1.alpha.Hydroxylation of 24-hydroxyvitamin D2 represents a minor physiological pathway for the activation of vitamin D2 in mammals

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1α-Hydroxylation of 24-Hydroxyvitamin D_2 Represents a Minor Physiological Pathway for the Activation of Vitamin D_2 in Mammals

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ABSTRACT: C_{24}-Hydroxylation was evaluated as a possible activation pathway for vitamin D_2 and vitamin D_3. Routine assays showed that 24-hydroxyvitamin D_2 and 1,24-dihydroxyvitamin D_2 could be detected in rats receiving physiological doses (100 IU/day) of vitamin D_2; however, 24-hydroxyvitamin D_3 could not be detected in rats receiving similar doses of vitamin D_3. In rats, 24-hydroxyvitamin D_2 was very similar to 25-hydroxyvitamin D_2 at stimulating intestinal calcium transport and bone calcium resorption. The biological activity of 24-hydroxyvitamin D_2 was eliminated by nephrectomy, suggesting that 24-hydroxyvitamin D_2 must undergo 1α-hydroxylation to be active at physiological doses. In vivo experiments suggested that when given individually to vitamin D deficient rats, 24-hydroxyvitamin D_2, 25-hydroxyvitamin D_2, and 25-hydroxyvitamin D_3 were 1α-hydroxylated with the same efficiency. However, when presented simultaneously, 24-hydroxyvitamin D_2 was less efficiently 1α-hydroxylated than either 25-hydroxyvitamin D_3 or 25-hydroxyvitamin D_2. 1,24-Dihydroxyvitamin D_2 was also approximately 2-fold less competitive than either 1,25-dihydroxyvitamin D_2 or 1,25-dihydroxyvitamin D_3 for binding sites on the bovine thymus 1,25-dihydroxyvitamin D receptor. These results demonstrate that 24-hydroxylation followed by 1α-hydroxylation of vitamin D_2 represents a minor activation pathway for vitamin D_2 but not vitamin D_3.

Vitamin D_3 and vitamin 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 mammals, whereas, vitamin D_2 is the major naturally occurring form of the vitamin in plants. Vitamin D_3 also occurs naturally in plants and may comprise as much as 1% of the total vitamin D in alfalfa (Horst et al., 1984a,b). Whether or not vitamin D_3 occurs naturally in other plant species is presently unknown. Nocturnal herbivores therefore would have evolved with vi-

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tamin D_2 as their major (if not only) source of vitamin D. Vitamin D_3, on the other hand, would have served as the major vitamin D source in most diurnal species.

Shortly after their discovery, it seemed apparent that vitamin D_2 and vitamin D_3 had similar biological activity in most mammals and that birds and new world monkeys discriminated against vitamin D_2 in favor of vitamin D_3 (Bethke et al., 1946; Steenbock et al., 1932; Hunt et al., 1967). Recent research, fostered by the discovery of sensitive analytical techniques and availability of high specific activity [^{3}H]vitamin D's, indicates that differences in the metabolism of vitamin D_2 and vitamin D_3 in mammals are perhaps widespread (Horst et al., 1982a,b). Most notable was the apparent discrimination against vitamin D_2 by pigs and cows and apparent preference for vitamin D_2 by rats. In these same experiments and in more recent studies (Hoy et al., 1988), it was shown that chicks discriminate against vitamin D_2 as a result of enhanced clearance of the vitamin D_2 metabolites 25-hydroxyvitamin D_2 (25-D_2)[1] and 1,25-D_2. These results corrected earlier research (Imrie et al., 1967) suggesting that discrimination against vitamin D_2 by chicks was a result of enhanced plasma clearance of the parent vitamin D_2 molecule.

Much is known about the side-chain metabolism of both vitamin D_2 and vitamin D_3. For example, to become active, each must undergo 25-hydroxylation in the liver followed by 1α-hydroxylation in the kidney. In addition, the major physiologic pathway of catabolism for the vitamin D_3 metabolites, 25-D_3 and 1,25-D_3, is initiated by C_{24}-oxidation (Holick et al., 1973; Kumar et al., 1978; Reinhardt et al., 1982). C_{24}-Oxidized metabolites then act as substrates for further oxidation at C_{23} (Napoli & Martin, 1984; Ishizuka et al., 1984; Mayer et al., 1983; Napoli & Horst, 1983; Reddy et al., 1987), leading to cleavage between C_{23} and C_{24} (Esvelt et al., 1979; Jones et al., 1983). Minor pathways of catabolism include C_{26}-oxidation and formation of lactone (Reinhardt et al., 1981; Tanaka et al., 1981; Horst et al., 1984a,b). Similarly, the 24-hydroxy derivatives of 25-D_2 and 1,25-D_2 are known (Jones et al., 1980; Horst et al., 1986; Reddy & Tserng, 1986). The metabolite 1,24,25-D_2 can be further hydroxylated at C_{28} or C_{26} (Reddy & Tserng, 1986). The C_{22} alkene and C_{24}-(*S*)-methyl group in vitamin D_2, however, may preclude the classical side-chain oxidation reactions known to occur in the vitamin D_3 metabolite series such as C_{24}-ketonization or C_{23}-oxidation.

The differences in side-chain chemistry between vitamin D_2 and vitamin D_3 offer opportunities for divergence in side-chain oxidation of the two sterols; for example, the 24-position in vitamin D_2 is a tertiary carbon, as is the 25-position for both vitamin D_2 and vitamin D_3. In addition, the 24-position in vitamin D_2 is an allylic carbon, making it a far more reactive site than the corresponding position on vitamin D_3. On the basis of these chemical differences, 24-hydroxylation of vitamin D_2 may be a quantitatively significant pathway for the further metabolism of vitamin D_2. In support of this argument are the data of Jones et al. (1980), who demonstrated that rats receiving pharmacological doses of vitamin D_2 24-hydroxylate vitamin D_2 to 24-D_2. A quantitative physiological evaluation of the 24-hydroxylation pathway, however, was not done by this group.

The present report evaluates 24-hydroxylation of vitamin D_2 as a possible first step in the activation of vitamin D_2. We present evidence to suggest that sequential formation of 24-D_2 and 1,24-D_2 represents an activation pathway for vitamin D_2 in mammals.

Materials and Methods

General. High-pressure liquid chromatography and mass spectrometry were conducted as previously described (Napoli et al., 1986; Koszewski et al., 1988).

Compounds. Vitamin D_2 was purchased from Sigma Chemical Co. (St. Louis, MO). Synthetic 25(*R*),26-D_2 and 25(*S*),26-D_2 were gifts from Dr. Dudley Williams (University Chemical Laboratory, Cambridge, England). Vitamin D_2 metabolites used for standards were isolated from plasma of a Jersey cow given excess vitamin D_2 (weekly intramuscular injections of 4×10^7 units). After 4 weeks of treatment, the cow was euthanized with phenobarbital. Blood (approximately 20 L) was collected in heparinized containers. Plasma was prepared by centrifugation and stored at −20 °C. Vitamin D metabolites were extracted, purified, and identified as described (Napoli et al., 1986; Koszewski et al., 1988).

Assays. Assays for the vitamin D_2 and vitamin D_3 metabolites were conducted as previously described (Horst et al., 1982a,b). The extraction and chromatographic conditions used to assay 25-D_2, 24,25-D_2, and 1,25-D_2 were also suitable for isolation and analysis of 24-D_2, 24,26-D_2, and 1,24-D_2, respectively. Competitive protein binding assays and the ability of 24-D_2 to stimulate intestinal calcium transport (ICT) and bone calcium resorption (BCR) were determined as previously described (Horst et al., 1986). Nephrectomy was done under isoflurane anesthesia just prior to introduction of experimental metabolites. In vitro 1α-hydroxylase assays were performed as previously described (Engstrom et al., 1984).

Metabolism and Biological Evaluation Studies. Standard 1,24-D_2 was prepared by incubating 10 μg of 24-D_2 in each of 10 flasks containing 5 mL of 20% kidney homogenates made from vitamin D deficient chicks (Engstrom et al., 1984). The 1,24-D_2 was isolated from lipid extracts of the homogenates by HPLC (Horst et al., 1982). The putative 1,24-D_2 was subjected to mass spectrometry for positive identification.

Experiments to study in vivo metabolism of various monohydroxylated vitamin D_2 substrates were performed in male weanling rats (Holtzman, Madison, WI). After 4 weeks on a vitamin D deficient diet (Teklad, diet no. TD81308), one group (six rats/group) of rats received daily oral supplementation (100 IU/day for the next 3 weeks) of vitamin D_2 (group I). Four additional groups (groups II–V) were maintained on a vitamin D deficient and calcium-deficient diet for 4 weeks. During the last 4 days of experimental diet, animals received 5 μg of either 24-D_2 (group II), 25-D_2 (group III), 25-D_3 (group IV), or a combination of 5 μg each of 24-D_2, 25-D_2, and 25-D_3 (group V). All treatments were given intraperitoneally (ip) once daily for 4 days in 100 μL of propylene glycol carrier.

Another set of experiments was designed to determine the influence of vitamin D_2 and vitamin D_3 supplementation on plasma concentrations of 25-D_2, 24-D_2, and 24-D_3. After 4 weeks on a vitamin D deficient diet, animals received 800 IU/day of either vitamin D_2 or vitamin D_3. After 3 weeks of treatment, blood was collected and plasma harvested for metabolite evaluation.

[1] Abbreviations: 25-D_3, 25-hydroxycholecalciferol; 25-D_2, 25-hydroxyergocalciferol; 24-D_2, 24-hydroxyergocalciferol; 24-D_3, 24,25-dihydroxycholecalciferol; 24,25-D_2, 24,25-dihydroxyergocalciferol; 24,25-D_3, 24,25-dihydroxycholecalciferol; 24,25-D_2, 24,25-dihydroxyergocalciferol; 24,26-D_2, 24,26-dihydroxyergocalciferol; 1,25-D_2, 1,25-dihydroxyergocalciferol; 1,25-D_3, 1,25-dihydroxycholecalciferol; 1,24-D_2, 1,24-dihydroxyergocalciferol; HPLC, high-performance liquid chromatography.

Table I: Bone Calcium Resorption (BCR) and Intestinal Calcium Transport (ICT) in Intact and Nephrectomized Rats Receiving Vitamin D_2 Metabolites

dose	serum Ca (mg/dL) (BCR)	^{45}Ca(serosal)/ ^{45}Ca(mucosal) (ICT)
Intact		
control	4.0 ± 0.3	2.8 ± 0.3
24-hydroxyvitamin D_2		
25 ng	4.5 ± 0.3[a]	4.3 ± 1.6[a]
50 ng	4.7 ± 0.2[a]	4.4 ± 0.5[a]
100 ng	4.6 ± 0.4[a]	5.8 ± 1.8[a]
25-hydroxyvitamin D_2		
25 ng	4.5 ± 0.2[a]	4.4 ± 4.5[a]
Nephrectomized		
control	2.8 ± 0.1	2.1 ± 0.3
24-hydroxyvitamin D_2		
50 ng	2.7 ± 0.1	1.9 ± 0.3
1,25-dihydroxyvitamin D_2		
12.5 ng	3.3 ± 0.1[a]	3.0 ± 0.5[a]

[a] Significantly different from controls ($P < 0.05$).

Results

Biological Evaluation. Plasma from a cow receiving excess vitamin D_2 contained a metabolite that was similar in concentration to, but slightly less polar than, 25-D_2 when analyzed by straight-phase HPLC chromatography (data not shown). This metabolite was identified as 24-D_2 by mass spectroscopy, NMR, and UV analysis (Napoli et al., 1986; Koszewski et al., 1987). The observation that this cow had similar plasma concentrations of 24-D_2 and 25-D_2 prompted us to quantitatively evaluate the biological significance of this pathway in animals receiving physiological doses of vitamin D_2.

The data in Table I summarize experiments done to determine the biological activity of 24-D_2 in rats. The data show that 24-D_2 and 25-D_2 are very similar in their ability to stimulate ICT and BCR. Nephrectomy abolished the activity of 24-D_2, which suggested that 24-D_2 must undergo 1α-hydroxylation in the kidney before becoming biologically active (Table I).

Isolation and Identification of 1,24-D_2. In order to study the in vivo 1α-hydroxylation of 24-D_2, standard 1,24-D_2 had to be synthesized. This was accomplished by incubating 24-D_2 with chick kidney homogenates made from vitamin D deficient chicks. Lipid extracts of the homogenates were subjected to HPLC and found to yield a peak slightly less polar than 1,25-D_2 on a straight-phase Zorbax (0.9 × 24 cm) HPLC column developed in 76/25/4 hexane/methylene chloride/2-propanol. In this system, 1,25-D_2 peaked at 35 mL, and the putative 1,24-D_2 peaked at 30 mL (data not shown). The 1,24-D_2 peak was collected and, in final preparation for mass spectral analysis, was further purified by using a Zorbax Sil column (0.45 × 25 cm) developed in 97/5/2 hexane/2-propanol/methanol (Figure 1). The final yield of product (~500 ng) was estimated by UV analysis. The mass spectrum (Figure 2) of the putative 1,24-D_2 showed a parent ion at m/z 428 consistent with the incorporation of a single hydroxyl group into the 24-D_2 structure. Successive losses of water from the parent ion were readily apparent in the mass spectrum as peaks at m/z 349 and 331. These data are in good agreement with the cleavage of the C_{24}/C_{25} bond observed in the spectrum of 24-D_2 (Jones et al., 1980; Engstrom & Koszewski, 1988). The loss of 141 amu further confirmed the presence of a single hydroxyl group in the side chain. The appearance of peaks at m/z 152 and 134, together with the peaks at m/z 269 and 251, indicated that the additional hydroxyl group was present in the A ring. This pattern is characteristic of 1α-hydroxylated vitamin D metabolites possessing an intact triene system (Napoli et al., 1986).

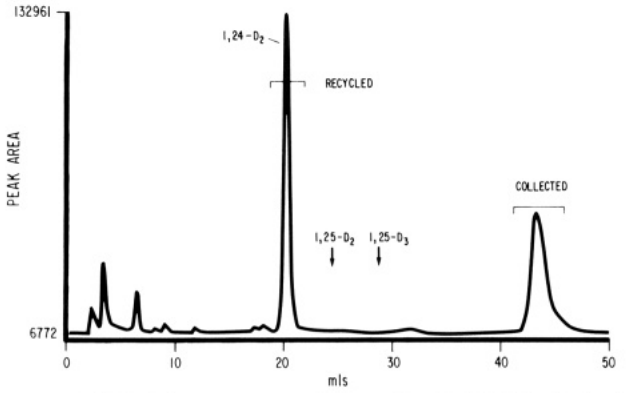

FIGURE 1: HPLC chromatogram of the semipurified 1,24-D_2 isolated from chick kidney homogenates outlined under Materials and Methods. The 1,24-D_2 was dried under N_2 and applied in toto to an HPLC column (Zorbax Sil 0.45 × 25 cm) developed in 97/5/2 hexane/2-propanol/methanol. The 1,24-D_2 was recycled one time for a total of two passes across the HPLC column. The elution volumes of 1,25-D_2 and 1,25-D_3 are also indicated.

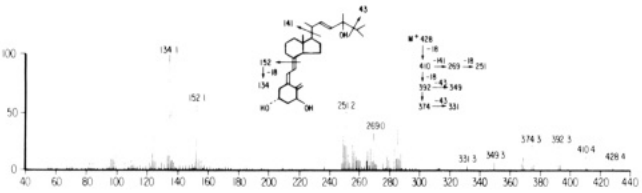

FIGURE 2: Low-resolution electron impact mass spectra of 1,24-D_2.

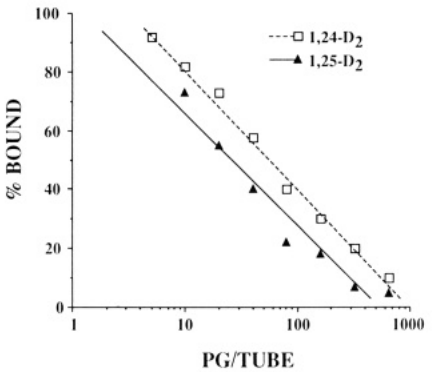

FIGURE 3: Relative binding of 1,24-D_2 and 1,25-D_2 to the 1,25-D receptor from bovine thymus. Radioactive 1,25-D_3 was used as tracer.

Competitive Protein Binding Assays. In vitro radioligand binding of the 1,24-D_2 suggested that this sterol was approximately 2-fold less competitive than 1,25-D_2 for [^{3}H]-1,25-D_3 binding sites on receptor from calf thymus (Figure 3).

Metabolism Studies. The plasma concentrations of the various mono- and dihydroxylated metabolites are displayed in Table II. The data show for the first time that 1,24-D_2 is a normal product of vitamin D_2 (group I) or 24-D_2 (groups II and V) metabolism in vivo. Also of interest in Table II are the data which show that when presented individually to vitamin D deficient animals, 24-D_2, 25-D_2, and 25-D_3 were 1α-hydroxylated with the same efficiency (groups II, III, and IV). Under these conditions, plasma 1,24-D_2, 1,25-D_2, and 1,25-D_3 circulated at 2397 ± 412, 2130 ± 130, and 1895 ± 326 pg/mL, respectively. Coadministration of 24-D_2, 25-D_2, and 25-D_3 (group V) resulted in preferential 1α-hydroxylation of 25-D_2 and 25-D_3 relative to 24-D_2. Under the conditions described for group V, the plasma 1,24-D_2, 1,25-D_2, and 1,25-D_3 were 193 ± 20, 1140 ± 75, and 772 ± 60 pg/mL,

Table II: Plasma Concentrations of Vitamin D Metabolites in Treated Rats and Cows[a]

		plasma concn (ng/mL)						plasma concn (pg/mL)		
sterol	group/diet[b]	24-D_2	25-D_2	25-D_3	24,25-D_2	24,26-D_2	24,25-D_3	1,24-D_2	1,25-D_2	1,25-D_3
D_2[c]	I/A	3.0 ±1.5	10.9 ±2.2	ND[d]	4.1 ±2.3	1.7 ±1.0	ND	8.8 ±4.1	151 ±44	ND
24-D_2[d]	II/B	177 ±25	ND	ND	9.1 ±1	16.3 4.0	ND	2397 ±412	ND	ND
25-D_2[e]	III/B	ND	214 ±48	ND	22.1 ±1.6	ND	ND	ND	2130 ±130	ND
25-D_3[e]	IV/B	ND	ND	115 ±23	ND	ND	14.9 ±1.8	ND	ND	1895 ±325
24-D_2, +25-D_2 +25-D_3[f]	V/B	48 ±12	97 ±22	84 ±12	19.9 ±4.7	14.9 ±2.1	12.0 ±1.4	193 ±20	1104 ±75	772 ±60
D_2 excess (cow)		173 ±69	141 ±33	13.7 ±2.5	106 ±36	14.7 ±88	3.35 ±1.1	ND	41 ±7	4.7 ±2.4

[a] Means ± SEM. $N = 4$–6. [b] All diets were vitamin D deficient. A = normal calcium (0.5%); B = low calcium (0.005%). [c] Oral supplement 100 IU/day for 3 weeks. [d] Not detectable. [e] Intraperitoneal injection, 5 μg/day for 4 days. [f] 5 μg of each given simultaneously, intraperitoneally for 4 days.

respectively. Both the plasma 1,24-D_2 and 1,25-D_3 were significantly lower ($P < 0.01$) than the 1,25-D_2. Similarly, the plasma 1,24-D_2 was significantly lower ($P < 0.01$) than the 1,25-D_3. Similar results were observed in in vitro assays using rat kidney homogenates. The results from these assays showed that when these substrates were presented simultaneously at saturating concentrations (5 μM), the renal 1α-hydroxylase enzyme produced similar amounts of 1,25-D_2 and 1,25-D_3 [1.9 and 1.95 pmol/(min·mg of tissue), respectively]. Production of 1,24-D_2, on the other hand, was <0.2 pmol/(min·mg of tissue). Due to the lack of adequate amounts of 24-D_2 substrate, the kidney hydroxylases could not be studied with each metabolite individually.

The plasma concentrations of 24,25-D_2, 24,25-D_3, and 24,26-D_2 are also presented in Table II. The data show that 24,26-D_2 (a pathway unique to vitamin D_2) is produced by rats receiving physiological doses of vitamin D_2. The data also indicate that 25-D_2 was preferentially metabolized to 24,25-D_2, whereas, 24-D_2 was preferentially metabolized to 24,26-D_2.

To determine if the 24-hydroxylation pathway may exist for vitamin D_3, studies were conducted in rats receiving 800 IU of either vitamin D_2 or vitamin D_3. The results (not shown) demonstrate that, in vitamin D_2 dosed rats, 25-D_2 and 24-D_2 circulated at 14.1 ± 3.2 and 15.9 ng/mL, respectively. Vitamin D_3 dosed rats, on the other hand, had no detectable 24-D_3, and 25-D_3 circulated at 26.3 ng/mL.

Discussion

This report identifies a new activation pathway for vitamin D_2 in mammals. The process involves initial C_{24}-hydroxylation of vitamin D_2 followed by 1α-hydroxylation. The presence of circulating 1,24-D_2 in vitamin D_2 and 24-D_2 dosed rats, the high affinity of 1,24-D_2 for receptor binding sites, and the similarities in biological activity of 25-D_2 and 24-D_2 support the argument that the pathway leads to vitamin D_2 activation. At the doses tested (800 IU/day for 3 weeks), the equivalent pathway could not be demonstrated for vitamin D_3. In that regard, Wichmann et al. (1981) have isolated small quantities of 24-D_3 from chick plasma. However, in their experiments, the chicks were dosed with pharmacological amounts (10 million IU) of vitamin D_3. Whether 24-D_3 was produced by chicks receiving physiological doses of vitamin D_3 was not addressed by these researchers. Although not yet demonstrated in biological systems, the compound 1,24-D_3 has been chemically synthesized and found to stimulate BCR and ICT (Smith et al., 1982). Additionally, 1,24-D_3 has been evaluated and found to be effective in the treatment of osteoporosis (Shiraki et al., 1985). Therefore, like 1,24-D_3, the metabolite

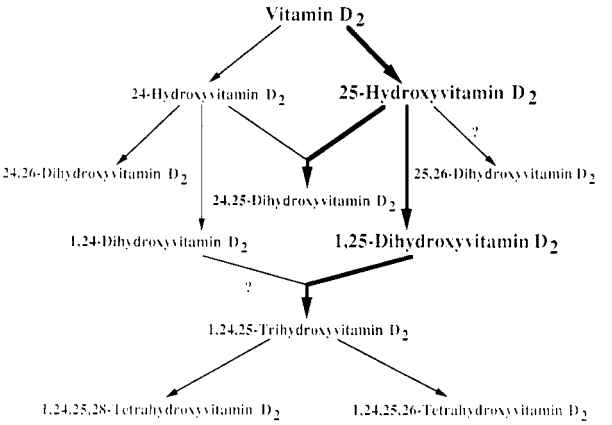

FIGURE 4: Known pathways of vitamin D_2 metabolism in mammals. The (?) denotes anticipated pathways not yet confirmed, and the bold arrows indicate the major physiological pathway for vitamin D_2 metabolism.

1,24-D_2 may have potential as a therapeutic agent in the prevention or treatment of some calcium-related diseases.

The presence of the C_{24}-activation pathway for vitamin D_2 therefore represents a deviation in the metabolic pathways for vitamin D_2 and vitamin D_3. The presence of an alternate activation pathway may aid in explaining the differences in efficacy between vitamin D_2 and vitamin D_3 for treatment of some bone diseases. For example, vitamin D_2 is superior to vitamin D_3 or 25-D_3 at curing bone lesions occurring in patients taking phenobarbitone/phenytoin (Christiansen et al., 1975; Tjellesen et al., 1985). These anticonvulsant treatments may target 25-D_3 or 1,25-D_3 to deactivation by stimulating target tissue catabolic enzymes; whereas, vitamin D_2 metabolites (24-D_2, 25-D_2, and 1,25-D_2) may be spared this process due to their different molecular configuration.

The major known pathways of vitamin D_2 metabolism in animals receiving normal dietary vitamin D_2 are depicted in Figure 4. As described, vitamin D_2 is preferentially hydroxylated at C_{25} with C_{24}-oxidation present as a minor pathway. At present, we are unable to pinpoint the major in vivo source of the vitamin D_2 C_{24}-hydroxylase; however, in vitro evidence from our laboratory suggests that 24-D_2 production in liver homogenates far exceeds production of 25-D_2 (Engstrom & Koszewski, 1988). Nevertheless, a major route of 25-D_2 metabolism subsequently results in the formation of 24,25-D_2. On the other hand, only a small amount of 24-D_2 is metabolized to 24,25-D_2, with hydroxylation at C_{26} to yield 24,26-D_2 being a major pathway (Koszewski et al., 1988). Activation of either 24-D_2 or 25-D_2 then results from C_1-hydroxylation with the major physiological pathway for ac-

tivation proceeding through C_1-hydroxylation of 25-D_2 to form 1,25-D_2. Relative to the vitamin D_2 activation process is our observation that 24-D_2 is less efficiently 1α-hydroxylated in the presence of 25-D_2 and 25-D_3 (Table II, group V). This phenomenon suggests that, in the normal vitamin D replete state, 24-D_2 is relatively ineffective at competing for the 1α-hydroxylase enzyme; however, in the absence of competitors, 24-D_2 is 1α-hydroxylated in vivo at an apparent rate equal to that of 25-D_2 and 25-D_3 (Table II, group II). Also proposed in Figure 4 is the converging of the two activation pathways to the formation of 1,24,25-D_2, a metabolite with little or no inherent BCR and ICT activity (Horst et al., 1986). Further metabolism of the 1,24,25-D_2 metabolite to tetrahydroxy metabolites has been demonstrated and reported by Reddy et al. (1986).

The C_{24}-activation pathway for vitamin D_2 therefore represents the most notable deviation to date from the well-understood pathways for vitamin D_3 metabolism. Although this information was collected largely from rat experiments, recent findings of 24-D_2 in humans undergoing vitamin D_2 therapy (Hollis et al., 1986) argues this pathway may also be active in other mammalian species. This information could be of potential significance in providing a more complete understanding of vitamin D activation in mammals, as well as providing possible explanations for described differences in biological activity between vitamin D_2 and vitamin D_3.

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Registry No. D_2, 50-14-6; D_3, 67-97-0; 24-D_2, 58050-56-9; 25-D_2, 21343-40-8; 25-D_3, 19356-17-3; 1,24-D_2, 124043-51-2; 1,25-D_2, 60133-18-8; 1,25-D_3, 32222-06-3; 24,26-D_2, 109028-06-0; 24,25-D_2, 58050-55-8; 24,25-D_3, 40013-87-4; Ca, 7440-70-2.

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