Recent progress in enzymology and molecular biology of enzymes involved in vitamin D metabolism

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The early studies of Huldschinsky (1), Mellanby (2), McCollum et al. (3), Steenbock and Black (4), Askew et al. (5), and others indicated that an antirachitic agent capable of curing rickets was present in cod-liver oil and in irradiated foods. The culmination of these investigations was the isolation and identification of vitamin D3 as the antirachitic factor followed by elucidation of its biosynthesis from cholesterol (6, 7).

In the ensuing three decades, relatively little additional work on vitamin D metabolism was done. Nonetheless, during this time there were great advances in methodology applicable to the study of vitamin D metabolism, which set the stage for a new era of vitamin D research. The advances included reverse-phase chromatography for vitamin D, development of a method for the biosynthesis of 14C-labeled vitamin D, and competitive binding assays for quantification of vitamin D (7, 8).

In 1968, Blunt, DeLuca, and Schnoes (9) reported the isolation of 25-hydroxyvitamin D3 (25-OH-D3). They also reported that this metabolite of vitamin D3 had a slightly greater biological activity than its precursor, vitamin D3. Until this time, all known metabolites of vitamin D3 isolated had been reported to be inactive (10). Lawson, Wilson, and Kodicek (11) studied the metabolism of 25-OH-D3 using a doubly labeled compound with a tritium label at carbon-1 and 14C at carbon-4. They observed that the tritium label, but not the carbon label, was lost in one of the metabolites of the compound found in intestinal nuclear fractions of chicks, a subcellular fraction known to retain the highest radioactivity.

Abbreviations, trivial names, and systemic names (in brackets): vitamin D3 [calcidol]; 25-OH-D3, 25-hydroxyvitamin D3 [calcidiol]; 1α-OH-D3, 1α-hydroxyvitamin D3 [calcitriol]; 1,25(OH)2D3, 1α,25-dihydroxyvitamin D3 [calciotriol]; 24-OH-D3, 24-hydroxyvitamin D3 [24-oxo-vitamin D3]; 24,25(OH)2D3, 24,25-dihydroxyvitamin D3 [24,25-oxo-vitamin D3]; 1,23,25(OH)3D3, 1α,23,25-trihydroxyvitamin D3 [.swing]

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of the labeled vitamin D3 administered. Kodicek, Lawson, and Wilson (12) then examined the effect of this metabolite on 45Ca transfer across the intestine. They showed that this compound was not only 2.9-times more active than vitamin D3 but was also 2.5-times more active than 25-OH-D3. The polarity of the compound and the loss of hydrogen from carbon-1 suggested that an oxygen-containing function had been introduced at the 1-position. The eventual identification of the metabolite as 1,25-dihydroxyvitamin D3 \([1,25(OH)_{2}D_3]\) was independently accomplished by Lawson, Fraser, and Kodicek (13), Holick, Schnoes, and DeLuca (14), and Norman et al. (15). The \(\alpha\) configuration of the 1-hydroxyl group was established by Semmler et al. (16), giving the definitive structure of 1\(\alpha\),25-dihydroxyvitamin D3. As no metabolite of vitamin D3 that is more active than 1,25(OH)\(_2\)D3 has so far been isolated, it is considered to be an ultimate form of vitamin D3. Meanwhile, another metabolite of vitamin D3, 24,25(OH)\(_2\)D3, was isolated by DeLuca’s group (17, 18). Subsequent study showed that this vitamin was formed in animals in a calcium-replete status, while 1,25(OH)\(_2\)D3 was formed in animals in a calcium-depleted status (19). Therefore, both compounds were considered to be involved in maintenance of calcium homeostasis.

The metabolic pathway of vitamin D3 is shown in Fig. 1. Enzymes responsible for hydroxylation in this pathway are members of the superfamily of P450 (20, 21). In this article, recent progress in enzymology and molecular biology of these hydroxylases is reviewed.

![Metabolic Pathway of Vitamin D](image)

**Fig. 1.** Metabolic pathway of vitamin D.

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I. VITAMIN D\(_3\) 25-HYDROXYLASE (25-HYDROXYLASE)

A. Subcellular localization of 25-hydroxylase

Horsting and DeLuca (22) showed that vitamin D3 is hydroxylated at C:25 by rat liver homogenate. Later, DeLuca (23) reported that this 25-hydroxylation was catalyzed by liver mitochondria. Two years later, however, Bhattacharya and DeLuca (24, 25) reported that vitamin D3 25-hydroxylase was present in microsomes and that its activity was dependent on the concentration of ionized calcium in serum. Björkhem and Holmberg (26, 27) reinvestigated the subcellular location of the enzyme using mass fragmentography for identification of metabolites. They found that NADPH-dependent 25-hydroxylation activity was present in both mitochondria and microsomes. In mitochondria NADPH could be replaced by isocitrate. Isocitrate is known to be an electron donor in mitochondria, as it can provide NADPH through oxidation by isocitrate dehydrogenase (21, 28). Madhok and DeLuca (29) reinvestigated the subcellular location of vitamin D3 25-hydroxylase. In accordance with their previous work (24, 25), they reported that the enzyme activity was present in both microsomes and mitochondria, when fortified with NADPH and cytosol. However, they attributed the mitochondrial activity to microsomal contamination, as they could not observe succinate-supported mitochondrial activity.
B. 25-Hydroxylase in liver microsomes

Confirmation that microsomal vitamin D3 25-hydroxylase was indeed a P450 enzyme was obtained by Andersson, Holmberg, and Wikvall (30). They isolated an electrophoretically homogeneous protein possessing vitamin D3 25-hydroxylation activity from rat liver microsomes and showed that the enzyme had spectral characteristics of a P450 enzyme. However, as they did not describe the amino acid sequence of the enzyme, it could not be ascertained whether the enzyme was new or was identical to other P450 enzymes already reported. Hayashi, Noshiro, and Okuda (31, 32) independently purified the microsomal P450 from rat liver that catalyzes vitamin D3 25-hydroxylation. They found that the enzyme had the same NH2-terminal amino acid sequence as CYP2C11 (33). This P450 is a constitutive P450 known to exist solely in male liver microsomes but not in the female. In fact, female liver microsomes showed a very low activity toward vitamin D3 25-hydroxylation catalyzed by a P450 different from CYP2C11 (about 1/5th that of male liver microsomes, 34).

C. Purification of 25-hydroxylase from liver mitochondria

Previously, Taniguchi, Hoshita, and Okuda (28) and Okuda (21) had established that liver mitochondria contain a P450 catalyzing 27-hydroxylation of 5β-cholestanetriol-3α,7α,12α-triol (27-hydroxylase). This finding was in conflict with previous claims as Sottocassa et al. (35) and Brunner and Bygrave (36) had reported that P450 was not observable spectrophotometrically in liver mitochondria. Björkhem and Holmberg (27) thought it quite possible that vitamin D3 25-hydroxylase was a different entity than cholestanetriol 27-hydroxylase based on some criteria (21). In order to solve these problems unequivocally, Masumoto, Ohyama, and Okuda (37) began to purify the mitochondrial vitamin D3 25-hydroxylase based on enzyme activity. The enzyme was extremely labile compared to other P450s and these workers found it useful to use a rapid HPLC technique that enabled them to complete the anion exchange column chromatography within 1 h. They isolated a homogeneous preparation with a turnover number of 0.36 min\(^{-1}\) toward vitamin D3 (and 1.6 min\(^{-1}\) toward 1α-OH-D\(_3\)) which were the highest values reported to date.

D. Identity of 25-hydroxylase with cholestanetriol 27-hydroxylase

In parallel with the work of Masumoto et al. (37), Okuda, Masumoto, and Ohyama (38) purified a mitochondrial hydroxylase that catalyzed 27-hydroxylation of cholestanetriol. The turnover number of their preparation for vitamin D3 25-hydroxylation was found to be the same as that of the enzyme purified by Masumoto et al. (37).

Ohyama et al. (39) performed additional experiments to test whether these two hydroxylation activities were due to a single enzyme. They concluded that both enzyme activities were due to a common active site of a single protein based on the following facts. 1) The two activities copurified using various column chromatographies. 2) 25-Hydroxylase was competitively inhibited by cholestanetriol. 3) Conversely, 27-hydroxylase was inhibited by 25-OH-D\(_3\) in the same manner. 4) Both enzyme activities were inactivated by controlled heat-denaturation, or chemical modification of enzyme protein. Independently, Dahlbäck and Wikvall (40) also reported that vitamin D3 25-hydroxylation activity was present in their preparation of cholestanetriol 27-hydroxylase. However, they raised the possibility that their results were due to contamination. Indeed, Dahlbäck (41) prepared a monoclonal antibody to cholestanetriol 27-hydroxylase, which inhibited the 27-hydroxylation of 5β-cholestanetriol-3α,7α,12α-triol but not the 25-hydroxylation of vitamin D3, and she postulated that the two enzymes were, in fact, different entities.

E. Cloning of 25-hydroxylase cDNA in liver mitochondria

Usui, Noshiro, and Okuda (42) prepared antibodies against rat liver mitochondrial vitamin D3 25-hydroxylase that had been isolated according to the method of Masumoto et al. (37). Using this antibody as a probe, they isolated a cDNA clone from a rat liver cDNA library. The isolated cDNA showed no similarity to any P450s known except to the cholestanetriol 27-hydroxylase isolated by Andersson et al. (43) from a rabbit liver cDNA library. This plasmid contained a mitochondria-specific presequence consisting of 50 amino acid residues. The mature enzyme consisted of 501 amino acid residues corresponding to a predicted molecular weight of 57,182.

F. Expression of a cDNA encoding rat liver mitochondrial vitamin D3 25-hydroxylase

To confirm whether a single enzyme is responsible for the two catalytic activities, vitamin D3 25-hydroxylation and cholestanetriol 27-hydroxylation, Usui et al. (44) prepared an expression plasmid encoding the rat liver mitochondrial vitamin D3 25-hydroxylase. This plasmid was transfected into COS cells and the mitochondrial fraction of the transfected cells was assayed for both hydroxylation activities. Usui et al. (44) observed both 25-hydroxylation of vitamin D3 and 27-hydroxylation of cholestanetriol in this fraction from transfected cells but not in that from control cells. The ratio of both activities
was roughly equal to that observed with the purified enzyme from rat liver mitochondria.

Akiyoshi-Shibata et al. (45) expressed the cDNA encoding 25-hydroxylase under the control of the yeast alcohol dehydrogenase I promoter and terminator in *Saccharomyces cerevisiae* cells. Mitochondria prepared from the transfected cells exhibited both 25-hydroxylase and 27-hydroxylase activities in a reconstituted system containing bovine adrenodoxin and NADPH-adrenodoxin reductase. They thus ruled out the formal possibility that the mammalian cell line might contain an endogenous cholestanetriol 27-hydroxylation activity (although only very low levels of this activity are found in most cultured cells). Su et al. (46) independently isolated a cDNA encoding a rat ovary mitochondrial P450 catalyzing both cholesterol 27-hydroxylation and vitamin D₃ 25-hydroxylation.

Recently, Guo et al. (47) isolated a cDNA for the human liver mitochondrial vitamin D₃ 25-hydroxylase using PCR products of a fraction of rat liver 25-hydroxylase cDNA as a hybridization probe. The structure of the isolated human cDNA was virtually identical to that of cDNA of human cholestanetriol 27-hydroxylase cDNA (48). When the cDNA was transfected into COS cells with an adrenodoxin cDNA, the transfected cells revealed a 10- to 20-fold higher 25-hydroxylation activity than nontransfected cells toward vitamin D₃ and 1α-OH-D₃. The transfected cells also showed a much higher 27-hydroxylation activity than 25-hydroxylation activity as in the case of rat liver mitochondrial enzyme. Vitamin D₂, which carries an ergosterol-like side chain, and dihydrotachysterol, a stereo-isomer of vitamin D₃, were hydroxylated at C-24 and 25, respectively, suggesting that the regioselectivity of the enzyme differs according to the structure of substrates (Fig. 2). Taken together with the finding of Cali and Russell (48), who observed that this cDNA expressed cholestanetriol 27-hydroxylation activity in COS cells, the evidence appears convincing that 25-hydroxylase and 27-hydroxylase are expressed by the same gene (CYP27) in humans as well.

However, these experiments do not exclude the possibility that the two enzymes differ by a posttranscriptional modification as suggested by Wikvall (49). Very recently, Axén et al. (50) reported that CYP27 of pig and human catalyzes not only 27-hydroxylation of cholestanetriol and 25-hydroxylation of vitamin D₃, but also 1α-hydroxylation of 25-hydroxvitamin D₃ (Fig. 2). As 1α-hydroxylase activity toward 25-OH-D₃ is much lower compared to the other two activities and liver does not seem to be involved in 1α-hydroxylation of 25-OH-D₃ at least in mammals, the physiological meaning of this activity remains to be established.²

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²Kobayashi's group observed that the major activity of 1α-hydroxylase exists in liver in fish (80) and in fetal rat (87). Whether 1α-hydroxylase activity and that of 25-hydroxylation are due to a common enzyme in these animal organs remains unsolved.

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G. Abnormal vitamin D₃ metabolism in cerebrotendinous xanthomatosis (CTX)

Cerebrotendinous xanthomatosis is caused by an anomaly of the gene structure of 27-hydroxylase (21, 51, 52). Berginer et al. (53) found that extensive osteoporosis and increased risk of bone fractures occurred in some CTX patients. The serum level of some vitamin D₃ metabolites such as 25-OH-D₃ and 24,25(OH)₂D₃ was lower than normal. In particular, the serum level of 24,25(OH)₂D₃ was more than 2-fold lower than the
normal, while that of 1,25(OH)$_2$D$_3$ was within normal limits. Affected subjects showed classic manifestations of CTX, including dementia, pyramidal and cerebellar insufficiency, peripheral neuropathy, cataracts, and tendon xanthoma, in association with elevated serum cholestanol concentrations. They therefore considered that extensive osteoporosis and increased risk of bone fractures were components of the inherited disease. It may be surmised that if 25-hydroxylase and cholestanetriol 27-hydroxylase are the same enzyme, the osteoporosis may be explained by low or null activity of liver mitochondrial vitamin D$_3$ 25-hydroxylation owing to the abnormal enzyme formed from the defective gene.

II. 1α-HYDROXYLATION OF 25-HYDROXYVITAMIN D$_3$ (1α-HYDROXYLASE)

The major location of 1α-hydroxylase is in the inner mitochondrial membrane of proximal convoluted cells of the kidney (54). Feeding a low calcium diet or a vitamin D-deficient diet to rats markedly stimulates 1α-hydroxylase (55).

A monoclonal antibody against this enzyme was prepared by Moorthy, Mandel, and Ghazarian (56) and Burgos-Trinidad et al. (57) from chick kidney. Moorthy et al. (56) immuno-purified both 1α-hydroxylase and 24-hydroxylase, and determined the NH$_2$-terminal amino acid sequence. The first 10 amino acid residues of the two enzymes were 100% homologous and amino acid compositions of the two enzymes were very similar. They thus suggested that 24-hydroxylase may be a proteolytic cleavage product of 1α-hydroxylase. However, Burgos-Trinidad et al. (57) who also immuno-purified the two enzymes noticed that NH$_2$-terminal amino acid sequences of 24-hydroxylase and 1α-hydroxylase were homologous but definitely different in some residues. Furthermore, amino acid compositions of the two enzymes were significantly different in contrast to the finding reported by Moorthy et al. (56). Ettinger, Ismael, and DeLuca (58), however, recently isolated a cDNA clone using the monoclonal antibody of Burgos-Trinidad et al. (57) as a probe and determined nucleotide sequence from which the amino acid sequence of the protein was deciphered. As a result it was found that it did not have homology to P450 enzymes and did not have a heme consensus sequence and was considered to be a vitamin D$_3$ hydroxylase-associated protein, VDHAP. The cloning of a cDNA encoding a true renal 1α-hydroxylase with the expected properties of a P450 enzyme has not yet been reported from any species.
III. 24R-HYDROXYLATION OF 25-HYDROXYVITAMIN D₃ (24-HYDROXYLASE)

A. Identification of 24R,25-dihydroxyvitamin D₃

24R,25(OH)₂D₃ was isolated by Suda et al. (17) from the plasma of pigs given large doses of vitamin D₃ and the structure was later established by Holick et al. (18) and Lam et al. (59). The compound is produced in kidney mitochondria when chicks are fed a high calcium diet, whereas 1,25(OH)₂D₃ was produced when animals were fed a low calcium diet (18). 24,25(OH)₂D₃ therefore seems to be involved in calcium homeostasis. It was also shown that the enzyme is located in intestine (60) and cartilage (61). However, the physiological function of this compound remains unresolved. Some authors consider that it is important for mineralization of bone matrix (62), as massive doses of 24,25(OH)₂D₃ stimulate bone formation without inducing hypercalcemia (63). Others consider that the real substrate of 24-hydroxylase is 1,25(OH)₂D₃ (64) owing to the fact that the enzyme shows a much higher affinity for 1,25(OH)₂D₃ than for 25-OH-D₃. With this hypothesis, 1,24,25(OH)₃D₃ is the end metabolic product formed whenever 1,25(OH)₂D₃, the active form of vitamin D₃, is present in excess in the target tissue. Nonetheless, if the extensive osteoporosis and increased risk of bone fracture of CTX patients is due to the low level of 24,25(OH)₂D₃, the physiological importance of this vitamin should be reconsidered.

B. Purification of 24-hydroxylase

To elucidate the role of 24-hydroxylation in calcium homeostasis at a molecular level, we have purified two enzymes involved in this reaction. Ohyama et al. (65, 66)
attempted to purify the 24-hydroxylase enzyme based on its catalytic activity toward 25-OH-D₃. Although several methods for the synthesis of 25-OH-D₃ had been reported, Ohyama et al. developed an efficient approach to the chemical synthesis of this compound (Fig. 3). In order to facilitate isolation of the enzyme from the kidney, Ohyama et al. (65, 66) induced the enzyme 8-fold by injecting a large amount of vitamin D₃. Lubrol and/or Tween 20 were found to be the most suitable detergents and pentyl-Sepharose 4B was better than other hydrophobic columns tested for enriching 24-hydroxylase in the early stage of purification. After 7 years of effort, Ohyama et al. (65, 66) obtained a homogeneous preparation of 25-OH-D₃ 24-hydroxylase from rat kidney mitochondria. An HPLC technique that permitted rapid anion exchange chromatography of the enzyme was a key component of the isolation procedure as it greatly reduced loss of enzyme activity. The purified 25-OH-D₃ 24-hydroxylase had a molecular weight of 53,000 and showed an absorption spectrum characteristic of P450 with a peak at 453 nm in the reduced CO-difference spectrum. Upon reconstitution with the adrenal mitochondrial electron donors, adrenodoxin and NADPH-adrenodoxin reductase, the purified enzyme catalyzed 24-hydroxylation of 25-OH-D₃ with a turnover number of 22 min⁻¹. Enzyme activity was inhibited by 7,8-benzoﬂavone, ketoconazole, and carbon monoxide, whereas it was only slightly inhibited by aminoglutethimide, metyrapone, and SKF-525A, which are well-known inhibitors of other species of P450. The enzyme not only 24-hydroxylated 25-OH-D₃ but also...
24-hydroxylated 1,25(OH)\(_2\)D\(_3\) with a lower turnover number (6.0 min\(^{-1}\)). However, the purified preparation did not catalyze \(\alpha\)-hydroxylation of 25-OH-D\(_3\). The enzyme followed Michaelis-Menten kinetics with a \(K_m\) of 2.8 \(\mu\)M toward 25-OH-D\(_3\).

The NH\(_2\)-terminal amino acid sequence was Arg-Ala-Pro-Lys-Glu-Val-Pro-Leu, which was different from any P450s reported to date, suggesting that it was a novel member of the P450 superfamily.

A specific monoclonal antibody and polyclonal antibodies were prepared against the purified enzyme. The latter inhibited the enzyme reaction about 70% and reacted with the protein in immunoblotting. The antibody did not react with any other P450 in rat liver microsomes or mitochondria, such as CYP2B1, CYP1A1, CYP7, CYP27.

C. Cloning of 24-hydroxylase cDNA

Using the monoclonal antibody as a probe, Ohyama, Noshiro, and Okuda (67) isolated a cDNA clone encoding 25-OH-D\(_3\) 24-hydroxylase from a rat kidney cDNA library. The isolated cDNA was 3.2 kb in length and contained an open reading frame encoding 514 amino acids. The deduced amino acid sequence contained a presequence in the NH\(_2\)-terminal region typical of mitochondrial enzymes. The amino acid sequence showed less than 30% similarity to those of all other P450s reported to date and is a member of a new P450 family. This enzyme was named CYP24 (trivial name, P450cc24; gene symbol CYP24) in the P450 superfamily (93).

Ohyama et al. (67) prepared an expression vector encoding the rat kidney mitochondrial 25-OH-D\(_3\) 24-hydroxylase and introduced this DNA into COS cells. The transfected cells produced a protein that was reactive to the monoclonal antibody. Solubilized mitochondrial fractions of the transfected cells hydroxylated 25-OH-D\(_3\) at C-24 in the presence of adrenodoxin and NADPH-adrenodoxin reductase, whereas mitochondria from mock-transfected cells had little activity (67).

Chen, Prahl, and DeLuca (68) recently isolated a cDNA of human 25-OH-D\(_3\) 24-hydroxylase from a human HL-60 cell cDNA library using PCR products representing a fragment of rat 24-hydroxylase cDNA as a hybridization probe. The human protein whose sequence was predicted from the cDNA was 90% homologous (82% identical) to that of the rat.

D. Induction of 24-hydroxylase by vitamin D\(_3\) metabolite

Ohyama et al. (67) injected 50,000 IU of vitamin D\(_3\) into rats and carried out Northern blot analysis of the kidney mRNA after 5 days using 24-hydroxylase cDNA as a probe. Messenger RNA for 24-hydroxylase markedly increased in the vitamin D\(_3\)-treated rats. Armbruch and Boltz (69) have shown that the induction occurred after a single administration of 1,25(OH)\(_2\)D\(_3\). They also found that 24-hydroxylase mRNA increased in the intestine of rats injected with 1,25(OH)\(_2\)D\(_3\).

The induction observed by Ohyama et al. (67) might be due to the 1,25(OH)\(_2\)D\(_3\) formed in vivo from the exogenous vitamin D\(_3\). Shinki et al. (64) demonstrated that induction of 24-hydroxylase mRNA by 1,25(OH)\(_2\)D\(_3\) was suppressed by the parathyroid hormone in the kidney but not in the intestine.

The induction of 24-hydroxylase by 1,25(OH)\(_2\)D\(_3\) was also observed in cultured human keratinocytes (70), the proliferation of which is known to be inhibited by 1,25(OH)\(_2\)D\(_3\) and differentiation is promoted. Roy et al. (71) studied the effect of phosphate deprivation of Hyp mice that exhibit rachitic bone disease, hypophosphatemia, impaired renal phosphate reabsorption, and abnormal regulation of renal 1,25(OH)\(_2\)D\(_3\) metabolism. They found that phosphate deprivation of Hyp mice resulted in a 3-fold increase in the maximum velocity of 24-hydroxylase activity, in the amount of 24-hydroxylase-immunoreactive protein, and 24-hydroxylase mRNA in kidney. Hyp mice also exhibited an appropriate increase in 24-hydroxylase mRNA and catalytic activity in response to increasing doses of 1,25(OH)\(_2\)D\(_3\). Induction of 24-hydroxylase was also observed in bone. Nishimura et al. (72) have shown that 24-hydroxylase activity and mRNA increased when osteoblast cells were incubated with 1,25(OH)\(_2\)D\(_3\).

E. Subcellular location of 24-hydroxylase and the induction-responsive site

Recently, Iwata et al. (73) characterized the intracellular location of 24-hydroxylase using an immunogold technique. They found that the enzyme was distributed in mitochondria along the renal tubules of rat kidney in normal rat. In vitamin D\(_3\)-treated rats, about a 12-fold increase in the amount of 24-hydroxylase was observed. Annbrecht and Bolz (69) have shown that the induction occurred after a single administration of 1,25(OH)\(_2\)D\(_3\). They also found that 24-hydroxylase mRNA increased in the intestine of rats injected with 1,25(OH)\(_2\)D\(_3\).
1,25(OH)\textsubscript{2}D\textsubscript{3}. They concluded that the following series of reactions are catalyzed by the 24-hydroxylase enzyme alone: 

i) \(25\text{-OH-D}\textsubscript{3} \rightarrow 24,25\text{(OH)}\textsubscript{2}D\textsubscript{3} \rightarrow 24\text{-oxo}-25\text{(OH)}D\textsubscript{3} \rightarrow 24\text{-oxo}-23,25\text{(OH)}\textsubscript{2}D\textsubscript{3}\); 

ii) \(1,25\text{(OH)}\textsubscript{2}D\textsubscript{3} \rightarrow 1,24,25\text{(OH)}\textsubscript{3}D\textsubscript{3} \rightarrow 24\text{-oxo}-1,25\text{(OH)}\textsubscript{2}D\textsubscript{3} \rightarrow 24\text{-oxo}-1,23,25\text{(OH)}\textsubscript{3}D\textsubscript{3}\). The enzyme thus catalyzes 24-hydroxylation, 24-\(\text{OH}\) dehydrogenation, and subsequent 23-hydroxylation of the 24-oxo compounds. Similar multiple-oxidations by P450 were also observed in the formation of estrogen by CYP19 (75), lanosterol de-
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