

Vitamin D: bringing light to the issue

By Lynn Stiff, BS, and Sharon M. Miller, PhC, MT(ASCP), CLS(NCA)

In recent years, a combination of widely publicized epidemiological studies, clinical trials, and impassioned written and verbal exchanges among researchers have contributed to the growing awareness of health professionals and the general public of the many roles played by vitamin D metabolites. Now, improved vitamin D status not only is linked to optimized functions of organs considered classical targets of the vitamin (e.g., bone, intestine, and kidney) but also to an impressive diversity of tissues.¹ As new studies reveal expanding roles for this vitamin, more clinicians are seeing the potential benefits of assessing vitamin D status among patients of all ages on a regular, continuing basis. The means by which vitamin D adequacy is judged depends upon accurate laboratory determination of serum 25OH vitamin D concentrations.

The presence of plasma membrane and intracellular vitamin D receptors (VDRs) has been reported in many cells.¹ Selective transcription of more than 200 genes is known to occur in response to the hormonal form of vitamin D. Gene transcription is regulated via the hormonal form of vitamin D (1,25(OH)₂D) binding with the intracellular VDR. The physiologic responses triggered by the targeted genes go far beyond actions related to calcium homeostasis. In addition to responding to the hormonal form of vitamin D produced by the kidneys and released into the blood, many “non-classical, D-influenced” tissues (e.g., colon, breast, prostate) are capable of producing local 1,25(OH)₂D from 25(OH)D, the major circulating form of vitamin D. The hormone acting within the tissues can then exert a remarkable variety of effects, including some associated with critical actions within tumor microenvironments. Cell proliferation may be inhibited, differentiation induced, angiogenesis blocked, and apoptosis promoted — all these effects contributing to suppression of tumor progression.^{2,3}

All in the “D” family

Vitamin D, a fat-soluble vitamin, is steroid-derived and is chemically described as a secosteroid because one of its four rings is open (see Figure 1).⁴ The vitamin and its metabolites exist in two forms.^{4,5} Those identified as originating from D₂ are derived from yeast and plant material that has been irradiated with ultraviolet

light B or UVB. This form is only obtained through supplementation and fortification. The D₃ forms are found in foods and supplements, and also can be produced by the body. Structurally, vitamin D₂ and vitamin D₃ differ in their side chains. The absence of an origin designation (e.g., D₂ or D₃) implies that the material being discussed is a mixture of both forms, that both forms undergo the process being described, or that the precise origin of the material to which reference is made is unknown and could be either D₂ or D₃.

The vitamin and its metabolites are transported in the blood bound to vitamin D-binding protein (DBP), an alpha-2 globulin.⁵ Although similar in molecular structure (see Figure 1), D₂-derived metabolites have been reported to be only about one-third to one-

half as active as D₃ forms of the vitamin in maintaining serum 25(OH)D serum levels.⁵ Recent evidence, however, appears to refute this statement of reduced effectiveness of D₂ derivatives.⁶

Location, location, location

Vitamin D is one of the few vitamins that can be produced by the body. When skin is exposed to UVB radiation (wavelengths of 290 nm to 320 nm), photolysis of cutaneous 7-dehydrocholesterol occurs to form vitamin D₃.⁸⁻¹⁰ About 20 minutes of sunlight exposure during the summer months can produce 20,000 IU of vitamin D₃.^{9,11} The active form of vitamin D (1,25(OH)₂D) is ultimately produced after two consecutive hydroxylations, first in the liver and then the kidneys (see Figure 2).

From about October through March, however, UVB radiation is insufficient to trigger this conversion at locations at or above 35° N latitude.⁹ Individuals who spend winter months in sunny locales and assume that the issue of insufficient cutaneous synthesis of D₃ does not concern them may be surprised to learn that limited studies have suggested the latitude effect

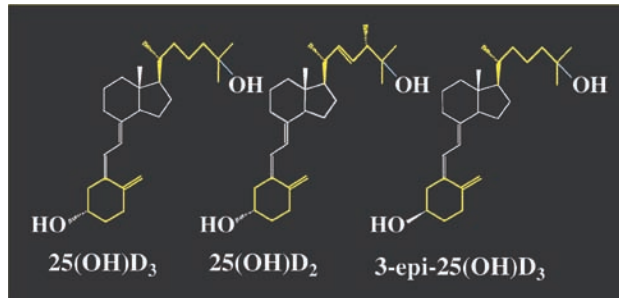


Figure 1. Forms of vitamin D. **Source:** Carter G, Jones J. 25-hydroxyvitamin-D (25-OHD) assays; “The state we’re in ...” Available at: www.deqas.org. Accessed March 21, 2009.

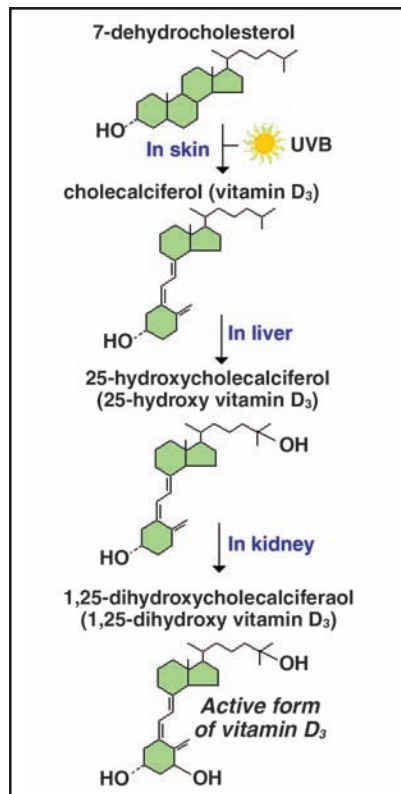


Figure 2. Reactions leading to the active form of vitamin D₃.⁶

may be a factor as far south in the United States as 32° N.¹²

About two-thirds of the U.S. population resides in regions at or above 35° N latitude (see Figure 3). Many studies have found a widespread prevalence of vitamin D insufficiency across North America, especially in the northern regions of the United States and in Canada.¹³⁻¹⁶ When discussing this topic, one physician replied, "It has been difficult to identify a population that is vitamin D sufficient."⁹

Because of diminished natural UVB exposure from fall through spring months, dietary intake of vitamin D is critical.⁹ Fatty fish such as salmon, mackerel, and tuna are the best sources, but fortified foods provide most of the vitamin D in the American diet.^{17,18} Because of the modest consumption of foods naturally high in vitamin D, supplementation is usually recommended when UVB exposure is insufficient.^{10,18} Regardless of the source, most individuals do not come close to meeting the current daily reference intake (DRI) for vitamin D through their diets. Presently, the DRI for adults <50 years of age is 5 µg (200 IU).¹⁹ In recent years, there has been much ardent debate over how much vitamin D is required for maintenance of adequate levels of 25(OH) D.¹³ Many authorities believe that higher intake recommendations for all gender and age categories are essential.^{5,9,11} The Institute of Medicine (IOM) is now beginning the process of reviewing vitamin D requirements, which many researchers feel are far too low.²⁰

Factors other than geographic latitude, seasonal fluctuations, and dietary practices impact an individual's vitamin D status. Individuals who are older (reduced level of vitamin D precursor in skin upon aging), have darker skin (higher melanin content), and whose clothing or sunscreen covers most of their skin, have a decreased ability to convert 7-dehydro-



Figure 3. Latitudes of continental U.S. population centers.

cholesterol to 25(OH)D₃, even when adequate sunlight is obtained.^{9,21} One study of elderly adults living in south Florida found approximately 40% of their sample population was vitamin D deficient during the winter months.²²

National Health and Nutrition Examination surveys (NHANES) have been conducted since 1971 with the goals of examining the health and nutritional status of non-institutionalized Americans. The NHANES III was conducted between 1988 and 1994 by the National Center for Health Statistics of the Centers for Disease Control and Prevention (CDC).²¹ The report of the survey exposed gender, racial, and ethnic differences in 25(OH) D concentrations in various population sectors; women had lower concentrations than men, non-Hispanic whites had higher concentrations than Mexican-Americans,

who in turn had higher concentrations than non-Hispanic blacks, and leaner and more active women had higher concentrations than heavier and less-active women (see Figure 4).²¹ Overall, about two-thirds of the U.S. population had serum levels indicating insufficiency.²³

Recently published data based from NHANES 2001-2004 indicates three of every four Americans have insufficient levels of 25(OH)D; 77% had insufficient levels of vitamin D.²⁴ Overall, the prevalence of 25(OH)D levels of 30 ng/mL or more decreased by approximately half from 45% in NHANES III to 23% in NHANES 2001-2004 (see Figure 5). Ninety-seven percent of non-Hispanic blacks and 90% of Mexican-Americans meet the criteria for being vitamin D insufficient. The suggestion has been made that minorities are at a markedly increased

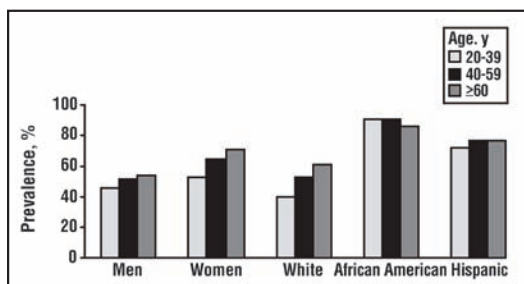


Figure 4. Prevalence of serum 25(OH)D levels <30 ng/mL, by race and ethnicity from NHANES data.²³

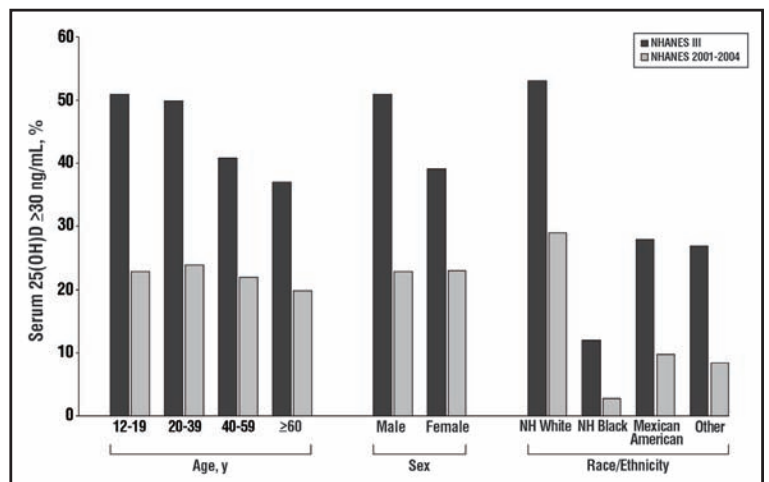


Figure 5. Stratified NHANES data depicting the prevalence of adequate serum 25(OH) D levels (>30ng/mL).²⁴

Poor vitamin D status may increase risk of:

- nervous system disease;
- multiple sclerosis;
- schizophrenia;
- depression;
- diabetes type 1 and 2;
- obesity;
- hypertension;
- muscle weakness (even in adolescents);
- frailty syndrome in older adults;
- heart failure;
- cardiovascular disease;
- cancers (i.e., colorectal, breast, prostate, non-melanoma skin);
- psoriasis;
- infectious diseases (i.e., tuberculosis, leprosy, seasonal epidemic influenza virus type A);
- asthma and allergy severity in childhood;
- polycystic ovary disease, menstrual problems and fertility;
- Crohn's and other inflammatory bowel diseases;
- bone disease (i.e., rickets, osteomalacia, osteoporosis); and
- periodontal disease.

Table 1. Diseases influenced by poor vitamin D status.¹

risk of health problems due — at least, in part — to poor vitamin D status.²⁴

Clinical applications

Approximately 35 tissues in humans have been identified as possessing VDRs.¹ Cells and tissues that are known to contain VDR receptors include the brain, heart, skin, gonads, prostate, breast, adipose tissue, adrenals, bone and bone marrow, cartilage, gastrointestinal tract and associated organs, immune cells (including activated lymphocytes, B and T cells), cardiac and smooth muscle cells, β -cells of the pancreas, thyroid gland, and parathyroid gland.¹ VDR binds several forms of D, but its affinity for $1,25(\text{OH})_2\text{D}_3$ is roughly 1,000 times that for 25OHD_3 .⁶ Table 1 identifies some conditions in which vitamin D insufficiency appears to play a role. As appreciation of the critical functions of vitamin D metabolites grows, practitioners in medical specialties such as geriatrics, orthopedics, physical medicine and rehabilitation, oncology, rheumatology, and family practice are increasingly likely to see major benefits in ordering testing of patients to assess their vitamin D status.^{5,25}

Measuring vitamin D

How much constitutes enough? Globally, approximately 1 billion individuals are deficient in or have insufficient circulat-

ing levels of $25(\text{OH})\text{D}$.¹⁸ The actual magnitude of vitamin D inadequacy is undoubtedly greater. As reflected in measurements of circulating levels of $25(\text{OH})\text{D}$, insufficiency of vitamin D has reached epidemic proportions in North America. Signs and symptoms linked to poor vitamin D status are often non-specific until deficiency is severe and bone integrity is compromised.²⁶ Patient complaints of chronic muscle and bone pain, depression, fatigue, cognitive difficulties, loss of muscle strength and mass, poor balance, and falls are attributable to multiple causes (or may simply be disregarded, especially if the patient is elderly).²⁶ Only accurate laboratory assessment of serum $25(\text{OH})\text{D}$ can focus attention on the true cause of the patient's difficulties and guide the clinician in taking corrective action.

Serum concentrations of $25(\text{OH})\text{D}$ correlate with substrate levels of vitamin D derived both from dietary intake and its cutaneous synthesis.⁸ A total of 100 IU ($2.5 \mu\text{g}$) of vitamin D raises the blood level of $25(\text{OH})\text{D}$ by 1 ng/mL.²⁷ The

biologically active form of vitamin D, $1,25(\text{OH})_2\text{D}$, is not routinely measured by the laboratory.⁸ Its level is tightly regulated by physiologic factors, and its half-life is only a few hours; conversely, serum $25(\text{OH})\text{D}$'s half-life is several weeks and is, therefore, a suitable reflection of the patient's actual vitamin D status.

Establishment of what serum level of $25(\text{OH})\text{D}$ is sufficient (or universally best) remains unsettled.^{9,10,13,18} Since the influence of vitamin D in the body is widespread, it becomes problematic to pinpoint the value that is adequate or, better yet, optimal year-round for all vitamin D-related physiological processes.¹ For instance, the value that is most favorable for calcium homeostasis and bone health may be different than that concentration that is deemed best for immune or cardiovascular function. Application of different criteria increases the difficulty of achieving consensus as to what constitutes the "ideal" serum level of $25(\text{OH})\text{D}$. Table 2 shows the typical values used to indicate various levels of vitamin D sufficiency.

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Testing frequency. Currently, no formal recommendations exist as to when and how frequently vitamin D testing should occur, so the testing is left to the discretion of clinicians. It is strongly recommended that all patients who are at risk of vitamin D deficiency be tested.¹⁰ Seasonal variations in the analyte concentration must be taken into account, so one test is inadequate to establish sufficiency of 25(OH)D. Testing should be conducted at least twice a year; measuring vitamin D levels during the fall through spring seasons would allow clinicians to assess the impact of “winter drop-off” on the adequacy of 25(OH)D circulating levels.

Automation, high sample throughput, and shortened turnaround time have contributed to a dramatic increase testing requests.²⁸ Predictions called for several million tests assessing vitamin D status to be conducted in the United States by the end of 2008. In fact, 25(OH)D testing has surged by as much as 80% to 90%.²⁹ A test that was once a rarity and thought to be suitable primarily for a research setting is now seen as a valuable diagnostic tool appropriate for the clinical laboratory. Despite this, there are serious issues associated with testing, such as the high variability in analyte measurement due to intermethod variability, laboratory-to-laboratory variation, and lack of standardization against reference materials.²⁵

Comparing assay technologies. Vitamin D assays originally were a time-consuming procedure, measuring DPB as the primary binding agent and a tritium-labeled tracer (³H-25(OH)D₃).⁴ Tests currently in use are much more appropriate to a high-throughput laboratory and include various forms of immunoassay, high-performance liquid chromatography (HPLC) and tandem mass spectrometry (MS/MS).^{8,30} The need to address the variability that exists among different assay methods to assess vitamin D status is a priority.³¹

■ **Immunoassays.** Immunoassays are extensively used and have been in previous large, ongoing studies, such as NHANES (CDC), the Harvard Nurses’ Study, the Harvard Health Professions Study, and the Framingham Study. LabCorp (Burlington, NC), the second largest reference laboratory-testing company in the United States, employs this method to assess 25(OH)D concentrations.

Level of sufficiency	Value
Severe deficiency	<10 ng/mL (25 nmol/L)
Vitamin D deficiency	<20 ng/mL (50 nmol/L)
Vitamin D insufficiency	21 ng/mL to 29 ng/mL (52 nmol/L to 72 nmol/L)
Adequate to derive health benefits	30 ng/mL to 100 ng/mL (75 nmol/L to 250 nmol/L)

Table 2. Levels of vitamin D sufficiency.^{5,9,17,18}

Clinical laboratories reporting serum measurements of 25(OH)D may use conventional units (ng/mL) or international system (SI) units (nmol/L). The conversion factor to SI units is: 1 ng/mL = 2.496 nmol/L.¹³

■ **Radioimmunoassay (RIA).** Developed in 1985, this method is produced and marketed by DiaSorin (Stillwater, MN). RIA accurately measures total 25(OH)D but is unable to separate D₂ and D₃. Despite this, RIA is frequently used because of its high correlation with HPLC and minimal procedural steps. Despite minimal processing, there can be a high cost per test.²⁵

■ **Chemiluminescent immunoassay (CLIA).** The LIAISON 25(OH)D assay has largely replaced the RIA and is also produced and marketed by DiaSorin. CLIA is fully automated and its results are highly correlated with RIA. Roche Diagnostics (Indianapolis, IN) has recently introduced the Elecsys 25(OH)D₃ assay for use with the company’s immunoassay analyzers.³² Serum 25(OH)D can also be measured using the Nichols ADVANTAGE 25(OH)D chemiluminescent assay (Nichols Institute, San Clemente, CA).

If previous reports of the D₂ derivatives being substantially less effective than the D₃ derivative in maintaining vitamin D status are correct (though as noted earlier recent evidence contradicts that assertion⁵), then a diagnostic dilemma may be created when an assay procedure does not distinguish between D₂ and D₃ levels. In theory, a patient could have a total 25(OH)D value fall within the acceptable range, but the bioactivity of the total 25(OH)D could actually be considerably less than ideal. A situation similar to a false-negative finding would exist, in which case, the patient would not then be treated for hypovitaminosis D when, in fact, clinically, they would be at less than a desirable level of the vitamin.

■ **LC-MS/MS.** Liquid chromatography tandem mass spectrometry (LC-MS/MS) is considered the “method of choice” by many, because this method is able to separate and individually quantify 25(OH)D₂ and 25(OH)D₃.

The technique, however, is not without shortcomings.^{14,29,33} There have been numerous reports of discrepancies between the results of LC-MS/MS and immunoassays, with LC-MS/MS reporting serum values up to 40% higher than those reported using RIA.³³ Survey data have indicated that labs performing 25(OH)D immunoassays report results ranging from 41 ng/mL to 96 ng/mL for a survey sample with a value of 75 ng/mL determined by LC-MS/MS.²⁹ Because of this, the method of testing is crucial to note when interpreting laboratory values.³³

Additional challenges with using this method properly include the need for each site to develop its own in-house standards (calibrators), since there is as yet no certified reference material to test method accuracy and a high-degree of expertise of laboratory personnel required to conduct the complex procedure.¹⁴ In addition, the lack of standardization among laboratories is a crucial concern.¹⁴ Not all laboratories have the same standard operating procedures, and LC-MS/MS interlaboratory value can vary by 20%.²⁹ Aliquots of one sample sent to seven different laboratories using LC-MS/MS to measure vitamin D reportedly generated seven different results in one study.³³ Mayo Medical Laboratories (Rochester, MN) employs this methodology, as does Quest Diagnostics (Madison, NJ), the largest reference laboratory-testing company in the United States.

Problems with testing

Not all test procedures are equivalent. This may explain the recent brouhaha over vitamin D testing conducted by Quest Diagnostics (Madison, NJ).³³ Quest produced its own assay for LC-MS/MS in 2006. Shortly thereafter, many physicians noticed the values were inconsistent with those obtained previously for specific patients. In late 2008, the corporation con-

tacted thousands of physicians, indicating values reported on patients might not have been accurate and offered retesting at no charge. Word spread rapidly, and the details of the unfolding saga were revealed in *The Dark Report* of December 22, 2008. The Quest retest offer became public knowledge when an article appeared in *The New York Times* on January 7, 2009, describing the astonishing situation. Serious questions regarding overall lab testing accuracy and oversight have since been raised.^{11,33}

While any well-run laboratory can experience an error in testing at some point, this error is usually quickly recognized and corrected.³³ In the case of Quest Diagnostics, the error went unnoticed by laboratory personnel for 18 months to 24 months. It has been suggested that the pressure on both the company's personnel and its LC-MS/MS platform due to the rapid increase in 25(OH)D testing that occurred at Quest could explain why the error went unremarked for so long. Problem areas identified were reagent preparation and inconsistencies in operating procedures. In a corporate statement, Quest claims to have corrected the problem by the end of 2008.

Despite the nature of this incident, it has value in serving as a dramatic reminder to all laboratories of the importance of strict adherence to quality-control and quality-assurance practices. It highlights the obligation of the laboratory to promptly identify and rectify the problem and to swiftly communicate with all clients the possibility of erroneous results having been reported. Credibility once questioned is not easily regained.

Improving testing

Calibration of D₂ and D₃ assays has been a continuing problem because there is no certified reference material available to test method accuracy.³⁴ The National Institute of Standards and Technology is currently creating a serum-based Standard Reference Material (SRM) to be used as a control material by labs. This could reduce the interlaboratory variability of measurements and enhance the accuracy of nutritional status data in the NHANES survey.³⁵ The standard, identified as SRM 729, was expected to be released by the end of 2008 but is not yet available. Some researchers suggest that using serum vitamin D levels from a healthy popula-

tion would be the best reference material for assessing adequacy.¹ The SRM will possess variability similar to that seen in a healthy vitamin D sufficient population, by consisting of four pools with varying levels of 25(OH)D₂ and 25(OH)D₃.³¹ One pool will also contain 3-epi-25(OH)D₃, a form of vitamin D that has been found to be present in infants (see Figure 1).

The UK-based Vitamin D Quality Assessment Scheme (DEQAS) has been monitoring the performance of 25(OH)D assays since 1989 and consists of approximately 400 participating laboratories worldwide.^{4,36} The overall aim of DEQAS is to ensure the analytical reliability of 25(OH)D and 1,25(OH)₂D assays.³⁷ DEQAS provides an opportunity to assess the accuracy and specificity of 25(OH)D methods as well as monitoring the analytical performance of a large number of their users.⁴

An improvement in vitamin D testing has recently been developed by ZRT Laboratory (Beaverton, OR) that will facilitate large-scale testing of vitamin D status.³⁸

Sample collection is via finger-stick rather than venipuncture, and the analysis of the dried blood spot is by LC-MS/MS. The test is reported to be convenient, cost effective, and reliable. The test was found to highly correlate with traditional LC-MS/MS testing and was also able to separate D₂ from D₃.

The future

The existence of an epidemic of vitamin D insufficiency in the United States is confirmed by measurements of circulating levels of 25(OH)D showing almost 75% of adults and adolescents possess serum 25(OH)D levels less than 30 ng/mL.²⁴ On the brighter side, a newly published article indicated that daily intakes of 3.5 µg of dietary vitamin D and 20 µg of vitamin D₃ supplements during winter was enough to achieve adequate serum 25(OH)D concentrations in 80% of premenopausal women in Maine by winter's end.³⁹

As more clinicians begin to see the medical importance of knowing a patient's vitamin D status — information readily



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acquired by ordering serum 25(OH)D testing for their patients — understanding the basics of vitamin D formation and its actions, and being aware of the challenges associated with meaningful laboratory assessment of this analyte are crucial.

Circulating 25(OH)D measurement offers an important clinical tool in the diagnosis, management, and prevention of a variety of disease states. Laboratory professionals and clinicians also need to be conscious of the technical challenges associated with both measuring and interpreting 25(OH)D levels. For maximum diagnostic accuracy, assay standardization must be achieved along with identification of a consistent reference range for circulating 25(OH)D levels. □

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3,000 tests...equipment...services...
tables of critical limits...reference intervals...

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