Metabolism and Molecular Mechanism of Action of Vitamin D: 1981

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Summary
Cholecalciferol must be regarded as a pro-hormone rather than a vitamin, since it is normally produced in skin under the influence of ultraviolet light. Cholecalciferol must be metabolized in liver to 25-hydroxycholecalciferol and subsequently to 1,25-dihydroxycholecalciferol before it can act on intestine, bone and kidney to provide calcium and phosphorus for bone mineralization and neuromuscular activity. 1,25-Dihydroxycholecalciferol is metabolized in liver and intestine to a C-23-carboxylic acid that is inactive. 25-Hydroxycholecalciferol is metabolized to a variety of metabolic products, including 23S,25R-25-hydroxycholecalciferol, 23S,25S-25-hydroxycholecalciferol-26,23-lactone, 24R,25R-25-dihydroxycholecalciferol and 25,26-dihydroxycholecalciferol. These metabolites are not involved in the known actions of vitamin D. 1,25-Dihydroxycholecalciferol localizes in the nuclei of target organs through a receptor mechanism. It is believed to initiate transcription of DNA that codes for calcium and phosphorus transport proteins, the nature of which is undetermined. Production of 1,25-dihydroxycholecalciferol is stimulated by low plasma calcium during pregnancy and lactation, 1,25-dihydroxycholecalciferol levels are greatly increased to meet calcium demands. However, vitamin D is not absolutely essential for reproduction. It is likely that some other hormone, possibly prolactin, functions at these periods to mobilize calcium. The clinical application of the vitamin D hormone and its analogues to the treatment of bone disease is presented to illustrate the application of basic science to medical practice. Evidence for each of these points is presented.

Introduction
In the early part of the century the disease rickets appeared in epidemic proportions in the children of North America and Western Europe (Hess, 1929). At this time the brilliant work of several nutritional biochemists led to the concept of accessory food factors called vitamins. These discoveries undoubtedly inspired Sir Edward Mellanby (1919) of the United Kingdom to seek evidence that rickets was, in fact, a dietary deficiency disease. He indeed was able to produce the disease in dogs by dietary means and prevented the disease by the administration of cod liver oil, a substance known to contain the fat-soluble vitamin A discovered originally by McCollum and his co-workers provided clear evidence that another substance also found in cod liver oil but different from vitamin A was responsible for the healing of rickets (McCollum et al., 1922). Sir Edward Mellanby therefore reasoned that the healing of rickets was yet another property of the fat-soluble vitamin A. Subsequent work by McCollum and his co-workers provided clear evidence that another substance also found in cod liver oil but different from vitamin A was responsible for the healing of rickets (McCollum et al., 1922). He termed this substance


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'vitamin D.' While Sir Edward Mellanby was making his magnificent contribution, Huldshinsky, a pediatrician in Vienna, and independently Chick, a pediatrician from the United Kingdom, demonstrated that ultraviolet light from sunlight or artificial sources could also heal rickets (Huldshinsky, 1919; Chick et al., 1923). Thus in 1922, it appeared that cod liver oil and ultraviolet light were equivalent in preventing this dietary deficiency disease. Steenbock & Black (1924), concluding several years of investigation into healing properties of ultraviolet light, provided the solution to this dichotomy. Their work and that of Hess & Weinstock (1924) demonstrated that ultraviolet light induced antirachitic activity in the sterol fraction of foods. This brilliant discovery provided the means whereby rickets could be eliminated as a major medical problem, inasmuch as the food supply could be enriched with vitamin D. It also provided a source of activated sterols for the ultimate isolation and identification, which was completed in 1931 by Askew et al. (1931) in the United Kingdom and somewhat later by Windaus and collaborators in Germany, culminating in the identification of vitamin D3 as the antirachitic vitamin (Windaus et al., 1932). Windaus and his collaborators subsequently prepared 7-dehydrocholesterol synthetically, and demonstrated its conversion into yet another important vitamin D substance, cholecalciferol (Windaus et al., 1936). Windaus & Bock (1937)
then isolated 7-dehydrocholesterol from pig skin, providing the presumptive evidence that cholecalciferol is the form of vitamin D produced in skin. This was only recently confirmed by the isolation and identification of cholecalciferol from skin of vitamin D-deficient rats irradiated with ultraviolet light (Esvelt et al., 1978; Holick & Clark, 1978). Thus, cholecalciferol normally is produced in skin by incident ultraviolet irradiation from sunlight and is not required in the diet under these circumstances. Because man does not always receive sufficient amounts of ultraviolet light, vitamin D has become a dietary essential. Thus cholecalciferol is in reality a pro-hormone rather than a vitamin.

The functions of vitamin D

A deficiency of vitamin D results in either one of two diseases: rickets in the young, growing, higher animal, and osteomalacia in the adult (Hess, 1929). In the former disease, the osteoblasts of bone and the chondroblasts of epiphyseal-plate cartilage elaborate approximately normal organic matrix, composed predominantly of collagen but including mucoproteins and mucopolysaccharides. Under normal circumstances, the collagen fibrils become mineralized with calcium and phosphorus ultimately in the form of hydroxyapatite (Neuman & Neuman, 1938). These calcium deposits impart the necessary rigidity to the collagen fibrils required for skeletal function. In vitamin D deficiency, the collagen fibrils fail to mineralize. The failure of mineralization of the soft and pliable collagen rope structures results in twisting and bending under the stress of weight and muscle operation. This results in the overt deformities characteristic of rickets. Ultimately, death would result of the organism because of impairment of internal organ function by collapse of the structural elements protecting the internal viscera (Sembrell & Harris, 1954; DeLuca, 1978a). The essence of vitamin D action, therefore, is the deposition of calcium and phosphorus in the collagen fibrils. Although it has been believed that vitamin D may play a role in the mineralization process itself, so far evidence on this is lacking. This idea, however, must remain, since it cannot be disproved and there is clinical evidence to support it (DeLuca, 1978a). The major reason for failure to mineralize the collagen fibrils in vitamin D deficiency is the insufficient supply of calcium and phosphorus to the calcification sites (DeLuca, 1967, 1978a). Blood is normally supersaturated with calcium and phosphorus and with regard to the hydroxyapatite of bone. In vitamin D deficiency, the calcium and phosphorus levels in blood are under-saturated and are thereby unable to provide the necessary substrate for the mineralization process. The essence of vitamin D action, therefore, in preventing the supervention of disease is the elevation of plasma calcium and phosphorus to levels that will support normal mineralization of bone. In addition to this important role of calcium and phosphorus, it must be recognized that calcium plays important roles in the neuromuscular system (DeLuca, 1977, 1978a). In the absence of adequate amounts of ambient calcium, the convulsive state of hypocalcaemic tetany results, a very serious disorder that will result in death unless rapidly corrected. In most species of adults, the epiphyseal plate is closed and, hence, endochondral elongation of bones no longer takes place. Nevertheless, bone is constantly being remodelled to keep the skeletal structure in good repair (Frost, 1966). Remodelling is a process whereby osteoclasts of bone resorb old bone, which is then followed by osteoblast elaboration of new matrix and mineralization. In vitamin D deficiency, the entire remodelling process is slowed down (Frost, 1966), but especially the mineralization of new organic matrix elaborated by osteoblasts. This results in weak bones that fracture easily. This disease is known as osteomalacia and is very closely related to the disease rickets from a biochemical point of view.

Vitamin D brings about the elevation of plasma calcium and phosphorus to levels that support normal mineralization by a variety of mechanisms (DeLuca, 1967, 1977, 1978a). Vitamin D is the only known hormone to stimulate active intestinal calcium absorption (DeLuca, 1978a). In this process, calcium is transported against an electrochemical potential gradient, with phosphorus the normal accompanying anion. The active form of vitamin D is responsible for this transportable calcium (DeLuca, 1978a). In addition to this process, the vitamin D hormone stimulates the active transport of phosphate (DeLuca, 1978a). These then represent two sources of calcium and phosphorus that ultimately support the plasma levels, and hence mineralization. However, the absorption of calcium is not a dependable process, since dietary levels of calcium vary considerably. To provide sufficient amounts of calcium for the neuromuscular functions, bone serves as a reservoir of calcium in addition to its structural role (DeLuca, 1978a). To mobilize calcium from the fluid immediately bathing crystals of bone, the membrane barrier between the plasma compartment and the bone fluid compartment is activated to transport calcium to the plasma compartment (DeLuca, 1978a). This stimulation is primarily under the direction of parathyrin, but in order to carry out this function the vitamin D hormone must also be present (DeLuca, 1978a). The two hormones then operate in concert to provide for the mobilization of calcium from bone to the plasma. Another possible site of action of the vitamin D hormone is in the distal renal tubule. The kidney reabsorbs 99% of the filtered load of calcium even in the absence of parathyrin and vitamin D (DeLuca, 1978a). However, the residual 1% of the filtered load is under control of these two humoral agents in the distal tubule (DeLuca, 1978a). It is believed that the vitamin D hormone acts in concert with parathyrin to bring about the reabsorption of calcium in the distal tubule. Vitamin D is not believed to act directly on the reabsorption of phosphorus in the renal tubule. These actions of the vitamin D hormone, therefore provide calcium and phosphorus for mineralization.

The metabolism of vitamin D

As a result of some 15 years of investigation, it is now well known that vitamin D must first be converted in the liver into 25-hydroxycholecalciferol [25(OH)D3] (DeLuca, 1967, 1977, 1978b; Kodeck, 1974; Haussler & McCaie, 1977). This compound must be subsequently activated further, a process taking place exclusively in the kidney, to form the final vitamin D hormone, 1,25-dihydroxycholecalciferol 1,25(OH)2D3, as illustrated in Fig. 1. These compounds were isolated, identified and chemically synthesized in our laboratory (DeLuca & Schnoes, 1976). However, it was the work of Fraser & Kodeck (1970) that clearly demonstrated that the kidney is the site of the second conversion. Since nephrectomized animals do not respond to physiological amounts of 25(OH)D3, but do respond to 1,25(OH)2D3 normally in terms of intestinal calcium transport and bone calcium mobilization, it became clear that 1,25(OH)2D3 is a vitamin that is the only source of calcium absorption (DeLuca, 1978a). Since 1,25(OH)2D3 is made exclusively in the
kidney in the non-pregnant animal and has its action in intestine and bone, it must be regarded as a hormone (DeLuca, 1974; Kodicek, 1974).

We have addressed the question of whether 1,25(OH)2D3 must be further metabolized before function. With the use of radioactive 1,25(OH)2D3 labelled in the side chain with 3H, it could be demonstrated that at the time the intestine responds to 1,25(OH)2D3, the only labelled substance of any significance found in the lipid fraction of that organ is 1,25(OH)2D3 (Frolk & DeLuca, 1971). Unfortunately, much of the label could not be accounted for in the lipid fraction, and synthesis of 4-14C-labelled 1,25(OH)2D3 led to the discovery of a side-chain-cleavage reaction (Kumar et al., 1976). The product of this metabolism was subsequently isolated and identified as the C-23-carboxylic acid termed "calcitroic acid", as shown in Fig. 2 (Esvelt et al., 1979). This metabolite of 1,25(OH)2D3 could account for some 40% of the 1-hydroxylated vitamin D compounds found in the intestine at the time of response (Esvelt & DeLuca, 1981).

However, inasmuch as it is biologically inactive, this pathway is considered as the major inactivation route of 1,25(OH)2D3. The intermediary metabolism of its formation has not yet been deduced. 1,25(OH)2D3 is also converted into 1,24,25-tri-hydroxycholecalciferol [1,24,25(OH)3D]3 in what is considered to be a minor pathway (Holick et al., 1973). Thus far, therefore, 1,25(OH)2D3 is the most potent metabolite of vitamin D known, and furthermore, its metabolites all show decreased, if not absent, biological activity. Thus, 1,25(OH)2D3 at this stage appears to be the active form of vitamin D in the mechanisms described above.

It is important to note that 1,25(OH)2D3 is rapidly metabolized, unlike other forms of vitamin D and unlike other steroid hormones. The lifetime of 1,25(OH)2D3 has been estimated to be between 2 and 5h in plasma (Coburn et al., 1981). Thus, in administering the compound for testing of biological activity or to provide it as a therapeutic agent, it is essential that it be administered in small doses frequently. There is also evidence that oral administration of this compound is not as effective as it is through the parenteral route (Tanaka et al., 1972).

Other metabolism of vitamin D

After the identification of 25(OH)D3 and chemical synthesis of it in the radioactive form, it became clear that a variety of metabolic products result from this compound (Cousins et al., 1970). This led to the isolation and identification of several metabolites of vitamin D.

All known metabolism of vitamin D progresses through the 1,25(OH)2D3 intermediate. Although much of the metabolism of 25(OH)D3 occurs in kidney, further metabolism is recognized in other tissues (DeLuca, 1978a). Fig. 3 demonstrates the known metabolic products of 25(OH)D3, originating in the kidney. Production of 1,25(OH)2D3 has been discussed above. In 1970, 25,26(OH)2D3 was isolated and identified (Suda et al., 1970). It has been chemically synthesized and its configuration demonstrated to be 25S,26(OH)2D3 (Redel et al., 1980). This compound has limited biological activity, and is produced in kidney and elsewhere (Tanaka et al., 1978). It is not likely to be a significant pathway of vitamin D metabolism and has recently been shown not to be an intermediate in the synthesis of the 25(OH)D3-26,23-lactone (Tanaka et al., 1990a). Although this compound is known to be made in kidney (Tanaka et al., 1978) and has little biological significance, it has recently been surprisingly rediscovered (Napoli et al., 1981). A new pathway of metabolism of cholecalciferol is the conversion of 25(OH)D3 into 23,25(OH)2D3 (Tanaka et al., 1981). The configuration of the 23-hydroxy group has been shown to be S. This compound is then subsequently converted in the kidney into another metabolite of considerable structural interest. This compound is 23S,25R-25(OH)2D3-26,23-lactone (Tanaka et al., 1978; Wichmann et al., 1979). Unfortunately, both metabolic products show no biological activity in the known systems that are responsive to vitamin D (C. Smith & H. F. DeLuca, unpublished work). This substance is present in normal plasma of animals and man, but is markedly increased under conditions of high vitamin D dosage (Shipard & DeLuca, 1980). Exactly what its role might be physiologically remains unknown. It is, however, a prominent metabolite of vitamin D.

The pathway that has received the most interest other than the 1-hydroxylation pathway is the formation of 24R,25(OH)2D3 (DeLuca, 1979a; DeLuca & Schnoes, 1976). This compound was isolated and identified in 1970 and the correct structure deduced in 1972. It was chemically synthesized and its configuration determined to be 24R,25(OH)2D3 (Tanaka et al., 1975a). This compound by itself has weak biological activity in birds (Boris et al., 1977; Holick et al., 1976), and has significant biological activity in vitamin D-deficient rats (Tanaka et al., 1975a). The production of this substance is regulated, and hence it had been supposed that this compound might have important biological activity (Boyle et al., 1973). Nevertheless, it could be demonstrated that this compound is inactive in vitamin D-deficient rats in the absence of kidneys (Boyle et al., 1973). It thus became clear that its activity was by virtue of its conversion into 1,24R,25(OH)2D3, which serves as an analogue of the hormonal form, 1,25(OH)2D3 (Holick et al., 1973). Nevertheless, 24R,25(OH)2D3 has been tested together with 1,25(OH)2D3 in a variety of systems, resulting in the suggestions that: (1) it is important as a mineralizing form of vitamin D (Ornoy et al., 1978), (2) it is required for hatchability of chick embryos (Henry & Norman, 1978), (3) it is required for suppression of parathyroid secretions (Henry et al., 1977), and (4) it is required for normal metabolism and function of epithelial-plate cartilage (Garabedian et al., 1978). These suggestions were based on either experiments in vitro on cartilage growth or on experiments in vivo in which 1,25(OH)2D3 was supplied orally on a once-a-day basis, resulting in incomplete response to 1,25(OH)2D3. This compound was then supplemented with 24,25(OH)2D3 in moderate to very high doses. Under these circumstances, 24R,25(OH)2D3 appeared to have additional action to the action of 1,25(OH)2D3. Unfortunately, these experiments in vivo did not take into account the widely differing pharmacokinetics of 1,25(OH)2D3 and 24,25(OH)2D3. Thus they do not take into account that some of the systems responsive to 1,25(OH)2D3 require stimulation every few hours, as for example in the bone mineralization area or in the elevation of plasma phosphorus phenomenon (Tanaka & DeLuca, 1974). These experiments, therefore, in my view, are suspect.
examine the question of whether 24-hydroxylation might have significant and specific activity, it was conceived that a compound blocked in the 24 position might provide the necessary experimental insight. Therefore, together with two Japanese groups, a compound, 24,24-difluoro-25-hydroxycholecalciferol [24,24(F,)-25(OH)D,] was prepared (Kobayashi et al., 1979; Yamada et al., 1979) and tested biologically. In addition, we prepared 24,24(F,)-1,25(OH)D, biologically (Tanaka et al., 1980b). The structures of these compounds are illustrated in Fig. 4. We therefore tested whether 24,24(F,)-25(OH)D, could provide all of the functions of vitamin D, despite the fact that it cannot be 24-hydroxylated. By using a specific receptor protein for 1,25(OH),D, and the plasma transport protein for vitamin D as well as the enzyme that metabolizes 25(OH)D, to 1,25(OH),D, it could be shown that the fluoro groups do not mimic hydroxy functions, but act as expected as analogues of hydrogen (Halloran et al., 1981). Furthermore, no 24,25(OH),D, could be detected in the plasma of vitamin D-deficient animals supported on the 24,24(F,)-25(OH)D, compound. In examining the biological responses, it became clear in both rats and chicks that the 24,24(F,)-25(OH)D, was equal in biological activity in intestinal calcium transport, in bone calcium mobilization, in the mineralization of bone, and in the elevation of plasma phosphorus concentration (Halloran et al., 1981; Tanaka et al., 1979a; Okamoto et al., 1981). We also submitted the resulting bone from both rats and chicks to Dr. Webster Jee and Dr. Scott Miller of the University of Utah to perform histomorphometry measurements and to Dr. Michael Parfitt and his group at Henry Ford Hospital for histomorphometric examination and histological examination. Bone mineralization is perfectly normal in the animals receiving the 24,24(F,)-25(OH)D, (Miller et al., 1981).

We then wished to examine whether any apparent lesion could be induced by supporting animals through an entire generation on either 1,25(OH),D, or 24,24(F,)-25(OH)D, as their sole source of vitamin D (R. J. Brommage, K. R. Jarnagin, & H. F. DeLuca, unpublished work). We obtained vitamin D-deficient rat pups born to mothers maintained on a vitamin D-free diet from weaning. These pups were supported by (1) 1,25(OH),D, provided parenterally by Alzet Minipumps, or (2) 25(OH)D, either orally or by minipump, or (3) 24,24(F,)-25(OH)D, orally, or (4) no vitamin D at all. The results were very clear. The animals supported on 1,25(OH),D, were perfectly normal in all respects and were able to reproduce normally. Pups born to these mothers continued to be normal throughout the examination period of an additional 6 weeks. Similar results were obtained when 24,24(F,)-25(OH)D, was provided. On the other hand, vitamin D-deficient animals did very poorly. The results in Fig. 5 illustrate that the bone mineral content of the female rats supported by this procedure was normal regardless of the source of vitamin D, whereas the deficient animals clearly have undermineralized bones. Furthermore, this phenomenon is evident in the pups born to mothers supported in this fashion and maintained for 6 weeks post partum on this regime. Thus these studies not only show that 24,24(F,)-25(OH)D, or 1,25(OH),D, support all the known functions of vitamin D but also provide strong evidence that there is no obvious physiological function of 24,25(OH),D,.

The results of improper mineralization of bone reported previously in animals given 1,25(OH),D, is undoubtedly related to the route of administration and frequency of administration of the compound. Furthermore, we have demonstrated quite clearly that the hatchability of eggs laid by hens supported on 1,25(OH),D, can be returned to normal with the 24,24(F,)-25(OH)D, as well as large doses of 24,25(OH),D,. Thus the necessity for 24-hydroxylation for hatchability of hens' eggs is also suspect (Henry & Norman, 1978). Fig. 6 demonstrates that 24,24(F,)-25(OH)D, is fully able to support normal egg
hatchability when provided to hens supported on 1,25(OH)\(_2\)D\(_3\) and who lay eggs showing only 20% of normal hatchability (S. Ameenuddin & H. F. DeLuca, unpublished work). Thus it is clear that 24,25(OH)\(_2\)D\(_3\) has no known function, and further, the only pathway of activation of the vitamin D molecule to a hormonal form is 25-hydroxylation followed by 1-hydroxylation.

It should be mentioned that 1-hydroxylation is not exclusively found in the kidney. It can be found in placenta (Tanaka et al., 1979b), and thus pregnant rats can produce 1,25(OH)\(_2\)D\(_3\) in the anephric state (Gray et al., 1979). Of considerable interest are the reports that cultures of bone and other tissues are capable of producing 1,25(OH)\(_2\)D\(_3\) (Turner et al., 1980). Unfortunately, these experiments are not supported by results in vivo. As shown by Fraser & Kodicek (1970) initially, confirmed in our own laboratory (Gray et al., 1971) and more recently with radioactive 25(OH)D\(_3\) of specific radioactivity 160Ci/mmol, no 1,25(OH)\(_2\)D\(_3\) is found in bone, intestine and other tissues when animals are in the anephric and non-pregnant state. Unfortunately, the reported culture experiments did not provide adequate identification of the product claimed to be 1,25(OH)\(_2\)D\(_3\). Thus there are two points to be made: (a) either the compound produced by the cultures is not 1,25(OH)\(_2\)D\(_3\), or (b) if it is 1,25(OH)\(_2\)D\(_3\), this expression of activity is not translated into the 'in vivo' state.

**Nature of the two significant hydroxylases responsible for vitamin D activation**

The 25-hydroxylation of vitamin D is not an exclusive property of the liver. It is known in hepatectomized animals or animals whose liver has been isolated from the circulation that some 25(OH)D\(_3\) can be produced from radioactive cholecalciferol (Olson et al., 1976). Tucker et al. (1973) first demonstrated the existence of some 25-hydroxylation in intestine and kidney. This was subsequently confirmed in our laboratory (Bhattacharyya & DeLuca, 1974a). However, since cholecalciferol specifically and rapidly accumulates in the liver (Ponchon & DeLuca, 1969), and since the isolation of the liver from the circulation decreases the amounts of 25(OH)D\(_3\) produced to very low levels (Olson et al., 1974), it is concluded that the liver is the most significant, if not the sole, source of production of 25(OH)D\(_3\) under physiological conditions. To carry out this reaction, two enzymes have been suggested. One is a microsomal enzyme (Bhattacharyya & DeLuca, 1974b) and another is mitochondrial (Björkhem & Holmberg, 1978). The microsomal enzyme is fuelled by NADPH and molecular oxygen and requires a cytoplasmic factor. This hydroxylase has a K\(_m\) for 25(OH)D\(_3\) of about 10\(^{-8}\)M (Madhok & DeLuca, 1979), in keeping with the low concentration of vitamin D provided to the animal physiologically. This system has been solubilized, resolved into two components and reconstituted (Yoon & DeLuca, 1980a). The mechanism of this hydroxylase is shown in Fig. 7. It is my view that this hydroxylase is the predominant

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**Fig. 6. Hatchability of chick embryos from eggs laid by hens supported on the indicated forms of vitamin D**

Laying hens were kept on the diets containing the indicated forms of vitamin D (Vit.D) until such time as the 1,25(OH)\(_2\)D\(_3\)-supplemented animals gave only 20-40% hatchability in their eggs. These animals were then shifted to diets containing 24,25(OH)\(_2\)D\(_3\), or 1,25(OH)\(_2\)D\(_3\) plus 24,25(OH)\(_2\)D\(_3\), or 24,24(F\(_{2}\))-25(OH)D\(_3\). Within 1 week of administration, those hens given 24,24(F\(_{2}\))-25(OH)D\(_3\) or the combination of 1,25(OH)\(_2\)D\(_3\) and 24,25(OH)\(_2\)D\(_3\) were producing normal eggs in terms of hatchability.

**Fig. 7. Mechanism of microsomal vitamin D 25-hydroxylation in liver**

Abbreviations: Fp, flavoprotein; P\(_{450}\), cytochrome P-450.
Abbreviation: Fp, flavoprotein.

Mechanism of action of 1,25(OH)2D3

The only significant work on mechanism of action carried out to date has been on the intestinal calcium-transport response to 1,25(OH)2D3. This response, however, is quite complex, as illustrated in Fig. 9 (Halloran & DeLuca, 1981a). As is evident in the time course of response of a vitamin D-deficient rat to a single injection of 1,25(OH)2D3, there are probably two mechanisms. The first is a very rapid response in which the intestinal calcium transport reaches a maximum at 6h and then decays to a minimum at 18h. The intestine then shows a second rebound response, reaching a maximum at 24–48h. The initial response can be re-induced after the 48h period, but the second is not further induced. It appears that the initial response is on existing villus cells and the second response may well be an effect on the crypt cells, programming them to transport calcium once they have migrated into the villus region. In any case, these two distinct mechanisms must be borne in mind when results from different laboratories are compared.

Initial biochemical measurements have revealed that the major localization of 1,25(OH)2D3 in intestine before the early response is in the nuclear fraction (Chen et al., 1970). Because of the problem of redistribution during homogenization and cell fractionation, this result was suspect. We therefore chemically synthesized radioactive 1,25(OH)2D3 labelled in the 26 and 27 positions with 3H of sufficient radioactivity to carry out frozen-section autoradiography (Napoli et al., 1980). We carried out these experiments in our laboratory (Zile et al., 1978) and together with Dr. Walter Stumpf at the University of North Carolina (Stumpf et al., 1979). The results shown in Fig. 10 demonstrate that it is the nuclei of the villus cells that accumulate 1,25(OH)2D3 within a 4h after injection and before the initiation of intestinal calcium transport, thus confirming that 1,25(OH)2D3, at least in part, must function through a nuclear mechanism (DeLuca et al., 1981). In accordance with this mechanism, as expected, the presence of a highly specific binding protein for 1,25(OH)2D3 can readily be demonstrated in washed intestinal-mucosal cells provided with adequate thiol protection from dithiothreitol and under conditions of high salt concentration (Franceschi et al., 1981). This receptor sediments at 3.7S on sucrose-gradient systems and has a Kd of 5 x 10^-11 M in the case of chick intestine. Many of the kinetic parameters have been determined in the crude state. This receptor is not found in such non-target tissues as smooth muscle, skeletal muscle, liver and fibroblasts. It is, however, found in tissues not previously appreciated to be targets of 1,25(OH)2D3 action, such as skin, parathyroid glands, stomach mucosa, pituitary glands and mammary glands (Franceschi et al., 1981). Very recently, we have been able to isolate this receptor molecule in pure form, as illustrated by the sodium dodecyl sulphate/polyacrylamide gels shown in Fig. 11 (Simpson & DeLuca, 1982). Tube III represents the final purified receptor protein, and tube I represents the crude nuclear extract. The receptor has a molecular weight of 67000 and is a single polypeptide chain. We are currently in the process of studying this protein biochemically, in the generation of antibodies, and in pursuit of further studies on nuclear interaction. Since it has a sedimentation coefficient of 3.7S with a molecular weight of only 67000, it is believed to be an oblong protein. It is likely that the availability of pure receptor will aid in our understanding of the nuclear sites where 1,25(OH)2D3 and its ligand bind in preparation for action.

Insight into the necessity for 1,25(OH)2D3 to interact with this receptor molecule before expression of activity has recently been obtained in young developing rats. Rat pups, when consuming mothers’ milk, show no dependency upon vitamin D...
in terms of intestinal calcium transport (Halloran & DeLuca, 1980a). Thus, intestines taken from vitamin D-deficient rat pups or pups maintained in the vitamin D-repleted state show no difference in intestinal calcium transport. Sensitivity of intestinal calcium transport to 1,25(OH)$_2$D$_3$ begins to appear at 14–18 days of life, and this exactly correlates with the appearance of the intestinal receptor for 1,25(OH)$_2$D$_3$ as illustrated in Fig. 12 (Halloran & DeLuca, 1981b). Furthermore, vitamin D-dependency rickets Type II, an experiment of nature in which there is a defect in target-organ responsiveness to 1,25(OH)$_2$D$_3$ (Bell et al., 1978), is likely to be a disease wherein the receptor to 1,25(OH)$_2$D$_3$ is absent. From these experiments it seems quite apparent that 1,25(OH)$_2$D$_3$ must interact with the receptor-like protein before it can express its action.

There has been considerable debate as to whether 1,25(OH)$_2$D$_3$ can exert its action on the intestine by a non-nuclear mechanism (Rasmussen & Gustin, 1978). These suggestions are based on experiments in which animals are given actinomycin D to block transcription of DNA. Under these circumstances, 1,25(OH)$_2$D$_3$ is able to stimulate intestinal calcium transport (Bikle et al., 1978). Unfortunately, actinomycin D given at 1μg/g body wt. does not block the synthesis of nuclear RNA in intestine by more than 30%. Thus, these experiments are in my view inconclusive. If more actinomycin D is given to the animals, death results, which prohibits any conclusions regarding the nature of the response to 1,25(OH)$_2$D$_3$. In short, experiments in which protein- and RNA-synthesis inhibitors are given in vitro cannot be used as evidence if they fail to block the responses in question. It became clear to us that these responses must be tested in an isolated environment such as a culture system where toxicity of other cell types would not be a problem. We therefore utilized the embryonic intestinal organ-culture system developed by Corradino (1973), in which intestines are taken from chick embryos 1 day before hatching and cultured in vitro. When 1,25(OH)$_2$D$_3$ is added in vitro at 75 nM, a marked response in carrier-mediated intestinal calcium uptake can be observed (Franceschi & DeLuca, 1981b). The $K_m$ for this response is about 1–2 μM, in exact agreement with the $K_m$ for calcium transport in young rats and chicks (DeLuca, 1978b). When cycloheximide is added in vitro to this system at 5 μM, it blocks totally the response to 1,25(OH)$_2$D$_3$ (Franceschi & DeLuca, 1981b). To eliminate the criticism that this is merely toxicity to the cells, the cycloheximide can be removed somewhat later and the response reappears. Similar experiments have been carried out with actinomycin D, and furthermore, other RNA-synthesis inhibitors such as anisomycin and α-amanitin block this response (Franceschi & DeLuca, 1981b). Thus it is apparent to us that intestinal calcium-transport response to 1,25(OH)$_2$D$_3$ is dependent on RNA and protein synthesis. It has been argued that this response might be true for embryonic intestine, but does not hold for young animals. We have, however, recently been able to administer actinomycin D and to block entirely the intestinal calcium transport response to 1,25(OH)$_2$D$_3$ without causing death of the animals (Kendrick et al., 1981). Thus, it is our current belief that the machinery activated by 1,25(OH)$_2$D$_3$ is, indeed, nuclear in nature, involving RNA and protein synthesis.
Fig. 11. Sodium dodecyl sulphate/polyacrylamide tube gels illustrating the purity of the isolated nuclear receptor molecule for 1,25(OH)\textsubscript{2}D\textsubscript{3}.

I, crude nuclear extract; II, nuclear extract after three column-chromatographic procedures; III, the preparation of chick intestinal cytosol after all purification procedures including an electrofocusing column; BSA, bovine serum albumin standard. Abbreviations: BPB, Bromophenol Blue; '24K' etc. signify mol.wts. of 24000 etc.

Fig. 12. Time course of appearance of the 1,25(OH)\textsubscript{2}D\textsubscript{3} receptor in the intestinal mucosa of developing rat pups

Fig. 13. Diagrammatic representation of the proposed mechanism of action of 1,25(OH)\textsubscript{2}D\textsubscript{3} (calcitriol) in intestinal villus cells

**Regulation of vitamin D metabolism**

An important aspect of the vitamin D endocrine system is the regulation of 1,25(OH)\textsubscript{2}D\textsubscript{3} production. After the isolation and identification of 1,25(OH)\textsubscript{2}D\textsubscript{3} in 1971 by our group and its demonstration of potent biological activity in the absence of kidneys came the realization that the production of this hormonal form of vitamin D might be regulated by the products that it seeks to affect, namely plasma calcium and phosphorus (Boyle et al., 1972). In experiments well known among physiologists, the plasma level of 1,25(OH)\textsubscript{2}D\textsubscript{3} is markedly affected by plasma calcium concentration. Under conditions of low plasma calcium, high levels of 1,25(OH)\textsubscript{2}D\textsubscript{3} are found. When plasma calcium levels are above normal, little or no 1,25(OH)\textsubscript{2}D\textsubscript{3} is produced. Thus the need for calcium markedly stimulates production of 1,25(OH)\textsubscript{2}D\textsubscript{3}. We therefore considered the possibility that the parathyroid glands might be responsible for this regulation (Garabedian et al., 1972). Parathyroidectomized rats do not produce 1,25(OH)\textsubscript{2}D\textsubscript{3} in response to a hypocalcaemic challenge (Garabedian et al., 1972). Parathyrin on the other hand, administered to these parathyroidectomized animals caused a marked increase of 1,25(OH)\textsubscript{2}D\textsubscript{3} production (Garabedian et al., 1972; Fraser & Kodicek, 1973). Thus it became clear that the parathyroid glands are the intermediary in regulation of 1,25(OH)\textsubscript{2}D\textsubscript{3} production in response to the need for calcium.

When serum calcium concentration falls below normal, parathyroid glands secrete parathyrin (DeLuca, 1974). Parathyrin proceeds to the kidney and bone, where it performs several functions. In the kidney, it blocks renal tubular reabsorption of phosphate, a non-vitamin-D-dependent process. It stimulates 25(OH)\textsubscript{D}\textsubscript{3} 1-hydroxylase by an unknown mechanism, and it acts together with 1,25(OH)\textsubscript{2}D\textsubscript{3} to promote renal reabsorption of calcium. 1,25(OH)\textsubscript{2}D\textsubscript{3} secreted from the kidney proceeds to the intestine, where it stimulates intestinal calcium transport, and to bone, where it works together with parathyrin.
to promote mobilization of calcium from bone (Garabedian et al., 1974). The rise in plasma calcium then shuts down production of 1,25(OH)2D3.

In the thyroparathyroidectomized animals, plasma phosphorus can also be shown to regulate plasma levels of 1,25(OH)2D3 (Tanaka & DeLuca, 1973). Thus, low plasma phosphorus concentrations stimulate production of 1,25(OH)2D3, or at least its accumulation, whereas high plasma phosphorus suppresses. Thus there are two physiological signals responsible for control of 1,25(OH)2D3 production, namely plasma calcium through the parathyrin system and plasma phosphorus. This therefore represents the vitamin D endocrine system, which has been well studied and documented.

Of considerable interest is that there are other factors that regulate production of 1,25(OH)2D3. One of these is that 1,25(OH)2D3 itself shuts down 1α-hydroxylase and stimulates 24-hydroxylation of 25(OH)D3 (Tanaka et al., 1975b). In fact, 1,25(OH)2D3 is considered the inducer of the 24-hydroxylase enzyme. The reason for this also remains unknown.

Of considerable importance are the situations in which large demands for calcium and phosphorus are evident. For example, during rapid phases of growth, plasma levels of 1,25(OH)2D3 are elevated, presumably because of the need for large amounts of calcium for mineralization of bone (Chesney et al., 1980a). This has been elegantly demonstrated by O'Riordan and his group (Papapoulos et al., 1980), who showed quite clearly that vitamin D-deficient patients given small amounts of vitamin D for healing of their osteomalacia show a very marked elevation of 1,25(OH)2D3 to the 200 pg/ml range during the mineralization process. This is to be compared with what is considered to be a normal value of about 30 pg/ml. Thus a statement of normal levels of 1,25(OH)2D3 is essentially meaningless unless the physiological circumstances under which this level is determined is described. Often clinical investigators will claim that plasma levels of 1,25(OH)2D3 are normal despite the presence of large amounts of uncalcified bone, using this as evidence that some other form of vitamin D must be necessary. However, when large amounts of uncalcified bone are present, plasma levels of 1,25(OH)2D3 must be very high to permit the rapid calcification necessary to heal the lesions.

One of the circumstances whereupon large amounts of calcium are required is in reproduction and lactation. There is no question that plasma 1,25(OH)2D3 rises to high levels during the terminal stages of pregnancy, when foetuses are being calcified, and during lactation, when large amounts of calcium are needed for milk production (Halloran et al., 1979; Pike et al., 1979). Upon learning this, we then raised the question of whether 1,25(OH)2D3 could support normal reproduction in the rat. We were surprised to learn that vitamin D is not absolutely required for reproductive activity in the rat (Halloran & DeLuca, 1979). As shown in Table 1, young rat pups born to vitamin D-deficient mothers have identical levels of calcium in their bodies as compared with the D-replete counterparts. These rat pups will develop normally until 1,25(OH)2D3 becomes necessary at about 7–14 days of neonatal life (Halloran & DeLuca, 1981c). This surprising lack of dependence of the female rat on vitamin D for reproduction led us to examine further their source of calcium. As shown in Fig. 14, bone loss is identical in vitamin D-deficient mothers and in mothers replete in vitamin D. After weaning of the rats, the vitamin D-deficient mothers do not regain their lost calcium, whereas the vitamin D-replete mothers do. As a result, vitamin D-deficient mothers cannot undergo a second pregnancy because of the lack of calcium available in bone (Halloran & DeLuca, 1980d). Of considerable interest and not shown here is that intestinal calcium absorption also increases during pregnancy and lactation in the vitamin D-deficient mother even in the absence of vitamin D (Halloran & DeLuca, 1980c). This result suggested that some other hormone must be involved in the mobilization of calcium during this period to provide calcium in the absence of the vitamin D and parathyroid systems. Although we have not conclusively demonstrated this, we have recently learned that prolactin, when injected into vitamin D-deficient male rats, can stimulate intestinal calcium transport (Pahuja & DeLuca, 1981). Thus we must begin to recognize that prolactin may directly affect intestinal calcium transport and bone calcium mobilization at critical periods during such conditions as pregnancy and lactation, and that this hormone may well become recognized as a calcitropic hormone during certain periods of life.

Clinical application of our understanding of the vitamin D endocrine system

Seldom in the field of basic science is one rewarded by having available a direct application of the findings to clinical medicine. The vitamin D system has been quite unique in this regard. Measurement of plasma levels of 25(OH)D3 is already widespread as an assessment of vitamin D status of a patient (Haussler & McCain, 1977). Thus, vitamin D deficiency is no longer a matter of question. It can be very quickly deduced by measuring plasma levels of 25(OH)D3. Furthermore, other kinds of disturbances can be more easily diagnosed by measurement of plasma levels of the vitamin D hormone, 1,25(OH)2D3 (Haussler & McCain, 1977). This can be measured either by radioimmunoassay or by radioreceptor assay. Both techniques require the isolation and purification of the vitamin D hormone before the competitive-binding measurement. In my view, there are advantages both for the radioimmunoassay and for the radioreceptor assay, depending upon the circumstances. Nevertheless, measurements of plasma 1,25(OH)2D3 levels are very useful in the diagnosis of renal osteodystrophy, hypoparathyroidism, osteomalacia and certain types of vitamin D-resistant rachitic conditions.

Of considerable importance is that 25(OH)D3, 1,25(OH)2D3, and its analogue, 1α(OH)D3, which was prepared as a synthetic exercise in our laboratory before the chemical synthesis of

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Table 1. Total body calcium of foetuses and pups from vitamin D-deficient mothers

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Ca (mg)</th>
<th>Total Ca (mg/g body wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(mg/g body wt.)</td>
<td></td>
</tr>
<tr>
<td>Foetuses (day 20 of pregnancy)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>7.3</td>
<td>1.9</td>
</tr>
<tr>
<td>+D</td>
<td>6.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Pups (60–72 h post partum)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>3.0</td>
<td>3.6</td>
</tr>
<tr>
<td>+D</td>
<td>2.8</td>
<td>3.7</td>
</tr>
</tbody>
</table>

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Fig. 14. Change in calcium content of femurs taken from pregnant vitamin D-sufficient (-----) and vitamin D-deficient (——) rats during pregnancy and lactation

Key: P-20, day 20 of pregnancy; L-14, day 14 of lactation; W, weaning; W + 3, 3 weeks post-weaning.
1,25(OH)₂D₃, are now used in the clinic. Management of hypoparathyroidism is now relatively efficient with the use of either 1α(OH)D₃ or 1,25(OH)₂D₃ (Neer et al., 1975). 1,25(OH)₂D₃ will directly stimulate intestinal calcium absorption without necessity for participation of parathyroid. Thus, oral calcium plus 1–5 μg of 1,25(OH)₂D₃ or an equivalent dose of 1α(OH)D₃ is very effective. Similar experience has been recorded for pseudohypoparathyroidism (Kooh et al., 1975). The most dramatic healing has been in the field of renal osteodystrophy, where there is a lack of the vitamin D hormone, at least in late stages of renal failure. It is likely that there are insufficient amounts of vitamin D hormone produced early in renal failure as a result of phosphate suppression of 1,25(OH)₂D₃ production and sheer loss of the organ responsible for 1,25(OH)₂D₃ production. Thus, the ratio of parathyroid to 1,25(OH)₂D₃ rises in the plasma early in this disease. In any case, renal-dialysis patients present with severe bone disease and, in the case of children, a lack of growth. Administration of 1,25(OH)₂D₃ at 1 μg per day given in small doses either twice or three times daily produces marked healing of bone in 90% of the cases (Haussler & McCain, 1977; Deluca, 1979). Approx. 10% of the renal osteodystrophic patients do not respond because of some problem in the mineralization system of bone and their hypersensitivity to 1,25(OH)₂D₃. Dramatic healing of bone and growth of children is illustrated in Fig. 15 (Chesney et al., 1980). A 3-year-old child with severe renal failure, lack of muscle tone, severe rickets and failure to thrive was administered 1,25(OH)₂D₃ at 0.5 μg per day for 14 months, giving rise to the clear growth and mineralization response.

Thus, the vitamin D endocrine system has been an exciting area of investigation with the added reward of clinical application, and will undoubtedly continue to be so in years to come. It is indeed a great honour and pleasure to put forth this interesting story in honour of a great chemist and biochemist, R. A. Morton of the University of Liverpool.

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