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COMMENTARY

VITAMIN D AS A PROHORMONE

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Almost since its discovery in 1922, vitamin D has been considered to act directly on the target tissues of intestine and bone without its further metabolism. However, the existence of vitamin D-resistant bone diseases in the medical world and the large lag between the time of vitamin D administration and the appearance of its first biological response appeared to argue against this concept [1, 2]. It was the introduction of sophisticated radiochemical syntheses, allowing the production of radioactive vitamin D of high specific activity [3] and the introduction of new chromatographic methods [4], which allowed the clear demonstration of the existence of biologically active metabolites of the vitamin [5, 6]. With the use of the new chromatographic systems coupled with sophisticated instrumentation such as mass fragmentometry and nuclear magnetic resonance spectroscopy has come the elucidation of the structure of the small amounts of vitamin D metabolites which could be isolated [7–9]. These metabolites have now been chemically synthesized, which has allowed the introduction of tritium of high specific activity (Ref. 10, and S. Yamada, H. K. Schnoes and H. F. DeLuca, unpublished results), permitting probes into the molecular mechanism of action of the active forms of the vitamin. Finally, the availability of large amounts of the active metabolites of vitamin D from synthetic sources [11–15] has permitted a complete elucidation of the vitamin D endocrine system and its interaction with other endocrine systems known to be involved in the control of calcium and phosphorus metabolism. The abundance of chemically synthesized vitamin D metabolites has also ushered in a new era in the treatment of metabolic bone disease utilizing the active forms of vitamin D [16, 17]. However, much remains to be learned concerning the mechanism of action of the active hormonal form of vitamin D, its further metabolism, and the mechanism whereby its production is regulated.

It is now known that vitamin D_3 upon intravenous administration, absorption from the small intestine or production in the skin by the photolysis of 7-dehydrocholesterol in the epidermis is rapidly accumulated in the liver where it undergoes hydroxylation on carbon 25 to produce 25-hydroxyvitamin D_3 (25-OH-D_3) [18]. This reaction is NADPH supported and is regulated in a feed back manner by the hepatic level of 25-OH-D_3 itself [19]. The 25-OH-D_3, however, does not act directly at physiologic concentrations in any target tissue. Instead it is the major circulating metabolite of vitamin D found at a level of about 20 ng/ml bound to an α-globulin of 51,000 molecular weight [20, 21]. The 25-OH-D_3 is taken up by the kidney where it undergoes a second hydroxylation on carbon 1 to yield 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] [8, 22, 23]. Because 1,25-$(OH)_2D_3$ is more potent than any other known form of the vitamin and because it is equally active in nephrectomized as well as intact vitamin D-deficient animals, whereas its precursors fail to act in the nephrectomized animal, it is apparent that 1,25-$(OH)_2D_3$ or one of its metabolites must be the metabolically active form of vitamin D in calcium transport reactions and in phosphate transport reactions [24, 25].

Because 1,25-$(OH)_2D_3$ is produced exclusively in the kidney and has its function in intestine and bone, it can be considered a hormone. In true hormonal fashion, its biosynthesis is regulated by the need for calcium or the need for phosphorus as revealed by hypocalcemia or hypophosphatemia [26]. Before such regulation occurs, however, 1,25-$(OH)_2D_3$ must induce some unknown change in kidney including the appearance of another hydroxylase, 25-OH-D_3-24-hydroxylase. This hydroxylase hydroxylates either 25-OH-D_3 or 1,25-$(OH)_2D_3$ on carbon 24 to produce the corresponding 24R-hydroxyl isomer [27, 28]. The function of the 24-hydroxylated forms of vitamin D is not understood at the present time, although current belief is that the 24-hydroxylation represents the initial step in the inactivation of the potent vitamin D molecule [15]. Other suggestions include a feedback regulation of the parathyroid glands, and anti-1,25-$(OH)_2D_3$ activity, but additional investigation will be required before these functions can be established.

The administration of 1,25-$(OH)_2D_3$ to vitamin D-deficient chickens not only causes the appearance of the 25-OH-D_3-24-hydroxylase, but it also suppresses the 25-OH-D_3-1-hydroxylase [28]. Thus, 1,25-$(OH)_2D_3$ itself plays a role in the feedback regulation of its own biogenesis. Exactly how 1,25-$(OH)_2D_3$ suppresses the 1-hydroxylase and stimulates the 24-hydroxylase remains unknown, although it appears to be an α-aminitin-inhibited response suggesting that transcription, and new protein synthesis is involved in this regulatory phenomenon [28, 29].

In animals given vitamin D (and thus have been induced by 1,25-$(OH)_2D_3$), the renal hydroxylases are regulated indirectly by serum calcium concentration [26, 30]. Low serum calcium concentration stimulates the 25-OH-D_3-1-hydroxylase and suppresses the 25-OH-D_3-24-hydroxylase, while hypercalcemia causes exactly the reverse to occur. It is generally believed that it is the parathyroid glands which are responsible for this regulation [31, 32]. Parathyroid tissue is known to secrete parathyroid hormone in response

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to hypocalcemia, and the parathyroid hormone can be shown to stimulate the 25-OH-D_3-1-hydroxylase and suppress the 25-OH-D_3-24-hydroxylase. Thus, the need for calcium can trigger parathyroid hormone secretion. This hormone stimulates 1,25-$(OH)_2D_3$ biosynthesis which in turn results in the elevation of intestinal calcium absorption, mobilization of calcium from bone and increased renal reabsorption of calcium [15, 26]. The resultant rise in serum calcium concentration suppresses parathyroid hormone secretion.

In addition to the calcium-parathyroid regulation, accumulation of 1,25-$(OH)_2D_3$ is apparently regulated by hypophosphatemia [33, 34]. Even in the absence of parathyroid glands, low blood phosphorus concentrations stimulate the appearance of 1,25-$(OH)_2D_3$ in the extracellular fluid. It also stimulates the 25-OH-D_3-1-hydroxylase as measured *in vitro*, although there appears to be a greater stimulation in the accumulation of 1,25-$(OH)_2D_3$ in blood and tissues than in the measured response of the 25-OH-D_3-1-hydroxylase [35, 36]. Since 1,25-$(OH)_2D_3$ stimulates the transport of phosphate across intestinal epithelium [25] and increases serum phosphorus concentration [37], it can also be considered a phosphate-mobilizing hormone. In its phosphate maneuvers, the 1,25-$(OH)_2D_3$ system does not interact with the parathyroid hormone, whereas its action is intimately associated with parathyroid hormone secretion in regulating serum calcium concentration. Thus, the selectivity of the 1,25-$(OH)_2D_3$ as a calcium-mobilizing hormone or as a phosphate-mobilizing hormone depends upon the presence or absence of the parathyroid hormone [25].

In addition to the above control mechanisms, it has recently been demonstrated that the sex hormones exert a marked controlling action on renal 25-OH-D_3-1-hydroxylase [38, 39]. This work has come about in relationship to the egg shell-forming systems of the bird. Shell-forming birds have high 25-OH-D_3-1-hydroxylase compared to their normal male counterparts. The injection of estradiol to mature males brings about a marked stimulation of the 25-OH-D_3-1-hydroxylase and a suppression of the 24-hydroxylase. In immature males, immature females or castrated males, testosterone or progesterone must also be given for estradiol to stimulate the 1-hydroxylase. Although it is unknown as to whether the sex hormones regulate vitamin D metabolism in mammals, the fact that they stimulate hydroxylases in birds is strongly suggestive that a generalized control of the 1-hydroxylase will be found in relation to the sex hormones. This is particularly important in view of the deteriorating bone disease known as postmenopausal osteoporosis which is associated with a disappearance of the estrogens and androgens following the menopause. Possibly a component of this disease is failure to make adequate amounts of 1,25-$(OH)_2D_3$ giving rise to failure to form new bone and the need to utilize existing bone to maintain serum calcium concentration in the absence of adequate calcium absorption. In any case, it is clear that, in true endocrine fashion, the vitamin D system which utilizes kidney as its endocrine organ interacts with more than one hormonal system in a complex fashion. The unraveling of these endocrine interrelationships will undoubedly give a much better understanding of metabolic bone disease and methods of treatment.

Vitamin D_2 is also metabolized to 25-hydroxyvitamin D_2 (25-OH-D_2) [40, 41] and 1,25-dihydroxyvitamin D_2 (1,25-$(OH)_2D_2$) [41, 42] before it can function. The well known discrimination against vitamin D_2 by birds is now believed to be due to a very rapid metabolism of compounds substituted on carbon 24 in this species to excretory products [41, 43]. Thus, 1,25-$(OH)_2D_2$ is just as effective as 1,25-$(OH)_2D_3$ when added to intestinal organ cultures (C. O. Parkes and H. F. DeLuca, unpublished results), and to cultures of bone (P. H. Stern and H. F. DeLuca, unpublished results). When they are injected *in vivo*, however, 1,25-$(OH)_2D_2$ is $\frac{1}{10}$ as active as 1,25-$(OH)_2D_3$ [44]. The exact nature of the discriminatory process against the 24 substituted vitamin Ds, such as vitamin D_2 in the bird, remains to be elucidated, however.

Extensive work has been carried out only on 25-OH-D_3-1-hydroxylase among the enzyme systems involved in the metabolism of vitamin D. This system has been clearly shown to be a mixed function oxidase which is dependent upon cytochrome P-450 [45]. This membrane bound system has been successfully solubilized and the three components of the hydroxylase isolated and recombined to give an active 25-OH-D_3-1-hydroxylase [45, 46]. Furthermore, two components of the hydroxylase are non-specific inasmuch as they can be replaced by similar enzymes from the beef adrenals. Thus, NADPH reduces a renal ferredoxin reductase (flavoprotein). This in turn reduces the iron–sulfur protein of 12,500 molecular weight (renal ferredoxin). Renal ferredoxin then reduces the cytochrome P-450, which carries out the specific hydroxylation of 25-OH-D_3 to form 1,25-$(OH)_2D_3$. In this system, the renal ferredoxin and the renal ferredoxin reductase can be replaced by adrenodoxin and adrenodoxin reductase from the beef adrenal glands. The 24-hydroxylase has not been studied further except to be shown to require NADPH, molecular oxygen and magnesium ions [47]. Presumably it is a mixed function oxidase but the oxygen 18 experiments which would demonstrate this have not been carried out.

Initial work has been carried out on the mechanism of action of 1,25-$(OH)_2D_3$ in the small intestine. There appears to be agreement that the nuclear fraction of intestine accumulates most of the 1,25-$(OH)_2D_3$ administered [48, 49]. Although the isolation of pure nuclei in good yield from the intestine has not been possible, analogy between vitamin D and the other steroid hormones argues strongly for a nuclear function of 1,25-$(OH)_2D_3$. In accord with this, a 3.7S cytosol protein, which binds specifically 1,25-$(OH)_2D_3$, has been discovered in the chick intestine [50, 51] and has been shown to facilitate the tranfer of radioactive 1,25-$(OH)_2D_3$ in a temperature-dependent process to isolated chromatin [52, 53]. The 3.7S cytosol-binding protein has now been stabilized and selectively studied revealing a high degree of specificity for 1,25-$(OH)_2D_3$ among the metabolites and analogs (B. E. Kream and H. F. DeLuca, unpublished results). Although only a 5–6S binding protein could be found in intestine from rats and other mammals, recently it has been possible to remove much

of the interfering 6S protein, and by means of new stabilization measures it has been possible to demonstrate the existence of a 3.2S specific binding protein for 1,25-$(OH)_2D_3$ in rat small intestine, embryonic rat and chick bone and rachitic chick bone, but not in other non-target tissues of 1,25-$(OH)_2D_3$ (B. E. Kream and H. F. DeLuca, unpublished results). Thus, it appears very likely that 1,25-$(OH)_2D_3$ exerts its action at least in part by interacting with a specific receptor protein which is then transferred to the nucleus bringing about transcription of specific messenger RNA which in turn codes for calcium and possibly phosphate transport proteins.

The specific calcium transport proteins remain unknown, although Wasserman *et al.* [54] have described a 24,000 molecular weight calcium binding protein in the cytosol of chick small intestine which has been believed to participate in calcium transport. In other tissues such as brain, this protein is not vitamin D dependent. Furthermore, the kinetics of its appearance in intestine does not argue strongly for its participation in the transport of calcium. Likely, additional factors are involved which have not been discovered. A 230,000 molecular weight protein has been discovered in the brush borders of small intestine in response to 1,25-$(OH)_2D_3$ [55]. This substance has alkaline phosphatase activity and binds calcium, whereas a 200,000 molecular weight protein found in the brush borders of rachitic chick small intestine does not bind calcium and disappears as the 230,000 molecular weight protein appears. It is not clear whether the 230,000 molecular weight substance is involved in the calcium transport process. Little is known concerning the phosphate transport process, and little is known concerning the calcium mobilization system of bone which is responsive to 1,25-$(OH)_2D_3$ except that it is blocked by the administration of actinomycin D [56], suggesting transcription of DNA as a mechanism.

The question of whether 1,25-$(OH)_2D_3$ is further metabolized before it functions in some of the systems is not entirely settled. Recently it has been discovered that 1,25-$(OH)_2D_3$ undergoes side chain oxidation to yield a new and unknown metabolite of the vitamin [57, 58]. This side chain oxidation, which can be detected by $C^{14}O_2$ exhalation after injection of 1,25-$(OH)_2$-[26,27-^{14}C]D_3, occurs early enough to be of significance at least in the phosphate transport reaction system. In addition, 1,25-$(OH)_2D_3$ undergoes 24-hydroxylation, but the further metabolism of 1,24,25-trihydroxyvitamin D_3 [1,24,25-$(OH)_3D_3$] remains largely unknown. The chief excretory route for vitamin D compounds is via the bile and feces [15, 26].

Much has been learned concerning the physiology of calcium metabolism in relation to the action of parathyroid hormone and 1,25-$(OH)_2D_3$. The ability of animals and man to adapt to low dietary calcium by increasing their intestinal absorption is by means of increasing 1,25-$(OH)_2D_3$ biosynthesis [30, 59]. Furthermore, it has now been shown that the parathyroid glands represent the primary signal in this situation. Thus, parathyroid hormone stimulation of intestinal calcium absorption is mediated by increased 1,25-$(OH)_2D_3$ biosynthesis [60]. On the other hand, the mobilization of calcium from bone is dependent on both 1,25-$(OH)_2D_3$ and parathyroid hormone [61].

The resurgence of interest in vitamin D chemistry because of the discovery of active forms has brought about the synthesis of new analogs [15], the most important so far being 1α-hydroxyvitamin D_3 (1α-OH-D_3) [62]. 1α-OH-D_3 functions by virtue of its conversion to 1,25-$(OH)_2D_3$ *in vivo* [63, 64]. Both 1,25-$(OH)_2D_3$ and 1α-OH-D_3 have been demonstrated to be of great value in the treatment of renal osteodystropy, hypoparathyroidism, pseudo-hypoparathyroidism, vitamin D dependency rickets, phenobarbital and dilantin-induced osteomalacia, and corticoid-induced osteoporosis [2, 15]. In addition, pharmacological amounts of 25-OH-D_3 are also useful in therapy of these conditions, probably by serving as an analog of 1,25-$(OH)_2D_3$ [15]. Of considerable interest is recent work which suggests that defective 1,25-$(OH)_2D_3$ biosynthesis could be involved in the genesis of post-menopausal and senile osteoporosis [65].

It is likely that the vitamin D system will now receive a great deal of attention from all scientists, ranging from the organic chemists seeking to make important and interesting new analogs of the active forms of vitamin D to physicians who will be anxious to try them in a variety of diseases involving calcium and phosphorus. It might, therefore, be expected that the pharmacology of these compounds will receive a great deal of attention in the immediate future.

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