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The D Vitamins and Bone

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"The adventure of the sun is the great natural drama by which we live."
Henry Beston, *The Outermost House*, 1928

I. Introduction

AWARENESS of the number and nature of compounds with vitamin D-like activity and the therapeutic usefulness of this class of substances has expanded dramatically during the past 15 years. The major factor in the evolution of our thinking has been the recognition that the native vitamin D molecule undergoes metabolic activation at specific tissue sites and that there is multifactorial control of these activation steps. Lesions at these tissue sites or in the control factors can result in symptoms of vitamin D deficiency or excess in target tissues.

Vitamin D was officially recognized as a pharmacologically important substance about 45 years ago as attested to by its inclusion in Cushny's textbook in 1934 (164) and in the 11th revision of the *U.S. Pharmacopoeia* in 1935 (684). Rickets and osteomalacia were described as sequelae of a vitamin D-deficient diet, and the effectiveness of vitamin D in their prophylaxis and treatment was recognized. The usefulness of vitamin D in treatment of hypocalcemias of diverse etiology and the risk of hypercalcemia with excessive amounts were also rapidly appreciated. During the late 1930s and the 1940s it was demonstrated that intestines from vitamin D-deficient animals exhibited defective calcium transport. A decade later it was shown that vitamin D could also mobilize calcium from bone. These studies were fundamental in establishing the major sites of action of the class of compounds. In the late 1960s the vitamin D field was revolutionized by the demonstration that the native compound is converted in the body to active products. Our subsequent expanding knowledge of vitamin D metabolism and its regulation has made the therapeutic implications and applications of vitamin D metabolites not only rational but unavoidable. The recent approval of 25-OH-vitamin D_3 and 1,25-$(OH)_2$ vitamin D_3 for therapeutic use attests to the clinical importance of the vitamin D metabolites and makes this an unusually appropriate time for the topic to be reviewed in PHARMACOLOGICAL REVIEWS.

This review will focus on one of the target tissues of vitamin D action, i.e. bone. Vitamin D deficiency leads

to undermineralized states of bone, i.e., *rickets* during growth and *osteomalacia* in the adult, causing deformities, increased susceptibility to fractures, and pain. Excess vitamin D results in resorption of bone with loss of both mineral and matrix. Release of bone mineral into the circulation is believed to be a major factor in the hypercalcemia of hypervitaminosis D. Normal bone remodeling as well as long-term calcium homeostasis is probably dependent upon vitamin D and its metabolites. The reader will find the rest of the review divided into four major sections. Section II is a broad overview of current thoughts regarding the processes that underly bone formation and resorption and is directed especially towards the reader whose experience is outside the calcified tissue field. Section III shifts the focus to "The D Vitamins," a term selected to include not only the native vitamin D compounds but also their recognized metabolites and theoretically and clinically important analogs. In Section IV the two threads are joined, as the effects of the D vitamins on the dual processes of bone formation and mineralization and the mechanisms of these effects are reviewed. It is hoped that this will provide the reader with the rational basis for the use of both classic vitamin D compounds and newer substances in bone disorders of widely ranging apparent etiology. It should be apparent that there are still many unresolved problems, even in terms of basic mechanisms, and these are addressed in section V.

Although the major emphasis of this review will be on bone, some aspects of the effects of the D vitamins on the intestine as well as current concepts of vitamin D metabolism will also be considered. I have chosen not to discuss the possible effects of the D vitamins on kidney, parathyroid glands, and muscle. For more information in these areas, as well as more detailed treatment of intestinal effects, metabolism of vitamin D, and clinical aspects, the reader is referred to other recent reviews (including 57, 115, 141, 144, 154, 176, 181a, 249, 346a, 359a), monographs (143, 358, 436a), and proceedings of workshops, conferences, and clinical symposia (18, 122, 445, 446, 477).

II. Molecular and Metabolic Events in Bone Formation and Resorption

A. Processes Involved in Bone Formation and Mineralization

The extensive extracellular compartment of bone consists largely of mineral and collagen, with smaller amounts of noncollagenous glycoproteins and mucopolysaccharides. The cartilage anlage, which is the precursor for most bones except those of the skull, is formed from type II collagen [three α_1 (II) chains, (413)] and noncollagenous glycoproteins. Mineralization involves vascular invasion in localized regions of the cartilage template, followed by appearance of osteoblasts and osteoclasts, initiation of synthesis of type I collagen [two α_1(I) and one α_2 chains (31, 412, 413)], and calcification of this collagen. In membrane bone formation, such as in the skull, type III collagen is deposited in the mesenchymal tissues and subsequently calcifies. X-ray diffraction evidence suggests that during bone calcification the mineral may undergo a change in state from amorphous calcium phosphate salts to the crystalline hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ (494). When mineralization fails to keep pace with bone growth, a zone of poorly mineralized matrix, or *osteoid*, results. Inadequate calcification leads to rickets in the growing child. The deformities in this disease result from pressure on the wide unmineralized cartilagenous zones. Those parts of the skeleton undergoing rapid growth seem to be the most affected, explaining both the localization and severity of the disease in different age groups (146). In the adult, where bone turnover is slower, deformities are less common, although bone pain can be severe and is postulated to result from stimulation of sensory nerve endings in the periosteum of undermineralized bone in response to pressure (146). Rickets and its relationship to vitamin D are discussed in more detail in section IV A.

The events that initiate mineralization in normal development are not yet fully understood, although investigations during the last few years have yielded some intriguing findings and models. The calcium × phosphate product of serum is insufficient for spontaneous precipitation in soft tissues (179). However, normal serum does appear to be supersaturated with respect to bone mineral (431). Specific organelles may be unique to mineralizing tissues. Several investigators have visualized and presented evidence consistent with a regulatory "*bone membrane*" composed of lining cells and osteoblasts that could either be a permeability barrier or a transporting surface (95, 516, 599). *Microspherules* (321) or *mitochondria* (399) within osteoblasts have been proposed to participate in mineral regulation. Another interesting concept that has experimental support is the *vesicle hypothesis*. In this model, cells in the region of cartilage calcification give rise to vesicles whose contents include lipids (486, 676) as well as specific enzymes (alkaline phosphatase, inorganic pyrophosphatase, 5′-AMPase, ATPase) that could, in the presence of appropriate concentrations of organic substrate, effect high concentrations of inorganic phosphate (4) and counteract the inhibitory effects of pyrophosphate on mineralization (180). The combination of this inorganic phosphate with calcium bound to the lipids has been postulated to serve as the nidus for crystallization. In this model, crystal growth causes rupture of the vesicles, and ions in the cartilage fluid then contribute to the further mineralization.

Investigations of the chemical composition of mineralizing tissues also have revealed some unique characteristics. In calcifying teeth, the appearance of specific *phosphoproteins* may play a role in the initiation of mineralization (641, 660). Bone *collagen* appears to have specific

nucleation sites that may be the regions where mineralization begins (208). Tissues that calcify have a unique *glycoprotein* composition (265) and a role for these substances in calcification has been postulated (298). Recently, calcium-binding proteins containing *γ-carboxyglutamic acid* residues have been demonstrated in hard tissue and implicated in soft tissue calcification (242, 496). The γ-carboxylation is vitamin K-dependent, similar to that shown for another class of extracellular calcium binding proteins, the clotting factors (596). These diverse phenomena have yet to be integrated into a scheme of "the mineralization process". Part of the difficulty in evaluating the significance of these factors in mineralization has been the lack of an in vitro system in which normal mineralization can be stimulated by physiologic or pharmacologic means. Vitamin D and its active metabolites, although they clearly enhance mineralization of rachitic bone when administered to the whole animal (see section IV A), have not been shown to have direct effects on any of the organelles or chemical characteristics described above. Several agents, including insulin and somatomedin, enhance bone collagen synthesis (91, 92) but have not been shown to stimulate mineralization. Elevated phosphate will stimulate the synthesis of collagen and increase mineralization of bone organ cultures (56). However, in vitro studies with this ion have to be interpreted with caution since elevated phosphate can produce nonphysiologic deposition of calcium phosphate, especially in the presence of tissue damage (512).

B. Processes Involved in Bone Resorption

Bone resorption involves the removal of both the mineral and the organic phase of the bone matrix. Physiologically, the process is probably of greatest importance during development when it may constitute a mechanism for contouring bones. However, it continues throughout life and may be accelerated during pathologic states, resulting in considerable loss of bone and weakening of the skeleton. Resorption is a cell-mediated activity, responsive to hormonal stimuli. Bone mineral turnover also occurs through physicochemical exchange, which is probably a surface reaction and may be independent of active cellular function. The postulated bone membrane (see section II A) has also been proposed as a site for hour-to-hour regulation of calcium flux (599).

As in the case of mineralization, the structures and processes bringing about resorption have not been fully defined. At the subcellular level, the *ruffled border* of the osteoclast (see II D, 233) probably plays a fundamental role in resorption, although its specific properties are still largely uncharacterized. Osteoclast *mitochondria* (399) and *lysosomes* (see below) may also participate in the osteolytic process.

In the earliest considerations of mechanism, it was noted that treatments that caused bone resorption increased the production of organic acids by the bone (5, 170, 366, 636). *Lactate* and *citrate* were the acids most commonly implicated, although the specific proportions or even the presence or absence of one or the other acid were not consistent findings (520). Acid per se could increase the solubility of the bone mineral (431), whereas citrate could remove mineral by chelation of calcium (431). It would be expected that if solubilization or chelation played a major role in resorption that there would be disproportionately more mineral than matrix released, at least initially. But this appears not to be the case; mineral and matrix seem to be lost in a parallel fashion (504). Release of *lysosomal enzymes* occurs in response to agents that cause resorption of bone (165, 504, 520, 635, 636). Although the specific substrates for the enzymes in bone have not been identified, it has been found that several agents that stabilize lysosomes inhibit bone resorption (165, 505, 511, 575). The release of lysosomal enzymes could be secondary to a decrease in pH (138) from acid production. On the other hand, release of lysosomal enzymes as the primary event could conceivably lead to subsequent loss of both mineral and matrix if the matrix were essential as a framework for the mineral.

Collagenase has been implicated in bone resorption (73, 189, 322, 500, 540, 638, 647). There has been some difficulty in studying the role of this enzyme since it may be tightly bound (540) and exist in bone as an inactive higher molecular weight precursor form (638). Addition of clostridial collagenase over a wide concentration range to bones in vitro does not produce resorption (580). Collagenase concentrations in whole bones do not change in parallel with resorption (73, 165), as lysosomal enzymes were shown to do. Recent studies indicate that parathyroid hormone (PTH) increases collagenolytic activity of cultured bone cells if the PTH receptors are protected during cell isolation (501). Another nonlysosomal enzyme implicated in bone resorption is *carbonic anhydrase*. This has been a difficult enzyme to study in bone and unique methods have been required to demonstrate its presence convincingly (135, 198). The major evidence implicating it in resorption is the correlation between the carbonic anhydrase and bone resorption inhibitory activities of several analogs of sulfanilamide (414, 644). The step at which carbonic anhydrase would act to stimulate resorption is uncertain, although possibilities include either the production of H^+ ions or of bicarbonate. Resorption has been shown to be dependent upon an adequate concentration of bicarbonate or CO_2 (389, 390), although again the mechanism by which this affects resorption is unclear. An action via the synthesis of nucleic acid precursors or citrate precursors has been proposed (388).

A number of biologic substances have been shown to stimulate resorption of bones directly in organ culture in vitro. These include PTH (33, 190, 502), prostaglandins (149, 331, 614), vitamin D metabolites and analogs (13, 266, 387, 509, 521, 581, 585, 591, 628), fatty acids (577, 583), and several partially characterized peptides and

lipopolysaccharides, including osteoclast activating factor (OAF) produced by hemagglutinin-activated peripheral leukocytes as well as some tumor cells (292, 374, 422, 423, 506, 627), a bone resorber from albumin (brA) (576, 586), some bacterial endotoxins (244, 245), and bacterial cell wall lipopolysaccharides (243). Thus far there does not seem to be a common mechanism by which all of these agents initiate the process, although increased cyclic AMP (cAMP) and stimulation of calcium flux have been implicated for some of them. In support of a role for *cyclic nucleotides* are the observations that phosphodiesterase inhibitors such as theophylline, methylisobutyl xanthine, and dibutyryl cAMP cause bone resorption (269, 332, 580, 637) and PTH and prostaglandins increase cAMP concentrations in bone and bone cells (102, 103, 258, 375, 392, 482, 530). Imidazole, which stimulates phosphodiesterase (87), inhibits resorption (16). However, the implication of cAMP as a mediator of resorption is complicated by the facts that (a) the PTH-induced increase in bone cAMP could be occurring predominantly in cells other than those mediating resorption (375); (b) calcitonin, dexamethasone, and verapamil, agents that inhibit bone resorption in vitro, either increase cAMP themselves (258, 530) or enhance the effects of PTH (107, 267, 268) on this process (the calcitonin-induced increase in cAMP appears to be more specific to bone resorbing cells than the PTH effect); (c) phosphodiesterase inhibitors increase cGMP as well as cAMP (9); (d) imidazole has effects other than to stimulate phosphodiesterase (151). The vitamin D metabolites per se have not been shown to increase cAMP in bone or bone cells in vitro (199, 386, 483, 670). In combination with PTH, they have been variously reported to inhibit (670), enhance (107, 386), or have no effect (483) on the action of PTH. These diverse findings have not been resolved, although dosage and specific tissue preparation may account for some of the disparities.

Another mechanism that has been proposed as an initiator for bone resorption is an influx of calcium (64, 158–160, 513, 598a, 669). A rapid transient decrease in serum calcium occurs when PTH is administered in vivo (472, 473). PTH also causes uptake of calcium by calvaria (473) and by isolated bone cells (160). However, the rapid uptake of calcium is reportedly not elicited by prostaglandins (157a) or vitamin D metabolites (156, 385a). In vitro, the calcium concentration of the incubation medium markedly affects the extent of resorption produced by a brief exposure to PTH, with optimum effects seen at 1.2 to 1.5 mM, and lesser effects at lower and higher calcium concentrations (510). Calcium can mimic effects of PTH and 1,25-$(OH)_2$ vitamin D_3 on bone cells (669). The divalent cation ionophore A23187, which, like PTH, stimulates calcium influx by isolated bone cells (159), has been found to enhance resorption of fetal rat limb bones over a narrow concentration range (158, 159, 165). It has not been possible to demonstrate this bone-resorbing effect of the ionophore in newborn mouse calvaria (308). Thus at the present time there is no common basis to explain the initiation of bone resorption by all agents, and it appears possible that they could act through several different trigger mechanisms.

C. Interrelationships between Bone Formation and Resorption

Although formation and resorption have generally been studied as isolated processes, there is evidence that there are interrelationships between the two. The major evidence is that under physiologic conditions and in some pathologic states changes in the two processes appear to occur simultaneously. In the normal developmental remodeling of bone, resorption and formation appear to act in concert to bring about the shaping and contouring of the individual bones (195). A dramatic example of the necessity for the dual processes is seen in mutants such as the *ia* rat or the osteopetrotic mouse, in which the resorption aspect of remodeling is deficient or absent (393, 645). These animals tend to have densely-mineralized, short, thick bones. In many metabolic bone disorders, including hyper- and hypoparathyroidism, acromegaly, and Paget's disease of bone, there appears to be a linear relationship between accretion and removal of calcium (236). Human metabolic bone disease can be viewed from the perspective of the balance between the numbers and activities of bone-forming and bone-resorbing cells (513). Two recent approaches may provide in vitro model systems for studying coupling. The first involves parallel determinations of proline incorporation and hydroxyproline release from 8-day embryonic chick calvaria maintained in long-term culture (295). A second new approach is the evaluation of the growth-promoting activities of conditioned media from cultured bones (93, 296).

D. The Cells of Bone and Their Putative Roles

Three basic types of mature differentiated cells have been characterized in bone. Precursor cells have also been identified in the tissue. *Osteoblasts* are defined by location, appearance, and function. In long bones they are generally present in the region of the hypertrophic cartilage as well as under the periosteum (467, 679). In the flat bones of the skull they are present between the periosteum and the bone. Osteoblasts have been described as ovoid, with an eccentric nucleus (466). Histochemically, they stain for alkaline phosphatase and may be the source of the alkaline phosphatase staining in the hypertrophic cartilage region of developing bones. The synthesis of collagen by osteoblasts is supported by autoradiographic evidence (364). Also, cells isolated from fetal calvaria, which contain a high proportion of osteoblasts (481), synthesize collagen in vitro (113, 481). Mineralization has been reported to occur in long-term cultures of such cells (55). A more homogeneous population of cells obtained from timed collagenase digestion of fetal mouse calvaria (termed "PT" or "OB" cells) was enriched in alkaline phosphatase, prolyl hydroxylase, and citrate decarboxylase as well as collagen-synthesizing activity

(375, 628, 670). The development of osteoblasts from precursor cells has been demonstrated by radioautography (327, 466, 677, 678). The same technique has shown that the cells eventually become surrounded by the matrix they synthesize (327, 466, 678). The cells are then termed osteocytes. For some period of time the osteocytes are connected to surface osteoblasts by fine connections whose structure has been described as similar to the "gap" junctions found in cardiac and smooth muscle (289) but whose purpose in bone has yet to be defined.

Osteoclasts are generally present on the endosteal surface in the region of bone spicules and trabeculae. In flat bones they appear to originate from the endosteal membrane. They are characteristically large cells, often multinucleate (233, 621) and, when active, exhibit a ruffled border in the region in apposition to mineralized bone (233). Osteoclasts, and most predominately the ruffled border, stain for acid phosphatase and other lysosomal enzymes (153). Although cultures of mature osteoclasts have been described (233), in general these have not been obtained in sufficient numbers for extensive study. A population enriched in cells that are osteoclast-like in enzyme composition but not in appearance has been isolated by timed collagenase digestion of fetal mouse calvaria (375). These cells, termed "CT" or "OC" cells, cause release of calcium when added to dead bone in culture.

The origin of the osteoclast has been the source of much investigation. A number of investigators have provided evidence that osteoclasts derive from fusion of precursor cells on the bone surface (260, 327, 547, 621, 677). Osteoclasts may arise from circulating blood cells. The evidence for this includes results of parabiotic and infusion studies in which cells from normal littermates restored resorption in osteopetrotic rats (646). Additional evidence derives from the occurrence of host-donor chimeras in the osteoclast population when rat bones were incubated on the amniotic membranes of chick embryos (317). Recently, it has been shown that circulating macrophages are capable of resorbing bone in vitro (318, 421). This intriguing finding will undoubtedly generate further research to determine the role of these cells in both pathologic and normal resorption of bone. Several years ago, it was suggested that there was an interconversion of osteoclasts to osteoblasts with the osteoclast undergoing formation of intracellular membranes, fragmenting, and becoming a bone-forming cell (513). Although this was attractively compatible with the sequence of events in bone remodeling, previous autoradiographic studies (466, 677) were inconsistent with such a mechanism, and no evidence has been found to support the concept.

E. Methodology for Evaluating Responses of Bone

Bone metabolism has been examined at multiple levels, from the apatite crystal and the collagen fiber to isolated bone cells, organ cultures, and in vivo systems. The scope and size of this review cannot permit a detailed description of all of these methods. Many of the systems for isolating and studying bone cells or for culturing long bones or calvaria have been alluded to above (113, 157, 234, 295, 375, 480, 503, 520, 576). These systems have permitted study of the actions of agents on bone isolated from the homeostatic control mechanisms provided by the parathyroid and thyroid glands and vitamin D metabolism and from other tissues that influence calcium and phosphorus metabolism, especially the intestine and kidneys. A number of experimental approaches that isolate effects occurring at the level of bone from those at other organs have been used to study bone function in vivo. These include perfusion techniques in which substances of interest are delivered to the bone via infusion into the nutrient artery and the bone response is evaluated from changes in the blood leaving through the interosseous vein (431, 640). Parathyroidectomy, thyroidectomy, and thyroparathyroidectomy as well as immunoassays and biologic assays for the hormones produced by these tissues have helped to dissociate or implicate PTH and calcitonin in observed bone changes. Nephrectomy has been an important tool in vitamin D research to establish both the site of the 1-hydroxylation as well as the importance of the 1-hydroxylation step for the biologic effects of vitamin D.

Among the parameters that are measured to assess bone responses in vivo, calcium and phosphate concentration in blood, urine, and feces are the most basic. Dietary restriction of calcium has been used to distinguish between dietary and skeletal origins of calcium. The use of labeled compounds is another indirect approach and one of the earliest applications of tracer kinetics was for studies of bone metabolism (215). The relative fates of orally and intravenously administered tracers of calcium have been used to distinguish absorption, resorption, and accretion (40, 255, 436, 598). These tracer approaches as well as the compartmental models that have been derived from them have been examined critically and some of their limitations have been fairly well established (256, 518). These include problems in interpretation under conditions where there is rapid bone turnover, local heterogeneity of bone turnover rates, nonosseous calcific deposits, and a dissociation between bone formation and mineralization, such as in osteomalacia and rickets. Other noninvasive techniques include measurement of substances derived from bone such as plasma alkaline phosphatase (493) and urinary hydroxyproline (330, 333). These approaches also have limitations. Hydroxyproline can derive from other collagenous tissues as well as bone. Bone alkaline phosphatase can be distinguished to some extent from that deriving from other tissues. One limitation with respect to this parameter is that the precise function of this enzyme and the meaning of changes in its levels are still not fully understood.

Several roentgenographic techniques have been applied to evaluate the state of the bones. Radiographs are useful in detecting qualitative changes in bone structure,

although a loss of 15% of bone mass is needed for changes to be detectable (17). Photon absorptiometry is a newer technique that assesses bone mineral mass and has been used for paired comparisons at the same site of the same individual after lapsed time or treatment (90). Neutron activation analyses has been applied to estimate total body calcium (293). Diagnosis of bone pathology often is derived from bone biopsy. If the tissue is analyzed by microradiography, resorbing and forming surfaces are characterized by the contours of the edges and the density of the image and quantified (88, 314). Tetracycline, which binds to divalent cations, is incorporated into bone as it mineralizes. The administration of two doses of tetracycline several weeks apart followed by assessment of the pattern of fluorescence has been used to evaluate growth and mineralization of bone (187). More recently, shorter interval tetracycline labeling has been proposed for measurement of accretion (599a). Another approach is the quantitative histologic evaluation of stained sections (88). Resorbing and forming surfaces and the percentage of mineralized and nonmineralized bone (osteoid) are assessed with this technique. Osteoclast counts are sometimes obtained and can be of diagnostic significance. It would seem that the variety of approaches would make assessment of in vivo changes in bone status relatively clear-cut and provide a good basis for evaluation of the effects of therapy. Unfortunately this is often not the case, due to the slow and subtle pattern of changes and the complex interrelationships between the processes.

III. The D Vitamins

A. Structural Characteristics

The D vitamins are a class of compounds all of which are secosteroids, i.e. steroids with an open ring (54). In the case of the vitamin D compounds the scission occurs between carbons 8 and 9 and is initiated by UV light of 290 to 320 nm. The photochemical reaction, first described by Windaus (668), occurs in the skin upon exposure to ultraviolet light (173, 275, 452). Epidermal 7-dehydrocholesterol photolyzes to previtamin D_3, which thermally equilibrates in the skin to vitamin D_3 (275). Thus far no endocrine or ionic regulation has been detected, although there is some evidence for feedback regulation (282), and skin pigment may affect the synthesis (282, 359a, 373). The various D vitamins, designated D_2 to D_6, differ from each other by substituents on the side chains (54, 426). Vitamins D_2 and D_3 are the substances of major biologic and therapeutic importance (see fig. 1). (In the course of this review, if no subscript is given, the reader can assume that the statement applies to both vitamin D_2 and vitamin D_3.) Vitamin D_2, ergocalciferol, seems to be produced mainly by fungi whereas vitamin D_3, cholecalciferol, was considered until recently to be solely an animal product. However, the identification of the dihydroxy metabolite 1,25-$(OH)_2D_3$ in the toxic shrub *Solanum malacoxylon* (252, 428) indicates that substances in the vitamin D_3 class of compounds, if

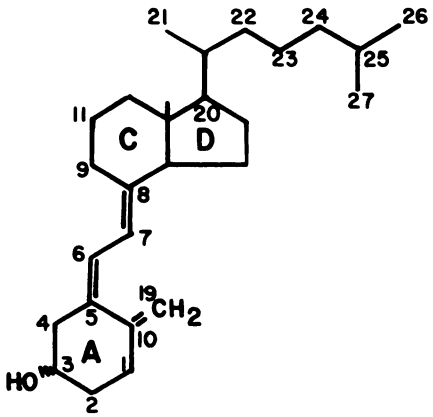


FIG. 1. Basic D vitamin structure. D_3 side chain is shown. D_2 side chain has 24-CH_3 group and 22-23 double bond.

not vitamin D_3 itself, occur in plants as well as animals. Vitamin D_2 and its metabolites are present in humans due to artificial fortification of the diet with vitamin D_2 in some countries. Where exposure to sunlight is limited and the diet is fortified, D_2 compounds may constitute the greater percentage of the vitamin D stores (220).

The conjugated double bond system, with an extinction coefficient of 18,200 at 265 nm, has facilitated quantitative analyses of the amount of these compounds in biologic fluids. Vitamin D_3 itself, as well as several of its metabolites, are present at high enough concentrations in plasma, for example, that they can be quantified by the UV absorption at 254 nm of extracts of serum purified by LH-20 and silicic acid high pressure liquid chromatography (169, 206, 337, 356, 543). Mass spectrophotometry has been the most valuable tool for proof of structure and, recently, nuclear magnetic resonance spectroscopy has gained in importance.

B. Biosynthesis and Metabolism

1. 25-Hydroxylation. A virtual revolution came about in the vitamin D field in the mid-1960s after the synthesis of high specific activity radiolabeled vitamin D. Before that time it was recognized that there was a considerable time lag before the effects of vitamin D became manifested. Also, neither vitamin D_2 nor vitamin D_3 applied in vitro in various vehicles and dosages had any effects on either of the recognized target tissues, intestine and bone. The time lag and the lack of direct effects suggested the possibility that vitamin D required bioactivation. Active metabolites were detected when 100 to 500 I.U. of randomly labeled ^{3}H-vitamin D (140) was injected into rats (441). The synthesis of higher specific activity vitamin D, 26,000 dpm/I.U. (432), permitted studies with physiologic doses of the vitamin. (1,2-^{3}H) vitamin D_3, 26,000 dpm/I.U., was injected into pigs and the metabolites were isolated (381). Of these metabolites, one designated peak IV proved to be faster-acting in promoting intestinal calcium absorption (418) and a more potent antirachitic substance (60). Furthermore, it stimulated bone resorption in vitro, which the native vitamin had

consistently failed to do (508, 628). The metabolite was identified as 25-hydroxycholecalciferol (25-OH-D_3) (60). The predominant, possibly the exclusive, site of synthesis of this metabolite in the rat was the liver (454). Further work indicated that the reaction occurred mainly in the microsomal fraction, required molecular oxygen and NADPH, and was inhibited by carbon monoxide and metyrapone, suggesting that it may be a P-450-mediated system (51, 383a). Recently a mitochondrial vitamin D–25-hydroxylase that also may be P-450-mediated has been reported in liver (485). Inhibition of the 25-hydroxylation of vitamin D_3 by pretreatment with the substrate has been described (49), although high concentrations of precursor can override the inhibition (222). The vitamin D analog dihydrotachysterol is likewise 25-hydroxylated in the liver. This hydroxylation is not reduced by pretreatment with either vitamin D_3 or dihydrotachysterol (50) and a different enzyme could be involved. The 25-hydroxylase is inhibited by several synthetic derivatives, including 24-nor-25-OH-D_3, 19-OH-dihydrovitamin D_3-II, 25-F-D_3, 24-dehydrocholecalciferol, and 25-dehydrocholecalciferol and 25-aza-vitamin D_3 (310, 438, 461, 462). 25-Hydroxylase antagonists that lack intrinsic biological activity could act as vitamin D antagonists in vivo. Such is the case with 24-nor-25-OH-D_3 in the chick (310).

2. 1-Hydroxylation. Further investigation revealed that 25-OH-D_3 was neither the most rapid-acting, nor the most potent metabolite of vitamin D_3. With the availability of tritiated 25-OH-D_3, a further metabolite was discovered (134, 250, 363) and shown to be the dihydroxylated product, 1,25-$(OH)_2$vitamin D_3 (1,25-$(OH)_2D_3$) (283, 360). In intact animals, this substance produced its effects on intestinal calcium transport and serum calcium much more rapidly than either 25-OH-D_3 or vitamin D_3 (246, 418, 457). In intact rats 10-fold as much vitamin D_3 and 3- to 5-fold as much 25-OH-D_3 were required to produce an effect equivalent to that obtained with 1,25-$(OH)_2D_3$ (610). The site of 1-hydroxylation was determined to be the kidney (182, 211, 442). Nephrectomized animals were unresponsive to concentrations of 25-OH-D_3 100-fold greater than those effective in intact animals, suggesting that the renal activation step was an obligatory one for effectiveness of the lower doses of 25-OH-D_3 and vitamin D_3 (71, 278, 673).

Subsequent investigations on the source of the oxygen, the action spectrum, and effects of inhibitors established that the renal 1-hydroxylation is effected by a mitochondrial cytochrome P-450-dependent mixed function oxidase for which NADPH is the electron donor (204, 205, 212, 263). The system bore marked similarities to the adrenal mitochondrial hydroxylases, and beef adrenal ferridoxin, adrenodoxin reductase, and flavoprotein could be effectively substituted for the corresponding renal constituents in a purified reconstituted system (205, 484). The enzyme system is located predominately in the proximal tubules (86, 411).

It is in the regulation of the 25-hydroxyvitamin D-1-hydroxylase that some of the possible physiologic and pathologic interrelationships are most apparent. 1,25-$(OH)_2D_3$ is a potent inhibitor of the 1-hydroxylase (212, 261, 357, 455, 458, 566, 626) and other metabolites, including 25-OH-D_3, 24R,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$, and 1,24,25-$(OH)_3D_3$ were also effective (261, 626). In contrast to the case with the 25-hydroxylase, this inhibition is not overcome by excess substrate and thus provides a very tight short-loop negative feedback control.

IONS, PARATHYROID HORMONE. Both decreased serum calcium (53, 301) and decreased serum phosphate (301) increase serum 1,25-$(OH)_2D_3$, providing a potential negative feedback system for regulating the serum levels of these ions. The feedback regulation has a time course measured in hours and thus would not provide a rapid mechanism for homeostasis but a slower continous one. The mechanism by which serum phosphate regulates serum 1,25-$(OH)_2D_3$ is not resolved, although there is evidence for increased renal 1-hydroxylation (37, 597, 604). The regulation of 1-hydroxylation by serum calcium (70, 178) appears to be indirectly mediated through PTH since the stimulatory effect of low calcium is lost in parathyroidectomized animals (183, 193). Direct effects of PTH on the 1-hydroxylase have been demonstrated in renal slices and isolated renal and cultured kidney cells (52, 262, 517, 626). The failure of some earlier attempts to show PTH effects (261, 552) may be due to growth factor requirements (262).

SEX HORMONES, PITUITARY HORMONES. Several other hormones enhance 1-hydroxylation in vivo. Estradiol (22–24, 601) and prolactin (568) stimulate the production of 1,25-$(OH)_2D_3$ in mammalian (23), avian (22, 24, 601), and amphibian (24) species. The estrogen antagonist, tamoxifen, decreased hydroxylation of 25-OH-vitamin D in the Japanese quail (21). Bromocriptine, a dopamine agonist that decreases prolactin secretion, lowered plasma 1,25-$(OH)_2D_3$ in rats (382). Whereas regulation by calcium and phosphate may be important for normal homeostatic control of these ions, regulation by the sex hormones could provide a mechanism for enhancing the absorption of dietary calcium at times in the life cycle when the calcium demand may be augmented. Evidence supporting this concept derives from the elevations in 1,25-$(OH)_2D$ noted during pregnancy (232, 347, 491, 633, 662) and lactation (62, 232, 347, 379, 491). The increase in 1,25-$(OH)_2D$ during lactation in rats was largely, but not entirely, abolished by parathyroidectomy (491). The probable importance of 1,25-$(OH)_2D$ during reproduction is reflected in the presence of extrarenal 1-hydroxylase in pregnant rats (214, 595, 612, 662). The dramatic increases in 1-hydroxylase during egg-laying in the bird (328) may be sex-hormonally regulated; however they could also represent a more direct response to the calcium demands of eggshell and medullary bone formation (28). Hypophysectomy decreases renal 1-hydroxylation in the rat (181) and the levels of 1,25-$(OH)_2D$ are restored to normal by growth hormone (GH) (567). Thus far, the findings in humans with regard to serum concentrations of 1,25-$(OH)_2D$ in acromegaly have been inconsistent

(78, 79, 350, 380). Moreover, GH replacement therapy in children with GH deficiency did not significantly change serum levels of 1,25-$(OH)_2D$ (202). At this time, the hormonal basis for the higher 1,25-$(OH)_2D$ concentrations during growth in children (112, 249) is unknown.

In addition to these physiologic regulators, certain exogenous agents affect the 1-hydroxylase. *Strontium*, which causes rickets in animals after prolonged exposure, blocks renal production of 1,25-$(OH)_2D_3$ (456). *Diphosphonates* such as ethane-1-hydroxy-1,1-diphosphonate (EHDP) inhibit the 1-α-hydroxylase and cause osteomalacia (38). However, in the case of the diphosphonates, there is a marked disparity between the doses that block the enzyme and those that affect bone. The actions of these agents to prevent mineralization may be due to direct effects on bone mineral metabolism, rather than to their action on the 1-hydroxylase (59). Diphosphonates, in addition to inhibiting mineralization, can prevent resorption, an effect that can be demonstrated in organ culture (523). Low doses of EHDP in vivo enhance calcium absorption (554), increase phosphate absorption (534), and stimulate the 1-hydroxylase (191).

DIABETOGENIC AGENTS. Alloxan and streptozotocin decrease the circulating concentrations of 1,25$(OH)_2D_3$ (546) and animals made diabetic by these compounds show diminished gastrointestinal absorption of calcium (545). Just how these agents interact with the enzyme has not been established. It is interesting, however, that glucose further decreases renal phosphate reabsorption in hypophosphatemic rickets (30), a disease apparently not due to decreased 1-hydroxylation but in which 1,25-$(OH)_2$ vitamin D levels have been reported to be at the low-normal to below-normal range (449). Ketogenic diets exaggerate the osteomalacia associated with anticonvulsant therapy (226) (see below). A clinical study has found normal 1,25-$(OH)_2D_3$ plasma concentrations in diabetic patients (257).

ANTICONVULSANTS. One group of agents that appear to have complex interactions with vitamin D metabolism are the anticonvulsants. This should come as no surprise to pharmacologists who are accustomed to dealing with the effects of these agents on drug metabolism. Phenobarbital and phenytoin can cause osteomalacia after long-term administration to epileptic patients (114, 145, 224, 227, 526, 565). The reported incidence of the disorder varies widely and estimates of as high as 30% have been cited (526). Risk factors that may influence the incidence and severity of the disease include vitamin D intake, sunlight exposure, physical activity, and coexisting diseases that modify availability of vitamin D metabolites to target tissue or their sensitivity to the metabolites (224). The mechanism of the anticonvulsant osteomalacia is not established. Some studies have found increases in 25-OH-D_3 in patients or animals treated with anticonvulsants (197, 398, 437), whereas others report depressed 25-OH-D_3 (65, 228). Increased 1,25-$(OH)_2D_3$ and increased 1α-hydroxylase have also been reported (316, 365). The hypothesis has been advanced that there may be depletion of vitamin D (560), perhaps due to enhanced catabolism of vitamin D to inactive products (225, 229). There are other complicating factors: anticonvulsants may decrease the absorption of vitamin D (398), although this has not been found in the rat (542). Also, there are direct end-organ effects of the anticonvulsants that could inhibit the actions of D metabolites on intestine (126) and bone (148, 230, 309) and contribute to the etiology of the disorder. Although phenytoin appears to inhibit collagen synthesis in fetal rat bone (148), it also can stimulate collagen synthesis, as observed in studies with isolated fibroblasts (551) and as inferred from the gingival hyperplasia that occasionally results from treatment with this agent (241). Phenytoin has been reported to accelerate fracture healing in mice (216). The role of altered collagen synthesis or breakdown in the accumulation of unmineralized osteoid after anticonvulsant treatment has not been fully explored. Some of the difficulty in resolving the mechanism of anticonvulsant osteomalacia derives from the lack of a good animal model. For example, intestines from vitamin D-deficient rats or D-repleted rats show enhanced calcium absorption when treated with phenytoin in vitro (147). Recently, two animal models that develop rickets with anticonvulsant treatment have been described (196, 642). Perhaps these models will provide information applicable to the human.

GLUCOCORTICOIDS. At the present time, the effects of cortisol seem at least as complex as those of the anticonvulsants. There are conflicting data as to whether glucocorticoids affect 1-hydroxylation and, if so, in what direction (108, 177, 334, 376). It has also been reported that cortisol accelerates the disappearance of radioactive 1,25-$(OH)_2D_3$ from the circulation (98). Cortisol probably affects the calcium metabolism by mechanisms independent of vitamin D as well, including inhibition of intestinal calcium uptake (177), inhibition of bone resorption (511, 575), and effects on bone growth and collagen synthesis (93, 481).

3. 24-Hydroxylation. In addition to 25- and 1-hydroxylation, 24-hydroxylation is another important pathway of vitamin D metabolism. This enzyme system appears to occur mainly in the kidney (213, 284, 617), although other tissue sites, including cartilage and embryonic chick calvarial cells, also possess some 24-hydroxylase activity (192, 213, 632). 24-Hydroxylation of both 25-OH-D and 1,25-$(OH)_2D$ occurs in animals (284, 600). 24,25-$(OH)_2$-Vitamin D_3 production is stimulated by 1,25-$(OH)_2D$ (606, 613) and inhibited by PTH (175, 193, 315). It is localized in the mitochondrial fraction and appears to be mediated by a mixed-function oxidase (335, 383). 24-Hydroxylation could serve as a means of terminating vitamin D action by two mechanisms. The first is that 24,25-$(OH)_2D_3$ is a much less potent compound than 1,25$(OH)_2D_3$ (see section III D 1 d). In vitro, its potency is approximately equal to that of 25-OH-D_3 (581). Secondly, 24-hydroxylation appears to accelerate the elimination of

vitamin D compounds in some species. This is seen dramatically in the chick where the 24-hydroxylated compounds have very little activity in vivo (273). It has been postulated that 24-hydroxylation may confer unique properties with regard to mineralization. This is discussed in more detail in later sections (III D 1 d).

4. Side-chain cleavage. Another pathway of termination of 1,25-$(OH)_2$-D action appears to be cleavage of the side chain (235, 348, 349). The occurrence of 1α-3β-dihydroxy-24-nor-5,7,10(19)-cholatrien-23-oic acid and 25-hydroxyvitamin D_3 26,23-lactone, vitamin D metabolites with modified side chains, has recently been reported (174, 667).

5. Conjugates. Polar metabolites of vitamin D, 25-OH-D, and 1,25-$(OH)_2D_3$ occur in the bile (10, 19, 46, 351). Some of these products appear to be glucuronide conjugates.

The major pathways of vitamin D metabolism are summarized in figure 2. The complete picture is obviously far more complex and will become even more so as the use of higher specific activity precursors and more sophisticated analytical methods reveal new metabolites that were not resolved by the older techniques (525).

C. Pharmacokinetics

1. Absorption. Vitamin D and its metabolites are absorbed from the small intestine. The speed of absorption seems to be considerably faster for 25-OH vitamin D than for cholecalciferol in the rat and the sites for maximal absorption of the metabolites differ, with the hydroxylated metabolites being absorbed from more proximal sites (288, 429). Absorption of vitamin D metabolites seems to require bile salts and is impaired in some cases of biliary cirrhosis (120, 340, 409, 563, 643). Also, there is diminished vitamin D absorption in patients who have had various portions of the intestine removed (3, 203, 487, 623, 675) and in patients with celiac disease (264). Osteomalacia has been reported in such patients. Vitamin D absorption decreases with aging (36). There is evidence that vitamin D and its metabolites undergo enterohepatic circulation (10, 351).

2. Protein binding. In the circulation of humans and rats the vitamin D compounds are bound with highest affinity by a protein, previously termed D-binding protein (306), calcifidiol-binding protein (223), or transcalciferin (67) and now generally called vitamin D-binding protein (DBP). Rat DBP has the mobility of an α-globulin (68, 528), a molecular weight of 52,000, an IEP of 4.8 (68), and a 4 S sedimentation coefficient (68, 163, 218). Human DBP has similar properties (305). It has been identified as a "group-specific component" protein (67, 136, 223, 306). The affinity of DBP for 25-OH-D_3 is greater than for vitamin D_3 or 1,25-$(OH)_2D_3$ (68, 359, 531). The K_d for 25-OH-D_3 is in the range of 5×10^{-8} to 5×10^{-9} M. There is a maximum binding capacity of one molecule of 25-OH-D per molecule of DBP (306). DBP levels appear to vary with hormonal and developmental states (34, 66, 69). At the reported serum concentrations of DBP (10 μM) (68, 306) and 25-OH-D (0.1 μM) (47, 206, 220) only a few percent of the binding sites would be filled with vitamin D metabolites (222). Also, most of the circulating vitamin D would be present in the bound form. A 6 S binding protein having immunologic cross-reactivity with DBP (341) and similar D metabolite affinities has been found in many tissues (162, 217, 254, 407). At the present time it is unresolved whether this 6 S binding protein derives from serum contamination of the tissue preparation (341, 639) or is a physiologically relevant factor in the transfer of the metabolite from plasma into the cells (58, 121).

3. Serum concentrations. Under physiologic conditions the human serum concentrations of vitamin D have been reported to be 1 to 5 ng/ml (356, 554); 25-OH-D, 25 to 40 ng/ml (47, 219, 222, 554); 24,25-$(OH)_2D$, 1 to 4 ng/ml (272, 291, 463, 554, 616); and 1,25-$(OH)_2D$, 20 to 40 pg/ml (85, 167, 300, 554, 584). Vitamin D levels have been quantified by UV absorption after organic extraction, LH-20, celite, and silica (high pressure) chromatography (356), by bioassay (651), and by GC mass fragmentography (139, 680). 25-OH-D has been measured by binding assays that utilize either the 4 S serum binding protein (47) or binding proteins from kidney (11, 219) or intestinal

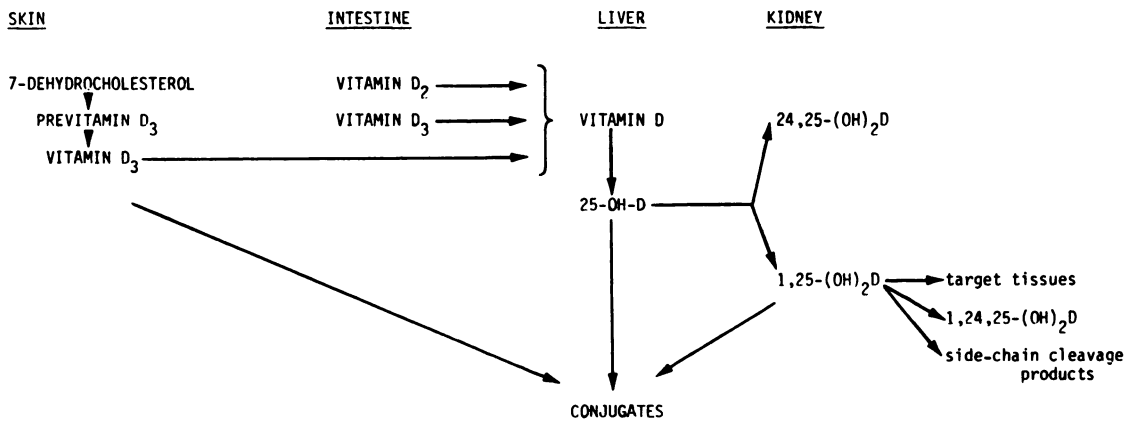


FIG. 2. Current concept of major pathways of vitamin D metabolism.

(300) cytosol. Most recently, methods for quantification based upon UV absorption of purified serum fractions have been described (169, 356, 543). Prepurification by chromatography (152) gives normal 25-OH-D values somewhat lower than those cited above (22.5 ± 2.8 ng/ml). 1,25-$(OH)_2$D has been quantified in serum by a radioreceptor assay involving the use of the 3.7 S receptor protein from intestine (84, 85, 167, 168), by radioimmunoassay (117, 544), and by bone resorption bioassay (584, 588), all of which require equivalent extensive prepurification of serum to eliminate potentially interfering substances. A metabolite of dihydrotachysterol gives artifactually elevated values, at least in the radioreceptor assay (210). Values obtained by the three methods are remarkably similar. 24,25-$(OH)_2$D has been assayed by competitive protein binding assay. The methods utilize the binding protein from rat kidney cytosol (345, 616) or from vitamin D-deficient rat serum (463). A metabolite of vitamin D_2 has been reported to cochromatograph with 24,25-$(OH)_2D_3$ and give artifactually high values (615). Also, 25-hydroxyvitamin D_3 26,23-lactone has been reported to comigrate with 24,25-$(OH)_2D_3$ (291).

4. Turnover. On the basis of tracer studies in animals, the turnover of 25-OH-D is slower than for vitamin D (221, 231) or 1,25-$(OH)_2$D vitamin D (672). Radioimmunoassay and tracer measurements of 1,25-$(OH)_2$D in human serum indicate a half-life of 5 to 8 hr (43, 211a, 396). Half-lives for 25-OH-D_3 (213) and 24,25-$(OH)_2D_3$ (396) are much longer, in the range of 2 to 3 weeks for 25-OH-D_3.

5. Receptor binding. At end-organ sites (intestine, bone) the metabolites bind specifically, with high affinity, and in a saturable manner to a 3.7 S protein complex that may represent the receptor (80, 81, 106, 344, 556, 630). The protein has resisted complete purification due to its lability (186, 401) but binding affinity of the vitamin D compound to the 3.7 S material correlates well with most other in vitro effects. The metabolites appear in the nuclei of target tissues (82, 83, 105, 362, 631, 657, 683), as well as the nuclei of cells not yet recognized as physiological targets for vitamin D action (8, 80, 248, 492, 592). A good correlation has been found between the time course of the effects of 1,25-$(OH)_2D_3$ and the presence of the compound in the intestine and bone after dosage with radioactive 1,25-$(OH)_2D_3$ (185, 631, 672).

D. Synthetic Analogs

A large number of vitamin D congeners have now been synthesized and tested for biologic activity both in vivo and in vitro. The in vivo studies have been carried out mainly in chicks and rats although several D vitamin compounds including vitamin D_2 and D_3, dihydrotachysterol (Hytakerol), 25-OH vitamin D_3 (calcifidiol, Calderol), 1α-OH vitamin D_3, 5,6-transvitamin D_3 (Delakmin), and 1,25-dihydroxyvitamin D_3 (calcitriol, Rocaltrol) have been employed or given preliminary tests as potential therapeutic agents. Animal studies have generally used the measurement of intestinal calcium transport in organs isolated from treated animals as the parameter of the intestinal effects of the compounds and elevation of serum calcium in animals on a low calcium diet as the parameter for measurement of mineral mobilization from bone. Phosphate transport or mobilization is less frequently determined. In some studies bone ash or the classic line test (133) is used to measure mineralization. For in vitro studies in the intestine, binding to the 3.7 S complex is the usual end point, although synthesis of intestinal calcium binding protein has also been used (127, 471). Studies with bone have most commonly been carried out in organ cultures where release of mineral or collagen products, collagen synthesis, and morphologic changes have been evaluated. Recently, affinity of analogs for bone cell receptors (106) and their relative effects on bone cell calcium transport (156) have been assessed. Compounds that have been evaluated in one or more of these systems include recognized naturally occurring metabolites of vitamin D_2 and D_3, i.e. the 25-hydroxy derivatives, the trihydroxylated 1,25-$(OH)_2$D, 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ compounds, and 1,24,25-$(OH)_3D_3$. Synthetic alterations that have been tested include deletion, substitution or stereoisomerization of one or more of the hydroxyl groups, rotation of the A ring, and shortening or lengthening of the side chain.

A large body of information has been obtained from these studies. Table 1 is a listing of the references to studies carried out on a variety of analogs in the most commonly used systems as described above. Table 2 presents the relative activities of a number of the analogs on intestinal binding and bone resorption, the most common in vitro systems. Potencies are given relative to 25-OH-D_3, which was assigned a potency of 1. It is difficult to reduce the in vivo data to table form, since time points and doses (as well as species and parameters) are not always comparable. In the text, we will summarize the effects of alterations in the molecule, compare in vivo and in vitro results as well as results from intestine and bone, and draw attention to significant disparities.

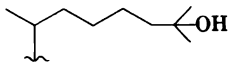
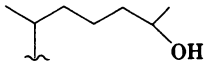
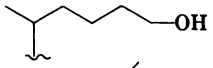
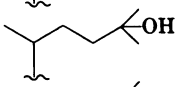
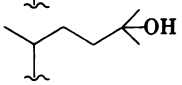
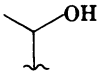
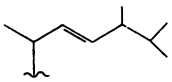
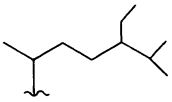
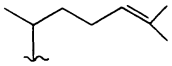
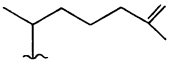
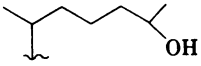
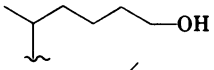
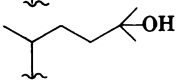
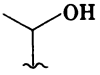
1. Modifications in hydroxyl groups. A. 1-HYDROXYL. Both in vivo and in vitro experiments indicate that the 1α-OH group contributes substantially to the potency of the vitamin D compounds. The difference in vitro between 25-OH-D_3 and 1,25-$(OH)_2D_3$ is on the order of 1000- to 5000-fold (13, 82, 342, 387, 498, 509, 521, 591). In vivo, 1,25-$(OH)_2D_3$ has been reported to be only 3 to 10 times more active than 25-OH-D_3 in various systems and species (249, 439, 610). This apparent disparity undoubtedly derives from the fact that in the intact animal 25-OH-D_3 undergoes 1-hydroxylation. Also, metabolic inactivation of 1,25-$(OH)_2D_3$ in vitro may contribute to the difference between in vivo and in vitro potency ratios.

TABLE 1: Part 1
Vitamin D analog studies*

Category	Compound	OH-Groups in Positions	In Vivo			In Vitro	
			Intestinal Ca Transport	Calcification	Bone Ca Mobilization	Bone Resorption	Intestinal Binding
Four OH groups	1,24R,25-$(OH)_3D_3$	1α 3β 24R 25	281, 649		281	585	166, 342
	1,24S,25-$(OH)_3D_3$	1α 3β 24S 25				585	166, 342
Three OH groups	1,25-$(OH)_2D_3$†	1α 3β 25	609, 610, 648	605, 609, 610	609, 610	387, 509, 522, 585, 591	82, 166, 300, 342, 444, 451, 498
	1,25-$(OH)_2D_2$	1α 3β 25		312		585	166, 300, 311a, 342
	1,25-$(OH)_2D_3$(3α)	1α 3α 25				585	
	1,24R-$(OH)_2D_3$	1α 3β 24R	323	323	323		
	1,24S-$(OH)_2D_3$	1α 3β 24S	323	323	323		
	24R,25-$(OH)_2D_3$	3β 24R 25	72, 415, 619, 648, 649	607	72, 415, 607	387, 478, 522, 581	166, 342, 498
	24S,25-$(OH)_2D_3$	3β 24S 25	607	607	607	387, 581	166, 342, 498
	25R,26-$(OH)_2D_3$	3β 25R26	354, 415, 593	593	354, 415, 593, 618, 619	478, 585	166
	25S,26-$(OH)_2D_3$	3β 25S26			618		
Two OH groups	1α-OH-D_3†	1α 3β	253, 280, 285, 323, 459, 478, 681	280, 323, 459	253, 280, 285, 323, 459, 478	387, 522, 591	82, 166, 342, 444, 450, 498, 681
	1α-OH-D_2	1α 3β	353, 519	353, 519	354, 519	580	
	3-Deoxy-1,25-$(OH)_2D_3$	1α 25	450			387	450, 498
	24R-OH-D_3	3β 24R	611	611	611	581	166, 342
	24S-OH-D_3	3β 24S	611	611	611	581	166, 342
	25-OH-D_3†	3β 25	519, 609, 610	519, 609, 610	519, 609, 610	387, 478, 522, 585, 591	82, 166, 342, 444, 450, 498
	25-OH-D_2	3β 25					342, 311a
One OH group	3-Deoxy-1α-OH-D_3	1α	352, 459	459	352, 451, 459	387, 591	166, 342, 444, 450
	Vitamin D_3†	3β	280	280, 323, 610, 611	280	387, 478, 522, 585, 591	82, 166, 342, 444, 450, 498
Ring rotation	5,6-Trans-25-OH-D_3	"1α" 25	276, 277			387, 522, 591	166, 444, 498
	5,6-Trans D_3	"1α"	276, 277		276, 277	591	498
	Isotachysterol	"1α"	274		274		
	25-OH-Dihydro-tachysterol	"1α" 25	231, 550		550	629	498
	Dihydrotachysterol	"1α"	238, 325		238, 325, 529	522, 629	444
Substitution	25-F-D_3	3β (25F)	462		462		
	1α-OH-25-F-D_3	1α 3β (25F)	425	425	425	424	424, 425
	1α-OH-24R-OH-25-F-D_3	1α 3β 24R (25F)	474	474		474	
	24R-OH-25-F-D_3	3β 24R (25F)	427	427	427		
Substitution	24,24-F_2-25-OH-D_3	3β ($24F_2$) 25	608	608	608		
	24,24-F_2-1,25-$(OH)_2D_3$	1α 3β ($24F_2$) 25	603		603		

* Active analogs.
† Since vitamin D_3, 25-OH-D_3, 1α-OH-D_3 and 1,25-$(OH)_2D_3$ have been studied in comparison with many of the congeners, only a few of the studies are cited.

TABLE 1: Part 2
Vitamin D Analog Studies

	Compound	Side Chain	In Vivo			In Vitro	
			Intestinal Ca Transport	Calcification	Bone Ca Mobilization	Bone Resorption	Intestinal Binding
	24-Homo-25-OH-D_3		440		440		440
	27-Nor-25-OH-D_3		279		279	522, 585	
	26,27-Bisnor-25-OH-D_3		279		279	522, 585	
	24-Nor-1,25-$(OH)_2D_3$					578	
	24-Nor-25-OH-D_3		276, 310, 440		310, 440	522, 585	440
	20-OH-pregnacalciferol		279		279		
	1α-OH-pregnacalciferol		355	355	355	355	
	22-27-Hexanor-20-OH-D_3		279		279	522	
	Vitamin D_2		426	426	426		
	Vitamin D_5		426	426	426		
	24-Dehydrocalciferol		462		462		
	25-Dehydrocalciferol		462		462		
Side chain modification	5,6-Trans-27-nor-25-OH-D_3		279		279		
Side chain modification	5,6-Trans-26-27-bisnor-25-OH-D_3		279		279		
Side chain modification	5,6-Trans-24-nor-25-OH-D_3		276				
Side chain modification	5,6-Trans-20-OH-pregnacalciferol		279		279		
Side chain modification	5,6-Trans,22-27-hexanor-20-OH-D_3		279		279		

The importance of the 1α-hydroxyl group is illustrated by the fact that anephric animals, which lack the 1-hydroxylase, are unresponsive to analogs lacking a 1-hydroxyl group at 10- to 100-fold the doses that are effective in intact animals. This has now been documented with many compounds including 25-OH-D_3 (72, 277), 24,25-$(OH)_2D_3$ (72, 619), 25,26-$(OH)_2D_3$ (354, 415), 27-nor-25-$(OH)_2D_3$ (279), and 26,27-bisnor-25-$(OH)_2D_3$ (279). Compounds with either a 1α-hydroxy group or a pseudo-1α-hydroxyl group (due to rotation of the A ring, such as 5,6-trans-D_3 derivatives or 25-hydrotachysterol) are active in nephrectomized animals at the same doses that give responses in intact animals (231, 276, 277). Under some conditions 1,25-$(OH)_2D_3$ is not as active as 25-OH-D_3 or even vitamin D_3. Specifically, when administered chronically by the oral route, 1,25-$(OH)_2D_3$ was less effective than 25-OH-D in maintaining serum calcium and promoting mineralization (609). Also, when given i.v. in a single dose, 1,25-$(OH)_2D_3$ did not have as prolonged an effect on serum phosphorus or as good an

TABLE 2

Relative potencies of vitamin D analogs on bone resorption and intestinal receptor binding*

Compound	Bone Resorption	Intestinal Binding
,25-$(OH)_2D_3$	1000, 5000	1000
,25-$(OH)_2D_2$	800	1000
,-Deoxy-1,25-$(OH)_2D_3$	100	100
,24R,25-$(OH)_3D_3$	100	100
,24S,25-$(OH)_3D_3$	50	10
4-Nor-1,25-$(OH)_2D_3$	10	
α-OH-D_3	10, 1	5, 1.5
α-OH-D_2	1	
5-OH-D_3	1	1
5-OH-D_2		1
4R-OH-D_3	1	1, 2
5-OH-dihydrotachysterol	1	10
α-OH-25-F-D_3	1	3
,25-$(OH)_2D_3$(3α)	1	
,6-Trans-25-OH-D_3	1. 0.01	1, 5
4R,25-$(OH)_2D_3$	0.5	0.3
5,26-$(OH)_2D_3$	0.1, 0.3	0.3
4S,25-$(OH)_2D_3$	0.08	0.05
α-OH-pregnacalciferol	0.05	0.08
4-nor-25-OH-D_3	0.01	
4S-OH-D_3	0.01	
6,27-Bisnor-25-OH-D_3	<0.05	
-Deoxy-1α-OH-D_3	<0.001	0.2
,6-Trans-D_3	<0.001	0.001
)ihydrotachysterol	<0.001	≤0.001
'itamin D_3	<0.001	≤0.001
'itamin D_2	<0.001	

* Potency estimates have been derived from the following sources: leferences 13, 81, 166, 300, 342, 387, 450, 478, 498, 509, 521, 580, 581, 85, 628. All are based upon direct or indirect comparison with 25-OH-)3, which was arbitrarily assigned a potency of 1. Except in cases of xtreme disparity, the value represents an average.

ffect on calcification as 25-OH-D_3 (605). The results uggest that the compound may be subject to fairly rapid nactivation or elimination, which could be an important onsideration in its therapeutic use.

B. 3β-HYDROXYL. This substituent, although impor-ant, appears to confer less activity than the 1α-OH roup. A comparison between the effects of 1α-OH-D_3 nd 3-deoxy-1α-OH-D_3 in several in vivo systems showed hat the presence of the 3β-OH group enhanced the ctivity 20- to 50-fold (459). This was consistent with ome in vitro data (166), although another study found nly a 5-fold difference on intestinal binding (498). 3-Deoxy-1,25-$(OH)_2D_3$ has been reported to have ⅛ the ctivity of 1,25-$(OH)_2D_3$ on intestinal binding in vitro 498) and 1/50 the activity on bone resorption in culture 387). Although extensive in vivo dose-response studies omparing these two compounds are lacking, data from time-course study (451) suggests that 1,25-$(OH)_2D_3$ is omewhat (perhaps as much as 3-fold) more potent than he 3-deoxy analog. Comparing the effects of the 1α-OH roup and the 3β-OH group, 3-deoxy-1α-OH-D_3 showed reater affinity for intestinal receptors than vitamin D_3 342, 450, 451, 498) and 3-deoxy-1,25-$(OH)_2D_3$ showed approximately 100 times the potency of 25-OH-D_3 on bone in vitro (387). Interestingly, when compounds with a pseudo-1α-OH group, such as 5,6-trans 25-OH-D_3 or 25-OH-dihydrotachysterol, were compared with 25-OH-D_3 in vitro, the pseudo-1α-OH group did not show a consistent effect on the potency of the compound (166, 387, 498, 591, 629), suggesting a possible deleterious influence of the methyl or the methylene group in the association with the receptor. The stereoisomer 3α-1,25-$(OH)_2D_3$ was several orders of magnitude less active than 1,25-$(OH)_2D_3$ (585), indicating that the orientation of the 3-OH groups is crucial and that the 3α-conformation of the group probably interferes with binding.

C. 25-HYDROXYL. Although it is clear that 25-OH-D_3 is more rapidly-acting than vitamin D_3 (381), potency differences between compounds differing only in the presence or absence of a 25-hydroxyl group have not always been apparent in vivo. This is probably due to the fact that compounds lacking the 25-OH group are generally rapidly 25-hydroxylated (286, 287, 681). In vitro, however, marked differences are seen. Vitamin D_3, dihydrotachysterol, 5,6-trans D_3, and 3-deoxy-1α-OH-D_3 are converted from inactive or relatively weak agonists in bone and intestinal systems to much more potent agents by the addition of a 25-hydroxyl group. Likewise, the potency of 1α-OH-D_3 is increased 100- to 1000-fold by the addition of a 25-hydroxyl group (82, 166, 387, 498, 591). A method for assessing the relative contributions of the hydroxyl groups to the vitamin D_3 molecule has been described (460). The 25-OH group fails to enhance activity if the molecule also has a 24-OH group. Thus 24R,25-$(OH)_2D$ is less active on intestinal receptor binding and bone resorption than either 24R-OH-D_3 or 25-OH-D_3 (166, 342, 387, 498, 581) and 1,24R,25-$(OH)_3D_3$ is less active than 1,25-$(OH)_2D_3$ (166, 342, 585). Presumably, the presence of the two adjacent hydroxyl groups impairs interaction with the receptor.

D. 24-HYDROXYL. Although 24R,25-$(OH)_2D_3$ is less potent in vitro than 25-OH-D_3, 24R-OH-D_3 is essentially equipotent with 25-OH-D_3 on bone resorption (581) or binding by chick intestinal cytosol (166). The function of the 24-OH group has been a matter of speculation. In the chick, the presence of a 24-OH group or a methyl in the 24-position (and a 22–23 double bond, i.e. vitamin D_2) markedly decreases in vivo activity (273, 307). The difference seen in the chick has been attributed to more rapid metabolism and excretion, rather than to a lack of effectiveness at the end organ (166, 300, 307, 311). Recently, it has been proposed that 24,25-$(OH)_2D$ may be a more effective promotor of mineralization than other vitamin D metabolites (320, 462). At the present time there is no evidence for a direct effect of 24,25-$(OH)_2D$ (or any vitamin D metabolite) on mineralization. Two in vitro systems in which 24,25-$(OH)_2D_3$ was reported to be more potent or more efficacious than either 25-OH-D_3 or 1,25-$(OH)_2D_3$ are the stimulation of rat growth plate chondrocytes to incorporate sulfate into proteoglycans (131) and the inhibition of PTH-induced lysosomal enzymes in mouse calvaria (368). However, there is as yet

no evidence that these events are directly involved in vitamin D-induced mineralization. Due to the different pharmacokinetic properties and potencies of the vitamin D metabolites, one could conceivably obtain superior effects with one exogenously administered metabolite compared to another by manipulating the treatment regimen with respect to dosage and frequency of administration (see IV a 5). The steric configuration of the 24-OH group influences the activity both in vivo and in vitro. In most studies, 24R-OH-D_3, 24R,25-$(OH)_2D_3$, and 1,24R,25-$(OH)_3D_3$ have been shown to be more potent than their 24 S epimers (166, 323, 342, 387, 581, 585, 607, 611). In the case of 24,25-$(OH)_2D_3$, the natural epimer has been shown to be the R stereoisomer (607).

E. FLUORO SUBSTITUTIONS. 25-F-D_3, 1α-OH-25-F-D_3, 24-OH-25-F-D_3, 1α-OH-24OH-25-F-D_3, 24,24-difluoro-25-(OH) D_3, and 24,24-difluoro-1,25-$(OH)_2$ D_3 have been synthesized and tested for biologic activity (424, 425, 427, 474, 603, 608). 1α-OH-25-F-D_3 and 24-OH-25-F-D_3 are substantially more active in vivo than in vitro, and it appears that they are converted to the common metabolite, 1α-OH-24R-OH-25-F-D_3 (427). The 24,24-difluoro compounds are of particular interest in view of the hypothesis (section d, above) that 24,25-$(OH)_2$D could play a unique role in mineralization. The stable difluoro compound 24,24-difluoro-25-OH-vitamin D_3 proved as effective as 24,25-$(OH)_2D_3$ in prevention of rachitogenesis and in mineralization, which is inconsistent with a significant function for the 24-hydroxyl group (608).

2. Side-chain modifications. Alterations in the length of the carbon side chain by as little as one carbon more or less affects activity dramatically both in vivo and in vitro. Removal of one methyl group to give 27-nor-25-OH-D_3 or one methylene to give 24-nor-25-OH-D_3 decreased bone resorption in vitro by 1 to 2 orders of magnitude (522, 585) and markedly decreased activity in vivo (276, 279, 310). Further shortening of the side chain resulted in compounds virtually devoid of activity (279, 355, 522, 585). The presence of a 1-α-OH group can still enhance the activity of the derivatives with shortened side chain, as can be seen in a comparison of the effects of 24-nor-25-OH-D_3 and 24-nor-1,25$(OH)_2D_3$ on bone in vitro (576, 585). Perhaps the most well-studied modification of the side chain is the effect of addition of a 24-methyl and a 22–23 double bond, giving vitamin D_2. As mentioned previously, this modification greatly decreases in vivo activity in the chick, probably due to a pharmacokinetic rather than an end-organ difference. In the rat, the D_2 and D_3 derivatives of vitamin D, 25-OH-D, and 1,25-$(OH)_2$D are essentially equipotent (61, 312, 585, 594). 1α-OH-D_2 may be a less potent bone-resorbing agent than 1α-OH-D_3 in vivo (519), although the two compounds were equally active as bone-resorbing agents in vitro (580).

3. Other compounds. A. NATURALLY-OCCURRING VITAMIN D GLYCOSIDES. A number of toxic plants, including *Solanum glaucophyllum* (*Solanum malacoxylon*), *Cestrum diurnum*, and *Trisetum flavescens* produce symptoms of hypercalcemia, hyperphosphatemia, bone demineralization, and soft tissue calcification (89, 346, 653, 674). In experimental studies, extracts of *S. malacoxylon* stimulated intestinal calcium and phosphorus absorption and increased intestinal calcium binding protein (130, 361, 400, 448, 634, 650, 652). They were also shown to compete with 1,25-$(OH)_2D_3$ for binding sites on intestinal chromatin (497), to stimulate bone resorption in vitro (370, 371, 561, 587), to inhibit the renal 1-hydroxylase when administered in vivo (497), to overcome the strontium-induced block in intestinal calcium transport (652), and to stimulate calcium transport in nephrectomized, vitamin-D deficient animals (650). Administered in vivo, *S. malacoxylon* gave rise to increased blood levels of 1,25-$(OH)_2D_3$ (428, 488). However, in contrast to vitamin D, the active principle from *S. malacoxylon* was soluble in water, methanol, ethanol, and 2-propanol and was not extractable into ether, chloroform, or benzene (303). The paradox was resolved when it was shown that treatment of aqueous extracts with glycosidase yielded a substance that cochromatographed with 1,25-$(OH)_2D_3$ and had a mass spectrum consistent with the identification (428, 488).

B. VITAMIN D MIMETICS. *Filipin* is a polyene antibiotic and structurally quite dissimilar to the vitamin D derivatives. Some polyenes have been shown to complex with membrane sterols and render the membranes leaky to low molecular weight substances (329). Filipin enhances the mucosal uptake of calcium by intestinal tissue and most dramatically by tissue from vitamin D-deficient animals (671). The mechanism of the effect remains to be elucidated, although several theories have been proposed (1, 64, 671). The divalent cation ionophore *A23187* has also been reported to stimulate mucosal uptake of calcium by vitamin D-deficient intestine (359a).

4. Comparison between in vivo and in vitro findings on bone. In the intact animal, there is opportunity for compounds to be activated through hydroxylation, as well as to be inactivated and excreted. Although some of these effects may occur to a limited extent in vitro (192, 632), the major activation pathways of 25-hydroxylation and 1-hydroxylation appear to be absent or insignificant in bone under the conditions used for testing resorption (509, 628). It is thus not surprising that vitamin D itself is inactive on bone in vitro. Compounds that could be activated by 1,3-, or 25-hydroxylation in the intact animal would tend to be more potent in vivo than would be predicted from in vitro results. For example, as mentioned previously, the greater difference between the potencies of 1,25-$(OH)_2D_3$ and 25-OH-D in vitro than in vivo (1000- to 5000-fold in vitro vs. 10-fold in vivo) may reflect continuous conversion of 25-OH-D to 1,25-$(OH)_2$D in vivo as well as more rapid inactivation and elimination of the 1,25-$(OH)_2$D. In contrast, some agents appear to be more active in vitro than would be predicted from in vivo results. This type of disparity is seen in the

case of two compounds with shortened side chain. 1α-OH-pregnacalciferol, although of low in vitro activity, was inactive in vivo at concentrations (355) at which one would predict effectiveness based on the in vitro results. 24-nor-1α-25-$(OH)_2D_3$ may represent another example of this phenomenon (576). Perhaps in the case of these two compounds there may be more rapid inactivation or elimination.

5. Comparison between effects on intestine and bone. Comparing data from bone organ cultures with binding to the 3.7 S fraction of intestinal cytosol indicates good agreement in the in vitro effects on bone and intestine, except that the responses in bone appear to require at least two hydroxyl groups (Table 2, 579). Compounds with only one hydroxyl group are inactive on bone in vitro at concentrations up to 10^{-4} M. Although this difference in affinity would seem to offer promise for a compound with selective intestinal action, further modification of the molecule would probably be necessary for this difference to be expressed in vivo because once the second hydroxyl is added, the difference in affinity between the intestinal receptor and the bone resorbing system is lost. It is interesting that a number of analogs, when tested in vivo, were inactive, or much less active on bone than on intestine (72, 277, 283, 323, 354, 607, 611, 619). The conditions for determining bone mobilization are somewhat different from those used to evaluate calcium transport, since the former is assessed in animals on a low calcium diet. Although low calcium enhances the intestinal effect (415) it may also decrease the sensitivity of bone to resorbing agents (15, 201, 510). Thus, it is possible that the experimental conditions under which bone mobilization is studied are producing a less responsive state. Since the in vitro studies indicate similar potencies on both tissues, the explanation for the disparity may be in factors such as distribution and metabolism. None of the modifications introduced into the vitamin D molecule has yielded a compound more potent on bone than the naturally occurring substance, 1,25-$(OH)_2D_3$. There are as yet no analogs that block the effects of 1,25-$(OH)_2D_3$ on bone. As mentioned previously, 24-nor-25-OH-D_3, a 25-hydroxylase blocker, could inhibit the effects of vitamin D_3 in the chick (310). The elucidation of its site of action could explain the earlier observation that 24-nor-25-hydroxy D inhibited bone resorption if the analog was injected in vivo and the bones subsequently cultured but did not inhibit when added to the bones in vitro (522).

IV. The Dual Effects of the D Vitamins on Bone

Bone is a major target tissue of vitamin D. Two distinct and as yet mechanistically unrelated effects are produced. One action is an increase in mineralization, observed morphologically as a decrease in the amount of osteoid. The other effect is the resorption of bone, with loss of both mineral and matrix. Both effects are elicited when vitamin D-deficient animals are treated with physiologic doses of vitamin D. In the vitamin D-deficient situation the effects of the diminished mineralizing activity are much more dramatically manifested than the loss in bone resorbing activity, although vitamin D-deficient animals are less responsive to the bone-resorbing effects of PTH, a topic that will be discussed below (IV B 2 b). In the converse situation, that is the excessive intake or production of vitamin D, toxicity is clearly manifested as an excessive amount of resorption. Overmineralization of bone is not seen with excess vitamin D.

A. Stimulation of Mineralization

1. Background. The physical deformities of rickets were recognized in antiquity. Although the etiology of the lesions was not recognized, a remarkable passage from Herodotus indicates that as early as 500 to 600 B.C. laymen believed that exposure to sunlight resulted in hardening of the skull:

> On the field where this battle was fought I saw a very wonderful thing which the natives pointed out to me. The bones of the slain lie scattered upon the field in two lots, those of the Persians in one place by themselves, as the bodies lay at the first—those of the Egyptians in another place apart from them: if, then, you strike the Persian skulls, even with a pebble, they are so weak, that you break a hole in them; but the Egyptian skulls are so strong, that you may smite them with a stone and you will scarcely break them in. They gave me the following reason for this difference, which seemed to me likely enough: The Egyptians (they said) from early childhood have the head shaved, and so by the action of the sun the skull becomes thick and hard. The same cause prevents baldness in Egypt, where you see fewer bald men than in any other land.* Such, then, is the reason why the skulls of the Egyptians are so strong. The Persians, on the other hand, have feeble skulls, because they keep themselves shaded from the first, wearing turbans upon their heads.
>
> Herodotus, *The Persian Wars*, Book III

In the middle to late 19th century the morphologic changes and alterations in ash content of rachitic bone were described (658). Several investigators reported on the epidemiology of rickets, commenting on its greater frequency in northern climates and heavily industrialized areas (e.g. 468). The squalor, filth, and darkness of the cities seemed almost symbolic of disease and one type of treatment for disease in general was to remove the patient to "fresh air and sunshine." Although in many cases this was ineffectual in halting a disease process, it was so remarkably effective in the treatment of rickets that it even may have inspired the child's novel, *Heidi*, published in 1921. Interestingly, at about the same time, a highly technological treatment was also found to be effective in reversing rachitic symptoms. This was the use of a newly discovered phenomenon, ultraviolet light irradiation (302). A simultaneous series of investigations on the role of diet in disease revealed that diets deficient in certain nutrients led to rickets (403, 406, 555, 557, 558). The unification of all of this theory came about with the

* As far as I am aware, there are no more modern studies on the possible effects of sunlight or vitamin D on hair growth. However, there may be a genetic linkage, as several cases of rickets with alopecia have been documented (25, 367, 533).

reports in 1924 by Steenbock and Black (573) and by Hess and Weinstock (270) that consumption of food that had been irradiated with ultraviolet light could prevent or cure the symptoms of rickets. Within the next decade the chemical nature of the D vitamins, their production through ultraviolet irradiation (7, 668), and their relationship to simple dietary rickets were finally appreciated. The period between the 1920s and 1960s was the era during which the physiologic mechanisms behind the antirachitic actions were elucidated. Orr et al. proposed that rickets might be due to poor calcium absorption (465). A high stool calcium in rachitic animals was consistent with the possibility that mineral absorption might be at fault (96, 313, 402, 434). Studies with isolated gut sacs revealed that if such tissues were taken from normal animals, calcium and phosphorus were concentrated on the serosal side of the tissue (433). In contrast, sacs from vitamin D-deficient animals were unable to maintain such a gradient. The inability of the intestines from vitamin D-deficient animals to transport ions actively offered a mechanism to explain their low serum calcium and phosphorus.

2. Direct vs. Indirect Mechanism for Mineralization. What was not clear from the early studies, and is still unresolved, is whether decreased intestinal transport of calcium and phosphorus is the sole basis of impaired bone mineralization in vitamin D-deficient animals. Evidence that diminished intestinal absorption may be the basic mechanism includes the observations that (a) either low calcium or low phosphorus diets per se can lead to impaired mineralization (297, 404, 470, 557, 559, 659); (b) the probability of the occurrence of rickets was predictable on the basis of the calcium × phosphate product (299); (c) rachitic bone, when placed in a solution containing normal calcium and phosphate, calcified (133); (d) vitamin D deficiency failed to produce changes in bone enzymatic activity (616) or osteoid maturation (294) if the animals were prevented from becoming hypocalcemic and hypophosphatemic (665); and (e) vitamin D or D metabolites have never been demonstrated to produce mineralization in vitro. On the other hand, there is some evidence suggesting a direct bone component in the effect, specifically clinical studies (572), as well as a report describing the presence of a nonionic mineralization-promoting factor in normal but not rachitic serum (663). Evidence consistent with, but not proof of, a direct effect on mineralization is the autoradiographic localization of administered 25-OH-D to osteoblasts, chondrocytes, and osteocytes (666) as well as the reported nuclear localization of administered $1,25\text{-}(OH)_2D_3$ in osteoblasts, chondrocytes, and osteocytes (141). It is equally conceivable that this localization could be related to inhibitory effects of the vitamin D metabolites on collagen synthesis or on proteoglycan metabolism (see Table 3). Thus, the weight of the evidence still suggests that the D vitamins enhance bone mineralization indirectly via increased intestinal calcium and phosphorus absorption.

3. Mechanism of the Intestinal Effect. As indicated earlier in this review, the intestinal effect will not be reviewed in detail here. However, in summary, it now appears that the D vitamins enhance calcium transport by facilitating an active transport process in the upper small intestine (541). A separate independent enhancement of phosphate transport also occurs (104, 304). There is some evidence suggesting that the stimulation of mineralization by the D vitamins may be more directly correlated to enhanced phosphate transport than to enhanced calcium transport, at least in the rat (602). Whether this observation can be extrapolated to other species is not yet known. The rat may be somewhat

TABLE 3

Relative potencies of vitamin D_3 metabolites on parameters of bone function in vitro.

Bone Function	Relative Potency	References
Subcellular		
Competition for $1,25(OH)_2D_3$ binding site (rat calvarial cell cytosol)	$1,25 > 25 > 24,25 > D_3 = 0$	106
Competition for 25-OH-D_3 binding site (rat calvarial cell cytosol)	$25 > 24,25 > 1,25 > D_3 > 0$	407
Cellular		
↓ Calcium influx (rat calvarial cells)	$25 \simeq 24,25 > 1,25 = D_3 = 0$	156
↑ Hyaluronate synthesis, acid phosphatase (mouse calvarial CT cells)	$1,25 > 24,25 \geq 0$	670
↓ Citrate decarboxylation, alkaline phosphatase, collagen synthesis (mouse calvarial PT cells)	$1,25 > 24,25 \geq 0$	670
$^{35}SO_4$ incorporation (rat growth plate chondrocytes)	$24,25 > 1,25 > 25 > D_3 = 0$	131
Whole bones, rudiments		
↑ Bone resorption (rat long bones, mouse calvaria)	$1,25 > 1,24,25 > 25 > 24,25 > D_3 = 0$	387, 509, 521, 522, 581, 585, 588, 628
↓ Collagen synthesis (rat calvaria)	$1,25 > 1,24,25 > 25 \simeq 24,25 \simeq D_3 = 0$	507

* $1,24,25 = 1,24,25\text{-}(OH)_3D_3$; $1,25 = 1,25\text{-}(OH)_2\text{-}D_3$; $24,25 = 24,25\text{-}(OH)_2\text{-}D_3$; $25 = 25\text{-OH-}D_3$; D_3 = vitamin D_3.

unique in that both a low phosphate diet and vitamin D deficiency are required to produce a rachitic state in this species (574). However, hypocalcemia and histologic evidence of osteomalacia were noted in pups of mothers fed vitamin D-deficient diets containing normal levels of phosphate (624a). The postulated receptor mechanisms in target tissues and the appearance of the active metabolites in the nuclei of target cells have been described previously (III C 5). The nuclear events in the intestine presumably would initiate the synthesis of the transport proteins. One candidate for such a protein is a vitamin D-dependent calcium-binding protein (CaBP) (655, 656). This protein is absent in vitamin D-deficiency and reappears upon repletion (655, 656). Addition of purified CaBP to intestinal cultures enhances calcium transport (129). Some studies on the kinetics of the appearance of the protein report a time-course incompatible with it playing a major role in the calcium transport (240, 569, 570). These studies showed increases in calcium transport at times when the CaBP is not yet measurable by sensitive radioimmunoassay techniques. Also, when vitamin D was withdrawn, calcium transport decayed much more rapidly than the levels of CaBP, and at a time when the absorptive capacity was lost, substantial levels of CaBP persisted. Other investigators do not observe this dissociation (128, 654). Recent as well as older work led to the consideration of several other membrane proteins as potential messengers for the effect (251, 339, 394, 419, 443). In addition to evaluation of kinetic relationships, the possibility of artifactual changes in vitamin D-deficient animals needs to be considered. The poor structural integrity of the vitamin D-deficient intestine was shown to result in the loss of Ca-ATPase from the membrane fraction during homogenization of the tissue (338).

4. Pattern of Bone Changes in Vitamin D-Deficiency. The state of the bones in vitamin D-deficiency has been evaluated by many of the techniques described earlier (II E). By radiographic analysis there is typically a widening of the metaphyseal growth plate and an irregular appearance of the end of the metaphysis due to uneven mineralization (146). Grossly, bulging or bowing may occur due to pressure on the undermineralized sites. The excess osteoid is evident histologically. However, since this can occur in other bone diseases as well, additional histologic criteria are needed (88). Quantitative histologic methods including tetracycline labeling and histochemical acid phosphatase determinations have indicated defects in both osteoid "maturation" and the rate of mineralization (39). The type I collagen in rachitic bone shows elevated hydroxylysine content (32, 622) and a consequent alteration in the crosslink pattern with an elevated ratio of dehydrodihydroxylysinonorleucine to dehydrohydroxylysinonorleucine (405). The high content of hydroxylysine-derived crosslinks may be a general consequence of hypocalcemia rather than a specific effect of vitamin D deficiency (188). Vitamin D_3 treatment results in an increase in the percentage of osteoid surface having a calcification front, a rise in plasma phosphate, and an increase in the osteoclast count (514). The cellular response of uremic, vitamin D-deficient animals to pharmacologic doses of vitamin D suggested enhanced metabolic activity of osteocytes and reduced osteoblastic activity (661).

5. Diseases of Vitamin D Deficiency and Their Treatment. There are a number of rachitic or osteomalacic disorders in which either vitamin D or one of the metabolites is a logical therapeutic agent. Simple *dietary* or *vitamin D-deficiency rickets* is prevented by assuring an adequate supply of vitamin D in the diet; 400 I.U./day is considered the normal requirement for the growing child (1 I.U. = 0.025 μg), and in many countries milk is fortified with this amount per quart. Although this has markedly decreased the incidence of dietary rickets, the disease has not disappeared entirely. Probably the best documented recent outbreaks have been among Asian immigrants to the British Isles (490, 495, 562). More symptoms were noted in older children, who were fed a native diet, than in infants, who received fortified milk (562). Other factors contributing to the occurrence of rickets and osteomalacia in this population are believed to be the lack of many days of bright sunlight in the country as well as the relatively darker pigment of the stratum corneum, which could be a barrier to ultraviolet light. An increasing number of cases of vitamin D-deficiency rickets has been reported in the United States recently (12, 20, 447). Contributing factors appear to include (a) inadequate exposure to UV light due to latitude, pollution, and ritual garments, and (b) inadequate infant intake of vitamin D in cases of underlying maternal vitamin D deficiency. Such deficiency has been reported to lead to congenital rickets (416). The current trend towards decreasing use of artificial fortified infant formulas has made this more probable. Another population at risk are the elderly (6, 99), especially those confined indoors in geriatric wards (124). An age-related decrease in absorption of vitamin D could play a role in the increased incidence of osteomalacia (36). The reported existence of normal 1,25-$(OH)_2$D concentrations and low 25-OH-D and 24,24$(OH)_2$D in patients with nutritional osteomalacia (160a) suggests that several active metabolites contribute to the normal mineralizing action of vitamin D in vivo.

Osteomalacia or rickets may accompany disorders of the tissues involved in the absorption or activation of vitamin D. Thus, *malabsorption syndromes* produced by intestinal disease or surgical resection as well as *hepatitis*, *obstructive jaundice*, and *cirrhosis* have been reported to result in diminished mineralization (3, 14, 36, 137, 203, 207, 264, 326, 487, 549, 563, 571, 623). Although 25-OH-D levels may be reduced in the hepatic syndromes (372, 409, 549, 564) and 25-OH-D has been shown to constitute more effective replacement therapy than vitamin D in that disproportionately lower doses are required (643), the actual defect in biliary cirrhosis may be in the ab-

sorption of vitamin D rather than its metabolism (410). The disproportionately greater effectiveness of 25-OH-D_3 could reflect the different patterns of absorption of vitamin D_3 and 25-OH-D_3 (288, 429) rather than a metabolic block. Low doses of 1,25-$(OH)_2D_3$ have been effectively used to produce healing of rickets in infants with neonatal liver disease (271). Calcium malabsorption and hypocalcemia have been noted in patients with *nephrotic syndrome* (172, 548) and it appears that the hypocalcemic state may derive from the loss of vitamin D binding protein in the urine and the consequent low levels of 25-OH-D in the circulation (35, 397).

A number of disorders seem to reflect deficiency or absence of the 1-hydroxylase in that they are effectively treated with small amounts (1 to 2 μg) of 1,25-$(OH)_2D_3$, but are refractory to vitamin D or 25-OH-D_3. Much larger doses of the 1,25-$(OH)_2D_3$ precursors are required than would be needed in simple deficiency states. Those diseases include *vitamin D-dependent rickets (VDDR)*, a recessive disorder in which the 1-hydroxylase may be deficient (184). In the treatment of *hypoparathyroidism*, low doses of 1,25-$(OH)_2D_3$ are effective in raising plasma calcium and reversing symptoms whereas substantially higher doses of vitamin D or 25-OH-D are required (26, 109, 336, 430, 532, 538). The disparity in dosage suggests that the hypocalcemia of hypoparathyroidism may be due at least in part to lowered 1α-hydroxylase activity in the absence of adequate PTH. This is supported by the findings that serum 1,25-$(OH)_2D$ is low in hypoparathyroidism and elevated in hyperparathyroidism (85, 589). *Pseudohypoparathyroidism* represents a state in which there is end-organ resistance to PTH. The 1-hydroxylase may represent one such resistant process, as several investigators have found lower than normal 1,25-$(OH)_2D$ concentrations in patients with this disorder (42, 155). There is disagreement as to whether plasma 1,25-$(OH)_2D$ concentrations are low in women with *postmenopausal osteoporosis* (247, 527). Administration of 1α-OH-D_3 or 1,25-$(OH)_2D_3$ has been reported to improve serum chemistry and bone histology in elderly osteoporotic patients (378).

Nephrectomized patients have been reported to have undetectable circulating 1,25-$(OH)_2D$ (84, 85, 167, 168, 584, 588), consistent with the kidney being the sole or at least the predominant site of the 1α-hydroxylase under normal conditions in the nonpregnant state. These individuals, as well as patients with *uremia* and markedly deteriorated renal function, frequently develop bone disorders, which include vitamin D resistant osteomalacia and osteitis fibrosa as well as occasional osteosclerosis (171, 377, 553). The vitamin D-resistance may be a consequence of decreased 1-hydroxylation due to either phosphate retention or loss of other renal cell function. Reduced 25-OH-D_3 may contribute to the pathogenesis in some patients (161). The osteitis fibrosa is believed to be due to secondary hyperparathyroidism in response to the hypocalcemia (171, 553). The pathogenesis of the osteosclerosis is unexplained. Treatment of these patients with 1,25-$(OH)_2D_3$, 1α-OH-D, dihydrotachysterol (DHT), 5,6-trans-D_3, or 25-OH-D_3 has been reported to increase their calcium absorption, thus lessening the secondary hyperparathyroidism (27, 48, 63, 74, 100, 101, 119, 123, 324, 384, 385, 479, 489, 536, 539, 624). Histologic and radiologic improvement of the bones have also been noted (2, 48, 100, 101, 110, 111, 324, 479, 536). However, some studies report lack of remineralization with 1,25-$(OH)_2D_3$ or 1α-OH-D_3 in some or all patients (63, 101, 384, 385, 489, 623). Factors that could contribute to an inadequate response include insufficient duration of therapy, coadministration of agents such as heparin (63) or anticonvulsants (101), that could interfere with or counteract the effect, phosphate depletion, excessive dosage, which could result in demineralization (see below), and the short half-life of the drug, which could result in erratic blood levels. It has been suggested that 25-OH-D_3 and 24,25-$(OH)_2D_3$ possess some specific effect to promote mineralization that 1,25-$(OH)_2D_3$ lacks (320, 464, 514). However, as discussed earlier (section III D 1 d), at the present time there is no experimental evidence to support the existence of such a specific mineralizing property. Moreover, consideration should also be made of the more rapid turnover of 1,25-$(OH)_2D_3$ as a factor in this reported difference in efficacy. The possibility of administering the drug in smaller divided doses might provide a better therapeutic effect.

Vitamin D metabolites are somewhat effective in the treatment of *hypophosphatemic rickets*. The primary defect in this sex-linked dominant disorder seems to be an impairment in renal phosphate conservation (209). 1,25-$(OH)_2D$ concentrations are not high, as would be expected with the hypophosphatemia, but are in the normal or low-normal range (247, 449). The bone disease may be a consequence of both low phosphate and depressed 1,25-$(OH)_2D$. Treatment with 1,25-$(OH)_2D$ alone did not reverse hypophosphatemia (75) and even pharmacologic doses of either 25-OH-D_3 or 1,25-$(OH)_2D_3$ resulted in only partial improvement in mineralization (118, 499, 537). *Vitamin D-dependent rickets, type II, (VDDR-type II)* is characterized by apparent end-organ resistance to 1,25-$(OH)_2D$ and high, 1,25-$(OH)_2D$ serum concentrations (77, 395, 682). This disorder has responded to higher doses of 1,25-$(OH)_2D$ (77, 395). A different type of apparent end-organ resistance has been described in a newborn infant with an exceedingly low dietary phosphate. In this case the rachitic state and elevated 1,25-$(OH)_2D$ levels were reversed by phosphate supplements (535).

B. Stimulation of Bone Resorption

1. Background. Toxicity due to ingestion of excess vitamin D is characterized by hypercalcemia, hyperphosphatemia, hypercalciuria, and hyperphosphaturia. The high serum calcium can result in tissue toxicity, most notably cardiac abnormalities and renal tubular damage.

The elevated calcium × phosphate product can result in soft tissue calcification. Progressive osteolytic lesions of the bones indicate that a large proportion of the excess calcium and phosphorus ultimately derives from bone. For a number of years, it was believed that only supraphysiologic levels of vitamin D led to loss of calcium from bone. With the advent of tracer kinetics, it was possible to prelabel bone and dietary calcium with different calcium isotopes and follow the fate of the two sources of calcium independently. By this approach, it was shown that even physiologic replacement doses of vitamin D caused mobilization of calcium from bone (96). This has been substantiated many times by the demonstration that the same doses of vitamin D metabolites that stimulate intestinal calcium absorption increase serum calcium in vitamin D-deficient animals on low calcium diets (e.g. 610).

2. Characteristics of the direct effect of D vitamins on bone. A. CONCENTRATION DEPENDENCE. The bone mineral-mobilizing or bone-resorbing effects of vitamin D metabolites are probably mediated at the bones themselves. Evidence supporting a direct effect derives from the sensitivity of isolated bones in organ culture to the bone-resorbing effects of the metabolites. Fetal rat bones undergo resorption in response to as little as 10^{-11} M 1,25-$(OH)_2D_3$ (584). Although 48 hr of culture are required for the effect of this concentration to be manifested, an exposure of 3 hr to 8×10^{-11} M 1,25-$(OH)_2D_3$ followed by 45 hr culture in a vitamin D-free medium elicits substantial resorption (580). Physiologic concentrations of 1,25-$(OH)_2D_3$ are in the 8×10^{-11} M to 10^{-10} M range. The in vitro and in vivo concentrations are not directly comparable because the concentrations of free hormone in the two situations are not known. As discussed previously, 1,25-$(OH)_2D$ is highly bound to a vitamin D-binding protein, and the amount of free hormone is probably no more than a few percent. In organ cultures, albumin is added to the medium and the binding of 1,25-$(OH)_2D_3$ by this protein could also be substantial. It is not possible to resolve this issue by simply incubating bones in 100% serum because there appear to be factors in serum that inhibit in vitro resorption (588).

B. RELATIONSHIP TO PARATHYROID HORMONE. In vivo experiments in rats and observations in patients have suggested that PTH is required for the bone-resorbing effects of vitamin D, and conversely that vitamin D is required for the bone-resorbing effects of PTH (97, 194, 201, 239, 408, 515). In contrast, either agent can produce resorption in vitro (590). This apparent disparity between in vivo and in vitro results could arise if the in vitro systems were not completely devoid of vitamin D or PTH. Alternatively, the resistance in vivo could be secondary to changes in serum calcium and phosphate or other factors. For example, it has been shown that increasing serum calcium overcomes the resistance to PTH shown by vitamin D-deficient animals and humans (15, 201). This observation has an in vitro counterpart since it has been shown that short-term exposure to PTH does not produce resorption if the medium calcium is too low (510). Other treatments that have restored the response of vitamin D-deficient rats to PTH include glucocorticoids (476) and calcitonin (417). The reported ineffectiveness of vitamin D in parathyroidectomized animals (194) could be attributable to their high serum phosphate. In addition to blocking the 1-hydroxylase, this high phosphate could also be inhibiting bone resorption (503). Likewise, the lack of PTH removes an important stimulator of the 1-hydroxylase. In some species, parathyroidectomy does not interfere with the effects of vitamin D, and in these studies, phosphate levels were not markedly altered (435, 524). Another factor decreasing the response to vitamin D in thyroparathyroidectomized animals may be the lack of thyroxin (475). Thus it seems possible that the in vivo interdependence between PTH and vitamin D for effects on bone may not reflect an interaction directly on bone. It has also been suggested that the observed interdependence is not at the level of osteoclastic resorption, but at the postulated bone membrane (142). This concept has yet to be tested experimentally.

C. MECHANISM OF ACTION. The mechanism of action of vitamin D on bone has been less extensively investigated than the action on the intestine. High affinity binding of 1,25-$(OH)_2D$ and 25-OH-D has been found in whole bone homogenates and isolated bone cells (106, 343, 391, 407). The 25-OH-D binding site does not appear to be the receptor for the action of vitamin D on bone resorption since there is poor correlation between the affinity of the various analogs for the binding protein and their bone-resorbing activity (407). The 1,25-$(OH)_2D$ binding protein could represent the bone cell receptor for the bone-resorbing effects of the D metabolites. It is not known in which cell type or types the binding protein is localized. Although a vitamin D-dependent CBP in bone was elusive for many years, such a binding protein has recently been reported in chick bone (116). The protein has a molecular weight of 34,000 d. When vitamin D was administered to rachitic chicks for 2 weeks, levels of the protein increased 25-fold. A further increase was observed when chicks were fed a low calcium diet. The role of calcium in vitamin D action on bone is still uncertain. In contrast to PTH, which effects a rapid influx of calcium into isolated bone cells (160), 25-OH-D_3 and 24,25-$(OH)_2D$ inhibited influx of calcium into bone cells (156). Indirect evidence for a role for calcium fluxes in 1,25-$(OH)_2D$ action are that inhibitors of calcium transport such as verapamil, ruthenium red, and cobalt block the bone-resorbing effects of both PTH and 1,25-$(OH)_2D$ (266, 580) and that the ionophore A23187 enhances them (158). As mentioned previously (section II B), there are at present conflicting reports regarding whether 1,25-$(OH)_2D$ potentiates (107, 386), inhibits (670), or has no effect (483) on PTH-induced increases in bone cAMP. All investigators appear to agree, however, that in con-

trast to what occurs in the intestine (125, 237), the D metabolites themselves do not directly increase cAMP in bone (199, 386, 483, 670). An in vivo study showed increases in cAMP concentrations in the calvaria of vitamin D-deficient rats 48 hours after vitamin D administration. The effect could not be reproduced by a lactose-containing high calcium diet, which elevated plasma calcium to levels even above those achieved with vitamin D treatment (319). A nuclear component to the direct effect of vitamin D on bone resorption is suggested both by localization (657) and the observation that actinomycin D prevents the bone-mobilizing effect of 1,25-$(OH)_2D_3$ (602, 657). 1,25-$(OH)_2D_3$, like PTH, increases lysosomal enzyme release from cultured bones (165, 625). Increases in osteoclast number, size, nuclear area, ruffled border, and clear zone have been described after vitamin D or 1,25-$(OH)_2D_3$ treatment (259, 290, 620).

3. Clinical aspects of vitamin D-induced bone resorption. Excess vitamin D produces hypercalcemia, most likely by enhancing bone resorption (434). Since the 1-hydroxylase appears to be tightly feedback-regulated and vitamin D per se is inactive on bone, it seems likely that the bone mobilization caused by excess vitamin D is attributable to a metabolite other than 1,25-$(OH)_2D$. The occurrence of vitamin D intoxication in an anephric patient (132) would clearly have to be produced by a metabolite other than 1,25-$(OH)_2D_3$. The most likely candidates of the known metabolites are 25-OH-D and 24,25-$(OH)_2D$. These two compounds are approximately equally potent in stimulating resorption in vitro (581), although 24R,25-$(OH)_2D_3$ has poor bone calcium-mobilizing activity in intact animals (273). Also, the presence of a 24-hydroxylase in cartilage (192) raises the question as to whether 25-OH-D acts in the form of the original compound or as the 24-hydroxylated metabolite. Occasional hypercalcemia has been reported in some patients treated with 1,25-$(OH)_2D_3$ at dosages of 1 to 3 μg/day (44, 74, 76, 336, 369, 378, 489, 532, 624). The symptoms have been noted in patients receiving the agent for treatment of the hypocalcemia of hypoparathyroidism, pseudohypoparathyroidism, or the osteomalacia of renal failure. In the case of two patients with pseudohypoparathyroidism, the increases in serum calcium were shown to correlate with elevations in serum levels of 1,25-$(OH)_2D_3$ (44). It is not yet established whether the increases in plasma 1,25-$(OH)_2D_3$ are due to cumulation from overloading of metabolic or excretory mechanisms, or are consequences of enhanced absorption or decreased degradation of the drug. It would be interesting to know whether administration of the agent in smaller divided doses will decrease the incidence of hypercalcemia. As mentioned previously, this might also be therapeutically more efficacious in promoting mineralization since it would provide a more physiologic pattern of 1,25-$(OH)_2D$ exposure. Elevated plasma concentrations of 1,25-$(OH)_2D$ have been found in patients with *hyperparathyroidism* (167, 247, 588). The relative contributions of PTH and 1,25-$(OH)_2D$ to the bone lesions and hypercalcemia have not been determined. It is of interest to note that the 1,25-$(OH)_2D_3$ concentrations in this disorder are in the range that would produce resorption when applied to bones in vitro, whereas the PTH concentrations are not. However, there may be other factors that render the isolated bones less sensitive to the resorbing effects of PTH.

Sarcoidosis is characterized by abnormal sensitivity to vitamin D in some patients (41). These individuals develop hypercalciuria and hypercalcemia upon exposure to vitamin D from dietary sources or excess sunlight. Recent studies indicate that hypercalcemic sarcoid patients have elevated plasma levels of 1,25-$(OH)_2D$ (45, 469). Elevated plasma 1,25-$(OH)_2D$ can be elicited in sarcoid patients by vitamin D regimens that have no effect in normal individuals (45, 582). Both the increased serum calcium and elevated plasma levels of 1,25-$(OH)_2D$ are reversed by cortisol treatment (45, 469). The kidney may not be the sole site of 1,25-$(OH)_2D_3$ production in sarcoidosis. Hypercalcemia and elevated 1,25-$(OH)_2D$ have been described recently in an anephric patient with sarcoidosis (29).

V. Important Unsolved Questions in the Actions of the D Vitamins on Bone

A. Is There a Direct Effect of Vitamin D to Promote Mineralization?

Obviously, one major unresolved question in the vitamin D field is whether the increased mineralization elicited by vitamin D in rachitic animals is mediated by a direct effect on bone. Although there is no definitive evidence that this is the case, and most of the evidence supports an indirect effect, it may be that the appropriate in vitro system for demonstrating a direct effect has not been devised. Table 3 indicates the rank order of potency of the vitamin D metabolites on various in vitro parameters of bone function. The potencies of the metabolites to stimulate resorption and to inhibit collagen synthesis correlate with the relative abilities to compete with cytosolic 1,25-$(OH)_2D$ binding. However, incorporation of $^{35}SO_4$ into proteoglycans, inhibition of calcium influx, and competition with 25-OH-D_3 binding show a different pattern of vitamin D metabolite responsiveness. Presumably, the 25-OH-D_3 binding represents binding to the 6 S protein described earlier. Although none of these parameters has been implicated in mineralization, they obviously represent direct effects on skeletal tissue that merit further investigation.

B. What Is the Function of the Effect of Vitamin D on Collagen Synthesis?

One of the most puzzling questions in the area of effects of vitamin D on bone is the matter of its action on collagen synthesis. The literature is still sparse, but is nonetheless confusing. In vitro studies with cultured bones from D-replete rat fetuses indicated an inhibition

of collagen synthesis, even at low concentrations of the D metabolites (507). In vivo studies in the vitamin D-deficient chick showed enhanced collagen synthesis in cartilage (94). Reduced rates of bone matrix formation or maturation have been noted in vitamin D-deficient rats (39, 453). Clinical reports of improved growth with vitamin D replacement (111) would imply enhanced collagen synthesis, but this was not measured directly. Conceivably, there could have been diminished collagen synthesis at the time when the osteoid was remineralizing followed by normal collagen synthesis when normal growth resumed.

C. What Role Do Changes in Alkaline Phosphatase Play?

Another area of uncertainty is the relationship of bone alkaline phosphatase to vitamin D action. Bone alkaline phosphatase activity increases in vitamin D deficient animals (150, 420, 664) as well as in animals on low phosphate diets (664). As mentioned earlier, this enzyme is postulated to play a role in mineralization. Since it could potentially have a regulatory function in the responses of bone to vitamin D, it is of considerable interest.

D. What Is the Function of Vitamin D in the Body?

Perhaps the ultimate unanswered question regarding vitamin D is a teleologic one, i.e., what is its overall role as distinguished from its specific actions. The answer is not obvious. Some of the actions, such as those on bone formation and resorption, seem opposite and paradoxical. The definition of the function of vitamin D should integrate all of these actions into a logical framework. The classic hypothesis is that the function of the vitamin is as an antirachitogenic factor. This concept would seem to be supported by the occurrence of rickets or osteomalcia in vitamin D-deficiency and their cure by administration of vitamin D or its active metabolites or analogs. The fact that vitamin D enhances calcium absorption is consistent with this role. On the other hand, the apparent lack of a direct effect on mineralization and the fact that vitamin D metabolites cause loss of bone through resorption, even in animals on low calcium vitamin D-deficient diets, seems inconsistent with such a function.

A second possibility is that vitamin D is a bone-remodeling hormone. Remodeling involves removal of calcium from one site (through resorption) and its local deposition. Clearly, vitamin D affects both processes. Serum concentrations of $1,25\text{-}(OH)_2D$ are higher during growth. Also consistent with a remodeling function is the evidence for regulation of the 1-hydroxylase in some species by such growth stimulatory factors as growth hormone and insulin. The apparent inhibitory effect of the D vitamins on collagen synthesis does not seem to fit logically, although it could be postulated that this is selective and prevents nondirected growth. Perhaps it is manifested so dramatically in organ culture because the tissues are sessile and not subject to piezoelectric forces that would direct growth and mineralization. The other unsatisfying aspect to the remodeling concept is that it seems inconsistent that the processes for making calcium available (i.e. intestinal calcium absorption and bone resorption) are under direct control whereas deposition per se seems most likely not to be.

A third possibility is that vitamin D is a homeostatic hormone in the sense of regulating calcium homeostasis and preventing hypocalcemia. By increasing intestinal calcium absorption and bone resorption, it would antagonize hypocalcemia. This effect would be feedback regulated, since low calcium would increase production of $1,25\text{-}(OH)_2D_3$ by stimulation of PTH release. The increase in $1,25\text{-}(OH)_2D_3$ production during periods of calcium stress (e.g. lactation, egg-laying) would be consistent with such a mechanism. There are several unsatisfying aspects to this function. The first is that the demonstrated effects of vitamin D are too slow to provide *rapid* homeostatic control of serum calcium. If the mechanism is related to specific events in the female reproductive cycle it would seem uneconomical that it would be so well developed in the male also. Another problem is that the vitamin D system occurs in organisms lacking PTH, which seems to be the major mechanism for calcium-mediated regulation of the 1-hydroxylase. There does not seem to be another means to turn off the hypercalcemic effect—the well-mineralized bone is not a sink for excess calcium.

A fourth possibility is that vitamin D is a phosphate-regulating hormone. The intestinal and bone effects, as well as effects on renal phosphate transport that have not been discussed here, all tend to increase serum phosphate. Vitamin D metabolism may be directly regulated by phosphate, in contrast to the indirect regulation by calcium. There could also be a short feedback loop at the tissue level since the effects of resorbing agents on bone are enhanced under conditions of low phosphate (468). Although a decline in phosphate can impair muscle activity and other tissue functions, this is an effect with a slow onset and not acutely life-threatening, and thus more compatible with time course of vitamin D action. Chronic vitamin D deficiency is characterized by muscle weakness and there is some evidence for defective phosphate metabolism in this state (57). Replacement of vitamin D restores muscle function and metabolism to normal. During growth and development there is additional phosphate demand, which could be reflected in stimulation of 1-hydroxylation by growth hormone, estrogen, and prolactin. This model of the role of vitamin D would view the effects on calcium and even the rickets as secondary aspects of a primary phosphate homeostatic mechanism. Calcium is affected because it is so commonly linked to phosphate transport; the body allows rickets to occur because even the mineralization of the skeleton is sacrificed in an attempt to maintain normal muscle function.

The ultimate answer may be that vitamin D does not have a simple unitary role, that it is a multifaceted substance with multiple functions. The dominance of one or another of these functions would be a reflection of the physiologic and developmental state of the organism. Within the past year, 1,25-$(OH)_2D_3$ receptors and effects have been described in the pancreas and pituitary gland (116a, 116b, 200, 248, 429a, 437a, 492, 592), tissues previously unrecognized as targets of vitamin D action. These findings suggest that we still have a great deal to learn before we will understand this substance.

VI. Summary and Conclusions

The research explosion in the vitamin D field during the past 10 to 15 years has revealed much that was not even suspected previously. The thrust has come predominately from studies on vitamin D metabolism and its regulation. A wide variety of metabolites and analogs have been introduced. These developments have had a dramatic impact in the area of metabolic bone disease and many puzzling disorders are now better understood and can be treated in a more logical manner. On the other hand, many fundamental problems related to the mechanisms of action of the D vitamins on bone remain to be solved. The mechanism whereby the D vitamins enhance mineralization, even whether there is a direct component to the effect, is still unknown. The interrelationship between synthesis of collagen and mineralization and the effects of the D vitamins on this are still mysteries. Even though the bone mobilization that produces hypercalcemia can be reproduced in vitro with the D vitamins, the molecular details of the process are still incompletely understood. Finally, we still cannot provide an unequivocal answer to the question of the ultimate physiologic role of vitamin D.

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