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CHAPTER 2

Vitamin D Metabolism

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I. INTRODUCTION

In 1919, when the field of experimental nutrition was still in its infancy, Sir Edward Mellanby conducted a classic experiment that for the first time associated the supplementation of various growth-promoting fats with the prevention of rickets [1]. He credited the cure to the presence of a fat-soluble substance called vitamin A. McCollum *et al.* [2], however, later discovered that the factor responsible for healing rickets was distinct from vitamin A. McCollum named this new substance vitamin D. It was also during this period when scientists realized that there were two antirachitic factors with distinct structures. As discussed by Norman [3], the first factor to be identified was designated vitamin D_2 (also known as ergocalciferol), whereas the structure of vitamin D_3 (cholecalciferol) became evident some 4 to 5 years later. Vitamins D_3 and D_2 are used for supplementation of animal and human diets in the United States. Vitamin D_3 is the form of vitamin D that is synthesized by vertebrates, whereas vitamin D_2 is the major naturally occurring form of the vitamin in plants. Animals that bask in the sun such as amphibia, reptiles, and birds therefore synthesize sufficient endogenous vitamin D_3 to meet their daily needs. However, herbivores may have evolved utilizing vitamin D_2 as their predominant source.

This chapter focuses on the general control and function of key enzymes involved in the regulation of vitamin D_2 and vitamin D_3 metabolism. Species differences in vitamin D metabolism, as well as vitamin D toxicity, are also discussed. The reader is also directed toward a number of additional reviews regarding vitamin D metabolism and action [3–8]. This chapter gives an overview of vitamin D metabolism; critical steps are discussed in further detail in the subsequent chapters of this section. Metabolism of vitamin D analogs is covered in Chapter 81.

II. VITAMIN D METABOLISM

A. Overview

Vitamin D refers to a group of compounds that possess antirachitic activity. Technically vitamin D is classified as a secosteroid. Secosteroids are those in which one of the rings has been broken; in vitamin D, the 9,10 carbon–carbon bond of ring B is broken, and it is indicated by the inclusion of "9,10-seco" in the official nomenclature. There are several known nutritional forms of vitamin D, however, the best known examples are cholecalciferol (vitamin D_3), which is produced in the skin, and ergocalciferol (vitamin D_2), which is derived from plant tissues (Fig. 1). Therefore, when reference is made to vitamin D, the lack of a subscript usually implies either vitamin D_2 or vitamin D_3.

The vitamin Ds are named according to the rules of the International Union of Pure and Applied Chemists (IUPAC) for steroid nomenclature [9]. Because vitamin D is derived from a steroid, the structure retains its numbering from the parent compound cholesterol. Configurations at asymmetric centers are designated by using the *R* and *S* notation applying the sequence-rule procedure [10]. Configuration of the double bonds are notated *E* for "entgegen" or *trans*, and *Z* for "zuzammen" or *cis* [11]. Thus the official name of vitamin D_3, by relation to cholesterol, is 9,10-seco(5*Z*,7*E*)-5,7,10(19) cholestatriene-3β-ol, and the official name of vitamin D_2 is 9,10-seco(5*Z*,7*E*)-5,7,10(19), 22-ergostatetraene-3β-ol.

Contemporary views categorize vitamin D_3 not as a vitamin but, rather, as a prosteroid hormone. This concept is supported by the fact that in mammals vitamin D_3 is derived from 7-dehydrocholesterol (the precursor of cholesterol) present in the skin. The direct action of sunlight on 7-dehydrocholesterol results in cleavage

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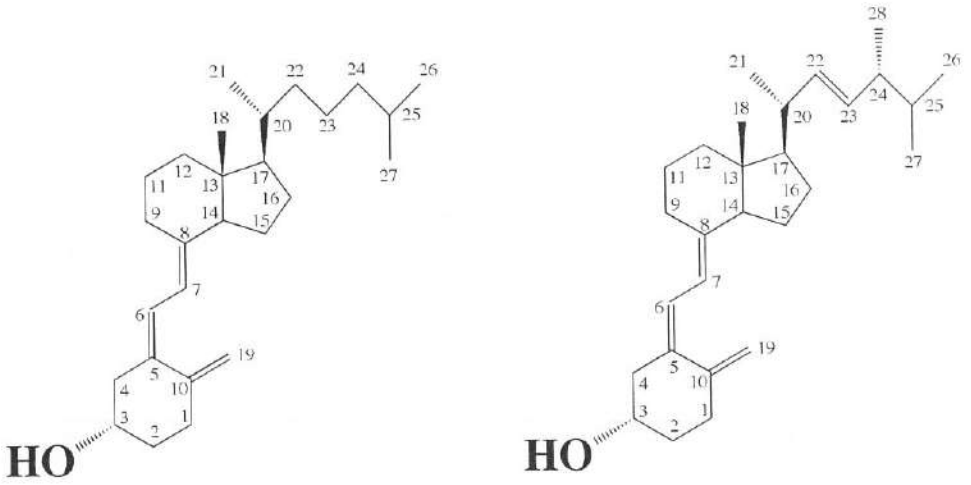

Vitamin D_3 Vitamin D_2

FIGURE 1 Important nutritional forms of vitamin D.

of the B ring of the steroid structure that on thermoisomerization yields vitamin D_3 (see Chapter 3). The significance of vitamin D as a prosteroid hormone became clearer in 1967 when Morii *et al.* [11a] isolated a new metabolite of vitamin D_3 from rats that was as effective as vitamin D_3 in healing rickets, raising blood calcium, and increasing intestinal calcium transport. This compound acted more rapidly than vitamin D_3, requiring only 8 to 10 hr after oral administration to initiate its response. This metabolite was identified as 25-hydroxyvitamin D_3 ($25OHD_3$) [11b]. The liver was demonstrated to be important in the production of this most abundant circulating form of vitamin D_3 that, under normal conditions, is present at 20 to 50 ng/ml [4]. Shortly following the discovery of $25OHD_3$, a number of laboratories showed that this metabolite is specifically hydroxylated at the 1α-position in the kidney to yield 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] [11c–13]. The latter metabolite is now generally accepted as the hormonally active form of vitamin D_3. Its importance is evidenced by the genetic disorder pseudo vitamin D–deficiency rickets (PDDR), which is caused by mutations in the 1α-hydroxylase gene. PDDR results in the inability to produce $1,25(OH)_2D$ leading to severe rickets (see Chapters 71 and 72). In normal human plasma, $1,25(OH)_2D_3$ circulates at approximately 1000-fold lower concentrations than $25OHD_3$ and is generally present at 20 to 65 pg/ml [14].

This simplistic picture outlined for vitamin D_3 activation is complicated by the fact that vitamin D_3 can be oxidatively metabolized to a variety of products. Most of these numerous metabolites have no identifiable biological function, and indeed many have been isolated from animals fed abnormally high amounts of vitamin D_3. Nevertheless, the evidence collected to date indicates that 25-hydroxylated vitamin D_3 metabolites are preferentially metabolized at the side chain. In particular, carbon centers C-23, C-24, and C-26 are readily susceptible to further oxidation. Figure 2 illustrates products of these oxidative pathways. As indicated, these pathways are shared by both $25OHD_3$ and $1,25(OH)_2D_3$, and their physiological importance is still a matter of controversy. For example, there is evidence that 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$] may function to stimulate bone mineralization [15,16], suppress parathyroid hormone (PTH) secretion [17], and maintain embryonic development [18]. For the most part, however, the C-24 hydroxylation and other side-chain modifications are generally considered to be catabolic in nature and play a key role in maintaining vitamin D homeostasis [19].

Although these side-chain oxidative pathways yield metabolites that are considered "nonfunctional," the presence of these compounds in circulation could pose serious problems in the analysis for $25OHD_3$ and $1,25(OH)_2D_3$ [20]. Further complicating the issue of understanding vitamin D activation, catabolism, and metabolite analysis is the presence of vitamin D_2. Vitamin D_2 has been shown to contribute significantly to the overall vitamin D status in humans and other mammals consuming supplemental vitamin D_2 [21–23]. Vitamin D_2 can also be metabolized in a similar fashion to produce several metabolites analogous to the vitamin D_3 endocrine system, including the hormonally

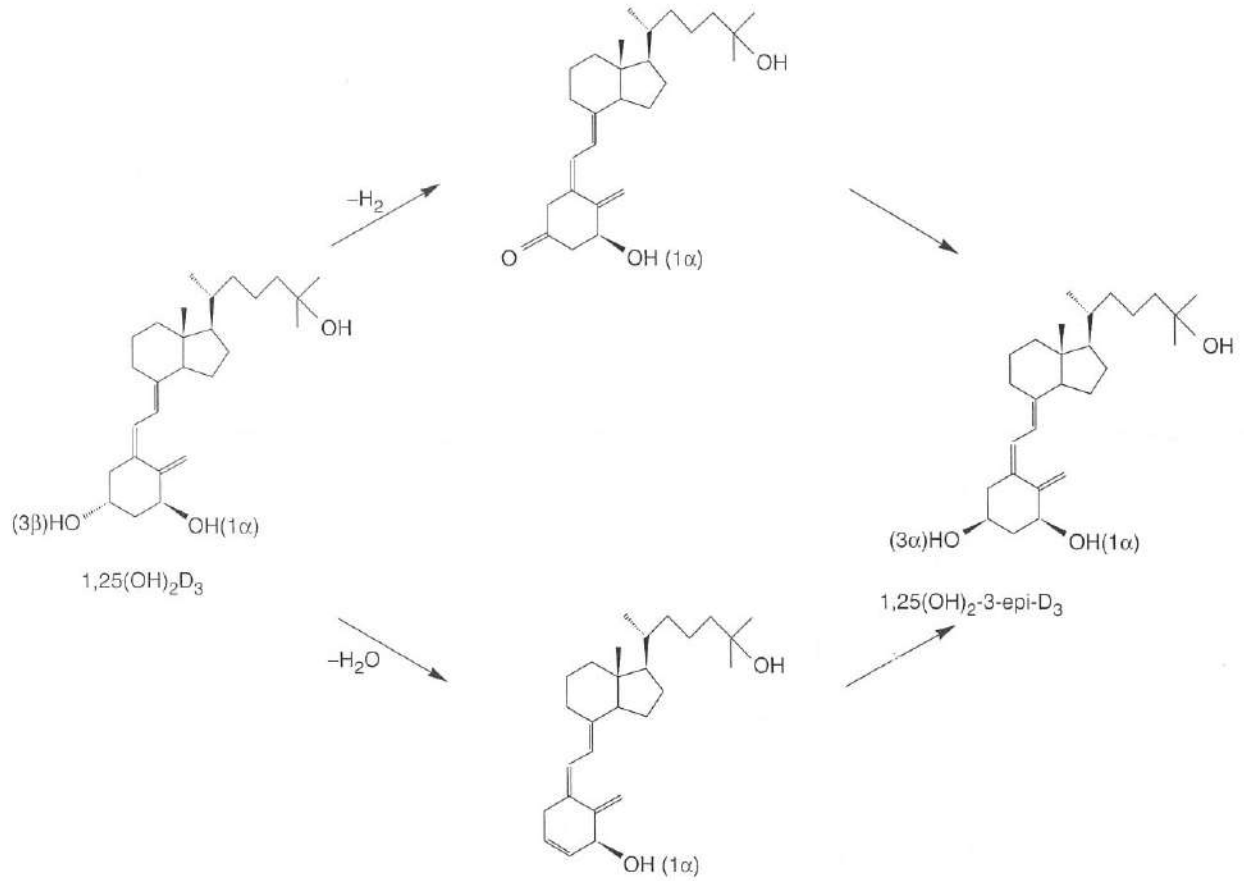

FIGURE 2 Pathways of vitamin D_3 metabolism.

active form of vitamin D_2, 1,25-dihydroxyvitamin D_2 [$1,25(OH)_2D_2$] [24]. Simple inspection of the side chain, however, would imply that differences between metabolism of vitamin D_2 and vitamin D_3 may exist. The presence of unsaturation at carbon centers C-22/C-23, along with the additional methyl group at C-24, would seem to preclude the existence of the same metabolic pathways for the two vitamins. Figure 3 outlines some of the known pathways of vitamin D_2 metabolism that have been shown to date. Deviations in the vitamin D_2 and vitamin D_3 pathways are discussed in detail in the following sections.

B. 25-Hydroxylase

The 25-hydroxylation of vitamin D is the initial step in vitamin D activation. The enzyme responsible for production of this metabolite is located in the liver (see Chapter 4). Extrahepatic sources of 25-hydroxylation have been described [25]; however, experiments with hepatectomized rats provided evidence that the liver is the major, if not the sole, physiologically relevant site of 25-hydroxylation of vitamin D [26]. Subsequent studies also described the existence of the 25-hydroxylase in both liver mitochondria and microsomes [27–31]. In early work, the microsomal enzyme was described as an enzyme of low capacity and high affinity and, therefore, the enzyme of greatest physiological importance [31]. In contrast, the mitochondrial enzyme was described as a high-capacity, low-affinity enzyme thought to be relevant only under conditions of high vitamin D concentration such as vitamin D toxicity [32]. Early evidence that the microsomal enzyme was the physiologically relevant enzyme came from experiments that suggested this enzyme could be regulated by vitamin D status [31]. It is now clear that liver production of 25-hydroxyvitamin D (25OHD) is not significantly regulated. 25OHD production is primarily dependent on substrate concentration. An important consequence of this lack of physiological regulation of 25OHD is that measurement of blood 25OHD is an excellent measure of vitamin D nutritional status.

The purification and cloning of putative liver 25-hydroxylases have been reviewed several times [33–37]. Examination of the literature shows that most of the focus is on the mitochondrial 25-hydroxylase

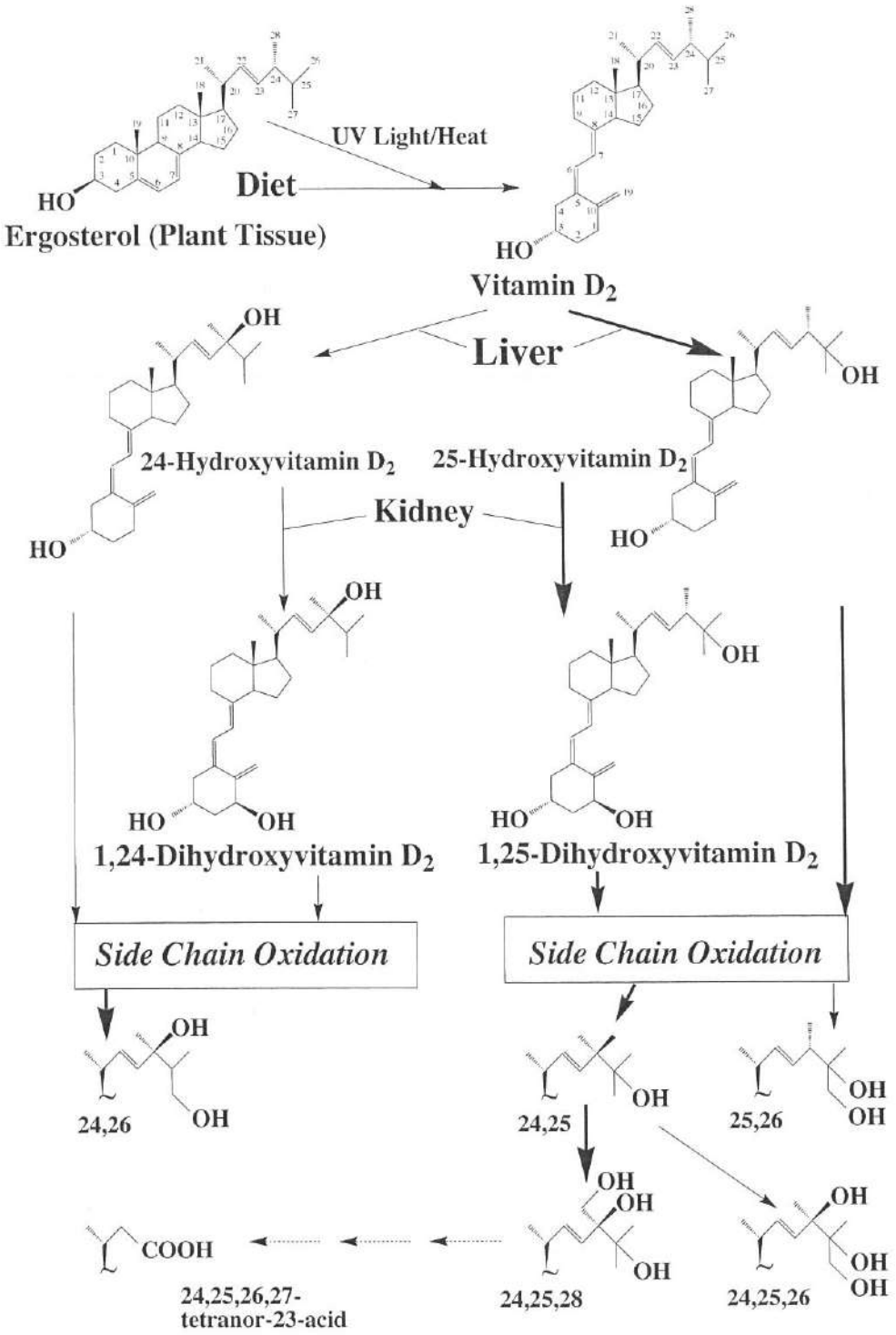

FIGURE 3 Pathways of vitamin D_2 metabolism.

designated CYP27A1, which is a cytochrome P450 capable of C-26(27) hydroxylation of sterols involved in bile acid synthesis and the 25-hydroxylation of vitamin D_3. The rat, rabbit, and human enzymes have been cloned [38–41]. The CYP27A1 clone has been expressed in COS cells [39,42], and its activity has been isolated from the mitochondria of these cells. The expressed enzyme was found to 27-hydroxylate cholestanetriol and 25-hydroxylate vitamin D_3. However, CYP27A1 does not 25-hydroxylate vitamin D_2 [39]. Rather, CYP27A1

was found to 24-hydroxylate and 26(27)-hydroxylate vitamin D_2. These activities could explain the presence of 24-hydroxyvitamin D_2 ($24OHD_2$), 1,24-dihydroxyvitamin D_2 [$1,24(OH)_2D_2$], and 24,26-dihydroxyvitamin D_2 [$24,26(OH)_2D_2$] [43–45] in the plasma of rats and cows. Since rats fed vitamin D–deficient diets and supplemented with physiological amounts of vitamin D_2 have $25OHD_2$ as their predominant monohydroxylated vitamin D_2 metabolite in the plasma [44] and targeted disruption of CYP27A1 does not decrease serum $25OHD_3$ [46], CYP27A1 is likely not the physiologic enzyme responsible for the 25-hydroxylation of vitamin D.

The rat liver microsomal 25-hydroxylase (CYP2C11) has also been studied, but it has been shown to be male-specific [47]. Data have also been presented indicating that microsomes do [48] or do not [49] possess 25-hydroxylase activity. Therefore, conclusions regarding the importance of the CYP27A1 and other ostensible microsomal 25-hydroxylases require additional research.

Data obtained studying pig liver 25-hydroxylation of vitamin D_3 [36,37,48,50,51] suggest that a third liver 25-hydroxylase exists that is microsomal in origin. In the pig, this enzyme, CYP2D25, is present equally in males and females and is markedly different from CYP27A1 and CYP2C11 based on a terminal amino acid sequence [51]. Most important is the finding that this pig microsomal enzyme 25-hydroxylates vitamin D_2 and vitamin D_3 equally. The 25-hydroxylation of vitamin D is not yet completely understood. Several enzymes may play a role in the 25-hydroxylation of vitamin D. Whether one enzyme is more physiologically relevant than others remains to be determined. Studies in primary cultures of pig hepatocytes suggest that both CYP2D25 and CYP27A1 can play a role in 25-hydroxylation of vitamin D_3 [51]. Nevertheless, it is clear that mammals can use vitamin D_2 as a sole source of vitamin D. Therefore, any 25-hydroxylase proclaimed as the key enzyme(s) in the 25-hydroxylation of vitamin D must be capable of 25-hydroxylating vitamin D_2 as well as vitamin D_3. If mammals, for example, possessed an enzyme with specificity for the vitamin D_2 side chain, this enzyme may be missed due to the almost exclusive use of vitamin D_3 or vitamin D_3 analogs as substrates for the 25-hydroxylating reaction.

C. 1α-Hydroxylase (CYP27B1)

In the late 1960s, $25OHD_3$ was believed to be the metabolically active form of vitamin D. However, the presence of a more polar metabolite, which accumulated in the intestinal mucosa chromatin of chicks administered ^3H-labeled vitamin D_3, suggested a new candidate for the active form of vitamin D [52]. Subsequent work by Lawson *et al.* [53] showed that during the formation of this metabolite the 1α-^3H was lost. This led them to suggest that the new metabolite had an oxygen function inserted at C-1 in addition to the hydroxyl group at C-25. The enhanced biological activity of this new metabolite was evident before its structure could be determined [54–56]. Fraser and Kodicek [56] demonstrated that nephrectomy abolished production of the new metabolite, and this active vitamin D compound was synthesized by kidney mitochondria. In 1971, three laboratories identified the active form of vitamin D as $1,25(OH)_2D_3$ [11,12,57]. Subsequently, the vitamin D_2 form was also isolated and identified [24].

The CYP27B1 is located in the inner mitochondrial membrane of the proximal convoluted tubule cells of the kidney [58] and is discussed in detail in Chapter 5. Extrarenal sites of 1α-hydroxylation have been reported in bone, liver, placenta, macrophages, and skin [59]. The physiological significance of these sites on systemic calcium metabolism is in doubt, as nephrectomy and/or severe renal failure results in very low to undetectable circulating $1,25(OH)_2D_3$ levels [60].

The regulation of $1,25(OH)_2D_3$ production is reciprocally regulated with respect to $24,25(OH)_2D_3$ [61]. Hypocalcemia caused by calcium-deficient diets, vitamin D deficiency, or pathological factors results in increased production of $1,25(OH)_2D_3$ [61–67]. This hypocalcemic-mediated induction of 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] production is secondary to increased PTH. Administration of PTH to animals results in increased $1,25(OH)_2D_3$ production [64,67,68]. PTH treatment *in vitro* induces CYP27B1 in renal slices [63] and cultured kidney cells [67,69] and is cAMP dependent [63,67,69,70]. Thyroparathyroidectomy (TPTX) or parathyroidectomy (PTX) results in the loss of the ability to synthesize $1,25(OH)_2D_3$. In humans, acute administration of PTH or primary hyperparathyroidism results in increased production of $1,25(OH)_2D$ [14,71], which is evidenced by elevations in plasma $1,25(OH)_2D$. However, in animal studies where PTH was administered chronically to goats and calves, a transient rise in plasma $1,25(OH)_2D$ was observed followed by a rapid decline to nearly undetectable levels [72,73]. These results could be attributed to hypercalcemic feedback on the renal CYP27B1. When plasma calcium in these animals reached 13 mg/dl, $1,25(OH)_2D$ production appeared to cease. This same group conducted similar experiments in rats and showed that chronic PTH infusion did not result in a reduction of plasma $1,25(OH)_2D_3$, but rather a

modest rise [74]. Clearly, there are varying degrees of direct calcium-mediated feedback on the renal CYP27B1. It is possible that species and age affect the set points at which plasma calcium becomes a direct negative regulator of $1,25(OH)_2D_3$ production.

In contrast to the indirect role of plasma calcium in inducing $1,25(OH)_2D_3$ production, the role of plasma phosphate appears more direct. As plasma phosphate declines, animals shift from $24,25(OH)_2D$ production to increased $1,25(OH)_2D_3$ production [75,76]. Since phosphate-deficient animals are hypercalcemic, serum PTH is down and therefore cannot be providing the signal to increase $1,25(OH)_2D_3$ production. Furthermore, TPTX phosphate-deficient animals produce $1,25(OH)_2D_3$ similarly to intact phosphate-deficient animals [66]. Gray [77] demonstrated that hypophysectomy abolished the increase in plasma $1,25(OH)_2D$ concentrations that normally accompanied dietary phosphate deprivation. Gray demonstrated that growth hormone or triiodothyronine replacement to hypophysectomized rats restored elevations in plasma $1,25(OH)_2D$ associated with low dietary phosphorus, therefore suggesting a permissive role of these hormones in regulation of the renal CYP27B1 during phosphorus deficiency.

A direct negative effect of $1,25(OH)_2D$ on its own production has been reported. The inhibitory effect of $1,25(OH)_2D_3$ on renal CYP27B1 activity occurs both *in vivo* and *in vitro* [64,78]. This repressive activity is probably indirect [79]. *In vivo*, this effect may be partially mediated through the ability of $1,25(OH)_2D_3$ to inhibit PTH secretion [80]. Similarly, Beckman *et al.* [81] showed that vitamin D toxicity mildly inhibited renal CYP27B1 activity while low-calcium diets significantly induced the CYP27B1. They further demonstrated that administration of toxic doses of vitamin D to animals fed a calcium-deficient diet reduced CYP27B1 activity by 90%. This result occurred in spite of the fact that these animals were hypocalcemic and had serum PTH levels equal to those of control animals receiving calcium-deficient normal vitamin D diets. These data suggest that high plasma concentration of vitamin D metabolites may act directly to suppress CYP27B1 activity.

There are many additional factors such as calcitonin (CT), acidosis, sex steroids, prolactin, growth hormone, glucocorticoids, thyroid hormone, and pregnancy that are potential regulators of $1,25(OH)_2D$ production. One of the most recent and interesting is the requirement for the endocytic receptor megalin in the proximal tubular cells to allow uptake of 25OHD for 1α-hydroxylation [82]. Discussion of these is beyond the scope of this general review of vitamin D metabolites. The characteristics and regulation of the CYP27B1 are described further in several reports [37,83–85].

The cloning of the renal CYP27B1 has been achieved [86–89]. Studies with CYP27B1 knockout mice [90,91] suggest that these animals have abnormalities similar to those observed in PDDR. These knockout models will undoubtedly provide new insight into the functions of $1,25(OH)_2D$ and are the subject of further review in Chapter 7.

D. 24-Hydroxylase (CYP24A1)

The 24-hydroxylation of $25OHD_3$ and $1,25(OH)_2D_3$ to form $24,25(OH)_2D_3$ [92] and $1,24,25(OH)_3D_3$ [93,94] is the primary mechanism and the first step in a metabolic pathway to inactivate and degrade these vitamin D metabolites.

It now appears that CYP24A1 is ubiquitous and may be present in every cell and tissue that contains the vitamin D receptor (VDR). In the kidney, CYP24A1 is found on the inner mitochondrial membrane of the renal tubules [95]. The primary regulators of renal CYP24A1 activity are PTH and $1,25(OH)_2D_3$. Normal and TPTX animals receiving injections or infusions of $1,25(OH)_2D_3$ show marked increases in both renal CYP24A1 mRNA levels and activity [63,74,96,97]. Administration of PTH partially or completely blocks expression of CYP24A1 mRNA and activity in these animals [63,68,74,96,97]. PTH acts on the kidney via adenylate cyclase and cAMP, and it has been shown that infusions of cAMP *in vivo* block $1,25(OH)_2D_3$-mediated inductions of the renal CYP24A1 [96,98]. Animals on calcium-deficient diets have elevated plasma $1,25(OH)_2D$ concentrations, which are accompanied by suppressed or undetectable renal CYP24A1 activity [78,96], as well as reduced VDR concentrations [99].

The reasons for the inability of $1,25(OH)_2D_3$ to up-regulate renal CYP24A1 during calcium deficiency are not clear. Iida *et al.* [100] have proposed that the down-regulation of renal VDR during calcium deficiency may be responsible for preventing the $1,25(OH)_2D_3$-mediated induction of renal CYP24A1. *In vivo* studies by Reinhardt and Horst [74], however, suggest that under these conditions PTH is probably the more important mediator of renal CYP24A1 regulation rather than down-regulation of VDR. In their experiments, Reinhardt and Horst [74] showed that $1,25(OH)_2D_3$ treatment of animals on normal calcium diets resulted in significant up-regulation of renal CYP24A1 as well as VDR. However, when PTH was infused simultaneously with $1,25(OH)_2D_3$, VDR up-regulation was still observed (albeit to a lesser degree), whereas CYP24A1 up-regulation was completely blocked. The importance of PTH in preventing the $1,25(OH)_2D_3$-mediated up-regulation of the renal CYP24A1 is also apparent

by observation in aged rats. With advancing age, renal PTH receptors are down-regulated [101,102], while VDR remains unchanged [103]. The reduction in renal PTH receptors makes the kidney less responsive to PTH [64], which is associated with significant elevations in CYP24A1 mRNA [103,104]. These data suggest that renal responsiveness to PTH, not a decline in VDR, is the major physiological regulator of the renal CYP24A1.

In the intestine, $1,25(OH)_2D_3$ is the primary regulator of CYP24A1. *In vivo* administration of $1,25(OH)_2D_3$ rapidly induces intestinal CYP24A1 activity [105]. This activity peaks by 6 hr postinjection, and rapidly declines thereafter to control levels 24 hr postinjection. Time-course experiments show that CYP24A1 mRNA peaks 4 to 6 hr post-injection and then rapidly disappears [104]. This is in contrast to the renal CYP24A1 mRNA, which peaks 12 to 24 hr post-$1,25(OH)_2D_3$ treatment and declines much more slowly. Shinki *et al.* [96] proposed that the intestinal CYP24A1 was 100 times more sensitive to $1,25(OH)_2D_3$ stimuli than the renal CYP24A1. However, they examined CYP24A1 mRNA only 3 hr after a $1,25(OH)_2D_3$ dose. Because renal CYP24A1 requires an additional 6 to 12 hr to reach peak expression, they likely underestimated the true sensitivity of the kidney to a $1,25(OH)_2D_3$ dose. In contrast to their effect in the kidney, TPTX, PTH administration, or cAMP infusion does not affect intestinal expression of CYP24A1 induced by $1,25(OH)_2D_3$ [96]. Animals fed low-calcium diets, with the associated secondary hyperparathyroidism and high plasma $1,25(OH)_2D_3$ concentrations, have marked inductions of both intestinal CYP24A1 mRNA and activity [96,105]. Another contrast between intestinal and renal CYP24A1 expression is seen in the aging rat model. Intestinal CYP24A1 mRNA and activity decline or change very little in the aged animal. This contrasts to the largely increased expression of renal CYP24A1 observed in the aged animal [103].

Calcitonin has been shown to be a potent suppressor of intestinal CYP24A1 expression [106]. In these experiments, Beckman *et al.* [81,106] showed that vitamin D toxicity was a potent inducer of CYP24A1 mRNA and enzyme expression in both intestine and kidney. They also showed that if the hypervitaminosis D_3-induced hypercalcemia was prevented by feeding low-calcium diets, the intestinal CYP24A1 expression was enhanced four-fold over hypercalcemic animals receiving the same toxic doses of vitamin D_3 but consuming a normal calcium diet. These observations prompted the examination of the possibility that CT released in response to the hypercalcemia may have suppressed the induced expression of the intestinal CYP24A1. In their series of experiments, Beckman *et al.* [106] clearly demonstrated that CT was a potent suppressor of intestinal CYP24A1 activity. Conceivably, the CT-mediated suppression of CYP24A1 activity could enhance $1,25(OH)_2D$-mediated activities by prolonging its half-life. This could exacerbate conditions that manifest hypercalcemia, such as hypervitaminosis D, by preventing 24-hydroxylation and catabolism of active vitamin D metabolites.

The CYP24A1 has been purified [107,108] and cloned [107,109] and the clone has been expressed [107,109]. Analysis of the amino acid sequence of the rat and human CYP24A1s showed that the sequences were 90% similar. The 21-amino acid heme binding region was found to be 100% identical [109]. Ohyama *et al.* [110] isolated the gene encoding the rat CYP24A1. This single-copy gene was approximately 15 kb and was composed of 12 exons. Several putative vitamin D response elements have been identified and are currently under study. Details of the purification and cloning of the CYP24A1 have been reviewed previously [35], and additional review of the molecular analysis and regulation of the CYP24A1 can be found in Chapter 6 in this book.

E. Physiological Role of CYP24A1

The major site for 24-hydroxylation appears to be the kidney. This is based on the observation that nephrectomy reduced or eliminated plasma $24,25(OH)_2D_3$ [111]. However, nephrectomy also eliminates the production of $1,25(OH)_2D_3$, the primary stimulator of CYP24A1. Therefore, the possibility remains that $24,25(OH)_2D_3$ may reappear in plasma of nephrectomized subjects treated with therapeutic doses of $1,25(OH)_2D_3$. Some studies suggest that $25OHD_3$ is the primary substrate for CYP24A1 [112]; however, it is now generally accepted that CYP24A1 is distributed throughout the body and that $1,25(OH)_2D_3$, rather than $25OHD_3$, is the preferred substrate for CYP24A1 [96,113,114].

Napoli *et al.* [115] and Napoli and Horst [116] identified the formation of 24-oxo-$1,25(OH)_2D_3$ and 24-oxo-1,23,25-trihydroxyvitamin D_3 [24-oxo-1,23, $25(OH)_3D_3$] from intestinal homogenates incubated with physiological amounts of $1,25(OH)_2D_3$. The formation of 24-oxo-$1,23,25(OH)_3D_3$ from 1,25-$(OH)_2D_3$ was enhanced by treatment of experimental animals with exogenous 1,25-$(OH)_2D_3$. They suggested that 24-hydroxylation, followed by C-23 oxidation, most likely represents a mechanism for terminating the cellular action of $1,25(OH)_2D_3$. In a review, Haussler [117] proposed a model for the cellular action of $1,25(OH)_2D_3$ in which he suggested that receptor-mediated, self-induced catabolism of $1,25(OH)_2D_3$ modulates the action of $1,25(OH)_2D_3$. The work of Lohnes and Jones [118], Reddy and Tserng [119], and Makin *et al.* [120] provided further support for this proposal by showing

the ubiquitous presence of catabolic pathways initiated by CYP24A1 in $1,25(OH)_2D_3$ target tissues and the complete destruction of $1,25(OH)_2D_3$ by these pathways. In fact, CYP24A1 has been shown to do more than just initiate this catabolic cascade. Akiyoshi-Shibata *et al.* [121] expressed the rat CYP24A1 cDNA in *Escherichia coli.* They found that this enzyme not only 24-hydroxylates but catalyzes the dehydrogenation of the 24-OH group and performs 23-hydroxylation resulting in 24-oxo-1,23,25$(OH)_3D_3$ production. Only the cleavage at C-23/C-24 resulting in the 24,25,26,27-tetranor-1OH,23COOHD_3 was not demonstrated. However, it is now recognized from the work of several groups that CYP24A1 is capable of the complete catabolism of 25OHD_3 and $1,25(OH)_2D_3$ via a five-step reaction process that includes 24-hydroxylation, 24-oxidation, 23-hydroxylation, side-chain cleavage, and subsequent production of the final degradative product, calcitroic acid [121–125].

Direct evidence that self-induced metabolism of $1,25(OH)_2D_3$ suppresses the action of $1,25(OH)_2D_3$ on target cells was reported by Pols *et al.* [126,127] and Reinhardt and Horst [128,129]. Both laboratories showed that ketoconazole inhibited $1,25(OH)_2D_3$-induced metabolism. This inhibition resulted in increased specific accumulation of $1,25(OH)_2D_3$ in target cells and a significant increase in the cellular half-life of $1,25(OH)_2D_3$-occupied VDR [129]. A result of blocking the self-induced metabolism of $1,25(OH)_2D_3$ was up-regulation of the VDR. Reinhardt and Horst [128] extended these studies by demonstrating that self-induced metabolism of $1,25(OH)_2D_3$ in target cells limits the response of target cells to a primary $1,25(OH)_2D_3$ stimulus by reducing occupancy of VDR by $1,25(OH)_2D_3$ and by preventing VDR up-regulation. Additionally, their data showed that entry of $1,25(OH)_2D_3$ into the cell is restricted due to extensive metabolism of the $1,25(OH)_2D_3$. In whole-cell VDR assays, hormone was degraded so rapidly that VDR binding was prevented. Reinhardt *et al.* [130] confirmed the inhibitory effects of self-induced induction of CYP24A1 on the cellular action of $1,25(OH)_2D_3$ *in vivo* by demonstrating that ketoconazole potentiates the $1,25(OH)_2D_3$ up-regulation of VDR in rat intestine and bone. Clearly, one of the primary roles of CYP24A1 catabolic pathway is terminating the actions of $1,25(OH)_2D_3$.

Recent work using CYP24A1 knockout mice has provided additional evidence for the role of CYP24A1 in the regulation of $1,25(OH)_2D$ activity via catabolism [19]. These mice were unable to clear $1,25(OH)_2D$, as was evidenced by abnormally high circulating concentrations of this metabolite. These data provided support to the contention that the primary role of CYP24A1 is initiating the catabolic pathway and terminating the actions of $1,25(OH)_2D$.

The role of CYP24A1 as an enzyme responsible for the production of a biologically active compound, i.e., $24,25(OH)_2D_3$, has been controversial. The work of St-Arnaud *et al.* [19] has also addressed part of this issue. Deficient mineralization of intramembranous bone was found in CYP24A1-ablated mice. The genetic cross of CYP24A1-ablated mice with VDR-ablated mice rescued the defective bone phenotype, strongly suggesting that the high concentrations of $1,25(OH)_2D$ acting through VDR and not $24,25(OH)_2D$ was the cause of the bone defect. Constitutive expression of CYP24A1 in transgenic rats surprisingly resulted in low plasma $24,25(OH)_2D$ and 25OHD with no effect on plasma $1,25(OH)_2D$ [131,132]. These rats developed albumineria and hyperlipidemia and suffered from reduced bone mass. The authors demonstrated that excreted albumin appeared to compete for the binding and reabsorption of the DBP-25-OHD_3 complex with megalin, resulting in a loss of 25OHD_3 into the urine and subsequent reduction of plasma $24,25(OH)_2D_3$. Supplementation of these rats with 25OHD_3 prevented the bone loss without changing plasma $1,25(OH)_2D$.

F. Other Vitamin D_3 Derivatives Functionalized at C-24

In a series of experiments conducted by Wichmann *et al.* [133,134], a number of 24-hydroxylated derivatives were isolated from plasma of chicks made toxic with vitamin D_3. These metabolites included 24OHD_3, 23,24,25-trihydroxyvitamin D_3 [23,24,25$(OH)_3D_3$], and 24,25,26-trihydroxyvitamin D_3 [24,25,26$(OH)_3D_3$]. These metabolites have not been described in animals receiving physiological amounts of vitamin D_3, and their biological significance is unknown; however, it is likely that 23,24,25$(OH)_3D_3$ and 24,25,26$(OH)_3D_3$ are metabolites of $24,25(OH)_2D_3$. $24,25(OH)_2D_3$ is also the probable precursor to the formation of the side chain cleavage product, 25,26,27-tri-norvitamin D_3-24-carboxylic [135]. This metabolite has been shown to be a product of *in vitro* kidney perfusion using 25OHD_3 as substrate. The analogous pathway, however, could not be demonstrated using $1,25(OH)_2D_3$ as substrate (S. Reddy, personal communication, 1996). Another metabolite isolated in the experiments of Wichmann *et al.* [133] was 23-dehydro-25OHD_3. The immediate precursor, site(s), and biological activity of this compound are unknown. Plausible sources of the 23-dehydro compound are dehydration of $24,25(OH)_2D_3$

or 23,25-dihydroxyvitamin D_3 [23,25$(OH)_2D_3$]. It is not certain if any of the metabolites are important under physiological conditions.

G. 23-Hydroxylation

The discovery of a C-23 oxidative pathway emerged much later than the other pathways and was ushered in by the identification of 23(*S*),25(*R*)25OHD_3-26,23-lactone [136,137], 23(*S*),25(*R*)1,25$(OH)_2D_3$-26,23-lactone [138], and their respective precursors 23(*S*),25$(OH)_2D_3$ and 1,23(*S*),25$(OH)_3D_3$ [139,140]. To date, there has been no specific 23-hydroxylase identified for the vitamin D system. Rather, like other side-chain modifications, 23-hydroxylation is likely carried out by CYP24A1 [122]. The compound, 25OHD_3-26,23-lactone, can be detected in plasma from normal rats, pigs, and chicks [140,141]. However, in several species, this metabolite is not expressed unless animals are consuming excessive amounts of vitamin D_3 [142]. This metabolite has unique activity in that it is three- to fivefold more competitive than 25OHD_3 for binding to the plasma vitamin D-binding protein (DBP) [136]. It was, therefore, the first modification of 25OHD_3 that led to enhanced binding to the plasma DBP. The metabolite, 1,25$(OH)_2D_3$-26,23-lactone, has also been demonstrated under normal conditions [143], with elevated plasma concentrations occurring during exogenous administration of pharmacological amounts of 1,25$(OH)_2D_3$ [144]. The major locus for formation of C-23 hydroxylated derivatives appears to be the kidney. Horst and Littledike [142] and Napoli *et al.* [140] demonstrated that nephrectomy eliminated or greatly impaired the biosynthesis of 25OHD_3-26,23-lactone when animals were treated with excess vitamin D_3 or 25OHD_3. They showed that this response was due to the inability of the animals to synthesize 23(S),25$(OH)_2D_3$. However, when 23(S),25$(OH)_2D_3$ was given to nephrectomized animals, the synthesis of 25OHD_3-26,23-lactone was restored. These data suggested that C-23-hydroxylation occurred predominantly, but not exclusively, in the kidney, whereas extrarenal tissues are quantitatively important in the pathway leading to 25OHD_3-26,23-lactone synthesis, which includes formation of the lactone intermediates 23,25,26$(OH)_3D_3$ [145] and 25OHD_3-26,23-lactol [146].

Although ambiguities remain regarding the biological effects of C-24 oxidation, 23-hydroxylation appears to clearly be a deactivation event. 23-Hydroxylation is the first side-chain modification of 25OHD_3 noted to substantially reduce its affinity for the plasma DBP [147]. 23-Hydroxylation of 1,25$(OH)_2D_3$ also leads to its increased plasma clearance and reduced VDR binding and biological activity [148].

The role of 23-hydroxylation as a primary oxidation event for the further metabolism of 25OHD_3 and 1,25$(OH)_2D_3$ is relatively minor to its role in the further metabolism of vitamin D_3 metabolites that have been previously oxidized at C-24. In other words, very little production of 23,25$(OH)_2D_3$ or 1,23,25$(OH)_3D_3$ would be expected under normal conditions. Rather, the convergences of the C-24 and C-23 oxidative pathways would lead predominantly to the formation of 24-keto-1,23,25$(OH)_2D_3$ and 24-keto-1,23,25$(OH)_3D_3$ [116], which subsequently cleave to form C-23 acids [119,149]. Therefore, as indicated in Fig. 2, the C-23 oxidative pathway can lead to two different patterns of side-chain modifications for both 25OHD_3 and 1,25$(OH)_2D_3$. One pathway, which is relatively minor under physiological conditions and more predominant during hypervitaminosis D_3, leads through 23-hydroxylation to formation of the lactones, whereas the other more physiologically significant pathway leads through 24-hydroxylation to 23-hydroxylation of 24-keto metabolites.

Other oxidized C-23 metabolites that have been identified include 23-keto derivatives of 25OHD_3 and 1,25$(OH)_2D_3$. 23-Keto-25-hydroxyvitamin D_3 was synthesized *in vitro* from 23(S)25$(OH)_2D_3$ and 23(R),25$(OH)_2D_3$ and has unique properties in that it binds with twofold higher affinity than 25OHD_3 for binding sites on the plasma DBP [150]. This affinity should be compared to that for 23(S),25$(OH)_2D_3$, which binds with 6- to 10-fold less affinity. 23-Keto-25OHD_3 is also about fourfold more competitive than 25OHD_3 for binding to the VDR. 23-Ketonization is, therefore, the first example of a side-chain modification enhancing the affinity of 25OHD_3 for the VDR. This high affinity of 23-keto-25OHD_3 for VDR prompted biosynthesis of 23-keto-1,25$(OH)_2D_3$ to determine if this modification might enhance binding of 1,25$(OH)_2D_3$ to VDR. Horst *et al.* [151] prepared this metabolite by incubating 23-keto-25OHD_3 in kidney homogenates prepared from vitamin D–deficient chicks. The major metabolite was isolated and identified as 23-keto-1,25$(OH)_2D_3$ and was shown to possess about 40% the activity of 1,25$(OH)_2D_3$ for VDR binding. 23-Ketonization of 1,25$(OH)_2D_3$, therefore, reduced the affinity of 1,25$(OH)_2D_3$ to VDR rather than increased binding. 23-Keto metabolites do not appear to be synthesized under physiologic conditions, as Napoli *et al.* [115] could not demonstrate the presence of these metabolites from rat intestinal homogenates incubated with 1,25$(OH)_2D_3$ or from intestinal extracts from rats dosed with 1,25$(OH)_2D_3$.

H. C-26 Hydroxylation

26-Hydroxylation of $25OHD_3$ and $1,25(OH)_2D_3$ produces $25,26(OH)_2D_3$ [152] and $1,25,26(OH)_3D_3$ [153], respectively. The natural product was originally assigned the 25(*R*) configuration. However, Partridge *et al.* [154] gave the assignment as 25(*S*). Ikekawa *et al.* [155] later discovered that the naturally occurring $25,26(OH)_2D_3$ actually existed as a mixture of 25(*S*) and 25(*R*) isomers. Although this assignment seems somewhat trivial, it was important in unraveling a controversy that existed regarding the physiological precursor to the *in vivo* synthesis of $25OHD_3$-26,23-lactone. Hollis *et al.* [156] demonstrated that $25,26(OH)_2D_3$ isolated from *in vivo* sources could act as a precursor to the formation of the $25OHD_3$-26,23-lactone. Subsequent research, however, suggested that synthetic $25(S),26(OH)_2D_3$ (which at the time was thought to be the natural configuration) did not act as precursor to the formation of $25OHD_3$-26,23-lactone [157], but synthetic $25(R),26(OH)_2D_3$ could act as a precursor [140,158]. As naturally occurring $25,26(OH)_2D_3$ is a mixture of the *R* and *S* isomers, this research validated the conclusion of Hollis *et al.* [156] suggesting that formation of $25OHD_3$-26,23-lactone could indeed proceed through $25,26(OH)_2D_3$. This pathway has been shown to be relatively minor [159,160], with the major pathway to $25OHD_3$-26,23-lactone synthesis proceeding through $23(S),25(OH)_2D_3$ [140,145,158].

The major locus for the 26-hydroxylase is unknown. Blood concentrations of $25,26(OH)_2D_3$ are not depressed in nephrectomized humans or pigs [142,161,162]. Therefore, production of these metabolites must take place at extrarenal sources. 26-Hydroxylase activity has, however, been demonstrated in microsomes isolated from rat and pig kidneys [163]. The only extrarenal source was reported in liver mitochondria [164]. The physiological role of the C-26 oxidative pathway remains elusive. However, $25,26(OH)_2D_3$ and $1,25,26(OH)_3D_3$ have been shown to possess biological activity with regard to stimulating bone calcium resorption and intestinal calcium absorption, albeit to a lesser degree than either $25OHD_3$ or $1,25(OH)_2D_3$ [152,165]. Therefore, it seems unlikely that 26-hydroxylation is essential for calcium uptake from the gut or release of calcium from bone.

I. C-3 Epimerization

Reddy *et al.* [159,160] have reported the metabolism of $1,25(OH)_2D_3$ in primary cultures of neonatal human keratinocytes and rat osteosarcoma cells into the novel A-ring modified metabolite, $1,25(OH)_2$-3-epi-D_3. This epimer is formed as a result of the change in the orientation of the C-3 hydroxy group from β to α. Other investigators also confirmed this finding [166,167]. Epimerization of hydroxy groups is a well-known phenomenon in bile acid metabolism [168] and the reaction is conducted by bile acid hydroxysteroid dehydrogenase. Figure 4 outlines the proposed pathways of C-3 epimerization of $1,25(OH)_2D_3$ as described by Reddy *et al.* [159]. Although there are two potential pathways for the production of $1,25(OH)_2$-3-epi-D_3 from $1,25(OH)_2D_3$, the most likely pathway is through keto intermediates. The C-3 epimerization has been shown to play a major role in hormone activation and inactivation in other steroid systems [169]. Indeed, $1,25(OH)_2$-3-epi-D_3 binds to the cellular $1,25(OH)_2D_3$ receptor (VDR) with less affinity [170,171] and has minimal activity at activating intestinal calcium transport and bone calcium resorption than $1,25(OH)_2D_3$ [170]. On the other hand, $1,25(OH)_2$-epi-D_3 is equipotent to $1,25(OH)_2D_3$ at suppressing parathyroid hormone secretion in bovine parathyroid cells [171] and at inhibiting keratinocyte proliferation [170,172]. While $1,25(OH)_2$-3-epi-D_3 does undergo side-chain metabolism [159,171], its conversion to C-23 and C-24 oxidized metabolites occurs at a slower rate than $1,25(OH)_2D_3$ [171]; therefore, enhanced metabolic stability of the $1,25(OH)_2$-3-epi-D_3 has been proposed as a possible explanation for the high *in vitro* activity in spite of its reduced binding affinity for VDR [171]. Thus, the enzyme(s) responsible for C-3 epimerization appears to play an important role not only in the regulation of intracellular concentration of $1,25(OH)_2D_3$ but also in the formation of metabolites with a different biological activity profile in specific target tissues. These differences in intracellular concentration of $1,25(OH)_2D_3$ and its metabolites from one tissue to another may be one possible explanation for the well-known tissue-specific actions of $1,25(OH)_2D_3$.

J. Unique Aspects of Vitamin D_2 Metabolism

The 24 position of vitamin D_2, in contrast to the similar position in vitamin D_3, can be considered to be highly reactive. It is a tertiary carbon as well as an allylic position, and the formation of a reactive intermediate (radical, cation) at this position would be highly stabilized. The proximity of this reactive center to the 25 position would afford the possibility of C-24-hydroxylation of vitamin D_2, but the presence of the C-24 methyl would preclude further oxidation to C-24-keto compounds as is known to occur in vitamin D_3 metabolism. Jones *et al.* [43] were the first to demonstrate C-24 oxidation when they isolated $24OHD_2$

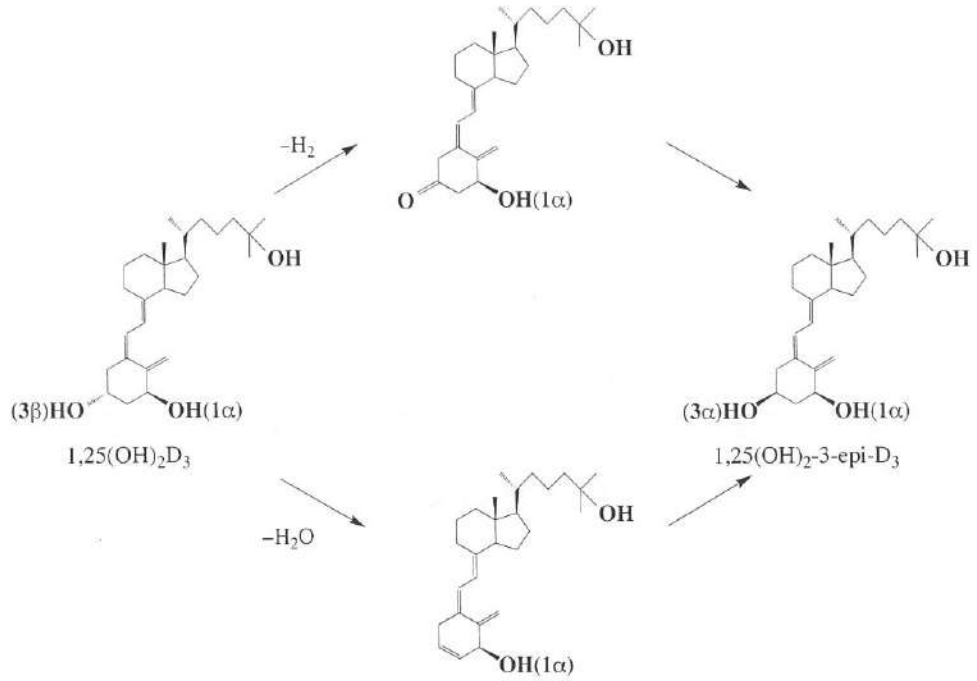

FIGURE 4 Possible pathways of epimerization at C-3 of 1,25(OH)$_2$D$_3$ (adapted from [159]).

from the plasma of male rats treated with 100 IU of radiolabeled vitamin D_2. Engstrom and Koszewski [173] have determined that production of 24OHD$_2$ can occur in liver homogenates from a variety of species, and actually exceeds the formation of 25OHD$_2$. Horst *et al.* [44] have shown that the concentration of 24OHD$_2$ in plasma was about 20% that of 25OHD$_2$ in rats receiving physiological doses of vitamin D_2, and was equivalent to 25OHD$_2$ in rats receiving pharmacological doses of vitamin D_2. They also demonstrated that 1α-hydroxylation of 24OHD$_2$ to form 1,24(OH)$_2$D$_2$ represented a minor but significant pathway for vitamin D_2 activation. In their experiments, they determined that 1,24(OH)$_2$D$_2$ rivaled both 1,25(OH)$_2$D$_2$ and 1,25(OH)$_2$D$_3$ in biopotency.

Both 24OHD$_2$ and 25OHD$_2$ and their 1α-hydroxylated metabolites can undergo subsequent hydroxylation to form 24,25-dihydroxyvitamin D_2 [24,25(OH)$_2$D$_2$] and 1,24,25-trihydroxyvitamin D_2 [1,24,25(OH)$_3$D$_2$]. The formation of 1,24,25(OH)$_3$D$_2$ represented an unequivocal deactivation of the vitamin D_2 molecule [174]. Conversely, the comparable vitamin D_3 metabolite, 1,24,25(OH)$_3$D$_3$, maintains significant biological activity and must undergo further side-chain oxidation to be rendered totally inactive [174]. Although vitamin D_2 is known to undergo side-chain oxidation, only recently has evidence emerged suggesting that vitamin D_2 (like vitamin D_3) undergoes side-chain cleavage. The paucity of information regarding vitamin D_2 side-chain metabolism is primarily due to the lack of appropriate radiolabeled and cold substrates. Zimmerman *et al.* [175] have used [9,11-^{3}H]-1,25-(OH)$_2$D$_2$ and cold 1,25-(OH)$_2$D$_2$ as substrates to determine if 1,25-(OH)$_2$D$_2$ undergoes side-chain cleavage. Similar experiments were done using [3α-^{3}H]-25OHD$_2$ as substrate [176]. In these metabolism experiments it became clear that aqueous-soluble metabolites were being produced from 1,25(OH)$_2$D$_2$ [175] and 25OHD$_2$ [176]. Furthermore, Zimmerman *et al.* [175] demonstrated that calcitroic acid was the major aqueous-soluble metabolite being produced from cell culture and kidney perfusion experiments. In a more recent study Horst *et al.* [177] studied the metabolism of 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_2$ using a purified rat CYP24A1 system. As expected, 1,23(OH)$_2$-24,25,26,27-tetranor D and calcitroic acid were the major lipid- and aqueous-soluble metabolites, respectively, when 1,25(OH)$_2$D$_3$ was used as substrate. However, when 1,25-(OH)$_2$D$_2$ was used as substrate, 1,24,25(OH)$_3$D$_2$ was the major lipid-soluble metabolite with no evidence for the production of either 1,23(OH)$_2$-24,25,26,27-tetranor D or calcitroic acid. Apparently, the CYP24A1 was able to 24-hydroxylate 1,25(OH)$_2$D$_2$, but was unable to cause further changes that would result in side-chain cleavage. When the analog 1,25-(OH)$_2$-22-ene-D$_3$ was used as substrate, CYP24A1 was again able to effect 24-hydroxylation

but not side-chain cleavage (R. Horst, S. Reddy, and J. Omdahl, 2003, unpublished data). Sunita Rao *et al.* [178] demonstrated that $1,25(OH)_2$-22-ene-D_3 could be metabolized to calcitroic acid by RWLue-4 cells and rat kidney. They suggested that the $1,25(OH)_2$-22-ene-D_3 was first hydroxylated at C-24, followed by further oxidation to $1,25(OH)_2$-24-oxo-22-ene-D_3 prior to side-chain, double-bond reduction to form $1,25(OH)_2$-24-oxo-D_3. The $1,25(OH)_2$-24-oxo-D_3 was then further metabolized to calcitroic acid, presumably by CYP24A1. The compound $1,25(OH)_2D_4$ [a.k.a. 22,23 dihydro-$1,25-(OH)_2D_2$] has also been shown to undergo side-chain oxidation similar to that of $1,25(OH)_2D_2$ *in vitro* [179] and metabolized to calcitroic acid *in vivo* [180]. These results suggest that metabolism of $1,25(OH)_2D_2$ to calcitroic acid clearly involves enzymes other than CYP24A1.

The compounds 25,26-dihydroxyvitamin D_2 [$25,26(OH)_2D_2$] and 1,25,26-trihydroxyvitamin D_2 [$1,25,26(OH)_3D_2$] have been chemically synthesized [181,182]. The metabolite $25,26(OH)_2D_2$ has also been tentatively, but not exhaustively, identified from *in vivo* sources [141,183], but it could not be demonstrated in kidneys perfused with $25OHD_2$ [184]. Similarly, $1,25,26(OH)_3D_2$ could not be demonstrated [185]. However, it is clear that 26-hydroxylation does occur when vitamin D_2 metabolites have been previously 24-hydroxylated. For example, when $24,25(OH)_2D_2$ and $1,24,25(OH)_3D_2$ were used as precursors, the metabolites $24,25,26(OH)_3D_2$ and $1,24,25,26(OH)_4D_2$, respectively, were produced in significant amounts [184,185]. Similarly, Koszewski *et al.* [45] and Jones *et al.* [186] demonstrated that C-26 hydroxylation was the major metabolic pathway for the further metabolism of $24OHD_2$ and $1,24(OH)_2D_2$. Oxidation at C-24, therefore, appears to be a prerequisite for C-26 oxidation of vitamin D_2 compounds. A similar situation also appears to exist for C-28 oxidation as demonstrated by Reddy and Tserng [184,185]. In their experiments, they isolated $24,25,28(OH)_3D_2$ and $1,24,25,28(OH)_4D_2$ from rat kidney perfusions and, through the use of various substrates, were able to show C-28 hydroxylation of vitamin D_2 metabolites occurs only after C-24 hydroxylation [187].

III. VITAMIN D TOXICITY

A. Overview

The first documented reports of vitamin D intoxication were made in the late 1920s by Kreitmeir and Moll [188] and Putscher [189]. These cases resulted from the ingestion of large quantities of vitamin D in the diet. Vitamin D intoxication, however, has never been reported following prolonged sunlight exposure. Holick *et al.* [190] suggested that nature has provided various control points that prevent the overproduction of vitamin D_3 by the skin. The most important point of control is the diversion of vitamin D_3 production from 7-dehydrocholesterol to non–biologically active over-irradiation products such as lumisterol and tachysterol. In addition, these authors suggested that skin pigmentation and latitude were also significant determinants (albeit to a lesser degree) that limit the cutaneous production of vitamin D_3.

As discussed earlier, once vitamin D is in circulation, the conversion to 25OHD is relatively uncontrolled. Normally, 25OHD circulates at 30 to 50 ng/ml in most species [141]. However, when vitamin D is given in excess, plasma 25OHD can be elevated to concentrations of 1000 ng/ml or greater [191,192], while plasma $1,25(OH)_2D$ remains at or below normal concentrations [193]. When circulating at very high concentrations, 25OHD can compete effectively with $1,25(OH)_2D$ for binding to the VDR. Therefore, during vitamin D toxicosis, 25OHD can induce actions usually attributed to $1,25(OH)_2D$ [193]. High circulating 25OHD can, therefore, explain how humans with low circulating concentrations of $1,25(OH)_2D$ can show signs of vitamin D toxicity [193], and why anephric humans [who are incapable of producing $1,25(OH)_2D$] can become vitamin D toxic [194]. Clinical aspects of vitamin D toxicity are discussed in Chapter 78.

Although it is generally accepted that $1,25(OH)_2D$ is reduced during hypervitaminosis, a notable exception to this generalization is the ruminant [195,196]. Horst and co-workers [195,197] have shown that vitamin D_3 intoxication initiated by giving 15 million IU of vitamin D_3 intramuscularly (i.m.) results in significant elevations in plasma $1,25(OH)_2D_3$. In contrast, pigs given the same i.m. dose showed a reduction in plasma $1,25(OH)_2D_3$, as was observed in other species [197]. Therefore, elevations in plasma $1,25(OH)_2D$ may play a significant role in the pathogenesis of vitamin D toxicity in ruminants.

B. Differences in Toxicity between Vitamins D_2 and D_3

Most research dealing with utilization of vitamins D_2 and D_3 assumes that these two forms are equally potent in most mammals. However, when large and potentially toxic doses were administered orally to rhesus monkeys [198] and horses [199], or were used in treating childhood osteodystrophy [200], vitamin D_2

presented fewer hypercalcemic side effects than vitamin D_3. In addition, $1OHD_2$, which is as effective as the $1OHD_3$ in standard bioassays, was shown to be five- to 15-fold less toxic than $1OHD_3$ [201].

Studies by Horst *et al.* [44] provide some insight into the difference between vitamin D_2 and vitamin D_3 toxicity. They demonstrated that under physiological conditions the predominant monohydroxylated form of both vitamins D_2 and D_3 is 25OHD. In the vitamin D_2–dosed rats, $24OHD_2$ accounted for approximately 20% of the monohydroxylated metabolites, whereas $24OHD_3$ could not be detected in the vitamin D_3–dosed rats. When a modest superphysiological dose (800 IU/day) of vitamin D_3 was given to rats, $25OHD_3$ remained the predominant metabolite in vitamin D_3–dosed rats and was present at 26.3 ng/ml. Under these conditions, there was still no evidence for the presence of $24OHD_3$. However, when the same amount of vitamin D_2 was given, the concentrations of $24OHD_2$ (14.1 ng/ml) nearly matched those of $25OHD_2$ (15.9 ng/ml). Interestingly, the combined concentrations of $24OHD_2$ and $25OHD_2$ in the vitamin D_2–dosed animals (~30 ng/ml) was similar to the $25OHD_3$ concentration (26.3 ng/ml) in the vitamin D_3–dosed rat. In standard assays, $25OHD_2$ and $25OHD_3$ are equipotent at displacing ^3H-$1,25(OH)_2D_3$ from the calf thymus VDR. However, $24OHD_2$ has been shown to have at least a twofold lower affinity for binding to the calf thymus VDR (R. L. Horst, unpublished data, 1996). Therefore, the reduced toxicity of vitamin D_2 is probably a result of diverting metabolism away from the production of $25OHD_2$ in favor of $24OHD_2$, which has a relatively limited affinity for VDR (a step necessary for the initiation of a biological response). Further differences between vitamins D_2 and D_3 were noted in their ability to up-regulate the VDR. Beckman *et al.* [202] found that VDR was significantly more enhanced in animals fed excess vitamin D_3 relative to those animals receiving an equivalent amount of vitamin D_2. Increased VDR would potentially accentuate toxic side effects by enhancing the responsiveness of intestinal tissue to the elevated 25OHD.

C. Factors Affecting Toxicity

The severity of the effects and pathogenic lesions in vitamin D intoxication depend on such factors as the type of vitamin D (vitamin D_2 versus vitamin D_3), the dose, the functional state of the kidneys, and the composition of the diet. Vitamin D toxicity is enhanced by a rich dietary supply of calcium and phosphorus, and it is reduced when the diet is low in calcium and phosphorus [203,204]. Toxicity is also reduced when the vitamin is accompanied by high intakes of vitamin A or by thyroxine injections [205]. The route of administration also influences toxicity. Parenteral administration of 15 million IU of vitamin D_3 in a single dose caused toxicity and death in many pregnant dairy cows [195]. On the other hand, oral administration of 20 to 30 million IU of vitamin D_2 daily for 7 days resulted in little or no toxicity in pregnant dairy cows [206]. Napoli *et al.* [207] have shown that rumen microbes are capable of metabolizing vitamin D to the inactive 10-keto-19-nor-vitamin D_3. Parenteral administration would circumvent the deactivation of vitamin D by rumen microbes and may partially explain the difference in toxicity between oral and parenteral vitamin D.

Various measures have been used in human medicine for treatment of vitamin D toxicity. These measures are mainly concerned with management of hypercalcemia. Vitamin D withdrawal is obviously indicated. It is usually not immediately successful, however, owing to the long plasma half-life of vitamin D (5 to 7 days) and 25OHD (20 to 30 days). This is in contrast to the short plasma half-life of $1OHD_3$ (1 to 2 days) and $1,25(OH)_2D_3$ (4 to 8 hr). Because intestinal absorption of calcium contributes to hypercalcemia, a prompt reduction in dietary calcium is indicated. Sodium phytase, an agent that reduces intestinal calcium absorption, has also been used successfully in vitamin D toxicity management in monogastrics [208]. This treatment would be of little benefit to ruminants because of the presence of rumen microbial phytases. There have also been reports that CT [209], glucagon [210], etidronate [211], and glucocorticoid therapy [212] reduce serum calcium levels or prevent the calcinosis resulting from vitamin D intoxication (see Chapter 78).

IV. SPECIES VARIATION IN VITAMIN D METABOLISM AND ACTION

Most concepts of vitamin D metabolism and function have been developed with the rat and/or chick as experimental models. Studying vitamin D metabolism is hampered by the paucity of data on the normal circulating levels of vitamin D metabolites in birds, mammals, and reptiles under normal conditions. Most recent research has focused on the analysis of 25OHD and $1,25(OH)_2D$ as indicators of vitamin D status or aberrant physiological states. Table I summarizes the concentrations of the two metabolites that have been reported for several species by various laboratories. Close inspection of the information suggests that some mammals (mole rat, wild wood vole, horse, and wild wood mouse) and aquatic species (lamprey, carp,

TABLE I Plasma 25-Hydroxyvitamin D and 1,25-Dihydroxyvitamin D Concentrations in Several Species of Animals

Species	Concentration		Ref.
	25OHD (ng/ml)	$1,25(OH)_2D$ (pg/ml)	
Human	32	31	[162]
Rhesus monkey	188	207	[231]
Rhesus monkey	50	95	[232]
Marmoset	90	400	[232]
Marmoset	64	640	[233]
Wild woodmouse	<5	<10	[234]
Wild bank vole	<5	<5	[234]
Mole rat	<2	17	[213]
Lamprey	ND[a]	274	[235]
Shark	ND	87	[235]
Leopard shark	56	3	[236]
Horned shark	33	6	[236]
Carp	ND	174	[235]
Bastard halibut	ND	192	[235]
Atlantic cod	<2	59	[237]
Bullfrog (mature)	2	21	[235]
Soft-shelled turtle	16	12	[235]
Turkey	26	52	[141]
Chicken	27	21	[141]
Cow	43	38	[141]
Sheep	27	36	[141]
Pig	76	60	[141]
Horse	6	19	[238]

[a]ND, Not done.

halibut, and bullfrog) appear to have very low or undetectable concentrations of 25OHD, and yet these animals appeared to be normal with no evidence of vitamin D deficiency. It is questionable whether some of these species have a requirement for vitamin D. The damara mole rat, for example, is a subterranean herbivore that in its natural habitat has no access to any obvious source of vitamin D and consumes a diet of roots and tubers [213]. These animals exhibit a high apparent calcium absorption efficiency (91%) and, like the horse and rabbit, actually use renal calcium excretion as the major regulator of calcium homeostasis [214,215]. In studies with rabbits consuming adequate amounts of calcium, it is very difficult to develop any overt or histological signs of vitamin D deficiency, and vitamin D may play a minor, if any, role in normal day-to-day functions in these animals [216].

In the wild, most animals do not have a dietary need for vitamin D, as sufficient vitamin D_3 can be synthesized in the skin on irradiation by sunlight. However, indoor confinement of humans and other animals has resulted in the diet becoming the main source of vitamin D, leading to considerable research to determine the amount of dietary vitamin D required to substitute for lack of exposure to sunlight. Photochemically produced vitamin D_3 enters the circulation and becomes immediately available, whereas dietary vitamin D_3 may undergo modifications prior to becoming available for use by the body. One species where significant modification of vitamin D occurs before absorption is the ruminant. Within 24 hr, as much as 80% of vitamin D can undergo metabolism *in vitro* in rumen incubation media [217]. At least four metabolites are produced by the rumen microbes [217,218]. Two of these metabolites have been identified as the cis (5*Z*) and trans (5*E*) isomers of 10-keto-19-norvitamin D_3 (Fig. 2) [207]. The trans isomer has also been identified in cow plasma (R. L. Horst, unpublished data, 1983). Neither compound has agonistic activity with regard to promoting bone calcium resorption [219] or intestinal calcium absorption [207]. Rather, this novel metabolism is likely a detoxification process, as evidenced by the ability of ruminants to tolerate large oral doses of vitamin D_3 that would be toxic if given parenterally. The presence of the rumen, therefore, represents a major control point in vitamin D metabolism that may differ from monogastrics. Such a control point may have survival value, because the ruminant evolved as a grazing animal with the opportunity for long periods of sunlight exposure, as well as consumption of large quantities of irradiated plants. If left uncontrolled, such a combination might result in vitamin D toxicity.

Shortly after the discovery of vitamin D, it seemed apparent that vitamins D_2 and D_3 had similar biological activities in most mammals and that birds and New World monkeys discriminated against vitamin D_2 in favor of vitamin D_3 [220,221]. More recent research, fostered by the discovery of sensitive analytical techniques and the availability of high specific activity ^3H-labeled vitamin D species, indicated that differences in the metabolism of vitamins D_2 and D_3 in mammals are perhaps widespread. Most notable were the apparent discrimination against vitamin D_2 by pigs [222], cows [218], and humans [223] and the apparent preference for vitamin D_2 by rats [222,224].

Vitamin D and its metabolites are transported in the blood of vertebrates attached to a specific protein commonly known as the vitamin D binding protein or DBP [225]. Baird *et al.* [226] have shown that protein binding increases the solubility of steroids and that the

metabolic clearance rate of steroids is in part dependent on their binding to specific plasma proteins. Affinity of metabolites to the plasma transport proteins may, therefore, provide a means for determining which species would utilize vitamin D_2 poorly. For example, if the binding protein showed lower affinity toward $25OHD_2$ relative to $25OHD_3$, then one would predict that $25OHD_2$ would be removed from the circulation faster than $25OHD_3$. This is indeed the case for the chick. Hoy *et al.* [227] showed that chick discrimination against vitamin D_2 was probably a result of enhanced clearance of the vitamin D_2 metabolites $25OHD_2$ and $1,25(OH)_2D_2$, and that the enhanced clearance was associated with weaker binding of these vitamin D_2 metabolites (relative to the vitamin D_3 forms) to DBP.

In one of the most comprehensive studies reported to date, Hay and Watson [228] studied the affinities of DBP for $25OHD_2$ and $25OHD_3$ in 63 vertebrate species. They found that the DBP in fish, reptiles, and birds discriminated against $25OHD_2$ in favor of $25OHD_3$, which is consistent (at least in birds) with the discrimination against vitamin D_2. One notable exception to this hypothesis, however, is the New World monkey. Hay and Watson [228] found that in New World monkeys, the plasma transport protein has equal affinity for $25OHD_2$ and $25OHD_3$, which is inconsistent with the well-documented discrimination against vitamin D_2. Factors other than affinity of the binding protein for 25OHD are, therefore, important in determining how efficiently the different forms of vitamin D can be utilized by animals.

Another example of species discrimination against the different vitamin D forms is in the rat. However, in this species, discrimination is against vitamin D_3 in favor of vitamin D_2 [222]. The rat DBP is known to have equal affinity for $25OHD_2$ and $25OHD_3$, but a lower affinity for vitamin D_2 relative to vitamin D_3 [229]. Reddy *et al.* [230] suggested that the lower affinity for vitamin D_2 resulted in its enhanced availability for liver 25-hydroxylation. Hence, in the presence of DBP, more $25OHD_2$ was made relative to $25OHD_3$ when equal amounts of vitamin D_2 or vitamin D_3 substrate were perfused into rat livers. This observation is consistent with the higher circulating concentrations of $25OHD_2$ observed in acute experiments with vitamin D–deficient rats dosed with equal amounts of vitamins D_2 and D_3 [222]. In the experiments conducted by Reddy *et al.* [230], if binding protein was eliminated from the perfusion media, equal amounts of $25OHD_2$ and $25OHD_3$ were synthesized. Collectively, these data suggest that discrimination against the different forms of vitamin D could likely result from variations in the affinity of DBP for the parent compound and/or one or more of their metabolites. Regardless of the mechanism for discrimination, it appears that these differences are present to afford animals the most efficient utilization of the most abundant antirachitic agents available in their environment.

V. CONCLUSION

Vitamin D metabolism still remains an exciting area of research with much more to be learned. Critical questions remain unanswered regarding complete elucidation of the vitamin D_2 metabolic pathway, and species differences between vitamins D_2 and D_3 metabolism are still virtually unexplored. The introduction of vitamin D analogs has also resulted in a totally different set of issues regarding their metabolism, tissue kinetics, mechanism of action, and potential therapeutic uses.

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