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Overview of Vitamin D

INTRODUCTION

Vitamin D, first identified as a vitamin early in the 20th century, is now recognized as a prohormone. A unique aspect of vitamin D as a nutrient is that it can be synthesized by the human body through the action of sunlight. These dual sources of vitamin D make it challenging to develop dietary reference intake values.

Vitamin D, also known as calciferol, comprises a group of fat-soluble seco-sterols. The two major forms are vitamin D₂ and vitamin D₃. Vitamin D₂ (ergocalciferol) is largely human-made and added to foods, whereas vitamin D₃ (cholecalciferol) is synthesized in the skin of humans from 7-dehydrocholesterol and is also consumed in the diet via the intake of animal-based foods. Both vitamin D₃ and vitamin D₂ are synthesized commercially and found in dietary supplements or fortified foods. The D₂ and D₃ forms differ only in their side chain structure. The differences do not affect metabolism (i.e., activation), and both forms function as prohormones. When activated, the D₂ and D₃ forms have been reported to exhibit identical responses in the body, and the potency related to the ability to cure vitamin D–deficiency rickets is the same (Fieser and Fieser, 1959; Jones et al., 1998; Jurutka et al., 2001). Experimental animal studies have indicated that vitamin D₂ is less toxic than vitamin D₃, but this has not been demonstrated in humans.

The activation steps involved in converting vitamin D from the diet and cutaneous synthesis are illustrated in Figure 3-1. Vitamin D, in either the D₂ or D₃ form, is considered biologically inactive until it undergoes two

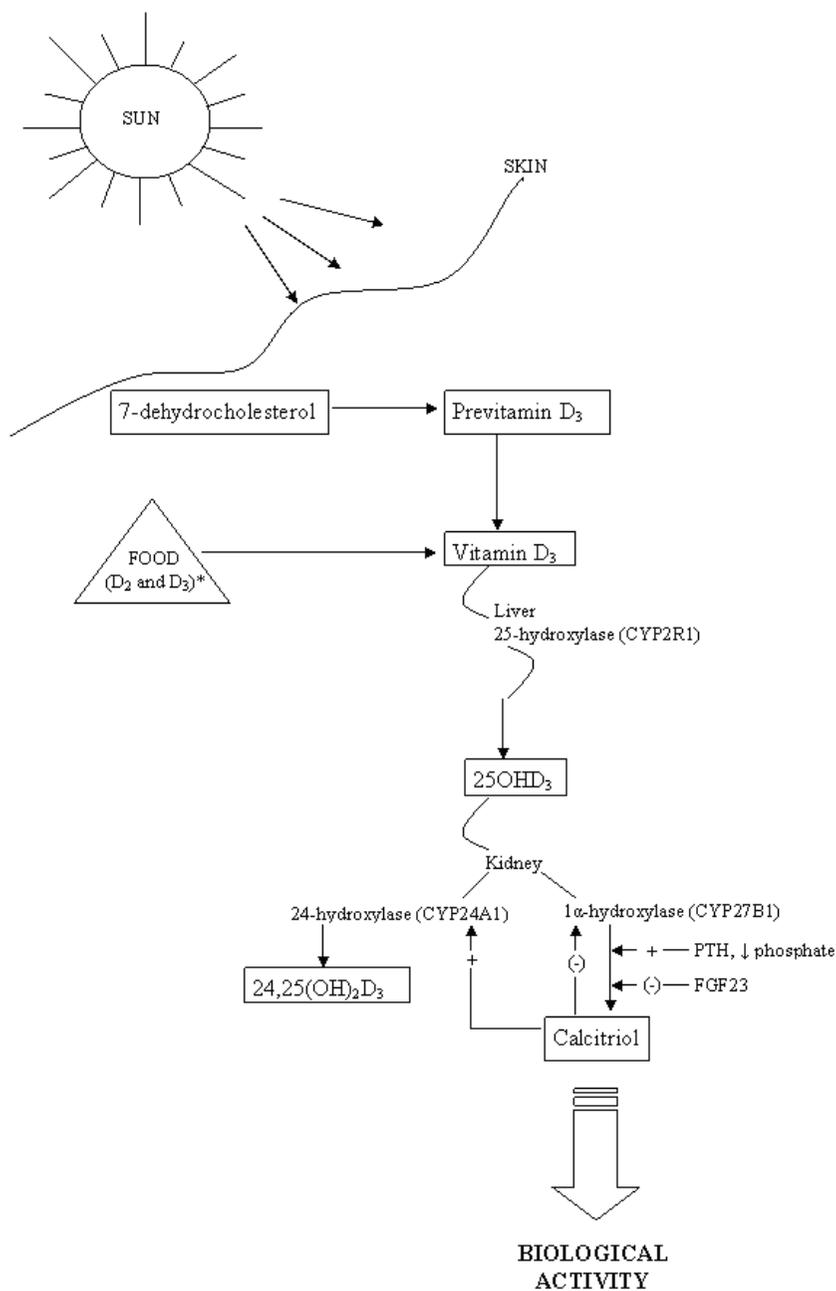


FIGURE 3-1 Overview of vitamin D synthesis, intake, and activation.

enzymatic hydroxylation reactions. The first takes place in the liver, mediated by the 25-hydroxylase (most likely cytochrome P450 2R1 [CYP2R1]) which forms 25-hydroxyvitamin D (hereafter referred to as 25OHD). The second reaction takes place in the kidney, mediated by 1 α -hydroxylase (CYP27B1), which converts 25OHD to the biologically active hormone, calcitriol (1,25-dihydroxyvitamin D). The 1 α -hydroxylase gene is also expressed in several extra-renal tissues, but its contribution to calcitriol formation in these tissues is unknown. 25OHD, the precursor of calcitriol, is the major circulating form of vitamin D; it circulates bound to a specific plasma carrier protein, vitamin D binding protein (DBP). DBP also transports vitamin D and calcitriol.

The renal synthesis of calcitriol is tightly regulated by two counteracting hormones, with up-regulation via parathyroid hormone (PTH) and down-regulation via fibroblast-like growth factor-23 (FGF23) (Galitzer et al., 2008; Bergwitz and Juppner, 2010). Low serum phosphorus levels stimulate calcitriol synthesis, whereas high serum phosphorus levels inhibit it. Following its synthesis in the kidney, calcitriol binds to DBP to be transported to target organs. The biological actions of calcitriol, involve regulation of gene expression at the transcriptional level, and are mediated through binding to a vitamin D receptor (VDR), located primarily in the nuclei of target cells (Jones et al., 1998; Jurutka et al., 2001). Additional hydroxylation reactions, such as that mediated by CYP24A1, as shown in Figure 3-1, result in more polar metabolites with greatly reduced or no apparent biological activity.

The classical actions of vitamin D—which by itself is inactive—are due to the functions of the active metabolite, calcitriol. These actions take the form of the regulation of serum calcium and phosphate homeostasis and, in turn, the development and maintenance of bone health (DeLuca, 1988; Reichel et al., 1989; Jones et al., 1998). Non-classical functions are less well elucidated. VDRs are found fairly ubiquitously throughout the body in tissues not involved with calcium and phosphate homeostasis, and the presence of VDRs in these tissues implies that calcitriol may play a more general role or that ligands other than calcitriol can activate the VDR. Furthermore, the specific vitamin D-responsive elements (VDREs), considered the hallmark of vitamin D action, are present in a large number of human genes involved in a wide range of classical and non-classical roles, such as the regulation of cell proliferation, cell differentiation, and apoptosis. It has been suggested that calcitriol exerts immunomodulatory and anti-proliferative effects through autocrine and paracrine pathways (Adams and Hewison, 2008). These wide-ranging actions of calcitriol have further been hypothesized to play a potential role in preventive or therapeutic action in cancer (Masuda and Jones, 2006) and chronic conditions such

as auto-immune conditions (including type 1 diabetes), cardiovascular disease, and infections (Holick et al., 2007).

Outside of the biological forms of vitamin D, a number of analogues based on the vitamin D structure have been synthesized for use as potential pharmacological agents. These are not, however, dietary or biosynthesized compounds; rather, they are designed for specific applications in research or clinical treatment. Examples of synthetic analogues that have gained importance in clinical medicine are briefly mentioned below.

The term vitamin D is generally used in this report to refer to both the D₂ and D₃ forms as well as their metabolites, although the two forms are distinguished when necessary for clarification (see Box 3-1 for definitions). Vitamin D levels in the diet—from foods and supplements—are expressed in International Units (IU), but may be expressed elsewhere in micrograms (µg). The biological activity of 1 µg of vitamin D is equivalent to 40 IU. Owing to the frequency with which serum 25OHD levels are included in this report text, the levels are expressed only as nanomoles per liter (nmol/L). As shown in Box 3-1, the nanomoles per liter measure can be converted to nanograms per milliliter (ng/mL) by dividing by a factor of 2.5.

BOX 3-1

Terms and Conversions Used in Reference to Vitamin D

Terms:

Vitamin D—also referred to as *calciferol*

Vitamin D₂—also referred to as *ergocalciferol*

Vitamin D₃—also referred to as *cholecalciferol*

25OHD—25-hydroxyvitamin D also referred to as *calcidiol* or *calcifediol*; indicates no distinction between D₂ and D₃ forms. When relevant, forms are distinguished as **25OHD₂** and **25OHD₃**

Calcitriol—1,25-dihydroxyvitamin D₃ (Note: *Ercalcitriol*—refers to 1,25-dihydroxyvitamin D₂, but in this report, the term “calcitriol” will be used for both)

24,25(OH)₂D—24,25-dihydroxyvitamin D

IU = International Unit is a measurement based on biological activity or effect; 1 IU of vitamin D is defined as the activity of 0.025 µg of cholecalciferol in bioassays with rats and chicks.

Conversions for Vitamin D₃:

[sources] 40 IU = 1 µg

[serum] 2.5 nmol/L = 1 ng/mL

SOURCES OF VITAMIN D

Diet

The dietary sources of vitamin D include food and dietary supplements; therefore, “total vitamin D intake” reflects the combined dietary contribution from foods and supplements. There are a few naturally occurring food sources of vitamin D. These include fatty fish, fish liver oil, and egg yolk. Some foods are, however, fortified with vitamin D. After vitamin D was recognized as important for the prevention of rickets in the 1920s (Steenbock and Black, 1924), vitamin D fortification of some foods was initiated on a voluntary basis.

In the United States, fluid milk is voluntarily fortified with 400 IU per quart (or 385 IU/L) of vitamin D (U.S. regulations do not specify the form) (FDA, 2009). In Canada, under the Food and Drug Regulation,¹ fortification of fluid milk and margarine with vitamin D is mandatory. Fluid milk must contain 35–45 IU vitamin D per 100 mL and margarine, 530 IU per 100 g. In addition, fortified plant-based beverages must contain vitamin D in an amount equivalent to fluid milk. In analyses conducted in the 1980s and early 1990s, a significant portion of milk samples in the United States were found to contain less than the specified amount of vitamin D (Tanner et al., 1988). Holick et al. (1992) found that 62 percent of milk sampled from five eastern states contained less than 80 percent and 10 percent contained more than 120 percent of the amount of vitamin D stated on the label. Chen et al. (1993) reported similar findings. A more recent report on vitamin D–fortified milk sampled in New York State over a period of 4 years showed that an average of only 47.7 percent of samples fell within the range of acceptable levels of vitamin D fortification (Murphy et al., 2001). However, recent surveys from the U.S. Department of Agriculture (USDA) indicate that these problems have been corrected. In a presentation to this committee, Byrdwell (2009) reported that a USDA survey of milk samples taken in 2007 from 24 locations across the United States showed that most samples had vitamin D levels within the range of 400 to 600 IU/quart.

In Canada, Faulkner et al. (2000) surveyed milk samples and found that 20 percent of skim milk, 40 percent of 2 percent fat milk, and 20 percent of whole milk, contained the recommended level of vitamin D. Samples collected by the Canadian Food Inspection Agency from 1999 through 2009 and analyzed for vitamin D indicated that during the last 4 years of sample collection, 47 to 69 percent were within the range specified by regulation (personal communication, S. Brooks, Health Canada, April

¹Available online at http://laws.justice.gc.ca/PDF/Regulation/C/C.R.C.,_c._870.pdf (accessed July 23, 2010).

30, 2010). In addition, over the past 5 years, the average vitamin D content of analyzed milk samples fell within this range. Over time, manufacturers in the United States have added vitamin D to other foods, and the food industry is increasingly marketing foods fortified with vitamin D (Yetley, 2008). Based on data from a U.S. Food and Drug Administration (FDA) survey that provides information on the labels of processed, packaged food products in the United States, Yetley (2008) reported that almost all fluid milks, approximately 75 percent of ready-to-eat breakfast cereals, slightly more than half of all milk substitutes, approximately one-quarter of yogurts, and approximately 8 to 14 percent of cheeses, juices, and spreads are fortified with vitamin D in the U.S. market. Many product labels included in the survey indicated that the form of added vitamin D was vitamin D₃. However, some milk substitutes are fortified with vitamin D₂. Cereal labels did not specify the form of added vitamin D. Levels of vitamin D ranged from 40 IU per regulatory serving for cereals and cheeses to 60 IU per regulatory serving for spreads and 100 IU per regulatory serving for fluid milk. Several food categories had within-category ranges of 40 to 100 IU of vitamin D per regulatory serving. Serum vitamin D and 25OHD have low penetrance into breast milk, together comprising 40 to 50 IU of antirachitic activity per liter, most of which is contributed by 25OHD (Leerbeck and Sondergaard, 1980; Hollis et al., 1981; Reeve et al., 1982; Specker et al., 1985). Data from the USDA report the vitamin D content of human milk to be 4.3 IU/100 kcal.² However, the vitamin D biological activity may be higher than the analyzed values, because human milk contains small amounts of 25OHD in addition to vitamin D₃ (Reeve et al., 1982); further, the biological activity of 25OHD is approximately 50 percent higher than that of vitamin D (Blunt et al., 1968).

The FDA has established that infant formula must contain 40 to 100 IU of vitamin D per 100 kcal.³ Commercial infant formulas contain approximately 60 IU of vitamin D per 100 kcal, as estimated by the USDA food composition database,⁴ and Yetley (2008) reported that commercial milk-based infant formulas collected between 2003 and 2006 contained 87

²USDA National Nutrient Database for Standard Reference Release 23. NBD No. 01107. Milk, human, mature, fluid. Available online at <http://www.ars.usda.gov/Services/docs.htm?docid=8964> (accessed August 3, 2010).

³USDA National Nutrient Database for Standard Reference Release 23. NBD No. 03946. Infant formula, ROSS, SIMILAC LACTOSE FREE ADVANCE, ready-to-feed, with ARA and DHA; and NDB no. 03815. Infant formula, MEAD JOHNSON, ENFAMIL LIPIL, with iron, ready-to-feed, with ARA and DHA. Available online at http://www.ars.usda.gov/main/site_main.htm?modecode=12-35-45-00 (accessed April 28, 2010).

⁴Available online at <http://www.nal.usda.gov/fnic/foodcomp/search/> (accessed March 16, 2010).

to 184 percent of label declarations. In Canada, infant formula is required by regulation to contain between 40 and 80 IU of vitamin D per 100 kcal.

In recent years, dietary supplements containing vitamin D have become more common and have been more frequently consumed. The form of vitamin D used in supplement products can be either vitamin D₂ or vitamin D₃. It would appear from informal observations of the market place that manufacturers are increasingly switching from vitamin D₂ to vitamin D₃, and some are increasing the vitamin D content of their products. Traditionally, many marketed dietary supplements have contained 400 IU per daily dose, but levels in supplements have been increasing. In the United States, vitamin D can now be found in multi-vitamin/multi-mineral formulations as well as a single supplement in a range of dosage levels, including 1,000 to 5,000 IU of vitamin D₃ per dose and even up to 50,000 IU of vitamin D₂ per dose. In Canada, dosage levels of vitamin D above 1,000 IU are obtainable only with a prescription.

Information about current national survey estimates of the intake of vitamin D from foods and supplements can be found in Chapter 7.

Synthesis in the Skin

Vitamin D₃ is synthesized in human skin from 7-dehydrocholesterol following exposure to ultraviolet B (UVB) radiation with wavelength 290 to 320 nm.⁵ The process of UVB-mediated conversion of 7-dehydrocholesterol to the previtamin D₃ form and subsequent thermal isomerization to vitamin D₃ occurring in the epidermis is illustrated in Figure 3-2.

The production of vitamin D₃ in skin is a function of the amount of UVB radiation reaching the dermis as well as the availability of 7-dehydrocholesterol (Holick, 1995). As such, the level of synthesis is influenced by a number of factors, as described below in the section entitled "Measures Associated with Vitamin D: Serum 25OHD," including season of the year, skin pigmentation, latitude, use of sunscreen, clothing, and amount of skin exposed. Age is also a factor, in that synthesis of vitamin D declines with increasing age, due in part to a fall in 7-dehydrocholesterol levels and due in part to alterations in skin morphology (MacLaughlin and Holick, 1985).

Toxic levels of vitamin D do not occur from prolonged sun exposure. Thermal activation of previtamin D₃ in the skin gives rise to multiple non-vitamin D forms, such as lumisterol, tachysterol and others (Holick et al., 1981; Webb et al., 1989), as illustrated in Figure 3-2; this limits the

⁵The chemical processes that lead to the formation of vitamin D₃ from its precursor are non-enzymatic and can take place ex vivo and in organic solvents, as well as in vivo. Therefore, vitamin D₃ can also be synthesized commercially.

formation of vitamin D₃ itself. Vitamin D₃ can also be converted to nonactive forms.

The absolute percentage of circulating 25OHD that arises from cutaneous synthesis versus oral intake of vitamin D in the free-living North American population cannot be clearly specified. Individuals living at Earth's poles during winter months and submariner crew members with very limited or no measurable UVB exposure have detectable levels of 25OHD in blood, arising from dietary sources and likely from previously synthesized and stored vitamin D. This topic is further explored in the section below that focuses on serum 25OHD.

METABOLISM OF VITAMIN D

Absorption

Owing to its fat-soluble nature, dietary vitamin D (either D₂ or D₃) is absorbed with other dietary fats in the small intestine (Haddad et al., 1993; Holick, 1995). The efficient absorption of vitamin D is dependent upon the presence of fat in the lumen, which triggers the release of bile acids and pancreatic lipase (Weber, 1981, 1983). In turn, bile acids initiate the emulsification of lipids, pancreatic lipase hydrolyzes the triglycerides into monoglycerides and free fatty acids, and bile acids support the formation of lipid-containing micelles, which diffuse into enterocytes. Early studies demonstrated that radiolabeled vitamin D₃ appeared almost exclusively in the lymphatics and in the chylomicron fraction of plasma; as well, subjects with impaired bile acid release or pancreatic insufficiency both demonstrated significantly reduced absorption of vitamin D (Thompson et al., 1966; Blomstrand and Forsgren, 1967; Compston et al., 1981). Subsequently, other clinical and experimental animal studies confirmed that vitamin D is most efficiently absorbed when consumed with foods containing fat (Weber, 1981; Johnson et al., 2005; Mulligan and Licata, 2010) and, conversely, that a weight-loss agent that blocks fat absorption also impairs the absorption of vitamin D (James et al., 1997; McDuffie et al., 2002). The optimal amount of fat required for maximal absorption of vitamin D has not been determined.

Within the intestinal wall, vitamin D, cholesterol, triglycerides, lipoproteins, and other lipids are packaged together into chylomicrons. Importantly, while a fraction of newly absorbed intestinal vitamin D is also transported along with amino acids and carbohydrates into the portal system to reach the liver directly, the main pathway of vitamin D uptake is incorporation into chylomicrons that reach the systemic circulation via the lymphatics. Chylomicron lipids are metabolized in peripheral tissues that express lipoprotein lipase, but particularly in adipose tissue and

skeletal muscle, which are rich in this enzyme. During hydrolysis of the chylomicron triglycerides, a fraction of the vitamin D contained in the chylomicron can be taken up by these tissues. Uptake into adipose tissue and skeletal muscle accounts for the rapid postprandial disappearance of vitamin D from plasma and probably also explains why increased adiposity causes sequestering of vitamin D and is associated with lower 25OHD levels (Jones, 2008). What remains of the original chylomicron after lipolysis is a chylomicron remnant, a cholesterol-enriched, triglyceride-depleted particle that still contains a fraction of its vitamin D content.

Metabolism to the Active Hormonal Form

Vitamin D, regardless of origin, is an inactive prohormone and must first be metabolized to its hormonal form before it can function. Once vitamin D enters the circulation from the skin or from the lymph, it is cleared by the liver or storage tissues within a few hours. The processes that follow are illustrated in Figure 3-3. Vitamin D is converted in the liver to 25OHD, a process carried out by a CYP enzyme that has yet to be fully defined but is likely CYP2R1 (Cheng et al., 2003). The crystal structure of CYP2R1 has been determined with vitamin D in the active site, and the enzyme has been shown to metabolize both vitamin D₂ and vitamin D₃ equally efficiently (Strushkevich et al., 2008). There is little, if any, feedback regulation of this enzyme. A large genome-wide association study of factors that might be determinants of the circulating 25OHD levels identified the human chromosomal 11p15 locus of CYP2R1 as a significant determinant, whereas the loci of the other enzymes purported to have 25-hydroxylase activity (e.g., CYP27A1 and CYP3A4) were not identified (Wang et al., 2010). The other determinants of serum 25OHD besides CYP2R1 have been reported to be DBP (also known as Gc protein), which has six common phenotypes (Laing and Cooke, 2005) as well as 7-dehydrocholesterol reductase and CYP24A1. Increasing intake of vitamin D results in higher blood levels of 25OHD, although perhaps not in a linear manner (Stamp et al., 1977; Clements et al., 1987).

At this point, 25OHD bound to DBP circulates in the blood stream and, when calcitriol is required due to a lack of calcium (or lack of phosphate), 25OHD is 1 α -hydroxylated in the kidney to form calcitriol, the active form, by the 1 α -hydroxylase enzyme (also known as CYP27B1) (Tanaka and DeLuca, 1983). This metabolic step is very tightly regulated by blood calcium and phosphate levels through PTH and the phosphaturic hormone, FGF23, and constitutes the basis of the vitamin D endocrine system that is central to maintaining calcium and phosphate homeostasis (see discussion below on functions and physiological actions). FGF23 acts by reducing the expression of renal sodium–phosphate transporters and reducing serum calcitriol levels.



FIGURE 3-3 The metabolism of vitamin D₃ from synthesis/intake to formation of metabolites. The process is the same for vitamin D₂ once it enters the circulation. NOTE: CYP = cytochrome P450 (a large and diverse group of enzymes). SOURCE: Reprinted with permission from Hector DeLuca.

Production of the CYP27B1 enzyme is stimulated by PTH, which is secreted in response to a lack of calcium. It is also stimulated by the hypophosphatemic action of FGF23 on renal phosphate excretion, but to a lesser extent. When PTH is suppressed, or FGF23, produced by osteocytes, is stimulated, 1 α -hydroxylation is markedly reduced (Liu et al., 2007; Quarles, 2008). Furthermore, calcitriol can act as a suppressor of CYP27B1, although the mechanism is not fully understood.

Calcitriol has its strongest metabolic activity in inducing its own destruction by stimulating the 24-hydroxylase enzyme (now known as CYP24A1;

Figure 3-1) (Jones et al., 1998). The enzyme CYP24A1 is found in all target tissues and is induced in response to calcitriol interacting with the VDR. CYP24A1 is largely responsible for the metabolic degradation of calcitriol and its precursor, 25OHD, and its deletion in the mouse results in 50 percent lethality at weaning and an inability to efficiently clear the active form of vitamin D (Masuda et al., 2005). CYP24A1 carries out a series of reactions resulting ultimately in production of calcitroic acid from calcitriol and 1-desoxycalcitroic acid from $24,25(\text{OH})_2\text{D}_2$, the major metabolite of 25OHD. These products are excreted through the bile into the feces (Jones et al., 1998); very little is eliminated through the urine (Kumar et al., 1976). The active forms of vitamin D_2 are also catabolized by CYP24A1 into a series of biliary metabolites, somewhat analogous to those of vitamin D_3 .

As described above, all naturally occurring vitamin D compounds interact with DBP. Calcitriol and vitamin D have significantly lower affinity for this protein than does 25OHD. Whereas vitamin D has an average lifetime in the body of approximately 2 months, 25OHD has a lifetime of 15 days, and calcitriol has a lifetime measured in hours (Jones et al., 1998). Aside from these key elements in vitamin D metabolism, more than 30 other metabolites have been found, including the 3-epi series of vitamin D compounds (DeLuca and Schnoes, 1983; Siu-Caldera et al., 1999). Their importance seems minimal and need not be discussed here.

Although the route of catabolism between $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_3$ differs beyond the initial 24-hydroxylation step, because 24-hydroxylation is primarily a deactivation step (Brommage and DeLuca, 1985; Horst et al., 1986; Lohnes and Jones, 1992; Jones et al., 1998), the rate of this initial step should be the important indicator of the loss of biological action. Comparisons of initial rate kinetics of the 24-hydroxylase enzyme (CYP24A1) activity toward $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_3$ and their precursors suggest that the rates of inactivation by CYP24A1 in vitro are virtually identical (Jones et al., 2009; Urushino et al., 2009). Although side-chain hydroxylation of $1\alpha,25(\text{OH})_2\text{D}_2$ represents the primary route of metabolism in the target cell, clearance of the metabolic products in vivo is complicated by additional non-specific liver CYPs (e.g., CYP3A4) (Gupta et al., 2004, 2005) that are inducible by $1\alpha,25(\text{OH})_2\text{D}_3$ in certain extra-hepatic tissues (Thompson et al., 2002) and also Phase II enzymes, including uridine diphosphate–glucuronosyl transferases, which are known to subject vitamin D metabolites to glucuronidation (LeVan et al., 1981; Hashizume et al., 2008). The pharmacokinetic consequence of the sum of these catabolic systems, as shown in studies in rats, is a slightly reduced half-life for $1\alpha,25(\text{OH})_2\text{D}_2$ compared with $1\alpha,25(\text{OH})_2\text{D}_3$ (Knutson et al., 1997).

There are reports that vitamin D_2 and vitamin D_3 are differentially susceptible to these non-specific inactivating modifications, such as those occurring in the liver in response to a variety of drugs. These enzymes in-

clude the liver and intestinal CYPs that are known to metabolize vitamin D compounds differently, such as CYP27A1, which 25-hydroxylates vitamin D₃ and 24-hydroxylates vitamin D₂ (Guo et al., 1993), and CYP3A4, which 24- and 25-hydroxylates vitamin D₂ substrates more efficiently than vitamin D₃ substrates (Gupta et al., 2004, 2005) and 23*R*- and 24*S*-hydroxylates 1 α ,25(OH)₂D₃ (Xu et al., 2006); the latter enzyme has recently been shown to be selectively induced by 1 α ,25(OH)₂D in the intestine (Thompson et al., 2002; Xu et al., 2006). Both CYP27A1 and CYP3A4 are known to have significantly lower Michaelis-Menten constants (K_m values) for 25OHD₃ compared with CYP2R1 (Guo et al., 1993; Sawada et al., 2000), in the micromole per liter range; this questions their physiological but not their pharmacological relevance. Recent work (Helvig et al., 2008; Jones et al., 2009) has shown that both human intestinal microsomes and recombinant CYP3A4 protein break down 1 α ,25(OH)₂D₂ at a significantly faster rate than 1 α ,25(OH)₂D₃, suggesting that this non-specific CYP might limit vitamin D₂ action preferentially in target cells, where it is expressed and when the substrate is in the pharmacological dose range. The same type of mechanism involving differential induction of non-specific CYPs may underlie the occasional reports of co-administered drug classes, such as anti-convulsants (Christiansen et al., 1975; Tjellesen et al., 1985; Hosseinpour et al., 2007), causing accelerated degradation of one vitamin D form over the other.

Storage

Adipose tissue stores of vitamin D probably represent “non-specific” stores sequestered because of the hydrophobic nature of vitamin D, but the extent to which the processes of accumulation or mobilization are regulated by normal physiological mechanisms remains unknown at this time. Rosenstreich et al. (1971) first identified adipose tissue as the primary site of vitamin D accumulation from experiments in which radiolabeled vitamin D was administered to vitamin D-deficient rats. Tissue levels of radioactivity measured during vitamin D repletion and during a subsequent period of deprivation showed that adipose tissue acquired the greatest quantity of radioactive compound and had the slowest rate of release. Work by Liel et al. (1988) suggested that there was enhanced uptake and clearance of vitamin D by adipose tissue in obese subjects compared with those of normal weights. Similarly, Wortsman et al. (2000) concluded that in obese subjects, vitamin D was stored in adipose tissue and not released when needed. Finally, Blum et al. (2008) found that, in elderly subjects supplemented with 700 IU of vitamin D per day, for every additional 15 kg of weight above “normal” at baseline, the mean adjusted change in 25OHD level was approximately 10 nmol/L lower after 1 year of supplementation.

The authors estimated that in order for subjects with body mass indexes (BMIs) above the normal range to obtain an increase in serum 25OHD level similar to that of subjects with weight in the normal range, an additional 17 percent increase in vitamin D above the administered dose of 700 IU/day would be needed for every 10 kg increase in body weight above baseline in their study population.

The implication of these studies is that vitamin D deposited in fat tissue is not readily available, and obese individuals may require larger than usual doses of vitamin D supplements to achieve a serum 25OHD level comparable to that of their normal weight counterparts. In support of the hypothesis that vitamin D is stored in adipose tissues, weight reduction studies show that serum 25OHD levels rise when obese individuals lose body fat (Riedt et al., 2005; Zitterman et al., 2009; Tzotzas et al., 2010). Conclusive statements regarding changes in serum 25OHD levels after gastric bypass surgery cannot be made, as a result of confounding factors, such as weight change, possible malabsorption, and diet. There is evidence of a rise in serum 25OHD levels after surgery (Mahdy et al., 2008; Aasheim et al., 2009; Goldner et al., 2009; Bruno et al., 2010), as well as evidence that there is no change after surgery (Riedt et al., 2006; Fleischer et al., 2008; Valderas et al., 2009). Gehrler et al. (2010) indicated that serum 25OHD levels decrease after gastric bypass surgery, although the quality of the methods used is questionable.

Excretion

As described previously, the products of vitamin D metabolism are excreted through the bile into the feces, and very little is eliminated through the urine. This is in part due to renal reuptake of vitamin D metabolites bound to DBP, as mediated by the cubilin–megalin receptor system (Willnow and Nykjaer, 2005).

Excess Intake

Excess intake of vitamin D—but not sun exposure, which is associated with a series of thermal and photoisomerization reactions (see Figure 3-2)—can lead to a state of vitamin D “intoxication” or “hypervitaminosis D.” Chemically synthesized vitamin D became available late in the third decade of the 20th century; reports of vitamin D intoxication were first found from 1928 to 1932 and continued throughout most of the 20th century (DeLuca, 2009). The condition of hypervitaminosis D leads to hypercalcemia and eventually to soft tissue calcification and resultant renal and cardiovascular damage (DeLuca, 1974). In the case of animal models, at necropsy, vitamin D–intoxicated rats show widespread calcification of organs and tissues.

The form of the vitamin implicated in the intoxication is 25OHD (Vieth, 1990; Jones, 2008). In fact, it has been shown in dietary supplementation studies using the CYP27B1 knockout mouse, which is incapable of making calcitriol, sufficiently high concentrations of serum levels of 25OHD can cause changes in vitamin D–dependent general expression even in the absence of calcitriol (Rowling et al., 2007; Fleet et al., 2008).

FUNCTIONS AND PHYSIOLOGICAL ACTIONS OF VITAMIN D

Calcium and Phosphate Homeostasis

The dominant function of vitamin D in its hormonal form (calcitriol or 1,25-dihydroxyvitamin D) is the elevation of plasma calcium and phosphate levels, which are required for mineralization of bone (DeLuca, 1979b; Holick, 1996). Furthermore, the elevation of plasma calcium to normal levels is also required for the functioning of the neuromuscular junction as well as vasodilatation, nerve transmission, and hormonal secretion.

Calcitriol—functioning as part of the endocrine system for maintaining serum calcium levels as outlined in Chapter 2—elevates plasma ionized calcium levels to the normal range by three different mechanisms (see Figure 2-1 in Chapter 2). The first mechanism, which does not require PTH, is the well-established role of calcitriol in stimulating intestinal calcium absorption throughout the entire length of the intestine, although its greatest activity is in the duodenum and jejunum. It is clear that calcitriol directly stimulates intestinal calcium and, independently, phosphate absorption.

In the second mechanism, calcitriol plays an essential role in the mobilization of calcium from bone, a process requiring PTH (Garabedian et al., 1972; Lips, 2006). It induces the formation and activation of the osteoclast to function in the mobilization of calcium from bone, as discussed in Chapter 2. In short, calcitriol facilitates the formation of osteoclasts by stimulating the secretion of a protein called receptor activator for nuclear factor κ B (RANK) ligand, which, in turn, is responsible for osteoclastogenesis and bone resorption (Suda et al., 1992; Yasuda et al., 2005).

In the third mechanism, calcitriol together with PTH stimulates the renal distal tubule reabsorption of calcium, ensuring retention of calcium by the kidney when calcium is needed (Sutton et al., 1976; Yamamoto et al., 1984). These well-known functions dominate vitamin D physiology and many of the functional proteins involved in these processes have been identified, although the exact molecular mechanisms of all of these systems have yet to be elucidated.

Thus, overall, calcitriol acts on the intestine, bone, and kidney as described above, and as illustrated in Figure 2-1 in Chapter 2, to elevate

serum calcium levels, closing the calcium loop. As serum calcium levels rise, PTH secretion drops. If serum calcium levels become too high, the parafollicular cells ("C" cells) of the thyroid secrete calcitonin, which blocks calcium resorption from bone and helps to keep calcium levels in the normal range. Calcitriol, through its receptor, the VDR, suppresses parathyroid gene expression and parathyroid cell proliferation, providing important feedback loops that reinforce the direct action of increased serum calcium levels (Slatopolsky et al., 1984; Silver et al., 1986).

Not shown in Figure 2-1 in Chapter 2 is the mechanism of action of vitamin D in regulating serum phosphorus levels, certain aspects of which remain obscure. What is known is that (1) a deficiency of phosphate stimulates CYP27B1 to produce more calcitriol, which in turn stimulates phosphate absorption in the small intestine; and (2) calcitriol can also induce the secretion of FGF23 by osteocytes in bone, which results in phosphate excretion in the kidney (Liu et al., 2008), as well as feedback on vitamin D metabolism.

Other Actions

It is noteworthy that the VDR is present in the nucleus of many tissues that are not involved in the regulation of calcium and phosphate metabolism. For example, the VDR has been clearly described in epidermal keratinocytes, in activated T cells of the immune system, in antigen-presenting cells, in macrophages and monocytes, and in cytotoxic T cells. Gene array studies in many cells and tissues show that calcitriol regulates several hundred genes throughout the body or as much as 5 percent of the human genome (Pike et al., 2008). However, exactly how calcitriol functions in these tissues and the physiological consequences are not clearly known.

Likewise, the importance of the paracrine or autocrine synthesis of calcitriol under non-disease conditions is unclear. The 1α -hydroxylase (CYP27B1) gene has been reported to be expressed in many extra-renal tissues (Hewison et al., 2007). In some cases, this is based upon in vitro production of calcitriol by cell lines as a consequence of culture conditions, but it also includes detection of the messenger ribonucleic acid (mRNA) transcript or protein for CYP27B1 in tissues in vivo (Hewison et al., 2007). There is no doubt that the kidney is physiologically the overwhelming site of production of calcitriol for the circulation, as chronic kidney disease or nephrectomy results in a significant fall in the serum calcitriol level (Martinez et al., 1995). The contribution of calcitriol to the maternal circulation stemming from production by the placenta is not clearly known; based on a case report for an anephric patient, it appears that the placenta produces calcitriol, but its contribution to the maternal circulation is low (Turner et al., 1988). The pregnancy-related rise in calcitriol is due to up-

regulation of the enzymes in the maternal kidney (Kovacs and Kronenberg, 1997). However, there may be other extra-renal 1α -hydroxylation sites that can act as intracrine systems primarily involved in regulation of cell or tissue growth: skin, gastrointestinal tract, or glandular tissue, such as prostate and breast (Diesing et al., 2006). In mice missing the *Vdr* gene (*Vdr*-null), calcitriol and the VDR play a role in lactational physiology; there is accelerated mammary development during pregnancy, but delayed involution of the mammary tissue after lactation (Zinser and Welsh, 2004). Extra-renal CYP27B1 may be up-regulated during inflammation (Ma et al., 2004; Liu et al., 2008) or down-regulated in cancerous tissue proliferation (Bises et al., 2004; Wang et al., 2004). Furthermore, extra-renal production of calcitriol is clearly found in certain pathological diseases, including granulomatous conditions such as sarcoidosis, lymphoma, and tuberculosis (Adams et al., 1989), which can be associated with hypercalcemia. If sarcoidosis is left untreated, the extra-renally produced calcitriol can enter the circulation, resulting in hypercalciuria and eventually hypercalcemia.

There is emerging evidence that calcitriol plays a role in the immune system that has not yet been clearly described. Exogenous calcitriol can suppress autoimmune diseases, but with hypercalcemia as an important side effect (DeLuca and Cantorna, 2001). It has been shown that the local conversion of 25OHD into calcitriol in monocytes or macrophages results in an increase in cellular immunity by stimulating the production of cathelicidin, an anti-microbial peptide capable of killing bacteria, particularly *Mycobacterium tuberculosis* (Liu et al., 2006). Recently, Stubbs et al. (2010) showed that renal dialysis patients treated with high-dose vitamin D₃ develop a population of immune cells with increased CYP27B1, VDR, and cathelicidin expression, although the role of these cells in vivo is unknown. Ironically, calcitriol has an opposite effect on the adaptive immune (B and T cell function) response. Calcitriol generally inhibits T helper cell proliferation and B cell immunoglobulin production. In contrast, calcitriol promotes the proliferation of immunosuppressive regulatory T cells and their accumulation at sites of inflammation (Penna et al., 2007).

A role for vitamin D in carcinogenesis evolved initially from in vitro studies as cell culture approaches became more widely available for the evaluation of the mechanisms of action of vitamin D and its metabolites (Masuda and Jones, 2006). The active hormone, calcitriol, was shown to consistently inhibit the growth of cancer cells and promote differentiation in vitro by regulating multiple pathways (Deeb et al., 2007; Kovalenko et al., 2010). Additional studies documented the presence of the VDR in a wide array of cancer cell types. Vitamin D orchestrates cell cycle progression via alterations in key regulators such as cyclin-dependent kinases, retinoblastoma protein phosphorylation, and repression of the proto-oncogene *myc* as well as by modulating growth factor receptor-mediated signaling

pathways (Koga et al., 1988; Kawa et al., 1996; Campbell et al., 1997; Xie et al., 1997; Yanagisawa et al., 1999; Sundaram et al., 2000; Gaschott and Stein, 2003; Li et al., 2004). In addition, calcitriol restores or enhances pro-apoptotic effects in cancer cells by several possible pathways, including repression of several pro-survival proteins such as Bcl2 and telomerase reverse transcriptase and by activating pro-apoptotic proteins Bax and μ -calpain (James et al., 1996; Diaz et al., 2000; Jiang et al., 2004; Kumagai et al., 2005). Evidence also supports an anti-angiogenic effect of vitamin D. Vascular endothelial growth factor (VEGF) expression by cancer cells is suppressed and endothelial cell responses to VEGF are inhibited by vitamin D, an observation supported by in vivo xenograft studies (Mantell et al., 2000; Bao et al., 2006). The immunoregulatory effects of vitamin D may also have an impact on cancer biology. Inflammation is a critical early step in the carcinogenesis cascade for many cancers, and the ability of vitamin D to exhibit anti-inflammatory effects on cancer cells by down-regulating the pro-inflammatory pathways, such as cyclooxygenase-2, may contribute to cancer inhibition (Moreno et al., 2005). In contrast, the role of vitamin D in cancer immunosurveillance of nascent or established cancers remains to be defined.

The encouraging in vitro findings, tempered with concerns about hypercalcemia, led to the development of many vitamin D analogues in the hope of retaining anti-cancer activity, but without increasing serum calcium, for the pharmacological therapy of cancer, as recently reviewed (Beer and Myrthue, 2004; Masuda and Jones, 2006; Trump et al., 2010).

Vitamin D₂ Versus Vitamin D₃

Vitamins D₂ and D₃, as described previously, differ only in their side chain structure. Physiological responses to both forms of the vitamin include regulation of calcium and phosphate homeostasis and regulation of cell proliferation and cell differentiation of specific cell types, as described above. Qualitatively, vitamins D₂ and D₃ exhibit virtually identical biological responses throughout the body (i.e., through gene expression) that are mediated by the VDR (Jones et al., 1998; Jurutka et al., 2001).

Regarding the potency of the two forms of vitamin D, there are reports that certain animals, such as avian species and New World monkeys (Chen and Bosmann, 1964; Drescher et al., 1969), discriminate against vitamin D₂. However, it has been assumed for several decades that the two forms are essentially equipotent in humans (Christiansen et al., 1975). Recent reports involving human dietary studies have argued for (Trang et al., 1998; Armas et al., 2004) or against (Holick et al., 2008) a metabolic discrimination against vitamin D₂, compared with vitamin D₃. Part of the apparent conflict between these different studies (Trang et al., 1998; Armas et al.,

2004; Holick et al., 2008) is almost certainly due to differences in size and frequency of dose (which have ranged from 1,000 IU daily doses to 50,000 IU in a single dose); the differences reported suggest a difference in pharmacokinetic parameters between vitamin D₂ and vitamin D₃.

This debate runs parallel to the suggestion that vitamin D₂ is less toxic than its vitamin D₃ counterpart. Experimental animal data from a number of mammalian species ranging from rodents to primates (Roborgh and de Man, 1959, 1960; Hunt et al., 1972; Sjoden et al., 1985; Weber et al., 2001), support the concept that the D₂ form is less toxic than D₃, but there is no evidence available in humans. Nonetheless, the implication of these diverse studies in several mammalian species is that vitamin D₂ compounds may show differences in pharmacokinetics that manifest as lower toxicity from high doses.

There is considerable evidence that most of the steps involved in the metabolism and actions of vitamin D₂ and vitamin D₃ are identical (Jones et al., 1998). The identification of the series of vitamin D₃ metabolites in the late 1960s and early 1970s was followed by the identification of their vitamin D₂ counterparts: 25OHD₂, 1 α ,25(OH)₂D₂, and 24,25(OH)₂D₂ (Suda et al., 1969; Jones et al., 1975, 1979, 1980a). Noteworthy here is the fact that the structural features unique to the vitamin D₂ side chain did not preclude either the 25- or 1 α -hydroxylation steps in activation of the molecule or the first step of inactivation, namely 24-hydroxylation. Studies have also shown that the steps in the specific vitamin D signal transduction cascade do not appear to discriminate discernibly between the two vitamin D homologues at the molecular level (e.g., binding to the transport protein, DBP [Hay and Watson, 1977; Jones et al., 1980a] or binding to the receptor, VDR [Jones et al., 1980b; Reinhardt et al., 1989]). Overall, it can be concluded that specific signal transduction systems designed to respond to vitamin D₃ respond to physiological doses of vitamin D₂ equally well.

At this time, firm conclusions about different effects of the two forms of vitamin D cannot be drawn; however, it would appear that at low doses, D₂ and D₃ are equivalent, but at high doses, D₂ is less effective than D₃. In essence, the potency of the two forms (as judged by the dose required to cure rickets) is assumed to be the same (Park, 1940). Differences in toxicity for humans, as judged by the dose to cause hypervitaminosis D, are unclear, but there is evidence from experimental animal data to suggest that D₂ is less toxic than D₃.

Skeletal Disorders

Vitamin D deficiency results in inadequate mineralization of the skeleton. Commonly referred to as rickets in children and osteomalacia in adults, this disorder has been described in Chapter 2 relative to calcium.

Vitamin D deficiency is characterized by aberrations in the mineralization of the bone. In children, the deficiency results in rickets (see also Chapter 2), in which the cartilage fails to mature and mineralize normally. Rickets is characterized by widening at the end of the long bones, rachitic rosary, deformations in the skeleton, including craniotabes and deformities of the lower limbs, known as bowed legs and knocked knees. In adults, the deficiency of vitamin D leads to osteomalacia in which the newly deposited bone matrix fails to mineralize adequately, and there are wide unmineralized bone matrix (osteoid) seams.

Vitamin D–dependent rickets type I (VDDR I) is an autosomal recessive trait that results in abnormally low calcitriol levels but normal serum 25OHD levels. The mutation in VDDR I affects the 1α -hydroxylase enzyme and leads to impaired intestinal calcium absorption and the resulting rickets (Fraser et al., 1973). VDDR I manifests in the first year after birth and is treated with calcitriol. Supplemental calcium and phosphate are usually not needed. The second disorder is vitamin D–dependent rickets type II (VDDR II), which results in hypocalcemia, tetany, convulsions, alopecia, and rickets. VDDR II is also an autosomal recessive trait, resulting from a mutation in the *Vdr* gene, which can appear in the second year after birth or go unrecognized until adulthood.

VITAMIN D ACROSS THE LIFE CYCLE

Overall, vitamin D's role at different life stages is less clearly age-related than that of calcium, and also less well understood, with numerous gaps in basic information. Although some aspects of vitamin D nutrition and physiology have been found to differ with life stage, most of the functions of vitamin D are quite consistent across life stages from infancy and childhood, to adolescence, adulthood, and old age. For all life stages highlighted below, specific studies and conclusions are detailed in Chapter 4.

Infancy

Healthy skeletal development in infancy requires adequate intakes of vitamin D as well as calcium. Inadequate vitamin D intake during periods of growth leads to development of vitamin D deficiency rickets, which when it occurs in North American populations typically manifests around 20 months of age (DeLucia et al., 2003). If rickets is diagnosed early, vitamin D therapy can cure it, but not if skeletal deformities are severe and growth plates have started to mature in puberty (DeLuca, 1979a). Infants at risk for developing rickets include those who are exclusively breast-fed, because vitamin D and 25OHD are normally present at low levels in breast milk (Bachrach et al., 1979; Ward et al., 2007). Health Canada currently

recommends that exclusively breast-fed infants receive a supplement of vitamin D,⁶ and the American Academy of Pediatrics guidelines support supplementation of breastfeeding infants with vitamin D (Gartner and Greer, 2003). Commercial infant formula contains vitamin D, as discussed previously in this chapter.

Childhood and Adolescence

This life stage is characterized by bone accretion. During the rapid growth phase of adolescence, almost 50 percent of the adult skeletal mass will be accumulated. The onset of puberty stimulates increased metabolism of 25OHD levels to calcitriol (Aksnes and Aarskog, 1982) and subsequent increased calcium intestinal absorption, decreased urinary calcium excretion, and greater calcium deposition into bone (Wastney et al., 1996). Information on relationships between 25OHD levels and optimal intestinal absorption of calcium or risk for rickets or fracture in children and adolescents is lacking, although Abrams et al. (2005) found evidence for an indirect relationship between low serum 25OHD and increased calcium absorption in young adolescents. Although a recent set of systematic reviews (Cranney et al., 2007; Chung et al., 2009), to be discussed in Chapter 4, did not report specifically on bone mass for this age group in relation to vitamin D nutriture, the reviews suggested the possibility of a relationship between serum levels of 25OHD and bone mineral density (BMD) in adolescents. A recent analysis of vitamin D intake and BMD in male and female adolescents and adults ages 13 to 36 years found positive correlations between vitamin D intake and bone density from adolescence into adulthood among male but not female subjects (van Dijk et al., 2009).

Adults

The life stages associated with younger adults, covering several decades, are characterized by a need for adequate nutrition for bone maintenance. The bone is constantly undergoing remodeling, and the maintenance of normal bone density reduces the risk of skeletal disorders ranging from osteomalacia to the onset of osteoporotic fractures later in life. It is also the time of pregnancy and lactation for some female members of this population.

Older adults, especially those characterized as frail, may have poor dairy and vitamin D intake, decreased sun exposure, reduced dermal conversion of 7-dehydrocholesterol to vitamin D₃ and secondary hyperparathy-

⁶Available online at http://www.hc-sc.gc.ca/fn-an/nutrition/infant-nourisson/vita_d_supp-eng.php (accessed September 1, 2010).

roidism, all of which contribute to increased risk for poor bone health and osteoporotic fractures. As discussed below, there is inconsistent evidence as to whether intestinal absorption of vitamin D declines with age. In women, bone loss occurs as a result of the decreased estrogen levels that accompany menopause. As aging continues, both men and women experience age-related bone loss. As is the case for calcium intake, it is not well established whether and to what extent intakes of vitamin D may mitigate the bone loss.

Pregnancy and Lactation

The role of vitamin D in pregnancy and fetal development is the focus of current attention. However, at present the role of vitamin D is not clear, and there are very few data by which to examine the questions surrounding the effect of the nutrient on pregnancy and lactation. Animal studies and inferential human data do not readily elucidate a specific function in fetal development, especially with respect to formation and mineralization of the fetal skeleton. Calcitriol levels increase during pregnancy, but factors other than vitamin D appear to stimulate the increased calcium absorption. Although a number of avenues are still being explored, the bulk of the evidence suggests that calcium is moved from the mother to the fetus without requiring calcitriol.

Breast milk is not normally a significant source of vitamin D for the infant and remains unchanged with supplementation at least up to 2,000 IU/day. Existing evidence suggests that vitamin D nutriture does not appear to affect the maternal processes of bone resorption that occur during lactation, nor its restoration post-lactation.

MEASURES ASSOCIATED WITH VITAMIN D: SERUM 25OHD

Serum 25OHD level is widely considered as a marker of vitamin D nutriture, and consideration of serum 25OHD measures for the purposes of nutrient reference value development has generated notable interest. There is agreement that circulating serum 25OHD levels are currently the best available indicator of the net incoming contributions from cutaneous synthesis *and* total intake (foods and supplements) (Davis et al., 2007; Brannon et al., 2008; Davis, 2008). Thus, the serum 25OHD level may function as a *biomarker of exposure*; it is a reflection of the supply of vitamin D to the body and can be a useful adjunct to examining the intake level of vitamin D if the confounders and the measure's variability depending upon a range of variables are kept in mind. However, what is not clearly established is the extent to which 25OHD levels serve as a *biomarker of effect*. That is, there is some question as to whether levels of 25OHD relate to

health outcomes via a causal pathway and can serve as predictors of such outcomes.

Research recommendations in the previous Dietary Reference Intake (DRI) review of vitamin D (IOM, 1997), as well as an Institute of Medicine (IOM) workshop on DRI research needs (IOM, 2007), called for studies to evaluate the intake requirements for vitamin D as related to optimal circulating 25OHD concentrations across life stage and race/ethnicity groups of U.S. and Canadian populations, taking into account variability in UVB radiation exposures. The issue of the role of serum 25OHD concentrations was also identified by the sponsors of this current study on vitamin D and calcium DRIs as central to the development of DRIs for vitamin D (Yetley et al., 2009). Much in the way of this information gap for serum 25OHD concentrations has not yet been addressed. Nonetheless, measures of serum 25OHD are important considerations in developing DRI values for vitamin D intake. The sections below highlight factors affecting serum 25OHD level and methodologies for its measurement. It is important to note that these discussions refer to 25OHD, not to calcitriol (i.e., 1,25-dihydroxyvitamin D). Calcitriol, the active hormonal form of the nutrient, has not been used typically as a measure associated with vitamin D nutriture or as an intermediate related to health outcomes. Calcitriol is not useful as such a measure, for several reasons. Its half-life is short (hours), its formation is not directly regulated by vitamin D intake, its levels are regulated by other factors (such as serum PTH), and, even in the presence of severe vitamin D deficiency the calcitriol level may be normal or even elevated as a result of up-regulation of the 1α -hydroxylase enzyme.

Factors Affecting Serum 25OHD Levels

Dietary Intake (Foods and Supplements)

Available literature demonstrates that serum 25OHD levels increase in response to increased vitamin D intake, although overall it can be concluded that the relationship is non-linear rather than linear. Factors that may affect the relationship between vitamin D intake and serum 25OHD levels are not entirely clear, and the reliability of such measures may be less than desirable. Moreover, there remains debate over the equivalence of vitamins D₂ and D₃ in the diet (Armas et al., 2004; Rapuri et al., 2004; Vieth, 2004), although it has been assumed that they are 25-hydroxylated at similar rates (see previous discussion of functions and physiological actions of vitamin D).

As part of the 2007 Agency for Healthcare Research and Quality (AHRQ) systematic review (Cranney et al., 2007, 2008), referred to hereafter as AHRQ-Ottawa, exploratory meta-regression analysis was conducted

of 16 trials in adults, which suggested an association between vitamin D dose and serum 25OHD concentrations. The analysis found that for each additional 100 IU of vitamin D₃, serum 25OHD concentrations rose by 1 to 2 nmol/L. Trials varied in their use of vitamin D₂ or vitamin D₃. A few of the studies reported different effects of vitamin D₂ and vitamin D₃ on serum 25OHD levels. Although the later AHRQ-Tufts analysis (Chung et al., 2009) did not identify newer (compared with AHRQ-Ottawa) randomized controlled trials related to intake of vitamin D and serum 25OHD levels, it did graphically evaluate the net changes in serum 25OHD concentrations against the doses of vitamin D supplementation using data from the trials in adults. The analysis confirmed the relationship between increasing doses of vitamin D and increasing net change in serum 25OHD concentrations in both adults and children, but it also concluded that the dose–response relationships differ depending upon study participants' baseline serum 25OHD levels (≤ 40 vs. > 40 nmol/L) and duration of the supplementation (≤ 3 vs. > 3 months).

In a recent study conducted by Smith et al. (2009), personnel stationed in the Antarctic in winter months (and thus presumed to obtain vitamin D from food and supplements only) were given graded doses of 400, 1,000, or 2,000 IU of vitamin D₃ per day for 5 months. Baseline levels of serum 25OHD rose from approximately 44 nmol/L to 57, 63, and 71 nmol/L, respectively, representing a change in 25OHD levels of 13, 19, or 27 nmol/L. Evident in this study is the continuing fall in 25OHD level in the “no pill” group (to 34 nmol/L) of men who were deprived of sunlight and received approximately 250 to 350 IU of vitamin D (which included foods and any non-study supplements) per day. A possible complicating factor in interpreting these data is that the subjects were consuming diets with a vitamin D content that ranged from 241 to 356 IU/day, in addition to the graded doses of vitamin D from administered supplements, although the amounts of vitamin D obtained from foods (or non-study supplements) were not significantly different between treatment groups. Therefore, any effect of these sources of vitamin D on serum levels would have been consistent between treatment groups.

Another study of serum 25OHD response to total intake under conditions of minimal sun exposure used two populations based in Cork, Ireland (51°N), and Coleraine, Northern Ireland (55°N). The study estimated the dose of vitamin D required to maintain 25OHD levels above certain chosen cutoff values (i.e., 25.0, 37.5, 50.0, and 80.0 nmol/L) during the winter months (Cashman et al., 2008). The researchers found that serum 25OHD levels that ranged between 65.7 and 75.9 nmol/L in late fall fell in all groups receiving 200, 400, and 600 IU of vitamin D₃ per day, as well as in the placebo group, in winter. The decrease in the 600 IU/day group was minimal, from 75.9 to 69.0 nmol/L. Cashman et al. (2008) went on to

plot the serum 25OHD levels attained after 5 months versus the estimated total vitamin D intake (approximately 0 to 1,400 IU/day) in 215 individuals, as shown in Figure 3-4. They concluded that, in these two populations at 51°N and 55°N, the wintertime intake required to achieve 25OHD cutoff levels of 37.5, 50.0, and 80.0 nmol/L were 796, 1,120, and 1,644 IU/day, respectively.

Importantly, the relationship between vitamin D intake and serum 25OHD response appears not to be linear, given evidence that increasing serum 25OHD level above 50 nmol/L requires more vitamin D intake than does increasing serum 25OHD levels when the starting point is less than 50 nmol/L (Aloia et al., 2008). Factors such as baseline serum 25OHD level in the population may be relevant. Further, there have been reports that the rise in serum 25OHD levels for a given dose tends to stabilize by week 6 (Harris and Dawson-Hughes, 2002; Holick et al., 2008) and that it does

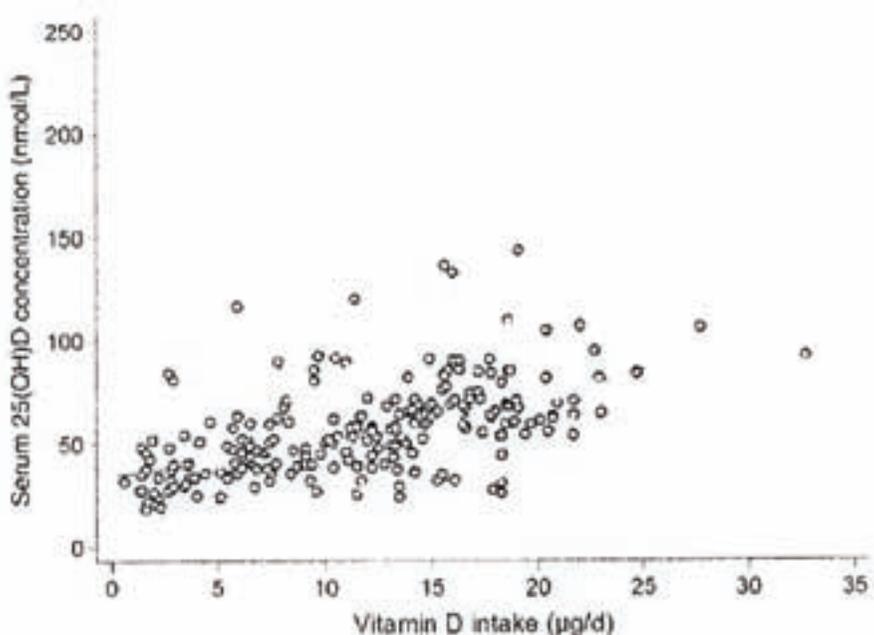


FIGURE 3-4 The relationship between serum 25-hydroxyvitamin D concentrations (in late winter 2007) and total vitamin D intake (dietary and supplemental) in 20- to 40-year-old healthy persons ($n = 215$) living at northern latitudes (51°N and 55°N).

SOURCE: Cashman et al. (2008). Reprinted with permission from the *American Journal of Clinical Nutrition* (2008, 88, 1535-42), American Society for Nutrition.

not vary with age at least up to 80 years of age (Harris and Dawson-Hughes, 2002; Cashman et al., 2008, 2009).

Sun Exposure

The cutaneous synthesis of vitamin D, and in turn its contribution to the concentration of serum 25OHD, is initially dependent upon the presence of 7-dehydrocholesterol in the skin. However, many variables can affect the cutaneous synthesis of vitamin D, making it difficult to estimate an average amount of vitamin D and, in turn, serum 25OHD levels that are produced by sun exposure in North America. There is, however, agreement that sun exposure is a significant source of the circulating serum 25OHD in summer for many North Americans, and is notably reduced as a contributor in the winter months. Early work from Webb et al. (1988, 1989) as well as a letter from Holick et al. (1989) have outlined the role of sunlight in regulating cutaneous synthesis of the vitamin and have implicated factors in vitamin D₃ synthesis in the skin to include aging, melanin pigmentation, season of the year, latitude, and use of sunscreen. Matsuoka et al. (1992) has discussed the role of clothing in preventing synthesis.

The 2007 AHRQ-Ottawa systematic review (Cranney et al., 2007) noted that the few available randomized clinical trials conducted between 1982 and the time of the analysis, which focused on the effect of UVB radiation on serum 25OHD level revealed little information about the impact of age, ethnicity, skin pigmentation, BMI, or latitude on serum 25OHD levels. However, UVB exposure increased serum 25OHD levels in vitamin D-deficient and -sufficient subjects with mean increases ranging from 15 to 42 nmol/L.

The difference in seasonal contributions to serum 25OHD level from sun exposure is discussed first. Next, factors affecting the synthesis of vitamin D—and, in turn, the levels of 25OHD in serum—are outlined.

Effect of season on circulating serum 25OHD level Sunlight exposure as a source of cutaneous synthesis of vitamin D is subject to a number of limitations. For example, excess exposure can lead to photo-degradation as a regulatory mechanism to avoid toxicity (Chen et al., 2007). In addition, latitude, time of exposure, and season all affect cutaneous synthesis, depending on the ability of UV rays to stimulate vitamin D production. Differences in seasonal exposure can vary by as much as 6 months at extreme northern and southern latitudes (Lucas et al., 2005; Kull et al., 2009).

A number of studies have examined serum 25OHD levels in different seasons. Van der Mei et al. (2007) compared cross-sectional data from men and women less than 60 years of age living in Australia and concluded that season was a strong determinant of serum 25OHD concentrations. Berry

et al. (2009) examined white adults ages 20 to 60 years living in the United Kingdom (UK) at latitude 53°N. In this study, women were found to have higher average serum 25OHD levels than men in both summer and winter (9 and 20 percent higher, respectively). In a study of young adults of diverse ethnic backgrounds living in Toronto, Gozdk et al. (2009) found that in winter, serum 25OHD levels of individuals with East and South Asian backgrounds were significantly lower than those of individuals with European ancestry. Kull et al. (2009) measured seasonal variance of 25OHD levels in adults ages 25 to 70 years living in Estonia in northern Europe, where dairy products are not fortified with vitamin D. During the winter, 73 percent of the study population had 25OHD levels that were below 50 nmol/L, and 8 percent had levels that were below 25 nmol/L, compared with 29 percent and 1 percent, respectively, during the summer. Rapuri et al. (2002) examined white, black, and Hispanic women 65 to 77 years of age in Omaha and reported mean serum 25OHD levels of 68 nmol/L in February and 86 nmol/L in August. These studies, despite variations in age, gender, and ethnicity, all suggest that seasonal change can affect cutaneous vitamin D synthesis. The winter low serum 25OHD concentrations and the summer high serum 25OHD concentrations from these studies are summarized in Table 3-1.

Although there are variations in the available data as well as a number of unknowns that may influence such values, they suggest a seasonal change in serum 25OHD concentrations between the winter nadir and the summer zenith of approximately 25 nmol/L. Free-living individuals in the latitudes studied appear to experience an approximately one-third seasonal increase in their circulating serum 25OHD levels as they moved from the winter months to the summer months.

The 25 nmol/L change in serum 25OHD level would appear to be similar in magnitude to change experienced by subjects given 2,000 IU/day in the Antarctic study (Smith et al., 2009). Although these data suggest that average cutaneous synthesis during the summer in northern latitudes equates to 2,000 IU/day, this may be a questionable conclusion given the many variables that come into play, ranging from feedback mechanisms to skin pigmentation to baseline levels of 25OHD. For example, recent work from Olds et al. (2008) suggested a curvilinear relationship between sun exposure and serum 25OHD levels, as well as variation depending upon initial concentrations of 7-dehydrocholesterol levels. At lower doses, vitamin D₃ production rises immediately in response to UV exposure, whereas at higher doses, the rate of production is lower and reaches an earlier plateau.

Effect of skin pigmentation on synthesis The presence of melanin in the epidermal layer is responsible for skin pigmentation. A number of

TABLE 3-1 Winter Low 25OHD Levels and Summer High 25OHD Levels Around the World

Location (Latitude)	Winter Baseline	Summer High
Toronto, Canada ^a 43°N	35 nmol/L	50 nmol/L ^b
Tasmania, Australia ^c 43°S	40 nmol/L	62 nmol/L
Estonia, northern Europe ^d 59°N	44 nmol/L	59 nmol/L
Salford/Manchester, UK ^e 53°N	46 nmol/L	71 nmol/L
Geelong region, Australia ^c 38°S	57 nmol/L	93 nmol/L
Omaha, USA ^f 41°N	68 nmol/L	86 nmol/L

^aGozdik et al., 2009.^bhigh value recorded in autumn.^cvan der Mei et al., 2007.^dKull et al., 2009.^eBerry et al., 2009.^fRapuri et al., 2002.

recent studies have reinforced the relationship of skin pigmentation to the capacity to produce vitamin D₃ after UV exposure, but the results are not all consistent. Armas et al. (2007) studied the incremental change in serum 25OHD levels in individuals with average 25OHD baseline levels of 52 nmol/L and with different skin pigmentation who were exposed to different daily doses of 20 to 80 mJ/cm² of UVB light three times per week for 4 weeks on 90 percent of their skin surface area. The work suggested that for individuals at the lighter pigmentation scale,⁷ exposed to similar UVB doses (20 to 80 mJ/cm²) resulted in twice the increase in serum 25OHD concentration compared with individuals at the opposite extreme (i.e., 62 vs. 32 nmol/L change) (Armas et al., 2007) (see modeled representation in Figure 3-5). However, other studies have shown that the response to UV dose is non-linear and dependent on genetic factors (Snellman et al., 2009), duration and dose rate of UV exposure, and baseline serum 25OHD levels (Bogh et al., 2010).

Another population study of 237 subjects at a single geographical location (Toronto, 43°N) also explored the effect of skin pigmentation on

⁷L*=70; the lightest skin tone of northern Europeans.

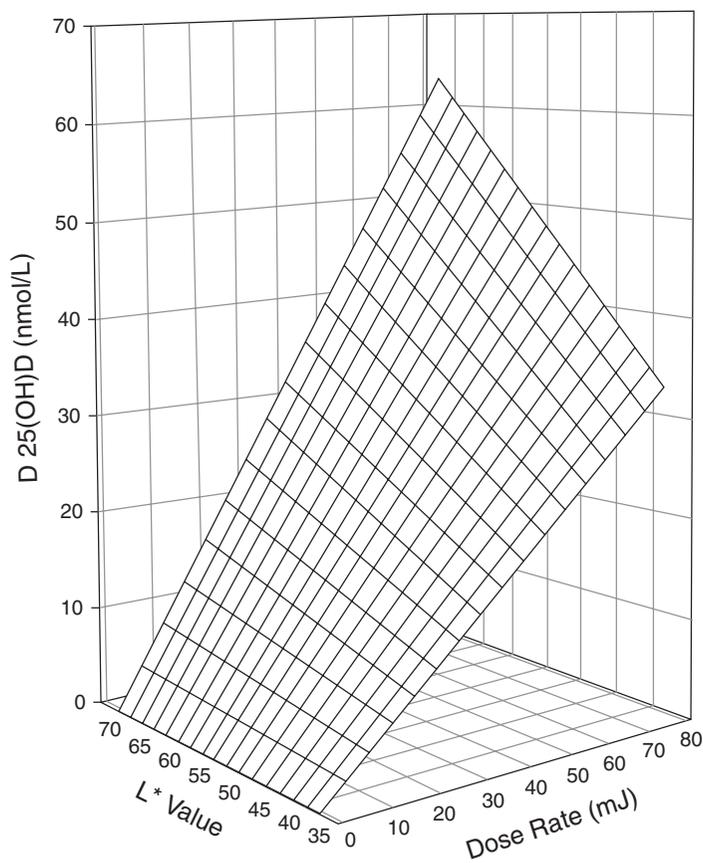


FIGURE 3-5 Three-dimensional scatter-plot of 4-week change in serum 25OHD concentration above baseline expressed as a function of both basic skin lightness (L^*) and UVB dose rate. Surface is hyperboloid, plotting equation 1, and was fitted to data using least squares regression methods.

NOTE: Equation 1 is $z = b * x * y$, where z is the induced increase in serum 25OHD level from baseline (in nmol/L); x is UVB dose (in mJ/cm^2 per session); y is skin lightness (expressed as the L^* score value from the reflective meter); and b is the sole parameter to be fitted.

SOURCE: Reprinted with permission from Robert Heaney.

25OHD synthesis (Gozdik et al., 2009). The mean serum 25OHD levels in Canadians of European, East Asian, and South Asian ancestry were 71.7, 44.6, and 33.9 nmol/L, respectively, in late fall; and 51.6, 28.1, and 26.5 nmol/L, respectively, in the winter. The authors found that differences between the three subgroups were strongly associated with skin pigmenta-

tion as well as amount of time spent outdoors and total vitamin D intake. A recent study of 182 individuals in Denmark, screened in January and February and selected to reflect wide ranges of baseline 25OHD levels and skin pigmentation, found that the increase in 25OHD levels after UVB exposure was inversely correlated with skin pigmentation as well as with baseline 25OHD (Bogh et al., 2010).

A number of small studies have reported serum 25OHD levels to be consistently lower in persons with darker skin pigmentation, and data from NHANES suggest that serum 25OHD levels are highest in whites, lowest in non-Hispanic blacks, and intermediate in Hispanic groups (Looker et al., 2008). Overall, there is considerable evidence that darker skin pigmentation is associated with a smaller increase in serum 25OHD concentration for a given amount of UVB exposure.

Effect of latitude on synthesis Early on, *in vitro* methods, such as exposure of sealed vials of 7-dehydrocholesterol to UVB radiation under “idealized conditions” at various geographical locations, were used to assess the effect of latitude, time of day, and season on the rate of vitamin D production (Webb et al., 1988). However, this approach cannot completely simulate the *in vivo* conditions in the body, where many factors serve to regulate this process. Furthermore, although the measurement of vitamin D concentrations in a mixture of irradiation products is analytically simple, vitamin D₃ levels in human serum are rarely used to estimate cutaneous synthesis of the vitamin, in part because of the transient nature of this blood parameter and the difficulty of measuring the low levels in serum (Holick, 1988; Hollis, 2008). Nevertheless, Holick and colleagues used a serum vitamin D₃ assay to augment *in vitro* methodology and suggested that, at latitudes above 43°N, cutaneous synthesis contributes little serum 25OHD to the system in the winter months between October and March in North America (Webb et al., 1988; Matsuoka et al., 1989).

More recent data may call into question current assumptions about the effect of latitude. In fact, Kimlin et al. (2007), using computer modeling, concluded that it may no longer be correct to assume that vitamin D levels in populations follow latitude gradients. Indeed, the relationship between UVB penetration and latitude is complex, as a result of differences in, for example, the height of the atmosphere (50 percent less at the poles), cloud cover (more intense at the equator than at the poles), and ozone cover. The duration of sunlight in summer versus winter is another factor contributing to the complexity of the relationship. Geophysical surveys have shown that UVB penetration over 24 hours, during the summer months at Canadian north latitudes when there are many hours of sunlight, equals or exceeds UVB penetration at the equator (Lubin et al., 1998). Consequently, there is ample opportunity during the spring, summer, and fall months in the far north for humans (as well as animals that serve as food

sources) to form vitamin D₃ and store it in liver and fat. These factors may explain why latitude alone does not consistently predict the average serum 25OHD level of a population.

Effect of sunscreen on synthesis Sunscreens are used to protect the skin from ultraviolet A (UVA) and UVB waveband exposure that is associated with deoxyribonucleic acid (DNA) damage—the same UVB exposure that is needed for vitamin D synthesis. Experimental studies suggest that sunscreens can decrease cutaneous vitamin D synthesis (Misra et al., 2008). However, emerging evidence suggests that although sunscreens are effective, many may not actually be blocking UVB because they are improperly or inadequately applied. Thus, sunscreen use may not actually diminish vitamin D synthesis in real world use, although further study is needed to verify its actual impact (Diehl and Chiu, 2010; Springbett et al., 2010).

Other variables affecting synthesis A number of other variables can impede sun exposure and thus inhibit cutaneous vitamin D synthesis. Clothing is an effective barrier to sun exposure and the UVB waveband, but the effectiveness of sun blocking depends on the thickness or weave of the fabric (Diehl and Chiu, 2010). Likewise, ethnic practices, such as extensive skin coverage with clothing, urban environments that reduce or block sunlight, air pollution, and cloud cover that reduces solar penetration can variously reduce sun exposure. In contrast, high altitude reduces the atmospheric protection against UVB waveband and can increase risk for sun damage as well as increase vitamin D synthesis (Misra et al., 2008). There may be a role for measures of physical activity in affecting vitamin D synthesis, although many covariates may be relevant, and some have suggested that genetics can account for some of the differences in synthesis of serum 25OHD.

Confounders Affecting Serum 25OHD Concentrations

Adiposity Interpreting data on serum 25OHD concentrations in obese and overweight persons is particularly challenging. Data from NHANES showed lower circulating levels of 25OHD among young adult obese non-Hispanic white women compared with their leaner counterparts; the relationship appeared to be weaker among non-Hispanic blacks. Differences in physical activity levels partially explain these differences (Looker, 2005, 2007). However, overweight and obese persons in NHANES also reported lower use of dietary supplements than did leaner persons of the same age or gender group (Radimer et al., 2004; Picciano et al., 2007), suggesting that lower dietary exposures could also contribute to the lower serum 25OHD levels in obese and overweight people.

Sequestration of vitamin D into fat likely also plays a significant role in

reducing the amount that can be presented to the liver for 25-hydroxylation. As noted previously, vitamin D is absorbed with fat as part of chylomicrons and is taken up first by peripheral tissues that express lipoprotein lipase, especially adipose tissue and skeletal muscle. This pathway predicts that increased adiposity should lead to lower serum 25OHD levels and, conversely, that weight loss should reduce peripheral sequestration and enable higher 25OHD levels. Consistent with this, not only does increasing adiposity correlate with lower 25OHD levels, but also a few studies of modest weight loss have found the circulating 25OHD levels to increase despite no increased intake of vitamin D from diet or sunlight exposure (Riedt et al., 2005; Reinehr et al., 2007; Zittermann et al., 2009; Tzotzas et al., 2010). The measured increase in serum 25OHD levels in overweight and obese individuals was about 1.5 nmol/L for a 100 IU/day vitamin D intake over 12 months (Sneve et al., 2008; Zittermann et al., 2009). Others found that obese subjects show a lower rise in serum 25OHD levels in response to both oral vitamin D intake and UVB exposure (Wortsman et al., 2000) or in a retrospective analysis in response to 700 IU/day vitamin D₃ supplementation (Blum et al., 2008). It is interesting that in severely obese individuals after malabsorptive gastric bypass surgery, vitamin D supplementation resulted in a marked rise in serum 25OHD level of approximately 3 nmol/100 IU intake when the dose was 800 to 2,000 IU/day, but only a 1 nmol/L rise when intake was increased to 5,000 IU/day (Goldner et al., 2009).

African American ancestry Serum 25OHD levels are lower in African Americans compared with light-skinned population groups (Looker et al., 2008), yet the risk for fracture is lower for African Americans than for other ethnic groups (Aloia, 2008). It should be noted, however, that there is a wide range of variability among individuals of any race or ethnicity (Aloia, 2008). Serum 25OHD levels in African Americans and whites have been shown to be similarly responsive to vitamin D supplementation at 40°N latitude (equivalent to Philadelphia or Indianapolis), increasing by 1 to 2 nmol/L per 100 IU/day at a dose of 3,440 IU/day (Aloia et al., 2008), although at doses below 2,000 IU/day, serum 25OHD levels do not increase above 50 nmol/L in African American girls (Talwar et al., 2007). The significance of maintaining a higher serum 25OHD level in African Americans is not understood at this time because of a lack of evidence on extra-skeletal effects of vitamin D.

Size and frequency of dose Dosing of vitamin D daily, weekly, or monthly has been tested, and there are reports of annual dosing as well. The results of a study by Chel et al. (2008) suggested that daily (600 IU/day) and weekly doses of vitamin D will increase serum 25OHD levels more than monthly doses, but Ish-Shalom et al. (2008) using a dose of 1,500 IU/day

found no difference due to timing of the doses. Ish-Shalom et al. (2008) suggested that the attenuated response to monthly dose in the Chel et al. (2008) study may have been due to poor compliance with a powdered monthly supplement compared with pills used for their daily and weekly doses. An alternative explanation is that only a lower (Chel et al., 2008) and not a higher (Ish-Shalom et al., 2008) dose is influenced by timing of the vitamin D supplement. Thus far, studies suggest that weekly and daily dosing give similar serum 25OHD responses.

Assays for Serum 25OHD

Serum 25OHD comprises the sum of 25OHD₂ and 25OHD₃. Because of the widespread use of both vitamin D₂ and vitamin D₃ in the United States and Canada, analysts must measure both 25OHD₂ and 25OHD₃ in order to provide the total 25OHD level in serum. This is in contrast to the situation in Europe where there has been a tradition of using only vitamin D₃ and where commercial methods that purport to measure only 25OHD₃ are available.

In North America, several assay types are currently in use, each with strengths and weaknesses (Makin et al., 2010). The two most common types of assays are

- Antibody-based methods, which use a kit or an automated clinical chemistry platform; and
- Liquid chromatography (LC)-based methods, which use automated equipment featuring either UV or mass spectrometric (MS)-detection.

As discussed below, both these methods are equivalent in terms of measuring the physiologically relevant parameter (total 25OHD level in serum), but there remains controversy over the performance of these assays in clinical and research laboratories. Moreover, reports in the literature for serum 25OHD measures should be interpreted with care, taking into account the type of assay employed, use of automation, year of analysis, and context of the analysis.

Overview of Assay Methodology

Assays for total 25OHD level in serum have existed for four decades since the metabolite was first discovered (Blunt et al., 1968). The earliest assays were competitive protein-binding assays (CPBAs), based upon the ability of either 25OHD₂ or 25OHD₃ to displace [³H]25OHD₃ from the plasma binding protein, DBP (Belsey et al., 1971; Haddad and Hahn,

1973). These assays incorporated extraction, chromatographic purification, and detection steps, but the assays were laborious and not easily simplified, owing to the presence of co-extracted interfering substances. They therefore fell out of favor. Non-chromatographic CPBA assays tended to read erroneously high, and some data from this period (1970s) are viewed as extremely questionable for this reason.

In the late 1970s LC-based assays emerged that used refined extraction and chromatographic steps with some form of fixed- or variable-wavelength UV detector to measure the distinctive native absorbance of the vitamin D metabolites with λ_{max} at 265 nm (Jones, 1978; Horst et al., 1979). These allowed for the separate estimation of 25OHD₂ and 25OHD₃ from serum samples. This type of assay has undergone much refinement over the years, with improvements in LC, improved automation, or the introduction of diode-array UV detectors that minimize the chance of picking up interfering substances (Lensmeyer et al., 2006). Although these assays require expensive equipment as well as sequential sample analysis, which make them somewhat time-consuming, they are still popular with a minority of analysts.

In the early to late 1980s, antibody-based assays were introduced, which use a proprietary antibody to a vitamin D molecular antigen, usually with a truncated vitamin D side chain. They are therefore devoid of the features that allow the resultant antibody to distinguish between 25OHD₂ and 25OHD₃ (Hollis and Napoli, 1985); this is of value, but at the expense of detecting other minor metabolites. Consequently, antibody-based methods measure only total 25OHD in serum. Various commercial assays differ because of the nature of the antibody used, some claiming an advantage that they do not discriminate between 25OHD₂ and 25OHD₃ (Hollis and Napoli, 1985), whereas others in fact do underestimate the 25OHD₂ pool and therefore provide correction factors to compensate for high 25OHD₂ content. Over the past decade, antibody-based 25OHD assays have become automated into a multiwell plate-format and the increased throughput has made them extremely popular. It is important to note that the majority of the data collected over the past 20 to 30 years have been analyzed using antibody-based assays.

Recently, LC-based assays have also undergone a radical transformation with the replacement of the UV detection step by a “universal detector” in the form of a tandem mass spectrometer (LC-MS/MS) (Jones and Makin, 2000). LC-MS/MS assays allow the analyst to discriminate between 25OHD₂ and 25OHD₃ and other serum lipids by their unique molecular masses and mass fragments. Accordingly, these methods measure 25OHD₂ and 25OHD₃, and therefore total 25OHD (Makin et al., 2010). Because these methods use short LC retention times, automated robotic extraction and LC separation steps, and computerized MS systems, they can be made relatively operator-free and provide high throughput. Further, their potential

advantages also include high specificity, high sensitivity, and better reproducibility (< 10 percent). The consensus among analysts is that LC-MS/MS assays will become the “gold standard” for assay performance in the future.

Thus, a variety of methods are available to determine serum 25OHD levels, each with its advantages and disadvantages that must be considered in evaluating the data arising from them.

Assay Performance Concerns

The methodologies used over four decades of assaying 25OHD levels in serum have each presented technical problems. These include, first, CPBA assays without chromatography that read high because of poorly defined “interferences” (Morris and Peacock, 1976; Stamp et al., 1976). Second, some LC-MS/MS assays with short LC run times read high because they cannot resolve 3-epi-25OHD₃, an isomer found in abundance in neonatal samples (Singh et al., 2006). Third, some antibody-based assays, particularly some of the automated versions, fail to recover or detect, and therefore underestimate 25OHD₂ (Carter et al., 2004). Fourth, some antibody-based assays overestimate values by detecting further metabolites of 25OHD (e.g., 24,25[OH]₂D and 26,23-lactone) particularly those found in hypervitaminotic D situations (i.e., 25OHD > 250 nmol/L) (Jones et al., 1987); and some antibody-based methods are sensitive to exogenous interferences such as the presence of dilutants (ethanol, non-human serum) used in quality control materials (Phinney, 2009). Thus, no assay is free from problems, and performance must be monitored vigilantly.

Performance has been a concern of analysts and clinicians in the vitamin D field for some time. Inter-laboratory comparisons have been conducted and published for two decades and suggest an unacceptable degree of variability in the different results (Jongen et al., 1984, 1989). This has led to the creation of external quality assurance schemes, such as the Vitamin D External Quality Assurance Scheme or DEQAS⁸ (Charing Cross Hospital, London, UK), similar to those used in other areas of clinical chemistry. Since its inception in the early 1990s, DEQAS has grown steadily, such that it now serves as a quarterly monitor of performance of analysts and 25OHD analytical methods for approximately 700 laboratories worldwide (Carter et al., 2010). Although initially DEQAS used gas chromatography-mass spectrometry (GC-MS) as its “gold standard,” more recently it has adopted an all-laboratory trimmed mean (ALTM) as the value it uses to judge performance. Using ALTM, DEQAS has served as an early-warning system for method and operator biases that have alerted the commercial kit manufacturers to modify their products or steps in their procedures or withdraw their kits. DEQAS has published performance characteristics (precision,

⁸Available online at <http://www.deqas.org/> (accessed March 9, 2010).

accuracy, and variability) regularly over the past decade (Carter et al., 2004; Carter, 2009; Jones et al., 2009). These publications have been augmented by other independent method comparisons (Glendenning et al., 2006; Lensmeyer et al., 2006; Roth et al., 2008).

In brief, these performance reports suggest some method biases in terms of accuracy and precision as well as variability as high as 15 to 20 percent. However, some skilled analysts can perform better than this with a coefficient of variation less than 10 percent. The recent introduction of the National Institute of Standards and Technology (NIST) reference standards⁹ calibrated using a “validated” LC-MS/MS method (Phinney, 2009) offers some hope that the variability of all methods can or will be improved in the future. Indeed, recent data suggest that an improvement is already occurring (Carter and Jones, 2009). At this time, however, serum 25OHD data in the literature must be viewed with care based upon the knowledge that they have been acquired using a variety of methods, each with its own shortcomings and subject to high variability.

Assay Shift and Drift: U.S. National Health and Nutrition Examination Survey

Recently, the National Center for Health Statistics (NCHS) posted an analytical note on its NHANES web page informing users about two issues that should be addressed when analyzing and using serum 25OHD data from NHANES.¹⁰ The first involved making direct comparisons between serum 25OHD levels from NHANES III (1988 to 1994) and those from the 2000 to 2006 NHANES because of a reformulation of the DiaSorin radioimmunoassay (RIA) kit¹¹ that resulted in shifts in assay results between the two time periods. Second, the note also cautioned that the data from the 2000 to 2006 NHANES were likely affected by drifts in the assay performance (method bias and imprecision) over time. A standard reference material (SRM 972, Vitamin D in Human Serum) and calibration solution (SRM 2972, 25-Hydroxyvitamin D₂ and D₃ Calibration Solutions) are currently available from the NIST. The NCHS plans to incorporate use of this SRM into future measures of 25OHD in NHANES. The web page also indicates that guidance concerning a correction factor and data interpretation regarding this shift and drift will be forthcoming from the agency. In the case of the Canadian survey of 25OHD levels in the Canadian population, the analytical methods used to determine 25OHD concentrations were the

⁹Available online at <http://ts.nist.gov/measurementservices/referencematerials/index.cfm> (accessed March 15, 2010).

¹⁰Available online at http://www.cdc.gov/nchs/data/nhanes/nhanes3/VitaminD_analytic_note.pdf (accessed March 17, 2010).

¹¹DiaSorin Radio-immunoassay (RIA) (Stillwater, MN).

Diasorin total 25OHD (Liaison) kit¹² (personal communication, S. Brooks, Health Canada, December 18, 2009).

MEASURES ASSOCIATED WITH VITAMIN D: PARATHYROID HORMONE

PTH, which, as described above, plays a role in vitamin D metabolism, is also a known marker for bone resorption, based upon the bone manifestations of secondary hyperparathyroidism—increased bone turnover, increased rates of bone loss, osteoporosis, and increased risk of fractures. Serum PTH level has thus been explored and suggested as a measure indicative of adequate vitamin D nutrition, notably on the basis of the level of serum 25OHD at which serum PTH level rises or, alternatively, the level of serum 25OHD at which serum PTH level no longer declines. This measure is discussed more fully in Chapter 4.

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¹²DiaSorin Liaison (Stillwater, MN).

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