabolism. Nutrition Research Reviews 4, 125-140.

Breves G, Ross R and Höller H 1985. Dietary phosphorus depletion in sheep: effects on plasma inorganic phosphorus, calcium, l,25-(OH)2-Vit.D3 and alkaline phosphatase and on gastrointestinal P and Ga balances. The Journal of Agricultural Science 105, 623-629.

Bringe AN, Jorgensen AN and DeLuca HF 1971. Efficacy of 25-hydroxycholecalciferol on prevention of parturient paresis in dairy cows. Journal of Dairy Science 54, 792.

Bringhurst FR, Stern AM, Yolts M, Mizrali N, Segre GV, Potts Jr JT 1988. Peripheral metabolism of PTH: fate of biologically active amino terminus in vivo. Amercian Journal of Physiological Endocrine Metabolism 255, E886-E893.

Bronner F 1987. Intestinal Calcium absorption: Mechanisms and applications. Journal of Nutrition 117, 1347-1352.

Brown AJ and Slatopolsky E 2007. Drug Insight: vitamin D analogs in the treatment of secondary hyperparathyroidism in patients with chronic kidney disease. Nature Clinical Practice Endocrinology and Metabolism 3, 134-144.

Bushinsky DA 2001. Acid-base imbalance and the skeleton. European Journal of Nutrition 40, 238-244.

Cai Q, Chandler JS, Wasserman RH, Kumar R and Penniston JT 1993. Vitamin D and adaptation to dietary calcium and phosphate deficiencies increase intestinal plasma membrane calcium pump gene expression. Proceedings of the National Academy of Sciences of the United States of America 90, 1345-1349.

Call JW, Butcher JE, Blake JT, Smart RA and Shupe JL 1978. Phosphorus influence on growth and reproduction of beef cattle. Journal of Animal Science 47, 216-225.

Call JW, Butcher JE, Shupe JL, Blake JT and Olson AE 1986. Dietary phosphorus for beef cows. American Journal of Veterinary Research 47, 475-481.

Carnagey KM, Huff-Lonergan EJ, Lonergan SM, Trenkle A, Horst RL and Beitz DC 2008. Use of 25-hydroxyvitamin D3 and dietary calcium to improve tenderness of beef from the round of beef cows. Journal of Animal Science 86, 1637-1648.

Chan PS, West JW and Bernard JK 2006. Effect of prepartum dietary Calcium on intake and serum and urinary mineral concentrations of cows. Journal of Dairy Science 89, 704-713.

Charbonneau E, Chouinard PY, Tremblay GF, Allard G and Pellerin D 2009. Timothy silage with low dietary cation-anion difference fed to nonlactating cows. Journal of Dairy Science 92, 2067-2077.

Chen XB and Gomes MJ 1992. Estimation of Microbial protein supply to sheep and cattle based on urinary excretion of purine derivatives: An overview of technical details. In International Food Resources Unit, Occasional Publication Rowett Research Institute, Aberdeen, United Kingdom.

Chizzotti ML, Valadares Filho SdC, Valadares RFD, Chizzotti FHM and Tedeschi LO 2008. Determination of creatinine excretion and evaluation of spot urine sampling in Holstein cattle. Livestock Science 113, 218-225.

Cho YM, Choi H, Hwang IH, Kim YK and Myung KH 2006. Effects of 25-hydroxyvitamin D3 and manipulated dietary cation-anion difference on the tenderness of beef from cull native Korean cows. Journal of Animal Science 84, 1481-1488.

Christakos S, Ajibade DV, Dhawan P, Fechner AJ and Mady LJ 2010. Vitamin D: Metabolism. Endocrinology and metabolism clinics of North America 39, 243-253.

Christenson RH 1997. Biochemical Markers of Bone Metabolism: An Overview. Clinical Biochemistry 30, 573-593.

Christian DG, Riche AB and Yates NE 2002. The yield and composition of switchgrass and coastal panic grass grown as a biofuel in Southern England. Bioresource Technology 83, 115-124.

Coates DB and Ternouth JH 1992. Phosphorus kinetics of cattle grazing tropical pastures and implications for the estimation of their phosphorus requirements. Journal of Agricultural Science 119, 401-409.

Coates DB 1994. The effect of phosphorus as fertiliser or supplement on pasture and cattle productivity in the semi-arid tropics of north Queensland. Tropical grasslands 28, 90-108.

Cohen R 1973. 2. Relation of pasture phosphorus to phosphorus content of blood, hair and bone of grazing steers. Australian Journal of Experimental Agriculture and Animal Husbandry 13, 5.

Cohen RDH 1980. Phosphorus in rangeland ruminant nutrition: A review. Livestock Production Science 7, 25-37.

Conrad HR, Sam LH and Hibbs JW 1956. Studies on milk fever in dairy cows. V. The effect of massive oral doses of vitamin D on absorption, excretion, retention and blood levels of Calcium and Phosphorus. Journal of Dairy Science 39, 1697-1705.

Cordell D, Drangert J-O and White S 2009. The story of phosphorus: Global food security and food for thought. Global Environmental Change 19, 292-305.

Cunningham JG 1997. Textbook of Veterinary Physiology. W.B. Saunders Company, Philadelphia.

Danisi G and Straub RW 1980. Unidirectional influx of phosphate across the mucosal membrane of rabbit small intestine. Pflügers Archiv European Journal of Physiology 385, 117-122.

Davies M, Mawer EB and Krawitt E 1980. Comparitive absorption of vitamin D_3 and 25-hydroxyvitamin D_3 in intestinal disease. Gut 21, 287-292.

DeGroot MA, Block E and French PD 2010. Effect of prepartum anionic supplementation on periparturient feed intake, health, and milk production. Journal of Dairy Science 93, 5268-5279.

Delaquis AM and Block E 1995. Acid-base status, renal-function, water, and macromineral metabolism of dry cows fed diets differing in cation-anion difference. Journal of Dairy Science 78, 604-619.

DeLuca HF 2004. Overview of general physiologic features and functions of vitamin D. American Journal of Clinical Nutrition 80, 1689-1696.

DeLuca HF 2008. Evolution of our understanding of vitamin D. Nutrition Reviews 66, 73-87.

Dohoo IR, Martin SW, McMillan I and Kennedy BW 1984. Disease, production and culling in Holstein-Friesian cows II. Age, season and sire effects. Preventive Veterinary Medicine 2, 655-670.

Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, Smith E, Bonadio J, Goldstein S, Gundberg C, Bradley A, Kersenty G 1996. Increased bone formation in osteocalcin-deficient mice. Nature 382, 448-452.

Duffield TF, Rabiee AR and Lean IJ 2008. A meta-analysis of the impact of monensin in lactating dairy cattle. Part 1. Metabolic effects. Journal of Dairy Science 91, 1334-1346.

Ebeling P, Sandgren M, DiMagno E, Lane A, DeLuca H and Riggs B 1992. Evidence of an age-related decrease in intestinal responsiveness to vitamin D: relationship between serum 1,25-dihydroxyvitamin D3 and intestinal vitamin D receptor concentrations in normal women. Journal of Clinical Endocrinoligy Metabolism 75, 176-182.

Ender F, Dishington IW and Helgebostad A 1962. Parturient paresis and related forms of hypocalcemic disorders induced experimentally in dairy cows. Acta Veterinaria Scandinavica 3, 1-52.

Espino L, Guerrero F, Suarez ML, Santamarina G, Goicoa A and Fidalgo LE 2003. Long-term effects of dietary anion-cation balance on acid-base status and bone morphology in reproducing ewes. Journal of Veterinary Medicine A 50, 488-495.

Etheridge RD, Pesti GM and Foster EH 1998. A comparison of nitrogen values obtained utilizing the Kjeldahl nitrogen and Dumas combustion methodologies (Leco CNS 2000) on samples typical of an animal nutrition analytical laboratory. Animal Feed Science and Technology 73, 21-28.

Fishwick G, Fraser J, Hemingway RG, Parkins JJ and Ritchie NS 1977. The effects of dietary phosphorus inadequacy during pregnancy and lactation on the voluntary intake and digestibility of oat straw by beef cows and the performance of their calves. The Journal of Agricultural Science 88, 143-150.

Fiske CH and Subbarow Y 1925. The colorimetric determination of phosphorus. Journal of Biological Chemistry 66, 375-400.

Fleischer P, Metzner M, Beyerbach M, Hoedemaker M and Klee W 2001. The relationship between milk yield and the incidence of some diseases in dairy cows. Journal of Dairy Science 84, 2025-2035.

Foote AP, Lambert BD, Brady JA and Muir JP 2011. Short communication: Phosphate transporter expression in Holstein cows. Journal of Dairy Science 94, 1913-1916.

Foote MR, Horst RL, Huff-Lonergan EJ, Trenkle AH, Parrish FC and Beitz DC 2004. The use of vitamin D3 and its metabolites to improve beef tenderness. Journal of Animal Science 82, 242-249.

Fortune CL, Farrugia W, Tresham J, Scoggins BA and Wark JD 1989. Hormonal regulation of osteocalcin plasma production and clearance in sheep. Endocrinology 124, 2785-2790.

Fraser D, Kooh SW, Kind PH, Holick MF, Tanaka Y and DeLuca HF 1973. Pathogenesis of hereditary Vitamin-D-Dependent rickets. New England Journal of Medicine 289, 817-822.

Fraser DR 1980. Regulation of the metabolism of vitamin D. Physiological Reviews 60, 551-613.

Fraser DR 1995. Vitamin D. The Lancet 104, 345.

Fredeen AH, DePeters EJ and Baldwin RL 1988. Effects of acid-base disturbances caused by differences in dietary fixed ion balance on kinetics of calcium metabolism in ruminants with high calcium demand. Journal of Animal Science 66, 174-184.

Gartner RJW, Murphy GM and Hoey WA 1982. Effects of induced, subclinical phosphorous deficiency on feed intake and growth of beef heifers. Journal of Agricultural Science 98, 23-29.

Gaynor PJ, Mueller FJ, Miller JK, Ramsey N, Goff JP and Horst RL 1989. Parturient hypocalcemia in Jersey cows fed Alfalfa haylage-based diets with different cation to anion ratios. Journal of Dairy Science 72, 2525-2531.

Geisert BG, Erickson GE, Klopfenstein TJ, Macken CN, Luebbe MK and MacDonald JC 2010. Phosphorus requirement and excretion of finishing beef cattle fed different concentrations of phosphorus. Journal of Animal Science 88, 2393.

GenStat 2008. GenStat for Windows, Release 11.1, Eleventh Edition. In VSN International Ltd, Oxford.

GenStat 2009. GenStat for Windows, Release 11.1, Eleventh Edition. In VSN International Ltd, Oxford.

Goff J 2003. Managing hypocalcemia and milk fever. Cattle Practice 11, 75-79.

Goff JP 2000. Pathophysiology of calcium and phosphorus disorders. Veterinary Clinics of North America-Food Animal Practice 16, 319-334.

Goff JP and Horst RL 1997. Physiological changes at parturition and their relationship to metabolic disorders. Journal of Dairy Science 80, 1260-1268.

Goff JP and Horst RL 1998. Use of hydrochloric acid as a source of anions for prevention of milk fever. Journal of Dairy Science 81, 2874-2880.

Goff JP and Horst RL 2003a. Milk fever control in the United States. Acta Veterinaria Scandinavica, 145-147.

Goff JP and Horst RL 2003b. Role of acid-base physiology on the pathogenesis of parturient hypocalcaemia (milk fever) - the DCAD theory in principal and practice. Acta Veterinaria Scandinavica 97, 51-56.

Goff JP, Littledike ET and Horst RL 1986. Effect of synthetic bovine parathyroid hormone in dairy cows: Prevention of hypocalcemic parturient paresis. Journal of Dairy Science 69, 2278-2289.

Goff JP, Kehrli Jr ME and Horst RL 1989. Periparturient hypocalcemia in cows: Prevention using intramuscular parathyroid hormone. Journal of Dairy Science 72, 1182-1187.

Goff JP, Reinhardt TA and Horst RL 1991a. Enzymes and factors controlling vitamin D metabolism and action in normal and milk fever cows. Journal of Dairy Science 74, 4022-4032.

Goff JP, Reinhardt TA and Horst RL 1995. Milk fever and dietary cation-anion balance effects on concentration of vitamin D receptor in tissue of periparturient dairy cows. Journal of Dairy Science 78, 2388-2394.

Goff JP, Ruiz R and Horst RL 2004. Relative acidifying activity of anionic salts commonly used to prevent milk fever. Journal of Dairy Science 87, 1245-1255.

Goff JP, Horst RL, Beitz DC and Littledike ET 1988. Use of 24-F-1, 25-dihydroxyvitamin D3 to prevent parturient paresis in dairy cows. Journal of Dairy Science 71, 1211-1219.

Goff JP, Brummer EC, Henning SJ, Doorenbos RK and Horst RL 2007. Effect of application of ammonium chloride and calcium chloride on alfalfa cation-anion content and yield. Journal of Dairy Science 90, 5159-5164.

Goff JP, Horst RL, Mueller FJ, Miller JK, Kiess GA and Dowlen HH 1991b. Addition of chloride to a prepartal diet high in cations increases 1,25-dihydroxyvitamin D response to hypocalcemia preventing milk fever. Journal of Dairy Science 74, 3863-3871.

Guard C 1996. Fresh cow problems are costly: Culling hurts the most. Proceedings of the Annual Veterinary Conference, Cornell University., Ithaca, New York. 100.

Gulay MS, Hayen MJ, Bachman KC and Head HH 2008. Prepartum feeding of cationic or anionic diets to Holstein cows given 30 or 60 day dry periods: Comparison of dry matter intake, physiological measures and milk production. Asian-Australasian Journal of Animal Sciences 21, 83-89.

Guo YD, Strugnell S, Back DW and Jones G 1993. Transfected human liver cytochrome P-450 hydroxylates vitamin D analogs at different side-chain positions. Proceedings of the National Academy of Sciences 90, 8668-8672.

Heron VS, Tremblay GF and Oba M 2009. Timothy hays differing in dietary cation-anion difference affect the capability of dairy cows to maintain their calcium homeostasis. Journal of Dairy Science 92, 238-246.

Hersom MJ, Hansen GR and Arthington JD 2010. Effect of dietary cation-anion difference on measures of acid-base physiology and performance in beef cattle. Journal of Animal Science 88, 374-382.

Hibbs JW and Pounden WD 1955. Studies on milk fever in dairy cows. IV. Prevention by short-time, prepartum feeding of massive doses of vitamin D. Journal of Dairy Science 38, 65-72.

Hibbs JW, Krauss WE, Monroe CF and Sutton TS 1946a. Studies on milk fever in dairy cows I. The possible role of vitamin D in milk fever. Journal of Dairy Science 29, 617-623.

Hibbs JW, Krauss WE, Pounden WD, Monroe CF and Sutton TS 1946b. Studies on milk fever in dairy cows. II. The effect of Vitamin D on some of the blood changes in normal and milk fever cows at parturition. Journal of Dairy Science 29, 767-781.

Hidiroglou M 1987. Kinetics of intravenously administered 25-hydroxyvitamin D3 in sheep and the effect of exposure to ultraviolet radiation. Journal of Animal Science 65, 808-814.

Hidiroglou M and Knipfel JE 1983. Plasma and milk concentrations of Vitamin D3 and 25-Hydroxy Vitamin D3 following intravenous injection of Vitamin D3 or 25-Hydroxy Vitamin D3. Canadian Journal of Complete Medicine 48, 78-80.

Hidiroglou M, Proulx JG and Roubos D 1979. 25-Hydroxyvitamin D in plasma of Cattle. Journal of Dairy Science 62, 1076-1080.

Hildmann B, Storelli C, Danisi G and Murer H 1982. Regulation of Na+-Pi cotransport by 1,25-dihydroxyvitamin D3 in rabbit duodenal brush-border membrane. American Journal of Physiology - Gastrointestinal and Liver Physiology 242, G533-G539.

Hodnett DW, Jorgensen NA and Deluca HF 1992. 1alpha-hydroxyvitamin D3 plus 25-hydroxyvitamin D3 reduces parturient paresis in dairy cows fed high dietary Calcium. Journal of Dairy Science 75, 485-491.

Hollis BW 2010. Assessment and Interpretation of Circulating 25-Hydroxyvitamin D and 1,25-Dihydroxyvitamin D in the Clinical Environment. Endocrinology & Metabolism Clinics of North America 39, 271-286.

Hollis BW, Conrad HR and Hibbs JW 1977. Changes in plasma 25-hydroxycholecalciferol and selected blood parameters after injection of massive doses of cholecalciferol of 25-hydroxycholecalciferol in non-lactating dairy cows. The Journal of Nutrition 107, 606-613.

Holmes WE 1990. Phosphorus and beef production in northern Australia. 11. A case study approach to the economic assessment of phosphorus supplementation. Tropical grasslands 24, 250-255.

Holtenius K and Ekelund A 2005. Biochemical markers of bone turnover in the dairy cow during lactation and the dry period. Research in Veterinary Science 78, 17-19.

Horst RL 1986. Regulation of calcium and phosphorus homeostasis in the dairy cow. Journal of Dairy Science 69, 604-616.

Horst RL, Goff JP and Reinhardt TA 1990. Advancing age results in reduction of intestinal and bone 1,25-dihydroxyvitamin D receptor. Endocrinology 126, 1053-1057.

Horst RL, Goff JP and Reinhardt TA 1994a. Calcium and vitamin D metabolism in the dairy cow. Journal of Dairy Science 77, 1936-1951.

Horst RL, Goff JP and Reinhardt TA 1994b. Symposium - Calcium-metabolism and utilization - Calcium and vitamin-D metabolism in the dairy-cow. Journal of Dairy Science 77, 1936-1951.

Horst RL, Goff J and Reinhardt TA 2003. Role of Vitamin D in Calcium homeostasis and its use in prevention of bovine periparturient paresis. Acta Veterinaria Scandinavica Supplement 97, 35-50.

Horst RL, Goff JP and Reinhardt TA 2005. Adapting to the transition between gestation and lactation: Differences between rat, human and dairy cow. Journal of Mammary Gland Biology and Neoplasia 10, 141-156.

Horst RL, Littledike ET, Riley JL and Napoli JL 1981. Quantitation of vitamin D and its metabolites and their plasma concentrations in five species of animals. Analytical Biochemistry 116, 189-203.

Horst RL, Goff JP, Reinhardt TA and Buxton DR 1997. Strategies for preventing milk fever in dairy cattle. Journal of Dairy Science 80, 1269-1280.

Hove K, Horst RL, Littledike ET and Beitz DC 1984. Infusions of parathyroid hormone in ruminants: Hypercalcemia and reduced plasma 1,25-dihydroxyvitamin D concentrations. Endocrinology 114, 897-903.

Hymøller L and Jensen SK 2010. Stability in the rumen and effect on plasma status of single oral doses of vitamin D and vitamin E in high-yielding dairy cows. Journal of Dairy Science 93, 5748-5757.

Jones RJ 1990. Phosphorus and beef production in northern Australia. 1. Phosphorus and pasture productivity - a review. Tropical grasslands 24, 131-139.

Joyce PW, Sanchez WK and Goff JP 1997. Effect of anionic salts in prepartum diets based on alfalfa. Journal of Dairy Science 80, 2866-2875.

Julien WE, Conrad HR, Hibbs JW and Crist WL 1977. Milk fever in dairy Cows. VIII. Effect of injected vitamin D3 and Calcium and Phosphorus intake on incidence. Journal of Dairy Science 60, 431-436.

Katai K, Miyamoto K, Kishida S, Segawa H, Nii T, Tanaka H, Tani Y, Arai H, Tatsumi S, Morita K, Taketani Y and Takeda E 1999. Regulation of intestial Na+-dependent phophate co-transporters by a low-phosphate diet and 1,25-dihydroxyvitamin D3. Biochemical Journal 343, 705-712.

Khorasani GR and Armstrong DG 1992. Calcium, phosphorus, and magnesium absorption and secretion in the bovine digestive tract as influenced by dietary concentration of these elements. Livestock Production Science 31, 271-286.

Knowlton KF, Taylor MS, Hill SR, Cobb C and Wilson KF 2007. Manure nutrient excretion by lactating cows fed exogenous phytase and cellulase. Journal of Dairy Science 90, 4356-4360.

Komisarczuk S, Merry RJ and McAllan AB 1987. Effect of different levels of phosphorus on rumen microbial fermentation and synthesis determined using a continuous culture technique. British Journal of Nutrition 57, 279-290.

Kurosaki N, Yamato O, Mori F, Imoto S and Maede Y 2007. Preventive effect of mildly altering dietary cation-anion difference on milk fever in dairy cows. Journal of Veterinary Medical Science 69, 185-192.

Larsen K 1972. Creatinine assay by a reaction-kinetic principle. Clinica Chimica Acta 41, 209-217.

Lauridsen C, Halekoh U, Larsen T and Jensen SK 2010. Reproductive performance and bone status markers of gilts and lactating sows supplemented with two different forms of vitamin D. Journal of Animal Science 88, 202-213.

Lean IJ, DeGaris PJ, McNeil DM and Block E 2006. Hypocalcemia in dairy cows: Meta-analysis and dietary cation anion difference theory revisited. Journal of Dairy Science 89, 669-684.

Liebholz J 1974. The flow of Calcium and Phoshorus in the digestive tract of the sheep. Australian Journal of Agricultural Research 25, 147-154

Liesegang A 2008. Influence of anionic salts on bone metabolism in periparturient dairy goats and sheep. Journal of Dairy Science 91, 2449-2460.

Liesegang A and Risteli J 2005. Influence of different calcium concentrations in the diet on bone metabolism in growing dairy goats and sheep. Journal of Animal Physiology and Animal Nutrition 89, 113-119.

Liesegang A, Sassi ML and Risteli J 2003. Diurnal variation in concentrations of various markers of bone metabolism in growing female goats and sheep. Animal Science 77, 197-203.

Liesegang A, Chiappi C, Risteli J, Kessler J and Hess HD 2007. Influence of different calcium contents in diets supplemented with anionic salts on bone metabolism in periparturient dairy cows. Journal of Animal Physiology and Animal Nutrition 91, 120-129.

Liesegang A, Sassi M-L, Risteli J, Eicher R, Wanner M and Riond J-L 1998. Comparison of bone resorption markers during hypocalcemia in dairy cows. Journal of Dairy Science 81, 2614-2622.

Liesegang A, Eicher R, Sassi ML, Risteli J, Kraenzlin M, Riond JL and Wanner M 2000. Biochemical markers of bone formation and resorption around parturition and during lactation in dairy cows with high and low standard milk yields. Journal of Dairy Science 83, 1773-1781.

Luebbe MK, Erickson GE, Klopfenstein TJ and Meyer N 2009. Effect of dieatary cation-anion difference on intake and urinary pH in high concentrate diets. In Nebraska Beef Report, pp. 94-95.

Lund J and DeLuca HF 1966. Biologically active metabolite of vitamin D_3 from bone, liver, and blood serum. Journal of Lipid Research 7, 739-744.

Mañas-Almendros M, Ross R and Care AD 1982. Factors affection the secretion of Phosphate in parotid saliva in the sheep and goat. Experimental Physiology 67, 269-280.

Mayer G, Marshak R and Kronfeld D 1966. Parathyroid effects on renal phosphorus excretion in the cow. American Journal of Physiology -- Legacy Content 211, 1366-1370.

McGrath J, Savage D and Nolan J 2011a. Comparison of urine pH and Ca excretion of multiparous Holstein cows and Brangus steers in response to anionic salt supplementation. In Recent Advances in Animal Nutrition - Australia (Ed. P Cronje), pp. 141-142. Animal Science, University of New England, Armidale.

McGrath J, Savage D, Nolan J and Elliott R 2011b. Anionic salt supplementation and intra-rumen administration of 25 hydroxycholecalciferol increase urinary calcium excretion. In Recent Advances in Animal Nutrition - Australia (Ed. P Cronje), pp. 139-140. Animal Science, University of New England, Armidale.

McGrath J, Savage D, Nolan J and Elliott R 2012a. Phosphorus and calcium retention in steers fed a roughage diet is influenced by dietary 25OH-vitamin D. Journal of Animal Production Science 52.

McGrath J, Savage D, Rodgers N, Nolan J, and Elliott R. 2012b. Anionic salts and dietary 25-hydroxy vitamin D stimulate calcium availability in steers. *ANIMAL*, (in press).

McHardy GJR and Parsons DS 1956. The absorption of inorganic phosphate from the small intestine of the rat. Experimental Physiology 41, 398-409.

McIvor J, Guppy C and Probert M 2011. Phosphorus requirements of tropical grazing systems: the northern Australian experience. Plant and Soil 349, 55-67.

McLean RW, Hendricksen RE, Coates DB and Winter WH 1990. Phosphorus and beef production in northern Australia. 6. Dietary attributes and their relation to cattle growth. Tropical grasslands 24, 197-208.

McNeill DM, Roche JR, McLachlan BP and Stockdale CR 2002. Nutritional strategies for the prevention of hypocalcaemia at calving for dairy cows in pasture-based systems. Australian Journal of Agricultural Research 53, 755-770.

Mellau LSB, Jorgensen RJ, Bartlett PC, Enemark JMD and Hansen AK 2004. Effect of anionic salt and highly fermentable carbohydrate supplementations on urine pH and on experimentally induced hypocalcaemia in cows. Acta Veterinaria Scandinavica 45, 139-147.

Montgomery JL, Parrish FC, Jr, Beitz DC, Horst RL, Huff-Lonergan EJ and Trenkle AH 2000. The use of vitamin D3 to improve beef tenderness. Journal of Animal Science 78, 2615-2621.

Moreira VR, Zeringue LK, Williams CC, Leonardi C and McCormick ME 2009. Influence of calcium and phosphorus feeding on markers of bone metabolism in transition cows. Journal of Dairy Science 92, 5189-5198.

Moore SJ, VandeHaar MJ, Sharma BK, Pilbeam TE, Beede DK, Bucholtz HF, Liesman JS, Horst RL and Goff JP 2000. Effects of altering dietary cation-anion difference on calcium and energy metabolism in peripartum cows. Journal of Dairy Science 83, 2095-2104.

Naito Y, Shindo N, Sato R and Murakami D 1990. Plasma osteocalcin in preparturient and postparturient cows: Correlation with plasma 1,25-dihydroxyvitamin D, Calcium, and inorganic Phosphorus. Journal of Dairy Science 73, 3481-3484.

NRC 1989. Nutrient Requirements of Dairy Cattle. National Academy Press, Washington, D.C.

NRC 2000. Nutrient Requirements of Beef Cattle. National Academy Press, Washington, D.C.

Oetzel GR, Olson JD, Curtis CR and Fettman MJ 1988. Ammonium-Chloride and Ammonium-Sulphate for prevention of parturient paresis in dairy-cows. Journal of Dairy Science 71, 3302-3309.

Oetzel GR, Fettman MJ, Hamar DW and Olson JD 1991. Screening of anionic salts for palatability, Effects on acid-base Status, and urinary Calcium excretion in dairy cows. Journal of Dairy Science 74, 965-971.

Ohyama Y, Noshiro M and Okuda K 1991. Cloning and expression of cDNA encoding 25-hydroxyvitamin D3 24-hydroxylase. FEBS Letters 278, 195-198.

Olson WG, Jorgensen NA, Schultz LH and Deluca HF 1973. 25-Hydroxycholecalciferol (25-OHD3) II. Efficacy of parenteral administration in prevention of parturient paresis. Journal of Dairy Science 56, 889-895.

Olson WG, Jorgensen NA, Bringe AN, Schultz LH and Deluca HF 1974. 25-hydroxycholecalciferol (25-OH-D3). III. Effect of dosage on soft tissue integrity and vitamin D activity of tissue and milk from dairy cows. Journal of Dairy Science 57, 677-682.

Omdahl JL, Morris HA and May BK 2002. Hydroxylase enzymes of the Vitamin D pathway: Expression, Function, and Regulation. Annual Review of Nutrition 22, 139-166.

Pannabecker TL, Chandler JS and Wasserman RH 1995. Vitamin D-Dependent Transcriptional Regulation of the Intestinal Plasma Membrane Calcium Pump. Biochemical and Biophysical Research Communications 213, 499-505.

Payne JM and Manston R 1967. The safety of massive doses of vitamin D_3 in the prevention of milk fever. Veterinary Record 81, 214-216.

Pehrson B, Svensson C, Gruvaeus I and Virkki M 1999. The Influence of acidic diets on the acid-base balance of dry cows and the effect of fertilization on the mineral content of grass. Journal of Dairy Science 82, 1310-1316.

Ponchon G and DeLuca HF 1969. The role of the liver in the metabolism of Vitamin D. Journal of Clinical Investigation 48, 1273-1279.

Poos MI, Hanson TL and Klopfenstein TJ 1979. Monensin Effects on Diet Digestibility, Ruminal Protein Bypass and Microbial Protein Synthesis. Journal of Animal Science 48, 1516-1524.

Ramberg CF, Jr, Johnson EK, Fargo RD and Kronfeld DS 1984. Calcium homeostasis in cows, with special reference to parturient hypocalcemia. American Journal of Physiology 246, R698-704.

Ramos-Nieves JM, Thering BJ, Waldron MR, Jardon PW and Overton TR 2009. Effects of anion supplementation to low-potassium prepartum diets on macromineral status and performance of periparturient dairy cows. Journal of Dairy Science 92, 5677-5691.

Rasmussen H, Fontaine O, Max EE and Goodman DB 1979. The effect of 1alpha-hydroxyvitamin D3 administration on calcium transport in chick intestine brush border membrane vesicles. Journal of Biological Chemistry 254, 2993-2999.

Reinhardt TA and Conrad HR 1980. Mode of action of pharmacological doses of cholecalciferol during parturient hypocalcemia in dairy cows. The Journal of Nutrition 110, 1589.

Rerat M, Philipp A, Hess HD and Liesegang A 2009. Effect of different potassium levels in hay on acid-base status and mineral balance in periparturient dairy cows. Journal of Dairy Science 92, 6123-6133.

Riond JL 2001. Animal nutrition and acid-base balance. European Journal of Nutrition 40, 245-254.

Risteli L and Risteli J 1993. Biochemical Markers of Bone Metabolism. Annals of Medicine 25, 385-393.

Rivera JD, Bachman SE, Hubbert ME, Branine ME, Horst RL, Williams SN and Galyean ML 2005. Short communication: Serum and tissue concentrations of vitamin D metabolites in beef heifers after buccal dosing of 25-hydroxyvitamin D3. Journal of Dairy Science 88, 1364-1369.

Roche JR 2003. Hypocalcaemia and DCAD for the pasture-based transition cow - a review. Acta Veterinaria Scandinavica 44, 65-74.

Roche JR, Dalley DE and O'Mara FP 2007. Effect of a metabolically created systemic acidosis on calcium homeostasis and the diurnal variation in urine pH in the non-lactating pregnant dairy cow. Journal of Dairy Research 74, 34-39.

Ross JG, Spears JW and Garlich JD 1994. Dietary electrolyte balance effects on performance and metabolic characteristics in growing steers. Journal of Animal Science 72, 1842-1848.

Rowell JG and Walters RE 1976. Analysing data with repeated observations on each experimental unit. Journal of Agricultural Science 87, 423-432.

Rowling MJ, Gliniak C, Welsh J and Fleet JC 2007. High Dietary Vitamin D Prevents Hypocalcemia and Osteomalacia in CYP27B1 Knockout Mice. The Journal of Nutrition 137, 2608-2615.

Sabbagh Y, O'Brien SP, Song W, Boulanger JH, Stockmann A, Arbeeny C and Schiavi SC 2009. Intestinal Npt2b Plays a Major Role in Phosphate Absorption and Homeostasis. Journal of the American Society of Nephrology 20, 2348-2358.

Schatz TJ and Hearnden MN 2008. Heifer fertility on commercial cattle properties in the Northern Territory. Australian Journal of Experimental Agriculture 48, 940-944.

Schneider KM, Boston RC and Leaver DD 1987. Quantitation of phosphorus excretion in sheep by compartmental analysis. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology 252, 720-731.

Schonewille JT, Vantklooster AT, Dirkzwager A and Beynen AC 1994. Stimulatory effect of an anion (chloride) - rich ration on apparent calcium-absorption in dairy-cows. Livestock Production Science 40, 233-240.

Schröder B, Rittmann I, Pfeffer E and Breves G 1997. In vitro studies on calcium absorption from the gastrointestinal tract in small ruminants. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 167, 43-51.

Scott D 1972. Excretion of phosphorus and acid in the urine of sheep and calves fed either roughage or concentrate diets. Experimental Physiology 57, 379-392.

Scott D and Beastall G 1978. The effects of intravenous Phosphate loading on salivary Phosphate secretion and plasma parathyroid hormone levels in sheep. Experimental Physiology 63, 147-156.

Scott D and Buchan W 1988. Effect of reduction in phosphorus intake on salivary phosphorus secretion and on duodenal digesta and dry-matter flow in sheep. The Journal of Agricultural Science 110, 411-413.

Scott D, Whitelaw FG and Kay M 1971. Renal excretion of acid in calves fed either roughage or concentrate diets. Experimental Physiology 56, 18-32.

Seifi HA, Mohri M and Zadeh JK 2004. Use of pre-partum urine pH to predict the risk of milk fever in dairy cows. Veterinary Journal 167, 281-285.

Shepard RM and Deluca HF 1980. Plasma concentrations of vitamin D3 and its metabolites in the rat as influenced by vitamin D3 or 25-hydroxyvitamin D3 intakes. Archives of Biochemistry and Biophysics 202, 43-53.

Shinki T, Jin CH, Nishimura A, Nagai Y, Ohyama Y, Noshiro M, Okuda K and Suda T 1992. Parathyroid hormone inhibits 25-hydroxyvitamin D3-24-hydroxylase mRNA expression stimulated by 1 alpha,25-dihydroxyvitamin D3 in rat kidney but not in intestine. Journal of Biological Chemistry 267, 13757-13762.

Sommerfeldt J, Horst RL, Littledike ET and Beitz DC 1979. In vitro degradation of cholecalciferol in rumen fluid. Journal of Dairy Science 62, 192.

Staric J and Zadnik T 2010. Biochemical markers of bone metabolism in dairy cows with milk fever. Acta Veterinaria Beograd 60, 401-410.

Stern J and Lewis WHP 1957. The colorimetric estimation of calcium in serum with o-cresolphthalein complexone. Clinica Chimica Acta 2, 576-580.

Stoffels K, Overbergh L, Bouillon R and Mathieu C 2007. Immune regulation of 1α-hydroxylase in murine peritoneal macrophages: Unravelling the IFNγ pathway. The Journal of Steroid Biochemistry and Molecular Biology 103, 567-571.

Suda T, Ueno Y, Fujii K and Shinki T 2003. Vitamin D and bone. Journal of Cellular Biochemistry 88, 259-266.

Takagi H and Block E 1991a. Effects of various dietary cation-anion balances on response to experimentally induced hypocalcemia in sheep. Journal of Dairy Science 74, 4215-4224.

Takagi H and Block E 1991b. Effects of manipulating dietary cation-anion balance on macromineral balance in sheep. Journal of Dairy Science 74, 4202-4214.

Takagi H and Block E 1991c. Effects of reducing dietary cation-anion balance on Calcium kinetics in sheep. Journal of Dairy Science 74, 4225-4237.

Tanaka Y and Deluca HF 1973. The control of 25-hydroxyvitamin D metabolism by inorganic phosphorus. Archives of Biochemistry and Biophysics 154, 566-574.

Taylor MS, Knowlton KF, McGilliard ML, Seymour WM and Herbein JH 2008. Blood mineral, hormone, and osteocalcin responses of multiparous Jersey cows to an oral dose of 25-Hydroxyvitamin D3 or Vitamin D3 Before parturition. Journal of Dairy Science 91, 2408-2416.

Termine JR and Posner AS 1967. Amorphouse/Crystalline interrelationship in Bone mineral. Calcified Tissue Research 1, 8-23

Ternouth JH 1990. Phosphorus and beef production in northern Australia. 3. Phosphorus in cattle - a review. Tropical grasslands 24, 159-169.

Ternouth JH and Coates DB 1997. Phosphorus homoeostasis in grazing breeder cattle. The Journal of Agricultural Science 128, 331-337.

Ternouth JH, Bortolussi G, Coates DB, Hendricksen RE and McLean RW 1996. The phosphorus requirements of growing cattle consuming forage diets. The Journal of Agricultural Science 126, 503-510.

Tomas FM 1974. Phosphorus homeostasis in sheep. II. Influence of diet on the pathway of excretion of Phosphorus. Australian Journal of Agricultural Research 25, 485-493.

Trummel CL, Raisz LG, Blunt JW and Deluca HF 1969. 25-hydroxycholecalciferol: Stimulation of bone resorption in tissue culture. Science 163, 1450-1451.

Tucker WB, Harrison GA and Hemken RW 1988a. Influence ofdietary cation anion balance on milk, blood, urine and rumen fluid in lactating dairy cattle. Journal of Dairy Science 71, 346-354.

Tucker WB, Xin Z and Hemken RW 1988b. Influence of dietary calcium-chloride on adaptive-changes in acid-base status and mineral metabolism in lactating dairy-cows fed a diet high in sodium-bicarbonate. Journal of Dairy Science 71, 1587-1597.

Tucker WB, Hogue JF, Adams GD, Aslam M, Shin IS and Morgan G 1992. Influence of cietary cation-anion balance during the dry period on the occurence of parturient paresis in cows fed excess calcium. Journal of Animal Science 70, 1238-1250.

Tucker WB, Hogue JF, Waterman DE, Swenson TS, Xin Z, Hemken RW, Jackson JA, Adams GD and Spicer LJ 1991. Role of sulfur and chlorine in the dietary cation-anion balance equation for lactating dairy-cattle. Journal of Animal Science 69, 1205-1213.

Vagg MJ and Payne JM 1970. The effect of Ammonium Chloride induced acidosis on calcium metabolism in ruminants. British Veterinary Journal 126, 531.

Vagnoni DB and Oetzel GR 1998. Effects of dietary cation-anion difference on the acid-base status of dry cows. Journal of Dairy Science 81, 1643-1652.

van Abel M, Hoenderop JGJ, van der Kemp AWCM, van Leeuwen JPTM and Bindels RJM 2003. Regulation of the epithelial Ca2+ channels in small intestine as studied by quantitative mRNA detection. American Journal of Physiology - Gastrointestinal and Liver Physiology 285, G78-G85.

Van Mosel M, Wouterse HS and Van't Klooster AT 1994. Effects of reducing dietary ($[Na^+ + K^+ - [Cl^- + SO_4]$) on bone in dairy cows at parturition. Research in Veterinary Science 56, 270-276.

Walton J and Gray TK 1979. Absorption of inorganic phosphate in the human small intestine. Clinical science (London, England : 1979) 56, 407-412.

Wang C and Beede DK 1992a. Effects of ammonium-chloride and sulfate on acid-base status and calcium-metabolism of dry Jersey cows. Journal of Dairy Science 75, 820-828.

Wang C and Beede DK 1992b. Effects of Magnesium on acid-base status and Calcium-metabolism of dry cows fed acidogenic salts. Journal of Dairy Science 75, 829-836.

Wasserman RH and Taylor AN 1973. Intestinal absorption of Phosphate in the chick: Effect of vitamin D3 and other parameters. Journal of Nutrition 103, 586-599.

Webb AR, DeCosta BR and Holick MF 1989. Sunlight Regulates the Cutaneous Production of Vitamin D3 by Causing Its Photodegradation. Journal of Clinical Endocrinology & Metabolism 68, 882-887.

Wertz AE, Knight TJ, Trenkle A, Sonon R, Horst RL, Huff-Lonergan EJ and Beitz DC 2004. Feeding 25-hydroxyvitamin D3 to improve beef tenderness. Journal of Animal Science 82, 1410-1418.

Wilkens MR, Mrochen N, Breves G and Schröder B 2010. Effects of 1,25-dihydroxyvitamin D3 on calcium and phosphorus homeostasis in sheep fed diets either adequate or restricted in calcium content. Domestic Animal Endocrinology 38, 190-199.

Wilkens MR, Oberheide I, Schroder B, Azem E, Steinberg W and Breves G 2012. Influence of the combination of 25-hydroxyvitamin D3 and a diet negative in cation-anion difference on peripartal calcium homeostasis of dairy cows. Journal of Dairy Science 95, 151-164.

Winks L 1990. Phosphorus and beef production in northern Australia. 2. Responses to phosphorus by ruminant - a review. Tropical grasslands 24, 140-158.

Winter WH, Coates DB, Hendricksen RE, Kerridge PC, McLean RW and Miller CP 1990. Phosphorus and beef production in northern Australia. 4. The response of cattle to fertilizer and supplementary phosphorus. Tropical grasslands 24, 170-184.

Won JH, Oishi N, Kawamura T, Sugiwaka T, Fukuda S, Sato R and Naito Y 1996. Mineral metabolism in plasma, urine and bone of periparturient cows fed anionic diets with different calcium and phosphorus contents. Journal of Veterinary Medical Science 58, 1187-1192.

Wright RD, Blair-West JR, Nelson JF, Tregear GW and Rosenblatt M 1984. Evaluation of the biological properties of parathyroid hormone and analogues in a vascularly isolated parotid gland-based assay. Journal of Endocrinology 102, 375-379.

Xu H, Collins JF, Bai L, Kiela PR and Ghishan FK 2001. Regulation of the human sodium-phosphate cotransporter NaPi-IIb gene promoter by epidermal growth factor. American Journal of Physiology - Cell Physiology 280, C628-C636.

Xu H, Uno JK, Inouye M, Xu L, Drees JB, Collins JF and Ghishan FK 2003. Regulation of intestinal NaPi-IIb cotransporter gene expression by estrogen. American Journal of Physiology - Gastrointestinal and Liver Physiology 285, G1317-G1324.