The structural characteristics of highly teratogenic retinoids comprise the following (117):

1. A polar terminus with an acidic pK_a
2. A lipophilic polyene side chain with good π electron delocalization, e.g., cis isomers are less active than the all-trans isomer
3. A fairly lipophilic ring with no set nature or dimensions opposite the polar terminus, and
4. Conformational restriction (for acidic retinoids only)

Interestingly, the two *least* teratogenic compounds, 4-hydroxyphenylretinamide (Fig. 3d) and retinoyl β-glucuronide (Fig. 1j) are conjugated, more polar molecules.

F. Safe Levels of Vitamin A Intake in Humans

This issue of crucial public health importance can be approached in two ways: (a) by assessing the no-effect level of vitamin A intake experimentally in various populations at risk and (b) by reviewing the formal recommendations made by expert groups. The major groups at risk are infants, young children, pregnant women, and lactating women. The issue is complicated in part by whether or not a given population will clearly benefit as well as be put at-risk by the ingestion of a large dose of vitamin A. In the following discussion, however, only the issue of toxicity will be considered.

Statistical issues are also crucial. It clearly is easier to define an appropriate maximal intake for a population in which an allowable percentage of that group, e.g., 1%, 3%, or 5%, is adversely affected than to specify an intake that presumably will affect nobody. As already mentioned, genetic vitamin A intolerance is known to exist in a handful of persons (38,110,121). However, recommendations for a healthy population certainly should not be based on the adverse reactions of a few hypersensitive individuals. Finally, some relatively nonspecific reactions to moderate doses might not be causally related to the vitamin A in the dose.

Nonetheless, despite these caveats, some useful guidelines can be defined for vitamin A intake. RDIs of vitamin A are completely safe insofar as we know. RDA values for infants, children 1–6 years, and pregnant women are 375, 400–500, and 800 μg retinol equivalents, respectively (35); (Table 2). These values, however, assume that intakes are a mixture of preformed vitamin A and corotenoids. Thus, the estimated amount of *preformed* vitamin A in the diet of infants, children 1–6 years, and pregnant women are approximately 340 μg (1133 IU_a), 300–375 μg (1000–1250 IU_a), and 600 μg (2000 IU_a), respectively.

1. Pregnant Women

Although additional vitamin A is not usually needed by healthy pregnant women, daily multivitamin tablets containing 5000 IU_a (1500 μg) of vitamin A are ingested by large numbers of women. Toxic reactions to their ingestion have not been reported.

Toxic reactions have been noted, however, in women, both pregnant and nonpregnant, ingesting daily doses of ≥18,000 IU_a (≥5400 μg) (106,110–113), whereas only sporadic claims, often poorly documented, have been made for toxic effects at lower daily doses. In a study of retinoid embryopathy in pregnant women, the lowest dose of 13-*cis* retinoic acid that was associated with birth defects was 10 mg/day (122). Thus, 1500 μg of vitamin A seems safe for essentially all pregnant women, whereas ≥5400 μg probably is not.

Responding to concerns about the teratogenicity of vitamin A, the Teratology Society recommended that women who might become pregnant limit their daily intake of preformed vitamin A to 8000 IU_a (2400 μg) and ingest provitamin A carotenoids as a primary source of dietary vitamin A (123). Furthermore, they recommended that the unit dose of commercially available vitamin A be limited to 5000–8000 IU_a and that the hazards of excessive intakes of vitamin A be indicated on the labels of such products. Similarly, the American Institute of Nutrition, the American Society for Clinical Nutrition, and the American Dietetic Association issued a joint formal statement that supplements of vitamins and minerals were not needed by well-nourished, healthy individuals, including pregnant women, except in some specific instances (124). The Council for Responsible Nutrition, a group sponsored by industry, has also advised that pregnant women, while needing to ensure an adequate intake of vitamin A, should prudently limit their intake of nutritional supplements of vitamin A to 5000–10,000 IU_a (125). Subsequently, they recommended that the unit dosage of retinol in commercial vitamin A preparations be limited to 10,000 IU_a.

In recognition of the fact that a deficiency of vitamin A in the mother can also cause abortion and fetal abnormalities, the International Vitamin A Consultative Group (IVACG) has recommended that the average daily diet of pregnant women should supply 620 μg retinol equivalents, in keeping with FAO/WHO recommendations (34). However, in areas of the world where this level of intake does not occur and little opportunity exists for dietary improvement, or in emergency situations in which food supplies are disrupted, IVACG recommends that daily supplements of 3000 μg retinol equivalents (10,000 IU_a) can be given safely anytime during pregnancy. They do not suggest, by the way, that well-nourished women take supplements.

To summarize, well-nourished healthy women of reproductive potential should include carotenoid-rich fruits and vegetables in their diet. They should also avoid taking supplements of preformed vitamin A during the first trimester of pregnancy, during which increased nutritional demands are small and the risk of fetal abnormalities is high. If supplements of vitamin A are subsequently taken, the daily dose should be carefully limited to 5000–10,000 IU_a.

2. Infants and Young Children

As already indicated, the probable daily amounts of preformed vitamin A in recommended diets of infants and young children are 1133 IU_a and 1250 IU_a, respectively. As a general public health measure, oral doses (200,000 IU_a) of all-*trans* retinyl palmitate in oil have been administered one to three times a year to preschool children, usually 1–6 years of age, in less industrialized countries (95). Side effects, e.g., nausea, vomiting, and bulging of the fontanelle in infants, have usually been reported in $<5\%$ of the treated children and have been transient (1–3 days) in nature. Because vitamin A toxicity is a function of weight, younger children tend to be most affected.

Because of international public health interest in combining vitamin A supplementation with the expanded program of immunization (EPI), the effects of dosing infants with vitamin A from 6 weeks to 9 months of age has been explored. When 50,000 IU_a of vitamin A was given orally in oil to Bangladesh infants at 6, 11, and 16 weeks of age, 11% showed transient bulging of the fontanelle. In a subsequent study in which 25,000 IU_a was given orally at approximately the same three times to Bangladesh infants, 8%, corrected for the 2.5% incidence in the placebo group, showed the same effect (126). In

this study, nearly all of the affected children had received three doses of vitamin A; none showed any toxicity after one dose. If we assume that the infants' daily intake from breast milk was approximately 100 μg (333 μg IU_a), the calculated mean daily increment from the dose is only an additional 715 IU_a, giving a total of 1048 IU_a, essentially the same as the recommended daily intake. A single oral dose of 50,000 IU_a given to Indonesian infants showed much less toxicity, i.e., 4.5% in the vitamin A–treated group and 2.5% in the control group (127). Furthermore, the cerebral fluid volume, but *not* the pressure, was transiently increased in these infants, and no lasting side effects were noted. Bulging of the fontanelle, in consequence, may be more of a transient physiological response to a dose of this magnitude than an indicator of toxicity.

As yet, the relationship between an acceptably safe dose and the age of infants has not been defined. Quite possibly, infants suffering from inadequate intakes of protein and calories may be more susceptible to vitamin A toxicity than better nourished infants. If so, the dose of vitamin A that is selected for a given country might well be based on anthropometric indices within that country.

Thus, a generally safe single dose for most infants, although currently undefined, probably will fall in the range of 4000–5000 IU_a per kilogram body weight with an interval between doses of ≥5 weeks.

IX. CAROTENOIDS

Unlike retinoids, including vitamin A, carotenoids are generally nontoxic. However, individuals who routinely ingest large amounts of carotenoids, either in tomato or carrot juice or in commercial supplements of β-carotene, can develop hypercarotenosis, characterized by a yellowish coloration of the skin and a very high concentration of carotenoids in the plasma. This benign condition, although resembling jaundice, gradually disappears upon correcting the excessive intake of carotenoids. The only known toxic manifestation of carotenoid intake is canthaxanthin retinopathy, which can develop in patients with erythropoietic porphyria and related disorders who are treated with large daily doses (50–100 mg) of canthaxanthin, the 4,4′-diketo derivative of β-carotene, for long periods (128). In most instances, however, these deposits of canthaxanthin disappear slowly upon termination of treatment (128). Canthaxanthin-containing supplements are not currently available in the United States. β-Carotene at similar doses is not known to cause retinopathy. Carotenoids, even when ingested in large amounts, are not known to cause birth defects or hypervitaminosis A, primarily because the efficiency of their absorption from the intestine falls rapidly as the dose increases and because their conversion to vitamin A is not sufficiently rapid to induce toxicity (39).

Quite apart from their function as precursors of vitamin A, carotenoids are distributed widely in mammalian tissues, can quench singlet oxygen, can serve as an antioxidant in tissues (particularly under conditions of low oxygen tension), and can stimulate the immune response (39,104).

Thus, by using provitamin A activity as the nutritional function and singlet oxygen-quenching and antioxidant activity as the biological action, four classes of carotenoids might be defined: those that are both nutritionally and biologically active, such as β-carotene; those that are nutritionally active and biologically inactive, such as 14′-β-apocarotenal; those that are nutritionally inactive but biologically active, such as lycopene and violaxanthin; and those that are both nutritionally and biologically inactive, such as phytoene.

Because over 90% of the 600 characterized carotenoids in nature are not precursors of vitamin A, their biological effects in mammalian physiology, independent of their provitamin A activity, are being followed with interest.

X. POTENTIAL HEALTH BENEFITS OF RETINOIDS AND CAROTENOIDS

A. Retinoids

The requirements for vitamin A and safe levels of intake have already been discussed. A positive response to chemopreventive treatment with large doses of vitamin A has been shown in leukoplakia and actinic keratosis (103). The retinoids most commonly used for therapy and chemoprevention, however, are 13-*cis* retinoic acid, all-*trans* retinoic acid, hydroxyphenylretinamide, etretinate, and acitretin (103). As indicated earlier, these agents have shown promising results in the prevention or treatment of some carcinomas (103). The major drawbacks in their use are their toxicity at highly efficacious doses and, in the case of APL, a rapidly developing resistance to the drug. Of the retinoids listed, all-*trans* retinoic acid and etretinate, because of the latter's slow turnover in the body, are the most toxic and hydroxyphenylretinamide the least. The search consequently continues to identify new retinoids with high efficacy but low toxicity. Retinoyl β-glucuronide, a naturally occurring metabolite of retinoic acid, shows these properties (99,118). It is active in treating acne but has not been tested against other diseases. In epidemiological surveys, the dietary intake of preformed vitamin A and the plasma concentration of retinol are rarely associated with a reduced incidence of chronic diseases.

B. Carotenoids

1. Cancer

One of the most dramatic and consistent observations in epidemiological studies is the inverse association between β-carotene intake and the incidence of *lung cancer* (104,129). These findings have stimulated intervention trials in two high-risk groups: asbestos workers in Tyler, Texas and middle-aged male smokers in Finland. The results of these intervention trials are disappointing. In asbestos workers, no differences in the prevalence of sputum atypia was noted between treated (50 mg β-carotene + 25,000 IU_a retinol every other day) and control groups over a 5-year period. In the Finnish study, the group treated daily with β-carotene (20 mg) showed a significantly higher incidence of lung cancer [relative risk (RR) = 1.18, 95% confidence interval (CI) = 1.03–1.36] and total mortality (RR = 1.08, 95% CI = 1.01–1.16) than did the placebo group. Supplemental β-carotene did not affect the incidence of other major cancers found in this population (129).

The unexpected negative finding in the Finnish study has several possible explanations: (a) Supplemental β-carotene is interfering with the intestinal absorption of other possible chemopreventive nutrients. In this regard, β-carotene inhibits the absorption in humans of lutein, which shows good antioxidant activity (42). In that same vein, α-carotene, which shows chemopreventive properties, might be similarly affected. (b) Supplemental β-carotene may be serving as a pro-oxidant in the well-oxygenated ambient of the lung (130). (c) The population of middle-aged male smokers is not representative of other groups, who might well benefit from a higher intake of carotenoids. (d) A comparison between treated and control subjects that fall only in the lowest quartile of initial plasma

carotene values might yield different results. (e) Vitamin C, which is low in the plasma of most Finns, may have played some role in the outcome. (f) Alcohol intake may also play an important role in the outcome.

Several other trials have provided results that support the findings in the Finnish study, namely, the Carotene and Retinal Efficacy Trial (CARET) and the Physician's Health Study (129).

The development of *head and neck cancers*, including those of the oral cavity, pharynx, and larynx, are influenced by many factors, including smoking, other uses of tobacco, alcohol, and diet (129). Serum carotene concentrations, adjusted for smoking, are inversely related to the incidence of these carcinomas. Supplements of β-carotene can markedly reduce leukoplakia, although the lesion returns upon cessation of treatment (129). A chemoprevention trial designed to assess the effect of daily supplements of β-carotene (50 mg) on the recurrence of head and neck cancer is currently under way (129).

The effects of various nutrient combinations on *esophageal cancer* and on *stomach cancer* was evaluated in Linxian, China, where the incidence of esophageal cancer is 100-fold higher than in the United States (129). Of four nutrient treatments, only one, involving supplements of β-carotene, selenium, and α-tocopherol, showed a positive effect: the reductions in total deaths, cancer deaths, esophageal cancer deaths, and gastric cancer deaths were 9% (RR = 0.91, 95% CI = 0.84–0.99), 13% (RR = 0.87, 95% CI = 0.75–1.00), 4% (RR = 0.96, 95% CI = 0.78–1.18), and 21% (RR = 0.79, 95% CI = 0.64–0.99), respectively (129). Although these results support the concept that diet influences cancer incidence, the general nutritional status of the population was poor. Thus, whether the mixed supplement, or one component of it, was protective as a result of generally improved health or of a more specific anticancer effect is not clear (129).

The dietary intake and serum concentrations of carotenoids are often inversely associated with the risk of *colorectal cancer* (129). However, by using adenomas as an indicator, supplemental β-carotene (25 mg/day) was found to be ineffective (RR = 1.01, 95% CI = 0.85–1.20) in preventing the recurrence of this lesion (129).

β-Carotene intake has been associated with an improved survival rate in *breast cancer* patients (129). Whether supplements of carotenoids reduce the incidence of breast cancer in a well-designed clinical trial is not known. In an ongoing trial, the effect of hydroxyphenylretinamide on the recurrence of breast cancer is being explored (103).

The risk of *cervical cancer* has been correlated with the prediagnostic serum levels of α-, β-, and total carotenoids (RR = 2.7–3.1, 95% CI = ≥1.1–≤8.1) (129). On the other hand, invasive cervical cancer among white women in the United States did not relate to any specific food group of the diet or to the use of supplements of vitamins A, C, and E and folic acid (131). However, cervical dysplasia, considered to be a precancerous lesion, did respond to β-carotene supplements (30 mg/day) (129).

The recurrence of skin cancer was not affected by β-carotene supplements (50 mg/day) over a 5-year period (RR 1.05, 95% CI = 0.91–1.22) (129).

Thus, a dichotomy exists. Most of the associations found between diseases in dietary or plasma level studies do not agree with the results of intervention trials. The former tend to show strong significant correlations and the latter, in large part, do not. Possible explanations are as follows: (a) β-Carotene, which is only one of approximately 600 known carotenoids, might not be the most active one, or indeed, might inhibit the absorption of other more chemopreventive carotenoids and other nutrients. (b) Carotenoids might be only one of a group of chemopreventive agents in foods that act synergistically in preventing carcinogenesis. The fact that the relative risk values for colored fruits and vegeta-

bles usually are less (more protective) than those for carotenoids or for any other component of the food supports this viewpoint. (c) Carotenoids may serve solely as a useful *marker* for a healthful lifestyle. (d) The preventive action of carotenoids might occur very early in disease progression but be ineffective later. Thus, subjects in identified high-risk groups, who often have had a primary tumor, may be resistant to nutritional supplements. (e) The associations found in observational epidemiology are not causal and can be confounded by a variety of unanticipated and unmeasured factors. In essence, the intervention trials may well be providing more valid answers (129).

2. Photosensitivity Disorders

Patients with erythropoietic porphyria and similar diseases benefit by ingesting supplements (180 mg/day) of β-carotene. Canthaxanthin, though also protective, is no longer used because of the reversible retinopathy that results (128). Although the concentrations of β-carotene and vitamin A are elevated in the livers of these patients, the side effects of β-carotene ingestion over a period of years are minimal (129).

3. Cardiovascular Disease

Epidemiologic studies suggest protective effects of carotenoid intake against both coronary events and stroke (104,129). In a European study (WHO/MONICA, i.e., monitoring cardiovascular disease), mortality from ischemic heart disease correlated inversely with serum vitamin E concentrations ($r^2 = 0.63$) but not with β-carotene levels ($r^2 = 0.04$) (132). If the 3 Finnish sites, which were outliers, of the 16 examined were excluded, however, the inverse correlation with β-carotene concentrations improved markedly ($r^2 = 0.50$). A mean serum β-carotene concentration in populations of 0.4 μmol/L or higher was associated with good health in the European studies, whereas a concentration <0.25 μmol/L in populations was related to an increased risk of coronary disease, stroke, and cancer (132). The risk of myocardial infarction was inversely related to adipose β-carotene content in smokers (RR = 2.62, 95% CI = 1.79–3.83) but not in nonsmokers (RR = 1.07) (129). Furthermore, physicians with stable angina or prior coronary revascularizations, who were supplemented with β-carotene for 5 years, showed a 51% reduction in the risk of major coronary events (129). β-Carotene did not show beneficial effects, however, in the total population enrolled in the Physicians Health Study (129). The incidence of cardiovascular deaths in the Finnish lung cancer study also was not affected by β-carotene supplementation.

The overall results, therefore, are somewhat mixed. The most likely mechanism of action of carotenoids, but by no means the only one, is a reduction in the oxidation of low-density lipoproteins, which seem to play a key role in atherogenesis (104). Of various antioxidants studied both in vivo and in vitro, however, β-carotene does not seem to be very protective, if at all. Thus, the relationship among dietary intakes of carotenoids, their plasma and tissue concentrations, and cardiovascular disease remains unclear.

4. Age-Related Macular Degeneration

The macular of the eye predominantly contains two pigments, lutein and zeaxanthin (133,134). Because these two pigments account for less than 25% of plasma carotenoids, their uptake from plasma and deposition in the macula show specificity. These pigments might consequently play a role in protecting the macula from damage caused by light and particularly by blue light. In a recent study with patients suffering from age-related macular degeneration (ARMD) vs. matched controls, subjects in the highest quintile of carot-

enoid intake had a 43% lower risk (RR = 0.57, 95% CI = 0.35–0.92) of suffering from ARMD than those in the lowest quintile (129). Of various carotenoids, the intake of spinach and collard greens, rich in these two carotenoids, was most strongly associated with reduced risk. However, not all studies support this finding (105). Nonetheless, higher plasma concentrations of lutein and zeaxanthin as well as β-carotene showed a significant trend toward a lower risk of developing ARMD. These interesting findings are currently being investigated.

5. Senile Cataract

Cataract consists of gradual opacification of the lens with aging, which may in part result from oxidative stress. Carotenoid intake, as well as that of vitamins C and E, has been associated with a reduced risk of cataract (105,129). In the main Linxian, China trial, however, combined supplements of β-carotene, selenium, and α-tocopherol were not associated with a reduction in the incidence of cataracts, and inconclusive results have been reported by others (105,129). Thus, whereas the concept that antioxidant nutrients might prevent oxidative damage to a fairly exposed structure, such as the lens, is highly feasible, the data supporting a protective role of dietary components in the process are mixed.

6. HIV Infection

In HIV infection T-helper (CD4) cells are destroyed, thereby impairing the immune response. In humans as well as in experimental animals, both β-carotene, which is a provitamin A carotenoid, and canthaxanthin, which is not, can enhance the immune response (135). Indeed, in HIV-infected patients, large doses of β-carotene increased the CD4/CD8 ratio, which is usually depressed in HIV infection, and improved the response to vaccines (135). UV light tends both to activate human HIV expression, at least in transgenic mice, and to reduce plasma carotenoid concentrations in humans. In phase II HIV-infected subjects, plasma carotenoid concentrations are reduced by 50%. AIDS patients treated daily with a combination of β-carotene supplementation (120 mg) and whole-body hyperthermia (42°C, 1 h) showed a better and longer lasting response than either treatment separately (136). Thus, carotenoids seem to ameliorate the condition of AIDS patients, probably, at least in part, by enhancing the immune response.

A quite different effect of a carotenoid has also been reported, namely, that halocynthiaxanthin (5,6-epoxy-3,3′-dihydroxy-7′,8′-didehydro-5,6,7,8-tetrahydro-β,β-carotene-8-one) strongly and rather specifically inhibits RNA-dependent DNA polymerase of the HIV virus (135). The use of carotenoids in the treatment of subjects with HIV infections clearly merits further attention.

XI. CONCLUDING REMARKS

Revising a chapter that was written approximately 9 years ago (137) is a valuable but humbling experience. The facts cited earlier have not changed, and many of the concepts have been modified only slightly; however, interests have shifted markedly and a whole new body of information and hypotheses—some quite clear, some conflicting—has arisen. Old observations are viewed in new ways, and a completely new set of research questions are being asked. Thus, in revising the chapter, the addition of a paragraph here and a reference there was just not feasible. As a consequence, the chapter is largely rewritten, and the reference list is in large part new. This new chapter and the previous one are, therefore, complementary to each other.

A major recent advance has been the discovery of the nuclear retinoid receptors and their impact on embryogenesis, cell differentiation, disease, and pharmacology. In truth, the paradigm of interpreting vitamin A actions has markedly changed. A new chemistry has arisen, with the focus on finding compounds that serve as specific agonists and antagonists for given retinoid receptors. The linkage between nutrition and molecular biology, seemingly such diverse fields, has been strengthened by the observation that the RXR receptors for vitamin A interact meaningfully with the thyroid receptor, dependent on iodine for its activity, and with the vitamin D receptor, dependent of course on another fat-soluble vitamin. The hint (not the demonstration) that α-tocopherol may also have specific nuclear effects adds further interest to this linkage.

Diet, admittedly along with a variety of other factors, is known to affect the onset and possibly the severity of major chronic diseases. In the past several years, an explosion of information about specific nutrients that may play roles in these processes has appeared. Despite the great care with which most of these studies have been done, these surveys have inherent constraints. Thus, the findings in large part have tantalized us rather than presented a coherent picture. Carotenoids, primarily together with vitamin E, have played a central role in these surveys.

Although these studies have great potential impact, some biases have arisen: namely, that carotenoids, vitamin A, vitamin E, vitamin C, and selenium all act solely as antioxidants in these processes. Indeed, the term ''antioxidant vitamins'' has become common parlance. To stimulate a broader, less constrained view of their potential actions, these nutrients, as well as many other naturally occurring compounds, both of endogenous as well as of dietary origin, might better be called ''physiological modulators'' (138). By so doing, the mechanism of action is not automatically inferred from the outset for whatever beneficial or adverse effect that they might show (138,139).

To keep the reference list within bounds, references are largely made to reviews, which in turn can serve as a guide to the primary research literature. Recent reviews, monographs, and articles of particular interest are cited in references 140–162. I regret the necessary omission of many specific research papers that have enriched our knowledge in this dynamic field.

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2

Vitamin D

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I. INTRODUCTION

Vitamin D designates a group of closely related compounds that possess antirachitic activity. The two most prominent members of this group are ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3). Ergocalciferol is derived from a common plant steroid, ergosterol, and is the form that was employed for vitamin D fortification of foods from the 1940s to the 1960s. Cholecalciferol is the form of vitamin D obtained when radiant energy from the sun strikes the skin and converts the precursor 7-dehydrocholesterol into vitamin D_3. Since the body is capable of producing cholecalciferol, vitamin D does not meet the classical definition of a vitamin. It is more accurate to call vitamin D a *prohormone*; thus, vitamin D is metabolized to a biologically active form that functions as a steroid hormone (1–4). However, since vitamin D was first recognized as an essential nutrient, it has historically been classified among the lipid-soluble vitamins. Even today it is thought of by many as a vitamin, although it is now known that there exists a vitamin D endocrine system that generates the steroid hormone 1α,25-dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$].

Vitamin D functions to maintain calcium homeostasis together with two peptide hormones, calcitonin and parathyroid hormone (PTH). Vitamin D is also important for phosphorus homeostasis (5,6). Calcium and phosphorus are required for a wide variety of biological processes (Table 1). Calcium is necessary for muscle contraction, nerve pulse transmission, blood clotting, and membrane structure. It also serves as a cofactor for such enzymes as lipases and ATPases and is needed for eggshell formation in birds. It is an important intracellular signaling molecule for signal transduction pathways, such as those involving calmodulin and protein kinase C. Phosphorus is an important component of

Table 1 Biological Calcium and Phosphorus

Calcium	Phosphorus
Utilization	
Body content: 70-kg man has 1200 g Ca^{2+}	Body content: 70-kg man has 770 g P
Structural: bone has 95% of body Ca	Structural: Bone has 90% of body P
Plasma [Ca^{2+}] is 2.5 mM, 10 mg %	Plasma [P_i] is 2.3 mM, 2.5–4.3 mg %
Muscle contraction	Intermediary metabolism (phosphorylated intermediates)
Nerve pulse transmission	
Blood clotting	Genetic information (DNA and RNA)
Membrane structure	Phospholipids
Enzyme cofactors (amylase, trypsinogen, lipases, ATPases)	Enzyme/protein components (phosphohistidine, phosphoserine)
Eggshell (birds)	Membrane structure
Daily requirements (70-kg man)	
Dietary intake: 700[a]	Dietary intake: 1200[a]
Fecal excretion: 300–600[a,b]	Fecal excretion: 350–370[a,b]
Urinary excretion: 100–400[a,b]	Urinary excretion: 200–600[a,b]

[a]Values in milligrams per day.
[b]Based on the indicated level of dietary intake.

DNA, RNA, membrane lipids, and the intracellular energy–transferring ATP system. The phosphorylation of proteins is important for the regulation of many metabolic pathways. Furthermore, the maintenance of serum calcium and phosphorus levels within narrow limits is important for normal bone mineralization. Any perturbation in these levels results in bone calcium accretion or resorption. Disease states, such as rickets, can develop if the serum ion product is not maintained at a level consistent with that required for normal bone mineralization. Maintaining a homeostatic state for these two elements is of considerable importance to a living organism.

Recently, $1\alpha,25(OH)_2D_3$ has been shown to act on novel target tissues not related to calcium homeostasis. There have been reports characterizing receptors for the hormonal form of vitamin D and activities in such diverse tissues as brain, pancreas, pituitary, skin, muscle, immune cells, and parathyroid (Table 2). These studies suggest that vitamin D status is important for insulin and prolactin secretion, muscle function, immune and stress response, melanin synthesis, and cellular differentiation of skin and blood cells.

There are a number of recent books (7–9) and comprehensive reviews (10–19) that cover many aspects of vitamin D, including its endocrinological aspects.

II. HISTORY

Rickets, a deficiency disease of vitamin D, appears to have been a problem in ancient times. There is evidence that rickets occurred in Neanderthal man about 50,000 BC (20). The first scientific descriptions of rickets were written by Dr. Daniel Whistler (21) in 1645 and by Professor Francis Glisson (22) in 1650. Rickets became a health problem in northern Europe, England, and the United States during the Industrial Revolution when many people lived in urban areas with air pollution and little sunlight. Prior to the discovery of vitamin D, theories on the causative factors of rickets ranged from heredity to syphilis (2).

Table 2 Distribution of $1,25(OH)_2D_3$ Actions[a]

Tissue Distribution of Nuclear $1,25(OH)_2D_3$ Receptor		
Adipose	Intestine	Pituitary
Adrenal	Kidney	Placenta
Bone	Liver (fetal)	Prostrate
Bone marrow	Lung	Retina
Brain	Muscle, cardiac	Skin
Breast	Muscle, embryonic	Stomach
Cancer cells	Muscle, smooth	Testis
Cartilage	Osteoblast	Thymus
Colon	Ovary	Thyroid
Eggshell gland	Pancreas β cell	Uterus
Epididymus	Parathyroid	Yolk sac (bird)
Hair follicle	Parotid	

Distribution of Nongenomic Responses	
Intestine	Transcaltachia
Osteoblast	Ca^{2+} channel opening
Osteoclast	Ca^{2+} channel opening
Liver	Lipid metabolism
Muscle	A variety

[a]Summary of the tissue location of the nuclear receptor for $1\alpha,25(OH)_2D_3$ (nVDR) (top) and tissues displaying ''rapid'' or membrane-initiated biological responses (bottom).

Some of the important scientific discoveries leading to the understanding of rickets were dependent on discoveries about bone. As reviewed by Hess (23), the first formal descriptions of bone were made by Marchand (1842), Bibard (1844), and Friedleben (1860). In 1885, Pommer wrote the first description of the pathological process taking place in the rachitic skeleton. In 1849, Trousseau and Lasque recognized that osteomalacia and rickets were different manifestations of the same disorder. In 1886 and 1890, Hirsch and Palm did a quantitative geographical study of the worldwide distribution of rickets and found that the incidence of rickets paralleled the incidence of lack of sunlight (23). This was substantiated in 1919 when Huldschinsky demonstrated that ultraviolet (UV) rays were effective in healing rickets (24).

In the early 1900s, the concept of vitamins was developed and nutrition emerged as an experimental science, allowing for further advances in understanding rickets. In 1919, Sir Edward Mellanby (25,26) was able to experimentally produce rickets in puppies by feeding synthetic diets to over 400 dogs. He further showed that rickets could be prevented by the addition of cod liver oil or butterfat to the feed. He postulated that the nutritional factor preventing rickets was vitamin A since butterfat and cod liver oil were known to contain vitamin A (26). Similar studies were also conducted by McCollum et al. (27).

The distinction between the antixerophthalmic factor, vitamin A, and the antirachitic factor, vitamin D, was made in 1922 when McCollum's laboratory showed that the antirachitic factor in cod liver oil could survive both aeration and heating to 100°C for 14 h, whereas the activity of vitamin A was destroyed by this treatment. McCollum named the new substance vitamin D (28).

Although it was known that UV light and vitamin D are equally effective in preventing and curing rickets, the close interdependence of the two factors was not immedi-

ately recognized. Then, in 1923, Goldblatt and Soames (29) discovered that food that was irradiated and fed to rats could cure rickets; food that was not irradiated could not cure rickets. In 1925, Hess and Weinstock demonstrated that a factor with antirachitic activity was produced in the skin upon UV irradiation (30,31). Both groups demonstrated that the antirachitic agent was in the lipid fraction. The action of the light appeared to produce a permanent chemical change in some component of the diet and the skin. They postulated that a provitamin D existed that could be converted to vitamin D by UV light absorption. Much more work ultimately demonstrated that the antirachitic activity resulted from the irradiation of 7-dehydrocholesterol.

The isolation and characterization of vitamin D was now possible. In 1932, the structure of vitamin D_2 was simultaneously determined by Windaus in Germany, who named it vitamin D_2 (32), and by Askew in England, who named it ergocalciferol (33). In 1936, Windaus identified the structure of vitamin D found in cod liver oil, vitamin D_3 (34). Thus, the ''naturally'' occurring vitamin is vitamin D_3, or cholecalciferol. The structure of vitamin D was determined to be that of a steroid or, more correctly, a *seco*-steroid. However, the relationship between its structure and its mode of action was not realized for an additional 30 years.

Vitamin D was believed for many years to be the active agent in preventing rickets. It was assumed that vitamin D was a cofactor for reactions that served to maintain calcium and phosphorus homeostasis. However, when radioisotopes became available, more precise measurements of metabolism could be made. Using radioactive $^{45}Ca^{2+}$, Linquist found that there was a lag period between the administration of vitamin D and the initiation of its biological response (35). Stimulation of intestinal calcium absorption required 36–48 h for a maximal response. Other investigators found delays in bone calcium mobilization and serum calcium level increases after treatment with vitamin D (36–40). The duration of the lag and the magnitude of the response were proportional to the dose of vitamin D used (37).

One explanation for the time lag was that vitamin D had to be further metabolized before it was active. With the development of radioactively labeled vitamin D, it became possible to study the metabolism of vitamin D. Norman et al. were able to detect three metabolites that possessed antirachitic activity (41). One of these metabolites was subsequently identified as the 25-hydroxy derivative of vitamin D_3 [$25(OH)D_3$] (42). Because $25(OH)D_3$ was found to have 1.5 times more activity than vitamin D in curing rickets in the rat, it was thought that this metabolite was the biologically active form of vitamin D (43). However, in 1968, Haussler et al. reported a more polar metabolite that was found in the nuclear fraction of the intestine from chicks given tritiated vitamin D_3 (44). Biological studies demonstrated that this new metabolite was 13–15 times more effective than vitamin D in stimulating intestinal calcium absorption and 5–6 times more effective in elevating serum calcium levels (45). The new metabolite was also as effective as vitamin D in increasing growth rate and bone ash (45). In 1971, the structural identity of this metabolite was reported to be the 1α,25-dihydroxy derivative of vitamin D[$1\alpha,25(OH)_2D_3$] (46–48), the biologically active metabolite of vitamin D.

In 1970, the site of production of $1\alpha,25(OH)_2D_3$ was demonstrated to be the kidney (49). This discovery, together with the finding that $1\alpha,25(OH)_2D_3$ is found in the nuclei of intestinal cells, suggested that vitamin D was functioning as a steroid hormone (44,50). Subsequently, a nuclear receptor protein for $1\alpha,25(OH)_2D_3$ was identified and characterized (50,51). Since the cDNA for the $1\alpha,25(OH)_2D_3$ nuclear receptor from several species has now been cloned and sequenced (52–55), the relationship between vitamin D and the other steroid hormones has been clearly established (56). The discovery that the biological

actions of vitamin D could be explained by the classical model of steroid hormone action marked the beginning of the modern era of vitamin D.

III. CHEMISTRY

A. Structure

As previously mentioned, vitamin D refers to a family of compounds that possess antirachitic activity. Members of the family are derived from the cyclopentanoperhydrophenanthrene ring system (Fig. 1), which is common to other steroids, such as cholesterol. However, vitamin D has only three intact rings; the B ring has undergone fission of the 9,10 carbon bond, resulting in the conjugated triene system of double bonds that is possessed by all D vitamins. The structure of vitamin D_3 is shown in Fig. 1. Naturally occurring mem-

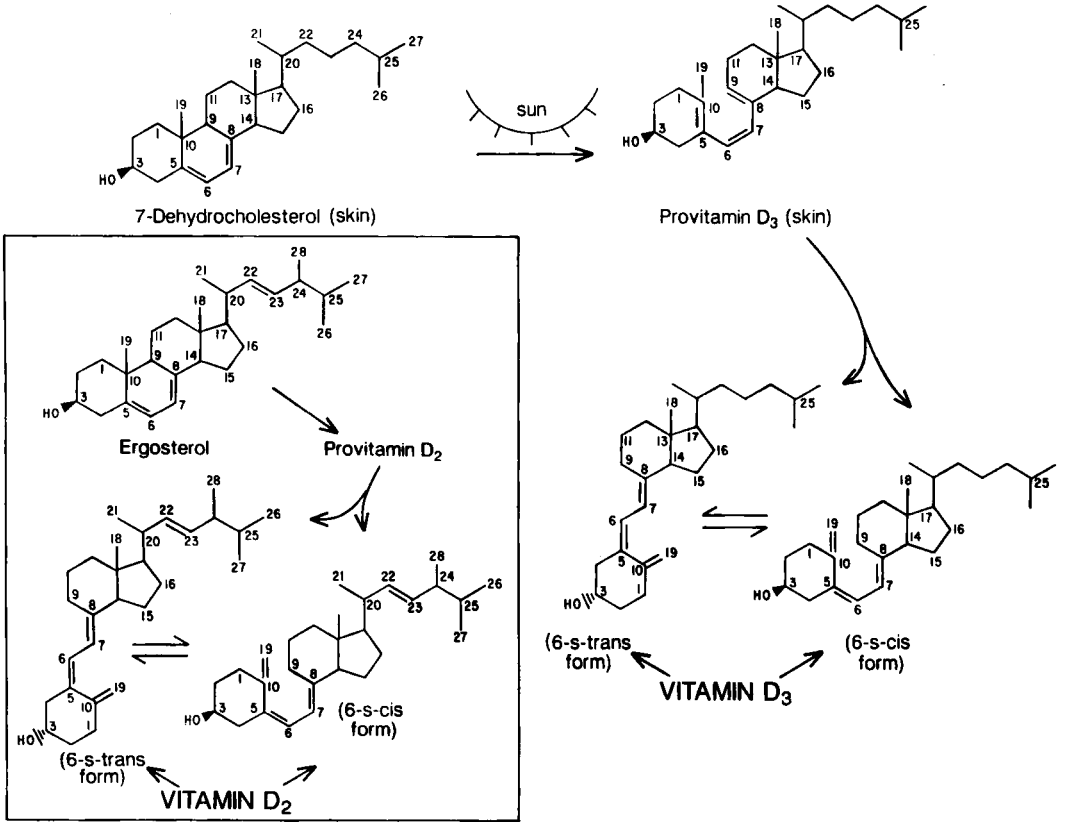
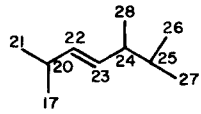
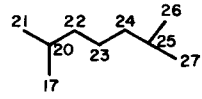
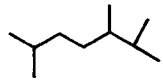
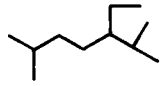
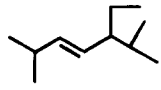
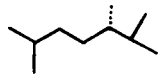


Fig. 1 Chemistry and irradiation pathway for production of vitamin D_3 (a natural process) and vitamin D_2 (a commercial process). In each instance the provitamin, with a $\Delta 5,\Delta 7$ conjugated double-bond system in the B ring, is converted to the *seco*-B previtamin, with the 9,10 carbon–carbon bond broken. Then the previtamin D thermally isomerizes to the ''vitamin'' form, which contains a system of three conjugated double bonds. In solution vitamin D is capable of assuming a large number of conformations due to rotation about the 6,7 carbon–carbon bond of the B ring. The 6-s-cis conformer (the steroid-like shape) and the 6-s-trans conformer (the extended shape) are presented for both vitamin D_2 and vitamin D_3.

Table 3 Side Chains of Provitamin D

Provitamin trivial name	Vitamin D produced upon irradiation	Empirical formula (complete steroid)	Side chain Structure
Ergosterol	D_2	$C_{28}H_{44}O$	
7-dehydrocholesterol	D_3	$C_{27}H_{44}O$	
22,23-dihydroergosterol	D_4	$C_{28}H_{46}O$	
7-dehydrositosterol	D_5	$C_{29}H_{48}O$	
7-dehydrostigmasterol	D_6	$C_{29}H_{46}O$	
7-dehydrocampesterol	D_7	$C_{28}H_{46}O$	

bers of the vitamin D family differ from each other only in the structure of their side chains; the side chain structures of the various members of the vitamin D family are given in Table 3.

From the x-ray crystallographic work of Nobel laureate Crowfoot-Hodgkin et al., it is now known that the diene system of vitamin D that extends from C-5 to C-8 is transoid and nearly planar (57,58). However, the C-6 to C-19 diene system is cisoid and not planar. The C-10 to C-19 double bond is twisted out of the plane by 60°. As a result, the A ring exists in one of two possible chair conformations. It is also known that the C and D rings are rigid and that the side chain prefers an extended configuration. In 1974, Okamura et al. reported that vitamin D and its metabolites have a high degree of conformational mobility (59). Using nuclear magnetic resonance (NMR) spectroscopy, they were able to detect that the A ring undergoes rapid interconversion between the two chair conformations, as shown in Fig. 2. This conformational mobility is unique to the vitamin D molecule and is not observed for other steroid hormones. It is a direct consequence of the breakage of the 9,10 carbon bond of the B ring, which serves to ''free'' the A ring. As a result of this mobility, substituents on the A ring are rapidly and continually alternating between the axial and equatorial positions.

B. Nomenclature

Vitamin D is named according to the new revised rules of the International Union of Pure and Applied Chemists (IUPAC). Since vitamin D is derived from a steroid, the structure retains its numbering from the parent steroid compound. Vitamin D is designated ''seco'' because its B ring has undergone fission. Asymmetrical centers are named using R, S

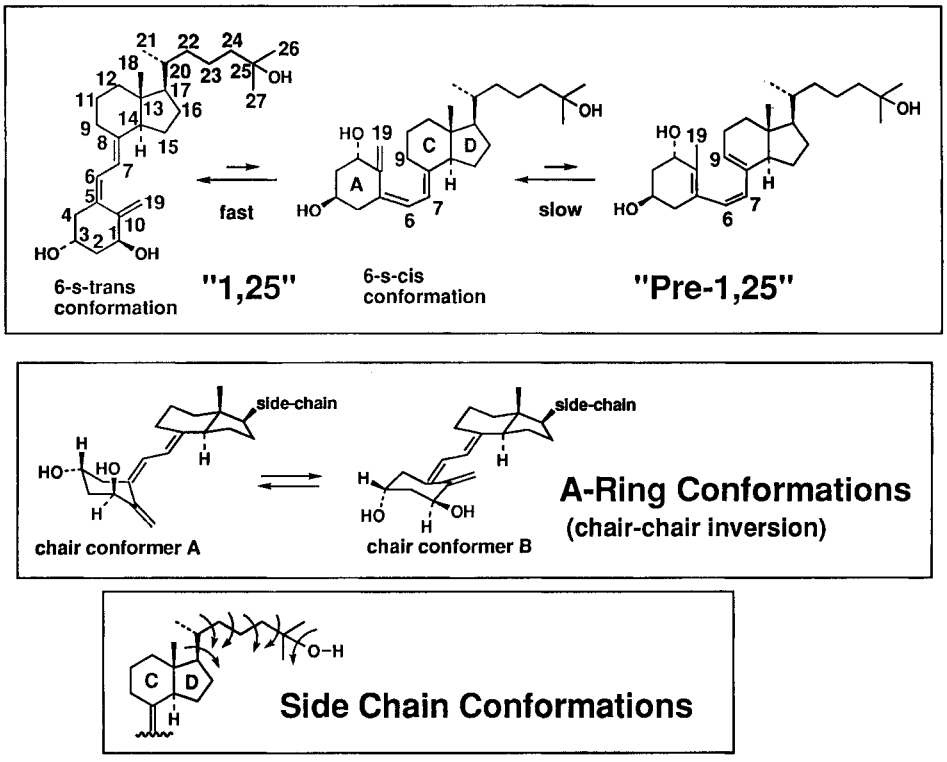


Fig. 2 The dynamic behavior of $1\alpha,25(OH)_2D_3$. The topological features of the hormone $1\alpha,25(OH)_2D_3$ undergo significant changes as a consequence of rapid conformational changes (due to single-bond rotation) or, in one case, as a consequence of a hydrogen shift (resulting in the transformation of $1\alpha,25(OH)_2D_3$ to pre-$1\alpha,25(OH)_2D_3$). (top) The dynamic changes occurring within the *seco*-B conjugated triene framework of the hormone (C5, 6, 7, 8, 9, 10, 19). All of the carbon atoms of the 6-s-trans conformer of $1\alpha,25(OH)_2D_3$ are numbered using standard steroid notation for the convenience of the reader. Selected carbon atoms of the 6-s-cis conformer are also numbered as are those of pre-$1\alpha,25(OH)_2D_3$. (middle) The rapid chair–chair inversion of the A ring of the secosteroid. (bottom) The dynamic single-bond conformational rotation of the cholesterol-like side chain of the hormone. The C/D *trans*-hydrindane moiety is assumed to serve as a rigid anchor about which the A-ring, *seco*-B triene, and side chain are in dynamic equilibrium.

notation and Cahn's rules of priority. The configuration of the double bonds is notated E, Z; E for ''trans,'' Z for ''cis.'' The formal name for vitamin D_3 is 9,10-*seco*(5*Z*,7*E*)-5,7,10(19)-cholestatriene-3β-ol and for vitamin D_2 is 9,10-*seco*(5*Z*,7*E*)-5,7,10(19),21-ergostatetraene-3β-ol.

C. Chemical Properties

1. Vitamin $D_3(C_{27}H_{44}O)$

Three double bonds
Melting point, 84–85°C
UV absorption maximum at 264–265 nm with a molar extinction coefficient of 18,300 in alcohol or hexane, $\alpha_D 20$ +84.8° in acetone

Molecular weight, 384.65
Insoluble in H_2O
Soluble in benzene, chloroform, ethanol, and acetone
Unstable in light
Will undergo oxidation if exposed to air at 24°C for 72 h
Best stored at 0°C

2. Vitamin $D_2(C_{28}H_{44}O)$

Four double bonds
Melting point, 121°C
UV absorption maximum at 265 nm with a molar extinction coefficient of 19,400 in alcohol or hexane, $\alpha_D 20 + 106°$ in acetone
Same solubility and stability properties as D_3

D. Isolation

Many of the studies that have led to our understanding of the mode of action of vitamin D have involved tissue localization and identification of vitamin D and its various metabolites. Since vitamin D is a steroid, it is isolated from tissue by methods that extract total lipids. The technique most frequently used for this extraction is that of Bligh and Dyer (60).

Over the years, a wide variety of chromatographic techniques have been used to separate vitamin D and its metabolites. These include paper, thin-layer, column, and gas chromatographic methods. Paper and thin-layer chromatography usually require long development times with unsatisfactory resolutions and have limited capacity. Column chromatography, with alumina, Floridin, Celite, silicic acid, and Sephadex LH-20 as supports, has been used to rapidly separate many closely related vitamin D compounds (2). However, none of the above methods are capable of resolving and distinguishing vitamin D_2 from vitamin D_3. Gas chromatography can separate these two compounds, but in the process vitamin D is thermally converted to pyrocalciferol and isopyrocalciferol, resulting in two peaks. High-performance liquid chromatography (HPLC) has become the method of choice for the separation of vitamin D and its metabolites (61,62). This powerful technique is rapid and gives good recovery with high resolution.

E. Synthesis of Vitamin D

1. Photochemical Production

In the 1920s, it was recognized that provitamins D were converted to vitamins D upon treatment with UV radiation (Fig. 1). The primary structural requirement for a provitamin D is a sterol with a C-5 to C-7 diene double-bond system in ring B. The conjugated double-bond system is a chromophore which upon UV irradiation initiates a series of transformations resulting in the production of the vitamin D *seco*-steroid structure. The two most abundant provitamins D are ergosterol (provitamin D_2) and 7-dehydrocholesterol (provitamin D_3).

2. Chemical Synthesis

There are two basic approaches to the synthesis of vitamin D. The first involves the chemical synthesis of a provitamin that can be converted to vitamin D by UV irradiation. The second is a total chemical synthesis.

Since vitamin D is derived from cholesterol, the first synthesis of vitamin D resulted from the first chemical synthesis of cholesterol. Cholesterol was first synthesized by two groups in the 1950s. The first method involves a 20-step conversion of 4-methoxy-2,5-toluquinone to a progesterone derivative, which is then converted in several steps to progesterone, testosterone, cortisone, and cholesterol (63). The other method uses the starting material 1,6-dihydroxynaphthalene. This is converted to the B and C rings of the steroid. A further series of chemical transformations leads to the attachment of the A ring and then the D ring. The final product of the synthesis was epiandrosterone, which could be converted to cholesterol (64). The cholesterol was then converted to 7-dehydrocholesterol and UV-irradiated to give vitamin D. The yield of vitamin D from photochemical conversion is normally 10–20%.

The first pure chemical synthesis of vitamin D, without any photochemical irradiation steps, was accomplished in 1967 (65). This continuing area of investigation allows for the production of many vitamin D metabolites and analogs without the necessity of a photochemical step. Pure chemical synthesis also allows for the synthesis of radioactive vitamin D and metabolites for the study of the metabolism of vitamin D.

Figure 3 summarizes some of the currently used synthetic strategies (14). Method A involves the photochemical ring opening of a 1-hydroxylated side-chain-modified deriv-

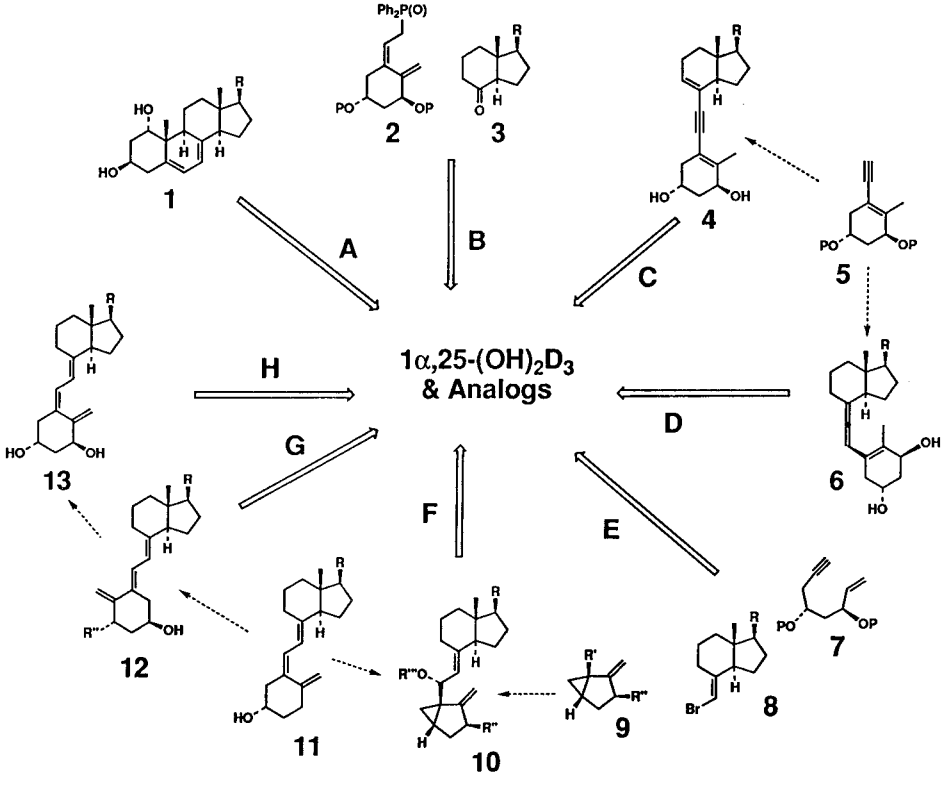


Fig. 3 Summary of approaches to the chemical synthesis of $1\alpha,25(OH)_2D_3$. The general synthetic approaches A–H, which are discussed in the text, represent some of the major synthetic approaches used in recent years to synthesize the hormone $1\alpha,25(OH)_2D_3$ and analogs of $1\alpha,25(OH)_2D_3$.

ative of 7-dehydrocholesterol **1** producing a provitamin that is thermolyzed to vitamin D (66,67). Method B is useful for producing side chain and other analogs. In this method, the phosphine oxide **2** is coupled to a Grundmann's ketone derivative **3**, producing the $1\alpha,25(OH)_2D_3$ skeleton (68,69). In method C, dienynes such as **4** are semihydrogenated to a previtamin structure that undergoes rearrangement to the vitamin D analog (70,71). Method D involves the production of the vinylallene **6** from compound **5** and the subsequent rearrangement with heat- or metal-catalyzed isomerization followed by sensitized photoisomerization (72). Method E starts with an acyclic A ring precursor **7** that is intramolecularly cross-coupled to bromoenyne **8**, resulting in the $1,25(OH)_2D_3$ skeleton (73,74). Method F starts with the tosylate of **11**, which is isomerized to the i-steroid **10**. This structure can be modified at C-1 and then reisomerized under sovolytic conditions to $1\alpha,25(OH)_2D_3$ or analogs (75,76). In method G, vitamin D derivatives **11** are converted to 1-oxygenated 5,6-*trans* vitamin D derivatives **12** (77). Finally, method H involves the direct modification of $1\alpha,25(OH)_2D_3$ or an analog **13** through the use of protecting groups, such as transition metal derivatives, or by other direct chemical transformations on **13** (78). These synthetic approaches have allowed the synthesis of more than 300 analogs of $1\alpha,25(OH)_2D_3$. For an article that gives an extensive review of all the synthetic approaches, see (14).

IV. METABOLISM

The elucidation of the metabolic pathway by which vitamin D is transformed into its biologically active form is one of the most important advances in our understanding of how vitamin D functions and the development of the vitamin D endocrine system. It is now known that both vitamin D_2 and vitamin D_3 must be hydroxylated at the C-1 and C-25 positions (Fig. 4) before they can produce their biological effects. The activation of vitamin D_2 occurs via the same metabolic pathway as does the activation of vitamin D_3, and the biological activities of both vitamin D_2 and vitamin D_3 have been shown to be identical in all animals except birds and the New World monkey. Apparently, these animals have the ability to discriminate against vitamin D_2 (79).

A. Absorption

Vitamin D can be obtained from the diet, in which case it is absorbed in the small intestine with the aid of bile salts (80,81). In rat, baboon, and human, the specific mode of vitamin D absorption is via the lymphatic system and its associated chylomicrons (82,83). It has been reported that only about 50% of a dose of vitamin D is absorbed (83,84). However, considering that sufficient amounts of vitamin D can be produced daily by exposure to sunlight, it is not surprising that the body has not evolved a more efficient mechanism for vitamin D absorption from the diet.

Although the body can obtain vitamin D from the diet, the major source of this prohormone is its production in the skin from 7-dehydrocholesterol. The 7-dehydrocholesterol is located primarily in the malpighian layer of the skin. Upon exposure to UV light, it is photochemically converted to previtamin D, which then isomerizes to vitamin D over a period of several days (85). Once formed, vitamin D is preferentially removed from the skin into the circulatory system by the blood transport protein for vitamin D, the vitamin D–binding protein (DBP).

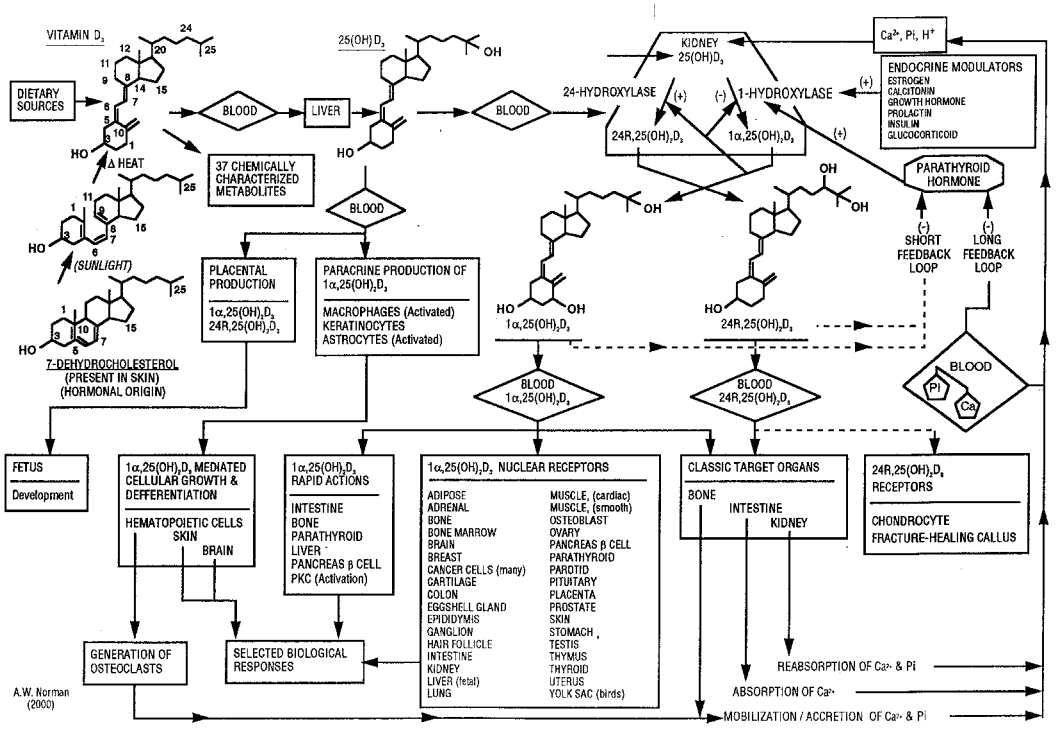


Fig. 4 Overview of the vitamin D endocrine and paracrine system. Target organs and cells for $1\alpha,25(OH)_2D_3$ by definition contain receptors for the hormone. Biological effects are generated by both genomic and nongenomic signaling pathways.

B. Transport

The actual site of transfer of vitamin D from the chylomicrons to its specific plasma carrier protein, DBP, is unknown. After an oral dose of radioactive vitamin D, the radioactivity becomes associated with the lipoprotein fraction of the plasma (86). As time passes, there is a progressive shift from this fraction to the γ-globulin fraction (87,88). It has been shown that the electrophoretic mobility of DBP is identical to that of γ_2-globulins and albumins (89,90).

In mammals, vitamin D, $25(OH)D_3$, $24R,25(OH)_2D_3$, and $1\alpha,25(OH)_2D_3$ are transported on the same protein, DBP (91,92). DBP, also known as group-specific protein (Gc protein), is a globulin protein with a molecular weight in humans of 58,000. It is a bifunctional protein, responsible both for the transport of vitamin D and its metabolites as well as functioning as a scavenger for actin which may be inappropriately present in the plasma. DBP possesses a high-affinity binding site for monomeric actin and forms a high molecular weight complex with it (93). DBP also possesses a high-affinity binding site for $25(OH)D_3$ and binds other vitamin D metabolites with somewhat lower affinity (92). Sequence analysis of the cDNA for DBP indicates that it shares homology with serum albumin and α-fetoprotein (94). DBP has been proposed to help in the cellular internalization of vitamin D sterols, and levels of DBP influence the concentration of ''bound'' and 'free' hormone

in the plasma (95). The concentration of the free hormone may be important in determining the biological activity of the hormone (95–99). Several review articles on DBP are available (97–99).

C. Storage

Vitamin D is taken up rapidly by the liver. Since it was known that the liver serves as a storage site for retinol, another fat-soluble vitamin, it was thought that the liver also functioned as a storage site for vitamin D. However, it has since been shown that blood has the highest concentration of vitamin D, in comparison with other tissues (100,101). From studies in rats it was concluded that no rat tissue can store vitamin D or its metabolites against a concentration gradient (82). The persistence of vitamin D in animals during periods of vitamin D deprivation may be explained by the slow turnover rate of vitamin D in certain tissues, such as skin and adipose tissue. During times of deprivation, the vitamin D in these tissues is released slowly, thus meeting the vitamin D needs of the animal over a period of time. In contrast, it was found that in pig tissue concentrations of $1\alpha,25(OH)_2D_3$, especially in adipose tissue, are three- to seven-fold higher than plasma levels (102).

Similarly, Mawer et al. carried out studies in humans on the distribution and storage of vitamin D and its metabolites (103). In human tissue, adipose tissue and muscle were found to be major storage sites for vitamin D. Their studies also indicated that adipose tissue serves predominantly as the storage site for vitamin D_3 and that muscle serves as the storage site for $25(OH)D_3$.

D. Metabolism

The parent vitamin D is largely biologically inert; before vitamin D can exhibit any biological activity, it must be metabolized by the body to its active forms. $1\alpha,25(OH)_2D_3$ is the most active metabolite known, but there is evidence that $24R,25(OH)_2D_3$ is required for some of the biological responses attributed to vitamin D (16,104,105). Both of these metabolites are produced *in vivo* following carbon-25 hydroxylation of the parent vitamin D molecule.

1. $25(OH)D_3$

In the liver, vitamin D undergoes its initial transformation, which involves the addition of a hydroxyl group to the 25-carbon. The metabolite thus formed is $25(OH)D_3$, which is the major circulating form of vitamin D. Although there is some evidence that this metabolite can be formed in other tissues, such as intestine and kidney, it is generally accepted that the formation of $25(OH)D_3$ occurs predominantly in the liver.

The production of $25(OH)D_3$ is catalyzed by the enzyme vitamin D_3 25-hydroxylase. The 25-hydroxylase is found in liver microsomes and mitochondria (106–109). It is a P450-like enzyme that is poorly regulated (110). Therefore, circulating levels of $25(OH)D_3$ are a good index of vitamin D status, i.e., they reflect the body content of the parent vitamin D_3 (111,112). Recent studies have suggested that the 25-hydroxylation of vitamin D is partially regulated by $1\alpha,25(OH)_2D_3$. Other studies suggest that 25-hydroxylation is dependent on intracellular calcium levels (113). However, the extent, nature, and physiological significance of any regulatory mechanism of this step in the metabolism of vitamin D remains uncertain. The 25-hydroxylase has been cloned (114) and expressed in yeast cells (115).

2. $1\alpha,25(OH)_2D_3$

From the liver, $25(OH)D_3$ is returned to the circulatory system where it is transported via DBP to the kidney. In the kidney, a second hydroxyl group can be added at the C-1 position. The enzyme responsible for the 1α-hydroxylation of $25(OH)D_3$ is the 25-hydroxyvitamin D_3-1α-hydroxylase (1-hydroxylase) (116).

1-Hydroxylase is located in the mitochondria of the proximal tubules in the kidney. The enzyme belongs to a class of enzymes known as mitochondrial mixed-function oxidases. Mixed-function oxidases use molecular oxygen as the oxygen source instead of water. 1-Hydroxylase is composed of three proteins that are integral components of the mitochondrial membrane: they are renal ferredoxin reductase, renal ferredoxin, and a cytochrome P450.

The most important point of regulation of the vitamin D endocrine system occurs through the stringent control of the activity of the renal 1-hydroxylase (117). In this way, the production of the hormone $1\alpha,25(OH)_2D_3$ can be modulated according to the calcium needs of the organism. Although extrarenal production of $1\alpha,25(OH)_2D_3$ has been demonstrated in placenta (118,119), cultured pulmonary alveolar and bone macrophages (120–122), cultured embryonic calvarial cells (123), and cultured keratinocytes (124,125), which can provide the hormone to adjacent cells in a paracrine fashion,the kidney is considered the primary source of circulating $1\alpha,25(OH)_2D_3$. Several regulatory factors have been identified that modulate 1-hydroxylase activity, but some are functional only in certain species and under certain experimental conditions. The major factors are $1\alpha,25(OH)_2D_3$ itself, PTH, and the serum concentrations of calcium and phosphate (126).

Probably the most important determinant of 1-hydroxylase activity is the vitamin D status of the animal. When circulating levels of $1\alpha,25(OH)_2D_3$ are low, the production of $1\alpha,25(OH)_2D_3$ in the kidney is high, and when circulating levels of $1\alpha,25(OH)_2D_3$ are high, synthesis of $1\alpha,25(OH)_2D_3$ is low (117). The changes of enzyme activity induced by $1\alpha,25(OH)_2D_3$ can be inhibited by cycloheximide and actinomycin D (127), which suggests that $1\alpha,25(OH)_2D_3$ is acting at the level of transcription. Another modulator of renal $1\alpha,25(OH)_2D_3$ production is PTH. PTH is released when plasma calcium levels are low, and in the kidney it stimulates the activity of the 1-hydroxylase and decreases the activity of the 24-hydroxylase. $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ also operate in a feedback loop to modulate and/or reduce the secretion of PTH. Other modulators of renal $1\alpha,25(OH)_2D_3$ production are shown in Fig. 4.

3. $24R,25(OH)_2D_3$

A second dihydroxylated metabolite of vitamin D is produced in the kidney, namely, $24R,25(OH)_2D_3$. Also, virtually all other tissues that have receptors for $1\alpha,25(OH)_2D_3$ can also produce $24R,25(OH)_2D_3$. There is some controversy concerning the possible unique biological actions of $24R,25(OH)_2D_3$. However, there is some evidence that $24R,25(OH)_2D_3$ plays a role in the suppression of PTH secretion (128,129), in the mineralization of bone (130,131), and in fracture healing (132,133). Other studies demonstrated that the combined presence of $24R,25(OH)_2D_3$ and $1\alpha,25(OH)_2D_3$ are required for normal egg production, fertility, and hatchability in chickens (104) and quail (134). From these studies it was apparent that only combination doses of both compounds were capable of eliciting the same response as the parent vitamin D. Thus, it appears that both $1\alpha,25(OH)_2D_3$ and $24R,25(OH)_2D_3$ may be required for some of the known biological responses to vitamin D.

The enzyme responsible for the production of 24*R*,25(OH)$_2$D$_3$ is the 25-hydroxyvitamin D$_3$-24*R*-hydroxylase (24-hydroxylase). Experimental evidence suggests that this enzyme is also a mixed-function oxidase. The activity of this enzyme is regulated so that when 1α,25(OH)$_2$D$_3$ levels are low, the activity of the 24*R*-hydroxylase is also low, but when 1α,25(OH)$_2$D$_3$ levels are high, the activity of the 24*R*-hydroxylase is high. Under normal physiological conditions both 1α,25(OH)$_2$D$_3$ and 24*R*,25(OH)$_2$D$_3$ are secreted from the kidney and circulated in the plasma of all classes of vertebrates.

In addition to these three metabolites, many other vitamin D$_3$ metabolites have been chemically characterized, and the existence of others appears likely. The chemical struc-

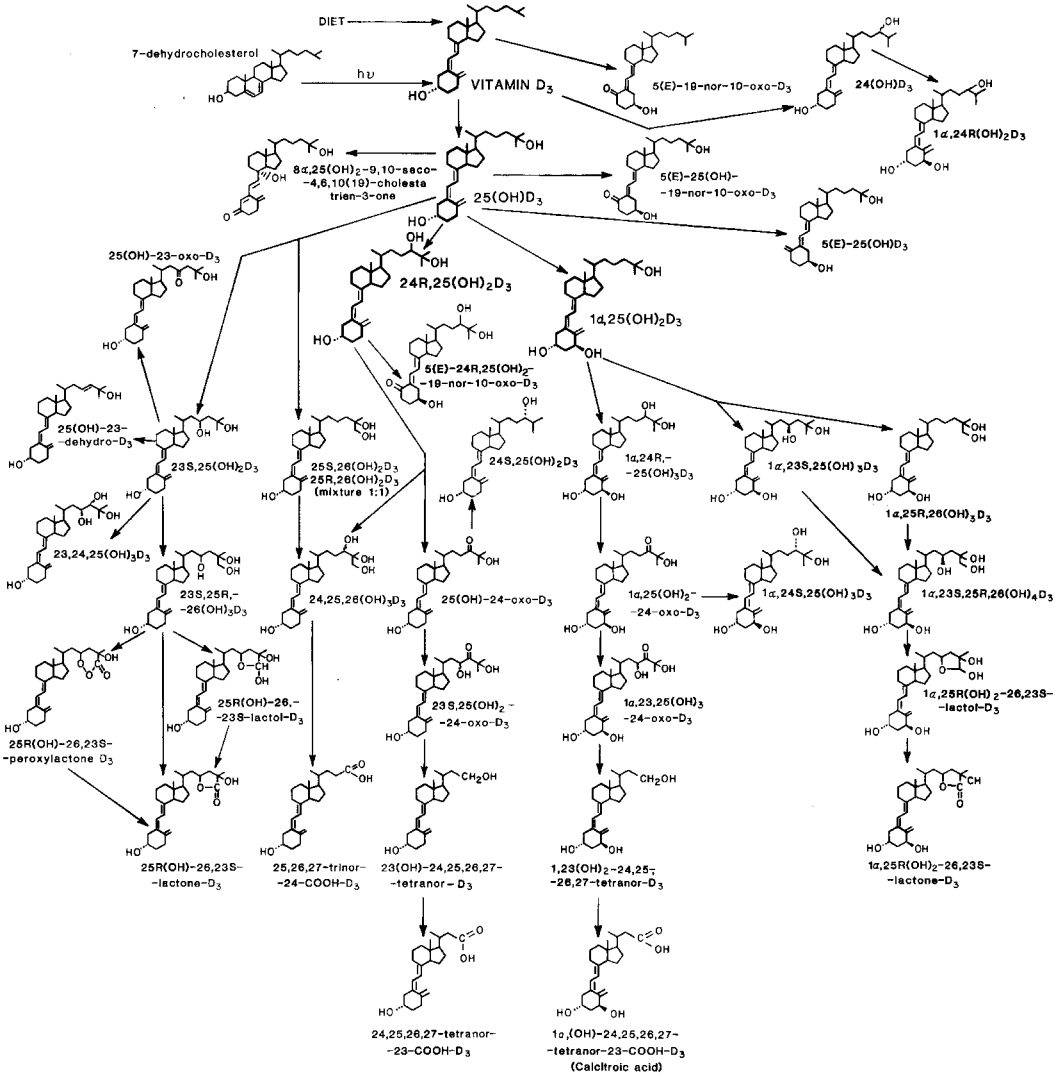


Fig. 5 Summary of the metabolic transformations of vitamin D$_3$. Shown here are the structures of all known chemically characterized vitamin D$_3$ metabolites.

tures of the 37 known metabolites are shown in Fig. 5. Most of these metabolites appear to be intermediates in degradation pathways of $1\alpha,25(OH)_2D_3$. None of these other metabolites have been shown to have biological activity except for the $1\alpha,25(OH)_2D_3$-26,23-lactone. The lactone is produced by the kidney when the plasma levels of $1\alpha,25(OH)_2D_3$ are very high. The metabolite appears to be antagonistic to $1\alpha,25(OH)_2D_3$ because it mediates a decrease in serum calcium levels in the rat. Other experiments suggest that lactone inhibits bone resorption and blocks the resorptive action of $1\alpha,25(OH)_2D_3$ on the bone (135), perhaps functioning as a natural antagonist of $1\alpha,25(OH)_2D_3$ to prevent toxic effects from overproduction of $1\alpha,25(OH)_2D_3$.

E. Catabolism and Excretion

Several pathways exist in humans and animals to further metabolize $1\alpha,25(OH)_2D_3$. These include oxidative cleavage of the side chain, hydroxylation of C-24 to produce $1\alpha,24,25(OH)_3D_3$, formation of 24-oxo-$1\alpha,25(OH)_2D_3$, formation of $1\alpha,25(OH)_2D_3$-26,23-lactone, and formation of $1\alpha,25,26(OH)_3D_3$ (Fig. 5). It is not known which of these pathways are involved in the breakdown or clearance of $1\alpha,25(OH)_2D_3$ in humans.

The catabolic pathway for vitamin D is obscure, but it is known that the excretion of vitamin D and its metabolites occurs primarily in the feces with the aid of bile salts. Very little appears in the urine. Studies in which radioactively labeled $1\alpha,25(OH)_2D_3$ was administered to humans have shown that 60–70% of the $1\alpha,25(OH)_2D_3$ was eliminated in the feces as more polar metabolites, glucuronides, and sulfates of $1\alpha,25(OH)_2D_3$. The half-life of $1\alpha,25(OH)_2D_3$ in plasma has two components. Within 5 min, only half of an administered dose of radioactive $1\alpha,25(OH)_2D_3$ remains in the plasma. A slower component of elimination has a half-life of about 10 h. $1\alpha,25(OH)_2D_3$ is catabolized by a number of pathways that result in its rapid removal from the organism (136).

V. BIOCHEMICAL MODE OF ACTION

The major classical physiological effects of vitamin D are to increase the active absorption of Ca^{2+} from the proximal intestine and to increase the mineralization of bone. This is achieved via two major signal transduction pathways: genomic and nongenomic.

A. Genomic

Vitamin D, through its daughter metabolite $1\alpha,25(OH)_2D_3$, functions in a manner homologous to that of steroid hormones. A model for steroid hormone action is shown in Fig. 6. In the general model, the hormone is produced in an endocrine gland in response to a physiological stimulus and then circulates in the blood, usually bound to a protein carrier (i.e. DBP), to target tissues where the hormone interacts with specific, high-affinity, intracellular receptors. The receptor–hormone complex localizes in the nucleus, undergoes some type of ''activation'' perhaps involving phosphorylation (137–140), and binds to a hormone response element (HRE) on the DNA to modulate the expression of hormone-sensitive genes. The modulation of gene transcription results in either the induction or repression of specific mRNAs, ultimately resulting in changes in protein expression needed to produce the required biological response. High-affinity receptors for $1\alpha,25(OH)_2D_3$ have been identified in at least 26 target tissues (12,16,141) and more than 50 genes are known to be regulated by $1\alpha,25(OH)_2D_3$ (142).

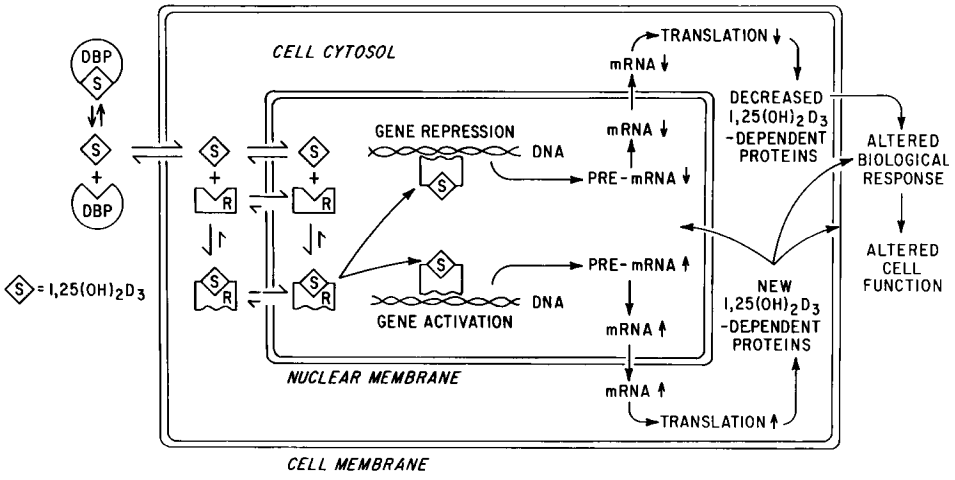


Fig. 6 General model for the mode of action of steroid hormones. Target tissues contain receptors for the steroid which confer on them the ability to modulate gene transcription. S, steroid; R, receptor protein, which may be present inside the cell in either the cytosol or nuclear compartment; SR, steroid–receptor complex; DBP, serum vitamin D–binding protein, which functions to transport the steroid hormone from the endocrine gland to its various target tissues.

Genes that have been shown to be transcriptionally regulated by $1\alpha,25(OH)_2D_3$ are listed in Table 4.

1. Nuclear Receptor

The $1\alpha,25(OH)_2D_3$ receptor was originally discovered in the intestine of vitamin-D deficient chicks (50,51). It has been extensively characterized and the cDNA for the nuclear receptor has been cloned and sequenced (52–55). The $1\alpha,25(OH)_2D_3$ receptor is a DNA-binding protein with a molecular weight of about 50,000 da. It binds $1\alpha,25(OH)_2D_3$ with high affinity with a K_D in the range of $1–50 \times 10^{-10}$ M (143–145). The ligand specificity of the nuclear $1\alpha,25(OH)_2D_3$ receptor is illustrated in Table 5. The $1\alpha,25(OH)_2D_3$ receptor protein belongs to a superfamily of homologous nuclear receptors (52). To date only a single form of the receptor has been identified.

The superfamily of ligand-dependent nuclear receptors includes receptors for glucocorticoids (GR), progesterone (PR), estrogen (ER), aldosterone, androgens, thyroid hormone (T3R), hormonal forms of vitamins A (RAR, RXR) and D (VDR), and several orphan receptors (141,146,147). Comparative studies of these receptors reveal that they have the common structural organization consisting of five domains (148), shown in Fig. 7. The different domains act as distinct modules that can function independently of each other (149–151).

The DNA binding domain, C, is the most conserved domain throughout the family. About 70 amino acids fold into two zinc finger–like motifs. Conserved cysteines coordinate a zinc ion in a tetrahedral arrangement. The first finger, which contains four cysteines and several hydrophobic amino acids, determines the DNA response element specificity. The second zinc finger, which contains five cysteines and many basic amino

Table 4 Genes Regulated by $1\alpha,25(OH)_2D_3$

Gene	Reg.	Evidence	Tissue/cell
α-Tubulin	Down	mRNA	Chick intestine
Aldolase subunit B	Up	mRNA	Chick kidney
Alkaline phosphatase	Up	mRNA	Rat intestine
			Chick intestine
			TE-85 cells
ATP synthase	Up	mRNA	Rat intestine
			Chick intestine
	Down	mRNA	Chick kidney
c-FMS	Up	mRNA	HL-60 cells
c-FOS	Up	mRNA	MG-63 cells
			HL-60 cells
c-KI-RAS	Up	mRNA	BALB-3T3 cells
c-MYB	Down	mRNA	HL-60 cells
c-MYC	Up	mRNA	MG-63
	Down	mRNA	U937 cells
		Transcription	HL-60 cells
			HL-60 cells
$Calbindin_{28K}$	Up	mRNA	Chick intestine
		Transcription	Mouse kidney
			Chick intestine
$Calbindin_{9K}$	Up	mRNA	Mouse kidney
		VDRE	Rat
Carbonic anhydrase	Up	mRNA	Marrow cells
		Transcription	Myelomonocytes
CD-23	Down	mRNA	PBMC
Collagen type I	Down	mRNA/VDRE	Rat
Cytochrome oxidase subunit I	Up	mRNA	Rat intestine
			Chick intestine
	Down	mRNA	Chick kidney
Cytochrome oxidase subunit II	Up	mRNA	Chick intestine
	Down	mRNA	Chick kidney
Cytochrome oxidase subunit III	Up	mRNA	Rat intestine
			Chick intestine
	Down	mRNA	Chick kidney
Cytochrome B	Down	mRNA	Chick kidney
Fatty acid–binding protein	Down	mRNA	Chick intestine
Ferridoxin	Down	mRNA	Chick kidney
Fibronectin	Up	mRNA	MG-63
			TE-85
			HL-60 cells
γ-Interferon	Down	mRNA	T lymphocytes
			PBMC
Glyceraldehyde-3-phosphate dehydrogenase	Up	mRNA	BT-20 cells

Table 4 Continued

Gene	Reg.	Evidence	Tissue/cell
GM-colony-stimulating factor	Down	mRNA	T lymphocytes
Heat shock protein 70	Up	mRNA	PBMC
Histone H4	Down	mRNA/ Ttranscription	HL-60 cells
1-Hydroxyvitamin D-24-hydroxylase	Up	mRNA	Rat kidney
		mRNA/ Transcription	Rat kidney
Integrin$_{\alpha v\beta 3}$	Up	mRNA/ Transcription	Avian osteoclast precursor cells
Interleukin-6	Up	mRNA	U937
Interleukin-4	Up	mRNA	U937 cells
Interleukin-2	Down	mRNA	T lymphocytes
Interleukin-3 receptor	Up	mRNA	MC3T3 cells
Matrix gla protein	Up	mRNA	UMR106-01, ROS 25/1, 25/4 cells
Metallothionein	Up	mRNA	Rat kertinocytes Mouse liver/kidney/skin Chick kidney
Monocyte-derived neutrophil-activating peptide	Up	mRNA/transcription	HL-60 cells
NADH DH subunit I	Down	mRNA	Chick kidney
NADH DH subunit III	Up	mRNA	Chick intestine
NADH DH subunit IV	Up	mRNA	Chick intestine
Nerve growth factor	Up	mRNA	L-929 cells
Osteocalcin	Up	mRNA	ROS 17/2.8 ROS 25/1
		VDRE	ROS 17/2.8 Rat
Osteopontin	Up	mRNA	ROS 17/2.8
		VDRE	ROS 17/2.8
Plasma membrane calcium pump	Up	mRNA	Chick intestine
Pre-pro-PTH	Down	mRNA	Rat
		mRNA/transcription	Bovine parathyroid
Prolactin	Up	mRNA	GH_4C_1 cells
Protein kinase inhibitor	Down	mRNA	Chick kidney
Protein kinase C	Up	mRNA/transcription	HL-60 cells
PTH	Down	mRNA	Rat parathyroid
PTH-related protein	Down	mRNA/transcription	TT cells
Transferrin receptor	Down	mRNA	PBMC
Tumor necrosis factor α	Up	mRNA	U937 cells
		Transcription	HL-60 cells
VDR	Up	mRNA	Rat intestine Rat pituitary MG-60 cells

Source: Ref. 142.

Table 5 Ligand Specificity of the Nuclear 1α,25(OH)$_2$D$_3$ Receptor

Ligand	Structural modification	RCI (%)[a]
1α,25(OH)$_2$D$_3$		100
1α,25(OH)$_2$-24-nor-D$_3$	Shorten side chain by 1 carbon	67
1α,25(OH)$_2$-3-epi-D$_3$	Orientation of 3β-OH altered	24
1α,25(OH)$_2$-24a-dihomo-D$_3$	Lengthen side chain by 2 carbons	24
1β,25(OH)$_2$D$_3$	Orientation of 1α-OH changed	0.8
1α(OH)D$_3$	Lacks 25-OH	0.15
25(OH)D$_3$	Lacks 1α-OH	0.15
1α,25(OH)$_2$-7-dehydrocholesterol	Lacks a broken B ring; is not a *seco* steroid	0.10
Vitamin D$_3$	Lacks 1α and 25-OH	0.0001

[a]The *R*elative *C*ompetitive *I*ndex (RCI) is a measure of the ability of a nonradioactive ligand to compete, under in vitro conditions, with radioactive 1α,25(OH)$_2$D$_3$ for binding to the nuclear 1α,25(OH)$_2$D$_3$ receptor (VDR)
Source: Ref. 14.

acids, is also necessary for DNA binding and is involved in receptor dimerization (146,150,152,153).

The next conserved region is the steroid binding domain (region E). This region contains a hydrophobic pocket for ligand binding and also contains signals for several other functions, including dimerization (154–157), nuclear translocation, and hormone-dependent transcriptional activation (149,150,158).

The A/B domain is also known as the immuno- or transactivation domain. This region is poorly conserved in amino acids and in size, and its function has not been clearly defined. The VDR has the smallest A/B domain (25 amino acids) of the known receptors; mineralocorticoid receptor has the largest (603 amino acids). An independent transcriptional activation function is located within the A/B region (146,150,151) that is constitutive in receptor constructs lacking the ligand binding domain (region E). The relative importance of the transcriptional activation by this domain depends on the receptor, the context of the target gene promoter, and the target cell type (159).

Region D is the hinge region between the DNA binding domain and the ligand bonding domain. The hinge region in the VDR contains 156 amino acids and has immunogenic properties. The VDR has the longest hinge region of the known receptors (160). Human GR and PR have hinge regions of 92 and 101 amino acids, respectively.

The VDR belongs to a subgroup of the receptors designated group II, which includes T3R, RAR, RXR, and several orphan receptors. All of the group II receptors can form heterodimers with RXR (161,162), and other heterodimeric interactions have also been

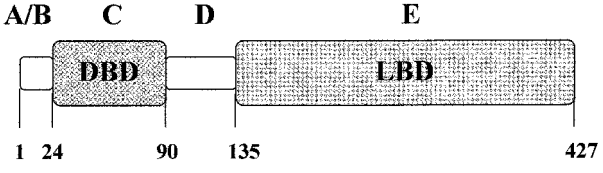


Fig. 7 Schematic representation of the human nuclear VDR. The DNA binding domain (C) and ligand binding domain (E) are boxed.

reported (163). T3R lacking the DNA binding domain can inhibit the transactivation of RAR (156) and VDR (152), but not a chimeric receptor containing the ligand binding domain of GR. The VDR can also form heterodimers with RAR (163,164). The ability to form heterodimers with other receptors allows for enhanced affinity for distinct DNA targets, generating the diverse range of physiological effects.

2. Calbindin D

One of the major effects of $1\alpha,25(OH)_2D_3$ in many of its target tissues is the induction of the calcium binding protein, calbindin D. In the mammalian kidney and brain and in avians, a larger form of the protein is expressed, calbindin D_{28K} (165), whereas in the mammalian intestine and placenta a smaller form is expressed, calbindin D_{9K} (166). The expression of calbindins in various tissues and species appears to be regulated to differing degrees by $1\alpha,25(OH)_2D_3$ (167).

Early experiments showed that actinomycin D and α-amanitin, transcriptional inhibitors, could block the induction of calbindin D_{28K} by $1\alpha,25(OH)_2D_3$ (168). Later experiments showed that $1\alpha,25(OH)_2D_3$ was able to stimulate total RNA synthesis in the chick intestine (169) in addition to specifically inducing the mRNA for calbindin D_{28K} (170). Nuclear transcription assays have shown that transcription of calbindin D_{28K} mRNA is directly induced by $1\alpha,25(OH)_2D_3$ in the chick intestine and is correlated to the level of occupied $1\alpha,25(OH)_2D_3$ receptors (171). The gene for calbindin D_{28K} has now been cloned and sequenced, but there is still much to learn about how $1\alpha,25(OH)_2D_3$ induces this gene (172).

B. Nongenomic Actions

Recent studies (173) suggest that not all of the actions of $1\alpha,25(OH)_2D_3$ can be explained by receptor–hormone interactions with the genome. $1\alpha,25(OH)_2D_3$ can stimulate the intestinal transport of calcium within 4–6 min, i.e., too quickly to involve genome activation. The rapid transport of calcium mediated by $1\alpha,25(OH)_2D_3$ in the intestine has been termed ''transcaltachia'' (''trans'' = across; ''cal'' = calcium; ''tachia'' = swiftly). Transcaltachia is not inhibited by actinomycin D but is inhibited by colchicine, an antimicrotubule agent, and by leupeptin, an antagonist of lysosomal cathepsin B. Transcaltachia induced by $1\alpha,25(OH)_2D_3$ in the intestine appears to involve the internalization of calcium in endocytic vesicles at the brush-border membrane, which then fuse with lysosomes and travel along microtubules to the basal lateral membrane where exocytosis occurs. Therefore, some of the actions of $1\alpha,25(OH)_2D_3$ may be mediated at the cell membrane or by extranuclear subcellular components.

Other effects of $1\alpha,25(OH)_2D_3$ that do not appear to be mediated by the nuclear receptor are phosphoinositide breakdown (174), enzymatic activity in osteoblast-derived matrix vesicles (175), certain secretion events in osteoblasts (176), rapid changes in cytosolic Ca^{2+} levels in primary cultures of osteoblasts and osteosarcoma cells (177–179), and increases in cyclic guanosine monophosphate levels in fibroblasts (180). These rapid effects appear to be mediated by a membrane receptor–like protein for $1\alpha,25(OH)_2D_3$ (181); a candidate membrane receptor for $1\alpha,25(OH)_2D_3$ has been proposed (182). Other steroid hormones, i.e., estrogen (183), progesterone (184–187), testosterone (188), glucocorticoids (189,190), corticosteroid (191), and thyroid (192,193), have been shown to have similar membrane effects (181). A model for the nongenomic signal transduction pathway is shown in Fig. 8.

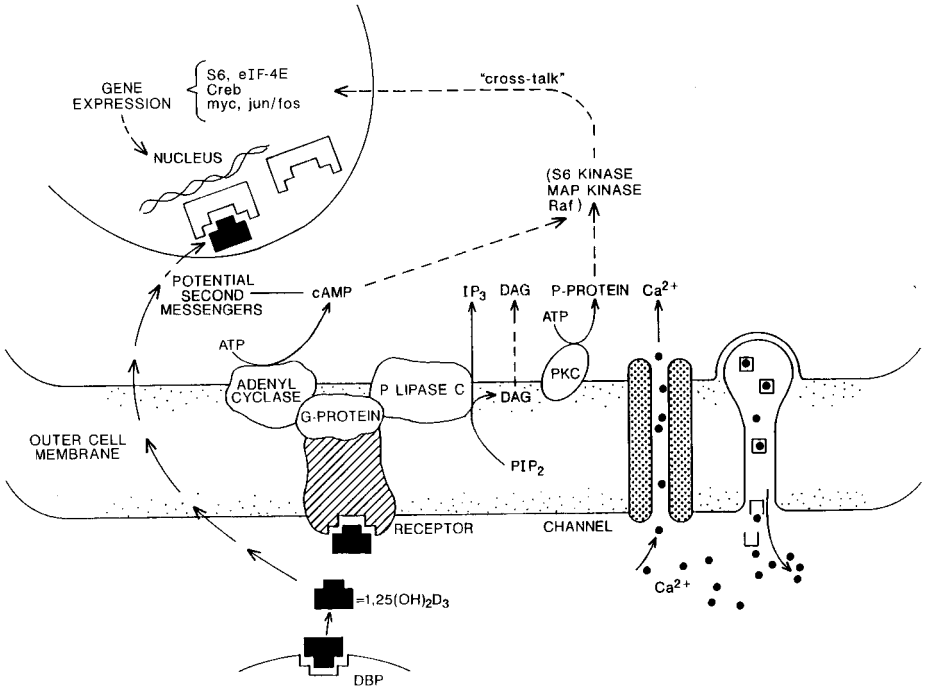


Fig. 8 Model describing the signal transduction pathways associated with the nongenomic response of transcaltachia. The general model of vesicular Ca^{2+} transport includes formation of Ca^{2+}-containing endocytic vesicles at the brush-border membrane, fusion of endocytic vesicle with lysosomes, movement of lysosomes along microtubules, and exocytotic extrusion of Ca^{2+} via fusion of the lysosomes with the basal lateral membrane of the intestinal enterocyte. The binding of $1\alpha,25(OH)_2D_3$ to a membrane receptor results in an increase of several second messengers, including IP3, cAMP, activation of PKC or intracellular Ca^{2+}, which may result in the transient opening of Ca^{2+} channels. The increased Ca^{2+} concentration may then initiate the exocytosis of the lysosomal vesicles.

VI. SPECIFIC FUNCTIONS OF $1\alpha,25(OH)_2D_3$

A. $1\alpha,25(OH)_2D_3$ and Mineral Metabolism

The classical target tissues for $1\alpha,25(OH)_2D_3$ are those tissues that have been found to be directly involved in the regulation of mineral homeostasis. In humans, serum calcium levels are normally maintained between 9.5 and 10.5 mg/100 mL, whereas the phosphorus concentration is between 2.5 and 4.3 mg/100 mL (2). Together with PTH and calcitonin, $1\alpha,25(OH)_2D_3$ maintains serum calcium and phosphate levels by its actions on the intestine, kidney, bone, and parathyroid gland.

In the intestine, one of the best characterized effects of $1\alpha,25(OH)_2D_3$ is the stimulation of intestinal lumen–to–plasma flux of calcium and phosphate (39,194,195). Although extensive evidence exists showing that $1\alpha,25(OH)_2D_3$ interacting with its receptor upregulates calbindin D in a genome-mediated fashion, the relationship between calbindin D and calcium transport is not clear (196). In the vitamin D–deficient state, both mammals and birds have severely decreased intestinal absorption of calcium with no detectable

levels of calbindin. There is a linear correlation between the increased cellular levels of calbindin D and calcium transport. When $1\alpha,25(OH)_2D_3$ is given to vitamin D–deficient chicks, the transport of calcium reaches maximal rates at 12–14 h, whereas calbindin D does not reach its maximal levels until 48 h (173). In one study employing immunohistochemical techniques, it was demonstrated that the cellular location of calbindin D_{28K} changed with the onset of calcium transport (197).

$1\alpha,25(OH)_2D_3$ treatment also is known to alter the biochemical and morphological characteristics of the intestinal cells (198,199). The size of the villus and the size of the microvilli increase upon $1\alpha,25(OH)_2D_3$ treatment (200). The brush border undergoes noticeable alterations of structure and composition of cell surface proteins and lipids, occurring in a time frame corresponding to the increase in Ca^{2+} transport mediated by $1\alpha,25(OH)_2D_3$ (201). However, despite extensive work, the exact mechanisms involved in the vitamin D–dependent intestinal absorption of calcium remain unknown (202–204).

The kidney is the major site of synthesis of $1\alpha,25(OH)_2D_3$ and of several other hydroxylated vitamin D derivatives. Probably the most important effect of $1\alpha,25(OH)_2D_3$ on the kidney is the inhibition of $25(OH)D_3$-1α-hydroxylase activity, which results in a decrease in the synthesis of $1\alpha,25(OH)_2D_3$ (205,206). Simultaneously, the activity of the $25(OH)D_3$-24-hydroxylase is stimulated. The actions of vitamin D on calcium and phosphorus metabolism in the kidney has been controversial, and more research is needed to clearly define the actions of $1\alpha,25(OH)_2D_3$ on the kidney.

Although vitamin D is a powerful antirachitic agent, its primary effect on bone is the stimulation of bone resorption leading to an increase in serum calcium and phosphorus levels (207). With even slight decreases in serum calcium levels, PTH is synthesized, which then stimulates the synthesis of $1\alpha,25(OH)_2D_3$ in the kidney. Both of these hormones stimulate bone resorption. Maintaining constant levels of calcium in the blood is crucial, whether calcium is available from the diet or not. Therefore, the ability to release calcium from its largest body store—bone—is vital. Bone is a dynamic tissue that is constantly being remodeled. Under normal physiological conditions, bone formation and bone resorption are tightly balanced (208). The stimulation of bone growth and mineralization by $1\alpha,25(OH)_2D_3$ appears to be an indirect effect of the provision of minerals for bone matrix incorporation through an increase of intestinal absorption of calcium and phosphorus. In bone, nuclear receptors for $1\alpha,25(OH)_2D_3$ have been detected in normal osteoblasts (209), osteoblast-like osteosarcoma cells, but not in osteoclasts. In addition, $1\alpha,25(OH)_2D_3$ can induce rapid changes in cytosolic Ca^{2+} levels in osteoblast and osteosarcoma cells by opening voltage-gated Ca^{2+} channels via a nongenomic signal transduction pathway (178,179).

Some of the actions of $1\alpha,25(OH)_2D_3$ in bone are related to changes in bone cell differentiation. $1\alpha,25(OH)_2D_3$ is known to affect a number of osteoblast-related functions. For example, $1\alpha,25(OH)_2D_3$ decreases type I collagen production (210), and increases alkaline phosphatase production and the proliferation of cultured osteoblasts (211); $1\alpha,25(OH)_2D_3$ increases the production of osteocalcin (212) and matrix Gla protein (213), and decreases the production of type I collagen by fetal rat calvaria (214).

$1\alpha,25(OH)_2D_3$ also affects the growth and differentiation of osteoclasts and osteoclast-like cells in vivo in rats (215) and in primate bone marrow cell cultures (216). Since the osteoclast does not have a nuclear VDR, $1\alpha,25(OH)_2D_3$ must affect osteoclasts indirectly or by nongenomic mechanisms. There is some evidence that a factor produced by osteoblasts promotes the formation of osteoclasts (217,218). It is possible that the only effect of $1\alpha,25(OH)_2D_3$ on the osteoclasts is to stimulate its generation from progenitor cells.

PTH is an important tropic stimulator of $1\alpha,25(OH)_2D_3$ synthesis by the kidney. High circulating levels of $1\alpha,25(OH)_2D_3$ have been shown to decrease the levels of PTH by two different mechanisms: an indirect mechanism due to the resulting increase in serum calcium levels, which is an inhibitory signal for PTH production, and a direct mechanism involving the interaction of $1\alpha,25(OH)_2D_3$ and its receptor, which directly suppresses the expression of the prepro-PTH gene.

During pregnancy and lactation, large amounts of calcium are needed for the developing fetus and for milk production. Hormonal adjustments in the vitamin D endocrine system are critical to prevent depletion of minerals leading to serious bone damage for the mother. Although receptors for $1\alpha,25(OH)_2D_3$ have been found in placental tissue and in the mammary gland, the role of vitamin D is not clear.

B. Vitamin D in Nonclassical Systems

In the 1970s and 1980s, nuclear receptors for $1\alpha,25(OH)_2D_3$ were discovered in a variety of tissues and cells not directly involved in calcium homeostasis. Thus, the role of the vitamin D endocrine system has expanded to include general effects on cell regulation and differentiation (12,18). Nuclear VDRs are present in muscle, hematolymphopoietic, reproductive, and nervous tissue, as well as in other endocrine tissues and skin. More than 50 proteins are known to be regulated by $1\alpha,25(OH)_2D_3$, including several oncogenes (56,142) (Table 2), which extend by far the classical limits of vitamin D actions on calcium homeostasis. In many of these systems it is not yet clear what the effect of vitamin D is on the tissue or its mode of action.

Skeletal muscle is a target organ for $1\alpha,25(OH)_2D_3$. Clinical studies have shown the presence of muscle weakness or myopathy during metabolic bone diseases related to vitamin D deficiency (19,22,219). These abnormalities can be reversed with vitamin D therapy. Experimental evidence has shown that $1\alpha,25(OH)_2D_3$ has a direct effect on Ca^{2+} transport in cultured myoblasts and skeletal muscle tissue. Furthermore, there is evidence that the action of $1\alpha,25(OH)_2D_3$ on skeletal muscle may be important for the calcium homeostasis of the entire organism because the hormone induces a rapid release of calcium from muscle into the serum of hypocalcemic animals. $1\alpha,25(OH)_2D_3$ receptors have been detected in myoblast cultures, and the changes in calcium uptake have been shown to be RNA- and protein synthesis–dependent, suggesting a genomic mechanism. $1\alpha,25(OH)_2D_3$ has also been shown to be important for cardiac muscle function (220–223).

In the skin, $1\alpha,25(OH)_2D_3$ appears to exert effects on cellular growth and differentiation. Receptors for $1\alpha,25(OH)_2D_3$ have been found in human (224) and mouse skin (225). $1\alpha,25(OH)_2D_3$ inhibits the synthesis of DNA in mouse epidermal cells (225). The hormone induces changes in cultured keratinocytes, which are consistent for terminal differentiation of nonadherent cornified squamous cells (226). Additional experiments have shown that human neonatal foreskin keratinocytes produce $1\alpha,25(OH)_2D_3$ from $25(OH)D_3$ under in vitro conditions (227), suggesting that keratinocyte-derived $1\alpha,25(OH)_2D_3$ may affect epidermal differentiation locally. Psoriasis is a chronic hyperproliferative skin disease. Some forms of psoriasis have been shown to improve significantly when treated topically with calcipotriol, a nonhypercalcemic analog of $1\alpha,25(OH)_2D_3$ (228–230). In mouse skin carcinogenesis, $1\alpha,25(OH)_2D_3$ blocks the production of tumors induced by 12-*O*-tetradecanoylphorbol-12-acetate (231).

In the pancreas, $1\alpha,25(OH)_2D_3$ has been found to be essential for normal insulin secretion. Experiments with rats have shown that vitamin D increases insulin release from the isolated perfused pancreas, in both the presence and the absence of normal serum

calcium levels (232–236). Human patients with vitamin D deficiency, even under conditions of normal calcemia, exhibit impaired insulin secretion but normal glucagon secretion, suggesting that $1\alpha,25(OH)_2D_3$ directly affects β-cell function (237).

Receptors for $1\alpha,25(OH)_2D_3$ have been found in some sections of the brain (238,239). However, the role of $1\alpha,25(OH)_2D_3$ in the brain is not well understood. Both calbindins D have been found in the brain, but neither the expression of calbindin D_{28K} nor that of calbindin D_{9K} appears to be directly modulated by vitamin D (238,239). In the rat, $1\alpha,25(OH)_2D_3$ appears to increase the activity of the choline acetyltransferase in specific regions of the brain (238). Other steroid hormones have also been shown to affect the metabolism of specific brain regions (240,241).

Also, normal, benign, hyperplastic and malignant prostatic epithelial and fibroblastic cells contain receptors for $1\alpha,25(OH)_2D_3$ (242). In hematopoietic tissue, $1\alpha,25(OH)_2D_3$ promotes the differentiation and inhibits proliferation of both malignant and nonmalignant hematopoietic cells. Human promyelocytic leukemia cells, HL-60, have been shown to have receptors for $1\alpha,25(OH)_2D_3$ and to differentiate toward macrophages upon treatment with $1\alpha,25(OH)_2D_3$ (243,244). Other effects of $1\alpha,25(OH)_2D_3$ on the immune system will be discussed in the next section.

C. Immunoregulatory Roles

In 1979, when the VDR was discovered in several neoplastic hematopoietic cell lines as well as in normal human peripheral blood mononuclear cells, monocytes, and activated lymphocytes (245,246), a role for $1\alpha,25(OH)_2D_3$ in immune function was suggested. Since then, $1\alpha,25(OH)_2D_3$ has been shown to affect cells of the immune system in a variety of ways. $1\alpha,25(OH)_2D_3$ reduces the proliferation of HL-60 cells and induces their differentiation to monocytes (243) and macrophages (244,247,248). The actions of $1\alpha,25(OH)_2D_3$ on normal monocytes are controversial, but it appears that the molecule may enhance monocyte function. $1\alpha,25(OH)_2D_3$ appears to reduce levels of HLA-DR and $CD4^+$ class II antigens on monocytes or macrophages with no effect on the expression of class I antigens (249). The enhancement of class II antigen expression is a common feature of autoimmunity and often precedes the onset of autoimmune diseases.

$1\alpha,25(OH)_2D_3$ also promotes the differentiation of leukemic myeloid precursor cells toward cells with the characteristics of macrophages (243). Subsequent experiments have shown that $1\alpha,25(OH)_2D_3$ does not alter the clonal growth of normal myeloid precursors but does induce the formation of macrophage colonies preferentially over the formation of granulocyte colonies (247). In addition, macrophages derived from different tissues can synthesize $1\alpha,25(OH)_2D_3$ when activated by γ-interferon (121). Also, $1\alpha,25(OH)_2D_3$ can suppress immunoglobulin production by activated B lymphocytes (250) and inhibit DNA synthesis and proliferation of both activated B and T lymphocytes (251–253). These findings suggest that a vitamin D paracrine system exists that involves activated macrophages and activated lymphocytes (Fig. 4).

$1\alpha,25(OH)_2D_3$ also affects some functions of T and B lymphocytes and natural killer (NK) cells. In T lymphocytes, the mitogen activation of lymphocyte proliferation is blocked in the presence of $1\alpha,25(OH)_2D_3$ (251,254,255), apparently by interference with cell cycle progression from early G1 to late G1 phase (254). $1\alpha,25(OH)_2D_3$ exhibits a permissive or enhancing effect on T-cell suppressor activity. In an in vitro model of transplant compatibility, the mixed lymphocyte reaction, $1\alpha,25(OH)_2D_3$ significantly enhanced T-cell suppressor activity (256). $1\alpha,25(OH)_2D_3$ also affects the cytotoxicity of NK and T-cytotoxic cells probably by interfering with their generation from precursor cells.

$1\alpha,25(OH)_2D_3$ has been shown to decrease mRNA levels for IL-2 (257), γ-interferon (258) and granulocyte-macrophase colony-stimulating factor (GM-CSF) (259,260). $1\alpha,25(OH)_2D_3$ also attenuates the inducing effect of T-helper cells on IgG synthesis by B cells (261).

Despite the wide range of actions of $1\alpha,25(OH)_2D_3$ on various immune cells, no general immunomodulatory role for $1\alpha,25(OH)_2D_3$ has been defined. In contrast to the serum calcium elevation observed in patients with sarcoidosis, lymphoma, and an anephric patients with end-stage renal disease, no systemic immunosuppressive activity of $1\alpha,25(OH)_2D_3$ has been described in these disease states to date, suggesting that $1\alpha,25(OH)_2D_3$ acts in an autocrine or paracrine fashion to modulate local immune function (261,262). $1\alpha,25(OH)_2D_3$ and cyclosporine, a potent immunosuppressive drug, appear to affect the immune system in a similar fashion. They both affect T lymphocytes during initial activation by antigen, select the generation of T-helper cells by inhibiting lymphokine production at a genomic level, and inhibit the generation of T-cytotoxic and NK cells. Both are involved in the enhancement of T-suppressor function, a key element in the efficacy of cyclosporine as a drug that reduces allograft tissue rejection (263). $1\alpha,25(OH)_2D_3$ appears to work synergistically with cyclosporine when the two compounds are used in combination (264,265).

The use of nonhypercalcemic $1\alpha,25(OH)_2D_3$ analogs can result in enhanced immunosuppressive effects without the toxicity risks of $1\alpha,25(OH)_2D_3$. Because of the synergistic effect when $1\alpha,25(OH)_2D_3$ is used with cyclosporine, synthetic $1\alpha,25(OH)_2D_3$ analogs may be used in the treatment of autoimmune diseases (266) or for transplantation (267) in combination with cyclosporine to reduce the toxicity of both compounds.

D. Structures of Important Analogs

In nephrectomized animals, vitamin D compounds cannot be hydroxylated at the C-1 position because the kidney is the site where this hydroxylation occurs. Researchers found that neither vitamin D nor 25(OH)D was able to elicit a significant biological response when administered in physiological doses to nephrectomized animals (48,268). Also, it was noted in the 1940s and 1950s that dihydrotachysterol$_3$ (a 5,6-trans analog of vitamin D_3) was biologically active under circumstances where the parent vitamin D demonstrated little or no biological activity. These findings raised the question of the functional importance of the various structural elements of the vitamin D molecule. Studies using analogs of vitamin D have been used to address this question. The ability of analogs to bind to the nuclear receptor for $1\alpha,25(OH)_2D_3$, to increase intestinal calcium absorption (ICA) and bone calcium mobilization (BCM), and to promote cellular differentiation are then determined. Because of recent advances in new vitamin D syntheses described above and in Fig. 3, analogs have been synthesized with modifications in the A ring, *seco*-B ring, C ring, C/D ring junction, D ring, and/or side chain (14, 269).

The importance of the configuration of the A ring has been studied by synthesizing 5,6-trans analogs. Because of the rotation of the A ring, these analogs cannot undergo 1-hydroxylation and have been found to be only 1/1000 as biologically effective as $1\alpha,25(OH)_2D_3$. The relative significance of the 3β-hydroxyl group has been assessed by preparing analogs such as 3-deoxy-$1\alpha,25(OH)_2D_3$. Although this analog is active in vivo, it is interesting in that it preferentially stimulates intestinal calcium absorption over bone calcium mobilization (270). Of all the analogs synthesized, only a few show such selective biological activity.

The effect of altering the length of the side chain has been studied. The 27-*nor*-25(OH)D_3 and 26,27-bis-*nor*-25(OH)D_3 are reportedly able to stimulate intestinal calcium absorption and bone calcium mobilization in both normal and anephric rats but are 10–100 times less active than 25(OH)D_3 (271). The 24-*nor*-25(OH)D_3 was found to have no biological activity (272), although it was able to block the biological response to vitamin D but not to 25(OH)D_3 or 1α,25(OH)$_2D_3$. This suggests that it might have anti–vitamin D activity.

One of the most interesting side-chain analogs of 1α,25(OH)$_2D_3$ is 1α(OH)D_3. This metabolite appears to have the same biological activity in the chick as 1α,25(OH)$_2D_3$ (273) and is approximately half as active in the rat (274). In an attempt to determine if the biological activity of 1α(OH)D_3 is the result of in vivo 25-hydroxylation, the 25-fluoro-1α(OH)D_3 derivative was prepared (275). The fluorine on C-25 prevents hydroxylation of this carbon. The fluoro compound was found to be one-fiftieth as active as 1α,25(OH)$_2D_3$, suggesting that 1α(OH)D_3 has some activity even without 25-hydroxylation.

From such studies, the particular attributes of the structure of 1α,25(OH)$_2D_3$ that enables it to elicit its biological responses are being defined. It is now known that the 3β-hydroxy group does not appear to be as important for biological activity as the 1α- or 25-hydroxyl groups; the cis configuration of the A ring is preferred over the trans configuration; and the length of the side chain appears critical, as apparently there is little tolerance for its being shortened or lengthened.

Analogs of 1α,25(OH)$_2D_3$ have been used to study the in vivo metabolism and mode of action of vitamin D compounds. There has also been widespread interest in developing 1α,25(OH)$_2D_3$ analogs to use as therapeutic agents in the treatment of osteoporosis, renal osteodystrophy, cancer, immunodeficiency syndromes, autoimmune diseases, and some skin disorders. Of particular interest are analogs that separate the calcemic effects from the proliferation and differentiation effects of 1α,25(OH)$_2D_3$.

One of the most successful analogs in terms of separating biological activities is a cyclopropyl derivative of 1α,25(OH)$_2D_3$, 1α,24*S*(OH)$_2$-22ene-26,27-dehydrovitamin D_3, designated calcipotriol; this analog has weak systemic effects on calcium metabolism but potent effects on cell proliferation and differentiation (276,277). It is rapidly converted to inactive metabolites in vivo (278,279) and is 200-fold less potent than 1α,25(OH)$_2D_3$ in causing hypercalciuria and hypercalcemia in rats (276). It is as effective in binding to the nuclear receptor as 1α,25(OH)$_2D_3$ and has similar effects on the growth and differentiation of keratinocytes (280,281). It is currently marketed as a topical treatment for psoriasis, a proliferative disorder of the skin (228,282–285).

Another analog that has potential as a therapeutic agent is 22-oxa-1α,25(OH)$_2D_3$. This analog has been shown to suppress the secretion of PTH and may be useful in the treatment of secondary hyperparathyroidism (286). It is 10 times more potent in suppressing proliferation and inducing differentiation than 1α,25(OH)$_2D_3$, with only 1/50 to 1/100 of the in vitro bone-resorbing activity of 1α,25(OH)$_2D_3$ (287).

Still another set of analogs of 1α,25(OH)$_2D_3$ with potential therapeutic applications are the compounds with a double bond at C-16 and/or a triple bond at C-23. The best characterized of these compounds is 1α,25(OH)$_2$-16ene-23yne-D_3 (288–290). This analog is 300-fold less active in intestinal calcium absorption (ICA) and bone calcium mobilization (BCM) and 10 to 15 times less active in inducing hypercalcemia in vivo in mice than 1α,25(OH)$_2D_3$. In three leukemia models, therapy with the analog resulted in a significant increase in survival (288,289). All of the 16-ene and or 23-yne analogs that have been tested are equivalent or more potent than 1α,25(OH)$_2D_3$ in the induction of HL-60 cell

differentiation and inhibition of clonal proliferation (289,291), and ten to two hundred fold less active in ICA and BCM (291,292).

Fluorinated analogs of $1\alpha,25(OH)_2D_3$ have been especially useful for studying the in vivo metabolism of $1\alpha,25(OH)_2D_3$. Fluorine groups have been substituted for the hydroxyls at C-25, C-1, and C-3 to study the importance of these hydroxylations for the biological activity of $1\alpha,25(OH)_2D_3$. Also, fluorine groups have been substituted for hydrogens at C-23, C-24, and C-26 to facilitate the study of $1\alpha,25(OH)_2D_3$ catabolism. The analog $1\alpha,25(OH)_2$-26,26,26,27,27,27-hexafluoro-D_3 has been shown to be 10 times more potent than $1\alpha,25(OH)_2D_3$ in calcium mobilization, with longer lasting effects due to its slower rate of catabolism and metabolic clearance (293). This analog is also 10 times more potent than $1\alpha,25(OH)_2D_3$ in suppressing proliferation and inducing differentiation of HL-60 cells (247,294,295).

VII. BIOLOGICAL ASSAYS

With the exception of vitamin B_{12}, vitamin D is the most potent of the vitamins (as defined by the amount of vitamin required to elicit a biological response). Consequently, biological samples and animal tissues usually contain very low concentrations of vitamin D. For example, the circulating plasma level of vitamin D_3 in humans is only 10–20 ng/mL, or $2–5 \times 10^{-8}$ M (296). In order to detect such low concentrations of vitamin D, assays that are specific for and sensitive to vitamin D and its biologically active metabolites are required.

A. Rat Line Test

From 1922 to 1958, the only official assay for determination of the vitamin D content of pharmaceutical products or food was the rat line test. The term ''official'' indicates that the reproducibility and accuracy of the assay are high enough that the results of the test can be accepted legally. This assay, which is capable of detecting 1–12 IU (25–300 ng) of vitamin D, is still widely used today to determine the vitamin D content of many foods, particularly milk (297–299). The rat line test for vitamin D employs recently weaned rachitic rats; these rats are fed a rachitogenic diet for 19–25 days until severe rickets develops. The rats are then divided into groups of 7–10 animals and are fed diets that have been supplemented either with a graded series of known amounts of vitamin D_3 as standards or with the unknown test sample. (Although vitamin D oils can be directly assayed, milk, vitamin tablets, and vitamin D–fortified foods must be saponified and the residue taken up into a suitable oil vehicle prior to assay.) The rats are maintained on their respective diets for 7 days. The animals are sacrificed and their radii and ulnae dissected out and stained with a silver nitrate solution. Silver is deposited in areas of bone where new calcium has been recently deposited. The regions turn dark when exposed to light. Thus, the effects of the unknown sample on calcium deposition in the bone can be determined by visual comparison with the standards. Typical results for the rat line test are shown in Fig. 9.

B. AOAC Chick Assay

Since the rat line test is done in rats, it cannot discriminate between vitamin D_2 and vitamin D_3. In the chick, vitamin D_3 is 10 times more potent than vitamin D_2, so it is important to accurately determine the amount of vitamin D_3 in poultry feeds. The Association of

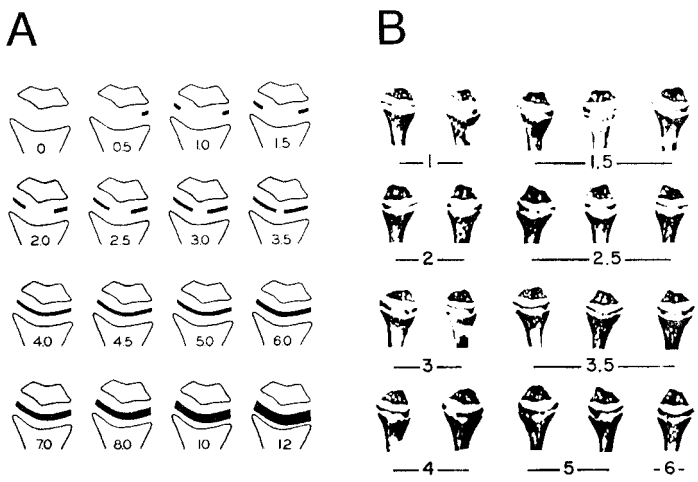


Fig. 9 Rat line test chart is shown in panel A. Photographs of radii sections scored according to the line test chart are shown in panel B.

Official Analytical Chemists (AOAC) chick test was developed to specifically measure vitamin D_3 (300,301).

Groups of 20 newly hatched chicks are placed on vitamin D–deficient diets containing added levels of vitamin D_3 (1–10 IU) or the test substance. After 3 weeks on the diet, the birds are sacrificed and the percentage of bone ash of their tibia is determined. A rachitic bird typically has 25–27% bone ash, whereas a vitamin D–supplemented has 40–45% bone ash. This assay is not used frequently because it is time consuming and expensive.

C. Intestinal Calcium Absorption

Other biological assays have been developed that make use of the ability of vitamin D to stimulate the absorption of calcium across the small intestine. Two basic types of assays measure this phenomenon: those that measure the effect of the test substance on intestinal calcium uptake in vivo (302) and those that employ in vitro methods (39,303). Each is capable of detecting physiological quantities, i.e., 2–50 IU (50–1250 ng; 0.13–3.2 nmol), of vitamin D.

1. In Vivo Technique

The in vivo technique for measuring intestinal calcium absorption uses rachitic chicks that have been raised on a low-calcium (0.6%), rachitogenic diet for 3 weeks. The birds are then given one dose of the test compound orally, intraperitoneally, or intracardially. Twelve to 48 h later, the chicks are anesthetized and 4.0 mg of $^{40}Ca^{2+}$ and approximately 6×10^6 dpm $^{45}Ca^{2+}$ are placed in the duodenal loop. Thirty minutes later, the chicks are killed by decapitation and serum is collected. Aliquots of serum are measured for $^{45}Ca^{2+}$ in a liquid scintillation counter (302).

2. In Vitro Technique

The general design of this technique is the same as that of the in vivo technique because vitamin D activity is measured in terms of intestinal calcium transport. In these assays,

a vitamin D standard or test compound is given orally or intraperitoneally 24–48 h before the assay. At the time of the assay, the animals are killed and a 10-cm length of duodenum is removed and turned inside out. A gut sac is formed by tying off the ends of the segment so that the mucosal surface is on the outside and the serosal surface on the inside. The everted intestinal loop is incubated with solutions of $^{45}Ca^{2+}$. The mucosal surface of the intestine actively transports the calcium through the tissue to the serosal side. The ratio of calcium concentration on the serosal vs. the mucosal side of the intestine is a measure of the ''active'' transport of calcium (39,304,305). In a vitamin D–deficient animal this ratio is 1–2.5; in a vitamin D–dosed animal it can be as high as 6–7. The chick in vivo assay is usually preferred because of the tedious nature of preparing the everted gut sacs. The in vitro technique is used primarily for studies with mammals rather than birds.

D. Bone Calcium Mobilization

Another assay for vitamin D activity that often is performed simultaneously with the chick in vivo intestinal calcium absorption assay is measurement of the vitamin D–mediated elevation of serum calcium levels. If 3-week-old rachitic chicks are raised on a zero-calcium diet for at least 3 days before the assay and then are given a compound containing vitamin D, their serum calcium levels will rise in a highly characteristic manner, proportional to the amount of steroid given (302). Since there is no dietary calcium available, the only calcium source for elevation of serum calcium is bone. By carrying out this assay simultaneously with the intestinal calcium absorption assay, it is possible to measure two different aspects of the animal's response to vitamin D at the same time.

E. Growth Rate

The administration of vitamin D to animals leads to an enhanced rate of whole-body growth. An assay for vitamin D was developed in the chick using the growth-promoting properties of the steroid (45,306). One-day-old chicks are placed on a rachitogenic diet and given standard doses of vitamin D_3 or the test compound three times weekly. The birds are weighed periodically, and their weight is plotted vs. age. In the absence of vitamin D, the rate of growth essentially plateaus by the fourth week, whereas 5–10 IU of vitamin D_3 per day is sufficient to maintain a maximal growth rate in the chick. The disadvantage of this assay is the 3- to 4-week time period needed to accurately determine the growth rate.

F. Radioimmunoassay and Enzyme-Linked Immunosorbent Assay for Calbindin D_{28K}

Additional biological assays utilize the presence of calbindin D_{28K} protein as an indication of vitamin D activity. Calbindin D_{28K} is not present in the intestine of vitamin D–deficient chicks and is only synthesized in response to the administration of vitamin D. Therefore, it is possible to use the presence of calbindin D_{28K} to determine vitamin D activity. A radioimmunoassay (RIA) and an enzyme-linked immunosorbent assay (ELISA), both capable of detecting nanogram quantities of calbindin D_{28K}, have been developed for this purpose (307).

A comparison of the sensitivity and working range of the biological assays for vitamin D is given in Table 6.

Table 6 Comparison of Sensitivity and Working Range of Biological Assays for Vitamin D

Assay	Time required for assay	Minimal level detectable in assay		Usual working range
		ng	nmol	
Rat line test	7 d	12	0.03	25–300 ng
AOAC chick	21 d	50	0.113	50–1250 ng
Intestinal Ca^{2+} absorption				
In vivo				
$^{45}Ca^{2+}$	1 d	125	0.33	0.125–25 g
$^{47}Ca^{2+}$	1 d	125	0.33	0.125–25 g
In vitro				
Everted sacs	1 d	250	0.65	250–1000 ng
Duodenal uptake of $^{45}Ca^{2+}$	1 d	250	0.65	250–1000 ng
Bone Ca^{2+} mobilization				
In vivo	24 h	125	0.32	0.125–25 g
Body growth	21–28 d	50	0.06	50–1250 ng
Immunoassays for calcium-binding protein	1 d	1	0.0025	1 ng

VIII. ANALYTICAL PROCEDURES

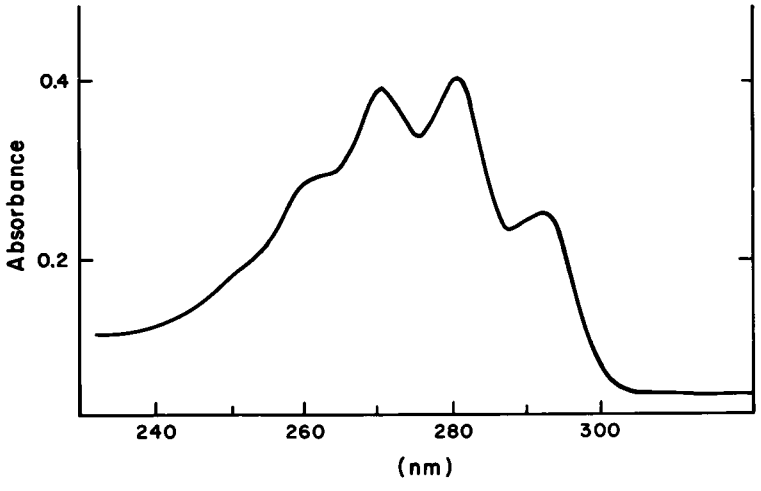
Although considerable progress has been made in the development of chemical or physical means to measure vitamin D, these methods at present generally lack the sensitivity and selectivity of the biological assays. Thus, they are not adequate for measuring samples that contain low concentrations of vitamin D. However, these physical and chemical means of vitamin D determination have the advantage of not being as time consuming as the biological assays and so are frequently used on samples known to contain high levels of vitamin D.

A. Ultraviolet Absorption

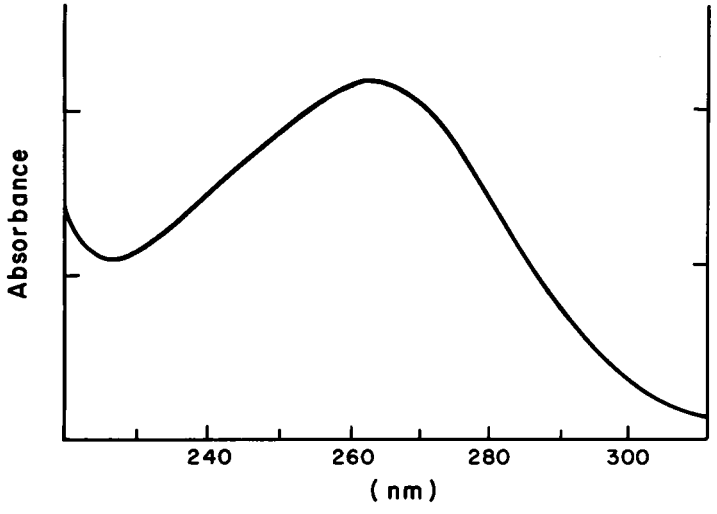
The first techniques available for quantitation of vitamin D were based on the measurement of the UV absorption at 264 nm. The conjugated triene system of double bonds in the vitamin D *seco*-steroids produces a highly characteristic absorption spectra (Fig. 10). The absorption maxima for vitamin D occurs at 264 nm, and at this wavelength the molar extinction coefficient for both vitamins D_2 and D_3 is 18,300. Thus, the concentration of an unknown solution of vitamin D can be calculated once its absorption at 264 nm is known. Although this technique is both quick and easy, it suffers from the disadvantage that the sample must be scrupulously purified prior to assay in order to remove potential UV-absorbing contaminants.

B. Colorimetric Methods

Several colorimetric methods for the quantitation of vitamin D have been developed over the years. Among these various colorimetric assays is a method based on the isomerization of vitamin D to isotachysterol. This procedure, which employs antimony trichloride, can detect vitamin D in the range of 1–1000 μg. Because it can detect such large amounts



A



B

Fig. 10 Ultraviolet spectrum of provitamin D and vitamin D. Panel A illustrates the characteristic UV absorption spectrum of provitamin D. The wavelengths of the several absorption maxima are 262, 271, 282, and 293 nm. The molar extinction coefficient at 282 nm is 11,500. Panel B illustrates the characteristic UV spectrum of vitamin D. The molar extinction coefficient at the 265- to 265-nm absorption maxima is 18,300.

of vitamin D, this assay is now used primarily to determine the vitamin D content of pharmaceutical preparations and has become the official U.S. Pharmacopoeia (USP) colorimetric assay for vitamin D_3.

In this assay, three types of tubes are normally prepared: one containing the standard vitamin D or unknown sample plus the color reagent; one containing only the solvent, ethylene chloride; and a third containing ethylene chloride, acetic anyhdride, and the color reagent. The absorbance at 500 nm is measured 45 s after addition of the color reagent.

The concentration of vitamin D in the assay tube is proportional to its absorbance, which is corrected for the solvent blank and for the tube containing the acetic anhydride. This procedure has been found to follow Beer's law for solutions containing 3.25–6.5 nmol vitamin D per milliliter assay solution. The major disadvantage to this assay is that the copresence of vitamin A, which is often present in pharmaceutical samples along with vitamin D, interferes with the assay. Thus, purification procedures that include adsorption chromatography and partition chromatography are required, making this assay procedure rather laborious and time consuming. Another disadvantage is the necessity for careful timing of the reaction because of the short time required for the appearance of maximal intensity of color. However, since this is the only direct chemical method routinely available, it has widespread industrial application (308).

C. Fluorescence Spectroscopy

There have been two reports in the literature describing assays that depend on the reaction of vitamin D with a substance capable of fluorescing (309,310). Both of these procedures are based on the fact that an acetic anhydride–sulfuric acid solution containing vitamin D is capable of fluorescence if the solution is activated by light of the correct wavelength. The lower limits of detectability of this assay are the same as those of the antimony trichloride colorimetric assay, and the same compounds that interfere with the colorimetric assay also interfere with this fluorescence assay. As a result, this assay is not normally used for the analytical determination of vitamin D concentration.

D. Gas Chromatography–Mass Spectrometry

One of the most powerful modern techniques available to steroid chemists for the analytical determination of samples containing mixtures of steroids is mass spectrometry (MS), or mass spectrometry coupled with prior separation by gas chromatography (GC). The gas chromatography–mass spectrometry (GC-MS) technique can be coupled to an on-line computer that collects information on the fragmentation patterns of steroids in the mass spectrometer. In this way a sophisticated quantitative assay can be developed with a sensitivity and selectivity approaching that of RIAs. There have recently appeared several GC-MS procedures applicable to vitamin D *seco*-steroids (311), but they are not yet widely employed.

E. High-Performance Liquid Chromatography

Several papers that describe the separation of vitamin D and its various metabolites by HPLC have appeared (312,313). This separation process has an exceedingly high resolving capability due to the large number of theoretical plates present in a typical column. Of equal importance to this technique is the sensitivity of the detector used for observing the separated compounds. All of the published procedures for the separation of vitamin D by HPLC have used an UV detector, and so their sensitivity is limited to approximately 5 ng. The chief advantage of using HPLC is the reduction in labor and time required to separate vitamin D and its metabolites. In the current official USP method for the determination of vitamin D, two prepurification steps, requiring up to 8 h, are necessary before the colorimetric analysis can be performed (314). However, with HPLC, reproducible separation of closely related compounds can be achieved in less than 1 h. HPLC has great

potential once a more sensitive detection method is developed. There are now available several official AOAC procedures for the HPLC determination of vitamin D in a variety of sample types, including vitamin preparations, multivitamin preparations, vitamin D oil concentrates, and fortified milk and milk powder (308).

F. Competitive Binding Assays

Various competition assays that can specifically quantitate the levels of $25(OH)D_3$, $24,25(OH)_2D_3$, or $1\alpha,25(OH)_2D_3$ in a sample are now available. Such assays were developed as a consequence of the discovery of specific vitamin D–binding proteins in the serum and tissues of mammals and birds, along with the availability of high specific activity tritiated $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$. Since these steroid competition assays are sensitive (they are capable of detecting picogram quantities), they are now routinely used to measure vitamin D metabolite levels in plasma.

Two different types of steroid competition assays have been developed for the detection of $1\alpha,25(OH)_2D_3$. The first employs incubation of intestinal mucosal cytosol plus the nuclear chromatin fractions with standardized amounts of tritiated $1\alpha,25(OH)_2D_3$. The $1\alpha,25(OH)_2D_3$ in the sample competes with the tritiated hormone for the binding sites of the $1\alpha,25(OH)_2D_3$ receptors present in the cytosol and chromatin fractions. By measuring the amount of tritiated $1\alpha,25(OH)_2D_3$ bound to the receptor, the amount of $1\alpha,25(OH)_2D_3$ in the sample can be determined. The first such assay developed that could be used to measure $1\alpha,25(OH)_2D_3$ levels in plasma was that of Brumbaugh and Haussler (315). Their technique requires a minimum of 10 mL plasma and involves a laborious three-stage chromatographic procedure. The final $1\alpha,25(OH)_2D_3$ peak is then assayed. Separation of bound from free steroid is achieved by filtration of the incubation media through glass filters. The steroid associated with the chromatin-cytosol receptor is specifically bound to these filters. A similar assay has been described by Procsal et al., except that separation of the bound from free steroid is achieved by high-speed differential centrifugation (316).

The second type of competition assay involves the use of calf thymus cytosol as the source of binding protein. Reinhardt et al. developed a radioreceptor assay for vitamin D_2, vitamin D_3, and their metabolites that does not require HPLC (317). Their technique includes the use of a stable $1\alpha,25(OH)_2D_3$ receptor preparation from calf thymus, nonequilibrium assay conditions, and solid-phase extraction of vitamin D metabolites from serum or plasma samples. This procedure requires 0.2–1.0 mL plasma. $1\alpha,25(OH)_2D_3$ is removed from the plasma on a C18-silica cartridge. The cartridge is first reverse-phase-eluted and then switched to normal-phase elution (318). The $1\alpha,25(OH)_2D_3$ is recovered and incubated with $[^3H]1\alpha,25(OH)_2D_3$ and reconstituted thymus receptor. Separation of receptor-bound hormone from free hormone is achieved by the addition of dextran-coated charcoal. Similar assays have been developed for $25(OH)D_3$ and $24,25(OH)_2D_3$ (146,319,320). This assay has a sensitivity of 0.7 pg.

IX. NUTRITIONAL REQUIREMENTS FOR VITAMIN D

A. Humans

The vitamin D requirement for healthy adults has never been precisely defined. Since vitamin D_3 is produced in the skin upon exposure to sunlight, a human being does not

have a requirement for vitamin D when sufficient sunlight is available. However, vitamin D does become an important nutritional factor in the absence of sunlight. In addition to geographical and seasonal factors, UV light from the sun may be blocked by factors such as air pollution and sun screens. In fact, as air pollution became prevalent during the industrial revolution, the incidence of rickets became widespread in industrial cities. It is now known that the rickets epidemic was partly caused by lack of sunlight due to air pollution. Thus, rickets has been called the first air pollution disease. Any condition that blocks sunlight from skin, such as the wearing of clothes, use of sunscreens, living indoors and in cities with tall buildings, or living in geographical regions of the world that do not receive adequate sunlight, can contribute to the inability of the skin to biosynthesize sufficient amounts of vitamin D. Under these conditions, vitamin D becomes a true vitamin in that it must be supplied in the diet on a regular basis.

Since vitamin D can be endogenously produced by the body and since it is retained for long periods of time by vertebrate tissue, it is difficult to determine minimum daily requirements for this substance. The requirement for vitamin D is also dependent on the concentration of calcium and phosphorus in the diet, the physiological stage of development, age, gender, degree of exposure to the sun, and the amount of pigment in the skin.

The current allowance of vitamin D recommended in 1989 by the National Research Council is 300 IU/day [1 IU = 0.025 μg vitamin D (321)] for infants from birth to 6 months in age; 400 IU/day for children, adolescents, and pregnant and lactating women; and 200 IU/day for other adults (322). Because rickets is more prevalent in preschool children, the Food and Agricultural Organization/World Health Organization (FAO/WHO) committee recommended that children receive 400 IU/day until the age of 6 years, after which the recommended daily allowance (RDA) is 100 IU/day.

In the United States, adequate amounts of vitamin D can readily be obtained from the diet and from casual exposure to sunlight. However, in some parts of the world where food is not routinely fortified and sunlight is often limited, obtaining adequate amounts of vitamin D becomes more of a problem. As a result, the incidence of rickets in these countries is higher than in the United States. Rickets was practically eradicated from the United States in the mid-1920s, but it has reappeared in the past two decades. The increase is predominantly due to changes in infant feeding and dietary preferences. Of 27 cases of infant rickets reported within the first year of life, 26 were found in breast-fed infants (323). Of 62 cases involving older children, 56 were from families following strict vegetarian diets that included no meat products, milk products, fish, or eggs.

B. Animals

The task of assessing the minimum daily vitamin D requirement for animals is no easier than it is for humans. Such factors as the dietary calcium/phosphorus ratio, physiological stage of development, gender, amount of fur or hair, color, and perhaps even breed, all affect the daily requirement for vitamin D in animals. Also, some animals, such as chicken and turkey, do not respond as well to vitamin D_2 as to vitamin D_3. As with humans, animals that are maintained in sunlight can produce their own vitamin D, so that dietary supplementation is not really necessary. For animals that are kept indoors or that live in climates where the sunlight is not adequate for vitamin D production, the vitamin D content of food becomes important. Sun-cured hays are fairly good sources of vitamin D, but

Table 7 Vitamin D Requirements of Animals

Animal	Daily requirements (IU)
Chickens, growing	90[a]
Dairy cattle:	
Calves	660[b]
Pregnant, lactating	5000–6000[c]
Dogs:	
Growing puppies	22[d]
Adult maintenance	11[d]
Ducks	100[d]
Monkey, growing rhesus	25[d]
Mouse, growing	167[d]
Sheep:	
Lambs	300[e]
Adults	250[e]
Swine:	
Breed sows	550[c]
Lactating sows	1210[c]
Young boars	690[c]
Adult boars	550[c]
Turkeys	400[a]

[a]IU required per pound of feed.
[b]IU required for 100 kg body weight.
[c]IU required per animal.
[d]IU required per kg body weight.
[e]IU required per 45 kg body weight.
Source: Published information by the Committee of Animal Nutrition, Agricultural Board (National Research Council).

dehydrated hays, green feeds, and seeds are poor sources. A brief list of the RDAs for animals is given in Table 7.

X. FOOD SOURCES OF VITAMIN D

For the most part, vitamin D is present in unfortified foods in only very small and variable quantities (Table 8). The vitamin D that occurs naturally in unfortified foods is generally derived from animal products. Saltwater fish, such as herring, salmon, and sardine, contain substantial amounts of vitamin D, and fish liver oils are extremely rich sources. However, eggs, veal, beef, unfortified milk, and butter supply only small quantities of the vitamin. Plants are extremely poor sources of vitamin D; fruits and nuts contain no vitamin D, and vegetable oils contain only negligible amounts of the provitamin. As a consequence, in the United Stated dietary requirements for vitamin D are met by the artificial fortification of suitable foods. Among these fortified foods are milk, both fresh and evaporated; margarine and butter; cereals; and chocolate mixes. Milk is fortified to supply 400 IU vitamin D per quart, and margarine usually contains 2000 IU or more per pound. A more complete listing of the vitamin D values of food is given by Booher et al. (324).

Table 8 Vitamin D Content of Unfortified Foods

Food source	Vitamin D (IU/100 g)
Beef steak	13
Beet greens	0.2
Butter	35
Cabbage	0.2
Cheese	12
Cod	85
Cod liver oil	10,000
Corn oil	9
Cream	50
Egg yolk	25
Herring (canned)	330
Herring liver oil	140,000
Liver:	
Beef (raw)	8–40
Calf (raw)	0–15
Pork (raw)	40
Chicken (raw)	50–65
Lamb (raw)	20
Mackerel	120
Milk:	
Cow (100 mL)	0.3–4
Human (100 mL)	0–10
Salmon (canned)	220–440
Sardines (canned)	1500
Shrimp	1150
Spinach	0.2

Source: Refs. 2 and 324.

XI. SIGNS OF VITAMIN D DEFICIENCY

A. Humans

A deficiency of vitamin D results in inadequate intestinal absorption and renal reabsorption of calcium and phosphate. As a consequence, serum calcium and phosphate levels fall and serum alkaline phosphatase activity increases. In response to these low serum calcium levels, hyperparathyroidism occurs. The result of increased levels of PTH, along with whatever $1\alpha,25(OH)_2D_3$ is still present at the onset of the deficiency, is the demineralization of bone. This ultimately leads to rickets in children and osteomalacia in adults. The classical skeletal symptoms associated with rickets, i.e., bowlegs, knock-knees, curvature of the spine, and pelvic and thoracic deformities (Fig. 11), result from the application of normal mechanical stress to demineralized bone. Enlargement of the bones, especially in the knees, wrists, and ankles, and changes in the costochondral junctions also occur. Since in children bone growth is still occurring, rickets can result in epiphysial abnormalities not seen in adult osteomalacia. Rickets also results in inadequate mineralization of tooth

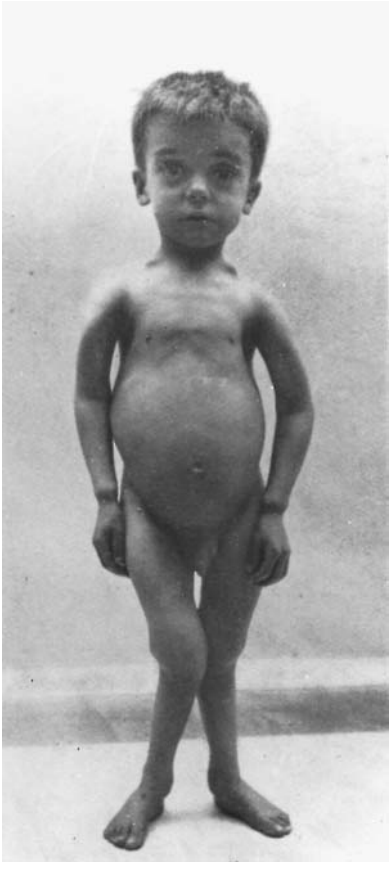


Fig. 11 Classic appearance of rickets in a child.

enamel and dentin. If the disease occurs during the first 6 months of life, convulsions and tetany can occur. Few adults with osteomalacia develop tetany.

Low serum calcium levels in the range of 5–7 mg per 100 mL and high serum alkaline phosphatase activity can be used to diagnose rickets and osteomalacia. Also, a marked reduction in circulating $1\alpha,25(OH)_2D_3$ levels in individuals with osteomalacia or rickets has been reported (325–327). Radiographic changes are also evident and can be used in diagnosis.

B. Animals

The response to vitamin D deficiency in animals closely resembles that in humans. Among the first symptoms of the deficiency is a decline in the plasma concentration of calcium and phosphorus. This is followed by an abnormally low growth rate and the characteristic alteration of bones, including faulty calcification of the bone matrix. As the disease progresses, the forelegs bend sideways and the joints become swollen. In laying birds, the eggs are thin-shelled, egg production declines, and hatchability is markedly reduced (328); classic symptoms of rickets develop, followed by tetany and death.

XII. HYPERVITAMINOSIS D

Excessive amounts of vitamin D are not available from natural sources. However, vitamin D intoxication is a concern in patients being treated with vitamin D or vitamin D analogs for hypoparathyroidism, vitamin D–resistant rickets, renal osteodystrophy, osteoporosis, psoriasis, some cancers, or in those who are taking supplemental vitamins. Hypervitaminosis D is a serious problem because it can result in irreversible calcification of the heart, lungs, kidneys, and other soft tissues. Therefore, care should be taken to detect early signs of vitamin D intoxication in patients receiving pharmacological doses. Symptoms of intoxication include hypercalcemia, hypercalciuria, anorexia, nausea, vomiting, thirst, polyuria, muscular weakness, joint pains, diffuse demineralization of bones, and disorientation. If allowed to go unchecked, death will eventually occur.

Vitamin D intoxication is thought to occur as a result of high 25(OH)D levels rather than high $1\alpha,25(OH)_2D$ levels (329,330). Patients suffering from hypervitaminosis D have been shown to exhibit a 15-fold increase in plasma 25(OH)D concentration as compared to normal individuals. However, their $1\alpha,25(OH)_2D$ levels are not substantially altered (331). Furthermore, anephric patients can still suffer from hypervitaminosis D even though they are for the most part incapable of producing $1\alpha,25(OH)_2D$. It has also been shown that large concentrations of 25(OH)D can mimic the actions of $1\alpha,25(OH)_2D$ at the level of the receptor (315,329,332,333).

In the early stages of intoxication, the effects are usually reversible. Treatment consists of merely withdrawing vitamin D and perhaps reducing dietary calcium intake until serum calcium levels fall. In more severe cases, treatment with glucocorticoids, which are thought to antagonize some of the actions of vitamin D, may be required to facilitate the correction of hypercalcemia. Since calcitonin can bring about a decline in serum calcium levels, it may also be used in treatment.

XIII. FACTORS THAT INFLUENCE VITAMIN D STATUS

A. Disease

In view of the complexities of the vitamin D endocrine system, it is not surprising that many disease states are vitamin D–related. Figure 12 classifies some of the human disease states that are believed to be associated with vitamin D metabolism according to the metabolic step where the disorder occurs.

1. Intestinal Disorders

The intestine functions as the site of dietary vitamin D absorption and is also a primary target tissue for the hormonally active $1\alpha,25(OH)_2D_3$. Impairment of intestinal absorption of vitamin D can occur in those intestinal disorders that result in the malabsorption of fat. Patients suffering from such disorders as tropical sprue, regional enteritis, and multiple jejunal diverticulosis often develop osteomalacia because of what appears to be a malabsorption of vitamin D from the diet (334). Surgical conditions, such as gastric resection and jejunoileal bypass surgery for obesity, may also impair vitamin D absorption. Also, patients receiving total parenteral nutrition in the treatment of the malnutrition caused by profound gastrointestinal disease often develop bone disease (335).

On the other hand, intestinal response to vitamin D can be affected by certain disease states. Patients suffering from idiopathic hypercalciuria exhibit an increased intestinal

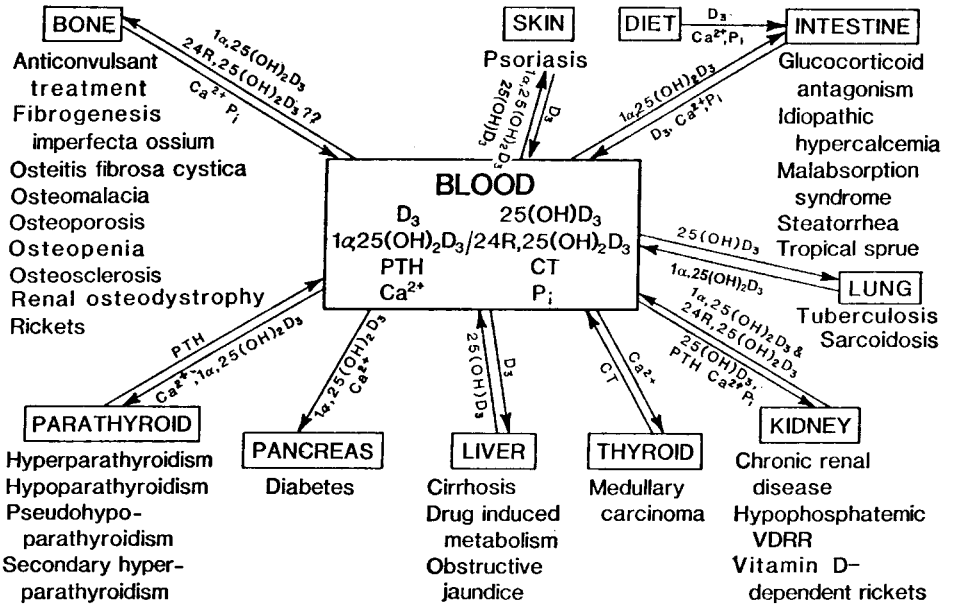


Fig. 12 Human disease states related to vitamin D. PTH, parathyroid hormone; CT, calcitonin; VDRR, vitamin D–resistant rickets; Pi, inorganic phosphate; Ca^{2+}, calcium.

absorption of calcium that may result from an enhanced intestinal sensitivity to $1\alpha,25(OH)_2D_3$ or from an overproduction of $1\alpha,25(OH)_2D_3$. The disease sarcoidosis also results in enhanced sensitivity to vitamin D. Sarcoidosis is characterized by hypercalcemia and hypercalciuria in patients receiving only modest amounts of vitamin D. Experiments have shown that these patients have elevated levels of serum $1\alpha,25(OH)_2D_3$. The excess $1\alpha,25(OH)_2D_3$ is likely of extrarenal origin and therefore not regulated by circulating levels of PTH (336). Other experiments have shown that macrophages from patients with sarcoidosis can produce $1\alpha,25(OH)_2D_3$ (120).

Other disease states that can result in extrarenal production of $1\alpha,25(OH)_2D_3$ are tuberculosis (337), leprosy (338), and some lymphomas (339).

2. Liver Disorders

The liver plays an important role in the vitamin D endocrine system; not only is it the primary site for the production of 25(OH)D, but it is also the source of bile salts that aid in the intestinal absorption of vitamin D. Furthermore, it is likely that the liver is the site where binding of 25(OH)D by vitamin D-binding protein occurs, and it may even be the site at which this binding protein is synthesized. Hence, malfunctions of the liver can possibly interfere with the absorption, transport, and metabolism of vitamin D. Malabsorption of calcium and the appearance of bone disease have been reported in patients suffering from either primary biliary cirrhosis or prolonged obstructive jaundice. The disappearance of radioactive vitamin D from the plasma of these patients is much slower than in normal humans (340), and their plasma 25(OH)D levels are reduced (341). Although these patients respond poorly to vitamin D treatment, they immediately respond if treated with

$25(OH)D_3$. Thus, it appears that the bone disease experienced by these patients results from their inability to produce 25(OH)D.

3. Renal Disorders

The kidney functions as the endocrine gland for $1\alpha,25(OH)_2D_3$. Thus, disease states that affect the kidney can concomitantly alter the production of this calcium homeostatic hormone. It is well known that patients suffering from renal failure also often suffer from skeletal abnormalities. Termed renal osteodystrophy, these skeletal abnormalities include growth retardation, osteitis fibrosa, osteomalacia, and osteosclerosis. It became apparent with the discovery that under normal conditions $1\alpha,25(OH)_2D_3$ is produced in the kidney that these skeletal abnormalities result from the failure of patients to produce $1\alpha,25(OH)_2D_3$. Support for this theory came from studies on the metabolism of radioactively labeled vitamin D in normal persons vs. patients with chronic renal failure. From these studies, anephric or uremic individuals appeared incapable of producing $1\alpha,25(OH)_2D_3$. Direct evidence for this came from the observation that circulating level of $1\alpha,25(OH)_2D_3$ in the normal subject is in the range of 30–35 pg/mL, whereas in chronic renal failure the levels have been reported as low as 3–6 pg/mL (342,343). However, after a successful renal transplant, $1\alpha,25(OH)_2D_3$ levels return to the normal range. Also, the administration of $1\alpha,25(OH)_2D_3$ to these patients results in the stimulation of intestinal calcium absorption and an elevation of serum calcium levels (344).

4. Parathyroid Disorders

As previously outlined, PTH influences the production of $1\alpha,25(OH)_2D_3$, so that any disease state that affects the secretion of PTH may, in turn, have an effect on the metabolism of vitamin D. Hyperactivity of the parathyroid glands, as in primary hyperparathyroidism, results in the appearance of bone disease resembling osteomalacia. Circulating $1\alpha,25(OH)_2D_3$ levels in these subjects have been reported to be significantly elevated (345), as is their intestinal calcium transport (346). On the other hand, in hypoparathyroidism, hypocalcemia occurs. In these patients, a slight reduction in circulating $1\alpha,25(OH)_2D_3$ levels has been reported (347). When these patients are treated with $1\alpha,25(OH)_2D_3$ their serum levels are increased to normal.

There are several published reviews on the role of vitamin D in disease (18,334,348).

B. Genetics

Vitamin D–resistant rickets (hypophosphatemic rickets) appears to be an X-linked, dominant genetic disorder. Winters et al. presented evidence that this disease is almost always inherited and is usually congenital (349). Males are usually more severely affected by this disease than females. Associated with the disease are skeletal abnormalities, such as rickets or osteomalacia, and a diminished renal tubular reabsorption of phosphate that results in hypophosphatemia. Individuals with this disease do not respond to physiological doses of vitamin D; treatments with $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ are also ineffective, although an increase in intestinal calcium absorption does occur (350). These patients have also been reported to have normal serum $1\alpha,25(OH)_2D_3$ levels. Thus, it appears that this disorder does not result from an alteration in the metabolism of vitamin D or from an impaired intestinal response to $1\alpha,25(OH)_2D_3$, but rather from a specific defect in renal tubular reabsorption of phosphate.

A genetic defect that interferes with vitamin D metabolism has also been suggested in vitamin D–dependent rickets type I. This ailment differs from rickets in that it appears in children who are receiving adequate amounts of vitamin D and requires pharmacological doses of vitamin D or $25(OH)D_3$ to reverse the harmful effect of disease on bone. However, the disease is responsive to physiological amounts of $1\alpha,25(OH)_2D_3$, which suggests that the defect occurs in the metabolism of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$. This disease state appears to be the result of an autosomal recessively inherited genetic defect (351). It is not known how this defect affects the metabolism of $25(OH)D_3$.

Vitamin D–dependent rickets type II also has a genetic basis. This ailment is similar to vitamin D–dependent rickets type I except that children do not respond to large doses of vitamin D, $25(OH)D_3$, or $1\alpha,25(OH)_2D_3$. The combination of symptoms, i.e., defective bone mineralization, decreased intestinal calcium absorption, hypocalcemia, and increased serum levels of $1\alpha,25(OH)_2D_3$, suggest end-organ resistance to the action of $1\alpha,25(OH)_2D_3$. Experiments have shown that these children have a single-point mutation in the nuclear receptor for $1\alpha,25(OH)_2D_3$ (352–355).

C. Drugs

Recent evidence suggests that prolonged use of anticonvulsant drugs, such as diphenylhydantoin or phenobarbital, can result in an impaired response to vitamin D; this results in an alteration of calcium metabolism and the appearance of rickets or osteomalacia. Serum $25(OH)D_3$ levels in patients receiving these drugs have been reported to be markedly reduced (356). Also, studies in animals suggest that these drugs stimulate the hepatic microsomal cytochrome P450 enzymes, which could lead to an increased catabolism of $25(OH)D_3$ (357). However, $1\alpha,25(OH)_2D_3$ levels have been shown to be normal or even increased after drug treatment (358). It appears that this drug-induced osteomalacia may not be the result of an effect of the drug on vitamin D metabolism. Studies on rat and chick duodena in organ culture indicate that anticonvulsant drugs may act on the gastrointestinal tract and affect the absorption of calcium (359). Anticonvulsant drugs have also been shown to inhibit calcium reabsorption in organ culture mouse calvaria (360). Further research is needed to determine the mechanism by which these anticonvulsant drugs affect calcium metabolism.

D. Alcohol

Persons suffering from chronic alcoholism exhibit a decrease in plasma $25(OH)D_3$ levels and in intestinal calcium absorption and bone mineral content. This is observed in patients with and without cirrhosis of the liver. Current evidence indicates that the impairment of intestinal calcium absorption is the result of low $25(OH)D_3$ levels (361,362). However, how chronic alcoholism results in low $25(OH)D_3$ levels is at present not understood.

E. Age

The fact that changes in the metabolism of vitamin D may occur with aging has been suggested by the observation that the ability to absorb dietary calcium decreases with age (363). In addition, loss of bone increases in the elderly and an age-related hypoplasia of bone cells occurs. $1\alpha,25(OH)_2D_3$ levels in the plasma decrease with age, possibly due to an age-related reduction of epidermal concentrations of 7-dehydrocholesterol, resulting in less photochemical production of $1\alpha,25(OH)_2D_3$ by the skin. Also, the 1α-hydroxylase

Table 9 Drug Forms of Vitamin D Metabolites

Compound name	Generic name	Commercial name	Pharmaceutical company	Effective daily dose (micrograms)[a]	Approved use
1α,25$(OH)_2D_3$	Calcitriol	Rocaltrol	Hoffmann-La Roche	0.5–1.0	RO, HP, O[b]
1α,25$(OH)_2D_3$	Calcitriol	Calcijex	Abbott	0.5 (i.v.)	HC
1α,24$(OH)_2$-19-nor-D_3	Paricalcitol	Zemplar	Abbott	2.8–7 (eod)	SHP
1α,24$(OH)_2D_3$	Tacalcitol	Bonalfa	Teijin Ltd.—Japan	40–80 (topical)	PP
1α,24S$(OH)_2$-22-ene-24-cyclopropyl-D_3	Calcipotriene	Dovonex	Leo—Denmark	40–80 (topical)	PP
1α,24S$(OH)_2$-22-ene-24-cyclopropyl-D_3	Calcipotriene	Dovonex	Westwood-Squibb	40–80 (topical)	PP
1α-OH-D_3	Alfacalcidol	One-Alfa	Leo—Denmark	1–2	RO, HP, O, VDRR
1α-OH-D_3	Alfacalcidol	Alpha-D_3	Teva-Israel	0.25–1.0	RO, O, HC, HP
1α-OH-D_3	Alfacalcidol	Onealfa	Teijin Ltd.—Japan	0.25–1.0	RO, O
1α-OH-D_3	Alfacalcidol	Onealfa	Chugai—Japan	0.25–1.0	RO, O
1α-OH-D_2	Doxercalciferol	Hectorol	Bone Care	10 3×/WEEK	SHP
25$(OH)D_3$	Calcifediol	Calderol	Organon-USA	50–500	RO
25$(OH)D_3$	Calcifediol	Dedrogyl	Roussel-Uclaf-France	50–500	RO
10,19-dihydrotachysterol$_3$	Dihydrotachysterol$_3$	Hytakerol	Winthrop	200–1000	RO

The key to the approved uses of the vitamin D analogs is as follows: RO, renal osteodystrophy; O, postmenopausal osteoporosis; PP, plaque psoriasis; HC, hypocalcemia (frequently present in patients with renal osteodystrophy who are subjected to hemodialysis); HP, hypoparathyroidism and associated hypocalcemia (which may be encountered in patients with hypoparathyroidism, pseudohypoparathyroidism, or in circumstances of postsurgical hypoparathyroidism); SHP, secondary hyperparathyroidism associated with renal osteodystrophy; VDRR, vitamin D-resistant rickets.

[a]Oral dose unless otherwise indicated; eod = every other day.

[b]The use of Rocaltrol for postmenopausal osteoporosis is approved in Argentina, Australia, Austria, Czech Republic, Colombia, India, Ireland, Italy, Japan, Malaysia, Mexico, New Zealand, Peru, Philippines, South Korea, South Africa, Switzerland, Turkey, and the United Kingdom.

enzyme is less responsive to induction by PTH due to the decrease of glomerular filtration with age (364).

F. Sex

Gray et al. demonstrated that men and women differ in their metabolism of vitamin D in response to various physiological stimuli (365). In women, they observed that dietary phosphate deprivation resulted in a decrease in serum phosphorus levels with a concomitant increase in plasma $1\alpha,25(OH)_2D_3$ concentrations. However, no change of either of these parameters was noted in men. Thus, the mechanism by which men and women respond to dietary phosphate deprivation seems to differ.

XIV. EFFICACY OF PHARMACOLOGICAL DOSES

Several ailments are known to respond to massive doses of vitamin D. For example, the intestinal malabsorption of calcium that results from chronic renal failure and the subsequent development of rickets or osteomalacia can be overcome by administration of 100,000–300,000 IU vitamin D per day (364). Patients suffering from hypoparathyroidism can usually be treated by giving 80,000–100,000 IU vitamin D per day (366). Also, children afflicted with vitamin D–dependent rickets type I can be treated with 10,000–100,000 IU per day (2). The therapeutic effect of such massive doses can be explained by the fact that 25(OH)D in sufficiently high concentrations will mimic the action of $1\alpha,25(OH)_2D_3$ at the receptor. However, as mentioned earlier, the administration of such pharmacological doses of vitamin D to patients over a prolonged period of time carries with it the danger of vitamin D toxicity.

Table 9 shows the drug forms of vitamin D metabolites that are currently available for the treatment of several disease states. Experiments are in progress to develop additional vitamin D analogs that will be useful pharmacological agents without the hypercalcemic side effect of $1\alpha,25(OH)_2D_3$.

XV. RECENT DEVELOPMENTS

Recently, the x-ray crystal structure of the ligand-binding domain (LBD) of VDR (367) and several other nuclear receptors have been determined. These LBDs are composed primarily of 11–13 α-helices that are folded to form three layers. The interior of the LBD is composed primarily of hydrophobic residues designed to bind lipophilic ligands like $1,25(OH)D_3$. Ligand binding to its cognate receptor induces conformational changes in the receptor, which increases its ability to activate gene transcription. The major structural difference between unoccupied and ligand-bound nuclear receptors is the repositioning of the C-terminal helix 12 containing the ligand-dependent transactivation domain, AF-2. Upon ligand binding, helix 12 moves from projecting away from the LBD to a position tightly packed against helix 3 of the LBD (368,369). Repositioning of the AF-2 domain results in the formation of new surfaces, including a hydrophobic cleft (370), needed to interact with other nuclear factors called coactivators. Coactivators are adapter proteins that link transcriptional activators such as nuclear receptors to basal transcription machinery resulting in increased gene transcription (371).

Three families of coactivator proteins are known to interact with nuclear receptors: SRC-1 (372)/NcoA-1 (373), TIF2 (374)/GRIP-1 (375), and ACTR/pCIP (376). These

proteins contain a conserved leucine-containing motif (LXXLL), important for interactions between coactivators and nuclear receptors (377). The coactivator complexes have activities associated with them such as histone acetyltransferase activity (378) or methyl transferase activity (379), which aid in the decondensation of chromatin to facilitate binding of RNA polymerase II transcription complex (RNA Pol II) to DNA. Some of the proteins in the coactivator complex can also interact with other proteins that are part of the RNA Pol II core complex. In this way, the coactivators can link upstream activators such as nuclear receptors to RNA Pol II and modulate gene transcription (380). Identifying these intermediary factors involved in transcriptional control of specific target genes is essential for understanding the biological actions of vitamin D and will continue to be an exciting area of research for several years.

XVI. CONCLUSION

Vitamin D, through its hormonally active metabolite $1\alpha,25(OH)_2D_3$, is known to act on bone and intestine to maintain calcium homeostasis. However, the actions of $1\alpha,25(OH)_2D_3$ are much broader than was originally thought. There is evidence that $1\alpha,25(OH)_2D_3$ is involved in the physiology of tissues not related to calcium homeostasis, such as skin, pancreas, pituitary, muscle, and hematopoietic cells. Figure 4 demonstrates the complexity of the vitamin D endocrine system as it is understood today. Although many advances have been made in the past decade, there is still much to learn about the detailed cellular and molecular mode of action of vitamin D and its metabolites.

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