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LITERATURE SURVEY

Vitamin D: An Update

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This article reviews the current status of the most promising areas of research and development on vitamin D during the last 15 years. Many pharmacists are involved in the manufacture and sale of vitamin D preparations, yet few are aware of the enormous scientific effort that was expended to find that vitamin D is inactive *per se* and that its biological activity is primarily due to one of its metabolites. Since this metabolite acts in true hormonal fashion vitamin D is now considered by biochemists as a prohormone. The findings of the mode of action of vitamin D are well documented.

In the early 1920's, cod liver oil was known to cure rickets and xerophthalmia. The name vitamin D was given to the antirachitic substance in cod liver oil (1–3), and differentiated from vitamin A by the fact that vitamin A activity in the oil was lost by heating while that of vitamin D was not. Hess and Steenbock discovered, independently (4–7), that the antirachitic activity of this vitamin could be induced by UV irradiation of rachitic animals or their food. Steenbock's discovery that antirachitic activity could be induced by irradiation of foods, particularly of the sterol fraction, ultimately led to the identification of the structure of vitamin D and to the eradication of rickets as a major medical problem (5, 6, 8).

It was later discovered that vitamin D occurs in two active forms, ergocalciferol and cholecalciferol. Ergocalciferol is the synthetic form derived by the irradiation of ergosterol and is designated as vitamin D_2 (9, 10). Cholecalciferol, the natural form, was identified (11, 12) and designated as vitamin D_3. Vitamins D_2 and D_3 are equally active in humans and other mammals, but D_2 is virtually inactive in birds. Thus, poultry feeds must be fortified with D_3.

BIOSYNTHESIS OF VITAMIN D_3

Vitamin D_3 is synthesized in the skin from 7-dehydrocholesterol (7-DHC) when the skin is exposed to sunlight. Okano *et al.* (13) first demonstrated this when they irradiated skins from vitamin D-deficient rats and isolated vitamin D_3 from saponified skin extracts. The sequence of steps leading to the cutaneous photosynthesis of vitamin D_3 from 7-DHC was recently demonstrated (14). In Caucasian skin, all of the epidermal strata contain 7-DHC, but the highest concentration is in the stratum basale and the stratum spinosum.

When irradiated, 7-DHC is photochemically converted by a fast reaction to previtamin D_3 throughout the epidermis and the dermis. Once formed in the skin, previtamin D_3 is isomerized to D_3 by a slow nonphotochemical rearrangement at a rate dictated by skin temperature. At

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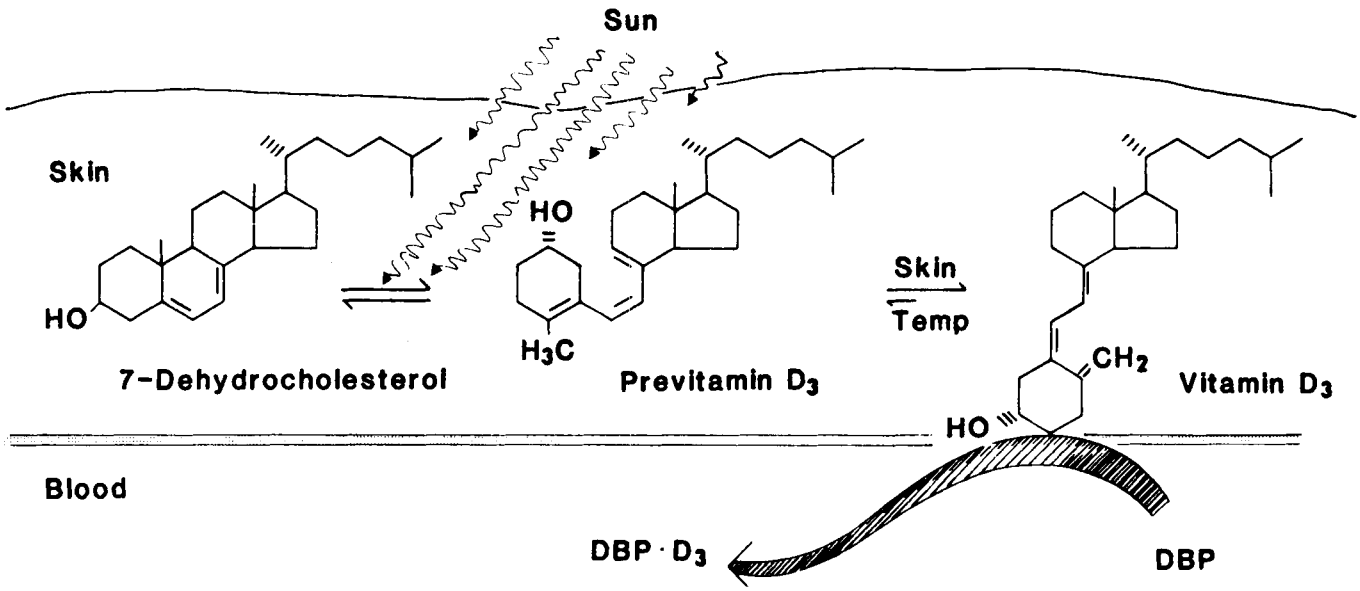


Scheme I—Diagram of the formation of previtamin D_3 in the skin, its thermal conversion to vitamin D_3, and transport by binding proteins (DBP) in plasma into the general circulation (Ref. 14).

37 ± 1° this thermal reaction is more rapid *in vivo* than *in vitro*. This difference is presumed to be due to the removal of vitamin D_3 by a specific binding protein from the skin into the circulating blood. Thus the skin serves as the site for the synthesis of 7-DHC, a reservoir for the storage of the primary photoproduct, previtamin D_3, and the organ where the slow thermal conversion of previtamin D_3 to D_3 occurs. The third process permits the skin to continuously synthesize D_3 and release it into the circulation for up to 3 days after a single exposure to sunlight. Scheme I illustrates this sequence of events.

COMMERCIAL VITAMIN D

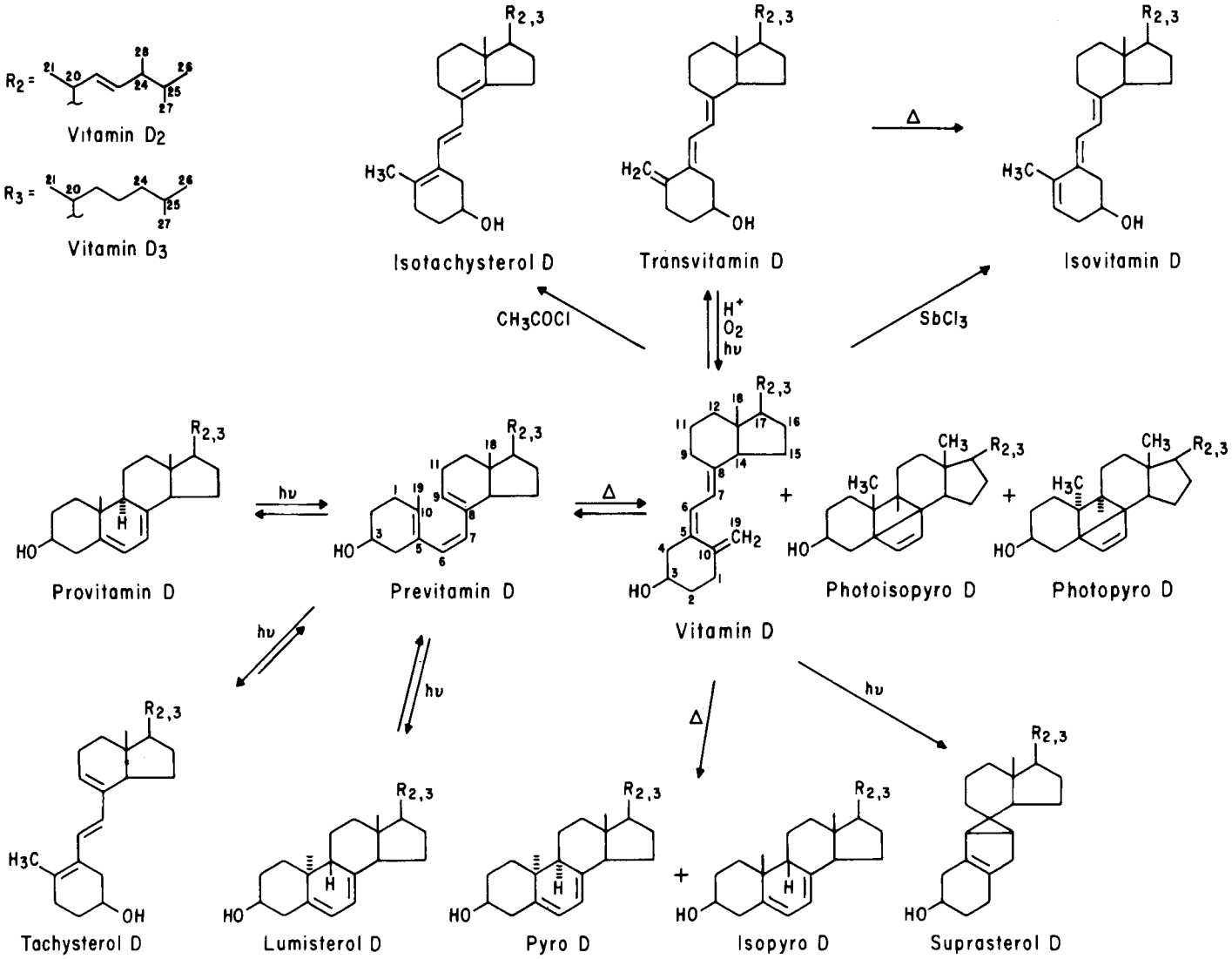
Vitamins D_2 and D_3 are prepared commercially by irradiation of the provitamins ergosterol and 7-DHC, respectively. The resulting previtamins are isolated and heated under carefully controlled conditions to produce the respective vitamins. A number of by-products are formed during these irradiation and heating steps, some of which are removed during the final cleanup steps. Scheme II shows the known degradation products (15, 16) that may be present in the synthetic vitamin D concentrates. Some of the by-products have biological activity, but others such as lumisterol, the suprasterols, and the pyro- and isopyrocalciferols have no antirachitic activity. Tachysterol and *trans*-vitamin D have only slight antirachitic activity. Thus, biological activity is primarily due to vitamin D and previtamin D (17). The biological activity of previtamin D is attributed to its *in vivo* conversion to vitamin D (18).

Vitamin D is a 9,10-seco steroid and is treated as such in the numbering of its carbon skeleton. The important aspects of vitamin D chemistry center about its *cis*-triene structure. This structure gives vitamin D and related compounds a characteristic UV absorption maximum at 265 nm and a minimum at 228 nm. An index of purity of the vitamin D compounds is a value of 1.8 for the ratio of the absorbance at 265 nm to that at 228 nm. The *cis*-triene structure makes vitamin D and related compounds susceptible to oxidation and other chemical transformations. Under even mild acidity, it isomerizes to form the 5,6-*trans*-isomer and isotachysterol. Upon mild heating, the 5,6-*trans*-vitamin D is converted to isovitamin D. At higher temperatures, vitamin D is converted to the pyro, isopyro and the supra forms. All of these transformation products have lower biological activity than vitamin D.

It was mentioned earlier that vitamin D exists in thermal equilibrium with previtamin D. Studies showed that the amount of previtamin formed and the rate of equilibration markedly increased with time between 0 and 100° (19, 20). Vitamin D concentrates are available commercially in a resin form, as a solution in peanut oil, and as a dry concentrate in a gelatin beadlet matrix. The vitamin D concentrates usually contain other fat soluble vitamins, especially vitamin A. Therefore, in the analysis of vitamin D concentrates and multivitamin products, the vitamin D must be released and separated from other fat-soluble vitamins prior to its determination. This is accomplished by an alkaline saponification followed by suitable chromatographic steps to purify the vitamin D from interfering ingredients.

Because of the thermal equilibrium with previtamin D, there is disagreement regarding the optimum saponification conditions. Most published reports show optimum saponification for 20–30 min under refluxing conditions. Thompson *et al.* (21), in their procedure for the analysis of vitamin D in fortified milk at the level of 8.8–11.7 ng/g, found that saponification overnight at room temperature produced negligible loss of vitamin D.

The instability of vitamin D has been a challenge in the isolation, identification, and quantitation of its metabolites from biological samples in which their concentrations are in the range of 0.02–30 ng/g of the tissues. However, there now are fairly reliable methods for the isolation and analysis of all major metabolites of vitamin D. Vitamin D and its metabolites can be kept stable in the dry form in an inert atmosphere and protected from light in a freezer. In solution it is stable in nonacidic vegetable oils and in certain organic solvents containing an antioxidant such as α-tocopherol (Vitamin E) and stored in the same conditions as the dry form.



Scheme II—Photochemical, thermal, and chemical reaction pathways in the synthesis of vitamin D (Refs. 15, 16, and 176).

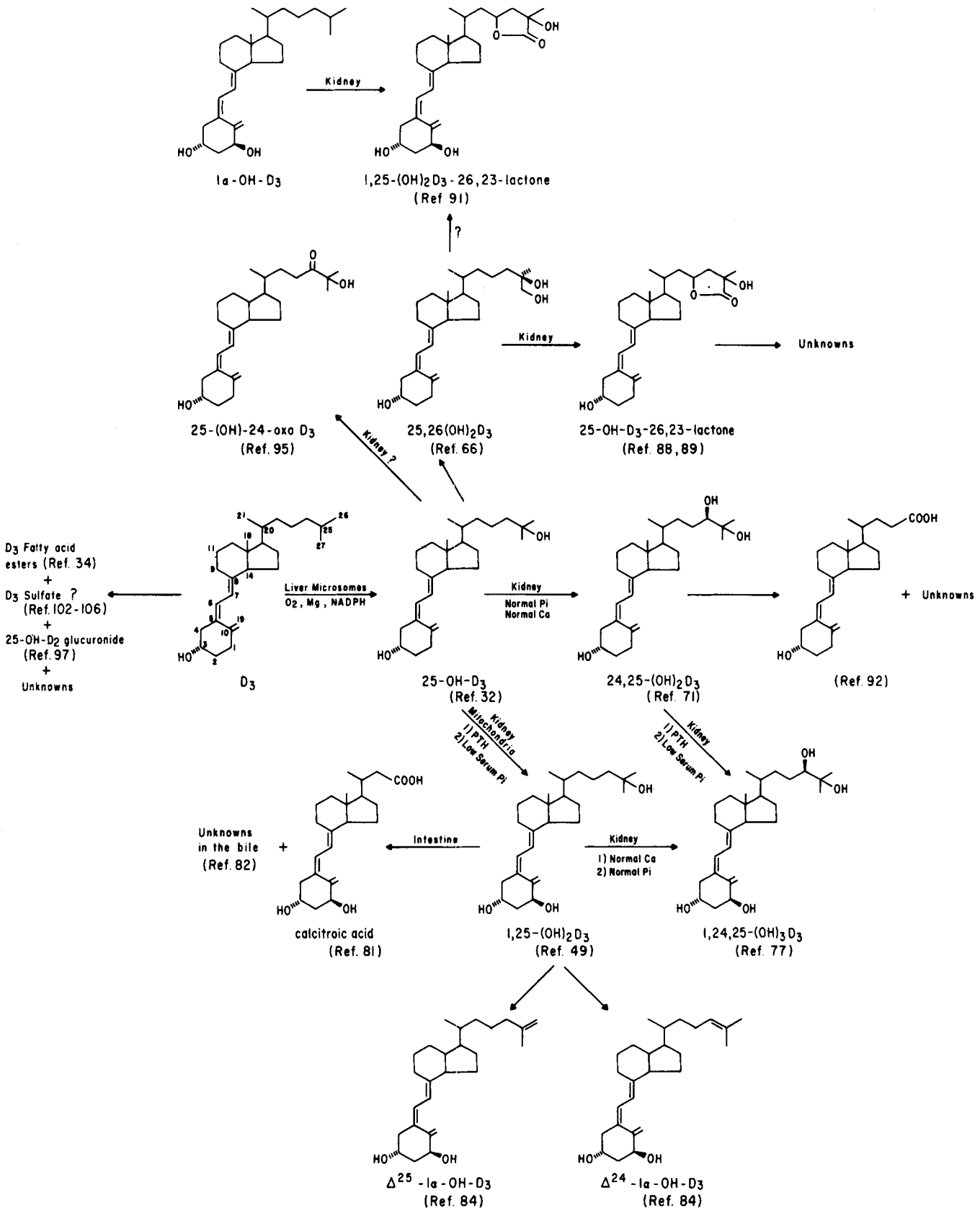
The original reference standard of vitamin D was a solution of irradiated ergosterol in olive oil. The present international standard is a solution of pure crystalline cholecalciferol in olive oil containing 0.025 μg/1 mg of solution. One international unit (IU) is equivalent to 0.025 μg of crystalline D_3 or 1 μg of D_3 is equivalent to 40 IU. The USP reference standards are crystalline ergocalciferol or cholecalciferol packed in sealed ampuls. The USP standards and the international standards are equivalent in that 1 μg of each is equivalent to 40 IU.

METABOLITES OF VITAMIN D_3

For 30 years following its discovery, relatively little was known about the synthesis of vitamin D or its metabolic fate in humans. It was thought that vitamin D acted directly on the target tissues of intestine and bone. However, the time lag reported by Carlsson (22) between the administration of vitamin D_3 and its physiological response appeared to argue against this concept. After intravenous administration of 10 IU of vitamin D_3, there was a lag of 10–12 hr before intestinal calcium transport response occurred (23). This led investigators to suspect that vitamin D_3 was metabolically altered before it became biologically active.

The transport of vitamin D in blood has been studied in considerable detail in animals. It has been shown that the chylomicrons and lipoproteins of the blood are initially important in the transport of vitamin D (24, 25). It was found (25) that upon incubation of blood with radioactive vitamin D or immediately following injection into blood, as much as 50% of the radioactivity in the blood was associated with the lipoprotein fractions. However, as time passed there was progressive shift of the radioactivity from the lipoprotein to the α_2-globulin fraction. The nature of the binding between vitamin D and this protein fraction is not known, but it appears that the vitamin is not easily dissociated from this protein. It was shown (26) that with time, after vitamin D absorption, there is a decreasing association of vitamin D with lipoproteins and an increased association with other protein fractions. The association of vitamin D activity with α_1- and α_2-globulin fractions of plasma was clearly demonstrated (27, 28).

25-Hydroxyvitamin D_3—Major developments in the metabolic study of vitamin D_3 came after radiolabeled compounds with high specific activities were synthesized. Selective ^3H-labeled and random ^{14}C-labeled vitamin D_3



Scheme III—Schematic pathway of vitamin D metabolism.

were prepared in the mid-1960's and used successfully in the isolation and identification of its major metabolites. Other factors that contributed to this success were the development of milder extraction procedures and novel chromatographic techniques.

Norman *et al.* (29) administered [^{3}H]vitamin D orally and intraperitoneally to rats and then extracted the kidney and intestinal tissues with a mixture of methanol–chloroform as described by Bligh and Dyer (30). After chromatography on silicic acid columns and thin-layer

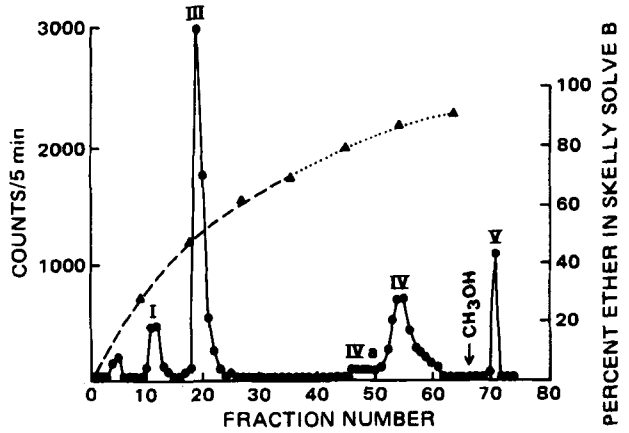


Figure 1—*Silicic acid chromatography of vitamin D_3 and its metabolites. Peaks III and I are vitamin D and its esters, respectively. Peaks IVa and V are unidentified at this time, and peak IV is 25-hydroxyvitamin D_3. (Ref. 32).*

plates, at least three other chloroform-soluble radioactive compounds were detected, all of which showed partial vitamin D activity. The aqueous methanol-soluble radioactive compounds were totally without vitamin D activity.

Biologically active metabolites of vitamin D were found (31) in the bone, liver, and serum of rats that had been given [^{3}H]vitamin D. As before (29), the aqueous-soluble metabolites from the tissues and the feces did not have vitamin D activity. At least three biologically active metabolites were isolated from the chloroform-soluble portion of the extract. One of these, designated as peak IV, was found in large amounts in the liver, blood, and bone. In 1938 Blunt *et al.* (32) conducted the classic experiment which led to the identification of this major metabolite as 25-hydroxy-vitamin D_3 (25-OH-D_3).

In this experiment, one hog was dosed with [^{3}H]vitamin D_3 and 800 ml of serum was collected when the peak IV radioactivity was expected to peak. Four other hogs were maintained on a diet supplemented with 250,000 IU of vitamin D_3 daily for 26 days. The plasma from these hogs (6.8 liters) was treated with ammonium sulfate to precipitate the proteins, which were then extracted with methanol–chloroform. This extract was combined with a similar extract from the serum of the hog that had received the [^{3}H]vitamin D_3 and subjected to chromatography on a 25-g silicic acid column eluted with an ether–Skellysolve B gradient followed by ether and finally stripping the column with methanol. This chromatographic system is reproduced in Fig. 1, which shows the radioactivity profile of the column eluate in order of increasing polarity of the metabolites. Peaks III and I are vitamin D_3 and its fatty acid esters, respectively.

The column eluate representing peak IV was purified further on a partition column using diatomaceous earth[1] as the solid support, 80:20 methanol–water as the stationary phase and Skellysolve B as the mobile phase. In this manner, 1.3 mg of the pure metabolite was obtained. This metabolite was positively identified as 25-OH-D_3 (32) by using GLC, UV, NMR, and mass spectrometry. This discovery was a milestone in the subsequent identification of other metabolites and in our current understanding of the physiological roles of these metabolites. Scheme III shows the metabolic pathway of vitamin D and all its currently known metabolites.

Two other groups of investigators (33, 34) independently found clues to the metabolic hydroxylation of vitamin D. It was soon established that 25-hydroxylation of vitamin D_3 takes place primarily in the liver (35, 36) and that 25-OH-D_3 is the major form of the circulating vitamin in human plasma (37) and is the primary metabolite of vitamin D_2 (38). This metabolite has been chemically synthesized (39).

Although the liver is the major site of 25-hydroxylation of vitamins D_2 and D_3, there is evidence suggesting that it is not the only site. It was shown (40) that homogenates of small intestine and kidney from the chick are capable of hydroxylating vitamin D_3 at position C-25. It was also shown (41) that hepatectomized rats can convert vitamin D_3 to 25-OH-D_3. Initially it was believed that the D_3-25-hydroxylase was located in the mitochondrial fraction of liver cells; however, careful investigation demonstrated that it is principally located in the liver microsomes, and that the enzymatic reaction is supported by reduced NADPH, molecular oxygen, and magnesium (42).

Initially, 25-OH-D_3 was considered to be the main biologically active metabolite of vitamin D. But soon it was discovered that physiological concentrations of 25-OH-D_3, like vitamin D_3, are incapable of stimulating either intestinal calcium transport or bone calcium mobilization (43–45).

1,25-Dihydroxyvitamin D_3—Earlier work (46) showed that the exhaled water from rats dosed with [1α-^{3}H]vitamin D_3 contained less radioactivity than the air from others dosed with vitamin D_3 labeled at the [24,25,26,27-$^3H_9$]-, [7-^{3}H]-, [6-^{3}H]-, and [3-^{3}H]- positions. Lawson *et al.* (34, 47), investigating the nature and localization of vitamin D metabolites by using [4-^{14}C,1α-^{3}H]-vitamin D_3, discovered a metabolite of vitamin D in the chick intestinal nuclei which was more polar than 25-OH-D_3 and which had lost its tritium group from position C-1. This metabolite, designated as peak P, was found in varying amounts in liver, kidney, bone, and blood and was localized in the intestinal nuclei.

It was postulated that the peak P metabolite was similar to the fraction designated "peak 4B" by Haussler *et al.* (33). The peak P metabolite had at least three times the biological activity of an equivalent amount of vitamin D_3 and was derived from 25-OH-D_3. However, the site of its synthesis was not obvious. Another study (48) established that this active metabolite was 1-oxygenated 25-OH-D_3 and that it was produced in the kidney. A short time later, Lawson *et al.* (49) identified peak P to be 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$]. This finding was confirmed by other investigators (50, 51) who found that 1,25-$(OH)_2D_3$ was part of peak V in the silicic acid column profile of vitamin D metabolites shown in Fig. 1.

It is now known that the formation of 1,25-$(OH)_2D_3$ is feedback regulated depending on the need for calcium in the blood. It is also known that 25-OH-D_3 is the main circulating form of all the known metabolites of vitamin D_3. There is as yet no reliable data on the concentration of vitamin D_3 in the blood, but it is believed to be very low (~2–3 ng/ml). The blood level of 25-OH-D_3 was initially determined by competitive protein binding methods

[1] Celite.

(52–54) which showed values of 15–38 ng/ml in normal humans. Since then, more reliable and precise high-performance liquid chromatographic (HPLC) methods have been developed (55–58) but the mean values were in the same range.

The endogenous level of 25-OH-D_3 in the cow was determined by HPLC (59, 60). It was generally in the range of 40–58 ng/ml of serum or plasma (mean 48 ± 5 ng/ml, n = 24), which is higher than that in the human. Following a single intravenous dose of 4 mg of 25-OH-D_3 to a cow, the average serum level declined from a high of 227 ng/ml at 1-hr postinjection, to 51 ng/ml after 51 days, showing an elimination half-life of ~10 days (61). These and other studies have confirmed the earlier findings that 25-OH-D_3 is the major circulating form of vitamin D in the blood of humans and cows.

The concentration of 1,25-$(OH)_2D_3$ in the human serum is very low, between 20 and 40 pg/ml. The concentration is determined by ligand binding methods after extensive cleanup of the serum extract. The most recent procedures use fractionization of the extract by HPLC to purify 1,25-$(OH)_2D_3$ from interfering materials prior to quantitation. Mean plasma values of 35 ± 3 (62), 34 ± 11 (63), and 31 ± 9 (64) pg/ml were reported in normal healthy adults. None was detected in the blood of anephric men (64), confirming that 1-hydroxylation takes place in the kidney. At the present time, 1,25-$(OH)_2D_3$ is considered to be the most potent known metabolite of vitamin D controlling calcium and phosphorus absorption from the intestine and mobilization of these elements from the bone when necessary.

Other Metabolites of Vitamin D_3—*25,26-Dihydroxyvitamin D_3*—It became known during the isolation and identification of 25-OH-D_3 that there were other metabolites in certain tissues and blood following administration of physiological doses of radioactive vitamin D. Suda *et al.* (65) prepared extracts of hog plasma containing these metabolites in a manner similar to the one used by Blunt *et al.* (32). The peak V portion in Fig. 1 was resolved into at least three components, designated V*a*, V*b*, and V*c*, on a second silicic acid column. The V*c* portion was rechromatographed on a diatomaceous earth[1] partition column specially designed for peak V metabolites (66). The V*c* peak was resolved into four peaks. The major peak V*c*3 was further purified on a Sephadex LH-20 column and a radiochemically pure fraction was obtained. This fraction was identified as 25,26-dihydroxyvitamin D [25,26-$(OH)_2D_3$]. This metabolite was about one-half as active as 25-OH-D_3 in the stimulation of intestinal calcium transport. The concentration of this metabolite is very low in the normal human; a value of 0.8 ± 0.4 ng/ml has been reported (67).

24,25-Dihydroxyvitamin D_3—Omdahl and DeLuca (68) reported that the inhibition of intestinal calcium transport due to dietary strontium results from a block of the kidney hydroxylase which produces 1,25-$(OH)_2D_3$ from 25-OH-D_3. They also found that when the synthesis of 1,25-$(OH)_2D_3$ was suppressed, a new metabolite appeared which behaved like the peak V*a* metabolite already described. This metabolite is made exclusively by the kidney (69, 70) and was identified as 24,25-dihydroxyvitamin D_3-[24,25-$(OH)_2D_3$] (71). This metabolite can be produced *in vitro* by incubating 25-OH-D_3 with kidney mitochondria from chickens fed a high calcium diet. It can also be produced *in vivo* in hogs.

Although the structure of 24,25-$(OH)_2D_3$ was determined (71) and confirmed by synthesis (72), the stereochemical configuration of the 24-hydroxyl group was not known. Tanaka *et al.* (73) showed that the trimethylsilyl ethers of 24*R*- and 24*S*-$(OH)_2D_3$ could be separated by HPLC and that the biologically active form was 24*R*,25-$(OH)_2D_3$. The concentration of this metabolite in the plasma of normal animals and humans is low. For example, Taylor *et al.* (74) reported a value of 1.68 ± 0.82 ng/ml among seven normal volunteers. In these subjects, the 25-OH-D_3 level was 8.8–35.7 ng/ml. Other investigators (62, 63, 67) have reported values of ~2 ng/ml of plasma.

It was demonstrated (75, 76) that under normal or hypercalcemic conditions, 24,25-$(OH)_2D_3$ is the major circulating metabolite of 25-OH-D_3. Like 1,25-$(OH)_2D_3$, it is capable of supporting growth, elevating serum calcium, and calcifying bones of rats on a normal calcium, normal phosphorus diet. Their data also demonstrated that 24,25-$(OH)_2D_3$ is capable of inducing intestinal calcium transport at dose levels similar to 1,25-$(OH)_2D_3$. The one major difference is that unlike the latter, 24,25-$(OH)_2D_3$ has little ability to mobilize calcium from the bone. The finding that nephrectomy blocks the biological activity of 24,25-$(OH)_2D_3$ led to the discovery that this metabolite can serve as a substrate for 25-OH-D_3-1-hydroxylase in the kidney, yielding a new, more polar metabolite, 1,24,25-trihydroxyvitamin D_3.

1,24,25-Trihydroxyvitamin D_3—1,24,25-trihydroxyvitamin D_3 [1,24,25-$(OH)_3D_3$] was first prepared (77) *in vitro* from tritium-labeled 24,25-$(OH)_2D_3$ by incubation with chicken kidney homogenates. A total of 50 μg of the labeled compound was incubated in 0.5 μg (120,000 dpm/μg) portions with 1.5 ml of the kidney homogenate at 37° for 30 min. The homogenates were combined and extracted as in previous experiments (71). The extract was subjected to several column chromatographic steps to obtain a radiochemically pure metabolite more polar than the starting material. This metabolite was identified as 1,24,25-$(OH)_3D_3$ by UV and mass spectrometry, and its reactivity to periodate treatment.

The biological activity of 1,24,25-$(OH)_3D_3$ parallels the biological activity reported for 24,25-$(OH)_2D_3$ (76). It is preferentially more active in inducing intestinal calcium transport than in mobilizing calcium from bone and is ~60% as effective as vitamin D_3 in the cure of rachitic lesions. Although on a weight basis it is less active both in the magnitude and duration of response than 1,25-$(OH)_2D_3$, it is probably much more active than 24,25-$(OH)_2D_3$ (77). There is no conclusive data on the concentration of 1,24,25-$(OH)_3D_3$ in the blood. It is known to be lower than that of 1,25-$(OH)_2D_3$ and, therefore, in the low picogram per milliliter range in the plasma.

1α-OH-24,25,26,27-Tetranor-D_3-23-carboxylic Acid (Calcitroic Acid)—During the studies on the metabolism of 1,25-$(OH)_2D_3$, the possibility that it might be converted to more active forms was investigated. In 1976, 26,27-^{14}C-labeled 25-OH-D_3 was synthesized and enzymatically converted to the corresponding 1,25-$(OH)_2D_3$ compound (78). When injected into vitamin D-deficient animals, this compound lost up to 20% of its 26,27-^{14}C in the expired air as carbon-14 dioxide within 24 hr, demonstrating the ex-

istence of a side-chain oxidation reaction of 1,25-$(OH)_2D_3$ (78, 79). This side-chain oxidation appeared to require 1α-hydroxylated metabolites, since nephrectomy abolished carbon-14 dioxide expiration from radiolabeled 25-OH-D_3 but did not affect the amount of carbon dioxide 14 expired from 1,25-$(OH)_2$[26,27-^{14}C]D_3-dosed rats (79). Removal of the jejunum, ileum, and colon drastically reduced the carbon-14 dioxide expired by these rats, implicating an intestinal site of side-chain oxidation (80).

In vivo experiments (81) using doses of 3α-^{3}H- and 26,27-^{14}C-labeled, 1,25-$(OH)_2D_3$ were conducted with examination of tissue extracts for metabolites relatively enriched in tritium. This study culminated in the isolation and identification of the major side-chain oxidized metabolite of 1,25-$(OH)_2D_3$ as 1α,3β-dihydroxy-24,25,26,27-tetranor-D_3-23-carboxylic acid, which was named calcitroic acid.

Calcitroic acid was found in the liver and intestinal tissue, but very little was present in the blood. The amount of calcitroic acid in the organic soluble extracts of the liver and intestine accounted for only 4–6% of the administered dose. It was recently found (82) that the water-soluble metabolites in rat bile are predominantly acids. As much as 13% of the dose present in bile after 24 hr was calcitroic acid. This means that substantial amounts of unknown metabolites of 1,25-$(OH)_2D_3$ remain in bile.

The function of the side-chain oxidation of 1,25-$(OH)_2D_3$ is not known. One possibility is that side-chain oxidation and biliary excretion are a route for inactivation and excretion of 1,25-$(OH)_2D_3$. Alternatively, metabolites of 1,25-$(OH)_2D_3$ in bile may be reabsorbed by the intestine and have biological function there (82). Calcitroic acid was chemically synthesized from 24-oxocholesterol acetate and its biological activity compared with 1,25-$(OH)_2D_3$ (83). It stimulated intestinal calcium transport 6 hr after intravenous administration, but the response was significantly less than that of an identical base of 1,25-$(OH)_2D_3$. It had no effect in the mobilization of calcium from the bone.

Δ^{24}-1α-Hydroxy- and Δ^{25}-1α-Hydroxyvitamin D_3—Onisko *et al.* (84), in their study of the metabolism of 1α,25-dihydroxy[3α-^{3}H]vitamin D_3 in the rat, found two minor radioactive compounds in the bile. These were identified as Δ^{24}-1α-hydroxyvitamin D_3 and Δ^{25}-1α-hydroxyvitamin D_3. The two compounds were present in the same ratio that is obtained by standard chemical dehydrations of the 25-hydroxy group. It is assumed that the isomers arise *via* a nonenzymatic elimination process in bile. The biological significance of these products is not known.

25-Hydroxyvitamin D_3-26,23-lactone—During the course of developing an assay procedure for 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ in human plasma, a previously unidentified compound was detected in chick plasma (85, 86). This compound was a contaminant in the assay of 24,25-$(OH)_2D_3$ or 25,26-$(OH)_2D_3$ when only a single chromatographic column was used for pre-assay purification; however, it could be resolved by HPLC on a microparticulate silica column (87). This metabolite was extracted from the plasma of chickens and was identified (88) as 25-hydroxyvitamin D_3-26,23-lactone (25-OH-D_3-26,23-lactone) from its UV, mass, Fourier transform IR, and PMR spectra. Hollis *et al.* (89) found that 25,26-$(OH)_2D_3$ is the precursor for 25-OH-D_3-26,23-lactone and the transformation takes place in the kidney, but its biological importance is not yet known.

1α,25-Dihydroxyvitamin D_3-26,23-lactone—After the discovery of 25-OH-D_3-26,23-lactone, Tanaka *et al.* (90) investigated if 1,25-$(OH)_2D_3$ would undergo a similar transformation to the corresponding lactone. They found that kidney extracts from chicks given large doses of vitamin D_3 readily converted 1,25-$(OH)_2D_3$ to 1,24-25-$(OH)_3D_3$, but produced only trace amounts of the lactone. However, when they incubated 25-OH-D_3-26,23-lactone with kidney homogenates from rachitic chickens known to be rich in 1-hydroxylase activity and deficient in other vitamin D hydroxylases, they isolated a compound which was identified as 1α,25-dihydroxyvitamin D_3-26,23-lactone [1,25-$(OH)_2D_3$-26,23-lactone]. They postulated that because 1α-hydroxylase activity is generally low under the conditions in which the lactone is formed, the *in vivo* 26,23-lactonization of 1,25-$(OH)_2D_3$ would likely be minimal.

Another investigator (91) fed the synthetic analog of vitamin D_3, 1α-hydroxyvitamin D_3 (1α-OH-D_3), to rats and isolated 1,25-$(OH)_2D_3$-26,23-lactone from the blood. The biological potency or significance of this metabolite is not established, and to the author's knowledge, it has not yet been isolated from human blood.

*25,26,27-Tris-*nor*-vitamin D_3-24-carboxylic Acid*—It was pointed out earlier that when 1-hydroxylation is inhibited, 25-OH-D_3 is metabolized to 24,25-$(OH)_2D_3$ (71), which in turn acts as the precursor for 1,24,25-$(OH)_3D_3$ (77). Recently, 24,25-$(OH)_2$ was found to rapidly metabolize to an acid with the loss of three terminal carbons on the side chain. This compound was identified as 25,26,27-tris-*nor*-vitamin D_3-24-carboxylic acid (92). The biological significance of this metabolite is not known.

25-Hydroxy-24-oxocholecalciferol—During the course of investigating renal 24-hydroxylase activity, it was found (93) that kidney homogenates from chicks supplemented with vitamin D_3 metabolized 25-OH[26,27-$^3H_6$]D_3 *in vitro* to three unknowns designated A, C, and E. Production of unknown A increased in parallel with the increase in the amount of 25-$(OH)D_3$ added as a substrate, while that of unknown C was fairly constant irrespective of substrate increase (94). Unknown A was less polar than 25-OH-D_3. This unknown was identified (95) as 25-hydroxy-24-oxocalciferol. The biological significance of this metabolite is unknown, and to this author's knowledge, there has been no confirmation of its presence *in vivo* in humans or animals.

25-Hydroxyvitamin D_2-25-β-D-glucuronic Acid—Previous studies (96) showed that the principal route of excretion of vitamin D metabolites in humans is through the bile. But the identity of most of the biliary metabolites is unknown. Avioli *et al.* (96) also found that in human subjects administered [^{3}H]vitamin D_3, only 8–9% of the biliary radioactivity was extractable with chloroform and that biliary excretion of vitamin D_3 was negligible. Upon treatment with β-glucuronidase, an additional 40% of the biliary radioactivity became chloroform soluble, but only ~5% of the liberated radioactivity appeared to be free vitamin D_3.

Examination of the biliary metabolites of vitamin D_2 in chickens revealed that the major metabolite was 25-hy-

droxyvitamin D_2-25-β-D-glucuronic acid (25-OH-D_2-25-glucuronide) (97). It has long been known that vitamin D_2 is much less active than vitamin D_3 in the chicken while the two forms are equally active in mammals. It was shown earlier (98) that vitamin D_2 is more rapidly removed from the chick blood than vitamin D_3 and excreted into the bile.

It was also reported (99) that the two forms of the vitamin are hydroxylated at C-25 equally by chick liver preparations. Several studies have long established that 25-OH-D_3 is the main circulating form of vitamin D_3 in humans and cows (55–61). But other studies (100, 101) demonstrated that the chick is unable to raise the blood levels of 25-OH-D_2 and other D_2 metabolites to the D_3 metabolite levels. Based on these facts, one may conclude that the facile formation of 25-OH-D_2-25-glucuronide in chicks and its removal from circulation is the reason that chicks and other birds discriminate against vitamin D_2. LeVan *et al.* (97) reported from their preliminary experiments that chicks dosed with [^{3}H]vitamin D_3 produce only small amounts of the 25-glucuronide. Experiments are in progress in their laboratories to see whether the 25-glucuronides of vitamin D metabolites occur in mammalian bile or if this pathway is peculiar to avian species.

Vitamin D Sulfate—Initial experiments with radiolabeled vitamin D showed very little radioactivity in the aqueous portion after the kidney, intestine, serum, or plasma was extracted with a mixture of chloroform and methanol (29, 31). However, the formation of water-soluble conjugates from vitamin D or its metabolites has always been suspected. Thus, several investigators reported the presence of vitamin D sulfate (D-sulfate) in human (102–104) and cow milk (102, 104, 105).

Lakdawala and Widdowson (103) collected milk from 34 women from 3–8 days postpartum and analyzed for D-sulfate. The D-sulfate concentration was 17.8 ng/ml between the 3rd and 5th days and 10.0 ng/ml between the 6th and 8th days, respectively. These authors noted that even in winter, breast milk protected the infants from rickets whereas cow milk did not. Sahashi *et al.* (102) found the concentration of D-sulfate in cow milk and human milk to be 204 and 950 IU/liter (5 and 24 ng/ml), respectively. These figures were all based on a nonspecific colorimetric method.

Very recently, (106) a more specific HPLC method was used for the analysis of human milk whey from six Caucasian mothers between 1 and 8 days postpartum using 1α,2α-[^{3}H]vitamin D sulfate as a tracer to monitor the recovery of the endogenous D-sulfate. No endogenous D-sulfate was found in any of the samples (detection limit, 1 ng/ml). This result was contradictory to that reported by others (102–105). Hollis's (106) contention was that the primary source of antirachitogenic activity in human milk is due to nonconjugated metabolites of vitamin D, primarily 25-OH-D_3. Yet, according to another report by Hollis *et al.* (107), the nonconjugated metabolites in human and cow milk are very low; human milk contained 320 ± 80 pg/ml (n = 5) of 25-OH-D_3. The concentrations of 24,25-$(OH)_2D_3$ and 1,24,25-$(OH)_3D_3$ in the same samples were 42 ± 3 and 12 ± 2 pg/ml, respectively. These results are puzzling when one considers the fact that the large majority of infants in the world are raised primarily on breast milk for up to 6–8 months without any gross manifestations of vitamin D deficiency.

Unidentified Metabolites of Vitamin D_3—Leading investigators are certain that there are many unidentified metabolites of vitamin D. For example, according to Onisko *et al.* (84), there remain substantial amounts of unknown metabolites of 1,25-$(OH)_2D_3$ in the bile. These are water-soluble, negatively-charged compounds, which are rendered chloroform-soluble after methylation. These are likely to be due to unknown carboxylic acid metabolites.

Most of the experiments leading to the discovery of the currently known metabolites have followed the injection of radioactive vitamin D_3 as a single dose to rachitic animals. Experiments with administration of radioactive vitamin D_3 daily for 1–2 weeks (108), have led to the discovery of a number of new metabolites of vitamin D_3, both polar and nonpolar, that have yet to be identified. Whether any of these will have important functions or whether they represent biochemical curiosities or inactivation products remains to be seen.

METABOLITES OF VITAMIN D_2

The only chemical difference between vitamins D_2 and D_3 is in the side chain. Vitamin D_2 has an extra methyl group on the 24th carbon and a double bond between the 22nd and 23rd carbons (Scheme II). The virtual inactivity of vitamin D_2 in birds has long been known. However, the major metabolic pathways of the two forms of vitamin D in birds and mammals are analogous. Thus, 25-OH-D_2 was isolated and identified from porcine blood (38). The 25-OH-D_2 is further converted in the kidney to 1,25-$(OH)_2D_2$, which was isolated and identified (109). The hydroxylases that act on the vitamin D_3 series have been shown to act on vitamin D_2 compounds as well (110). Thus, the liver possesses vitamin D_2-25-hydroxylase and the kidney possesses 1α-hydroxylase. The current opinion is that the primary metabolites of vitamin D_2 would undergo further metabolism like vitamin D_3, but little work has been carried out with humans or animals, primarily because of the unavailability of suitable radiolabeled tracers.

SUMMARY OF THE METABOLIC SCHEME OF VITAMIN D

The currently known metabolic pathway of vitamin D is shown in Scheme III. Vitamin D_3 from the diet or that generated in the skin is absorbed into the blood. There is as yet no reliable figure on its concentration in blood. A portion of the vitamin D is converted into fatty acid esters (34) which are distributed in all vital organs and blood. The fatty acid esters have lower biological potency than vitamin D. Not much research has been conducted on the role of fatty acid esters in the total picture of vitamin D metabolism. A portion of the vitamin D is believed to be available in lactating mammals as D-sulfate (102–105), but this theory has been contradicted by other investigators (106, 107).

Vitamin D is stored to a considerable extent in the body. The liver is thought to be a storage organ, but more recent studies have shown that fat is the major storage site (111). Depletion of vitamin D in the animals depends on the turnover of the fat depots, which usually takes considerable time.

Vitamin D_3 *per se* has no biological activity. It is hydroxylated at carbon 25 to 25-OH-D_3 (35, 36) in the liver. The liver 25-hydroxylase system requires NADPH, mo-

lecular oxygen, and magnesium and is located in the microsomal fraction. Initially, it was thought that this 25-hydroxylation was feed-back regulated (112) to reduce the chances of toxicity to over-intake of vitamin D_3. However, later studies (113) with rats that received weekly or triweekly doses of vitamin D_3 showed that the amount of 25-OH-D_3 in plasma correlated with an increase in vitamin D intake, irrespective of the dose administered. Thus, it seems that vitamin D is efficiently mobilized to 25-OH-D_3 in the liver and then bound to a plasma-binding protein where it acts as a reservoir in the blood. However, other 25-hydroxylases are also present in the liver. These act on cholesterol, dihydrotachysterol, and other analogs of vitamin D such as 1α-hydroxyvitamin D_3 (114–116).

The major circulating form of vitamin D metabolites in the blood is 25-OH-D_3 (37, 55–58). It is transported to the kidney by a specific protein and further hydroxylated on carbon C-1 to 1,25-$(OH)_2D_3$ or on C-24 to 24,25-$(OH)_2D_3$ depending on the circumstances. Under conditions of hypocalcemia, the parathyroid glands are stimulated to secrete parathyroid hormone (PTH), which in turn stimulates synthesis of 1,25-$(OH)_2D_3$. The 1,25-$(OH)_2D_3$ immediately proceeds to the intestine where it functions to stimulate intestinal calcium absorption without PTH. In addition, 1,25-$(OH)_2D_3$ together with the PTH hormone functions to mobilize calcium from previously formed bone. These two functions result in the elevation of serum calcium to normal levels that suppress PTH secretion and, hence, 1,25-$(OH)_2D_3$. Like hypocalcemia, hypophosphatemia directly stimulates synthesis of 1,25-$(OH)_2D_3$, which then independently stimulates the elevation of serum inorganic phosphate.

The mechanism of action of 1,25-$(OH)_2D_3$ was proved (50, 117) after techniques were developed for making [^{3}H]-1,25-$(OH)_2D_3$ from [^{3}H]-25-OH-D_3 *in vitro* from kidney homogenates. Using this technique, these investigators were able to correlate the presence of 1,25-$(OH)_2D_3$ in the intestine and bone, which accounted for 98 and 80% of the total radioactivity in the respective target tissues, with the simultaneous induction of intestinal calcium transport and bone calcium mobilization. These data strongly supported the earlier findings that 1,25-$(OH)_2D_3$ is the biologically active form of vitamin D_3 responsible for maintaining calcium and phosphorus homeostasis and that no further metabolism is required.

Under normocalcemic or hypercalcemic conditions, renal 1α-hydroxylation is inhibited and side-chain hydroxylation of 1,25-$(OH)_2D_3$ occurs, resulting in the formation of the most polar metabolite known so far, 1,24*R*,25-$(OH)_3D_3$ (77). Under these conditions 25-OH-D_3 also undergoes side-chain oxidation to form 24*R*,25-$(OH)_2D_3$ (71) and 25,26-$(OH)_2D_3$ (66). Of these two, 24*R*,25-$(OH)_2D_3$ is the more prominent. There is evidence suggesting that the intracellular concentration of phosphorus is a more important regulator than PTH for determining whether the hydroxylation of 25-OH-D_3 in the kidney occurs on either the C-1α or the C-24 positions (118). The ability of 24*R*,25-$(OH)_2D_3$ to stimulate both intestinal calcium transport and a weak bone mineralization response is believed to be dependent on its 1α-hydroxylation in the kidney to form 1,24*R*,25-$(OH)_3D_3$.

Thus, under normal conditions, one of the end products of both 1,25- and 24*R*,25-dihydroxyvitamin D is the same, *i.e.*, 1,24*R*,25-$(OH)_3D_3$. 1,24*R*,25-$(OH)_3D_3$ is only ~10% as active as 1,25-$(OH)_2D_3$ on a molar basis. It is more effective in stimulating calcium transport than bone mineralization (77, 119).

The low biological activity of 24*R*,25-$(OH)_2D_3$ and 1,24*R*,25-$(OH)_3D_3$ and their rapid metabolism and excretion in the chick prompted speculation that the purpose of 24-hydroxylation of 25-OH-D_3 and 1,25-$(OH)_2D_3$ is to introduce a hydroxyl group proximal to the 25 position to provide facile oxidative cleavage (120). Yet, it is puzzling that these two 24-hydroxylated metabolites are prominent circulating metabolites. Recently, it was reported (121) that small doses of 24*R*,25-$(OH)_2D_3$ increased intestinal calcium transport in anephric patients, suggesting that the compound *per se* is active and 1α-hydroxylation is not absolutely necessary.

When the body does not need 24*R*,25-$(OH)_2D_3$ or 1,24*R*,25-$(OH)_3D_3$, the former is converted to 25,26,27-tris-*nor*-vitamin D_3-24-carboxylic acid (92). The 25,26-$(OH)_2D_3$ undergoes more extensive transformations. After it was synthesized, its biological activity was studied in vitamin D-deficient rats and their nephrectomized counterparts. The results suggested that, like 24*R*,25-$(OH)_2D_3$, it requires renal 1α-hydroxylation before it can stimulate an intestinal calcium transport response (122). As discussed earlier it is also oxidized to calcitroic acid (81) and to 25-OH-D_3-26,23-lactone (88).

VITAMIN D AS A PROHORMONE

Vitamin D is virtually absent from the plant world and is found only in specific substances such as fish liver oils, egg yolk, and to a very small degree, in unfortified milk. A notable exception to this is the existence of conjugated forms of 1,25-$(OH)_2D_3$ in the South American plant *Solanum glaucophyllum* and *Cestrum diurnum* in the southern United States (123, 124). Vitamin D is unique in that under normal circumstances it is formed in the skin in sufficient quantities when humans and animals are exposed to sunshine, and extra supplementation is unnecessary. The previtamin D_3 that is formed in the skin is slowly released into the body as vitamin D_3. This process permits the skin to continuously synthesize and release vitamin D_3 into circulation for up to 3 days after a single exposure to sunlight (Scheme I and Ref. 14).

Because of changes in modern lifestyle and because the urban areas around the world are contaminated with UV-absorbing materials, it may be that insufficient amounts of vitamin D are produced in the skin. Vitamin D_3 is also unique in that it has to be metabolized in sequence to 25-OH-D_3 and to 1,25-$(OH)_2D_3$ or to some as yet unidentified metabolite before it becomes physiologically active, not only in its ability to prevent and cure rickets, but also to maintain calcium and phosphorus homeostasis. In this respect, 25-OH-D_3 only acts as a precursor for 1,25-$(OH)_2D_3$ and also for 24*R*,25-$(OH)_2D_3$ which is also biologically active.

Since 1,25-$(OH)_2D_3$ acts on tissues remote from its production site, it meets the criteria and definition of a hormone. In true hormonal form, its biogenesis is regulated by hypocalcemia or hypophosphatemia. PTH plays a vital role in the biochemistry of vitamin D_3. If 1,25-$(OH)_2D_3$ is accepted as a hormone, then vitamin D_3 can be considered as a prohormone and 25-OH-D_3 as a prehormone. Since the

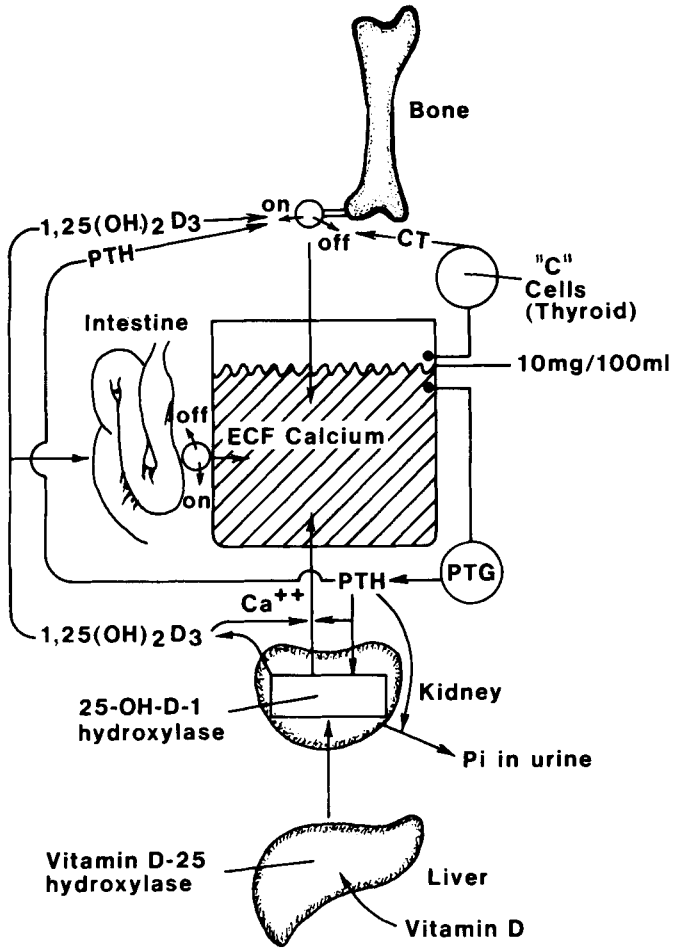


Figure 2—*Diagram of the calcium homeostatic mechanisms involving the vitamin D endocrine system (Ref. 132).*

kidney is the sole site of manufacture of 1,25-$(OH)_2D_3$, the kidney can be considered as the endocrine organ.

REGULATION OF CALCIUM HOMEOSTASIS AND VITAMIN D METABOLISM

The normal serum calcium level is maintained at ~10 mg/100 ml of ionized calcium. The accompanying anion is inorganic phosphorus. When the calcium level falls, the parathyroid glands which serve as the hypocalcemic detection organ are immediately stimulated to produce PTH. PTH is transported to the kidney, liver, and bone (125, 126). In the kidney it causes a phosphate diuresis which is vitamin D independent (127). PTH also stimulates renal reabsorption of calcium. Whether vitamin D is involved in this process is not absolutely established, but available evidence suggests that it is dependent on the existent serum 1,25-$(OH)_2D_3$ (128, 129).

Another, less rapid, calcium-conserving process occurs when PTH stimulates renal 25-OH-D-1-hydroxylase to produce 1,25-$(OH)_2D_3$ (130) and 1,24*R*,25-$(OH)_3D_3$; 1,25-$(OH)_2D_3$ stimulates intestinal calcium absorption without PTH involvement (128, 129). PTH is initially secreted in response to hypocalcemia within minutes, and its lifetime is measured in minutes (125, 126). In contrast, the action of 1,25-$(OH)_2D_3$ requires hours and its lifetime is measured in hours. Thus, PTH serves in a short-term renal process for restoring the calcium level, whereas the action of 1,25-$(OH)_2D_3$ is more prolonged. In the bone, the secreted PTH, together with 1,25-$(OH)_2D_3$, stimulate the transfer of calcium from the bone fluid compartment to the extracellular fluid compartment and into general circulation.

The three sources of calcium described restore serum calcium to the normal level, at which time PTH secretion is suppressed, shutting down the entire calcium mobilizing system. On the other hand, in cases of hypercalcemia, C cells of the thyroid secrete calcitonin, which suppresses mobilization of calcium from the bone and probably stimulates excretion of calcium and phosphorus in the kidney (131). In this manner the vitamin D endocrine system efficiently controls calcium homeostasis as shown in Fig. 2 (132).

In addition to phosphate, PTH, and 1,25-$(OH)_2D_3$, sex-related regulatory phenomena are known to affect calcium absorption. For example, there is a great need for calcium in egg-laying birds. Egg-laying birds have a high level of 1-hydroxylase activity in their kidneys, while nonlaying mature females or males have little 1-hydroxylase activity but higher 24*R*-hydroxylase activity (133, 134). During pregnancy and lactation, plasma 1,25-$(OH)_2D_3$ is elevated in both rats and humans (135, 136) to meet increased calcium demand. Growth hormone and prolactin are believed to provide the stimuli for increased 1-hydroxylase activity (137). These data suggest that sex hormones and other endocrine systems are also involved in the calcium regulatory processes.

Several review articles discuss the biogenesis of vitamin D, its metabolism, the mechanism of action of the metabolites in maintaining calcium and phosphorus homeostasis, and the clinical applications of vitamin D metabolites (132, 138–149).

CLINICAL APPLICATIONS OF VITAMIN D METABOLITES

It is beyond the scope of this article to go into a detailed discussion of the clinical applications of vitamin D metabolites. Of all the metabolites, 25-OH-D_3 and 1,25-$(OH)_2D_3$ are the most important and both are available commercially as prescription products in the United States and West European and Scandinavian countries. The clinical uses of these two compounds are for bone diseases related to abnormal calcium and phosphorus deficiencies in the blood.

As already mentioned, 25-OH-D_3 is the major circulating form of vitamin D_3 and has to be converted to 1,25-$(OH)_2D_3$ to function in the restoration of calcium and phosporus serum levels. The normal serum level of 25-OH-D_3 is 20–40 ng/ml, whereas that of 1,25-$(OH)_2D_3$ is only 20–40 pg/ml. The serum level of 25-OH-D_3 can go up depending on the vitamin D intake or exposure to sunshine, whereas that of 1,25-$(OH)_2D_3$ is constant and regulated according to the calcium level in the serum. In addition, 25-OH-D_3 is much less toxic than 1,25-$(OH)_2D_3$; therefore, 25-OH-D_3 is preferred in all calcium deficiency diseases where renal 1-hydroxylase activity is not impaired by lack of PTH stimulation or the inability of the kidney to produce this enzyme.

Since the discovery of the major metabolites of vitamin D_3, several synthetic analogs were made and researched not only to elucidate the mechanism of vitamin D_3 metabolism, but also as possible substitutes for 25-OH-D_3 and

1,25-$(OH)_2D_3$. Of these, the most prominent one is 1α-hydroxyvitamin D_3 (1α-OH-D_3) which is hydroxylated very readily in the liver to 1,25-$(OH)_2D_3$ (150). Except for a few specific diseases, the rationale for the use of these D_3 metabolites in the treatment of bone diseases is clouded by the interplay of the other less active metabolites and also by the several hormones and enzymes involved in the bone mineralization and bone formation processes. For example, 24,25-$(OH)_2D_3$ was until recently considered as the first of a series of metabolites in the excretionary route for 25-OH-D_3. In normocalcemic animals, 24-25-$(OH)_2D_3$ is the predominant dihydroxylated metabolite, and even though only a few humans have been treated to date with this metabolite, it was reported (149, 151) that it may have an important adjunctive role in the treatment of bone diseases.

Rickets—Rickets was originally thought of as a bone disease, but later found to be a blood disease in the sense that it appeared when the calcium and phosphorus levels in the blood were low. This led scientists to believe that the product of calcium and phosphorus concentrations in the blood was the determining factor in the production of rickets and this measure was used as a diagnostic test. With the mandatory practice of fortifying fresh and evaporated milk, rickets as a nutritional disease has almost been eliminted in the western world.

In addition to vitamin D-deficiency rickets, there are the vitamin D-dependent and vitamin D-resistant hypophosphatemic rickets. Vitamin D-dependent rickets is an inherited disease in children that occurs even when the patients take the daily requirement of vitamin D. These children develop aminoaciduria. The disease is treated by high doses of vitamin D (152), pharmacological doses of 25-OH-D_3 (153), or as little as 1 μg/day of 1,25-$(OH)_2D_3$ (153, 154). It is now believed that this disease is a defect of 1-hydroxylation of 25-OH-D_3. The cure with large doses of vitamin D_3 or 25-OH-D_3 is probably due to the 25-OH-D_3 interacting directly with the receptors.

Vitamin D-resistant hypophosphatemic rickets is more common and is characterized by low blood phosphorus, normal calcium serum concentration, and either normal or reduced intestinal calcium absorption. Studies using a strain of mice having the same genetic and phenotypic characteristics as the human in the development of this disease demonstrated that there is a generalized defect in phosphate transport reactions, especially in the kidney and the intestine (155, 156). Patients with this disease are given large doses of phosphate to counteract the large losses in the urine. Large doses of phosphate can bring about excessive secretion of PTH, which has to be corrected by the administration of 1,25-$(OH)_2D_3$ or 1α-OH-D_3. Thus, the treatment of this type of rickets is a complicated system of checks and balances.

Hypoparathyroidism—PTH secreted by the parathyroid gland senses hypocalcemia (Scheme III). Hypoparathyroid patients do not have this ability and therefore do not metabolize 25-OH-D_3 to 1,25-$(OH)_2D_3$. As a result, the serum calcium levels drop while inorganic phosphate may rise. Previously, such patients were treated with large doses of vitamin D, but now these cases can be treated better with 1,25-$(OH)_2D_3$ or 1α-OH-D_3 at doses of 0.68–2.7 μg/day plus sufficient dietary calcium (157–159). These small doses caused a marked increase in serum calcium concentration and urinary calcium excretion, without significant changes in renal calcium clearance or urinary hydroxyproline excretion. These results suggest that the correction of hypocalcemia involved primarily a stimulation of intestinal calcium absorption rather than a stimulation of skeletal calcium absorption.

Pseudohypothyroidism—Pseudohypothyroid patients secrete sufficient amounts of PTH in response to hypocalcemia, but the kidney and bone do not respond. The result is that patients become hypocalcemic in the presence of large circulating levels of PTH. These patients are treated satisfactorily with oral 1,25-$(OH)_2D_3$ or 1α-OH-D_3 plus calcium. In pharmacological amounts 25-OH-D_3 has also been used successfully for this condition.

Renal Osteodystrophy—Patients with renal osteodystrophy have lost the ability to make 1,25-$(OH)_2D_3$, the calcium-mobilizing hormone. This disease is complicated by the fact that the kidney fails to excrete phosphate, thereby causing a secondary hyperparathyroidism. This in turn can cause excessive erosion of bone calcium giving rise to osteitis fibrosa. Inadequate amounts of 1,25-$(OH)_2D_3$ may also lead eventually to bone resistance to PTH (160). Patients with renal osteodystrophy have been successfully treated with 1,25-$(OH)_2D_3$, 25-OH-D_3, and 1α-OH-D_3 (161–164).

Both 25-OH-D_3 and 1,25-$(OH)_2D_3$ are approved in the United States for the treatment and management of patients undergoing renal dialysis for diseases associated with chronic renal failure or hypocalcemia. At the present time, these are the only clinical conditions for which the two compounds have received approval from the Food and Drug Administration.

The cause for renal osteodystrophy is multifunctional, involving more than excess PTH and/or 1,25$(OH)_2D_3$ deficiency. The exact reasons for the development of one form of bone disease *versus* another remains to be clarified. Although the availability of vitamin D metabolites has substantially improved the therapeutic outlook, the relative merits of 1,25-$(OH)_2D_3$ and 25-OH-D_3 in the management of this bone malfunction remain controversial.

Three predominant forms of renal osteodystrophy are recognized. These are osteitis fibrosa, osteomalacia, and a combination of the two. From available but unpublished clinical data, one might make the following conclusions.

1. Osteitis fibrosa patients with serum calcium <10 mg/dl and phosphorus <5 mg/dl respond about equally to either 1,25-$(OH)_2D_3$ or 25-OH-D_3. Patients on 1,25-$(OH)_2D_3$ treatment may require closer observation because the fluctuation in serum calcium is probably greater than during treatment with 25-OH-D_3. Treatment with either compound is contraindicated for patients with serum calcium >11 mg/dl. If the hypercalcemia is marginal (10–11 mg/dl), 25-OH-D_3 is preferable to 1,25-$(OH)_2D_3$ because it may be less likely to cause an abrupt rise in serum calcium.

2. For patients with pure osteomalacia and serum phosphorus levels <5 mg/dl, 25-OH-D_3 is clearly the treatment of choice. If the calcium level in these patients is >10 mg/dl, a low calcium diet may permit sufficient doses of 25-OH-D_3 to be administered without provoking significant hypercalcemia (>11 mg/dl).

3. In patients with mixed osteomalacia and osteitis fibrosa (serum phosphorus <5 mg/dl), positive responses

have been reported with both 1,25-$(OH)_2D_3$ and 25-OH-D_3. Overall mineralization might be promoted better with 25-OH-D_3 than with 1,25-$(OH)_2D_3$. This is probably because of the role played by 24,25-$(OH)_2D_3$ (149, 151) generated from 25-OH-D_3 in the bone formation process.

Steroid-Induced Osteoporosis—Administration of glucocorticoids results in the thinning of the bones or osteoporosis. The exact reason for this disease is not known. Even though reports on the effect of glucocorticoids on the metabolism of vitamin D conflict, some indicate that administration of either 25-OH-D_3 or 1,25-$(OH)_2D_3$ is beneficial in correcting this problem (165).

Postmenopausal and Senile Osteoporosis—There is convincing evidence that sex hormones may affect vitamin D metabolism and calcium absorption (133–137). It has been shown that intestinal calcium absorption and serum levels of 1,25-$(OH)_2D_3$ diminish with age (165, 166). It has also been suggested that osteoporotic patients may have lower levels of 1,25-$(OH)_2D_3$ in their plasma than controls (167). In addition, there is evidence that the intestinal calcium absorption of osteoporotic patients can be enhanced by 1,25-$(OH)_2D_3$, but not by vitamin D_3 itself. This suggests that age- and sex-related factors prevent the metabolism of vitamin D_3 to its active forms. Several clinical trials are ongoing with 25-OH-D_3, 1,25-$(OH)_2D_3$, and 1α-OH-D_3 for the treatment of this fairly common disorder.

In one study (168), oral administration of 25-OH-D_3 for 3 months to six patients with postmenopausal osteoporosis was effective in raising low intestinal calcium absorption and in augmenting the production of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. If 24,25-$(OH)_2D_3$ is important for bone formation as has been suggested (149, 151) then 25-OH-D_3 may be preferred over 1,25-$(OH)_2D_3$, since the latter is not converted to 24,25-$(OH)_2D_3$ in the body.

Anticonvulsant Drug-Induced Osteomalacia—There is a high incidence of osteomalacia among patients treated with the antiepileptic drug combination of phenytoin[2] and phenobarbital. This condition is generally treated with vitamin D (~4000 IU daily). It is obvious that this disease can be treated with lower doses of the more active metabolites. Stamp *et al.* (169) reported good results with 25-OH-D_3.

Hepatic Disorders—Since vitamin D is first hydroxylated in the liver, it seems that 25-OH-D_3 would be indicated for people with hepatic disorders. Cirrhosis of the liver does not appear to cause markedly reduced 25-OH-D_3 levels in the blood. If patients with hepatic disorders also have bone diseases associated with low serum calcium levels, it is quite likely that they would benefit from 25-OH-D_3 administration.

TOXICITY OF VITAMIN D AND ITS METABOLITES

Vitamin D_3 is toxic at high doses. Since it is converted in sequence to 25-OH-D_3 and to 1,25-$(OH)_2D_3$, and since 1,25-$(OH)_2D_3$ is the most biologically active form, it is not surprising that the margin of safety is lowest for 1,25-$(OH)_2D_3$ and highest for vitamin D_3. The recommended daily intake of vitamin D is 400 IU (10 μg). There is a considerable safety factor between the normal intake and the toxic dose, yet there are diseases like sarcoidosis and idiopathic hypercalcemia in which patients are overly sensitive to vitamin D. However, because there is an ample concentration of 25-OH-D_3 in normal human blood, there is no need to take more than 400 IU/day unless recommended by a physician. Continued daily intake exceeding 100,000 IU produces toxic manifestation. The symptoms include generalized weakness, nausea, vomiting, constipation, polyuria, dehydration, and elevated serum calcium and phosphorus levels. These adverse effects are reversible if the vitamin is withdrawn in time.

The crucial steps in the metabolism of vitamin D_3 are tightly controlled and feedback regulated. However, large doses of vitamin D_3 can overcome control of the 25-hydroxylation step. Haddad and Stamp (170) found a concentration of >600 ng/ml of 25-OH-D_3 in the serum of patients on long-term treatment with 5 mg (200,000 IU) of vitamin D for hypoparathyroidism or sex-linked hypophosphatemic rickets. So it is possible that the toxic effect of excessive intake of vitamin D_3 in normal individuals may be due to the abnormally high level of 25-OH-D_3 in the blood. The toxic symptoms are calcification of the heart, lungs, kidneys, and other soft tissues. Large doses of vitamin D will cause a marked demineralization of the bone at the same time that it causes hypercalcemia and nephrocalcinosis.

ANALYSIS OF VITAMIN D AND ITS METABOLITES

Biological and Chemical Methods—Biological and chemical methods are the oldest and probably the most widely accepted methods for the analysis of vitamin D. The biological methods can be divided into three groups: curative, prophylactic, and those based on calcium absorption into the blood stream. All are based on the administration of measured doses of a standard vitamin D preparation to a group of test animals and comparison of the biological responses with a similar group given the substance under test. A third group of test animals is used as controls. However, these methods are costly, do not distinguish vitamin D from other biologically active isomers and metabolites, and individual variations in the responses of the test animals are high. These tests and specific methods are detailed elsewhere (171–173).

The most widely used chemical method is the antimony trichloride colorimetric method. Antimony trichloride also reacts with vitamin A, and because vitamin A occurs along with D in many biological samples and is also an ingredient in many commercial products, it is necessary to remove it and other interfering substances prior to reaction with the reagent. This is generally achieved by saponification with alcoholic potassium hydroxide, extraction of the unsaponifiable fraction with petroleum ether, and cleanup on chromatographic columns. The procedure for the determination of vitamin D in pharmaceutical preparations is detailed elsewhere (172).

This method is applicable with suitable modifications to biological samples like fish liver oils, fortified milk, *etc.*, but the colorimetric procedures are being replaced when possible by more specific instrumental methods, usually HPLC. To the author's knowledge, there are no reported colorimetric procedures for vitamin D metabolites.

GLC—Although GLC procedures have been used extensively for the determination of numerous physiologically important steroids and hormones, only a few inves-

[2] Dilantin, Parke-Davis.

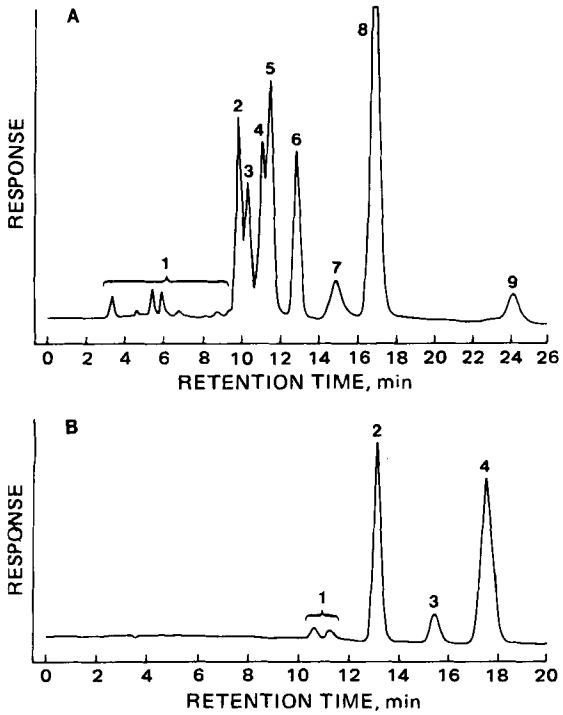


Figure 3—*Chromatogram of a synthetic mixture of photochemical isomers and reaction products of vitamin D_3 (A). Key: 1, unknowns; 2,* trans-*vitamin D_3; 3, previtamin D_3; 4, lumisterol$_3$; 5, isotachysterol$_3$; 6,* p-*dimethylaminobenzaldehyde (internal standard); 7, tachysterol$_3$; 8, vitamin D_3; and 9, 7-dehydrocholesterol. Chromatogram of a typical vitamin D_3 resin (B). Key: 1, resin impurities; 2,* p-*dimethylaminobenzaldehyde (internal standard); 3, tachysterol$_3$; and 4, vitamin D_3 (Ref. 176).*

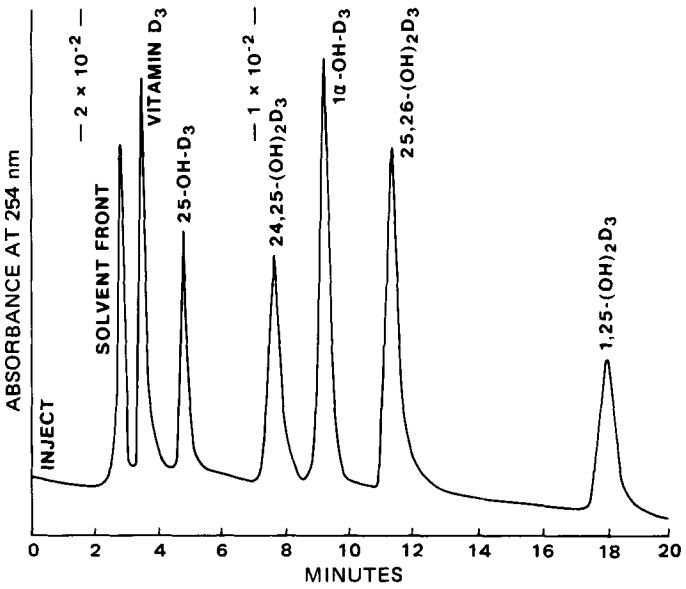


Figure 4—*Chromatogram of vitamin D_3 and its metabolites. A mixture of 40 ng of vitamin D_3, 30 ng of 25-OH-D_3, 25 ng of 24,25-$(OH)_2D_3$, 40 ng of 1α-OH-D_3, 40 ng of 25,26-$(OH)_2D_3$, and 25 ng of 1,25-$(OH)_2D_3$ were injected in 10 μl of 10% isopropanol in Skellysolve B using a U-6 K injector (Waters). With 10% isopropanol in Skellysolve B at 3000 psi pressure and two Zorbax-SIL (DuPont) (2.1 mm × 25 cm) columns in series, a flow rate of 0.5 ml/min was achieved (Ref. 177).*

tigators have attempted to develop similar techniques for vitamin D. The major problems with the GLC analysis of vitamin D are due to its open ring structure incorporating three conjugated double bonds in a 5,6-*cis* configuration. The 5,6-*cis* double bond causes less controllable thermal cyclization into the pyro- and isopyrocalciferols at operating GLC temperatures, resulting in two peaks. Another reason for the lack of interest in GLC analysis is that vitamin D concentration in biological systems is low and the samples generally contain structurally similar compounds requiring extensive sample cleanup. GLC methods for vitamin D analysis were reviewed previously (174, 175). In recent years, HPLC has become the most popular technique for analyzing vitamin D and 25-OH-D_3 and also serves as an important purification step prior to the ligand-binding methods for 1,25-$(OH)_2D_3$,24,25-$(OH)_2D_3$, and 25,26-$(OH)_2D_3$.

Competitive Protein-Binding Methods—It was once believed that 25-OH-D_3 was the most important biologically active metabolite of vitamin D. This belief, coupled with the fact that 25-OH-D_3 is the most abundant of all metabolites in the blood, stimulated a great deal of interest in its analysis.

Proteins with high binding capacity for 25-OH-D_3 were discovered and were utilized in the development of several competitive protein-binding assay procedures (52–54). Similar competitive protein binding methods are also available for vitamin D_3 and its more polar metabolites with much lower concentrations in the blood.

Several different binding proteins have been discovered with either greater binding capacity or greater specificity for a particular metabolite. Under standardized conditions, competitive protein-binding methods are used extensively because the sample volume is very small (0.1–1 ml), sensitivity is unattainable by other methods, sample manipulation is minimal, and a large number of samples can be analyzed simultaneously. The disadvantages are interference from related compounds that compete with the binding-proteins and lack of precision. The interference from related compounds is now minimized greatly by the use of HPLC.

HPLC—The main advantage of HPLC over GLC is that the problems associated with thermal cyclization and degradation are eliminated. Vitamin D and related compounds have a molar extinction coefficient of ~15,000 at 254 nm which allows the use of the most common and stable spectrophotometric detector available for HPLC. Therefore, HPLC is currently the technique of choice for vitamin D analysis. It is invaluable for the analysis of 25-OH-D_3 in biological samples and is also used extensively for purifying the more polar metabolities prior to their analysis by ligand binding methods. Since HPLC is nondestructive the fractions of interest can be collected and used for further tests if necessary.

The photochemical and thermal byproducts of vitamin D synthesis are shown in Scheme II. Until the advent of HPLC it was almost impossible to determine the actual vitamin D content of such a complex synthetic mixture. The separation achieved by Tartivita *et al.* (176) is shown in Fig. 3. Figure 3A is the chromatogram of a crude synthetic mixture and Fig. 3B is of commercial vitamin D in the resin form. These chromatograms were obtained on a 30-cm × 4-mm i.d. commercial microparticulate silica column. The mobile phase was a 70:30:1 mixture of chloroform (free from ethanol and water), *n*-hexane, and tetrahydrofuran at a flow rate of 1 ml/min. Using this system, vitamin D_3 was quantitated in a resin sample containing

Table I—Representative HPLC Systems for the Analysis of Vitamin D and Metabolites

Column No.	Mobile Phase	Compounds Separated or Analyzed	Sample Matrix	Reference
1[a], 50 cm × 2.1 mm	10% isopropanol in Skellysolve B	All known metabolites of D_2 and D_3	Synthetic mixture	177
2[b], 5 μm, 25 cm × 2.1 mm	Acetonitrile–methanol–water (90:5:5)	25-OH-D_3	Human serum	55
2, 5 μm, 22 cm × 6.2 mm	1.5% Water in methanol	D_2 and D_3	Human serum	58
1, 5 μm, 22 cm × 6.2 mm	9% Water in methanol	25-OH-D_3 and 25-OH-D_2	Human serum	58
3[c], 10 μm, 60 cm × 6 mm	*n*-Hexane–isopropanol (88:12)	1,25-$(OH)_2$D	Human serum	62
1, 22 cm × 6.2 mm	10% Isopropanol in hexane	25-OH-D, 24,25-$(OH)_2$D, 1,25-$(OH)_2$D	Human serum	63
1, 25 cm × 4.6 mm	Isopropanol–hexane (1:24 v/v)	25-OH-D and 25-OH-D_3	Human serum	64
1, 25 cm × 4.6 mm	Isopropanol–hexane (1:9 v/v)	24,25-$(OH)_2$D, 25,26-$(OH)_2$D and 1,25-$(OH)_2$D	Human serum	64
2, 25 cm × 4.6 mm	Water–methanol (1:49 v/v)	D_2 and D_3	Human serum	64
1, 6 μm, 25 cm × 6.2 mm	*n*-Hexane–isopropanol–methanol (87:10:3)	25-OH-D_3, 24,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$	Human serum	178
1, 25 cm × 4.5 mm	10% Isopropanol in hexane	25-OH-D, 24,25-$(OH)_2$D, 25,26-$(OH)_2$D, 1,25-$(OH)_2$D	Human serum	67
1, 25 cm × 4.5 mm	2.5% Isopropanol in hexane	25-OH-D_2 and 25-OH-D_3	Human serum	67
4[d], 25 cm × 2.6 mm	Acetonitrile–methanol (1:1)	D_3	Fortified milk	179
5[e], 10 μm, 25 cm × 4 mm	1.25% Isopropanol in cyclohexane	D_3	Instant nonfat dried milk	180
6[f], 50 cm × 4.6 mm	Chloroform–*n*-hexane–acetic acid (70:30:1)	D_3	Cod liver oil	181
7[g], 25 m × 3 mm	5% Water in methanol	D_3	Cod liver oil	182
3, 30 cm × 4 mm	0.4% Ethanol in chloroform	D_3	Livestock feed supplement	183
8[h], 25 cm × 4.2 mm	5% Water in methanol	D_3	Livestock feed supplement	184
5, 25 cm × 4.2 mm	30% Chloroform in *n*-hexane	D_3	Livestock feed supplement	185
2, 5 μm, 25 cm × 2.1 mm	Acetonitrile–methanol–water (90:5:5)	25-OH-D_3	Bovine tissues and chicken egg yolk	186, 187
3, 30 cm × 4 mm	Chloroform–hexane–tetrahydrofuran (70:30:1)	Photochemical and thermal isomers	Synthetic mixture and commercial D_3 resin	176
9[i], 5–10 μm, 20–30 cm × 4.6 mm	0.3–0.53% Amyl alcohol in *n*-hexane	D	Commercial D resin powder	188
9, 5–10 μm, 15–60 cm × 3–6.25 mm	0.15–0.7% Amyl alcohol in *n*-hexane	D	Multivitamin preparations	189

[a] Zorbax-Sil, Dupont de Nemours, Wilmington, Del. [b] Zorbax ODS, Dupont de Nemours, Wilmington, Del. [c] μPorasil, Waters Associates, Milford, Mass. [d] ODS-HC-Sil-X-1, Perkin-Elmer Corp., Norwalk, Conn. [e] Lichrosorb NH_2, E. M. Reagents, Montreal, Canada. [f] Partasil, 10 PXS, Whatman, Inc., Clifton, N.J. [g] Lichrosorb 10, RP_{18}, Chromapack, Holland. [h] μBondapack C_{18}, Waters Associates, Milford, Mass. [i] Microparticulate silica

20×10^6 IU/g with a relative standard deviation of 1.37%.

HPLC has been frequently used for the quantitative analysis of 25-OH-D_3 in human serum (55–58). It has also been used extensively for the separation of minor constituents into purer fractions prior to their determination by any one of the appropriate protein-binding assays. Figure 4 shows the separation of a synthetic mixture of all the important metabolites of vitamin D_3 obtained by Jones and DeLuca (177). This separation was achieved using two 25-cm × 2.1-mm i.d. microparticulate silica columns in series and 10% isopropanol in Skellysolve B as the mobile phase at a flow rate of 0.5 ml/min. On the same column, they showed the difficult separation of 25-OH-D_3 from 25-OH-D_2 using 2.5% isopropranol in Skellysolve B at a flow rate of 0.7 ml/min

HPLC also has been used for the analysis of vitamin D and all its metabolites in a variety of matrixes. These include milk and milk products, cod liver oil, animal feed supplements, vitamin D concentrates, multivitamin preparations, chicken egg yolk, cow tissues (liver, kidney, and muscle), cow blood, and human blood. A summary of representative HPLC systems from the literature is shown in Table I.

CONCLUSION

After three decades of comparative inactivity, a surge of interest in vitamin D during the last 15 years has resulted in our understanding of the biogenesis of vitamin D_3, the identification of the major metabolites, the establishment of the metabolic pathway, and the hormonal nature of the metabolites. Vitamin D_3 *per se* is now known to be biologically inactive, while 1,25-$(OH)_2D_3$ (or some other as yet unidentified metabolite) has been established as the principal hormone responsible for maintaining calcium and phosphorus homeostasis. The kidney is the endocrine organ in the vitamin D hormonal functions. Extensive research is still underway in elucidating the role of other endocrine organs in the regulation of vitamin D metabolism and its physiological functions. Other unanswered questions deal with the mechanisms of (*a*) the active forms of vitamin D in the transport of calcium from the intestine into general circulation, (*b*) bone calcium mobilization, and (*c*) renal control of phosphorus concentration in the blood. Meanwhile, two principal metabolites, 25-OH-D_3 and 1,25-$(OH)_2D_3$, are available as prescription products in several western countries for the treatment of vitamin D deficiency diseases.

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RESEARCH ARTICLES

Quantitation of Lipophilic Chloroethylnitrosourea Cancer Chemotherapeutic Agents

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Received February 27, 1981, from the **Brain Tumor Research Center, Department of Neurological Surgery, School of Medicine, University of California, San Francisco, CA 94143* and the ‡*Department of Medicinal Chemistry, School of Pharmacy, Purdue University, West Lafayette, IN 47907.* Accepted for publication June 2, 1981.

Abstract □ A simple and rapid quantitative method for the derivatization and determination of lipophilic chloroethylnitrosoureas is described. This procedure involves the ether extraction of the chloroethylnitrosourea from plasma and conversion of the parent drug to an *O*-methylcarbamate by reaction in anhydrous methanol. The product *O*-methylcarbamate may be separated with gas chromatography (GC) and detected with nitrogen-specific GC detectors or with mass spectrometry using multiple-ion detection. The lower limit of detection for each method was ~100 ng/ml plasma.

Keyphrases □ Cancer chemotherapeutic agents—lipophilic chloroethylnitrosoureas, quantitation □ Chloroethylnitrosoureas—quantitation by gas chromatography □ Gas chromatography—quantitation of lipophilic chloroethylnitrosourea cancer chemotherapeutic agents

The described assay was applied to 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine, I), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine, II), 1-(2-chloroethyl)-3-(4-*trans*-methylcyclohexyl)-1-nitrosourea (semustine, III), 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosourea (IV), and 1-(2-chloroethyl)-3-(4-amino-2-methyl-5-pyrimidinyl)methyl-1-nitrosourea (V)[1]. The clearance curve for IV from rat plasma is presented as an example of the application of this method to the determination of biodistribution parameters.

BACKGROUND

The chloroethylnitrosoureas are a class of highly effective and widely used cancer chemotherapeutic agents (1–3). Analysis of these drugs in plasma at therapeutically significant concentrations has proven to be difficult. Although several methods of analysis have been reported, none are generally applicable to patient pharmacokinetic analyses. As a consequence, only carmustine pharmacokinetics has received detailed study (4).

Chloroethylnitrosoureas are thermally labile and decompose at or below 100° so that the parent compounds cannot be analyzed with gas chromatography (GC) (5). Compounds of this class are readily separated with high-performance liquid chromatography (HPLC) (6); however, the low wavelength and molar absorptivity of the nitrosourea chromophore

[1] These compounds are commonly referred to by their respective code designations BCNU, CCNU, Methyl-CCNU, PCNU, and ACNU.

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