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NUCLEAR MECHANISM OF ACTION OF 1α,25-DIHYDROXYVITAMIN D₃

by

Joseph Edward Zerwekh

A Dissertation Submitted to the Faculty of the
COMMITTEE ON BIOCHEMISTRY (GRADUATE)
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1976
I hereby recommend that this dissertation prepared under my direction by Joseph Edward Zerwekh entitled Nuclear Mechanism of Action of 1α,25-Dihydroxyvitamin D₃ be accepted as fulfilling the dissertation requirement of the degree of Doctor of Philosophy.

Mark R. Haussler
Dissertation Director

After inspection of the final copy of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:

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This approval and acceptance is contingent on the candidate's adequate performance and defense of this dissertation at the final oral examination. The inclusion of this sheet bound into the library copy of the dissertation is evidence of satisfactory performance at the final examination.
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SIGNED: Joseph C. Zenueh
Dedicated to my wife,

JoAnn Graham Zerwekh
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ABSTRACT

1α,25-dihydroxyvitamin D₃ is the metabolite of vitamin D₃, which mediates the biochemical events that result in increased calcium absorption from the intestine. Analysis of the nuclear mechanism of action of this vitamin D metabolite in the chick intestinal mucosa cell nucleus has been undertaken to begin to characterize the nuclear events which culminate in an increased calcium absorption in this tissue. Both in vivo and in vitro experiments have been utilized, with not only 1α,25-dihydroxyvitamin D₃ and its natural precursors vitamin D₃ and 25-hydroxyvitamin D₃, but also the synthetic vitamin D analog, 1α-hydroxyvitamin D₃. This synthetic sterol is demonstrated to act with the same rapidity and efficacy as the natural hormone in promoting intestinal calcium absorption and bone calcium resorption in vivo. However, in vitro receptor studies show 1α-hydroxyvitamin D₃ is approximately three orders of magnitude less efficacious than 1α,25-dihydroxyvitamin D₃. This disparity is accounted for by demonstrating that 1α-hydroxyvitamin D₃ is rapidly metabolized to 1α,25-dihydroxyvitamin D₃ in vivo.

Physiological amounts of 1α,25-dihydroxyvitamin D₃ given to rachitic chicks in vivo stimulates the activity of the endogenous nucleoplasmic (form II) DNA-dependent RNA polymerase activity of intestinal nuclei 2-fold within 2 hours after administration of the sterol. No changes are observed in the nucleolar (form I) RNA polymerase activity within this time period. At this sterol concentration the increased RNA
polymerase activity is both tissue and sterol specific and only 1α-hydroxyvitamin D₃ is able to elicit the same increase in enzyme activity. Solubilization of nuclear protein and resolution of the RNA polymerases on DEAE-Sephadex reveals a similar increase in polymerase II activity when assayed on exogenous template.

1α,25-dihydroxyvitamin D₃ administration to rachitic chicks also results in an increase in the chromatin template activity of intestinal target tissue assayed in vitro using either Escherichia coli or wheat germ RNA polymerase. The maximum stimulation of template activity is 12 to 20% over control values and occurs 2 hours after administration of the sterol. This rapid effect precedes the biologic response to 1α,25-dihydroxyvitamin D₃ in the intestine and is not observed in other tissues such as liver or kidney. The in vivo enhancement of intestinal chromatin template activity is specific for the 1α,25-dihydroxyvitamin D₃ hormone in that equivalent doses of 25-hydroxyvitamin D₃ or vitamin D₃ did not elicit a response in 2 to 3 hours. Only 1α-hydroxyvitamin D₃ was able to mimic the natural hormone in vivo.

Further characterization of the nuclear mechanism of action of 1α,25-dihydroxyvitamin D₃ is performed by pre-incubating the hormone with target tissue cytosol at 0° for 1 hour to form hormone-receptor complexes which are subsequently incubated with purified intestinal nuclei for an additional hour at 25°. Chromatin isolated from these nuclei displays a 12 to 24% increase of template activity over similar preparations not containing hormone when assayed in vitro. The response is specific for
intestinal cell cytosol and for physiologic levels of 1α,25-dihydroxyvitamin D₃, but also occurred with pharmacologic doses of 25-hydroxyvitamin D₃.

These data imply that the nuclear mechanism of action of 1α,25-dihydroxyvitamin D₃ at its target tissue is similar to the molecular events of steroid hormone action and that through a specific alteration in gene transcription, 1α,25-dihydroxyvitamin D₃ is able to ultimately exert its physiologic response of enhanced calcium transport.
CHAPTER I

INTRODUCTION

Rickets, a disease characterized by abnormal bone ossification, has been known for many centuries. Little was known about this disease until 1919 when Mellanby (1) first demonstrated that it was caused by the lack of a dietary trace constituent. In 1922, McCollum et al. (2) cured rickets in dogs with a fat soluble constituent from cod liver oil and termed it vitamin D. Subsequently, it was discovered that antirachitic activity could be induced in foods (3) and rickets cured in children (4) by ultraviolet irradiation. This finding led to the isolation and identification of vitamin D$_2$ in 1932 (5) and vitamin D$_3$ in 1936 (7) in the laboratories of Windhaus and colleagues. Figure 1 pictures the formulae of these vitamins and their immediate precursors.

An animal can obtain vitamin D exogenously from a dietary supplement or endogenously via irradiation of its precursor, 7-dehydrocholesterol, present in the skin. Although the minimum daily requirement for the vitamin is not well defined, it is estimated to be 100 to 400 i.u.$^2$ in adult humans, and approximately 10 i.u. daily in the chick to prevent the symptoms of rickets (9). However, provided that an animal is

1. The chemical name of vitamin D$_3$ is 9,10-seco-5,7,10(19)-cholestatrien-3β-ol (6).

2. One international unit (i.u.) of vitamin D$_3$ or vitamin D$_2$ is defined (8) as 0.025 µg.
Figure 1. Compounds of the vitamin D family.

Ultraviolet radiation of provitamin D results in the production of vitamin D. The various combinations of side chains (R) with the sterol nuclei pictured comprise the respective provitamins, vitamins, and dihydrotachysterols.
exposed to sufficient sunlight, it requires no dietary vitamin D, thus making the classification of this sterol as a vitamin tenuous.

It is now known that vitamin D prevents rickets by maintenance of adequate serum calcium and phosphorus concentrations. This is accomplished by stimulation of intestinal calcium absorption (10) and intestinal phosphorus absorption (11) and the mobilization of calcium and phosphorus from bone (12). There is also evidence that vitamin D improves the renal re-absorption of calcium and phosphorus (13, 14). However, further studies are needed in this area before vitamin D can be considered to have a major biological function in the kidney. The best established function of vitamin D is to increase intestinal calcium transport (15-17). Since this is partly an active process, metabolic energy must be supplied by the cells carrying out the absorption in the intestinal villi. Although phosphate is the normal accompanying anion of this active process, it is now known that vitamin D stimulates a phosphorus transporting mechanism independent of that for calcium (11, 18-21). Vitamin D also plays an important role in the mobilization of calcium and phosphorus from previously formed bone and may also have an effect on bone formation (22) although the mechanism of this effect on bone is unknown (23). Thus, the intestine is a primary target organ for vitamin D while the skeleton is considered a second major target tissue.

One important characteristic of the action of vitamin D on calcium mobilization, first realized by Irving in 1944 (24), is the time lag between administration of the sterol and the resulting physiological response. The lag is apparently in part due to the necessity for
metabolic conversion of the vitamin to an activated form which performs
the biochemical functions attributed to the parent vitamin (25, 26, 27).
The lag may also be caused by the slow transportation of the active
metabolite to the target organ and to its subcellular receptor site(s).
Evidence will be presented in this dissertation which indicates that the
lag is also a consequence of the active metabolite participating in an
induction process involving the expression of new genetic information,
which is eventually utilized to elicit the physiological effect of the
vitamin.

Between 1964 and 1971 it was established that vitamin D is the
precursor of several biologically active metabolites. In 1968, the pre­
dominant vitamin D₃ metabolite in blood was isolated by Blunt, DeLuca,
and Schnoes (27), who determined its structure to be 25-hydroxyvitamin D₃
(25-OHD₃)
³. Since this metabolite displayed greater potency than vitamin
D₃ in curing rickets (28) and was observed to function directly upon
isolated intestine and bone to stimulate calcium translocation (29-31),
it was originally thought to be the active form of vitamin D₃ (32, 33).
However, 25-OHD₃ is now known to be the product of the initial step in
the activation of the vitamin and serves as the precursor to other active
vitamin D metabolites. Also, in 1968 Haussler, Myrtle, and Norman (34),
working in the laboratory of Norman, reported the detection of a polar
metabolite of vitamin D₃ and proposed it to be the active principle in

3. Abbreviations in this dissertation are: 25-hydroxyvitamin D₃
(25-OHD₃), 1α-hydroxyvitamin D₃ (1α-OHD₃), 1α,25-dihydroxyvitamin D₃
(1α,25-(OH)₂D₃), calcium binding protein (CaBP), ribonucleic acid (RNA),
messenger ribonucleic acid (mRNA), deoxyribonucleic acid (DNA), and
trichloroacetic acid (TCA).
the target organ, based upon its specific binding to intestinal mucosal chromatin and its biological activity equivalent to vitamin D₃. In 1969, Lawson, Wilson, and Kodicek (35) proposed that this new vitamin D metabolite contained an additional hydroxyl group at the 1-alpha position based upon a decrease in the ³H/¹⁴C ratio of the more polar metabolite following simultaneous administration of [1α-³H] vitamin D₃ and [4-¹⁴C] vitamin D₃. Furthermore, this metabolite was present in the liver, kidney, and intestines of vitamin D-deficient chicks, but only in the intestinal cell nuclei was the lowest ³H/¹⁴C ratio found, indicating preferential accumulation of this metabolite at the nucleus (36).

Haussler and coworkers isolated 3.5 µg of this metabolite from chickens and determined the purified material to be at least 5 times as effective as vitamin D₃ in stimulating intestinal calcium absorption and to act 3 times faster than either vitamin D₃ or 25-OHD₃ on an equal weight basis (37). This active vitamin D metabolite was then isolated from chick intestine (38) and subsequently from renal homogenates (39-41) and identified as 1α,25-dihydroxyvitamin D₃ [1α,25-(OH)₂D₃]. In addition to stimulating intestinal calcium absorption, 1α,25-(OH)₂D₃ was also found to be more active than vitamin D₃ or 25-OHD₃ in causing bone calcium resorption (42-44).

Thus, before vitamin D₃ can function in the stimulation of calcium transport it must undergo two enzymatic hydroxylations. 25-hydroxylation occurs in the liver, kidney, and intestine (45, 46), and the 25-OHD₃ intermediate is then converted to 1α,25-(OH)₂D₃ exclusively in the kidney (47). Since it is known that the renal synthesis of
$1\alpha,25-(\text{OH})_2\text{D}_3$ is feedback regulated by the calcium requirements of the animal (48), this active metabolite can be classified as a steroid hormone that mediates calcium and phosphate transport in target tissues such as intestine and bone.

Synthesis of $1\alpha,25-(\text{OH})_2\text{D}_3$ is not the only conversion $25\text{-OHD}_3$ may undergo. Several other vitamin D metabolites have been identified, indicating the existence of a more complex metabolic pathway for the vitamin, as illustrated in Figure 2. Either $24,25$-dihydroxyvitamin $\text{D}_3$ [24,25-(OH)$_2\text{D}_3$] or $25,26$-dihydroxyvitamin $\text{D}_3$ [25,26-(OH)$_2\text{D}_3$] can be produced from $25\text{-OHD}_3$ (49, 50). However, the physiological role of these other dihydroxy-D-vitamins is not presently understood, and they may represent initial products in the catabolism of $25\text{-OHD}_3$; however, both of these products display significant biological activity in the chick and rat (50-52). The synthesis and biological assay of various synthetic vitamin D analogs has been recently reviewed (53). These synthetic analogs may prove to be applicable in the treatment of patients with possible defects in the metabolism of vitamin D. One of these analogs in particular, $1\alpha$-hydroxyvitamin $\text{D}_3$ ($1\alpha\text{-OHD}_3$), has been shown to act as rapidly and with equal potency as $1\alpha,25-(\text{OH})_2\text{D}_3$ in stimulating intestinal calcium absorption and bone mineral resorption in both the rachitic chick (54) and vitamin D deficient rat (55). Evidence to be presented in this dissertation will show that $1\alpha\text{-OHD}_3$ undergoes rapid $25$-hydroxylation and can serve as an effective precursor of $1\alpha,25-(\text{OH})_2\text{D}_3$ (56). Therefore, $1\alpha\text{-OHD}_3$ is useful in studying $1\alpha,25-(\text{OH})_2\text{D}_3$ mediated events because of this rapid conversion.
Figure 2. Metabolic pathway for physiological amounts of vitamin D₃ in the chick.
Numerous lines of evidence have led to the conclusion that \( \alpha,25\text{-}(\text{OH})_2 \text{D}_3 \) is the active form of vitamin \( \text{D}_3 \). In addition to its being the most potent form of the vitamin in the stimulation of calcium mobilization \textit{in vivo} (37, 42-44, 57, 58) and \textit{in vitro} in isolated perfused intestines (31), intestinal organ culture (59) and embryonic bone culture (60), chronic administration of \( \alpha,25\text{-}(\text{OH})_2 \text{D}_3 \) to animals raised on a rachitogenic diet completely prevents all radiologic and histologic signs of rickets (61, 62). Thus, \( \alpha,25\text{-}(\text{OH})_2 \text{D}_3 \) mediates normal calcium metabolism and bone development and apparently carries out all the functions ascribed to vitamin \( \text{D}_3 \).

An understanding of the molecular mechanism of action of \( \alpha,25\text{-}(\text{OH})_2 \text{D}_3 \) in the small intestine is of great importance in the comprehension of mineral homeostasis in animals. One hypothesis for the mechanism of action of \( \alpha,25\text{-}(\text{OH})_2 \text{D}_3 \) in the intestine is that this sterol facilitates the expression of genetic information and that the specific protein products of this induction process, such as intestinal calcium binding protein (CaBP) (63), function in the transport of calcium. In addition to CaBP, vitamin D dependent alkaline phosphatase (64) and calcium stimulated ATPase (65) have been proposed as functioning in the uptake of calcium by the intestinal mucosal cell. The mechanism by which these proteins might enhance calcium transport is at present unknown.

The proposed manner in which \( \alpha,25\text{-}(\text{OH})_2 \text{D}_3 \) mediates its molecular mechanism of action in the target cell is similar to the "two-step" method that has been proposed for the mechanism of action of other steroid hormones at their respective target tissues (66-68). According
to this hypothesis, the hormone enters the target cell and interacts reversibly but with high association to an intracellular protein which is located only in the target organ. The association of the hormone with this cytoplasmic receptor is followed by a temperature-dependent redistribution of the steroid from the cytoplasm to the nucleus, as demonstrated in vitro, where it associates with the chromatin and directs the expression of genetic information. The basic tenets of the nuclear mechanism as applied to steroid hormones are as follows: 1) after association of the hormone-receptor complex with the nuclear chromatin, the deoxyribonucleic acid duplex (DNA) becomes unwound so that previously covered initiation sites are exposed; 2) there is a subsequent increase in the binding of DNA-dependent ribonucleic acid (RNA) polymerase to the initiation sites; and 3) an increase in RNA synthesis results, presumably for heterogenous RNA (HnRNA), which is then translated into proteins functioning in the physiological response to the hormone in its target tissue. Increased RNA synthesis has been found in response to aldosterone in the isolated toad bladder (69), glucocorticoids in liver tissue (70, 71), estrogen in chick oviduct (72-74), progesterone in uterus (75), dihydrotestosterone in rat prostate (76, 77), and thyroid hormone in rat liver (78).

A variety of experimental evidence has provided strong support for the mechanism of action for 1α,25-(OH)₂D₃. In 1973 Brumbaugh and Haussler (79) reported the existence of a specific 1α,25-(OH)₂D₃ binding protein in the cytoplasm of intestinal mucosal cells from rachitic chicks. This observation was subsequently confirmed by Tsai and Norman
in the chick and extended to the rat by Chen and DeLuca (81). Brumbaugh and Haussler (82-84) have isolated and characterized this specific 1α,25-(OH)2D3 binding protein and have shown it to have the following properties: 1) sediments at 3.7 S in high-salt sucrose gradients, 2) has an apparent molecular weight of 47,000 based upon agarose gel filtration, 3) is identified as a protein-hormone complex by selective destruction with proteases, 4) has a dissociation constant of \(2 \times 10^{-9}\) M for the hormone, and 5) migrates to the chromatin in a temperature-dependent process to yield a nuclear receptor which is indistinguishable from the cytosol hormone-receptor complex by sucrose gradient and agarose gel filtration analysis. Intracellular receptors have also been identified for estrogen (85, 86), progesterone (87, 88), androgens (89), aldosterone (90), and cortisol (91). Thus, the site and mechanism of the initial action of 1α,25-(OH)2D3, like that of the steroid hormones, appears to be the binding of the hormone to its specific cytoplasmic receptor and then subsequent association of this complex with the nuclear chromatin.

The nuclear mechanism of action of 1α,25-(OH)2D3 in the mucosal cell has been investigated in several laboratories. Even before the demonstration that vitamin D3 must be converted to more polar metabolites, Eisenstein and Passavoy (92) demonstrated that actinomycin D, a potent inhibitor of transcription, when administered prior to vitamin D3, could inhibit the ensuing hypercalcemia induced by vitamin D3. It was then shown (93) that actinomycin D given one hour before vitamin D3 in rats completely prevented the rise in serum calcium and increased calcium
transport by this vitamin in everted gut sacs, in vitro. Actinomycin D inhibition of increased calcium transport in response to vitamin D₃ was also reported to occur in chicks (94) at this same time. These findings were supported by the observation that 5-fluoro-orotic acid, another transcriptional antimetabolite, blocked the increased serum calcium response to vitamin D₃ and that the observed inhibition of calcium transport by these antibiotics did not appear to be due to an inhibition of the absorption and distribution of vitamin D₃ nor to cell necrosis as shown by light microscopy of the intestinal villi (95). Recently, Tsai, Midgett, and Norman (96) reported that actinomycin D can block the increase in calcium transport by 1α,25-(OH)₂D₃ as measured by intestinal ⁴⁵Ca transport. Furthermore, actinomycin D administration did not inhibit the localization of the hormone in the intestine, suggesting that the 1α,25-(OH)₂D₃ stimulated increase in calcium transport is mediated through a transcriptive mechanism.

In 1967, Stohs, Zull, and DeLuca (97) demonstrated that within three hours after the administration of 2,000 i.u. of vitamin D₃ to D-deficient rats, the incorporation of ³H-orotic acid into intestinal RNA is stimulated two- or three-fold. Furthermore, the response produced by 2,000 i.u. of vitamin D₃ was blocked by actinomycin D and when 10 i.u. of vitamin D₃ was administered, labelling of the RNA occurred in eight hours. Norman found similar results in the rachitic chick in 1966 (98). In his study he observed that maximal labelling of the intestinal RNA by ³H-uridine occurred in one-half hour after administration of 5,000 i.u. of vitamin D₃ and by five hours, a time at which maximal stimulation of
calcium absorption was reached, the amount of radioactively labelled RNA had returned to control values. In addition, when the dose of vitamin D₃ was progressively lowered, the time of maximal stimulation of 3H-uridine incorporation into RNA progressively increased, correlating well with the fact that the lag in calcium transport progressively increased with decreasing concentrations of vitamin D₃. Although these early studies were performed with little knowledge of vitamin D₃ metabolism, the results are indicative of early stimulation of RNA synthesis by the vitamin and support the notion that this sterol may function by altering the transcriptive capacity of the target cell nucleus. Tsai and Norman (99) have reported that administration of 5 i.u. of 1α,25-(OH)₂D₃ to rachitic chicks produces maximal labelling of intestinal RNA six hours after the hormone is administered. Again, this time of RNA labelling supports the hypothesis that 1α,25-(OH)₂D₃ may be increasing nuclear RNA synthesis prior to physiological responses in the target organ. In 1971, Corradino and Wasserman (100) demonstrated that addition of vitamin D₃ to embryonic chick duodena in organ culture medium was able to induce the synthesis of CaBP, as measured by a specific immuno precipitation with CaBP antibody. Utilizing this in vitro system, Corradino further demonstrated that 1α,25-(OH)₂D₃ was much more potent in stimulating the production of CaBP and increasing ⁴⁷Ca uptake than the parent sterol and that the addition of actinomycin D or α-amanitin to the medium prevented the observed increases in ⁴⁷Ca uptake and calcium binding protein production stimulated by 1α,25-(OH)₂D₃ (101, 102). The inhibition by α-amanitin indicates that intestinal RNA synthesis is essential to the nuclear
action of $1\alpha,25-(OH)_2D_3$ since $\alpha$-amanitin has been shown to specifically inhibit nucleoplasmic RNA polymerase activity in isolated nuclei from a variety of eukaryotic sources (103).

Further support for the notion that vitamin $D_3$ is functioning similarly to other steroid hormones in stimulating messenger RNA (mRNA) synthesis has been provided by two additional lines of experimental evidence. Hallick and DeLuca (104) reported that 2,000 i.u. of vitamin $D_3$ could increase the template capacity of intestinal nuclear chromatin, as measured with *E. coli* RNA polymerase, in vitamin $D$-deficient rats three hours after administration of the sterol. Similar results have been reported by Haussler (105) using the rachitic chick. Perhaps the strongest support for increased mRNA synthesis by vitamin $D_3$ has been reported by Emtage, Lawson, and Kodicek (106), who demonstrated increased polysomal mRNA activity specific for chick intestinal CaBP in response to the administration of vitamin $D_3$ to rachitic chicks. Although the above data were not obtained with $1\alpha,25-(OH)_2D_3$, the results are suggestive that vitamin D induces CaBP synthesis by specifically regulating gene expression.

The preponderance of experimental evidence indicates that the nuclear mechanism of action of $1\alpha,25-(OH)_2D_3$ involves the expression of genetic information via a transcriptive process, similar to the action of steroid hormones. However, another mechanism of action for $1\alpha,25-(OH)_2D_3$ at its target cell has been proposed.

Early investigations of the actinomycin D sensitivity of the $1\alpha,25-(OH)_2D_3$ mediated increase of intestinal calcium transport by Tanaka
and associates in the vitamin D-deficient rat (107) and Haussler in the rachitic chick intimated that this antibiotic was ineffective in preventing the 1α,25-(OH)₂D₃-mediated increase in calcium transport. Cycloheximide administration, however, was effective in blocking the physiological response. These findings suggest that perhaps the active sterol mediates its effects at the target cell by influencing translational events which would lead to the production of an active CaBP. Also, the mechanism may be similar to that reported for vitamin K in which the vitamin is necessary for the carboxylation of certain glutamic acid residues in prothrombin during the synthesis of this clotting factor (108). The lack of actinomycin D sensitivity observed by the above investigators was probably due to the fact that recovery of transcription had occurred during the seven-to-eight-hour interval between actinomycin D administration and the calcium transport assay. This is based on studies in which greater than 95% inhibition of RNA polymerase activity is necessary for actinomycin D to block hormone-mediated transcription (T. J. Lindell, personal communication) and that the inhibition of transcription by the antibiotic is temporary with recovery of transcription beginning shortly after maximal inhibition (109). This is supported by the work of Tsai and associates (96), who used four doses of actinomycin D at two-hour intervals during administration of the active hormone to block the increase in calcium transport, suggesting that they inhibited

4. Inhibition of the 1α,25-(OH)₂D₃-stimulated increase in calcium transport was observed 3 out of 24 times when one dose of actinomycin D was administered 1 to 3 hours before 1α,25-(OH)₂D₃ to rachitic chicks. The calcium transport response was then quantitated 5 hours later.
transcription by greater than 95% while not inhibiting the localization of 1α,25-(OH)₂D₃ in the intestine.

Freund and Bronner recently reported (110) that 1α,25-(OH)₂D₃ produced detectable levels of CaBP and stimulated calcium uptake in intestinal cells from vitamin D-deficient rats in vitro after only a 90-minute exposure to the active hormone. This is the most rapid stimulation of CaBP production and calcium uptake in vitro reported and is consistent with the "two-step" mechanism of 1α,25-(OH)₂D₃ action. Corradino has reported that a relatively high level of 1α,25-(OH)₂D₃ in the culture medium produced a significant increase in cyclic AMP (cAMP) concentration after only 30 minutes using the embryonic chick intestine in organ culture. This is one of the earliest detectable actions of this sterol in this system (111). This report suggests the involvement of cAMP-dependent protein kinases in the mechanism of action of 1α,25-(OH)₂D₃. Presumably these kinases are active in the phosphorylation of nonhistone proteins, nuclear RNA polymerases, and other nuclear proteins, such that transcription of previously covered sequences occurs, leading to the production of specific mRNA(s).

The experiments reported in this dissertation have been designed to elucidate the sequence of nuclear events immediately after the interaction of 1α,25-(OH)₂D₃ with its target organ, the intestine. The basic finding of the work to be reported here is that there is now direct evidence for a nuclear mechanism of action of this hormone which can be observed by examining the intestinal nuclei of rachitic chicks after administration of this hormone. It is assumed in these studies that a
target organ for $1\alpha,25-(OH)_2D_3$ contains a finite number of specific receptor sites which selectively retain the hormone, where this complex retention in the nucleus is presumed to effect transcription and induce the synthesis of functional proteins which participate in calcium translocation. The sequence of biochemical events occurring before the association of the hormone-receptor complex with the chromatin, including the binding of the hormone to its receptor and the events which occur after synthesis of the putative mRNA, including translation, will not be investigated in the present experiments.

In summary, this dissertation will detail the characterization of the nuclear events prior to the stimulation of calcium absorption by $1\alpha,25-(OH)_2D_3$ in the intestine of rachitic chicks. The results indicate that $1\alpha,25-(OH)_2D_3$ interacts with its target cell nucleus in a manner similar to action of steroid hormones in their respective target organs, i.e., through alteration of RNA polymerase and chromatin template activities. Several reports of this work have already appeared in the literature (112-116).
 CHAPTER II

MATERIALS AND METHODS

Animals

White Leghorn cockerels were used in all experiments. One-day-old chicks were obtained regularly as a generous gift from the Arizona State Hatchery, Tucson, Arizona, or from Demler Farms, Anaheim, California, and then raised on a vitamin D-deficient diet which has been previously described (61). The calcium content of this diet was determined to be 0.67%. Unless otherwise noted, the chicks were used for experiments when their growth plateaued at a weight of about 150 g, usually between their third and fourth week of development. Chicks grown on this same D-deficient diet, but orally supplemented with 100 i.u. of vitamin D_3 per week, exhibited normal increased growth during this period and weighed about 250 g. Rachitic animals routinely had plasma calcium concentrations of 5.5 to 6.0 mg per 100 ml plasma and phosphorus concentrations of 5.1 mg per 100 ml of plasma as determined with a Technicon Autoanalyzer. The percent bone ash of the rachitic animals was 27%. Vitamin D_3-supplemented chicks had normal plasma calcium concentrations of 10.4 to 11.0 mg per 100 ml plasma, phosphorus concentrations of 6.3 mg per 100 ml, and bone ash percentages of 45 to 50% (61, 62). Radiographic examination of the distal femur and proximal tibia of rachitic chicks were evaluated and demonstrated considerable unossified cartilage, decreased density of mineral, and a lack of bony trabeculations at the
growing ends of the bones. The vitamin D-supplemented chicks showed normal bone density and ossification (61, 62). In addition, histological examination of stained sections of the epiphyseal region of the tibias showed widened and diffuse growth plates in the bones of the rachitic chicks and a sharply defined and calcified growth plate in the bones of the vitamin D-supplemented chicks (61).

Radiochemicals

25-hydroxy[26,27-methyl-3H] vitamin D₃ (6.0 to 9.8 Ci/m mole) was purchased from Amersham Searle Company, Chicago, Illinois. The radiochemical purity of the 25-OH[3H]D₃ was 95%, determined following purification on Celite liquid-liquid partition chromatographic columns, and its specific activity was determined by ultraviolet absorbance spectrophotometry at 265 nm. 5,6-[3H]UTP (22.2 to 47 Ci/m mole) and ⁴⁵Ca (carrier free, 15 Ci/g calcium) were purchased from New England Nuclear Corp., Boston, Massachusetts. ⁴⁵Ca was diluted so that 0.2 ml contained 7.4 mg of ⁴⁵CaCl₂ and 3 x 10⁶ cpm of ⁴⁵Ca.

Sterols and Other Chemicals

Crystalline, non-radioactive sterols were used without further purification except where indicated and were obtained as follows: vitamin D₃ was purchased from Calbiochem, Los Angeles, California; 25-OHDC₃ was a gift from Dr. John C. Babcock of the Upjohn Co., Kalamazoo, Michigan; and 1α-OHDC₃ was a gift from Dr. Maurice M. Pechet of the Research Institute for Medicine and Chemistry, Cambridge, Massachusetts.
Chemicals used in the enzymatic generation of 1α,25-(OH)₂D₃ from 25-OHD₃ were obtained from Sigma Chemical Co., Saint Louis, Missouri, and included: L-malic acid, monosodium salt; D-glucose-6-phosphate, monosodium salt; and nicotinamide adenine dinucleotide phosphate, monosodium salt (NADP⁺). Enzymes and standard proteins were obtained as follows: bovine serum albumin (fraction V) from Sigma Chemical Co., Saint Louis, Missouri; *Escherichia coli* DNA-dependent RNA polymerase (E.C. 2.7.7.6) from *Escherichia coli* strain K₁₂ (200 to 600 units/mg protein) from Sigma Chemical Co., Saint Louis, Missouri, or prepared from *E. coli* strain W3110 (400 units/mg protein) according to the procedure of Burgess (117). Wheat germ DNA-dependent RNA polymerase was prepared according to the procedure of Jendrisak and Burgess (118) and had a specific activity of 210 units/mg protein.

Triton X-100 and phenylmethylsulfonylfluoride (PMSF) were obtained from Sigma Chemical Co., Saint Louis, Missouri. Liquifluor, Omnifluor, and Aquasol, a premixed liquid scintillation solution, were purchased from New England Nuclear Corp., Boston, Massachusetts. Deoxyribonucleic acid (Type I, sodium salt from calf thymus), tris-(hydroxymethyl) aminomethane (tris, Sigma 7-9), and ethylenediaminetetraacetic acid (EDTA, tetrasodium salt) were obtained from Sigma Chemical Co., Saint Louis, Missouri. Diphenylamine was supplied by Eastman Organic Chemicals, Rochester, New York. Orcinol (monohydrate) was obtained from Fisher Scientific Co., Fair Lawn, New Jersey; and Lowry phenol reagent (Folin and Ciocalteu) were purchased from Harleco, Philadelphia, Pennsylvania. Alpha-amanitin was obtained from Henley and
Co., Inc., New York, New York. Filters (Whatman 3MM and DE 81) were purchased from Reeve-Angel, Clifton, New Jersey. All solvents were reagent grade, and those employed in chromatographic procedures were glass distilled before use. All other chemicals were analytical grade unless otherwise noted.

**Chromatography Materials**

Silicic acid (Bio-Sil HA, minus 325 mesh) was obtained from Bio-Rad Laboratories, Richmond, California. Sephadex LH-20-100 (Lipophilic, particle size 25-100 µ) was purchased from Sigma Chemical Co., Saint Louis, Missouri. Celite, which served as a support medium for liquid-liquid partition chromatography was supplied by Johns Manville Co., Lompoc, California. Diethylaminoethyl cellulose (Whatman DE 52, microgranular preswollen) and phosphocellulose (Whatman P11) were obtained from Reeve-Angel, Clifton, New Jersey. Diethylaminoethyl Sephadex (A-25, particle size 40-120 µ) was supplied by Sigma Chemical Co., Saint Louis, Missouri.

**Buffers**

The buffers used were:

1. Buffer A = 0.05 M Tris-HCl, pH 7.4, 0.025 M KCl, 5 mM MgCl₂;
2. Buffer B = 0.008 M EDTA, 25 mM NaCl, pH 8.0, 12 mM β-mercaptoethanol;
3. Buffer C = 0.01 M Tris-HCl, pH 7.5, 12 mM β-mercaptoethanol;
4. Buffer D = 0.01 M Tris-HCl, pH 7.4, 5 mM MgCl₂, 1 mM PMSF; and
5. Buffer E = 0.05 M Tris-HCl, pH 7.9, 25% glycerol (v/v), 5 mM MgCl$_2$, 1 mM EDTA.

Exposure of Animals to Sterols, In Vivo

The appropriate amount of sterol was dissolved in 0.2 ml of 1,2-propanediol (practical grade, Baker Chemical Co., Philadelphia, Pennsylvania) prior to administration. The sterol (in 100% solution) was first dried under nitrogen (dry, 99.9%) and then dissolved in a small volume of diethyl ether, mixed with 1,2-propanediol, and then nitrogen was bubbled through the solution at room temperature to remove the ether. Doses were made up immediately before use and administered orally. After the appropriate time interval, the chick was killed by decapitation.

Preparation of Subcellular Fractions

All operations involving the preparation of subcellular components were carried out at 1-3 degrees. Immediately following sacrifice of the animal, the tissues of interest were excised and rinsed in ice-cold, 0.25 M sucrose-Buffer A. Prior to homogenization, intestinal mucosa was scraped free from the serosa on an inverted petri dish with a microscope slide, and livers or kidneys were minced. For studies of the endogenous RNA polymerase activities, subcellular fractions were prepared by a modification of the procedure of Blobel and Potter (119). The tissue to be subfractionated was homogenized in 0.25 M sucrose-Buffer A containing 0.1% Triton X-100 (v/v) and 1 mM PMSF (10 volumes/g of tissue) with 5 passes using a motor-driven, Potter-Elvehjem homogenizer equipped with a Teflon pestle. During homogenization, tubes were kept in an ice
bucket in a 1-3° room. The homogenate was filtered through glass wool in a 50 cm³ disposable plastic syringe. The homogenate was then centrifuged at 1500 x g in a RC-2B refrigerated centrifuge (Sorvall SS-34 rotor) for 10 minutes. The resulting crude nuclear pellet was resuspended in 6 ml of 0.25 M sucrose-Buffer A plus 1 mM PMSF, mixed with two volumes of 2.3 M sucrose-Buffer A plus 1 mM PMSF, and mixed by homogenization in a Potter-Elvehjem homogenizer as before. The homogenate was transferred to a cellulose nitrate ultracentrifuge tube, underlaid with 15 ml of 1.7 M sucrose-Buffer containing 1 mM PMSF, and the samples were then centrifuged at 81,800 x g (Beckman SW-27 rotor) for 30 minutes in a Beckman L3-40 ultracentrifuge. Pelleted nuclei were suspended in 1.0 M sucrose-Buffer D to give a final concentration of 30 μg of DNA per 50 μl.

For studies of template activity, nuclei of the tissue to be subfractionated were prepared as follows: 6 g of the tissue to be studied were homogenized in 24 ml of 0.25 M sucrose-Buffer A containing 12 mM β-mercaptoethanol as described above. The homogenates (25% w/v) were centrifuged at 1200 x g for 10 minutes in a Sorvall RC-2B refrigerated centrifuge to pellet crude nuclei. The supernatant was centrifuged at 100,000 x g for 1 hour in a Beckman L3-40 ultracentrifuge (Type 42 rotor) to yield a mitochondrial-microsomal pellet and a final supernatant fraction (cytosol). The crude nuclear pellet was resuspended in homogenization buffer and filtered through glass wool as before. The filtrate was then recentrifuged at 1200 x g for 10 minutes and the nuclear pellet was then resuspended in 1.7 M sucrose-Buffer A to yield a 20% homogenate
(w/v). Purified nuclei were harvested by centrifugation at 27,000 x g for 30 minutes in a Sorvall RC-2B refrigerated centrifuge (SS-34 rotor).

Chromatin was prepared from crude or purified nuclei by homogenizing successively in one 25 ml portion of Buffer E and one 25 ml portion of 1% Triton X-100-Buffer C with the crude chromatin being harvested by sedimentation at 30,000 x g for 10 minutes after each wash. The chromatin was homogenized in 25 ml of Buffer C and sedimented at 30,000 x g for 10 minutes. The resulting gelatinous pellet was mixed with enough 1.0 M sucrose-Buffer D, without PMSF but containing 12 mM \( \alpha \)-mercaptoethanol, to give a final DNA concentration of 5 to 16 \( \mu \)g/25 \( \mu \)l.

**Preparation of 1α,25-Dihydroxyvitamin D₃**

Nonradioactive 1α,25-(OH)₂D₃ was produced in vitro by a modification (41) of the method of Lawson et al. (39). Fourteen grams of kidney from rachitic chicks were homogenized in 0.3 M sucrose. The homogenate (140 ml) was mixed with a phosphate buffer, pH 7.4, containing Mg\(^{2+}\) and a NADPH generating system. In a final volume of 540 ml, the mixture contained: 0.2 M KH\(_2\)PO\(_4\), 3.2 mM MgCl\(_2\), 3.7 mM L-malate, 1.7 mM glucose-6-phosphate, 0.15 mM NADP\(^+\), and 75 units of glucose-6-phosphate dehydrogenase. Three hundred and twenty-five nanomoles of 25-OHD₃ containing 0.325 nanomoles of 25-hydroxy[26(27)-methyl-\(^3\)H] vitamin D₃ (6.5 Ci/mmol) were added to the reaction mixture and incubation was carried out at 37° for 2 hours (radioactive 25-OHD₃ was added to the reaction mixture in order to determine the elution profile of the active metabolite during the ensuing purification on silicic acid, Sephadex LH-20, and Celite column chromatography). Termination of the reaction occurred with the
addition of 3.2 volumes of methanol-chloroform (2:1 v/v) and the mixture stirred for 10 minutes. After standing for 15 minutes, the solution was filtered to remove the residual protein and the sterols extracted according to the method of Bligh and Dyer (120). One volume of chloroform and one-half volume of water were added to the extracts to cause a phase separation into an aqueous-methanol phase and a lower chloroform phase which contained 85 to 95% of the sterols. This chloroform phase was flash evaporated to dryness and the resulting lipids solubilized in diethyl ether. After centrifugation at 12,000 x g for 10 minutes to clarify the solution, the diethyl ether was evaporated under a stream of nitrogen and the 1α,25-(OH)₂D₃ was then purified by column chromatography on silicic acid, Sephadex LH-20, and Celite. 1α,25-(OH)₂D₃ was stored in distilled ethanol at -20° and contained approximately 900 dpm of radioactivity per unit as opposed to the radioactive 1α,25-(OH)₂D₃ which contained 938,000 dpm/unit. Therefore, 1α,25-(OH)₂D₃ prepared as above is termed nonradioactive 1α,25-(OH)₂D₃.

Chromatographic Methods

Silicic Acid Column Chromatography

Silicic acid was activated by heating in a vacuum oven to 120° for 24 hours just prior to use. Thirty grams of activated silicic acid were then suspended in hexane and poured into a column (1.8 x 18 cm) with the aid of 3 pounds nitrogen pressure for packing. Samples for purification were applied in a small volume of hexane-diethyl ether (1:1 v/v). Ten fractions were then collected from columns which were batch eluted.
with 5% acetone in diethyl ether as follows: fractions number 1, 2, and 6-10, 50 ml; fractions 3-5, 20 ml (Figure 3A, page 35). 1α,25-(OH)₂D₃ eluted in fractions 4 through 8. Each column was run with 3 pounds of nitrogen pressure and with a flow rate of 7 ml per minute.

Sephadex LH-20 Column Chromatography

Liquid-gel partition chromatography on Sephadex LH-20 was performed by the method of Holick and DeLuca (121). Columns were eluted with 65% chloroform in hexane, and run under 3-5 pounds nitrogen to obtain a flow rate of about 1 ml/minute. Seven ml fractions were collected from columns (1 x 33 cm, containing 11 g Sephadex LH-20, Figure 3B). 1α,25-(OH)₂D₃ eluted in fractions 12 through 18.

For purification of 1α-OHD₃, 5 ml fractions were collected from columns (1 x 60 cm, containing 20 g Sephadex LH-20) which were batch eluted with 50% chloroform-hexane.

Celite Column Chromatography

Celite was washed with concentrated HCl and organic solvents, and the fine particles were removed prior to use (122). Column chromatography was by the procedure of Haussler and Rasmussen (43). For resolution of dihydroxy-D-vitamins, 5 volumes of 10% ethyl acetate in hexane were equilibrated with 1 volume of 45% water in ethanol. The lower water-ethanol phase served as a stationary phase and was mixed with 11 g Celite (1:1 v/w). The Celite was then suspended in excess ethyl acetate-hexane (upper phase, mobile) and packed with a glass rod into a homogeneous column (1 x 35 cm). Columns were run under 3 pounds nitrogen.
pressure to achieve flow rates of about 0.5 ml per minute. Five ml fractions were collected (Figure 3C).

For purification of 1α-OHD₃, 500 ml hexane (mobile phase) were equilibrated with 250 ml of 15% water in methanol (stationary phase). Columns (1 x 36 cm, containing 11 g Celite) were packed and eluted with the mobile phase under 3 pounds nitrogen pressure. Five ml fractions were collected, and the flow rates were about 0.5 ml per minute. Samples were applied to all Celite Columns in 0.4 ml of the mobile phase.

DEAE-Sephadex Column Chromatography

All chromatographic procedures were carried out at 1-3°C. DEAE-Sephadex (A-25) was suspended in 30 volumes (w/v) water and the pH adjusted to 7.8 with 1 M Tris-base. After standing overnight, the slurry was washed two times with 30 volumes of 0.5 M (NH₄)₂SO₄ (pH 7.9) by settling and decanting. This was followed by three washes with 30 volumes of 0.05 M Tris-HCl (pH 7.9) and two washes with Buffer E. The resulting slurry was poured into a 50 cm³ disposable syringe which served as the column (1 ml of column material per 4 g of protein) and equilibrated with five bed volumes of 0.05 M (NH₄)₂SO₄ in Buffer E. Soluble nuclear protein was isolated from frozen intestinal nuclei used in RNA polymerase experiments according to the procedure of Roeder and Rutter (123), except that no (NH₄)₂SO₄ precipitation of the "F2" supernatant was performed. Rather, the supernatant was diluted to 0.05 M (NH₄)₂SO₄ and applied directly to the column and eluted with a linear gradient of 0.05 M (NH₄)₂SO₄ to 0.4 M (NH₄)₂SO₄. Fractions of 0.6 ml
were collected in plastic tubes and assayed for polymerase activity using the "low UTP" assay described below.

DEAE-cellulose (Whatman DE 52) was prepared for chromatographic use as previously described (117). Columns were prepared and protein eluted as previously described for the purification of *E. coli* RNA polymerase (117) or wheat germ polymerase (118).

Phosphocellulose (Whatman P11) was prepared and utilized for column chromatography as previously described by Jendrisak and Burgess (118).

**Measurement of Intestinal Nuclear RNA Polymerase Activities**

RNA Polymerase Assay in Isolated Intestinal Nuclei

The standard reaction mixture for RNA polymerase (I and II) activities contained in a final volume of 125 µl is: 7 µmol of Tris-HCl (pH 7.9); 0.75 µmol of NaF; 0.75 µmol each of GTP, CTP, and ATP; 0.0125 µmol of unlabelled UTP (high UTP); 1 µCi of 5-$^3$H[UTP] (22.2 Ci/mmol); 0.2 µmol of MnCl$_2$; 6.62 µmol of (NH$_4$)$_2$SO$_4$; and 50 µl of each nuclear suspension. Assays (in triplicate) were performed in the presence and absence of α-amanitin (0.1 µg per assay). All reaction mixtures were incubated for 5 minutes at 20°. Reactions were stopped by pipetting 100 µl aliquots onto Whatman DE-81 filter discs (2.3 cm diameter). The filter paper discs were then washed seven times in 5% Na$_2$HPO$_4$ (4 ml per filter), twice in distilled water followed by two 95% ethanol washes and one diethyl ether wash. Form II RNA polymerase activity was determined
by subtracting the incorporated cpm plus α-amanitin from those minus α-amanitin. In addition, zero time controls were performed for each nuclear sample (also in triplicate) by addition of nuclei (0°) to the assay mixture plus α-amanitin and immediately pipetting aliquots to the DE-81 filters and then to the phosphate wash solution. This value was subsequently subtracted from the assays for nucleolar form I polymerase (plus α-amanitin). Unless otherwise noted, the results are expressed as pmoles of UMP incorporated into RNA per 5 minutes per mg DNA.

RNA Polymerase Assay for Soluble Nuclear Protein

For the determination of RNA polymerase activities after chromatographic resolution on DEAE-Sephadex, the standard assay described above was utilized except that it contained 0.00125 μmol of unlabelled UTP (to insure high specific activity of the labelled UTP), no (NH₄)₂SO₄, and 20 μg of calf thymus DNA per assay as the template. Aliquots (50 μl) of solubilized RNA polymerases were incubated for 10 minutes at 30°. Unless otherwise noted, the results are expressed as pmoles of UMP incorporated into RNA per 10 minutes.

Measurement of Intestinal Nuclear Chromatin Template Activity

Assay for Chromatin Template Activity after In Vivo Administration of 1α,25-(OH)₂D₃

The standard reaction mixture for nuclear chromatin template activity contained in a final volume of 240 μl: 12 μmol of Tris-HCl (pH 7.9); 0.75 μmol of NaF; 0.075 μmol each of GTP, CTP, and ATP; 0.0125 μmol
of unlabelled UTP; 1 μCi of \(5,6-^3\text{H}[\text{UTP}]\) (42.5 Ci/mmol); and 0.2 μmol of McCl\text{2}. Alpha-amanitin was added to one-half of the reaction mixture (0.1 μg/assay) and each reaction was started by the addition of 25 μl of \(E.\ coli\) RNA polymerase (130 μg) or \(\text{wheat germ RNA polymerase}\) (52 μg) and 25 μl of DNA (10 to 16 μg) as chromatin. This concentration of \(E.\ coli\) polymerase was used to insure a saturating amount of enzyme in the reaction mixture for DNA concentrations of up to 25 μg/25 μl. Bacterial RNA polymerase was omitted from those assays with α-amanitin. Transcription was allowed to proceed for 10 minutes at 37° and the assays (in quintuplicate) were then terminated by pipetting the entire reaction mixture onto Whatman No. 3MM filters, which were immediately placed in a 10% TCA wash solution (4 ml/filter) at 1-3°. Filters were then washed three times in 5% TCA at 1-3° followed by one wash each of distilled water, 95% ethanol, and diethyl ether. The cpm incorporated in the reactions containing α-amanitin represent transcription by endogenous RNA polymerases I and III. The cpm obtained from this control were subtracted from those without α-amanitin to yield the \(E.\ coli\) RNA polymerase supported template activity. The results are expressed as nmoles of UMP incorporated into RNA per 10 minutes per mg DNA unless otherwise noted.

Assay for Chromatin Template Activity after \(\text{In Vitro}\) Addition of \(1α,25-(\text{OH})_2\text{D}_3\)

For studies of \(\text{in vitro}\) transcription, cytosol was prepared as above and various sterols (in 50 μl of ethanol/10 ml cytosol) were added to a final concentration of 17 nM in sterol. Control cytosols contained 50 μl of ethanol. Sterols were incubated with the cytosol for 60
minutes at 0°, following which the purified nuclei were reconstituted with the pre-incubated cytosol-sterol mixture (nuclei from 3 gm of mucosa containing 6 mg of DNA/12 ml of cytosol representing 10 mg/ml of protein) by gentle homogenization (glass Teflon) and allowed to incubate at 25° for 60 minutes. At the end of the incubation, nuclei were harvested by centrifugation at 1200 x g and chromatin was then prepared from the nuclei. The standard assay mixture as described above was then utilized for measurement of chromatin template activity. Unless otherwise noted, the results are expressed as nmoles of UMP incorporated into RNA per 10 minutes per mg of DNA.

**Liquid Scintillation Counting**

Liquid scintillation counting of all radioactive samples ($^3$H or $^{45}$Ca) was carried out after addition of the appropriate liquid scintillation counting solution. Dried filters from the RNA polymerase and chromatin template assays were placed directly in the scintillation vials and counted with counting solution A. This consisted of 4 g of 2,5-diphenyloxazole and 80 mg of p-bis-(o-methylstyrlyl) benzene per liter of toluene. To this mixture was added 29 ml of NCS solubilizer-water mixture (25 ml of NCS and 4 ml water previously mixed by stirring). For aqueous plasma sample counting, as for $^{45}$Ca, Aquasol (New England Nuclear) was utilized as the liquid scintillant in the ratio of 50 to 1, scintillant to sample. For nonaqueous sample counting, counting solution B, consisting of 4g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-2-(5-phenyloxazolyl) benzene per liter of toluene, was used. Samples were counted to 2% error in a Beckman LS-250 ambient temperature or a Beckman
LS-230 refrigerated liquid scintillation spectrometer. Efficiencies for $^3$H ranged from 35 to 45% and efficiency for $^{45}$Ca was 70%. In most cases, cpm were converted to nmoles of nucleoside incorporated based on the known specific activity of the labelled nucleoside triphosphate, assuming no conversion of nucleoside.

**Spectrophotometric Determinations**

In quantitating the concentration of sterol solutions, absorbance measurements and spectra were made on a Beckman DB spectrophotometer equipped with a 10-inch Beckman recorder. The absorbance at 280 nm of the eluate of DEAE-cellulose or phosphocellulose columns was made by measurement of individual fractions using a Gilford model 240 spectrophotometer equipped with a model 2443-A rapid sampler and a model 4008 data lister.

**Mass Spectrometry of Sterols**

A model RMU-6E Hitachi double focusing mass spectrometer was used to analyze 5-10 µg portions of vitamin D$_3$, 25-OHD$_3$, 1α-OHD$_3$, and 1α,25-(OH)$_2$D$_3$ to confirm chemical structures. Samples were directly introduced on the probe and continuous scanning was carried out from 80-150° above ambient.

**Biological Assay of Vitamin D Metabolites and Analogs**

A bioassay for the various vitamin D metabolites and analogs was carried out by a modification of the procedure of Coates and Holdsworth (124). The compound of interest, dissolved in 0.2 ml of 1,2-propanediol,
was administered to rachitic chicks. Control chicks received 0.2 ml of 1,2-propanediol alone. At the appropriate time interval 0.2 ml of a $^{45}$Ca solution, consisting of 1.5 Ci $^{45}$Ca and 7.4 mg CaCl$_2$ in 50 ml distilled H$_2$O, was administered to the chicks (0.2 ml/chick). Forty-five minutes later the chicks were sacrificed by decapitation and 3-5 ml of blood were collected in heparinized centrifuge tubes. After sedimentation of the red cells by centrifugation at 12,000 x g for 10 minutes, aliquots (0.2 ml) of plasma were added to liquid scintillation vials containing 10 ml Aquasol scintillation counting solution, and radioactivity of the same energy as $^{14}$C was determined using the $^{14}$C isotope window.

**Chemical Assays**

**Deoxyribonucleic Acid**

Deoxyribonucleic acid (DNA) in various cell fractions was assayed by the following method. Nucleic acids (and proteins) in a 50 µl aliquot of the cell fraction were precipitated with 2 ml cold 10% TCA. Following a second cold 10% TCA wash and centrifugation at 4,000 x g for 5 minutes, the pellet was washed once with 2 ml of 95% ethanol. The delipidized precipitate was then extracted with hot (90°) 5% TCA for 15 minutes to solubilize the nucleic acids, and the residual protein was separated by centrifugation. A 0.5 ml aliquot of the extract was assayed for DNA by the diphenylamine method of Burton (125). One ml of diphenylamine reagent, consisting of 1 g diphenylamine dissolved in 100 ml glacial acetic acid, 10 ml concentrated sulfuric acid, and 0.5 ml of 2% acetaldehyde, was added to the extract and the mixture allowed to stand overnight.
Ribonucleic Acid

Nucleic acids in 1 ml aliquots of cell fractions were solubilized as described above, and a 1 ml aliquot of the nucleic acid extract was analyzed for RNA by the orcinol procedure (126). A standard curve for DNA in orcinol was also prepared so that the contribution of DNA in the orcinol assay could be subtracted.

Protein

Protein was determined by the method of Lowry et al. (127); bovine serum albumin was employed as the protein standard.
CHAPTER III

RESULTS

Preparation and Purity of 1α,25-Dihydroxyvitamin D₃ and Related Sterols

Generation and Purification of 1α,25-(OH)₂D₃

Since 1α,25-(OH)₂D₃ had not been chemically synthesized and was not available commercially, it was necessary to biosynthesize this compound from 25-OHD₃. 1α,25-(OH)₂D₃ was prepared from 25-OHD₃ by means of an in vitro preparation of the chick renal 25-OHD₃-1α-hydroxylase enzyme. In this procedure, a kidney homogenate from vitamin D-deficient chicks is incubated with 25-OHD₃ in the presence of a NADPH generating system by a modification (41) of the procedure of Lawson et al. (39). The stereospecific hydroxylation of 25-OHD₃ in the kidney mitochondria to yield 1α,25-(OH)₂D₃ is mediated by cytochrome P-450 (128), and the activity of this enzyme is markedly enhanced in animals that have a low calcium status. Therefore, it was possible to achieve high conversion of 25-OHD₃ to 1α,25-(OH)₂D₃ in renal homogenates from chicks raised on a vitamin D-deficient diet for 3-4 weeks prior to sacrifice (129). As shown in Figure 3A, the hydroxylation of 25-OHD₃ is very efficient in this system, and the active hormone is one of the predominant vitamin D metabolites in the methanol-chloroform extract of the kidney homogenate. The radioactivity in the elution position of 1α,25-(OH)₂D₃ comprises 40%
Figure 3. Chromatographic purity of 1α,25-dihydroxyvitamin D₃.

1α,25-(OH)₂D₃ was prepared from 25-OHD₃ as described under "Materials and Methods." A. The liquid extract from the kidney homogenate was chromatographed on a silicic acid column (1.8 x 18 cm). B. Fractions 6 to 10 from the silicic acid column were rechromatographed on a Sephadex LH-20 column (1 x 33 cm). C. A Celite liquid-liquid partition column. 1α,25-(OH)₂D₃ migrated in fractions 18-22.
Figure 3. Chromatographic purity of 1\alpha,25-dihydroxyvitamin D\textsubscript{3}.
of the total eluted radioactivity following separation of the hormone from the 25-OHD$_3$ precursor by chromatography on a silicic acid column. Further purification of the 1$\alpha$,25-(OH)$_2$D$_3$ was carried out by column chromatography on Sephadex LH-20 and Celite (Figures 3B and 3C). Use of this purification sequence resolves the sterol hormone from all known vitamin D metabolites, including 24,25-(OH)$_2$D$_3$ and 25,26-(OH)$_2$D$_3$ (42). The final yield of 1$\alpha$,25-(OH)$_2$D$_3$ varied from 16 to 24% and the final 1$\alpha$,25-(OH)$_2$D$_3$ product was judged to be free of contaminating materials by ultraviolet absorption spectrophotometry and mass spectrometry (Figures 4 and 5).

In all experiments, 1$\alpha$,25-(OH)$_2$D$_3$ concentration was quantitated by measurement of absorbance at 264 nm. The mass spectra of the other nonradioactive sterols used in this dissertation -- vitamin D$_3$, 25-OHD$_3$, and 1$\alpha$-OHD$_3$ -- were determined in collaboration with Mr. Peter Baker of the Department of Chemistry, The University of Arizona, and are shown in Figure 5. Respective parent molecular ions of m/e 384, 400, and 416 are seen for vitamin D$_3$ and its mono- and di-hydroxylated derivatives. Both 1$\alpha$-OHD$_3$ and biologically prepared 1$\alpha$,25-(OH)$_2$D$_3$ yield fragments at m/e 287, 269 (287-H$_2$O), 251 (287-2H$_2$O), 152 and 134 (152-H$_2$O), indicating that 1$\alpha$-OHD$_3$ and the natural hormone contain an additional oxygen atom in the A-ring. The A-ring fragments of vitamin D$_3$ and 25-OHD$_3$ occur at m/e of 271, 253 (271-H$_2$O), and 136, indicating one less oxygen atom. In addition to the ultraviolet absorption spectra of these compounds, the mass spectra in Figure 5 further verify the purity of the nonradioactive compounds employed in this study.
Figure 4. Ultraviolet absorption spectra of nonradioactive vitamin D sterols.

A. Spectrum of crystalline 25-OHD₃ provided by Dr. John C. Babcock of the Upjohn Company (Kalamazoo, Michigan). B. Spectrum of 1α,25-(OH)₂D₃ prepared in kidney homogenates of rachitic chicks, in vitro, as described under "Materials and Methods."
Figure 5. Mass spectra of vitamin D sterols, vitamin D$_3$, 25-hydroxyvitamin D$_3$, 1a-hydroxyvitamin D$_3$, and 1a,25-dihydroxyvitamin D$_3$. 
Purification and Biological Activity of 1α-Hydroxyvitamin D₃

Although 1α,25-(OH)₂D₃ had not been chemically synthesized, 1α-OHD₃ had been by Barton et al. (130) and could have the same biological activity as the natural hormone in stimulating calcium absorption. Therefore, this synthetic sterol was investigated for its ability to be used as a substitute for the more laboriously generated 1α,25-(OH)₂D₃. Synthetic 1α-OHD₃ was provided as a gift for biological assay determinations. Before proceeding with the biological activity determinations, it was necessary to further purify the sterol since the ultraviolet absorption spectrum (Figure 6A) indicated the presence of contaminating materials. Therefore, approximately 750 µg was applied to a Celite column as described in "Materials and Methods" and the fractions containing the sterol (as determined by ultraviolet absorption spectrophotometry) were collected. Figure 6B shows that after Celite chromatography considerable purification had been obtained and the spectrum appeared to resemble that for a vitamin D analog. The sample was then rechromatographed on Sephadex LH-20 as described in "Materials and Methods" and the location of the sterol again determined by ultraviolet absorption spectrophotometry. Figure 6C depicts the final ultraviolet absorption spectrum of 1α-OHD₃ after Celite and Sephadex LH-20 column purification. Its spectrum is identical to that of 1α,25-(OH)₂D₃ with a maximum at 265 nm and a characteristic trough at 228 nm. Further verification of the structure and purity of this compound was accomplished by mass spectrometric analysis, as shown in Figure 5.
Figure 6. Ultraviolet absorption spectra of 1α-hydroxyvitamin D₃.

A. Spectrum of 1α-OHD₃ provided by Dr. Maurice M. Pechet of the Research Institute for Medicine and Chemistry (Cambridge, Massachusetts). B. Spectrum of 1α-OHD₃ after chromatography on a Celite liquid-liquid partition column. C. Spectrum of 1α-OHD₃ after chromatography on a Celite column and on a Sephadex LH-20 column as described under "Materials and Methods."
1α-hydroxyvitamin D₃ was compared with 1α,25-(OH)₂D₃ in its ability to increase calcium absorption within 9 hours after administration of the sterols (Table 1). This time was predicted on the basis that 1α,25-(OH)₂D₃ is capable of increasing calcium absorption with a maximum effect at 5-10 hours (39-42). As little as 0.16 nmol of either sterol causes a significant increase in calcium absorption; maximal effects are seen at a dose of 0.65 nmol of the sterols. Of major significance is the equivalence in activity between the two sterols since, on a mole basis, 1α-OHD₃ and 1α,25-(OH)₂D₃ produce virtually identical biologic responses. Raising the dose of 1α-OHD₃ to a level of 6.5 nmol does not significantly increase the response.

The time course of action of 0.5 nmol of vitamin D₃, 1α,25-(OH)₂D₃, and 1α-OHD₃ in stimulating calcium absorption is depicted in Figure 7. The synthetic sterol induced a rapid increase in calcium absorption with a maximum effect occurring in 5-10 hours. The temporal response of the 1α-OHD₃ is coincident with that of 1α,25-(OH)₂D₃ and both sterols induced changes in calcium transport far faster than did vitamin D₃. These results indicate that there is about a 2-hour lag in response to 1α-OHD₃. However, the availability of large quantities of the synthetic 1α-OHD₃ allowed a further probe of this latent period. Figure 8 demonstrates the time course of action of a relatively large dose of 6.5 nmol administered orally. There was a doubling of calcium absorption in only 2-3 hours with this dose of 1α-OHD₃. Although this represents the most rapid and dramatic increase in calcium absorption thus far reported for a vitamin D-sterol, there existed a distinct lag of 60-90 minutes in
Table 1. Comparison of 1α-hydroxyvitamin D₃ and 1α,25-dihydroxyvitamin D₃ in their ability to rapidly promote increased intestinal calcium absorption in the chick.

1α-OHD₃ or 1α,25-(OH)₂D₃ were orally administered to rachitic chicks at the concentrations indicated 9 hours prior to the assessment of calcium absorption as described in Materials and Methods. Each point represents the average of 5 measurements ± S.D. All groups treated with sterols are significantly different from rachitic controls at P < 0.01 by the Student-t test.

<table>
<thead>
<tr>
<th>Sterol administered</th>
<th>Amount of sterol (nmoles)</th>
<th>Calcium absorption (cpm ⁴⁵Ca/0.2 ml plasma)</th>
<th>% of maximal response</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Rachitic control)</td>
<td>0</td>
<td>315 ± 77</td>
<td>-</td>
</tr>
<tr>
<td>1α-OHD₃</td>
<td>0.16</td>
<td>533 ± 84</td>
<td>38</td>
</tr>
<tr>
<td>1α,25-(OH)₂D₃</td>
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<td>562 ± 58</td>
<td>43</td>
</tr>
<tr>
<td>1α-OHD₃</td>
<td>0.65</td>
<td>812 ± 156</td>
<td>86</td>
</tr>
<tr>
<td>1α,25-(OH)₂D₃</td>
<td></td>
<td>834 ± 81</td>
<td>90</td>
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<tr>
<td>1α-OHD₃</td>
<td>6.5</td>
<td>895 ± 145</td>
<td>100</td>
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</table>
Figure 7. Time course of induction of intestinal calcium absorption in rachitic chicks by 1α-hydroxyvitamin D₃, 1α,25-dihydroxyvitamin D₃, and vitamin D₃.

Rachitic chicks were orally dosed with 0.5 nmol of 1α-OHD₃ (●), 1α,25-(OH)₂D₃ (▲), or vitamin D (○) at the prescribed times prior to sacrifice. The intestinal calcium transport response was then assayed as described under "Materials and Methods." Each point is the average of 5 separate animals ± S.D.
Figure 8. Early time course of induction of intestinal calcium absorption in rachitic chicks by \( \alpha \)-hydroxyvitamin D\(_3\) and vitamin D\(_3\).

Rachitic chicks were orally dosed with 6.5 nmol of either \( \alpha \)-OHD\(_3\) (●) or vitamin D\(_3\) (○) at the prescribed times prior to sacrifice. The intestinal calcium transport response was then assayed as described under "Materials and Methods." Each point represents the average of 5 separate animals ± S.D.
the response to 6.5 nmol of 1α-OHD₃, which may be consistent with the functioning of this sterol through the control of gene expression and protein synthesis.

The bone calcium resorbing activity of 1α-OHD₃ was assessed by administering the sterol to rachitic chicks that had been placed on a low calcium diet for four days. An increase in plasma calcium concentration of such chicks is largely a measure of the bone calcium mobilizing activity of vitamin D sterols (131). Both 1α,25-(OH)₂D₃ and 1α-OHD₃ are considerably more active than vitamin D₃ 8 hours after administration (Table 2). Since 0.65 nmol of the natural hormone or 1α-OHD₃ elicits a greater increase in plasma calcium than 6.5 nmol of vitamin D₃ (Table 2), the hydroxylated derivatives are estimated to be at least 10 times as active as the parent vitamin D₃ within the 8-hour dosage period. 1α-hydroxyvitamin D₃ appears to be equal to 1α,25-(OH)₂D₃ in bone calcium mobilizing activity (Table 2) as well as in stimulating calcium transport from the intestine (Table 1). With a larger dose of 6.5 nmol of 1α-OHD₃, significant increases in plasma calcium are seen as early as two hours, and by 16 hours the plasma calcium level is strikingly increased to a near normal level of 9.7 mg/100 ml (Table 2).

Metabolism of 1α-OHD₃ to 1α,25-(OH)₂D₃

In order to determine if 1α-OHD₃ had to be converted to 1α,25-(OH)₂D₃ before exerting its biological effects, a series of in vivo biological activity experiments were performed since radioactive 1α-OHD₃ was not available. These studies were designed to test whether or not, when limiting amounts of these two sterols are administered to rachitic
Table 2. Bone calcium mobilizing activity of 1α-hydroxyvitamin D₃ in rachitic chicks.

Sterols were orally administered to rachitic chicks that had been raised on a calcium diet for 4 days and plasma calcium concentrations determined as described under "Materials and Methods." Each point represents the average of 5 measurements ± S.D. All values are significantly different from the control at P < 0.05 using the Student-t test.

<table>
<thead>
<tr>
<th>Sterol administered</th>
<th>Amount of sterol (nmole)</th>
<th>Time (hr)</th>
<th>Plasma calcium concentration (mg/100 ml)</th>
<th>Increment above control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (rachitic-low calcium control)</td>
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<td>-</td>
<td>5.3 ± 0.7</td>
<td>-</td>
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<tr>
<td>Vitamin D₃</td>
<td>6.5</td>
<td>8</td>
<td>6.4 ± 0.6</td>
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<td>1α,25-(OH)₂D₃</td>
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<tr>
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<td>6.8 ± 0.4</td>
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<tr>
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<tr>
<td>1α-OHD₃</td>
<td>6.5</td>
<td>16</td>
<td>9.7 ± 0.7</td>
<td>4.4</td>
</tr>
</tbody>
</table>
chicks, 1α-OH D$_3$ would appear to have a longer time course of action by virtue of its metabolism to 1α,25-(OH)$_2$D$_3$. The time course of calcium absorption in rachitic chicks pursuant to an oral dose of 162.5 pmol of either 1α-OH D$_3$ or 1α,25-(OH)$_2$D$_3$ is shown in Figure 9. At this limiting dose of sterol, which produces approximately 40% of the maximum possible response at 9 hours, the time courses of stimulation of calcium transport with the two sterols are practically identical. 1α,25-(OH)$_2$D$_3$ may function slightly more rapidly than 1α-OH D$_3$ which has a slightly more sustained action, but at no time are the data statistically different for the two sterols (Figure 9). As has been previously reported (54, 55), the hormone and its synthetic analog rapidly stimulate calcium absorption, following a 2-3 hour lag period, to yield a maximal effect 6-9 hours after administration of the sterols. The decay in the response is less dramatic than has been previously demonstrated by others (57, 58), but is consistent with an earlier report (37) of a 50% decay by 40 hours.

The dose-response relation was next quantitated at 9 hours after administration of 1α-OH D$_3$ or 1α,25-(OH)$_2$D$_3$ at various doses (20-650 pmol) and is shown in Figure 10. Virtually identical activity was obtained for the two sterols at all doses tested and at no dose were the two sterols significantly different in their ability to increase calcium transport. Thus, regardless of the sterol dose or the time of assay, 1α-OH D$_3$ and 1α,25-(OH)$_2$D$_3$ are virtually indistinguishable in these in vivo experiments. The data in Figures 9 and 10 are consistent with the concept that these two sterols function by identical mechanisms, but do not eliminate
Figure 9. Time course of action of 1α-hydroxyvitamin D3 and 1α,25-dihydroxyvitamin D3 in stimulating intestinal calcium absorption.

162.5 pmol of either 1α-OH-D3 (○) or 1α,25-(OH)2D3 (▲) was administered orally and calcium transport from intestine measured, in vivo, at the indicated time after dosing. Absorption of calcium was measured as described under "Materials and Methods." Each point is the average of determinations on 5 separate animals ± S.E.M.
Figure 10. Calcium absorption response of rachitic chicks to various doses of 1α-hydroxyvitamin D₃ and 1α,25-dihydroxyvitamin D₃.

Doses of 0.02–0.65 nmol of 1α-OHD₃ or 1α,25-(OH)₂D₃ were administered to rachitic chicks and calcium absorption was measured 9 hours later. Calcium absorption is expressed as increment (Δ cpm of ⁴⁵Ca) above the transport in untreated controls. Each bar represents the average of 6 separate animals ± S.E.M.
the possibility that the conversion of 1α-OHD₃ to 1α,25-(OH)₂D₃ is sufficiently rapid to precede the slower induction of calcium absorption.

When administered daily for a 3-week period to chicks raised on a rachitogenic diet, the results expressed in Table 3 illustrate that, as in single dose experiments, the biological activity of 1α-OHD₃ is equivalent to or greater than that of 1α,25-(OH)₂D₃. Of the four parameters tested, 1α-OHD₃ was somewhat more potent than the natural hormone in three cases: growth promotion, maintenance of plasma calcium, and increase of percent bone ash. Again, these results provide evidence for the equivalence of the synthetic sterol and the natural hormone in vivo. However, the greater efficacy of chronically administered 1α-OHD₃ is also suggestive of its serving as a precursor for 1α,25-(OH)₂D₃ with 1α,25-(OH)₂D₃ having a shorter biological half life.

Since no differences were noted in biological activity between 1α-OHD₃ and 1α,25-(OH)₂D₃ in vivo, an examination of the relative ability of 1α-OHD₃ to compete with radioactively labelled 1α,25-(OH)₂D₃ for the intestinal receptor was undertaken. The evidence correlating this receptor system with the biological response to the vitamin has been discussed previously (82, 83, 132, 133). By assessing the ability of various vitamin D sterols to compete with radioactively labelled 1α,25-(OH)₂D₃ for this intestinal receptor, this receptor system was used as an assay for vitamin D activity at the molecular level. When 1α-OHD₃ was tested in such a competitive binding assay, it competed with the radioactive hormone two to three orders of magnitude less efficiently than the nonradioactive 1α,25-(OH)₂D₃ (Figure 11). Thus, although 1α-OHD₃ is
Table 3. Activity of chronically administered 1α-OHD₃ and 1α,25-(OH)₂D₃ in chicks.

All sterols were orally administered daily for 3 weeks to rachitic chicks. Relative activity is computed by determining the daily dose of sterol required to produce a 50% curative effect and then comparing it to the level of vitamin D₃ necessary to elicit an equivalent response. Vitamin D₃ potency is set at 1.0 for all parameters tested. Experiment performed by Mr. Douglas Cork (The University of Arizona, Department of Chemistry).

<table>
<thead>
<tr>
<th>Sterol tested</th>
<th>Promotion of growth</th>
<th>Maintenance of plasma calcium</th>
<th>Increase in % bone ash</th>
<th>Alleviation of radiologic signs of rickets</th>
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<td>5.1</td>
<td>3.4</td>
<td>3.7</td>
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</table>
Figure 11. Relative ability of 1α-hydroxyvitamin D₃ and 1α,25-dihydroxyvitamin D₃ to compete with 1α,25-dihydroxyvitamin D₃ for intestinal chromatin receptor sites, in vitro.

Varying amounts of 1α-OHD₃ (o) or 1α,25-(OH)₂D₃ (Δ) were added to a reconstituted cytosol-chromatin receptor system from chick intestinal mucosa and incubated in the presence of 5 nM 1α,25-(OH)₂-[³H]D₃ (Brumbaugh et al., 134). The amount of tritium associated with the chromatin (trapped on filters) in the absence of nonradioactive competitor is taken as 100% and the displacement data with nonradioactive sterols expressed as the percent of this control binding. The concentration of nonradioactive competing sterol to radioactive 1α,25-(OH)₂D₃ is depicted on the abscissa. The possibility that nonradioactive 1α-OHD₃ is converted to 1α,25-(OH)₂D₃ by the intestinal 25-hydroxylase enzyme is eliminated by the fact that the 25-hydroxylase resides in the microsomal fraction and only cytosol and Triton X-100 washed chromatin fractions are present in the incubation. Experiment performed by Peter Brumbaugh (Department of Biochemistry, The University of Arizona).
equipotent to 1α,25-(OH)$_2$D$_3$ in all in vivo assays, it is strikingly less active at the molecular level. The data pictured in Figure 11 suggest that 1α-OHD$_3$ must be transformed to 1α,25-(OH)$_2$D$_3$ before acting upon the target intestine.

It was, therefore, deemed necessary to demonstrate this metabolic conversion in the chick directly. The experiment was performed by administering 0.65 nmol of 1α-OHD$_3$ to each of twenty rachitic chicks and vehicle alone to twenty rachitic chicks as the control. Three hours later, the animals were sacrificed and preparation of the chromatin and extraction of the sterols was carried out as described under "Materials and Methods." Putative 1α,25-(OH)$_2$D$_3$ fractions were isolated by silicic acid and Celite chromatography from chromatin extracts of control chicks and 1α-OHD$_3$-treated chicks. Assessment of loss of nonradioactive 1α,25-(OH)$_2$D$_3$ during the purification was done by monitoring tracer 1α,25-(OH)$_2[^3]H$D$_3$ added at the beginning of the isolation. Figure 12 pictures the results of purifications on the control and 1α-OHD$_3$-treated chromatin extracts. Final recovery of tracer 1α,25-(OH)$_2[^3]H$D$_3$ was 60% for both control and test groups. The Celite purified 1α,25-(OH)$_2$D$_3$ fractions from both control and 1α-OHD$_3$-treated chromatin extracts were then assayed via radioreceptor assay as previously described (56, 134). Figure 13A is the isotope dilution standard curve for authentic nonradioactive 1α,25-(OH)$_2$D$_3$. Figure 13B indicates that the 1α,25-(OH)$_2$D$_3$ fraction from control chicks produces no deflection in the assay while the 1α,25-(OH)$_2$D$_3$ fraction from 1α-OHD$_3$-treated chicks yields a significant competition for receptor binding sites. When the level of 1α,25-(OH)$_2$D$_3$
Figure 12. Chromatographic purification of suspected 1α,25-dihydroxyvitamin D₃ from intestinal chromatin of rachitic chicks given 1α-hydroxyvitamin D₃.

Twenty rachitic chicks each received 0.65 nmol of nonradioactive 1α-OHD₃ and were killed three hours later. Preparation of chromatin and extraction of sterols was carried out as described under "Materials and Methods." After addition of marker 1α,25-(OH)₂D₃ (17,000 dpm), purification of this extract ("1α-OHD₃-treated") was performed on successive silicic acid and Celite columns as depicted in the top panels. In separate experiments, crystalline 1α-OHD₃ (monitored by absorbance at 265 nm) was found to migrate approximately to fraction 5 on identical silicic acid columns and to fraction 6-7 on identical Celite columns. Thus, 1α-OHD₃ is completely resolved from 1α,25-(OH)₂-[³H]D₃ on these columns. Details of the chromatographic procedures are presented under "Materials and Methods." The bottom two panels illustrate the purification of a "control" extract prepared from 20 chicks that received dosing vehicle alone. Final yields of tracer 1α,25-(OH)₂-[³H]D₃ were 60% in both 1α-OHD₃-treated and control extracts. Experiment performed in collaboration with Peter Brumbaugh.
Figure 12. Chromatographic purification of suspected 1α,25-dihydroxyvitamin D₃ from intestinal chromatin of rachitic chicks given 1α-hydroxyvitamin D₃.
Figure 13. Radioreceptor assay of suspected 1α,25-dihydroxyvitamin D₃ isolated from chicks treated with 1α-hydroxyvitamin D₃.

A. Standard curve obtained with authentic 1α,25-(OH)₂D₃. The details of this assay have been previously reported (134). B. Assay of purified chromatin extracts (see Figure 12) from control and 1α-OHD₃-treated chicks; 5-, 10-, and 20-µl aliquots (in triplicate) of the suspected 1α,25-(OH)₂D₃ peaks (in 2.0 ml of ethanol) were assayed simultaneously with standard amounts of sterol as depicted in Panel A. Each assay was carried out in triplicate and the results are ± S.E.M. Experiments performed in collaboration with Peter Brumbaugh.
is calculated from the results in Figure 13B (taking into account the purification losses), each chick intestinal chromatin (15 mg of DNA equivalent) contains 26 pmol of $\alpha,25-(OH)_{2}D_3$ 3 hours subsequent to a physiologic dose of $\alpha$-$OHD_3$. This saturation value is in close agreement with the level of 25 pmol previously determined following direct administration of labelled hormone (83). Therefore, sufficient $\alpha,25-(OH)_{2}D_3$ is produced from $\alpha$-$OHD_3$ to saturate the physiologic receptors in the intestine. Since this phenomenon occurs prior to the initiation of calcium transport increases by $\alpha$-$OHD_3$, this strongly suggests that $\alpha$-$OHD_3$ functions by metabolism to the natural hormone. This metabolic conversion was also demonstrated in vitro in intestinal mucosa homogenates (Table 4) and indicates that the intestinal vitamin $D_3$-25-hydroxylase enzyme is capable of catalyzing the conversion of $\alpha$-$OHD_3$ to the natural $\alpha,25-(OH)_{2}D_3$ hormone. In addition, if the homogenate is boiled prior to the addition of $\alpha$-$OHD_3$, there is no conversion of the synthetic sterol to $\alpha,25-(OH)_{2}D_3$ in this system (Table 4), indicating that the conversion is not a result of $\alpha$-$OHD_3$ being nonenzymatically oxidized to $\alpha,25-(OH)_{2}D_3$ during extraction and chromatographic isolation of suspected $\alpha,25-(OH)_{2}D_3$ fractions for radioreceptor assay.

Thus, by virtue of its metabolism to $\alpha,25-(OH)_{2}D_3$, $\alpha$-$OHD_3$ is an effective precursor of the natural hormone and since it can be easily synthesized and in greater quantities than $\alpha,25-(OH)_{2}D_3$ it should prove to be useful in studies which up to now required the natural hormone. Figure 14 represents a summary of the structures and metabolic interrelationship of vitamin $D_3$ and the biologically active metabolites and
Table 4. Production of 1α,25-dihydroxyvitamin D₃ from 1α-hydroxyvitamin D₃ by intestinal mucosa in vitro.

Intestinal mucosa from rachitic chicks was homogenized (10%) in 0.25 M sucrose-Buffer A. Homogenate (0.5 ml/flask) was diluted to 10 ml with phosphate buffer, pH 7.4 and an NADPH generating system, and incubations were carried out following addition of 1α-OHD₃ in 50 μl ethanol. Methanol-chloroform (2:1) extracts of homogenates were purified by silicic acid, Sephadex LH-20, and Celite chromatography to isolate suspected 1α,25-(OH)₂D₃. Filter assay was carried out as previously described (134). Experiment performed by Mr. Peter Brumbaugh (Department of Biochemistry, The University of Arizona).

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<th>Time of incubation (min)</th>
<th>1α,25-(OH)₂D₃ produced (pmole)</th>
<th>Percent conversion</th>
</tr>
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<td>100°C for 20 min</td>
<td>30</td>
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</tbody>
</table>
Figure 14. Structures and metabolic pathways of vitamin D₃, 25-hydroxyvitamin D₃, 1α,25-dihydroxyvitamin D₃, and 1α-hydroxyvitamin D₃.

The natural metabolic pathway of vitamin D₃ in the chick is indicated with the dark arrows while the conversion of synthetic 1α-OHD₃ to 1α,25-(OH)₂D₃ is indicated with a light arrow.
analog used in this dissertation. It is evident that 1α-OHD₃ does not need to undergo the slower rate-limiting 1α-hydroxylation⁵ that is necessary for the conversion of 25-OHD₃ to 1α,25-(OH)₂D₃ and thus represents an effective precursor of the active metabolite.

**Assay of Intestinal Cell Nuclei DNA-Dependent RNA Polymerase Activity**

In order to study the influence of 1α,25-(OH)₂D₃ and related vitamin D₃ sterols on the RNA polymerase activities of mucosal cell nuclei, it was necessary to first devise an assay system for these enzymes. Prior to studies of transcription in intestinal nuclei, the ammonium sulfate optima for polymerases I and II were determined, since these optima vary in different types of nuclei (T. J. Lindell, personal communication). This study (Figure 15) indicated that 0.05 M ammonium sulfate [(NH₄)₂SO₄] is optimal for both forms I and II in these nuclei. At this (NH₄)₂SO₄ concentration, nuclei are still intact (Figure 16), which thus reduces the variability of the individual assays.

Figure 17 depicts the time course of incorporation of UMP into RNA catalyzed by forms I and II polymerase in isolated rachitic chick mucosal nuclei. There is an absolute requirement for all four nucleoside triphosphates since the omission of one (-ATP) results in no incorporation of UMP by the form II RNA polymerase. The lack of linearity is not due to substrate limitations in the assay since additions of multiples of the respective nuclear samples, incubated for the standard 5 minutes,

---

⁵. 25-hydroxylation is usually rapid as opposed to the slower, rate-limiting 1α-hydroxylation of vitamin D sterols (37, 135).
Figure 15. Effect of ammonium sulfate on RNA polymerase I and II activities in isolated intestinal nuclei.

Purified intestinal nuclei were prepared and assayed for RNA polymerase I (o) and II (●) activities in the presence of increasing ammonium sulfate concentrations using the standard RNA polymerase assay system described under "Materials and Methods." Each point represents triplicate determinations ± S.E.M.
Figure 16. Purified intestinal cell nuclei under phase contrast microscopy.

Nuclei were prepared by treatment with a 0.1% Triton X-100-sucrose buffer followed by sedimentation through 1.7 M sucrose as described under "Materials and Methods." Phase contrast microscopy was performed with a Zeiss phase contrast microscope using a x100 oil immersion objective to give a total magnification of x1000.
Figure 17. Time course of UMP incorporation into chick intestinal nuclei by RNA polymerases I and II.

Purified intestinal nuclei were prepared and assayed for polymerase I (o) and II (●) activities as described under "Materials and Methods." Incorporation of UMP into RNA by the form II polymerase when ATP is deleted from the assay mixture is also shown (○). Each point represents the average of 3 determinations ± S.E.M. As indicated, the numbers on the ordinate have been multiplied by 10^{-2}; units are expressed per mg DNA.
reveal a linear dependence on DNA (nuclei) concentration (Figure 18). Furthermore, UMP incorporation by the form II enzyme was greater at 20° than at 30° or 37° (Figure 19), suggesting that the nuclear preparations may contain high RNase levels. However, the addition of a 100-fold excess of unlabelled UTP to the reaction mixture causes only a 10% loss of label already incorporated into an acid insoluble product (RNA) by the form II enzyme. Therefore, the effects of endogenous RNases on the linearity of UMP incorporation are negligible.

Other attempts to improve the linearity of UMP incorporation by the form I and II RNA polymerases were unsuccessful and, therefore, Figure 17 represents the best obtainable linearity for the assay. The time course of UMP incorporation by the nucleoplasmic enzyme into vitamin D₃-treated chick mucosal cell nuclei is not any more linear than that of Figure 17 (Figure 20). Although there is an increased incorporation at all time points, the curve is identical to that of Figure 17. Therefore, the standard assay conditions for the determination of RNA polymerase activities were 5 minutes of incubation at 20°.

Effect of Vitamin D₃ and Related Sterols In Vivo on Polymerases I and II Activity

The initial studies of RNA polymerase activity were performed with vitamin D₃ rather than the active metabolite 1α,25-(OH)₂D₃ since it was known that the parent vitamin would be metabolized to the natural hormone in vivo. Therefore, 1.3 μg of vitamin D₃ or vehicle alone (control) was orally administered to rachitic chicks at different intervals and the animals sacrificed. Intestinal nuclei were prepared and
Figure 18. Dependency of UMP incorporation into chick intestinal nuclei on the RNA polymerase I and II concentrations.

Purified intestinal nuclei were prepared and assayed for RNA polymerase I (●) and II (○) activities as described under "Materials and Methods." Increasing concentrations of DNA (RNA polymerase I and II) were incubated for the standard 5-minute reaction period. Each point represents one determination at the respective DNA concentrations indicated.
Figure 19. Temperature dependency of UMP incorporation into chick intestinal nuclei by RNA polymerase II.

Purified intestinal nuclei were prepared and assayed for RNA polymerase II activity as described under "Materials and Methods" at temperatures of 37° (□), 30° (○), and 20° (●). In one of the assays, a 100-fold excess of nonradioactive UTP was added to one-half of the reaction mixture after 5 minutes of incubation (■) and aliquots removed at the indicated times for assessment of the activity of endogenous RNases. Each point represents the average of 3 determinations ± S.E.M.
Figure 20. Time course of UMP incorporation by RNA polymerase II into control and vitamin D₃-treated intestinal nuclei.

Animals were orally dosed with 1.3 µg of vitamin D₃ (•) or carrier alone (○) (control) and 6 hours later purified intestinal nuclei were prepared and assayed for form II polymerase activity as described under "Materials and Methods." Each point represents the composite average of 3 determinations on 3 animals per control or test group ± S.E.M.
assayed for RNA polymerase activities as described in "Materials and Methods." Figures 21 and 22 depict the time courses of polymerase I and II activities, respectively. Form I polymerase activity begins increasing 6-8 hours after administration of the sterol and is significantly elevated (p < 0.05) by 20 hours. The form II enzyme activity is significantly elevated (p < 0.05) at 6-12 hours after dosing with the sterol. By 20 hours, a time at which the vitamin's physiologic response (increased calcium transport) has begun, the form II polymerase activity has declined and is no longer significant. These results contradict the earlier report of Lawson et al. (136) of no increase in RNA polymerase activities after 125 µg of vitamin D₃. However, those authors used a relatively insensitive assay for measuring the RNA polymerase activities and examined the nuclei for enzyme activity at time intervals after dosing which were too soon or too long to see changes in the enzyme activities. Since this parent sterol is metabolized to 1α,25-(OH)₂D₃, these results prompted an examination of the time course of polymerase activities after administration of the 1α,25-(OH)₂D₃ hormone.

Preliminary investigations into the effect of 1α,25-(OH)₂D₃ on RNA polymerase activities were performed between 0 and 12 hours. This time was selected because 1α,25-(OH)₂D₃ produced its maximal stimulation of calcium transport at 9 hours (Figure 7). Rachitic chicks were dosed with 0.27 µg of 1α,25-(OH)₂D₃ and the polymerase activities in isolated intestinal nuclei examined at 6 and 12 hours after administration of the sterol. Unlike the stimulation of form I RNA polymerase activity produced by vitamin D₃ (Figure 21), it is apparent that no significant
Figure 21. Time course of activity of RNA polymerase I after vitamin D₃ in chick intestinal nuclei.

Animals were orally dosed with 1.3 μg of the sterol at the indicated times before sacrificing. Purified intestinal nuclei were prepared and assayed for RNA polymerase I activity as described under "Materials and Methods." The numbers in parentheses indicate the number of animals per group. Each point represents the composit average of 3 assays per animal ± S.E.M. *Represents p < 0.05 as performed by the Student-t test on paired samples.
Figure 22. Time course of activity of RNA polymerase II after vitamin D₃ in chick intestinal nuclei.

Animals were orally dosed with 1.3 µg of vitamin D₃ at the indicated times before sacrificing. Purified intestinal nuclei were prepared and assayed for RNA polymerase II activity as described under "Materials and Methods." Each point represents the composite average of 4 animals per group (3 assays/animals) ± S.E.M. *Represents p < 0.05 as performed by the Student-t test on paired samples.
changes resulted in form I polymerase activity up to 12 hours after dosing with the active metabolite (Figure 23). Similarly, form II enzyme activity (Figure 24) did not appear to be increased after 1α,25-(OH)₂D₃ administration. However, a closer examination of the temporal response of these two enzymes to 1α,25-(OH)₂D₃ was performed, nonetheless, with greater replication at each time period.

Figure 25 summarizes the results of three experiments designed to show the time course of mucosal cell nuclei RNA polymerase I and II activities subsequent to administration of the hormone. It is evident that 1α,25-(OH)₂D₃ produced a rapid enhancement of polymerase II activity in 2-3 hours (p < 0.05) which returned to control values by 5-10 hours. These results demonstrate a near 2-fold increase in the activity of this enzyme after administration of the hormone. In contrast, the activity of the nucleolar form I enzyme did not change throughout the time studied as was previously seen (Figure 23). Comparable results were also obtained when 1α-OHD₃ was used in place of 1α,25-(OH)₂D₃ (Figure 26) and a shorter time course performed. The results indicate that again at 2 hours a significant (p < 0.025) increase in form II activity is evident. There is no change in form I activity. Since 1α-OHD₃ is very rapidly metabolized to 1α,25-(OH)₂D₃, these results essentially represent another determination of the time course of activation of RNA polymerase II by the active metabolite and demonstrate that the earliest observable increase in the activity of this enzyme is at 2 hours.

Further verification of the 1α,25-(OH)₂D₃-mediated stimulation of endogenous form II activity was performed by examining the tissue and
Figure 23. Time course of activity of RNA polymerase I after 1α,25-dihydroxyvitamin D3 in chick intestinal nuclei.

Animals were orally dosed with 0.27 μg of the sterol at the indicated times before sacrificing. Purified intestinal nuclei were then prepared and assayed for RNA polymerase I activity as described under "Materials and Methods." Each point represents the composite average of 3 animals per group (3 assays/animal) ± S.E.M.
Figure 24. Time course of activity of RNA polymerase II after 1α,25-dihydroxyvitamin D₃ in chick intestinal nuclei.

Animals were orally dosed with 0.27 µg of the sterol at the indicated times before sacrificing. Purified intestinal nuclei were then prepared and assayed for RNA polymerase II activity as described under "Materials and Methods." Each point represents the composite average of 3 animals per group (3 assays/animal) ± S.E.M.
Figure 25. Detailed time course of activity of RNA polymerases I and II after 1α,25-dihydroxyvitamin D₃ in chick intestinal nuclei.

Animals were orally dosed with 0.27 μg of the sterol at the indicated times before sacrificing. Purified intestinal nuclei were prepared and assayed for RNA polymerase I (●) and II (○) activities as described under "Materials and Methods." Each point represents the composite average of three separate experiments (4 animals per point per experiment, 3 assays per animal) ± S.E.M. *Represents p < 0.05 as performed by the Student-𝑡 test on paired samples.
Figure 26. Time course of activity of RNA polymerases I and II after 1α-hydroxyvitamin D₃ in chick intestinal nuclei.

Animals were orally dosed with 0.27 μg of the sterol at the indicated times prior to sacrificing. Purified intestinal nuclei were prepared and assayed for RNA polymerase I (•) and II (○) activities as described under "Materials and Methods." Each point represents the composite average of 3 animals per group (3 assays/animal) ± S.E.M. *Represents p < 0.05 as performed by the Student-t test on paired samples.
sterol specificity of this response. Since it was possible that considerable amounts of the administered sterols could be lost due to poor absorption or rapid catabolism, the experimental protocol was changed to insure that each tissue received saturating amounts of sterol. Three doses of $1\alpha,25-(OH)_2D_3$ (0.27 $\mu$g/dose) were administered to rachitic chicks at three-hour intervals. The animals were sacrificed three hours after the last dose and the appropriate tissues removed and nuclei prepared and assayed for polymerase activity as described in "Materials and Methods." Table 5, experiment 1, depicts the results of an experiment which indicates that when rachitic chicks are dosed once with the natural hormone the activity of the form II enzyme is identical to that of the control when assayed 9 hours after administration of the sterol, as expected (Figure 25). When three doses of the hormone are administered, there is a significant ($p < 0.025$) increase in the form II activity although the magnitude of stimulation (28%) is not as high as that seen previously. Experiment 2 indicates that $1\alpha,25-(OH)_2D_3$ produced significant increases in the form II activity of target tissue nuclei and not in the nuclei of liver. Further support for the role of $1\alpha,25-(OH)_2D_3$ in stimulating the form II polymerase activity was obtained by an examination of the sterol specificity of this response. Table 6 summarizes two experiments in which the active hormone and its biological precursors, either vitamin D$_3$ or 25-OHD$_3$, were administered in three doses (0.27 $\mu$g/dose) and the intestinal nuclei then assayed for polymerase activity. Experiment 1 indicates that vitamin D$_3$ did not produce any increase in the polymerase activity over control values while the active metabolite
Table 5. Tissue specificity of 1α,25-(OH)₂D₃-stimulated increase in form II DNA-dependent RNA polymerase activity.

1α,25-(OH)₂D₃ was orally administered to rachitic chicks (0.27 μg/dose) either once or 3 times at 3-hour intervals. Intestinal or liver nuclei were then prepared and assayed for endogenous RNA polymerase activity as described under "Materials and Methods." Each value represents the average of 5 animals (3 assays/animal) ± S.E.M.

<table>
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<th>Experiment No.</th>
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<th>% stimulation</th>
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Table 6. Sterol specificity of 1α,25-(OH)₂D₃-stimulated increase in form II DNA-dependent RNA polymerase activity.

All sterols were orally administered in 3 doses (0.27 µg/dose) at 3-hour intervals to rachitic chicks. Intestinal nuclei were then prepared and assayed for endogenous RNA polymerase activity as described in "Materials and Methods." Values represent the average of 5 animals (3 assays/animal) ± S.E.M.

<table>
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<th>Experiment No.</th>
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<td></td>
<td>vitamin D₃ (x3)</td>
<td>1379 ± 101</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>1α,25-(OH)₂D₃ (x3)</td>
<td>1924 ± 206 (p &lt; 0.050)</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>none</td>
<td>1124 ± 135</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>25-OHD₃ (x3)</td>
<td>1487 ± 73 (p &lt; 0.050)</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>1α,25-(OH)₂D₃ (x3)</td>
<td>1839 ± 75 (p &lt; 0.005)</td>
<td>63</td>
</tr>
</tbody>
</table>
did, as previously seen in Table 5. When 25-OHD$_3$ was administered, it did produce a significant increase in the form II polymerase activity (experiment 2) although not as great as for 1α,25-(OH)$_2$D$_3$. These results support the notion that at the physiological levels of vitamin D$_3$ employed in these experiments insufficient 1α,25-(OH)$_2$D$_3$ was probably synthesized during the 9-hour dosing interval. Consequently, there was no increase in the polymerase activity. However, the administration of 25-OHD$_3$ may have produced enough 1α,25-(OH)$_2$D$_3$ in 9 hours to cause saturation of the target tissue with the hormone and thus increase the form II polymerase activity.

These results, therefore, imply that stimulation of the form II polymerase activity by 1α,25-(OH)$_2$D$_3$ in the intestinal mucosa cell nucleus is one of the early events which may lead to the expression of new genetic material and ultimately to the physiologic response.

**DEAE-Sephadex Column Chromatography of RNA Polymerases I and II**

To determine if the increased activity of the form II polymerase was due to a change in the chromosomal template or in the enzyme itself, identical aliquots of soluble nuclear protein from control and 2-hour 1α,25-(OH)$_2$D$_3$-treated animals (Figure 25) were chromatographed on DEAE-Sephadex and assayed for RNA polymerase activities as described in "Materials and Methods." As shown in Figure 27, this increase in activity is reflected as an increase in the activity of soluble polymerase II and the two-fold increase of hormone-treated relative to control soluble polymerase II activity is similar to that seen in the
Figure 27. DEAE-Sephadex chromatography of soluble nuclear protein from control and 1α,25-dihydroxyvitamin D₃-treated chicks.

Soluble nuclear protein was isolated from pooled samples of the zero and 2 hour 1α,25-(OH)₂D₃-treated nuclei from Figure 25 as described under "Materials and Methods." Each column represents 18 mg of protein from a pooled sample (4 animals per sample). Assays were performed as described under "Materials and Methods" in the presence of α-amanitin (Δ) and in the absence of α-amanitin (o). The solid line represents the ammonium sulfate gradient.
nuclear assays (Figure 25). It is again apparent that there is no increase in the nucleolar polymerase I activity. DEAE-Sephadex chromatography of polymerases I and II was also performed on soluble nuclear protein from the control, 6-hour, and 24-hour vitamin D$_3$-treated nuclei (Figure 28) used in the experiments depicted in Figure 20 and 21. It can be seen that the form II to I ratio increases from 3.1 for the control to 4.5 for the 6-hour treated soluble nuclear protein. This magnitude of stimulation of the form II enzyme as assayed with exogenous template is comparable to that observed when intact nuclei are assayed. By 24 hours, a time at which the nuclear polymerase II activity is no longer significantly elevated, the activity of the soluble enzyme has returned to values which give a II to I ratio of 2.9, comparable to the control ratio. No significant changes were observed in the soluble nucleolar form I enzyme. Since the augmented RNA polymerase II activity is still evident when assayed on exogenous template, the amount and/or activity of nucleoplasmic RNA polymerase, per se, is enhanced by the action of 1α,25-(OH)$_2$D$_3$.

Effect of 1α,25-Dihydroxyvitamin D$_3$ In Vitro on RNA Polymerases I and II

To determine if the stimulation of the endogenous form II RNA polymerase could be reconstructed in vitro, purified nuclei from rachitic chicks were incubated for 60 minutes with hormone-receptor complexes. The 1α,25-(OH)$_2$D$_3$-receptor complexes were formed as described in "Materials and Methods." After incubation, the nuclei were then collected and assayed for RNA polymerase activities as described in
Figure 28. DEAE-Sephadex chromatography of soluble nuclear protein from control and vitamin D$_3$-treated chicks.

Soluble nuclear protein was isolated from pooled samples of the (A) zero, (B) 6 hour, and (C) 24 hour vitamin D$_3$-treated intestinal nuclei of Figures 21 and 22 as described under "Materials and Methods." Each column represents 20 mg of protein from a pooled sample (3 animals per sample). Assays were performed as described in "Materials and Methods" in the presence of $\alpha$-amanitin (A) and in the absence of $\alpha$-amanitin (o). The dashed line in each panel represents the ammonium sulfate gradient.
Figure 28. DEAE-Sephadex chromatography of soluble nuclear protein from control and vitamin D$_3$-treated chicks.
"Materials and Methods." Using this protocol, it was found that both form I and II RNA polymerase activities were significantly decreased, even in the control nuclei. A time course of endogenous nuclear polymerase activities (Figure 29) incubated in the presence and absence of 1α,25-(OH)₂D₃ clearly shows the rapid loss of both enzyme activities from nuclei in the absence of PMSF while nuclei incubated in the presence of PMSF retain their enzyme activities. By 15 minutes of incubation time, approximately 30% of the form II RNA polymerase activity has been lost and by 60 minutes, the time at which the nuclei are assayed, only 17% of the original enzyme activity remains. The results indicate the need for including PMSF in all solutions during the preparation and assay of endogenous nuclear RNA polymerase activities since this agent has been reported to prevent nonspecific losses of form II RNA polymerase activity (137). However, initial studies in which PMSF was included in a reconstituted system of 1α,25-(OH)₂D₃-receptor complexes and intestinal chromatin indicated that PMSF significantly inhibited (50%) the association of the hormone-receptor complex with the chromatin. These results precluded the use of PMSF in experiments involving the in vitro transfer of hormone-receptor complex to the chromatin. Other attempts to reconstruct the 1α,25-(OH)₂D₃-mediated increase in form II RNA polymerase activity in vitro by incubating intestinal mucosa cell nuclei from rachitic chicks with the hormone-receptor complexes in the presence of the RNA polymerase assay system or in the presence of form II avian RNA polymerase collected from DEAE-Sephadex chromatography and added to the incubation system gave essentially negative results. One explanation for
Figure 29. Effect of phenylmethylsulfonylfluoride on the loss of nuclear RNA polymerase I and II activities from intestinal nuclei in vitro.

Purified intestinal nuclei were incubated in the presence (■, ●) or absence (□, ○) of 1α,25-(OH)₂D₃-receptor complexes in vitro as described under "Materials and Methods." Form I RNA polymerase activity (○, ●) or form II polymerase activity (□, ■) were assayed as described in "Materials and Methods" in the absence of phenylmethylsulfonylfluoride (PMSF). Form II RNA polymerase activity from control nuclei was also quantitated in the presence of PMSF (Δ). Each point represents the polymerase activity of a 100 µl aliquot removed from the reaction mixture at the times indicated.
the lack of any observed changes in the form II RNA polymerase activity in vitro by 1α,25-(OH)₂D₃ is that this sterol may be inducing the form II enzyme and that no changes in the activity of this enzyme would be observed in the in vitro system since this induction would not be reconstructed in the in vitro assay system.

Assay for Intestinal Nuclear Chromatin Template Activity

Preparation of Escherichia coli RNA Polymerase

Bacterial RNA polymerase was prepared from E. coli W3110 (donated by Dr. Christopher Mathews, University of Arizona) which had been grown to 3/4 log phase in enriched medium. The purification of this enzyme, according to the procedure of Burgess (117), is summarized in Table 7. Fifty-eight grams of E. coli paste was used and an overall purification of 400-fold was attained. This prokaryotic RNA polymerase was used in most template assays.

Preparation of Wheat Germ RNA Polymerase

The purification of wheat germ RNA polymerase is summarized in Table 8 from 500 g of starting material, utilizing the procedure of Jendrisak and Burgess (118). Since the enzymatic and physical properties of wheat germ RNA polymerase have been shown to be similar, if not identical, to the analogous enzymes isolated from other eukaryotic sources (138, 139), it was used in some template assays as a substitute for avian intestinal form II enzyme. Wheat germ RNA polymerase was purified approximately 3,000-fold.
Table 7. Summary of *Escherichia coli* W3110 RNA polymerase purification.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total activity (units)*</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase-treated extract</td>
<td>6592</td>
<td>10,712</td>
<td>1.6</td>
<td>100</td>
</tr>
<tr>
<td>High speed supernatant</td>
<td>3978</td>
<td>8,658</td>
<td>2.2</td>
<td>81</td>
</tr>
<tr>
<td>Ammonium sulfate fraction</td>
<td>1480</td>
<td>7,900</td>
<td>5.3</td>
<td>74</td>
</tr>
<tr>
<td>Pooled DEAE-cellulose peak</td>
<td>12</td>
<td>4,650</td>
<td><strong>390.0</strong></td>
<td>43</td>
</tr>
</tbody>
</table>

*One activity unit of enzyme incorporates 1 nanomole of UMP in 10 minutes of incubation under the conditions described in "Materials and Methods."
Table 8. Summary of wheat germ RNA polymerase purification.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total activity* (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>66,000</td>
<td>5270</td>
<td>0.079</td>
<td>100</td>
</tr>
<tr>
<td>Polymin P eluate</td>
<td>3,400</td>
<td>6580</td>
<td>1.9</td>
<td>125</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>440</td>
<td>3530</td>
<td>8.0</td>
<td>67</td>
</tr>
<tr>
<td>precipitate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose peak</td>
<td>15</td>
<td>1350</td>
<td>90.0</td>
<td>26</td>
</tr>
<tr>
<td>Phosphocellulose peak</td>
<td>8.5</td>
<td>1600</td>
<td>190.0</td>
<td>30</td>
</tr>
</tbody>
</table>

* One activity unit of enzyme incorporates 10 picomoles of UMP in 15 minutes of incubation under the conditions described in "Materials and Methods."
Kinetics of Chromatin Template Assay

Before assaying the chromatin template activity of 1α,25-(OH)₂D₃-treated animals, it was necessary to determine the kinetics of incorporation of UMP into RNA by the exogenous RNA polymerases. Figures 30 and 31 demonstrate the time course of incorporation of UMP into RNA by the bacterial and wheat germ enzymes, respectively, using chick intestinal chromatin as the template. Linear kinetics are seen for both enzymes for ten minutes, the reaction time for the template assay. In addition, the kinetics of incorporation of UMP had a linear dependence on the enzyme concentration for both enzymes, as shown in Figures 32 and 33. Furthermore, E. coli RNA polymerase displayed rifampicin (100 µg/assay) sensitivity and the wheat germ polymerase was sensitive to α-amanitin (0.1 µg/assay) (Figures 32 and 33). It is also evident in these figures that when 25 µg of DNA as intestinal chromatin is used as the template, saturating levels of bacterial and wheat germ enzyme are computed to be 130 and 30 µg, respectively. Consequently, these levels of enzyme were used in all template assays containing 25 µg or less of DNA as chromatin. All template assays were performed for 10 minutes at 37°.

Effect of 1α,25-Dihydroxyvitamin D₃ and Related Sterols In Vivo on Intestinal Chromatin Template Activity

To assess whether 1α,25-(OH)₂D₃ administration affects intestinal chromatin template activity, the hormone was given (0.27 µg/animal) to rachitic chicks 2 hours prior to preparation of the chromatin. This time was selected because saturation of the chromatin with the hormone and maximal stimulation of endogenous RNA polymerase II activity occur at
Figure 30. Time course of UMP incorporation by *Escherichia coli* RNA polymerase using chick intestinal chromatin as the template.

Chick intestinal chromatin (8-12 µg of DNA) and 130 µg of *Escherichia coli* RNA polymerase were allowed to synthesize RNA under conditions described under "Materials and Methods" for the times indicated.
Figure 31. Time course of UMP incorporation by wheat germ RNA polymerase using chick intestinal chromatin as the template.

Chick intestinal chromatin (8-12 µg of DNA) and 52 µg of wheat germ RNA polymerase were allowed to synthesize RNA under conditions described under "Materials and Methods" for the times indicated.
Figure 32. Dependency of $[^3\text{H}]\text{UMP}$ incorporation by chick intestinal chromatin upon *Escherichia coli* RNA polymerase concentration.

Increasing amounts of *Escherichia coli* RNA polymerase were added to the standard template assay system described under "Materials and Methods" in the absence (○) or presence (●) of rifampicin (100 µg/assay) and 25 µg of chick intestinal chromatin as the template.
Figure 33. Dependency of $[^3H]$ UMP incorporation by chick intestinal chromatin upon wheat germ RNA polymerase concentration.

Increasing amounts of wheat germ RNA polymerase were added to the standard template assay system described under "Materials and Methods" in the absence (o) or presence (●) of α-amanitin (0.1 µg/assay) and 25 µg of chick intestinal chromatin as the template.
this time (Figures 25 and 26) (113), and the ensuing physiologic response in calcium transport which occurs in 3-8 hours (37). The results of three experiments depicted in Figure 34 indicate that administration of the active hormone significantly increased \( p < 0.05 \) the template activity of intestinal chromatin by 12 to 20\% over control values in these experiments.

Further characterization of this template response was accomplished by examining the time course of intestinal chromatin template activation. The results are expressed in Figure 35 as the ratio of the template activity of the sterol-treated animals to that of the controls. The data are expressed as a ratio to correct for interexperimental variation since each time point represents one separate experiment and its appropriate control. These values are tabulated in the legend of Figure 35. Significant stimulation \( p < 0.05 \) of the template activity was evident at 2 hours and the magnitude of stimulation (16\%) was in agreement with the values previously determined (Figure 34). At three hours, the stimulation had dropped from 16 to 10\% and was no longer statistically significant. By 5 hours, a time at which the physiologic response begins, the template activity had dropped to 5\% above that of the control.

**Tissue and Sterol Specificity of 1α,25-Dihydroxyvitamin D<sub>3</sub> Stimulation of Intestinal Chromatin Template Activity In Vivo**

The observed increase in chromatin template activity was selective for intestinal nuclear chromatin, as shown in Table 9. Chromatin isolated from nuclei of liver or kidney tissue of hormone-treated animals
Figure 34. Effect of 1α,25-dihydroxyvitamin D3 on chick intestinal chromatin template activity in vivo.

Rachitic chicks were orally dosed with either vehicle (control) or 0.27 μg of 1α,25-(OH)2D3 and two hours later intestinal chromatin prepared as described under "Materials and Methods." Chromatin (10 to 15 μg of DNA) was then assayed in the presence of excess (130 μg) Escherichia coli RNA polymerase as described under "Materials and Methods." Each control and test group represents 5 animals. Values are the composite average of determinations on five separate animals ± S.E.M.; each chick chromatin preparation was assayed in quintuplicate to obtain the template activity value in that animal. Significance was calculated using the two-tailed, Student-t test. The numbers in parentheses represent the percent stimulation of the template activity of the test group over that of the control.
Figure 34. Effect of 1α,25-dihydroxyvitamin D3 on chick intestinal chromatin template activity in vivo.
Figure 35. Time course ofintestinal chromatin template activation by 1α,25-dihydroxyvitamin D₃ in vivo.

Rachitic chicks were orally dosed with 0.27 μg of the sterol or carrier alone to the control animals for the times indicated. The results are expressed as the ratio of the sterol-treated animals' template activity to that of the controls. The average values (nmol of UMP incorporated per mg DNA) of template activity ± S.E.M. for the control and test groups, respectively, at each time point were 1 hour, 12.3 ± 0.6 and 12.6 ± 0.3 (p > 0.1); 2 hours, 11.0 ± 0.6 and 12.8 ± 0.6 (p < 0.05); 3 hours, 14.8 ± 0.4 and 16.5 ± 0.9 (p > 0.05); and 5 hours, 20.1 ± 0.8 and 21.8 ± 1.0 (p > 0.1). The standard assay for template activity is described under "Materials and Methods." Five animals per control and experimental group were assayed in quintuplicate as described in Figure 34. *Indicates p < 0.05 as calculated by Student-t test.
Table 9. Tissue specificity of chick chromatin template activation produced by 1α,25-(OH)₂D₃ in vivo.

For template activity measurements in vivo, various tissue chromatinins (8-12 μg of DNA) and 130 μg of RNA polymerase were allowed to transcribe RNA as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Sterol treatment*</th>
<th>[³H] UMP incorporated into RNA/mg DNA** (nmol)</th>
<th>Increase over control animals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine</td>
<td>none</td>
<td>11.8 ± .2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+1α,25-(OH)₂D₃</td>
<td>13.9 ± .4 (p &lt; 0.050)***</td>
<td>18</td>
</tr>
<tr>
<td>Liver</td>
<td>none</td>
<td>15.8 ± .4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+1α,25-(OH)₂D₃</td>
<td>15.9 ± .5 (p &gt; 0.100)</td>
<td>-</td>
</tr>
<tr>
<td>Kidney</td>
<td>none</td>
<td>13.6 ± .3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+1α,25-(OH)₂D₃</td>
<td>14.3 ± .4 (p &gt; 0.100)</td>
<td>5</td>
</tr>
</tbody>
</table>

*0.65 nmole of sterol was orally administered to rachitic chicks two hours prior to preparation of chromatin from each tissue.

**Values are the average of determinations on 5 separate animals ± S.E.M.; each chick chromatin preparation was assayed in quintuplicate to obtain the template activity value in that animal.

***Significance was calculated using the two-tailed, Student-t test.
showed no significant stimulation over control animals while intestinal nuclei from the same animals showed significant stimulation, indicating that the hormone-mediated template response is specific for the target tissue chromatin.

Table 10 presents the results of three experiments designed to test the sterol specificity of chick intestinal mucosa cell chromatin template activation. 1α,25-dihydroxyvitamin D₃, its natural precursors vitamin D₃ and 25-OHD₃, and the synthetic precursor 1α-OHD₃ were tested. Since vitamin D₃ and 25-OHD₃ must be metabolized to 1α,25-(OH)₂D₃, their time course of action is slower than that of the natural hormone by several hours (39). Consequently, both vitamin D₃ and 25-OHD₃ failed to elicit an increase in chromatin template activity when assayed 2 hours after in vivo exposure of the chicks, as shown by experiments 1 and 2. However, 1α,25-(OH)₂D₃ produced an 18% stimulation of template activity, as previously seen at the 2-hour period. When 1α-OHD₃ was examined at 2 hours, a 5% stimulation was observed which was not significant. Since 1α-OHD₃ is rapidly metabolized to 1α,25-(OH)₂D₃ (Figure 13), it was anticipated that the synthetic sterol should also produce a template activation. Experiment 3 indicates that if the synthetic sterol is administered for 2.5 hours a significant increase of 16% in the template activity is observed. A detailed examination of the time course of chromatin template activation by 1α-OHD₃ showed that consistent stimulation was seen only between 2 and 3 hours after the dose. The slightly slower action of 1α-OHD₃, in enhancing the chromatin template activity,
Table 10. Sterol specificity of intestinal mucosa chromatin template activation in vivo.

Intestinal mucosa cell chromatin (8-12 μg of DNA) and 130 μg of RNA polymerase were allowed to synthesize RNA under conditions described in "Materials and Methods" for template activity measurements in vivo.

<table>
<thead>
<tr>
<th>Sterol*</th>
<th>Time treatment (hr)</th>
<th>$[^3]H$ UMP incorporated into RNA/mg DNA** (nmol)</th>
<th>Increase over control chromatin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>2</td>
<td>11.2 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>1α,25-(OH)$_2$D$_3$</td>
<td>2</td>
<td>13.2 ± 1.0 (p &lt; 0.05)** 18</td>
<td></td>
</tr>
<tr>
<td>25-OHD$_3$</td>
<td>2</td>
<td>11.2 ± 0.8 (p &gt; 0.100) 0</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 2:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>2</td>
<td>15.6 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>1α-OHD$_3$</td>
<td>2</td>
<td>16.5 ± 0.7 (p &gt; 0.100) 5</td>
<td></td>
</tr>
<tr>
<td>vitamin D$_3$</td>
<td>2</td>
<td>15.2 ± 0.8 (p &gt; 0.100) -</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 3:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>2.5</td>
<td>15.8 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>1α-OHD$_3$</td>
<td>2.5</td>
<td>18.2 ± 0.6 (p &lt; 0.05) 16</td>
<td></td>
</tr>
</tbody>
</table>

*0.65 nmoles of various vitamin D analogs were administered orally to rachitic chicks for the times indicated prior to preparation of the intestinal cell chromatin.

**Values are the average of determinations on 5 separate animals ± S.E.M.; each chick chromatin preparation was assayed in quintuplicate to obtain the template activity value in that animal.

***Significance was calculated using the two-tailed, Student-t test.
may be due to the additional time required for metabolic conversion to produce a sufficient amount of $1\alpha,25-(OH)_2D_3$ prior to association with chromatin.

Effect of $1\alpha,25$-Dihydroxyvitamin D$_3$ and Related Sterols In Vitro on Intestinal Chromatin Template Activity

To further verify that $1\alpha,25$-(OH)$_2$D$_3$ could affect the intestinal chromatin template activity, an in vitro system of purified mucosal cell nuclei reconstituted with the hormone-receptor complexes was utilized. $1\alpha,25$-dihydroxyvitamin D$_3$ has been shown by Brumbaugh and Haussler (83, 84) to associate at $0^\circ$ with a 3.7 S receptor protein in the intestinal cell cytosol fraction. At $25^\circ$, this hormone-receptor complex subsequently undergoes a temperature-dependent transfer to the nuclear chromatin (82-84). Figure 36 summarizes three experiments involving the in vitro transfer of hormone to the nucleus by preincubating the hormone with the cytosol receptor fraction at $0^\circ$ for 1 hour and then reconstituting purified nuclei (from rachitic chicks) with the hormone-receptor complexes by incubation at $25^\circ$ for 1 hour. When chromatin from these nuclei was assayed for template activity, it was evident that the sterol produced a 12 to 23% increase over control values. The third experiment indicates that the variation between chromatin preparations is less than 4% for both the control and experimental groups when six separate nuclear and cytosol fractions are prepared and reconstituted simultaneously in a single experiment.
Figure 36. Effect of 1α,25-dihydroxyvitamin D3 on reconstituted chromatin template activity in vitro.

Intestinal mucosa cell cytosol was prepared as described under "Materials and Methods" and then preincubated with either ethanol (50 μl/10 ml cytosol) for the control group or 50 μl of ethanol containing 1α,25-(OH)2D3 (0.17 nmol/10 ml cytosol) to give a final hormone concentration of 17 nM for the test group. Hormone-receptor complexes were allowed to equilibrate at 0° for 1 hour. Purified intestinal nuclei were then reconstituted with the preincubated cytosol via homogenization and allowed to incubate at 25° for 1 hour. Chromatin was then prepared from the incubated nuclei and assayed for template activity as described under "Materials and Methods." Experiment 3 was performed to determine the inter-preparation variation when nuclei from rachitic chicks were divided into six equal portions and treated separately with cytosol containing ethanol only or 1α,25-(OH)2D3 in ethanol. Values are the average of seven assays per chromatin preparation ± S.E.M. Significance was calculated using the two-tailed, Student-t test. The numbers in parentheses represent the percent stimulation of the template activity of the test group above that of the control.
Figure 36. Effect of 1α,25-dihydroxyvitamin D₃ on reconstituted chromatin template activity in vitro.
Cytosol and Sterol Specificity of 1α,25-Dihydroxyvitamin D₃ Stimulation In Vitro of Intestinal Chromatin Template Activity

Table 11 shows that the in vitro template response has an absolute requirement for intestinal cytosol. Neither liver nor kidney cytosol was able to produce an increase in the chromatin template activity when these fractions were preincubated with 1α,25-(OH)₂D₃ and subsequently reconstituted with intestinal nuclei. These results demonstrate a requirement for the target tissue cytosol receptor to mediate the effect of the hormone on increased template activity in this system.

The sterol specificity of the in vitro template response was determined by using the various vitamin D metabolites and synthetic analogs as previously described in the in vivo experiments. The results are summarized in Table 12, experiment 1, and indicate that 25-OHD₃ was unable to stimulate template activity when used at the same concentration as 1α,25-(OH)₂D₃ (17 nM). However, when the concentration of 25-OHD₃ was increased 500-fold, a concentration at which 25-OHD₃ can compete with 1α,25-(OH)₂D₃ equally for the receptor (140), a 23% stimulation of template activity is seen. This change in chromatin template activity is comparable to that produced by the active hormone. When 1α-OHD₃ was used at the same concentration as 1α,25-(OH)₂D₃, it produced no increase in template activity in vitro, as shown in experiment 2, indicating a lack of conversion to 1α,25-(OH)₂D₃ in this in vitro system.⁶ Therefore, the

⁶. Even though the 25-hydroxylase is known to be present in the intestine (46), it is a microsomal enzyme (141) and therefore would not be expected to be present in the cytosol or purified nuclear fractions.
Table 11. Cytosol specificity of various reconstituted chick tissue chromatin for template activation by 1α,25-(OH)₂D₃ in vitro.

Intestinal chromatin (8-12 μg of DNA) and 130 μg of RNA polymerase were allowed to synthesize RNA under conditions described in "Materials and Methods" for template activity measurements in vitro.

<table>
<thead>
<tr>
<th>Cytosol source</th>
<th>Sterol treatment*</th>
<th>[³H] UMP incorporated into RNA/mg DNA** (nmol)</th>
<th>Increase over control chromatin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestine</td>
<td>none</td>
<td>12.7 ± .4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1α,25-(OH)₂D₃</td>
<td>15.7 ± .3 (p &lt; .005)**</td>
<td>24</td>
</tr>
<tr>
<td>Kidney</td>
<td>none</td>
<td>13.9 ± .2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1α,25-(OH)₂D₃</td>
<td>13.0 ± .3 (p &gt; .100)</td>
<td>-</td>
</tr>
<tr>
<td>Liver</td>
<td>none</td>
<td>14.3 ± .3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1α,25-(OH)₂D₃</td>
<td>11.9 ± .5 (p &lt; .010)</td>
<td>-</td>
</tr>
</tbody>
</table>

*Either ethanol (50 μl/10 ml cytosol) for the control or 1α,25-(OH)₂D₃ (0.17 nmole/10 ml cytosol) for the experimental group was preincubated with the tissue cytosols listed above at 0° for 1 hour. Purified intestinal cell nuclei were then reconstituted with the preincubated cytosol and allowed to incubate at 25° for 1 hour. The template assay was then performed on chromatin isolated from the purified nuclei as described in "Materials and Methods."

**Values are the average of 7 assays per chromatin preparation ± S.E.M.

***Significance was calculated using the two-tailed, Student-t test.
Table 12. Sterol specificity of intestinal mucosa chromatin template activation after reconstitution in vitro.

Intestinal mucosa cell chromatin (8-12 µg of DNA) and 130 µg of RNA polymerase were allowed to synthesize RNA under conditions described under "Materials and Methods" for template activity measurements in vitro.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Sterol treatment*</th>
<th>Sterol concentration (nM)</th>
<th>[^{3}H] UMP incorporated into RNA/mg DNA**</th>
<th>Increase over control animals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>none</td>
<td>-</td>
<td>14.5 ± .2</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1α,25-(OH)(_2)D(_3)</td>
<td>17</td>
<td>17.9 ± .4 (p &lt; .005)**</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>25-OH-D(_3)</td>
<td>17</td>
<td>14.1 ± .4 (p &gt; .100)</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>25-OH-D(_3)</td>
<td>8500</td>
<td>17.9 ± .5 (p &lt; .005)</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>none</td>
<td>-</td>
<td>16.6 ± .2</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>1α-OH-D(_3)</td>
<td>17</td>
<td>16.0 ± .3 (p &gt; .100)</td>
<td>-</td>
</tr>
</tbody>
</table>

*Either ethanol (50 µl/10 ml cytosol) for the control or the sterols at the final concentrations listed above were incubated with the intestinal cytosol fraction at 0° for 1 hour. Purified intestinal nuclei were then reconstituted with the cytosol preparations and incubated at 25° for 1 hour. The template activity assays were then performed with mucosa cell nuclear chromatin prepared from the previously incubated nuclei as described in "Materials and Methods."

**Values are the average of 7 determinations per chromatin preparation ± S.E.M.

***Significance was calculated using the two-tailed, Student-t test.
increase in template activity is specific for 1α,25-(OH)₂D₃ in the *in vitro* reconstitution system and only larger doses of 25-OHD₃ function in a comparable manner.

**Temperature Dependence of 1α,25-Dihydroxyvitamin D₃ Stimulation *In Vitro* of Intestinal Chromatin Template Activity**

Experiments were performed to determine if the 25° incubation temperature was necessary for the observed 1α,25-(OH)₂D₃-mediated increase in intestinal chromatin template activity *in vitro*. After the hormone-receptor complexes had been formed at 0° for 60 minutes, purified intestinal nuclei from rachitic chicks were then reconstituted with the hormone-receptor complexes or with the cytosol fraction alone and incubated an additional 60 minutes at either 25 or 0°. Table 13 indicates that the stimulation of the template activity is consistently seen only when a 25° incubation temperature is utilized. These data indicate that the increased template response is dependent upon the migration of the hormone-receptor complexes into the nucleus, which does not occur at 0°.

**Effect of 1α,25-Dihydroxyvitamin D₃ Concentration on *In Vitro* Stimulation of Intestinal Chromatin Template Activity**

Various levels of 1α,25-(OH)₂D₃ were preincubated with the cytosol fraction at 0° and then subsequently reconstituted with purified nuclei. When chromatin from these nuclei was assayed for template activity it was observed that a saturating amount of the sterol (17 nM) was necessary for maximal stimulation of the template activity.
Table 13. Temperature dependency of 1α,25-(OH)₂D₃-stimulated increase of chick intestinal chromatin template activity in vitro.

Intestinal mucosa cell chromatin (8-12 µg of DNA) and 130 µg of RNA polymerase were allowed to synthesize RNA under conditions described under "Materials and Methods" for template activity measurements in vitro.

<table>
<thead>
<tr>
<th>Incubation temperature*</th>
<th>Sterol</th>
<th>nmoles UMP incorporated into RNA/mg DNA**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>none</td>
<td>13.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>1α,25-(OH)₂D₃</td>
<td>14.9 ± 0.6 (p &gt; 0.100)***</td>
</tr>
<tr>
<td></td>
<td>1α,25-(OH)₂D₃</td>
<td>14.7 ± 0.8 (p &gt; 0.100)</td>
</tr>
<tr>
<td>25</td>
<td>none</td>
<td>16.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>1α,25-(OH)₂D₃</td>
<td>17.8 ± 0.7 (p &lt; 0.050)</td>
</tr>
<tr>
<td></td>
<td>1α,25-(OH)₂D₃</td>
<td>18.2 ± 0.4 (p &lt; 0.025)</td>
</tr>
</tbody>
</table>

*Either ethanol (50 µl/10 ml cytosol) for the control or 1α,25-(OH)₂D₃ at a final concentration of 17 nM in ethanol (50 µl/10 ml cytosol) were incubated with the intestinal cytosol fraction at 0°C for 1 hour. Purified intestinal nuclei were then reconstituted with the cytosol preparations and either incubated at 0°C or 25°C for an additional hour. The template activity assays were then performed with mucosa cell nuclear chromatin prepared from the previously incubated nuclei as described in "Materials and Methods."

**Values are the average of 7 determinations per chromatin preparation ± S.E.M.

***Significance was calculated using the two-tailed, Student-t test.
(Figure 37). This value correlates well with the report that at a 1α,25-(OH)$_2$D$_3$ concentration of 10-15 nM, the intestinal chromatin in vitro is saturated with radioactively labelled 1α,25-(OH)$_2$D$_3$ (82). Although significant increases in template activity are observed at a sterol concentration of 6 nM, maximal stimulation occurs with a sterol concentration of 17 nM. Therefore, a maximal increase in chromatin template activity requires a saturating level of 1α,25-(OH)$_2$D$_3$ in the chromatin.

Substitution of Chromatin for Nuclei in
the In Vitro Template Response

Further characterization of the in vitro template response was accomplished by substituting intestinal chromatin from rachitic chicks for purified nuclei during the 25° incubation. Table 14 indicates that when chromatin is used directly an 18% stimulation of template activity is observed. This indicates that for the in vitro reconstitution system utilized in this study the intact nucleus is not necessary for the association of the hormone-receptor complex with the intestinal chromatin or for stimulation of the template activity. These data suggest a direct effect of the 1α,25-(OH)$_2$D$_3$-receptor complex on the composition and/or structure of the chromatin, resulting in an altered ability to act as a template for mRNA synthesis.

Use of Wheat Germ RNA Polymerase in Assays for
Intestinal Chromatin Template Activity

In the above studies, bacterial RNA polymerase was used to estimate the extent of template activation. At the present time, a controversy exists as to whether a bacterial enzyme can transcribe eukaryotic
Figure 37. Effect of 1α,25-dihydroxyvitamin D₃ concentration on reconstituted intestinal chromatin template activity.

Increasing concentrations of 1α,25-(OH)₂D₃ were added to intestinal mucosa cell cytosol as previously described for Figure 36 to obtain the sterol concentrations indicated. After formation of the hormone-receptor complexes at 0° for 1 hour, purified intestinal nuclei were reconstituted with the hormone-receptor complexes via homogenization and incubated for an additional hour at 25°. Chromatin was then prepared from these incubated nuclei and assayed for template activity as described under "Materials and Methods." A sterol concentration of 17 nM was taken as the maximal response since concentrations higher than 17 nM did not give greater stimulation of template activity.
Figure 37. Effect of 1α,25-dihydroxyvitamin D₃ concentration on reconstituted intestinal chromatin template activity.
Table 14. Effect of 1α,25-(OH)₂D₃ on the in vitro reconstituted chromatin template activity when chromatin is used directly for purified intestinal nuclei.

Intestinal chromatin (8-12 μg of DNA) and 130 μg of RNA polymerase were allowed to synthesize RNA under conditions described under "Materials and Methods" for template activity measurements in vitro. Chromatin from rachitic chicks was reconstituted with the hormone-receptor complexes and incubated at 25° for 1 hour. The template assay was then performed on the chromatin as described in "Materials and Methods." Values are the average of 7 determinations per chromatin preparation ± S.E.M. Significance was calculated using the Student-t test.

<table>
<thead>
<tr>
<th>Sterol</th>
<th>nmoles UMP incorporated into RNA/mg DNA</th>
<th>Group average</th>
<th>Percent stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>13.1 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.2 ± 0.5</td>
<td>13.6 ± 0.8</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>15.2 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1α,25-(OH)₂D₃</td>
<td>15.6 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.2 ± 0.6</td>
<td>15.9 ± 0.2 (p &lt; 0.050)</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>15.9 ± 0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
chromatins with the same fidelity as the endogenous enzyme. In order to determine if the use of *E. coli* RNA polymerase was valid for the above studies, an examination of the time course of incorporation of UMP by chromatins treated with and without 1α,25-(OH)$_2$D$_3$ in *vitro* was performed using both *E. coli* RNA polymerase and wheat germ RNA polymerase. The ability of wheat germ polymerase to function in the template assay has already been shown (Figures 31 and 32). Figure 38 represents a typical time course of UMP incorporation for both enzymes in *vitro*. Although the wheat germ enzyme incorporates approximately three orders of magnitude less UMP than does the bacterial enzyme, quantitative differences exist between the control and 1α,25-(OH)$_2$D$_3$-treated chromatins. This indicates that the 1α,25-(OH)$_2$D$_3$-stimulated template activity is also evident when a eukaryotic-like enzyme is used. The low degree of incorporation by this enzyme may represent high specificity for a limited number of initiation sites on the chromatin. Table 15 summarizes the results of the experiments depicted in Figure 38 as determined at the 10-minute incubation time point for the bacterial enzyme and at the 30-minute incubation time for wheat germ polymerase. It is apparent that the 1α,25-(OH)$_2$D$_3$-mediated increase in chromatin template activity is manifested when either RNA polymerase enzyme is utilized.
Figure 38. Time course of UMP incorporation of control and 1α,25-dihydroxyvitamin D₃-treated chromatin by *Escherichia coli* and wheat germ RNA polymerases.

Chromatin (8 to 15 µg of DNA) was prepared from purified intestinal nuclei that had been incubated with cytosol containing either ethanol (control) (●) or 1α,25-(OH)₂D₃ (▲), described under "Materials and Methods." These chromatin preparations were then assayed for template activity as described under "Materials and Methods" using either (A) 130 µg of *Escherichia coli* RNA polymerase, or (B) 52 µg of wheat germ RNA polymerase. Values represent two assays that were performed on each chromatin preparation at the times indicated.
Figure 38. Time course of UMP incorporation of control and 1α,25-dihydroxyvitamin D3-treated chromatins by Escherichia coli and wheat germ RNA polymerases.
Table 15. Summary of 1\alpha,25-(OH)\textsubscript{2}D\textsubscript{3}-stimulated increases in chick intestinal chromatin template activity using Escherichia coli or wheat germ RNA polymerases.

Chick intestinal chromatin (8-12 µg of DNA) treated with ethanol (control) or 1\alpha,25-(OH)\textsubscript{2}D\textsubscript{3} (17 nM) and 130 pg of E. coli RNA polymerase or 52 µg of wheat germ RNA polymerase were allowed to synthesize RNA for 40 minutes under conditions described under "Materials and Methods" for template activity measurements in vitro.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Experiment No.</th>
<th>Sterol</th>
<th>nmoles UMP incorporated into RNA/mg DNA</th>
<th>Percent stimulation</th>
<th>Group average (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli*</td>
<td>1</td>
<td>none</td>
<td>19.9</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1\alpha,25-(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>22.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>none</td>
<td>19.0</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1\alpha,25-(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>22.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>none</td>
<td>17.1</td>
<td>30</td>
<td>29</td>
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<td></td>
<td>1\alpha,25-(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>22.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>none</td>
<td>18.5</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1\alpha,25-(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>28.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>none</td>
<td>18.2</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1\alpha,25-(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>24.6</td>
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<td></td>
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<tr>
<td>Wheat germ**</td>
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<td>none</td>
<td>9.0</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1\alpha,25-(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>13.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>3.75</td>
<td>60</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>1\alpha,25-(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>6.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>none</td>
<td>9.0</td>
<td>33</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1\alpha,25-(OH)\textsubscript{2}D\textsubscript{3}</td>
<td>12.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme source</td>
<td>Experiment No.</td>
<td>Sterol</td>
<td>Nmoles UMP incorporated into RNA/mg DNA</td>
<td>Percent stimulation</td>
<td>Group average (%)</td>
</tr>
<tr>
<td>---------------</td>
<td>---------------</td>
<td>--------</td>
<td>----------------------------------------</td>
<td>--------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Escherichia coli*</td>
<td>1</td>
<td>none</td>
<td>19.9</td>
<td>22.7</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1α,25-(OH)₂D₃</td>
<td>19.0</td>
<td>22.4</td>
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<tr>
<td></td>
<td>3</td>
<td>none</td>
<td>17.1</td>
<td>22.4</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1α,25-(OH)₂D₃</td>
<td>18.5</td>
<td>28.1</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>none</td>
<td>18.2</td>
<td>24.6</td>
<td>33</td>
</tr>
<tr>
<td>Wheat germ**</td>
<td>1</td>
<td>1α,25-(OH)₂D₃</td>
<td>9.0</td>
<td>13.5</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>none</td>
<td>3.75</td>
<td>6.0</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1α,25-(OH)₂D₃</td>
<td>9.0</td>
<td>12.0</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>none</td>
<td>3.75</td>
<td>5.6</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1α,25-(OH)₂D₃</td>
<td>3.75</td>
<td>6.0</td>
<td>60</td>
</tr>
</tbody>
</table>

*Values for the incorporation by E. coli RNA polymerase represent the 10-minute point of the 40-minute time course.

**Values for the incorporation by wheat germ RNA polymerase represent the 30-minute point of the 40-minute time course.
CHAPTER IV

DISCUSSION

The experiments reported in this dissertation have been designed to elucidate the mechanism of action of \(\alpha,25-\text{(OH)}_2\text{D}_3\) in its target organ, the intestine. By examining the activities of the endogenous DNA-dependent RNA polymerases and the template activity of intestinal chromatin after administration of \(\alpha,25-\text{(OH)}_2\text{D}_3\) and its biologic, and synthetic precursors, an attempt has been made to delineate the nuclear mechanism of action of this steroid hormone. A basic principle involved in the design and interpretation of the studies is that a target organ for \(\alpha,25-\text{(OH)}_2\text{D}_3\), which undergoes a primary physiologic response following administration of the sterol, contains a finite number of specific sites which selectively retain the hormone. It has been shown that the \(\alpha,25-\text{(OH)}_2\text{D}_3\)-receptor complex originates in the cytoplasm and can undergo a temperature-dependent transfer to the nuclear chromatin. At the chromatin, the hormone-receptor complex then effects the changes in polymerase and template activity reported in this dissertation.

There are several important prerequisites for a study of the nuclear mechanism of action of \(\alpha,25-\text{(OH)}_2\text{D}_3\). An experimental animal which is consistently devoid of endogenous vitamin D is required. In such a system, administration of the active hormone would produce detectable transcription changes in the nucleus prior to the initiation of calcium transport. Presumably, these changes would not be of
sufficient magnitude to be detectable after administration of the sterol to vitamin D-replete animals. The preferred animal is the chick which is readily made vitamin D-deficient and develops the classic symptoms of rickets within three weeks on a rachitogenic diet (61). By contrast, the rat does not develop rickets unless the ratio of calcium to phosphorus in the diet is drastically altered (142).

Also essential is the availability of pure $1\alpha,25-(\text{OH})_2D_3$. This active metabolite was prepared in vitro from $25\text{-OHD}_3$ by incubating the precursor sterol with kidney homogenates from vitamin D-deficient chicks. Due to the high activity of the 25-hydroxyvitamin $D_3$-1-hydroxylase in animals raised on a rachitogenic diet, efficient conversion of $25\text{-OHD}_3$ to the hormone can be effected (Figure 3). Purification of $1\alpha,25-(\text{OH})_2D_3$ was carried out by column chromatography on silicic acid, Sephadex LH-20, and Celite. When this purification scheme is applied to 5 $\mu$g quantities of $1\alpha,25-(\text{OH})_2D_3$, a preparation of sufficient purity for analysis by mass spectrometry and ultraviolet spectrophotometry is obtained (Figures 4B and 5).

In 1968, Haussler reported the first definitive study of the subcellular distribution of vitamin D and its metabolites in the intestine of the chick, in vivo (34, 105). Following intracardial injection of rachitic chicks with physiological doses of radioactive vitamin $D_3$, 35% of the radioactivity isolated from the intestinal mucosa was located in the nuclear chromatin fraction. The association of the radioactivity with intestinal mucosa chromatin was determined to be specific and 87% of the chromatin-bound radioactivity existed as a polar metabolite of
vitamin D₃, subsequently shown to be 1α,25-(OH)₂D₃. These initial results suggested that the mechanism of action of 1α,25-(OH)₂D₃ in increasing calcium transport may be through the alteration of genetic expression. This hypothesis was substantiated by several investigators who have reported experiments in various animal model systems that suggest that the mechanism of vitamin D action involves cellular regulation of protein synthesis. Following administration of 1α,25-(OH)₂D₃, one of the earliest detectable events is the interaction of the hormone with a cytoplasmic macromolecular binding component (80-82), followed by changes in RNA synthesis and nuclear RNA metabolism (99) after association of the hormone-receptor complex with the chromatin (83). In addition, the response of the intestine to 1α,25-(OH)₂D₃ can be blocked by the RNA synthesis inhibitors, actinomycin D and α-amanitin (96, 101). The purpose of the present investigation is to extend these initial results by examining the 1α,25-(OH)₂D₃-mediated nuclear events which occur after association of the hormone-receptor complex with the chromatin and prior to the physiological response. The rationale behind such a study is based on the premise that if 1α,25-(OH)₂D₃ regulates nuclear gene transcription, thereby altering the synthesis of specific types of RNA(s), such that the basic questions related to this purported nuclear mechanism of action of this hormone may be answered in the investigation of its interaction with the intestinal cell nucleus. These questions include: 1) What is the biochemical mechanism which enables a small molecular weight sterol to selectively identify and activate specific genes? 2) To what extent is the enzymology of transcription involved in
the 1α,25-(OH)₂D₃-mediated changes in RNA synthesis? and 3) Is the chronology of the nuclear events in agreement with the appearance of the physiological response? The experiments reported in this dissertation represent a logical progression from the studies of the chromatin binding of the 1α,25-(OH)₂D₃-receptor complex to experimental examination of the above theoretical considerations.

Prior to studies of the nuclear mechanism of action of the hormone, it was necessary to purify and determine the biological activity of the synthetic analog 1α-OHD₃, which was used extensively in the studies reported in this dissertation. After purification of the crude synthetic material on Celite and Sephadex LH-20 columns (Figure 6), the data showed that, in chicks, 1α-OHD₃ is equal in efficacy to 1α,25-(OH)₂D₃, the active metabolite of vitamin D₃. This synthetic sterol exerts an effect on both of the established vitamin D-target organs, the intestine and bone (Tables 1 and 2, Figure 7), and its rapid activity in stimulating intestinal calcium absorption and bone mineral mobilization suggested that it may be a useful analog of the vitamin.

The possibility then arose that 1α-OHD₃ may exert its hormonal effect largely through conversion into 1α,25-(OH)₂D₃. This notion was supported by two lines of experimental evidence. Haussler et al. (37) showed that 25-OHD₃ acts only slightly faster than vitamin D₃, while 1α,25-(OH)₂D₃ functions far more rapidly than either sterol. This finding suggests that the rate-limiting step in vitamin D metabolism in vivo is 1α-hydroxylation and that 25-hydroxylation proceeds at a relatively rapid rate. Also, vitamin D₃-25-hydroxylase has been found in
several tissues, including the intestine (46). It was not known whether 1α-OHD$_3$ could serve as a substrate for the 25-hydroxylase enzyme, but a similar analog, dihydrotachysterol$_3$, is converted to 25-hydroxy-dihydrotachysterol$_3$ in experimental animals and suggested the same might be true for 1α-OHD$_3$.

A comparison of 1α-OHD$_3$ and 1α,25-(OH)$_2$D$_3$ in biological activity experiments in vivo (Figures 9 and 10), and receptor binding studies in vitro (Figure 11), displayed a striking disparity between the relative effectiveness of 1α-OHD$_3$ in vivo and in vitro. This disparity is accounted for by demonstrating that 1α-OHD$_3$ is metabolized to 1α,25-(OH)$_2$D$_3$ in vivo (Figure 13). Moreover, this conversion occurs rapidly enough to saturate the physiologic receptors in intestine with 1α,25-(OH)$_2$D$_3$ prior to eliciting an increase in calcium absorption. Differential absorption of the sterols does not appear to have influenced the observed temporal and dose-response relationships (Figures 9 and 10); equal doses produced equal responses. The 25-hydroxylation of 1α-OHD$_3$ is apparently rapid, as shown by in vitro intestinal mucosa studies (Table 4). Following our report that 1α-OHD$_3$ is converted to 1α,25-(OH)$_2$D$_3$ (56), confirmation came from independent studies in the perfused rat liver (143) and for the rat and chick in vivo (144, 145) using radioactively labelled 1α-OHD$_3$.

Thus, 1α-OHD$_3$ is an effective precursor of 1α,25-(OH)$_2$D$_3$ and since it is considerably easier and less expensive to synthesize than 1α,25-(OH)$_2$D$_3$ it may become the sterol of choice in the therapeutic management of such metabolic bone diseases as renal osteodystrophy (146).
and hypoparathyroidism (147). Furthermore, the use of this potent synthetic 1α,25-(OH)₂D₃ precursor in elucidating the molecular mode of action of vitamin D has proven to be of value in the experiments reported in this dissertation.

Evidence in this dissertation indicates that 1α,25-(OH)₂D₃, the active form of vitamin D₃, administered to rachitic chicks enhances the activity of the nucleoplasmic DNA-dependent RNA polymerase II in intestinal nuclei. This observation, coupled with previous reports of a specific cytosol-chromatin receptor (83) and the induction of calcium-binding protein (59) provides support for the concept that 1α,25-(OH)₂D₃ mediates calcium transport by regulating gene activity. The present finding, that 1α,25-(OH)₂D₃ rapidly leads to stimulation of the RNA polymerase which transcribes mRNA, appears to be an important step in the mechanism of action of vitamin D.

The time course of this increased RNA polymerase activity (Figure 25) is compatible with that of other hormone systems, which indicates that the primary event for increased polymerase activity is the migration of the hormone-receptor complex into the nucleus. The increase in RNA polymerase II activity closely parallels the saturation of the chromatin by the hormone-receptor complex (Figure 39). Likewise, a decrease in RNA polymerase II activity follows the decrease in the amount of hormone-receptor complex on the chromatin. It would appear that maximal occupancy of the chromatin by the hormone-receptor combination provides the stimulus for enhanced RNA polymerase II activity in the intestine. This effect occurs 2-3 hours after administration of
Figure 39. Time course of 1α,25-dihydroxyvitamin D₃-mediated events in the chick intestinal cell.

The dose of 1α,25-(OH)₂D₃ for all parameters depicted was 0.27 μg.
$1\alpha,25-(OH)_{2}D_{3}$ and precedes the physiologic response to increase calcium absorption in the intestine. Calcium transport is not significantly increased until 5 hours after 0.27 µg of $1\alpha,25-(OH)_{2}D_{3}$ and the maximum transport increase occurs in about 9 hours (Figure 39). By 12 hours, a time at which calcium transport is well underway, the form II polymerase activity has returned to control values (Figure 25). In addition, the early increase in form II RNA polymerase is also evident when $1\alpha$-OHD$_{3}$ is administered (Figure 26).

The increase in endogenous form II RNA polymerase activity was also specific for intestinal nuclei. Nuclei from a non-target tissue, such as liver, displayed no increase in the form II RNA polymerase activity after administration of three doses of $1\alpha,25-(OH)_{2}D_{3}$ (Table 5). Administration of the sterol in this manner resulted in significant stimulation of the polymerase activity in the intestine although the magnitude of stimulation is somewhat reduced compared with that previously seen. This may be due to the inability to repeatedly stimulate the form II enzyme activity maximally and that during the dosing interval the polymerase activity may decay and reach a plateau of stimulation at approximately 30% higher than that of the control. Table 6 indicates that stimulation of the form II enzyme is also sterol specific. When three doses of 25-OHD$_{3}$ were given (0.27 µg/dose), a significant increase in the form II enzyme activity resulted. Although this increase was not as great as with the natural hormone, it indicates that 25-OHD$_{3}$ was probably metabolized to $1\alpha,25-(OH)_{2}D_{3}$ during the dosing interval and that enough $1\alpha,25-(OH)_{2}D_{3}$ was produced to saturate the target tissue with the
hormone and thereby produce the observed increase in polymerase activity.

Vitamin D$_3$, however, produced no changes in the form II polymerase activity when administered in three doses (0.27 µg/dose) during a 9-hour interval. This is in contrast to the data obtained when 1.3 µg of vitamin D$_3$ is administered once and the form II enzyme activity is significantly elevated between 6-9 hours after dosing (Figure 22). The enhancement of RNA polymerase II activity by 6-9 hours is consistent with the lag time for metabolism of the native vitamin to 1α,25-(OH)$_2$D$_3$ when a large dose is orally administered and supports the finding of a direct effect of the hormonal sterol in 2-3 hours. Therefore, insufficient 1α,25-(OH)$_2$D$_3$ was probably produced from three low doses of vitamin D$_3$, which resulted in the observed lack of increased polymerase activity.

Specific stimulation of RNA polymerase II activity in chick intestinal nuclei suggests that only enhanced mRNA synthesis is required for the action of 1α,25-(OH)$_2$D$_3$ on calcium absorption since no increases have been observed for RNA polymerase I during the 9-hour period subsequent to administration of the hormone. The effectiveness of the inhibitors actinomycin D (96) and α-amanitin (101) also supports a specific role of mRNA in the induction of calcium-binding protein and calcium transport. Yet other steroid hormones, such as testosterone (148) and aldosterone (149), have been shown to affect RNA polymerase I and ribosomal RNA (rRNA) synthesis. Several hormones modulate both RNA polymerase I and II (150, 151), and estrogen has recently been shown to initially stimulate uterine RNA polymerase II, followed by a second phase
of control of RNA polymerase activity in which both polymerases I and II are increased (152).

Most steroid hormones cause an early increase in mRNA synthesis in respective target organs, but a common feature is a concomitant rise in the synthesis of rRNA caused by an increased nucleolar RNA polymerase activity. Initial DNA transcription in these tissues may, therefore, produce a protein factor which is responsible for increasing nucleolar associated rRNA synthesis (153-155). In the present studies involving a single dose of 1\alpha, 25-(OH)_{2}D_{3}, enhancement of nucleolar RNA polymerase activity did not appear to be a requirement for induction of calcium absorption (Figures 23 and 25). A possibility is that 1\alpha, 25-(OH)_{2}D_{3} only elicits the synthesis of a limited amount of mRNA, which may be a characteristic of this highly differentiated tissue. Therefore, any increase in polymerase II activity allows an increase in specific mRNA synthesis without increasing rRNA synthesis. Presumably, enough cytoplasmic ribosomes are present to accommodate this increased mRNA synthesis for calcium binding protein induction after administration of one physiologic dose of 1\alpha, 25-(OH)_{2}D_{3}. This is supported by the observation that RNA polymerase I is eventually increased after treatment with vitamin D_{3} (Figure 21), which suggests that continual influx of hormone from its vitamin precursor may be involved in ultimately sustaining the intestinal calcium absorption response.

More studies are required to clarify the involvement of increased nucleoplasmic RNA polymerase activity in the action of 1\alpha, 25-(OH)_{2}D_{3}. The mechanism for this increase in RNA polymerase II is not known, but
the possibility that there is *de novo* synthesis of the enzyme or phosphorylation of the enzyme by a protein kinase is not ruled out. Also, the hormone-receptor complex could activate or release a stimulatory factor or itself function as a stimulatory factor to increase enzyme activity. Whatever the mechanism, the augmented RNA polymerase II activity is still manifest in solubilized nuclear proteins fractionated on a DEAE-Sephadex column (Figures 27 and 28). Thus, when partially purified and assayed using an exogenous template, RNA polymerase II is still stimulated approximately 2-fold by prior treatment of the animal with 1α,25-(OH)$_2$D$_3$ or vitamin D$_3$. This indicates that an increase in template activity alone cannot explain these data. Furthermore, this conclusion is similar to that reached for the stimulation of uterine nuclei by estrogen on the basis of experiments with exogenous template (156). Therefore, the amount and/or activity of nucleoplasmic RNA polymerase, in addition to changes in the chromatin template activity, may be influenced by the action of 1α,25-(OH)$_2$D$_3$.

Previous investigations of the influence of vitamin D$_3$ on the activity of the endogenous RNA polymerase of chick intestinal nuclei indicated that the vitamin was without effect on the enzymes (136). Although Lawson's (136) study was performed without knowledge of the active hormonal form of vitamin D$_3$, increases in the RNA polymerase activities should have been observed due to the very large (125 μg) dose of vitamin D$_3$ administered to the animals. However, the investigators used a relatively insensitive assay for the polymerases, differentiating the two enzyme activities by assaying in the presence of low salt and
Mg$^{2+}$ ion (form I) or high salt and Mn$^{2+}$ ion (form II). This method is not as sensitive or as accurate as the assay system employed in this dissertation, in which α-amanitin is used to distinguish between the two enzyme activities. Therefore, the activity of one enzyme may have been masked by the activity of the other. In spite of this, it is doubtful that changes in the polymerase activities would have been observed by Lawson et al. (136) since they examined the intestinal nuclei for enzyme activity at only 1 hour after administration of the vitamin. Based on the present data, this time is known to be too early to observe any significant increases in endogenous polymerase activities. Therefore, the present data may more accurately reflect the effect of 1α,25-(OH)$_2$D$_3$ and its precursors on nuclear RNA polymerase activities. The time course of this change in the enzymology of transcription is in agreement with both the chronology of binding of the hormone-receptor complexes to the chromatin and the ensuing physiological response (Figure 39).

The data presented in this dissertation indicate that the nuclear mechanism of action of 1α,25-(OH)$_2$D$_3$ is similar to that for other steroid hormones. Numerous steroid hormones are now known to affect chromatin template activity. For example, in vivo administration of hydrocortisone (157) and testosterone to rats (158) and estrogen to both ovariectomized rats (159) and immature chicks (160) elicits increases in the chromatin template activity of liver, prostate, and uterus, respectively. In the present studies, 1α,25-(OH)$_2$D$_3$ was examined for its ability to produce changes in chick intestinal nuclear chromatin template activity in vivo and in vitro using Escherichia coli and wheat germ RNA polymerases.
The rapid increase in template activity by 1α,25-(OH)₂D₃ reported in this dissertation (Figures 34, 35, and 39) appears prior to the physiologic response by 3 to 8 hours and appears to closely parallel the \textit{in vivo} binding of the hormone to the chromatin and the increase in the endogenous nucleoplasmic DNA-dependent RNA polymerase activity for this hormone, as shown in Figure 39. In addition, the 1α,25-(OH)₂D₃-mediated increase in intestinal chromatin template activity is found only in target tissue chromatin (Table 9) after administration of 1α,25-(OH)₂D₃ or the synthetic precursor 1α-OHD₃ (Table 10). This demonstrates that the response is both tissue and sterol specific in these \textit{in vivo} experiments.

Although increases in template activity have been reported to occur for most steroid hormones \textit{in vivo}, very few studies of hormone effects on template capacity and RNA synthesis have been reported utilizing \textit{in vitro} reconstitution of the hormone-receptor complex with chromatin. Purified uterine nuclei exposed to the estradiol-receptor complex of uterine cytosol demonstrate an increased RNA synthetic capacity as measured by increased endogenous DNA-dependent RNA polymerase activity (161). Recently, the \textit{in vitro} stimulation of prostatic endogenous RNA polymerase by 5 α-dihydrotestosterone-receptor complexes has also been reported (148, 162). In these other studies the increase in the endogenous nuclear DNA-dependent RNA polymerase activity was used as a measure of increased RNA synthetic activity. In addition, O'Malley and Schrader (163) have been able to reconstitute \textit{in vitro} purified oviduct cytosol receptors and progesterone to form hormone-receptor
complexes. When these complexes are subsequently added to chick oviduct chromatin in the presence of E. coli RNA polymerase and the four nucleoside triphosphates in vitro, there is a significant increase of RNA synthesis as compared to preparations lacking hormone (163). This dissertation reports that reconstitution of the 1α,25-(OH)$_2$D$_3$-receptor complex with purified intestinal cell nuclei significantly increases the chromatin template activity as assayed in vitro (Figure 36). There have been no detectable in vitro 1α,25-(OH)$_2$D$_3$ effects on the endogenous RNA polymerase activities in mucosal cell chromatin when assayed in the presence and absence of α-amanitin. However, a very low level of endogenous RNA polymerase II activity was observed due to losses of the enzyme during the preparation of the chromatin since all buffers lacked PMSF, an agent known to prevent nonspecific losses of form II polymerase (137) (Figure 29). Thus, the data with isolated chromatin do not contradict the finding of an in vivo 1α,25-(OH)$_2$D$_3$-mediated alteration in RNA polymerase II activity in whole nuclei. Nevertheless, the present study represents the first report of a 1α,25-(OH)$_2$D$_3$-stimulated increase in chromatin template activity both in vivo and in vitro, as measured with exogenous Escherichia coli RNA polymerase.

The 1α,25-(OH)$_2$D$_3$-mediated stimulation of intestinal chromatin template activity closely follows and supports previous reports concerning characterization of the cytosol and nuclear receptor proteins for this hormone (79, 81-84). The absolute requirement of target tissue cytosol for the chromatin template response (Table 11) correlated with the unique presence of this receptor in intestinal cytosol. Further, the
utilization of the temperature-dependent migration of the hormone-receptor complex to the chromatin in the in vitro reconstitution experiments is in agreement with the reported mandatory requirement for temperatures of 25° to allow the binding of the (transformed) receptor to chromatin (84) and consequently the increased template activity (Table 13). These data indicate that the cytoplasmic receptor molecule is a functional mediator of increased intestinal cell nuclear activity by 1α,25-(OH)_{2}D_{3}. In all of the in vitro studies it was assumed that addition of the sterol to the intestinal cytosol fraction produced the hormone-receptor complex which, in turn, was responsible for the increased template activity. Thus, although there is strong evidence that the hormone is forming a complex with its functional receptor in the present experiments, unequivocal proof that the complex is affecting transcription requires the use of pure 1α,25-(OH)_{2}D_{3}-receptor in combination with hormone. Until purified receptor is available, it will have to remain an assumption that the complex is the mediator of increased intestinal chromatin template activity. Ultimately, use of the purified receptor protein and characterization of the mRNA produced in the in vitro system via a protein synthesizing system should provide a true test of the exact role of increased intestinal chromatin template activity in the nuclear action of 1α,25-(OH)_{2}D_{3}.

It has been previously reported that vitamin D_{3} administration at reasonably large doses of 130 nmol to vitamin D-deficient rats (104) and 325 nmol to rachitic chicks (105) produces an increase in intestinal chromatin template activity 3 hours after administration of the sterol.
In the present study, vitamin D$_3$ and 25-OHD$_3$, unlike the hormone, were ineffective at low levels \textit{in vivo} (0.65 mmol) and \textit{in vitro} (17 nM), whereas a 500-fold excess of 25-OHD$_3$, which is effective \textit{in vitro} in other isolated systems such as intestine (30) and bone (31), was active in stimulating chromatin template activity \textit{in vitro} (Table 12). These findings, plus the report that a 500-fold excess of 25-OHD$_3$ over 1α,25-(OH)$_2$D$_3$ is required to compete efficiently with 1α,25-(OH)$_2$D$_3$ for the intestinal receptor (133, 140), suggest that in the previously reported experiments with large doses of vitamin D$_3$, a sufficient amount of 25-OHD$_3$ was rapidly produced which effectively bound to the receptor to simulate physiologic hormone levels and produce the observed increase in template activity. Moreover, since vitamin D$_3$ and 25-OHD$_3$ require a considerably longer time (15 to 40 hours) than 1α,25-(OH)$_2$D$_3$ (4 to 8 hours) to enhance intestinal calcium transport (37), it might be expected that both precursor sterols could elicit chromatin template activation in low doses, but at later time periods than the 2-to-3-hour effect seen with the hormone. Thus, slow metabolism of vitamin D$_3$ and 25-OHD$_3$ to sufficient quantities of 1α,25-(OH)$_2$D$_3$ may account for the lack of effect of small doses of these sterols at 2 hours. This time restriction is not placed upon the synthetic precursor 1α-OHD$_3$, however, for this sterol acts very rapidly (4 to 8 hours) to stimulate calcium absorption (54, 55) because it is quickly and efficiently converted into the 1α,25-(OH)$_2$D$_3$ hormone (56, 143-145). Therefore, \textit{in vivo}, a small dose of 1α-OHD$_3$ is capable of stimulating intestinal chromatin template activity in 2 to 3 hours (Table 10). But in reconstituted cytosol and purified nuclei where
metabolism to the hormone is precluded, the synthetic precursor does not affect template activity at concentrations where 1α,25-(OH)₂D₃ produces its maximum effect (Table 12). Furthermore, maximal stimulation of the template response is seen only when saturating levels of 1α,25-(OH)₂D₃ (17 nM) are used in the reconstituted cytosol and purified nuclei system (Figure 37). Significant stimulation of the template activity is seen with hormone concentrations as low as 6 nM. However, maximal stimulation of the template response occurs only with saturating levels of 1α,25-(OH)₂D₃. This requirement correlated well with the reported concentration of 1α,25-(OH)₂D₃ (15 nM) required to saturate intestinal chromatin with radioactively labelled hormone (82). Therefore, it appears that a direct relationship exists between the level of chromatin template activation and the degree of chromatin saturation by 1α,25-(OH)₂D₃. In summary, the \textit{in vivo} operation of various sterols on chromatin template activity is dependent upon the size of the dose and the time required for metabolism to 1α,25-(OH)₂D₃, whereas \textit{in vitro} the response is selective for the active hormone and can only be demonstrated with higher concentrations of other vitamin D sterols which simulate the hormone at the receptor level.

In the present study, bacterial RNA polymerase was used to estimate the extent of template activation. Butterworth, Cox, and Chesterton (164) have reported that rat liver chromatin is a more efficient template for rat liver RNA polymerase II than for RNA polymerase from \textit{Micrococcus luteus}. They also reported that the mammalian and bacterial enzymes bind to and transcribe from different sites on the chromatin. Recently it has
been reported that the restriction of *E. coli* RNA polymerase activity on chromatin is mainly due to a decrease in available polymerase initiation sites (165). These findings suggest that the use of bacterial RNA polymerase could yield measurements of template activity that are not biologically relevant and that homologous avian enzyme may be ideal for the template assays. However, based on the following studies, *E. coli* RNA polymerase appears to be an acceptable substitute. Bacterial RNA polymerase and the endogenous tissue polymerase have been shown to transcribe equally well with respect to (a) RNA sequences synthesized on a chromatin template versus those in intact cells with both repetitive (166) and unique sequences (167); (b) inability of the enzymes to transcribe repressed globin genes in brain chromatin (167); and (c) measurement of the number of RNA polymerase binding and initiation sites on DNA and chromatin (168). In order to assess whether the use of *E. coli* RNA polymerase was producing biologically relevant measurements of template activity, in the *in vitro* reconstituted system, the experiments were repeated using wheat germ RNA polymerase. Since this enzyme has properties identical to the nucleoplasmic enzyme from eukaryotes (138, 139), its use in the *in vitro* reconstitution system allowed a comparison to be made of the bacterial and wheat germ's enzyme transcriptive ability on intestinal chromatin. The results (Figure 38 and Table 15) indicate that when wheat germ RNA polymerase is used in the *in vitro* assay quantitative differences exist in the amount of UMP incorporated by control and 1α,25-(OH)$_2$D$_3$-treated chromatins for each chromatin. Although the degree of incorporation for the wheat germ enzyme is approximately 1/1000 of
that for the bacterial enzyme, the 1α,25-(OH)$_2$D$_3$-mediated increase in chromatin template activity is approximately 1.8 times greater than that for the *E. coli* enzyme when an analysis is made at the 30-minute time point for the wheat germ enzyme (Table 15). It is possible that the low degree of incorporation by wheat germ polymerase may represent transcription from a limited number of specific initiation sites on the chromatin and the 1α,25-(OH)$_2$D$_3$-mediated effect is a larger fraction of this more specific total incorporation. Therefore, the hormone's effect on stimulation of transcription is greater with this more selective polymerase. At present, then, use of wheat germ RNA polymerase in the template activation experiments indicates that *E. coli* RNA polymerase is an acceptable substitute for endogenous avian enzyme when examining control and 1α,25-(OH)$_2$D$_3$-treated chromatin for template activity differences. The question as to whether the *in vitro* initiation sites on chromatin are identical with those that are operative in the intact intestinal nuclei remains to be answered.

The present results are consistent with the "two-step" mechanism for steroid hormone binding in target tissues. This sequence of events involves initial binding of the hormone to a cytoplasmic receptor protein, located only in the respective target tissue, which is subsequently transported into the nucleus. Evidence presented in this dissertation indicates that, once in the intestinal cell nucleus, the 1α,25-(OH)$_2$D$_3$-receptor complex specifically activates the genome. This results in simultaneous increased activity of nuclear DNA-dependent RNA polymerase II and increased intestinal chromatin template activity, presumably for
the synthesis of mRNA(s) specific for the synthesis of the intestinal calcium binding protein. Figure 40 depicts a model of the probable "two-step" mechanism in the intestinal cell after 1\alpha,25\text{(OH)}_2D_3 administration. It will be observed that in order for the cytoplasmic receptors to interact with acceptor sites on the nuclear chromatin they first must be complexed to 1\alpha,25\text{(OH)}_2D_3 and then allowed to undergo a temperature-dependent transformation, presumably resulting in a specific structural modification of the receptor prior to association with the chromatin.

The similarities in the time course of hormone binding, the template response, and the increase in nucleoplasmic RNA polymerase activity, all prior to the physiologic response (Figure 39), suggest that an interrelationship between these events may exist. The nature of the interaction of the nuclear 1\alpha,25\text{(OH)}_2D_3-receptor complex with the chromatin is presently a matter of speculation. However, it has been suggested that DNA (169, 170) and/or the nonhistone proteins (171, 172) are intimately involved in the recognition of estrogen and progesterone steroid hormone receptors. \[^3\text{H}\]-estradiol has been shown to be released from uterine nuclei upon DNase treatment (173), and binding of the estrogen receptor to DNA, in vitro, has been observed (169, 174). The receptor-DNA interaction was of high affinity (Kd 2 x 10^{-10} \text{ M}), and saturation was observed (500 receptor complexes/cellular DNA equivalent, assuming one estradiol molecule per complex) (169). However, binding of the uterine receptors to DNA was not specific in that they interact with DNA isolated from rat uterus (169), calf thymus (169, 175), salmon sperm, E. coli, and B. subtilis (174, 176). No direct evidence has been
Figure 40. Proposed model of 1α,25-dihydroxyvitamin D₃-mediated two-step mechanism in the chick intestinal cell.

1α,25-(OH)₂D₃ enters the cell and binds to a specific cytoplasmic protein receptor (R). The resulting hormone-receptor complex migrates into the nucleus where it binds to chromatin, possibly to the non-histone protein fraction, which results in an "uncovering" of the DNA template such that RNA polymerase II (RNA POLYMERASE) may begin transcription from these previously covered initiation sites. This results in the synthesis of specific mRNA(s) which is then translated by the polysomal fraction in the cytosol resulting in the synthesis of calcium binding protein (CaBP).
reported to support the notion that steroid hormone receptor complexes may bind to specific DNA sequences.

Elegant studies of the specific interaction of the progesterone receptor with experimentally altered forms of chromatin have been performed in the laboratory of O'Malley. Removal of histones from oviduct chromatin and substitution with histones from non-target tissues resulted in little change in chromatin acceptor capacity for the $^3$H progesterone-receptor complex (177, 178). However, when the nonhistone proteins were removed from the chromatin, binding of the progesterone-receptor complex was blocked. In addition, reconstitution of oviduct nonhistone proteins with erythrocyte DNA imparted acceptor capacity to this "hybrid" chromatin, while chromatin containing DNA from oviduct and erythrocyte nonhistone proteins had a low affinity for progesterone-receptor complexes. Thus, the acceptor capacity for the hormone receptor, in vitro, was dependent on the presence of the nonhistone proteins from the chromatin of the target organ. It could be speculated that the $1\alpha,25-(OH)_2D_3$-receptor complex associated with the acceptor sites on chromatin that are determined by the presence of the nonhistone proteins. This would then lead to an "uncovering" of the genome and to an increase in the number of available RNA polymerase binding sites and transcription of new mRNA sequences (Figure 40). This appears to be the nuclear mechanism of action for estrogen. Schwartz et al. (160) have shown that the estrogen mediated increase in template capacity of oviduct target tissue is due to an increased number of RNA polymerase binding and initiation sites on the chromatin template under conditions where reinitiation is blocked. A
further possibility for the nuclear mechanism of action of \(1\alpha,25-(OH)_2D_3\), not shown in Figure 40, is that the hormone-receptor complex in some way directly activates (and/or induces) the endogenous RNA polymerase II such that the activated (and/or induced) enzyme may recognize appropriate initiation sites, leading to increased mRNA transcription. This model is consistent with the increased RNA polymerase I activity observed by Sajdel and Jacob (179). Moreover, it is interesting that Muller, Totsuka, and Zuhn (180) have recently obtained preliminary data suggestive of association between estradiol receptor and quail oviduct RNA polymerase I as determined by DEAE-cellulose chromatography, gel filtration, and sucrose gradient centrifugation. However, to date there is no conclusive proof of association between RNA polymerases and steroid hormone receptors. Moreover, the evidence published thus far (179, 180) suggests only a relationship between the receptor and RNA polymerase I. Thus, this model cannot explain the increased RNA polymerase II activity which apparently precedes the increase in polymerase I activity and is probably responsible for the transcription of new RNA sequences. In addition, the activity of the form II RNA polymerase may be regulated by a direct phosphorylative mechanism. Jungmann, Hiestand, and Schweppes (181) have reported the stimulation of both calf ovary RNA polymerases I and II, present in partially purified extracts, by the addition of cyclic-AMP (cAMP) dependent protein kinase from calf ovary cytosol. Martelo and Hirsch (182) have also reported the stimulation of isolated rat liver RNA polymerase I by a nuclear protein kinase. Recently, Dahmus (183) has reported that the activity of purified RNA polymerase II from
Novikoff ascites tumor cells is stimulated 5-7 fold by a protein factor having extensive protein kinase activity. Support for the direct phosphorylation of the endogenous RNA polymerase II is provided by the observation that the increased RNA polymerase II activity reported in this dissertation is still apparent when the solubilized enzyme is assayed on exogenous template (Figure 27). This observation, coupled with the report of a rapid increase in the production of cAMP in intestinal organ culture by 1α,25-(OH)_2D_3 (111) intimate that a cAMP-dependent protein kinase may be activating the form II enzyme in the presence of the 1α,25-(OH)_2D_3-receptor complex. However, this does not rule out the possibility that de novo induction of enzyme synthesis may also be occurring. More studies will be required of this aspect of polymerase activation.

Alternatively, the hormone-receptor complex may in some way enhance chain elongation by the endogenous RNA polymerases to enable these enzymes to more freely traverse certain DNA sequences which in its absence act as termination regions for the growing polyribonucleotide chain.

Another possibility for the nuclear mechanism of action of 1α,25-(OH)_2D_3 is that the hormone-receptor complex may affect intranuclear processing and/or transport of RNA from the nucleus to the cytoplasm. This model of post-transcriptional control of genetic expression calls for continuous synthesis of mRNA produced from an "open" gene. The intracellular concentration of this mRNA would then increase via subsequent steroid hormone control of its degradation (184). However, support
for this post-transcriptional control of genetic expression in response to steroid hormones has diminished since pools of mRNA specific for ovalbumin were not observed in animals that had been withdrawn from estrogen treatment (185, 186).

It is proposed in this dissertation that association of the 1α,25-(OH)₂D₃-receptor complex with intestinal chromatin initiates a series of nuclear events which leads to the stimulation of calcium transport. There are several lines of evidence that support the hypothesis that 1α,25-(OH)₂D₃ exerts its primary effect at the level of transcription:

1. By two hours after administration of the hormone, there is a significant increase in the chromatin template activity (Figure 34) of target tissue chromatin and not in the template activity of non-target chromatins. This time course of increased template activity follows closely the appearance of radioactively labelled hormone on the chromatin and precedes the observed physiologic response by 3-7 hours.

2. By two hours following administration of the hormone there is a significant increase in the activity of the form II RNA polymerase, presumably for the synthesis of new mRNA species which are specific for the intestinal calcium binding protein (106). The ensuing physiologic response occurs 3 to 7 hours after the appearance of the increased polymerase activity and is dependent upon the presence of the hormone on the chromatin.
3. **In vitro** reconstitution of the $\text{1\alpha,25-(OH)\textsubscript{2}}\text{D}_3$-mediated increase in template activity indicates the mandatory requirement for saturating levels of $\text{1\alpha,25-(OH)\textsubscript{2}}\text{D}_3$ and for the target tissue cytosol receptor macromolecule.

In order to gain more information about the interrelationships between nuclear binding of the hormone and increased polymerase and template activities, it will be necessary to purify the cytoplasmic and nuclear $\text{1\alpha,25-(OH)\textsubscript{2}}\text{D}_3$ receptors. Investigation of the number of nuclear $\text{1\alpha,25-(OH)\textsubscript{2}}\text{D}_3$-receptor complexes and RNA polymerase initiation sites in chick intestinal chromatin during stimulation with the hormone using purified receptor may clarify the relationship between template and RNA polymerase activities. In addition, use of an **in vitro** reconstitution system in which hybrid chromatin is used directly in place of purified intestinal nuclei (Table 14), along with purified avian polymerase II, may provide some insight into the nature of the chromatin site occupied by the hormone-receptor complex and the involvement of nonhistone proteins in the recognition of appropriate initiation sites. Ultimate verification of the nuclear mechanism of action of $\text{1\alpha,25-(OH)\textsubscript{2}}\text{D}_3$ will require reconstitution of the purified nuclear components such that a specific mRNA for calcium binding protein is produced, isolated, purified, and then translated in an **in vitro** protein synthesizing system (106).

Through these studies, it should be possible to gain an understanding of the biochemical events which may be defective in pathological conditions of mineral metabolism such as vitamin D-resistant rickets, renal
osteodystrophy, idiopathic hypercalciuria, hypoparathyroidism, hypoparathyroidism, and vitamin D-dependent rickets.


60. Raisz, L. G., Trummler, C. L., Holick, M. F., and DeLuca, H. F. (1972), Science 175, 768.


76. Fujii, T., and Villu, C. (1968), Endocrinology 82, 463.


90. Herman, T. S., Fimognari, G. M., and Edelman, I. S. (1968), 
J. Biol. Chem. 243, 3849.

U.S.A. 65, 709.

117, 77.

Science 149, 182.


96. Tsai, H. C., Midgett, R. J., and Norman, A. W. (1973), Arch. 
Biochem. Biophys. 157, 339.

97. Stohs, S. J., Zull, E., and DeLuca, H. F. (1967), Biochemistry 6, 
1304.


Commun. 54, 622.


103. Lindell, T. J., Weinberg, F., Morris, P. W., Roeder, R. G., and 

U.S.A. 63, 528.

105. Haussler, M. R. (1968), Ph.D. Dissertation, University of 
California, Riverside, Riverside, California.

246, 100.


110. Freund, T., and Bronner, F. (1975), Science 190, 1300.


