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1,25-DIHYDROXYVITAMIN D: HORMONAL REGULATION OF BIOSYNTHESIS AND PURIFICATION OF ITS INTESTINAL RECEPTOR

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1,25-DIHYDROXYVITAMIN D:
HORMONAL REGULATION OF BIOSYNTHESIS AND
PURIFICATION OF ITS INTESTINAL RECEPTOR

by

John Wesley Pike

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF BIOCHEMISTRY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1979
I hereby recommend that this dissertation prepared under my direction by John Wesley Pike entitled 1,25-Dihydroxyvitamin D: Hormonal Regulation of Biosynthesis and Purification of its Intestinal Receptor be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Mark R. Hausler
Dissertation Director

11/14/79

As members of the Final Examination Committee, we certify that we have read this dissertation and agree that it may be presented for final defense.

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Final approval and acceptance of this dissertation is contingent on the candidate's adequate performance and defense thereof at the final oral examination.

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SIGNED: John Wesley Pike
Dedicated to

MY PARENTS
    for their continued faith and encouragement

MY WIFE
    for her love and support during this endeavor
ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Dr. Mark R. Haussler for his invaluable guidance and support throughout the course of these investigations. His continued interest and encouragement were essential to the completion of this work.

I would also like to thank those esteemed scientists who collaborated with me on portions of the work found in Chapter 3: Dr. Svein U. Toverud, Dr. Agna Boass, Dr. Iain MacIntyre, and Dr. Evangelos Spanos. Without their significant contributions most of the studies on the hormonal regulation of 1,25-dihydroxyvitamin D biosynthesis could not have been accomplished.

Special thanks go to Dr. Andrew M. Goldner, who demonstrated a special interest in my progress throughout graduate school.

Sincere gratitude is also expressed to the original members of the Vitamin D Research Group — Toni A. McCain, Joseph E. Zerwekh, and Mark R. Hughes; and to later members — Adrienne Sainten, Patricia G. Jones, Elizabeth F. Safilian, Jeffrey B. Parker, Lisa Gooze, Laura A. Hagan, and John S. Chandler.

Finally, I would like to express my warmest appreciation to my wife, Christine Ann, for her constant faith and moral support during this study and for her direct contribution to this work (Chapter 3, Figure 3-16). The most important result of this event was the birth of a special little boy, Collin Andrew Pike.

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ABSTRACT

The renal biosynthesis of 1,25-dihydroxyvitamin D (1,25-(OH)₂D) is known to be stringently regulated by extensive ionic and hormonal controls. Quantitation of this mineral regulating hormone by radio-receptor assay has recently enabled a study of its influence and synthetic control during physiological states of growth and reproduction. The data reveal that 1,25-(OH)₂D is strikingly elevated during those situations, most likely to mediate the enhanced absorption of mineral. Since the elevation of this sterol hormone dynamically coincides with the peak mineral demands of pregnancy and lactation, it suggests a rapid and precise modulation of 1,25-(OH)₂D biosynthesis.

Regulatory control of 1,25-(OH)₂D apparently involves both pituitary and sex hormones. This is supported by the observation that hypophysectomy in growing rats leads to a dramatic fall in the sterol hormone, a depression reversed by growth hormone therapy. Further, estrogen (DES) or prolactin administration in chicks enhances circulating 1,25-(OH)₂D. Conversely, inhibition of prolactin secretion in lactating rats limits the synthesis of the sterol hormone. Although the inextricable relationship between 1,25-(OH)₂D synthesis and parathyroid hormone is also demonstrated, the results lend strong support to other regulators of 1,25-(OH)₂D biosynthesis.

A primary target organ for 1,25-(OH)₂D is the intestine, where the hormone is known to bind a specific cytoplasmic receptor prior to its effects on the enhancement of intestinal mineral absorption.
Several chromatographic and electrophoretic techniques have been developed to further characterize this specific 1,25-(OH)\(_2\)D receptor. The protein binds to a large number of ion exchange and group selective affinity resins, including DNA-cellulose, blue dextran-Sepharose, and heparin-Sepharose, and displays specific characteristics upon elution from these resins. These interactions imply a function for the 1,25-(OH)\(_2\)D receptor in binding to DNA in vivo, and support the current concept that the receptor acts to alter the expression of specific genes.

The use of these techniques is important in the identification and characterization of 1,25-(OH)\(_2\)D receptors in other tissues. By employing DNA-cellulose chromatography and sucrose gradient analysis, a binding component for 1,25-(OH)\(_2\)D identical to that found in chick intestine has been observed in pituitary, placenta, pancreas, and parathyroid glands. Since each of these tissues elaborates a hormone(s) which regulates the biosynthesis of 1,25-(OH)\(_2\)D, it is reasonable to speculate that these sterol "receptors" mediate negative feedback regulation, resulting in a depression in hormone synthesis. Regardless, the identification of these new "receptors" for 1,25-(OH)\(_2\)D suggests a much wider role for vitamin D than was originally envisioned.

Group selective affinity resins can also be employed in a sequential scheme to purify the receptor for 1,25-(OH)\(_2\)D from rachitic chick intestine. The receptor has been precipitated from intestinal cytosol with Polymin P, resolubilized, and has successively been chromatographed on DNA-cellulose, Sephacryl, blue dextran-Sepharose, and heparin-Sepharose. The receptor has been purified to approximately 50%
of homogeneity by these techniques, and has been shown to be present in
the purified material along with several contaminating species. The
present level of receptor purification appears adequate to initiate
studies of its function within the intestinal cell nucleus.
CHAPTER 1

INTRODUCTION

The isolation and chemical identification of vitamin D$_2$ and vitamin D$_3$, antirachitic factors collectively described earlier by McCollum et al. (1), were finally achieved in 1937 by Windaus and his collaborators (2), and Windaus, Schenck, and von Werder (3). This work represented the culmination of an extensive research effort begun almost two decades before, when Mellanby (4) first demonstrated that the disease rickets was due to the lack of a specific dietary constituent. Figure 1-1 summarizes the various compounds and their precursors which represent the basic vitamin D family. Although further metabolism of vitamin D occurs, these compounds comprise the ones which must be provided either in the diet or by exposure to ultraviolet radiation.

The disease rickets, characterized by abnormal bone ossification, involves an imbalance in the ion product of plasma calcium and phosphate at a level consistent with normal bone mineralization. Although the absolute amount of calcium found in extracellular fluids represents less than 1% of the entire body calcium (the endoskeleton contains the remaining 99%), this component serves a primary role in some of the most fundamental of life processes, including nerve

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1. The chemical name of vitamin D$_3$ or cholecalciferol is 9,10-seco-5,7,10(19)-cholestatrien-3β-ol.
Figure 1-1. Compounds of the Vitamin D Family

The production of vitamin D from its respective provitamin is accomplished by irradiation with ultraviolet light. The various combinations of side chains (R) with the sterol nuclei pictured comprise the respective provitamins, vitamins, and dihydrotachysterols.
conduction, blood clotting, muscle contraction, enzyme activation, regulation of membrane integrity, and the coupling between stimulation and release of polypeptide hormones. As a result, all organisms expend a substantial amount of energy in the stringent regulation of extracellular, and thus intracellular, calcium. This regulation, in general terms, is depicted in Figure 1-2. It represents the contemporary view of control of plasma calcium (in man) through a complex interplay between vitamin D, parathyroid hormone (PTH), and calcitonin (CT) on three major target organs, the intestine, bone, and kidney. Plasma calcium is maintained at 10 mg%, primarily through the intestinal absorption of this ion from the diet (5), a process facilitated by vitamin D. The relationship between this vitamin and absorption was definitively elucidated in 1940 by Nicolaysen and Eeg-Larsen (6). This vitamin D-dependent process has subsequently been shown to be an energy requiring active transport which occurs against an electrochemical gradient across the intestinal epithelial cells (7,8). Vitamin D also acts in concert with the polypeptide hormone PTH to mediate the resorption of calcium from bone (9) and kidney (10,11), although neither of these processes is as well understood as the classic action of vitamin D on intestinal calcium absorption (12,13). If plasma calcium falls below 10 mg%, the parathyroid glands are capable of sensing a calcium deficiency and elaborating the calcium elevating hormone PTH. If an active hypercalcemia occurs, the thyroid gland responds by secreting CT, a hormone whose current primary role appears to be the inhibition of bone calcium mobilization (14), thereby lowering plasma calcium. The results of these regulated actions is the strict
Figure 1-2. Normal Calcium Homeostasis.

Circulating plasma calcium (Ca) concentration is controlled by the concerted effects of vitamin D (Vit. D), parathyroid hormone (PTH), and calcitonin (CT). The plasma pool of calcium is supplied by the absorption of the ion from the intestine and the resorption of the ion from the endoskeleton; both of these processes are dependent upon vitamin D. Bone resorption is also dependent upon PTH and CT, the former acting as a positive modulator and the latter as a negative regulator of this process. The removal of calcium from the circulation is principally accomplished by bone accretion and calciuria. The net effect is a stringently regulated mechanism which maintains plasma calcium at 10 mg percent.
maintenance of normal plasma calcium. Extracellular phosphate is also similarly regulated by vitamin D, since it is known that a calcium independent phosphate transport process exists in intestinal epithelia (5, 15,16). It also appears to involve a specific carrier (17,18) different from that for calcium. Thus, the homeostasis of both calcium and phosphate is critical to the well-being of all organisms, and vitamin D plays a key role in the regulation of these ions.

Irving (19) originally noted that a substantial time lag existed between the administration of vitamin D and the resulting physiological response of calcium and phosphate mobilization. It is now known that there are several components responsible for this delayed action of the sterol. The primary factor is the necessity for considerable metabolic conversion of the vitamin: first to an hydroxylated intermediate in the liver, and then to a dihydroxylated active form in the kidney, a form capable of initiating the biological functions originally ascribed to the parent compound (20-22). The lag may also be caused by transport of the metabolites: the intermediate form to its site of specific hydroxylation, and the final hormonal form(s) to its specific target tissue where biological action is precipitated. Additional delay is also attributed to an apparent interaction between the active metabolite and its subcellular receptor site, and to the subsequent participation of this sterol-protein complex in the expression of new genetic material (23,24). These products are then used by as yet unknown means to elicit the physiological effects of the original vitamin, i.e., mineral transport.
The concept that vitamin D is a precursor or prohormone to a host of metabolites possessing biological activity equal to or greater than the parent compound came in the late 1960's, and work in this area still represents a major research thrust on the action of vitamin D$_3$. The major circulating metabolite of vitamin D$_3$ was isolated from the plasma of pigs by Blunt, DeLuca, and Schnoes (22), and determined to be chemically identical to 25-hydroxyvitamin D$_3$ (25-OHD$_3$). It was later shown that this metabolite was synthesized predominantly in the liver (25-27), although subsequently, Tucker, Gagnon, and Haussler (28) provided some evidence that 25-OHD$_3$ could be produced in the kidney and intestine of chicks. Since this metabolite displayed greater potency than vitamin D$_3$ in curing rickets (29), and was observed to function directly upon isolated intestine and bone to stimulate calcium translocation (30-32), it was originally thought to be the active form of the vitamin (33,34). However, the measurement of 25-OHD$_3$ in patients under high vitamin D intake suggested the level of this metabolite fluctuated dramatically (35,36) and that its concentration was predominately a function of diet or sunlight (37), observations which were inconsistent with a compound whose primary function was to regulate

2. Abbreviations in this dissertation are: IU (international unit, equals 0.25 μg of vitamin D$_2$ or vitamin D$_3$); 25-hydroxyvitamin D$_3$ (25-OHD$_3$); 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$); 1,25-dihydroxyvitamin D$_2$ and 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$); 24,25-dihydroxyvitamin D$_3$ (24,25-(OH)$_2$D$_3$); 25,26-dihydroxyvitamin D$_3$ (25,26-(OH)$_2$D$_3$); calcium-binding protein (CaBP); parathyroid hormone (PTH); thyroid hormone (TSH); diethylstilbestrol (DES); growth hormone (GH); prolactin (PRL); calcium (Ca); phosphorus (P); messenger RNA (mRNA); vitamin D$_3$-25-hydroxylase (25-OHase); 25-hydroxyvitamin D$_3$-1-hydroxylase (1-OHase); sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE), 25-hydroxyvitamin D-24-hydroxylase; 24-OHase.
mineral homeostasis. It is now known that 25-OHD$_3$ represents an intermediate in the activation of vitamin D$_3$ and serves as an immediate precursor to other specifically hydroxylated active forms. During the initial investigations of 25-OHD$_3$, Haussler and Norman (38), and Haussler, Myrtle, and Norman (39) detected a more polar metabolite of vitamin D$_3$ called peak 4B which they proposed to be the most active principle in the target intestinal tissue. This was based upon its specific binding to intestinal mucosal chromatin and its biological activity. Shortly afterwards, Lawson, Wilson, and Kodicek (40) also observed this metabolite, and suggested that it was modified at the 1-alpha carbon due to the loss of 1alpha-3H from [1alpha,3H]-vitamin D$_3$ during biosynthesis. Further, this metabolite was present in a number of tissues in rachitic (D-deficient) chicks, but only in intestinal cell nuclei was the loss of 1alpha-3H most evident, indicating a preferential accumulation of this metabolite in the nucleus (41). Haussler and coworkers (42) then isolated several ug of this metabolite from chickens and determined the purified material to be more rapid and five times more effective than vitamin D$_3$ or 25-OHD$_3$ on an equal mass basis. This derivative of vitamin D$_3$ was subsequently isolated from chick intestine (43) and renal homogenates (44,45) and identified chemically as 1alpha,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$). Further, the compound was found to be more active than either its immediate precursor 25-OHD$_3$ or its parent sterol vitamin D$_3$ in causing bone calcium resorption (46-48). Thus, the activation of vitamin D$_3$ appears to require two enzymatic hydroxylations before it is capable of stimulating biological activity.
The first, 25-hydroxylation, occurs in liver, kidney, and intestine (26-28); the second, 1α-hydroxylation, occurs exclusively in the kidney (44).

25-Hydroxyvitamin D₃ represents a precursor product for several metabolites other than 1,25-(OH)₂D₃. Current thought on the metabolism of vitamin D₃ is illustrated in Figure 1-3. As previously discussed, the native vitamin is converted to 25-OHD₃ and then to 1,25-(OH)₂D₃. However, at least two other dihydroxylated compounds are also synthesized, although little is known about their significance. They include 25,26-dihydroxyvitamin D₃, not found in the major target tissues after vitamin D administration (49), and 24,25-dihydroxyvitamin D₃ (24,25-(OH)₂D₃), a metabolite less active than its non-24-hydroxylated precursors (50). Thus, these compounds may represent initial products in the degradative metabolism of 25-OHD₃. However, 24,25-(OH)₂D₃ has demonstrated considerable activity in rats (51), and has recently been implicated as an active metabolite in bone (52-54). Thus its dismissal as a catabolic by-product may not be entirely warranted. Further, there is some evidence to suggest that a newly discovered trihydroxylated metabolite, 1,24,25-trihydroxyvitamin D₃, may be active in normal vitamin D₃ function. On an equal mass basis, it appears to be almost equivalent to 1,25-(OH)₂D in stimulating biological activity in rats (55), although it is relatively inactive in chicks (56). Despite this, its circulating concentration still remains unknown and thus its physiological relevance must await further research.

Most evidence marshalled to date convincingly supports the idea that 1,25-(OH)₂D₃ is the principle, if not the only, functional
Figure 1-3. The Metabolism of Vitamin D
metabolite of vitamin D$_3$. Chronic administration of 1,25-(OH)$_2$D$_3$ to animals raised on rachitogenic diets completely prevents all histologic and radiologic signs of rickets (57,58). It maintains normal mineral metabolism (59) and does so faster and more efficiently than either of its precursor forms (42). Further, not only is 1,25-(OH)$_2$D$_3$ the most potent form of the vitamin in the stimulation of calcium and phosphate mobilization in vivo (42,49,60-63), but it is also the most efficacious metabolite in vitro in isolated perfused intestines (32), intestinal organ culture (64), and embryonic bone culture (46). Importantly, its circulating level has been measured by radioreceptor assay in a host of situations, both physiological and pathological (65-68), and these data indicate that 1,25-(OH)$_2$D fluctuates in a positive fashion with the intestinal absorption of calcium which it is known to mediate. Finally, the observation that 1) 1,25-(OH)$_2$D$_3$ acts on specific tissues distant from its origin and 2) its biosynthesis, unlike that of vitamin D$_3$ and 25-OHD$_3$, is tightly regulated by both feedback control and an extensive number of ionic and hormonal factors involved in mineral homeostasis (see discussion below), support the concept that the 1,25-(OH)$_2$D$_3$ metabolite is indeed an active hormone.

The enzymes which are responsible for the conversion of vitamin D$_3$ to its various metabolites, their tissue loci, and the multitude of controls which regulate their activity represent another primary focal point of research in the field of vitamin D. The 25-hydroxylase (25-OHase), which metabolizes vitamin D$_3$ to 25-OHD$_3$, was first observed in the liver by Horsting and DeLuca (27). In subsequent work, Tucker et al. (28) confirmed this finding, and extended the enzymes' activity to
both chick kidney and intestine. However, although extrahepatic 25-hydroxylation does occur, it is generally considered that the liver represents the predominant site of 25-OHD$_3$ synthesis. The 25-OHase is present in the liver microsomal fraction of all species, but apparently requires a soluble cytoplasmic factor for maximum activity (69). Further, its activity in vitro requires both glucose-6-phosphate and magnesium ion, and can be dramatically enhanced by the addition of an NADPH-generating system (28). However, the enzyme does not appear to contain cytochrome P450, since inhibitors of the complex such as carbon monoxide have no effect on its activity.

In contrast, the 25-hydroxy-1α-hydroxylase (1-OHase) occurs exclusively in the kidney, and is specifically located in the inner membrane of the mitochondria (70). The enzyme is an NADPH-dependent, cytochrome P450-containing, mixed function oxidase, as supported by the observations that 1) molecular oxygen is incorporated into the substrate (71) and 2) the enzyme is blocked with carbon monoxide (72) and white light (73). This enzyme has been extensively purified (74,75), and is currently being characterized as to its physical properties.

The 25-hydroxy-24-hydroxylase (24-OHase) is less well understood. It is found in the kidney (76) and possibly elsewhere (77). Although it is located in intact mitochondria, utilizes molecular oxygen, and is blocked by inhibitors of oxidative phosphorylation, it does not appear to be dependent on cytochrome P450 since it is not sensitive to carbon monoxide (76). This enzyme remains to be investigated as thoroughly as its counterpart, the 1-OHase, or as the 25-OHase.
The regulation of these enzymes provides the primary control of the metabolism of vitamin D₃, and is consistent with the view that 1,25-(OH)₂D₃ represents the hormonal specifier of most biological activity. Although DeLuca (78) originally suggested that 25-hydroxylation represented a point of endocrine regulation, most subsequent evidence has proven to the contrary. The chick 25-OHase is not feedback regulated by its product in vitro (28), a hallmark of endocrine regulation networks. Further, if 25-OHD₃ is measured in the serum of animals after vitamin D challenge, little significant regulation of the circulating level of this metabolite is registered (35,36). In striking contrast, it is generally agreed that the kidney 1-OHase represents the major pivotal control point in vitamin D metabolism. A general model for regulation of 1,25-(OH)₂D₃ biosynthesis is depicted in Figure 1-4. The factors which, in endocrine fashion, influence the activity of the 1-OHase and the biosynthesis of 1,25-(OH)₂D₃ are extensive, and new regulators continue to be identified. The 1-OHase appears to be negatively feedback regulated by the product of its activity, 1,25-(OH)₂D₃. In D-deficiency, there is a marked increase in activity of the 1-OHase (28,41,79), and if physiological doses of 1,25-(OH)₂D₃ are administered, the enzyme eventually disappears (58). The question of whether this is a direct effect of 1,25-(OH)₂D₃ on the enzyme remains open. The synthesis of 1,25-(OH)₂D₃ is also determined by both the plasma calcium and phosphate status of the animal. In the case of calcium, hypocalcemia appears to have a smaller effect on 1,25-(OH)₂D₃ biosynthesis if the parathyroid glands are removed (80,81), implicating the intermediary, PTH. Certainly the administration of PTH can directly increase the
Figure 1-4. The Regulation of Vitamin D.

25-Hydroxyvitamin D₂ is converted to 1,25-(OH)₂D₃ by the action of the renal 1-OHase. This conversion is positively regulated (solid lines) by calcium (via PTH) and phosphorus. The product, 1,25-(OH)₂D₃, also regulates its own synthesis (dashed lines) via a negative feedback effect on the parathyroid gland. 1,25-(OH)₂D₃ in turn exhibits an action on both intestine and bone to elevate calcium and phosphorus.
Figure 1-4. The Regulation of Vitamin D.
production of 1,25-(OH)2D3 (82,83), supporting the concept that low serum calcium serves to stimulate the secretion of PTH which in turn regulates the 1-OHase enzyme. Conversely, phosphate ion exhibits an effect on 1,25-(OH)2D3 synthesis independent of PTH. Low phosphorus diets are able to cause a dramatic increase in the production of 1,25-(OH)2D3 (84,85) and in the circulating concentration of 1,25-(OH)2D3 (86,87), regardless of whether intact parathyroid glands are present. This influence may be mediated by fluctuations in intracellular phosphate, or indirectly, by unknown humoral intermediates. Calcitonin (CT) is the factor opposing PTH and whose effect is to depress the level of serum calcium. However, conclusive experiments have yet to be performed to demonstrate a negative effect of CT on the synthesis of 1,25-(OH)2D3. Thus, the above regulators represent the major influences which impinge upon the 1-OHase to control the biosynthesis of 1,25-(OH)2D3 and maintain the homeostasis of calcium. Interestingly, those factors which exert a positive influence on the 1-OHase have a converse effect on the 24-OHase, i.e., when the 1-OHase is activated, the 24-OHase is inhibited. This suggests an inverse regulation and elaboration of 1,25-(OH)2D3 and 24,25-(OH)2D3 (88-90). However, further work will be necessary to more fully define the nature of these observations.

The previous discussion has considered the mechanisms and events of normal mineral homeostasis. However, several physiologic situations exist in which calcium and phosphorus are metabolized at considerably higher rates. These periods are growth, pregnancy, lactation, and avian egglaying, and are characterized by typically elevated rates of intestinal mineral absorption (91-93). A major portion of this dissertation
deals with a study of 1,25-(OH)₂D and its regulation during these periods of mineral stress. With the recent development of a specific and sensitive radioreceptor assay for this hormone (35,36,65,66,94), it has been possible to directly determine the total serum activity of 1,25-(OH)₂D₃. Direct measurement of the hormone alleviates the problems associated with experiments which heretofore have measured only enzyme activities or the ability of animals to metabolize radiolabeled 25-(OH)D₃ to dihydroxy-derivatives. Since the latter techniques measure the conversion of 25-(OH)[³H]D₃ to 1,25-(OH)₂[³H]D₃, they are affected by the pool size of 25-OH-D₃ precursor at the time of experiment, and probably do not reflect the steady-state concentration of plasma hormone. The results described in this dissertation also support the identification of new regulators of 1,25-(OH)₂D₃ biosynthesis in growth and reproduction. These factors, whose primary roles are the endocrine development and maintenance of the above physiologic states, include the steroid hormone estrogen and the polypeptides growth hormone, prolactin, and possibly placental lactogen. Experiments designed to elucidate these factors include the measurement of 1) vitamin D₃ metabolizing enzymes and 2) circulating 1,25-(OH)₂D₃ hormone after in vivo administration or depletion (by ablation of the tissue source or by chemical inhibitors) of the specific hormone of interest. Although the mechanism of action of these new hormonal influences on vitamin D₃ metabolism remains to be elucidated, it is hoped that the results of these investigations will add an interesting new dimension and complexity to research on the action of the fat soluble D-vitamin.
An understanding of the molecular mechanism of action of hormonal 1,25-(OH)$_2$D$_3$ at the level of specific target cells represents the third extremely fertile area of research in the investigation of vitamin D action. The bulk of evidence supports the concept that 1,25-(OH)$_2$D$_3$ regulates the expression of genes which ultimately code for proteins active in mineral transport (see review in 23,24). This induction process is the cornerstone which characterizes the action of steroid hormones such as estrogen (95,96), progesterone (97), androgens (98), aldosterone (99), and cortisol (100). The progressive series of events which lead to the synthesis of new proteins involve the binding of hormone to a high affinity cytoplasmic receptor, a redistribution of this complex to the nucleus where it associates with the genome, selective gene activation, and the biosynthesis of new mRNA which codes for biologically active proteins. The discovery that 1,25-(OH)$_2$D$_3$ participates in a similarly characterized scheme in the intestine strongly suggests that this vitamin D$_3$ metabolite is a novel sterol hormone which acts similar to other steroid hormones.

Specific support for the above mechanism of action of 1,25-(OH)$_2$D$_3$, depicted in model form in Figure 1-5, resides predominantly in the following observations. Both biochemical (101-104) and autoradiographic (105,106) localization of the hormone reveals its presence in the intestinal nucleus following injection of 1,25-(OH)$_2$[^3]H]D$_3$ into the chick. In the case of biochemical studies, the localization is rapid, specific for the chromatin fraction, saturable, and precedes the 1,25-(OH)$_2$D$_3$-elicited absorption of calcium. Further, biochemical studies now confirm the presence of a specific, high affinity receptor
Figure 1-5. Proposed Mechanism of Action of 1,25-(OH)_2D_3 within the Intestinal Nucleus.

1,25-Dihydroxyvitamin D diffuses into the cell and binds to a specific receptor molecule. The complex translocates to the nucleus where it binds to chromatin, activates RNA polymerase II, and influences the expression of genetic material. The result is increased translation of proteins, e.g., CaBP, biologically active in mineral uptake. \( R \) = cytoplasmic receptor, \( R_n \) = nuclear receptor.
Figure 1-5. Proposed Mechanism of Action of 1,25-(OH)₂D₃ within the Intestinal Nucleus.
protein in the cytoplasm of intestinal cells, which serves to convey the hormone to the nucleus (101-103). The component sediments at 3-3.7S in high salt sucrose gradients, binds 1,25-(OH)$_2$D$_3$ with high affinity (Kd = 2 x 10$^{-9}$M) and specificity (25-OHD$_3$ = 1/500 as well as 1,25-(OH)$_2$D$_3$), and is present in very low titers only in target tissues. Importantly, Brumbaugh and Haussler have shown in vitro that the sterol hormone first binds to the receptor in intestinal cytosol, and then subsequently accumulates within the nucleus bound to chromatin (102, 103). Since the discovery of this receptor in chick intestine, similar receptors have been observed in other target tissues, including rat intestine (107), bone (108), and parathyroid glands (109,110). Once bound to chromatin, the receptor-hormone complex apparently influences the biosynthesis of nuclear RNA. Tsai, Midgett, and Norman (101) provided initial evidence in the chick in vivo that the administration of 1,25-(OH)$_2$D$_3$ enhanced RNA synthesis. However, it was Zerwekh, Lindell, and Haussler (111) who reconstituted the crude cytosol receptor-hormone complex with intestinal nuclei from unstimulated rachitic chicks, and were able to effect a positive change in RNA synthesis. Additional observations by Zerwekh et al. (111,112) demonstrate that upon administration of 1,25-(OH)$_2$D$_3$, RNA-polymerase II activity is elevated and there is an increase in the capacity of intestinal chromatin to support RNA biosynthesis. Thus, although specific details of 1,25-(OH)$_2$D$_3$ action remain to be elucidated, the hypothesis that this sterol can actively influence intestinal transcription seems well supported.
Several proteins are known to appear in response to 1,25-(OH)$_2$D$_3$, including alkaline phosphatase (AP) (113) and calcium-binding protein (CaBP) (8). Although the kinetics of appearance of AP do not correlate well with calcium transport, the appearance of CaBP is in much closer agreement. Also, several other proteins, as yet unclassified, have been recently identified by Wilson and Lawson (114,115). Presumably, one or all of these proteins is involved in calcium or phosphate transport, although these processes remain to be fully characterized.

The direct action of 1,25-(OH)$_2$D$_3$ on the synthesis of mRNA's for the above proteins has yet to be demonstrated. Spencer et al. (116,117) have isolated polysomal RNA from D-deficient and 1,25-(OH)$_2$D$_3$-treated chicks and demonstrated, by cell free in vitro translation and antibody precipitation techniques, that translatable mRNA for CaBP was present only in the polysomes of the treated animals. Further, they observed that the appearance of CaBP-mRNA preceded the appearance of calcium absorption. However, these studies do not preclude the possibility of preexisting mRNA and that the effects of 1,25-(OH)$_2$D$_3$ are post transcriptional, i.e., to process or enhance the translatability of mRNA. Definitive proof that 1,25-(OH)$_2$D$_3$ serves to initiate transcription must await two developments: 1) purification of CaBP-mRNA and the subsequent development of a complimentary DNA probe capable of directly measuring the appearance of CaBP-mRNA in the intestinal nucleus and 2) the purification of the intestinal receptor for 1,25-(OH)$_2$D$_3$ in a form capable of initiating RNA biosynthesis in vitro. Once these objectives are met, specific experiments, as
reviewed by Parker (118), should provide the evidence required to substantiate or refute the current hypothesis of steroid hormone action for 1,25-(OH)$_2$D$_3$.

The second major portion of this dissertation encompasses an investigation of receptors for 1,25-(OH)$_2$D$_3$. By using a combination of unique chromatographic procedures and sucrose gradient analysis, specific binding components for 1,25-(OH)$_2$D$_3$ have been identified and partially purified and characterized from chick pituitary, pancreas, and rat placenta. The previously reported parathyroid gland receptor (110) was also reexamined. These observations have been used to develop hypotheses as to the function of 1,25-(OH)$_2$D$_3$ within these tissues, a function which, although similar in action to that in intestine, may lead to substantially different physiological results.

The final thrust of this dissertation is the purification of the intestinal form of the 1,25-(OH)$_2$D$_3$ receptor. This endeavor is made possible by the application of a series of unique chromatographic procedures developed to study the 1,25-(OH)$_2$D receptor, which are also capable of extensively purifying it. This protein, along with its counterpart in other tissues, exists in extremely low cellular titers, and requires greater than a 200,000-fold purification to homogeneity from intestinal cytosol. Also, its extreme lability during isolation further complicates the effort. Nevertheless, the results reported in this dissertation document its purification to near homogeneity, and then define several of its physical properties. This purification should eventually allow functional studies of this receptor's action on the intestinal nuclear genome, providing further
insights into the mechanism of action of vitamin D. Hopefully, these results will also lead to a more complete understanding of the general mechanisms of eukaryotic gene regulation.
CHAPTER 2

MATERIALS AND METHODS

Animals and Diets

Chicks

White Leghorn cockerels were obtained as one day old chicks from Demler Farms, Anaheim, CA, and were raised in an ultraviolet free environment for four to six weeks. Light Sussex–Rhode Island Red cockerels were also obtained as one day old chicks, and raised under similar conditions. Both groups of chicks were fed a standard basal rachitogenic (vegetable protein) diet (57) containing 0.34-1% Ca and 0.4-0.5% P or supplemented with vitamin D₃ by the addition of either vitamin D₃ to the diet (2 IU/g diet) or by crop injection of 40-50 IU vitamin D₃ in 1,2-propanediol every three days. Mature normal hens were obtained from the University of Arizona Poultry Department, Tucson, AZ during the physiological state of egg laying, and had been maintained on a standard nutritional diet containing 1-2% Ca. Chicks fed rachitogenic diets without supplementation became rachitic when their growth plateaued at 150 g. They were characterized by plasma calcium concentrations of 5.5 to 6.0 mg per 100 ml plasma, phosphorus concentrations of 5.1 mg per 100 ml plasma, and percent bone ash values of 27 to 29%. Vitamin D supplemented chicks demonstrated normal increases in growth during the four to six week period, and weighed approximately 250 g. Further, they maintained normal plasma
concentrations of calcium at 11.0 mg per 100 ml and phosphorus at 6.3 mg per 100 ml, and bone ash percentages of 45–50% (57). Radiographic examination of the distal femur and proximal tibia of the chicks were evaluated for extent of enchondral bone ossification, amount of mineral present, and bone development organization. Rachitic chicks demonstrated considerable unossified cartilage, decreased density of mineral and a lack of bony trabeculations at the growing ends of bones. Vitamin D-supplemented chicks were normal with respect to these parameters (57, 58). Histological examination of stained sections of the epiphyseal region of the tibias showed widening 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 (57).

Rats

Rats were obtained from Holtzman Company, Madison, WI as young growing rats of either sex, or as normal, pregnant, or lactating adult females. Rats were also obtained from Wistar. All animals arrived in the laboratory several days prior to the experiments, and were maintained on a basal diet consisting of 75% whole wheat flour, 13% casein, 4% corn oil, 2% salt mixture (free of Ca and P), 1.3% vitamin D-free vitamin mixture, and 0.8% CaCO₃. Final concentration of calcium was 0.37% and phosphorus was 0.32%. In some cases (hypophysectomy experiments) calcium and phosphorus concentrations were increased to 1% and 0.9% respectively. When vitamin D was supplemented in the diet, it was added at 2-5 IU/g diet as vitamin D₃.
Bovine

Bovine animal tissue was obtained courtesy of Marks Slaughter-house, Tucson, AZ.

Patients

Blood was drawn from female donors either as control ($\bar{x} = 25$ yr), pregnant ($\bar{x} = 25$ yr, 20–38 weeks pregnant), or as lactating ($\bar{x} = 30$ yr, six weeks lactating) women.

Radiochemicals

25-Hydroxy[26(27)-methyl-$^3$H]-vitamin D$_3$ (6.7 Ci/mmole) was purchased from Amersham Searle Company, Chicago, IL. The radiochemical purity of 25-OH[3H]D$_3$ was 95%, as assessed following purification on Celite liquid-liquid partition chromatography. Its specific activity was determined by ultraviolet absorbance spectrophotometry at 265 nm. High specific activity 25-hydroxy[3H]-vitamin D$_3$ (110 Ci/mmole) was also obtained from Amersham Corporation, Arlington Heights, IL. The radiochemical purity of this compound was 95% as assessed following purification on Celite liquid-liquid partition chromatography or on a Dupont Zorbax-Sil column under high performance liquid chromatography (HPLC). Specific activity was assessed as above.

Biochemicals and Fine Chemicals

General Biochemicals

Chemicals used in the enzymatic generation of 1,25-(OH)$_2$D$_3$ from 25-OH$_3$D$_3$ were obtained from Sigma Chemical Company, St. Louis, MO, and included L-malic acid, monosodium salt; D-glucose-6-phosphate,
monosodium salt; and nicotinamide adenine dinucleotide phosphate, monosodium salt (NADP⁺). Heparin (Type II), disodium salt, and riboflavin (B₂) were also obtained from Sigma Chemical Company. Bromocriptine (CB-154) was a gift of Sandoz Pharmaceuticals, Hanover, NJ. Blue dextran was purchased from Pharmacia, Piscataway, NJ.

Standard Proteins and Enzymes

Phosphorylase-a (rabbit muscle), bovine serum albumin (Fraction V), pyruvate kinase (Type II, rabbit muscle), aldolase (Grade III, rabbit muscle), ovalbumin (Grade V), carbonic anhydrase (Rabbit erythrocytes), chymotrypsinogen-A (Type II, bovine pancreas), myoglobin (Type II, whale skeletal muscle), lysozyme (Grade III, egg white), ribonuclease (Type II-A, bovine pancreas), and glucose-6-phosphate dehydrogenase (Type VII, baker's yeast) were all purchased from Sigma Chemical Company, St. Louis, MO.

Sterols

Crystalline 25-OH₃ was a generous gift from Dr. Jack Hinman and Dr. John Babcock of the Upjohn Company, Kalamazoo, MI. 1,25-(OH)₂D₃ was provided by Dr. M. Uskokovic, Hoffmann-La Roche Inc., Nutley, NJ.

Hormones

Diethylstilbestrol, testosterone, progesterone, and prolactin (PRL, ovine) were all obtained from Sigma Chemical Company, St. Louis, MO.
Deoxyribonucleic Acid

Deoxyribonucleic acid (calf thymus) was purchased from Worthington Biochemicals, Freehold, NJ.

General Fine Chemicals

Triton X-100 was obtained from Rohm and Haas Company, Philadelphia, PA. Sodium dodecyl sulfate (SDS, sodium salt) was obtained from Sigma Chemical Company or from Bio-Rad, Richmond, CA. Sodium deoxycholate (disodium salt), \( N,N,N^1,N^1 \)-tetramethyl-ethylenediamine (TEMED), monothioglycerol, tris-(hydroxymethyl) aminomethane (Tris, Sigma 7-9), and ethylenediaminetetraacetic acid (EDTA, tetrasodium salt) were obtained from Sigma Chemical Company. 2-Mercaptoethanol was obtained from Matheson, Coleman and Bell, Norwood, OH. Ammonium sulfate (ultrapure) was obtained from Schwartz-Mann, Orangeburg, NY. Polymin P (polyethyleneimine) was purchased from Miles Laboratories, Elkhart, IN. Glycine, acrylamide (electrophoresis grade), \( N,N^1 \)-methylene-bisacrylamide (Bis, electrophoresis grade), and Coomassie Brilliant Blue R-250 were obtained from Eastman Chemicals, Rochester, NY. Bromophenol Blue and ammonium persulfate (electrophoresis grade) were obtained from Bio-Rad, Richmond, CA. Folin and Ciocalteau reagent was obtained from Harleco, Gibbstown, NJ. Liquifluor, a concentrated liquid scintillation solution consisting of 100 g of 2,5-diphenyloxazole and 1.25 g of \( p \)-bis-[2-(5-phenyloxazoyl)] benzene/liter toluene, and aquasol, a premixed liquid scintillation solution were purchased from New England Nuclear Corporation, Boston, MA. All solvents were reagent grade and glass
distilled prior to use when employed in chromatographic procedures. All other reagents were of analytical grade or better.

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**Chromatographic and Filtration Media**

Silicic acid (Bio-Sil HA, minus 325 mesh) was obtained from Bio-Rad, Richmond, CA. Sephadex LH-20 (Lipophilic, particle size 25-100µ) was purchased from Sigma Chemical Company, St. Louis, MO. Celite, a support medium for liquid-liquid partition chromatography, was supplied by Johns-Manville Company, Lompoc, CA. High pressure liquid chromatography was performed on a Dupont Zorbax Sil (25 cm x 4.6 mm) column, Dupont Company, Wilmington, DE. Phosphocellulose (P-11) and DEAE-cellulose (DE-52) were purchased from Whatman, Inc., Clifton, NJ. Sepharose (4B), CNBr-activated Sepharose (4B), and Sephacryl (S-200) were purchased from Pharmacia, Piscataway, NJ. Hydroxylapatite (Bio Gel-HTP), Affi-Gel blue, and cellulose (Cellex N-1) were obtained from Bio-Rad, Richmond, CA. Glass fiber filters (Type A/E) were obtained from Gelman, Ann Arbor, MI. Whatman DEAE filters (DE-81, 2.5 cm) and #1, #2, #3 filters were purchased from Whatman, Inc., Clifton, NJ. Millipore filters (HAWP 025 00, HA 0.45µ) were obtained from Millipore Corporation, Bedford, MA.

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**Buffers**

Buffers used were:

1) Isotonic sucrose (IS) = 0.25 M sucrose.

2) STKM = 0.25 M sucrose, 0.05 M Tris-HCl, pH 7.4, 0.025 M KCl, 0.005 M MgCl$_2$, 0.012 M thioglycerol, 0.001 M EDTA.

3) EDTA-Buffer B = 0.008 M EDTA, 0.025 M NaCl, pH 8.0.
4) Triton-Buffer C = 0.01 M Tris-HCl, pH 7.5, 1% Triton X-100.
5) Tris-Buffer D = 0.01 M Tris-HCl, pH 7.5.
6) KETT-Buffer = 0.01 M Tris-HCl, pH 7.4, 0.001 M EDTA, 0.012 M thioglycerol, and various concentrations of KCl. KETT-0 = KETT-buffer, no KCl; KETT-0.2 = KETT-0, 0.2 M KCl; etc.
7) HAP-Buffer = 0.05 M Tris-HCl, pH 7.4, 0.1 M KCl, 0.012 M thioglycerol, 0.001 M EDTA, and various concentrations of KH$_2$PO$_4$ ranging from 0.01-0.3 M. HAP-0 = HAP-buffer, no KH$_2$PO$_4$; HAP-0.3 = HAP-0, 0.3 M KH$_2$PO$_4$.
8) Sephacryl Buffer = KETT-0.3, 0.02% NaN$_2$.
9) Tris-Glycine Buffer = 0.005 M Tris, 0.38 M glycine, pH 8.3 (diluted 10x with distilled water).
10) Tris-Glycine-SDS-Buffer = 0.10 M Tris, 0.76 M glycine, 4% sodium dodecyl sulfate (SDS), pH 8.3 (diluted 4x with water).
11) Sample Buffer = 10% glycerol, 5% 2-mercaptoethanol, 3% SDS, 0.0125 M Tris, 0.095 M glycine, pH 8.3, 0.1% bromophenol blue.

Preparation of Radioactive 1,25-Dihydroxyvitamin D$_3$

1,25-(OH)$_2$[^3H]D$_3$ was produced in vitro by a modification of the method described by Lawson et al. (44). Each preparation of 1,25-(OH)$_2$[^3H]D$_3$ (either 6.7 Ci/mmole or 110 Ci/mmole) involved the following procedures: The kidneys of 10 rachitic chicks were excised, weighed (12 g), and homogenized immediately in 100 ml of ice cold 0.3 M sucrose with a Potter-Elvehjem homogenizer at 1-4°C. The homogenate was then mixed with a phosphate buffer system containing an NADPH generating
system containing the following: 0.16 M KH₂PO₄, 0.0032 M MgCl₂, 0.0078 M L-malate, 0.0037 M glucose-6-phosphate, 0.0003 M NADP⁺, 150 units of glucose-6-phosphate dehydrogenase in a final volume of 410 ml (divided equally into five 250 ml flasks, 82 ml each). 400 μl of 25-(OH)[³H]D₃ substrate (7 nmoles in ethanol) was added to each flask (total 35 nmoles), swirled, placed in a 37°C shaking incubator, and incubated exposed to air with gentle rotary shaking for 90 minutes. The reaction was stopped by the addition of 3.5 volumes (1400 ml) of methanol-chloroform (2:1 v/v) to the contents of the flasks. After allowing the denatured protein to settle out, the supernatant was harvested by decanting into a large separatory funnel. The last few milliliters of supernatant was collected after brief centrifugation of the denatured protein residue. Sterols were then extracted by the method of Bligh and Dyer (119). Briefly, one-fifth volume of chloroform and one-tenth volume of deionized water were added to the supernatant (approximately 1800 ml), the mixture shaken, and the phases allowed to separate. The lower phase (CHCl₃, containing 85-95% of the sterols) was harvested and then combined with a 750 ml chloroform wash of the upper aqueous-methanol phase. This phase contained both unreacted 25-(OH)[³H]D₃, the generated 1,25-(OH)₂[³H]D₃ product, and other degradative metabolites of 1,25-(OH)₂[³H]D₃. The volume was flash-evaporated to dryness, and the resulting lipids and sterols resolubilized in diethyl ether. After brief centrifugation (12,000 x g for 10 minutes) to clarify the solution, the diethyl ether was evaporated under nitrogen to approximately 10 ml, and the 1,25-(OH)₂[³H]D₃ purified by column chromatography to be described.
Silicic Acid Column Chromatography

Twenty five grams of silicic acid were activated by heating in a vacuum oven to 120° for 24 hours just prior to use. The cooled resin was then slurried in diethyl ether, poured into a 1.8 x 18 cm column, and packed with the aid of nitrogen pressure at 2.5 psi. The 10 ml sample was applied to the column, and after several 5 ml ether washes of the sample vial, the column was eluted with 5% acetone in ether under approximately 2.5 psi of nitrogen pressure. 100 ml fractions were collected, and the peak of 1,25-(OH)₂[³H]D₃ (second peak) located by counting a small (20 µl) aliquot of each fraction. The peak fractions were harvested, combined, flash-evaporated to dryness, and then resolubilized in 400 µl of 65% chloroform in hexane (v/v).

Sephadex LH-20 Column Chromatography

Liquid-gel partition chromatography was performed on Sephadex LH-20 as described by Holick and DeLuca (120). Five grams of resin were slurried in 65% chloroform in hexane (v/v), and poured into a 1 x 15 cm column. After sample application (and 2-400 µl vial washes) the column was eluted with the slurry solvent. 25-(OH)[³H]D₃ emerged between 0-40 ml elution volume, whereas 1,25-(OH)₂[³H]D₃ eluted between 40-95 ml. The second fraction (40-95 ml) was harvested and roto-evaporated to dryness.

Celite Column Chromatography

Celite was first washed with concentrated HCl, organic solvents, and defined as described by Engel et al. (121). The chromatography was then performed by the procedure of Haussler and Rasmussen (49). Fifty ml
ethyl acetate was diluted to 500 ml with hexane (upper mobile phase) and then cross-equilibrated for 10 minutes with 45 ml water diluted to 100 ml with ethanol (lower stationary phase). One ml of stationary phase per gram Celite was "cake" mixed with a stirring rod, and then excess mobile phase added to create a slurry. The mixture was "fluffed" for 10 minutes by stirring with a magnetic stirrer, and then packed tightly into a 1 x 36 cm column with the aid of a glass rod. The dried sample was resolubilized in 200 µl mobile phase and applied, along with three vial washes (200 µl, 2-400 µl), under nitrogen pressure. The sterols were eluted with mobile phase under nitrogen pressure, and the 1,25-(OH)₂[³H]D₃ emerged in 125 ml volume. The peak was located by counting a small aliquot (10 µl) from each 5 ml fraction. The final pooled peak was taken to dryness under nitrogen, resolubilized in distilled ethanol or ethanol-toluene (1:1, v/v) at -20°. The final yield ranged from 30-50%.

Assessment of 1,25-(OH)₂[³H]D₃

The post-Celite sample should be free of residue prior to solvent resolubilization, and then adjusted to 1-2 ng/ml with appropriate solvent: 6.7 Ci/m mole 1,25-(OH)[³H]D₃, ethanol; 110 Ci/m mole 1,25-(OH)₂[³H]D₃, ethanol-toluene (1:1, v/v). These solutions represent stock solutions from which dilutions were formulated. Further, 110 Ci/m mole 1,25-(OH)₂[³H]D₃ was often reduced to 7 Ci/m mole by the addition of nonradioactive 1,25-(OH)₂D₃, and then stored as 2 ng/ml in ethanol at -20°.
Modifications of 1,25-(OH)$_2$[${}^3$H]D$_3$ Purification Procedure

High pressure liquid chromatography was often employed as a procedure for isolating the 1,25-(OH)$_2$[${}^3$H]D$_3$ product, as an alternative to Celite chromatography (122). The post LH-20 sample was nitrogen dried, resolubilized in 100 μl of 15% isopropanol in hexane (v/v), and the sample applied to a 25 cm x 4.6 mm Dupont Zorbax-Sil-850 column injected by means of a Rheodyne Model 70-10 Sample Injection Valve and a Model 70-11 Loop-filler port with a 200 μl sample loop. The column, equilibrated in 15% isopropanol in hexane (v/v), was eluted with the same solvent at a flow rate of 1 ml/min, and the 1,25-(OH)$_2$[${}^3$H]D$_3$ emerged between 14-17 minutes from time of injection. The product was stored as previously described and judged >95% radiochemically pure.

**Animal Surgery**

**Parathyroidectomy**

Parathyroid glands were removed by excision from one group of rats: rats suckling ten 12-13-day-old pups, lactating rats and non-lactating rats of similar age were sham-operated. Completeness of parathyroidectomy was assumed when a postoperative (48 hour) drop in serum calcium of at least 3 mg per 100 ml was observed.

**Hypophysectomy**

Male rats were hypophysectomized by the transsphenoidal route as described by Ingle and Griffiths (123).
Administration of Hormones and Sterols

In the majority of cases, vitamin D₃ sterols were administered in the diet. However, in some cases vitamin D₃ was administered orally dissolved in 1,2-propanediol (practical grade). The sterol was first dissolved in diethyl ether, mixed with vehicle, and then bubbled with nitrogen (dry, 99.9%) to eliminate ether. Doses were made up just prior to administration, and administered orally via injection into the crop (in total volume of 0.2 ml). Ovine prolactin (oPRL, 26 μ/mg, 3 mg) was dissolved in 0.3 ml of 0.002 M NaOH, diluted to 6 ml with physiologic saline, and delivered by subcutaneous injection twice daily at a dose of 10 μg or 50 μg oPRL. Human growth hormone (HGH) was similarly prepared and administered at doses of 50 μg or 250 μg twice daily. The human GH preparation contained less than 0.1% of other pituitary hormones and has a potency of 2.5 IU/mg. Progesterone, testosterone, and diethylstilbestrol were dissolved in either arachis oil, sesame oil, or ethanol, and administered subcutaneously in dose ranging from 100 μg to 5 mg. In all hormone injection experiments, control animals received vehicle injected alone.

Preparation of Tissue Subcellular Fractions

Tissues (parathyroid glands, intestine, liver, pancreas, pituitary, and placenta) were excised and immediately placed in STKM at 0-4°C. Intestines were routinely rinsed with either STKM or IS, slit longitudinally, and then blotted dry. The mucosa was generally scrapped free of the underlying muscular serosa and weighed, although for large scale purification the entire gut was used. Other tissues were blotted
free of buffer and immediately weighed. Tissue was then homogenized in 2.5-10 volumes of STKM (specified), depending upon the purpose of the experiment. Homogenization was achieved by a variety of means: 6 passes with a Potter-Elvehjem homogenizer fitted with a Teflon pestle; 3-15 second bursts on setting 6 with a Polytron (P-10, Brinkman); 2-60 second bursts with a Waring blender. Homogenization was routinely performed on ice. The crude homogenates were then centrifuged 1200 x g for 10 minutes in a Sorvall SS-34, HB-4, or GSA rotor in a Model RC-2B or RC-5 (Sorvall Instruments Division of E.I. Dupont, Incorporated) and the sediment designated the crude nuclear fraction. The mitochondrial fraction was prepared by centrifuging the previous supernatant at 8000 x g for 20 minutes, and harvesting the pellet. The post-mitochondrial supernatant was then centrifuged 100,000 x g for 90 minutes in a Spinco TY-30, 220,000 x g for 45 minutes in a Spinco 50.2, or 360,000 x g for 30 minutes in a Spinco TY65 rotor in a Beckman L5-65 Ultracentrifuge and the resultant supernatant designated the tissue cytoplasmic fraction (cytosol). The microsomal pellet from this procedure was discarded.

Chromatin was prepared from crude nuclei by resuspending the crude nuclear pellet, via homogenization, successively in 12.5 volumes (w/v) of EDTA, Triton, and Tris buffers. The particulate material was harvested by sedimentation at 30,000 x g for 10 minutes after each homogenization, and the final gelatinous chromatin pellet was resuspended in various buffers as the experiment dictated.
1,25-Dihydroxyvitamin D Assay

Sterol Extraction from Plasma

Two to ten milliliters of plasma (or serum) were routinely required for triplicate assay. Several different methods were used for the extraction of sterol. Method 1 was a modification of the Bligh and Dyer (119) technique, and was performed as described in the section dealing with the generation of 1,25-(OH)$_2$[H]$^3$D$_3$. Method 2 was more rapid and facile, and resulted in excellent yield of sterols (80-95%). Plasma volume (5-10 ml) was measured, transferred to a 250 ml centrifuge bottle, and 1000-2000 cpm 1,25-(OH)$_2$[H]$^3$D$_3$ added in ethanol as tracer sterol. Seven volumes of acetone:1,2-dichloroethane (3:1, v/v) was added to the plasma sample followed by vigorous shaking for 30 minutes at room temperature. The solutions were then centrifuged at 5000 RPM in a Sorvall GSA rotor for 10 minutes to pellet protein. The supernatant was flash-evaporated to 0.5-1.0 ml of lipid-water suspension, and then transferred via three-five ml diethyl ether washes to a scintillation vial. The solution was thoroughly mixed, and after phase separation occurred, the lower aqueous phase was carefully pipetted away and discarded. The upper ether phase, containing lipid and sterols, was subsequently dried for further purification. Method 3 was employed with two ml plasma samples utilizing an extremely sensitive assay procedure to be described. Five volumes of acetone were added to the sample, shaken vigorously for five minutes and then centrifuged 10,000 x g for 10 minutes. The resulting extract was then dried directly under nitrogen to approximately 1 ml of aqueous-lipid solution, covered with 15 ml of
diethyl ether, shaken, and then allowed to extract overnight. The lower aqueous phase was then removed and the sample dried as described in Method 2.

Chromatography

Sephadex LH-20 chromatography was performed as described in (120) and detailed under Preparation of Radioactive 1,25-Dihydroxyvitamin D₃. The fraction containing 1,25-(OH)₂D₃ was dried by flash-evaporation, resolubilized in 0.5 ml of diethyl ether, and then applied to a 0.8 x 6.5 cm silicic acid column (1.5 g of silicic acid, activated by heating to 120°C for 24 hours in a vacuum oven, and prepared in diethyl ether). After 2-0.5 ml washes of the sample vial, the column was eluted successively with an additional 12 ml of diethyl ether and then 10 ml of acetone; the hormone emerged in the second fraction with acetone. Final purification of the 1,25-(OH)₂D₃ was achieved with Celite liquid-liquid partition chromatography (49), as described in Preparation of Radioactive 1,25-Dihydroxyvitamin D₃. The only modification was a reduction in column size to 0.8 x 8 cm (1 g of "fluffed" Celite equilibrated in upper mobile solvent). Sample from the previous purification procedure was dried under a stream of nitrogen, resolubilized in Celite mobile phase, and applied in 0.2 ml volume. After several sample vial washes, the column was eluted with mobile phase and the hormone emerged between 7 and 22 ml solvent. The latter two columns were prepared in 10 ml glass pipettes fitted with glass wool plugs. All purified samples after Celite chromatography were dried under nitrogen, resolubilized in 0.4 ml ethanol. Determination of yield of the 1,25-(OH)₂D hormone was achieved
by counting a 50 μl aliquot of the purified sample for tritium (yields ranged from 50-75%). When 12 samples were processed simultaneously, the total time for purification was approximately 2 hours/sample/technician using one of the outlined extraction procedures, standard LH-20 chromatography, and micro-silicic acid and micro-Celite chromatography.

Modifications of Purification Chromatography

Two major modifications of the hormone purification scheme, as detailed above were employed when 2 ml of plasma (or serum) was used as 1,25-(OH)$_2$D source. Sephadex LH-20 column chromatography was modified by using 2.3 g of resin, slurried in 65% chloroform in hexane (v/v), and poured into a 10 ml glass pipette (0.8 x 12 cm resin column). The sample was applied exactly as described for the larger LH-20 column, except that the hormone emerged between 20 and 40 ml elution volume of the 65% solvent. The second modification was the use of high pressure liquid chromatography (HPLC) instead of Celite chromatography, as a final purification procedure. HPLC was performed exactly as described in Preparation of Radioactive 1,25-Dihydroxyvitamin D$_3$. The purification of the hormone using these modifications as carried out in the following sequence: silicic acid, Sephadex LH-20, and then high pressure liquid chromatography. Yields of hormone were routinely 50-65%.

Preparation of Reconstituted Cytosol and Chromatin

Reconstituted intestinal cytosol and chromatin were used for the competition binding assay to detect 1,25-(OH)$_2$D. Intestines were excised, and treated (including mucosal scrapping) as described in the section on Preparation of Tissue Subcellular Fractions. Two grams of
mucosa were then homogenized in 25 ml of STKM by six passes with a Potter-Elvehjem. During homogenization and subsequent steps in tissue preparation, all tubes, flasks, pipettes, rotors, and centrifuges were rigorously kept at 0-4° by the use of an ice-slush bucket. Chromatin and cytosol were then prepared (as described previously) and the resultant chromatin pellet combined and gently homogenized with one-half of the resulting cytosol (12.5 mls). The "reconstituted Cytosol-chromatin" receptor system was then passed through a 22 G needle, and used either fresh, or frozen in liquid nitrogen and stored at -80° for later use. For assay employing high specific activity \(1,25-(OH)_2[^3H]D_3\) (110 Ci/mmol), this reconstituted material was diluted upon thawing with seven volumes of STKM.

Radioreceptor Binding Assay

New glass culture tubes (borosilicate; 13 mm x 100 mm) were used for individual assay incubations. Basically two assays for \(1,25-(OH)_2D\) were performed, differing only in sensitivity of hormone detection and made possible by the utilization of radioactive \(1,25-(OH)_2[^3H]D_3\) of either 6.7 Ci/mmol or 110 Ci/mmol specific activity. To each tube, 20 µl of radioactive \(1,25-(OH)_2[^3H]D_3\) (either 175 pg or 20 pg) was added in ethanol. The standard curve was constructed by adding various volumes (0-100 µl) of nonradioactive \(1,25-(OH)_2D_3\) (7 ng/ml or 100 pg/ml). For plasma samples, 10-100 µl of purified sample was used (adjusted relative to the concentration of \(1,25-(OH)_2D\) determined in the first assay).
The sterol in each assay tube was dried under a stream of nitrogen, resolubilized in 10 μl of distilled ethanol, and then 100 μl of reconstituted cytosol-chromatin system added (either as standard, 80-100 μg DNA and 400 μg protein or as one-eighth diluted solution, 10 μg DNA and 50 μg protein). The ethanol was added just prior to the addition of the cytosol-chromatin mixture, and aided in solubilizing the sterols to achieve a higher binding efficiency and reduced non-specific binding. The final concentration of 1,25-(OH)$_2$[H]$^3$D$_3$ was either 4.3 nM or 0.48 nM. After incubation (30 minutes or 60 minutes) at 25° with shaking in a water bath, the quantity of labeled sterol bound to chromatin was determined by collecting the chromatin on a glass fiber filter. To each tube, 1 ml of cold Triton buffer was added, the mixture applied to a Gelman type A/E glass fiber filter (presoaked for 30 minutes in deionized water and prerinsed in Triton buffer), and filtered at 1 ml/minute under vacuum. The tube was then washed with 2 ml of Triton buffer, and the wash then applied to the filter under the same vacuum pressure. Following filtration, the filters were placed in scintillation vials, extracted with 5 ml of acetone, and dried at 250° under air. The filter was then covered with 5 ml of nonaqueous based counting solution to resolubilize the radioactive sterols and the samples counted in a Beckman LS-250 liquid scintillation counter. Routinely, 900-1400 cpm of the approximately 3000 or 6000 cpm of 1,25-(OH)$_2$[H]$^3$D present in the assay tube (added sterol) were recovered as bound sterol in the absence of competing nonradioactive 1,25-(OH)$_2$D$_3$ or serum 1,25(OH)$_2$D. The first assay (3000 cpm 1,25-(OH)$_2$[H]$^3$D$_3$ added) demonstrated 30-40% efficiency in binding and the second assay (6000
cpm 1,25-(OH)$_2$[sup][3]H]D$_3$ added) provided 20% binding recovery. Interassay variation was 10-15%.

**25-Hydroxyvitamin D$_3$-1-Hydroxylase Assay**

25-Hydroxyvitamin D-hydroxylase enzymes were assayed as described (124). Chicks were killed by decapitation, and the kidney tissue removed immediately and placed on ice. Homogenates (2% v/v) were then prepared with aid of a Potter-Elvehjem in ice cold Tris-Acetate Buffer. Portions of the homogenate (3 ml) were preincubated at 27° for 5 minutes, and the substrate 25-hydroxy [26(27)-Me$^3$H]-D$_3$ (6.7 Ci/mmol) added in 20 μl of ethanol to a final concentration of 5nM. The reaction mixtures were flushed with 100% O$_2$ for 30-60s and then incubated for 10 minutes at 37° with gentle shaking. The reaction was then stopped with the addition of 9 ml of 65% methanol/chloroform (v/v) and the mixtures shaken overnight at 4°. The mixture was then treated by the techniques of Bligh and Dyer (119) to extract 25-OHD$_3$ and its metabolites, and the extracted sterols resolved by Sephadex LH-20, as described in Preparation of Radioactive 1,25-Dihydroxyvitamin D$_3$. Under the assay conditions described, production of 1,25-(OH)$_2$D$_3$ and 24,25-(OH)$_2$D$_3$ was linear with time up to 20 minutes, falling off rapidly beyond this until at 80 minutes there was no further increase in metabolite production. Further, 1,25-(OH)$_2$D and 24,25-(OH)$_2$D synthesis were proportional to the homogenate concentration in the range of 2-10% (w/v), and no enzyme activity was evident after the homogenate had been boiled for 20 minutes. Results are expressed as femtomoles of
$1,25-(\text{OH})_2[^{3}\text{H}]D_3$ or $24,25-(\text{OH})_2[^{3}\text{H}]D_3$ product formed per minute per milligram of homogenate protein ± standard deviation for triplicate assay.

**Receptor Preparations**

**Preparation of Tissue**

Rachitic chicks (1-100) were sacrificed by decapitation and the intestines quickly removed, rinsed in either ice cold STKM or IS, and then placed in ice cold STKM. The guts were blotted, and the mucosa scraped free of serosa and then homogenized in 2.5 volumes (w/v) of STKM with six passes in a Potter-Elvehjem homogenizer (with intermittent cooling on ice). The homogenate was then centrifuged in Sorvall rotor to prepare a post-mitochondrial supernatant. The resultant solution was decanted through several layers of cheese cloth, and then used to prepare the high speed cytoplasmic fraction (cytosol). When large scale preparations of receptor were needed, the whole intestine was used, by first rinsing in STKM, slitting, and then homogenizing in 2.5 volumes (w/v) of STKM with a Polytron (PT-10, Brinkman) with 3-15 second bursts on setting six with 30 second cooling periods between bursts. Generally 100 g batches of intestine were homogenized in 250 ml STKM. The resulting homogenate was then centrifuged to prepare a post-mitochondrial supernatant and this solution used to prepare cytosol.

**Precipitation of Receptors**

The receptor for $1,25-(\text{OH})_2D$ was often precipitated by several means prior to assay or purification chromatography. Preground
ammonium sulfate crystals were added to intestinal cytosol to 40% of saturation, and after stirring for 20-30 minutes, the precipitate was sedimented at 16,000 x g for 15 minutes. This pellet was then frozen at -80° and stored from up to 6-8 months without loss of receptor binding activity, permitting the accumulation of large quantities of receptor containing material (125). The pellets could be resolubilized in one-fourth to one-fifth the original volume of cytosol, with a subsequent yield of receptors of approximately 70% and a purification on a protein basis of 2-3-fold. Receptors were also precipitated from cytosol with Polymin P (polyethyleneimine, Miles Laboratories). Cytosol was made up to 0.04% Polymin P, stirred for 10 minutes, and the solution centrifuged 16,000 x g for 15 minutes. The resulting supernatant, containing the receptor, was then made up to 0.08% Polymin P (the solution was brought to an additional 0.04%), stirred for 15 minutes, and then centrifuged 16,000 x g for 15 minutes. The receptor, now in the pellet, was extracted with the original cytosolic volume of KETT-0.5 by gentle homogenization and stirring for 15 minutes, and the insoluble material repelleted at 16,000 x g for 15 minutes. The supernatant, containing protein, receptor, and residual Polymin P, was then made 40% saturated with preground ammonium sulfate and treated as described for salt precipitation as above. The resulting pellet contained receptor free of residual Polymin P, and was frozen at -80° and storable for 6-8 months without loss of receptor binding activity. The pellets were resolubilized in approximately 4-5% of the original volume of cytosol, and demonstrated a yield of receptor of approximately 50-60%.
DEAE-Filter Technique

The receptor for $\text{1,25-}(\text{OH})_2\text{D}$ was assayed routinely by a modification (125,126) of the DEAE-filter technique of Santi et al. (127). Aliquots of nascent receptor solutions (10-500 μl) were first incubated with various concentration of labeled $\text{1,25-}(\text{OH})_2[\text{H}]\text{D}_3$ or incubated in parallel with both labeled $\text{1,25-}(\text{OH})_2[\text{H}]\text{D}_3$ and a 100-fold excess of nonradioactive hormone (Borosilicate tubes, 13 x 100 mm). The solutions contained 10% ethanol (v/v) to aid in the solubilization of sterols, and were incubated either at $0^\circ$ for 2-12 hours, or for 15-30 minutes at $25^\circ$. After incubation, the samples were diluted with 1-3 ml of Triton buffer (so final salt concentration was less than 0.1 M KCl), and then filtered through 2 DEAE-filters (Whatman, DE-81) which had been pre-soaked in deionized water and equilibrated with Triton buffer. Filtration was carried out at approximately 1 ml/minute, with 2-2 ml washes of assay tube and filter with the same buffer. The filters were extracted in scintillation vials with 5 ml of acetone, dried under a stream of air at $250^\circ$, and then the radioactive sterols resolubilized in aqueous counting solution. The vials were counted for tritium with an efficiency of 33%. Assay was generally done in duplicate, with an assay variation of 10-15%, and an efficiency of the filtration procedure of 75%, assessed by measurements of binding at low hormone concentrations. Receptor preparations which were already labeled were also assayed as above by omitting the incubation step. However, once labeled, estimates of non-specific binding (5-10%) could not be measured.
Hydroxylapatite

Receptors were also assayed by hydroxylapatite essentially as described by Williams and Gorski (128). After incubation as previously described, two volumes of a 50% slurry of hydroxylapatite (Bio-Gel HTP, Bio-Rad) equilibrated in HAP-0.01 was added and mixed. The resin was washed three times with HAP-0.01, the resin collected by centrifugation, and then extracted in 1 ml ethanol. The supernatant was removed, after brief centrifugation, dried, and then counted by liquid scintillation as just described.

DNA-Cellulose

Receptors were also assayed with affinity resins such as DNA-cellulose. After incubation of receptor with sterol as above (salt concentration less than 0.1 M KCl), the incubate was applied to a 1 ml micro-column of DNA-cellulose resin equilibrated in KETT-0.1. After washing the resin with 5 ml of KETT-0.1, the receptor was eluted with 5 ml of KETT-0.5. An aliquot of this eluate was then counted with an aqueous counting solution via liquid scintillation. Since independent assessment of purification of the 1,25-(OH)$_2$D receptor demonstrated that DNA-cellulose was far superior to either DEAE or hydroxylapatite, this method was the most quantitative and the most accurate for determinations of 1,25-(OH)$_2$D-receptor binding activity (129).

Sucrose Density Gradient Analysis

Sucrose gradient analysis (see below) was also used for the determination of specific macromolecular-bound 1,25-(OH)$_2$D$_3$. However, since recovery of tritium was 40-60% in this non-equilibrium
situation, due to extensive hormone-receptor dissociation, the technique provided low estimates of receptor binding activity. As a result, the procedure was generally employed only as an assay for receptor identification, because the macromolecular-bound tritium sedimented in a very specific fashion.

**Sucrose Gradient Ultracentrifugation**

Linear gradients of 5-20% sucrose prepared in KETT-0.3 were made with a Buchler gradient mixer, Auto-Densi Flow, and Polystaltic Pump. Aliquots (0.1-0.4 ml) of labeled receptor-containing solutions were layered gently onto cold gradients, and then centrifuged either with a Spinco 50.1 rotor at 220,000 x g for 22.5 hours or a Spinco SW 60 rotor at 369,000 x g for 16 hours in a Beckman L5-65 Ultracentrifuge. Fractions (5 or 6 drops) were collected from the top, and counted with 5 ml of aqueous scintillation cocktail. Sedimentation coefficients were calculated by comparison with standard protein markers: chymotrypsinogen, 2.5 S; ovalbumin, 3.67 S; and bovine serum albumin (BSA), 4.4 S.

**Chromatography of Receptors**

**Preparation of Chromatography Media**

**DNA-cellulose.** DNA-cellulose was prepared by a modification of the technique described by Alberts and Herrick (130). Nonionic cellulose (Cellex N-1, Bio-Rad) was extensively washed successively with 95% ethanol, 0.01 N NaOH, 0.001 N EDTA, 0.01 HCl, and deionized water, and then dried. Calf thymus DNA (Worthington) was dissolved in KETT-.1 buffer with gentle shaking at 4° for 5-6 days (5 ml chloroform added to
prevent microbial growth). The dissolved DNA was then mixed with the washed cellulose in batches, and the resultant solution allowed to dry. The dried DNA-cellulose mixture was then resuspended in 100% ethanol, positioned in front of an ultraviolet light, and exposed with stirring to UV irradiation for 15-20 minutes. After exposure, the solution was filtered to remove ethanol, washed extensively with KETT buffer, and frozen at -20°. DNA uptake onto cellulose was approximately 75% with 1.4-1.6 mg DNA bound per ml of cellulose. The appropriate amount of DNA-cellulose was then extensively washed with KETT-0.05 prior to its packing into columns.

Blue dextran-Sepharose. Blue dextran-Sepharose was prepared as described by Ryan and Vestling (131). Blue dextran (Pharmacia) was dissolved in 0.001 M NaHCO₃ buffer and then added to a slurry of CNBr-activated Sepharose (15 g dried Sepharose, Pharmacia). The mixture was then gently stirred overnight in a cold room at 4°. Free blue dextran was separated from coupled blue dextran-Sepharose, by extensive washing with 1.0 M KCl, KETT-1, and then KETT-0.05. The appropriate volume of resin was then packed into a column and washed with one-fourth volume of 2% BSA in KETT-0.05 prior to chromatography of receptors.

Heparin-Sepharose. Heparin-Sepharose was prepared as described by Molinari et al. (132). The polysulfate-containing mucopolysaccaride heparin (Sigma) was dissolved in 0.001 M NaHCO₃, combined with CNBr-activated Sepharose 4B (15 g slurried in 0.01 N HCl), and gently mixed overnight at 4°. The heparin-coupled gel was separated from free heparin as described for blue dextran-Sepharose, and then equilibrated in KETT-0.05.
Hydroxylapatite. Bio-Gel HTP (Bio-Rad) was washed extensively with HAP-0.3 and then HAP-0.01 prior to its equilibration in column form in HAP-0.

DEAE-cellulose. Diethylaminoethyl cellulose (DE-52, Whatman) was extensively equilibrated in KETT-0, until the resin wash and the equilibrating buffer had identical pH and conductivity. During the washes, the resin was allowed to settle and was defined to improve flow rate. It was then equilibrated in KETT-0.05.

Phosphocellulose. Phosphocellulose (P-11, Whatman), was processed by precycling and defining the resin successively in water, 0.5 M NaOH, water, 0.5 M HCl, deionized water, and then adjusted to pH 7.4 with the addition of solid Tris (Sigma). The resin was then extensively washed with 1 M KCl, KETT-1, and then equilibrated with KETT-0.05.

Sephacryl S-200. Sephacryl S-200 (Pharmacia) was obtained as a preequilibrated slurry, reequilibrated in Sephacryl buffer, deaerated, and then packed into a column (see below). Standard proteins were BSA, ovalbumin, chymotrypsinogen, and ribonuclease.

Preparation of Columns

Sephacryl resin was packed into a 1.6 x 60 cm Pharmacia column according to the instructions provided by the manufacturer, and equilibrated with Sephacryl buffer at a flow rate of 60 ml/hour. All other affinity and ion exchange resins (excluding hydroxyapatite) used primarily for analytical work were packed with KETT-0.05 into various dimension columns made from plastic disposable syringes (5-50 ml) fitted with plastic resin or steel (Swinnex) support disks and flow rate
valves. The resin was packed at a flow rate of approximately 40-80 ml/hour and covered with Whatman #1, 2.5 cm diameter, filter papers. Column dimensions were generally 1-2.5 cm x 2-6 cm. Hydroxylapatite resin, equilibrated in degassed HAP-0, was poured into similar columns, fitted with Whatman filter disks above and below the resin. Prepara­tive columns for large DNA-cellulose or blue dextran-Sepharose resins were prepared in 5 x 15 cm Glenco or Pharmacia columns fitted with a lower flow adapter. The resin was equilibrated in KETT-0.05 at a flow rate of 200 ml/hour.

Chromatography

**Sephacry S-200.** 0.5-1 ml samples of receptor (derived from either cytosol, precipitates, or various stages of purification) solu­bilized in Sephacryl buffer were applied to the column and resolved with the same buffer at a flow rate of 60 ml/hour. Fractions of 2 or 3 ml were collected, aliquots (1-20%) were taken, and then counted for tritium by liquid scintillation techniques.

**Affinity and Ion Exchange Resins.** Receptor-containing samples were applied to these resins under low salt conditions at a flow rate of 40-60 ml/hour. Unlabeled receptors derived from ammonium sulfate or Polymin P precipitation were first resolubilized in KETT-0 (residual ammonium sulfate provides a conductivity equivalent to between 0.1-0.4 M KCl) for 15-30 minutes by gentle stirring, centrifuged 16,000 x g for 15 minutes to pellet insoluble particulate matter, and then labeled with a saturating concentration of 1,25-(OH)$_2$[^3H]D$_3$ (7 or 110 Ci/mmol, added in ethanol to 5%, v/v) for 2-12 hours at 0-4°C. After labeling, the
receptor sample was centrifuged 16,000 x g for 15 minutes to remove ethanol precipitates, and then diluted with KETT-0 to the appropriate salt concentration which allowed receptor binding to the particular resin. The sample was applied to the resin at 40–100 ml/hour, depending upon column size, and the resin washed extensively with low salt KETT-buffer which cleared weak or noninteracting protein species from the resin. The labeled receptor was then eluted from the resin during a linear KCl gradient of 3–10 resin bed volumes performed in KETT-buffer. Most gradients were formed with the aid of a 250 ml, 500 ml, or 1 liter Kontes Gradient Maker. Receptor-containing samples were also applied to hydroxylapatite resin, washed as above with HAP-0.01, and then receptor eluted during a $\text{KH}_2\text{PO}_4$ gradient between 0.01 and 0.3 M. Fractions of 1–15 ml were collected from all the columns and then aliquots (1–20%) taken and counted for tritium. Conductivity was used to determine KCl or $\text{KH}_2\text{PO}_4$ gradients, using the appropriate salt as standard. Proteins were measured at 280 nm using a Pharmacia Flow-Through Monitor and a Beckman Recorder, or at 750 nm (see below).

Concentration of Receptor

Pooled peaks of receptor eluted from either Sephacryl S-200 or the affinity, or ion exchange resins, were pooled in several ways. When protein concentrations were greater than 20µg/ml, the solution was precipitated with ammonium sulfate at 40% of saturation. Amicon Ultrafiltration Stirred Cells, using PM-10 membrane (Amicon), were used at later stages of purification to concentrate receptor for subsequent electrophoretic gel analysis. Often, at intermediate purification
steps, the pooled receptor peak required only salt dilution (using KETT-0) prior to application on the subsequent gel. Receptor solutions were also concentrated by dialysis in KETT-0.1 in 2 M sucrose or by laying the dialysis bag containing receptor solution on a bed of sucrose crystals at 4°C. Hydroxylapatite was also used for concentrating and desalting receptor preparations, particularly after the final purification procedure. Hydroxylapatite (0.8 x 1.0 cm) was equilibrated in HAP-0, and then receptor sample applied. This resin is unaffected by KCl, but the receptor was eluted in a volume of 1-2 ml with HAP-0.3.

Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed essentially as described by Laemmli (133). Protein samples derived from crude cytosol or various stages of purification were precipitated with 6.5% final concentration trichloroacetic acid (TCA) (when protein concentration was greater than 10 μg/ml), the small pellets washed gently with 1-2 ml of diethyl ether to remove TCA, and the samples solubilized in 40 μl Sample buffer. Complete solubilization was achieved by heating to 90-100°C for 2 minutes. The sample was then applied to a 11% polyacrylamide, 0.25% Bis slab gel with a stacking gel of 2.5% polyacrylamide and 2.5% Bis. The samples were electrophoresed at a constant voltage of 60V in Tris-Glycine-SDS buffer until the bromophenol blue tracking dye entered the resolving component of the gel. The voltage was then increased to 120V constant, 20-30 mA. When the dye reached the end of the gel, the slab gel was removed and
stained according to the techniques described by Fairbanks, Steck, and Wallach (134). It was destained by diffusion in 10% acetic acid, and then mounted and dried on paper using a slab gel dryer (Hoeffer Scientific). Standard proteins were resolved in accompanying lanes and MW were represented by: phosphorylase a, 94,000; BSA, 68,000; pyruvate kinase, 57,000; ovalbumin, 43,000; aldolase subunit, 40,000; carbonic anhydrase, 29,000; chymotrypsinogen, 25,500; myoglobin, 18,000; lysozyme, 14,400.

Nondenaturing Gel Electrophoresis

Native gel electrophoresis of the 1,25-(OH)\(_2\)D receptor was performed as described by Davis (135). Appropriately concentrated 1,25-(OH)\(_2\)[\(^3\)H]D\(_3\)-labeled receptors were electrophoresed in 5.5 x 12 cm pyrex tubes using an electrophoresis system. The tubes were kept rigorously cold (1\(^\circ\)) by ethylene glycol in a circulating bath (Neslab Incorporated). Resolving gels (8 cm) were made 6.5% polyacrylamide, 0.25% Bis, and were chemically polymerized in the cold at 1-4\(^\circ\). Stacking gels (1.0 cm) were prepared as 3.2% polyacrylamide, 0.8% Bis, and were photopolymerized with riboflavin at 1-4\(^\circ\). Samples of receptor (100-400 \(\mu l\)) were layered under the Tris-Glycine running buffer onto the gel (in 10-40% sucrose, 10 \(\mu l\) bromophenol as tracking dye) and then electrophoresed with the same upper and lower Tris-Glycine Buffer at 1 mA per tube constant current until the tracking dye entered the resolving gel. The amperage was then increased to 2 mA per tube until the dye reached approximately 1 cm from the end of the gel. Gels were "rimmed" out with water at 1-4\(^\circ\), and then either stained by the techniques of Fairbanks
et al. (134) and scanned with a Quick Scan R & D (Helena Laboratories) or sliced into 2 mm sections with a gel slicer (Bio-Rad). Slices were extracted for two hours with acetone, dried at 250° under a stream of air, and then counted for tritium after the addition of 5 ml nonaqueous scintillation cocktail. Yield of receptor-hormone complexes varied between 10-20%.

**Liquid Scintillation Counting**

Liquid scintillation counting of all radioactive sterols was performed after the addition of the appropriate liquid scintillation counting cocktail. All non-aqueous samples were counted with LC-Solution A, consisting of 4 g 2,5-diphenyloxazole and 50 mg 1,4-bis 2-(5-phenyloxazoyl) benzene per liter of toluene, or Aquasol (New England Nuclear). Efficiency of counting with these solutions of 40-50%. Aqueous samples were counted using LC-Solution B, consisting of 3 g 2,5-diphenyloxazole and 38 ml 1,4-bis 2-(5-phenyloxazoyl) benzene per liter of toluene: Triton X-100 (3:1 v/v) or Liquiflour (New England Nuclear). Efficiency of counting with LC-Solution B was 33%. Samples were counted to 5% error in a Beckman LS-230 refrigerated liquid scintillation spectrometer. Generally, cpm was used for calculations of sterol or receptor concentration, although when a calculation of dpm was necessary, it was determined by the addition of an internal standard ([3H]-toluene, specific activity 21,000 dpm per 10 μl, New England Nuclear), and recounted for tritium.
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 column eluates was generally measured with the use of a Pharmacia Flow-Through Monitor equipped with a 10-inch Beckman Recorder. However, during some experiments, particularly sucrose gradient analysis of standard protein, the absorbance at either 235 nm or 280 nm was determined directly with a Gilford model 240 spectrophotometer equipped with a model 2443-A Rapid Sampler and a 4008 Data Lister.

Protein Determinations

Protein was determined by the method of Lowry et al. (136) or by a modification of that technique (137,138). The modification involved solubilization of all protein samples with 100 μl of 0.15% sodium deoxycholate per ml of sample, and then precipitation of the protein with 6.5% TCA (final concentration). This technique will quantitatively precipitate protein down to 1 μg/ml and was effective in the measurement of small amounts of protein. The precipitated protein was resolubilized as described (137) and the absorbance at 750 nm compared to an appropriately constructed standard curve generated with bovine serum albumin (Sigma).
CHAPTER 3

HORMONAL REGULATION OF 1,25-DIHYDROXYVITAMIN D BIOSYNTHESIS

This chapter will relate new advances in the role and biosynthetic regulation of 1,25-(OH)_2D. The unique physiologic settings of growth and certain reproductive states are used as a format in which to study the interrelationships between 1,25-(OH)_2D, intestinal calcium and phosphate absorption, and certain hormones which have been documented to influence calcium metabolism. Since it is difficult to isolate the independent effect of single hormones within these systems, the results stress the complexity of events which accompany a study of the regulation of vitamin D metabolism. Therefore, the conclusions to these experiments must be tempered with the need for additional corroborative evidence. Nevertheless, the observations made in this chapter reveal certain noteworthy features: 1) a dynamic elevation in 1,25-(OH)_2D occurs in response to the elevated needs of calcium and phosphate during growth and reproduction, probably to mediate the enhanced uptake of these ions and 2) hormones such as growth hormone, prolactin, and estrogen contribute to the activation of enzymes which result in the increased biosynthesis of this sterol hormone. These data provide a framework for subsequent comprehensive studies of the hormonal events which surround vitamin D metabolism during growth, pregnancy, and lactation.
Results

Measurement of 1,25-Dihydroxyvitamin D₃

In 1974, Brumbaugh and Haussler (102,103) identified a cytoplasmic receptor for 1,25-(OH)₂D₃ in the intestinal mucosa of rachitic chicks. This high affinity receptor demonstrated a unique specificity for 1,25-(OH)₂D, and was subsequently used as the basis for a sensitive, competitive binding assay for circulating 1,25-(OH)₂D₃ (65,66,94). Since the receptor-hormone complex was observed to interact with intestinal chromatin, the isolation of bound sterol was achieved by trapping the ternary complex through filtration on glass fiber filters. However, purification of hormone was difficult and time consuming due to the extensive chromatographic procedures required to isolate 1,25-(OH)₂D₃ free of lipid contamination. These problems were partially relieved by Hughes et al. (35) who reported an improved 1,25-(OH)₂D assay in 1976 based upon a similar assay methodology, but more rapid hormone isolation procedures. Micro-chromatography was developed for the collective isolation of 1,25-(OH)₂D (D₂ and D₃) using standard Sephadex LH-20, micro-silicic acid, and micro-Celite columns (see Methods). These procedures were initially used for the isolation and quantitation of 1,25-(OH)₂D (both D₂ and D₃) in this dissertation, and are summarized in Figure 3-1 (chromatographic methodology) and Figure 3-2 (binding assay methodology). Depicted in Figure 3-3 are the results of a standard curve generated by these techniques which demonstrates a sensitivity of approximately 17 pg of hormone.
Figure 3-1. Chromatographic Methodology for the Purification of 1,25-(OH)₂D₃.

1,25-Dihydroxyvitamin D is purified from 10 ml of plasma as outlined in Methods. The hormone is subsequently assayed by radioreceptor assay in triplicate.
Figure 3-2. Radioreceptor Assay Methodology for the Measurement of 1,25-(OH)₂D₃.

The three phases of the radioligand receptor assay are illustrated.
Figure 3-3. Radioreceptor Competitive Binding Assay Standard Curve (6.7 Ci/mmol).

A sample of 1,25-(OH)$_2$[H]D$_3$ (175 pg; 6.7 Ci/mmol) was incubated with increasing amounts of non-radioactive 1,25-(OH)$_2$D$_3$ in 0.1 ml of reconstituted cytosol-chromatin. Bound tritiated compound (as determined by filtration) is plotted as a function of increasing amounts of non-radioactive 1,25-(OH)$_2$D$_3$ in the incubation mixture. These data represent a typical example of a standard curve for the quantitation of 1,25-(OH)$_2$D. Points represent the average ± SEM for a triplicate assay.
One of the primary disadvantages of the radioreceptor assay devised by Brumbaugh and Haussler (65,66) and Hughes et al. (35) was the use of cytosol-chromatin mixtures prepared fresh just prior to assay. This practice was not only time consuming with respect to the assay procedure, but demanded the constant availability of rachitic chicks from which the cytosol-chromatin was derived. Thus, an experiment was performed to test the possibility of using rapidly frozen cytosol-chromatin mixtures in the binding assay. The data in Figure 3-4 indicate that aliquots of this mixture can be frozen in liquid nitrogen, rapidly thawed, and then successfully utilized in the receptor-chromatin binding assay for 1,25-(OH)₂D. These observations led to low temperature (-80°) routine storage of extensive amounts of cytosol-chromatin mixtures, dramatically facilitating the measurement of 1,25-(OH)₂D₃.

With the advent of higher specific activity 25-(OH)₂[³H]D₃ (11.2 Ci/mmol, 110 Ci/mmol) which was converted to 1,25-(OH)₂[³H]D₃ (see Methods), the purification and assay of 1,25-(OH)₂D₃ was extensively modified. These changes involved the following:

1) Extraction of 2-5 ml plasma with acetone (see Methods).

2) Chromatographic isolation of 1,25-(OH)₂D₃ employing sequential micro-silicic acid, micro-Sephadex LH-20, and high pressure liquid chromatography.

3) Development of a more sensitive assay with a detection limit of 1 pg of competing unlabeled hormone.

Figure 3-5 demonstrates the chromatography and subsequent resolution of 25-(OH)D₃, 24,25-(OH)₂D₃, and 1,25-(OH)₂D₃ eluted with 65% chloroform in hexane (v/v) on a micro-Sephadex LH-20 column (0.8 x 8.0 cm, 2.3 g dry
Figure 3-4. Test of Frozen Cytosol-Chromatin in Radioreceptor Binding Assay.

Standard curves for the measurement of 1,25-(OH)₂D₃, as described in Figure 3-3, were generated using 0.1 ml of a freshly prepared cytosol-chromatin mixture (○—○), the same mixture after liquid nitrogen freezing and storage at -80° for 24 hours (□—□), and the same frozen mixture after storage at -80° for 2 weeks (△—△). Points represent the average of a triplicate assay.
Figure 3-5. Resolution of $25$-$\text{OH}[^{3}\text{H}]D_3$, $1,25$-$\text{(OH)}_2[^{3}\text{H}]D_3$, and $24,25$-$\text{(OH)}_2[^{3}\text{H}]D_3$ on Mini-Sephadex LH-20 Chromatography.

$25$-$\text{OH}[^{3}\text{H}]D_3$ (6.7 Ci/mmol, 2000 cpm), $1,25$-$\text{(OH)}_2[^{3}\text{H}]D_3$ (6.7 Ci/mmol, 3000 cpm), and $24,25$-$\text{(OH)}_2D_3$ (2000 IU) were applied to a column of Sephadex LH-20 (6 ml) equilibrated in 65% chloroform in hexane. The column was eluted with the same solvent and fractions (1 ml) were collected. One half the fraction (0.5 ml) was dried and counted for tritium (○—○) and the other half dried, resolubilized in ethanol, and analyzed via UV absorbance at 264 nm (□—□).
Sephadex LH-20). This experiment suggested that if a plasma sample (2-5 ml, extracted with acetone) was chromatographed on a micro-silicic acid column, satisfactory resolution of vitamin D metabolites on the smaller, more rapid, micro-Sephadex LH-20 could be achieved. Instead of Celite chromatography, the final step for the purification of the hormone was the use of high pressure liquid chromatography (HPLC) as described by Jones and DeLuca (122). Purified 1,25-(OH)$_2$D$_3$ from 2 ml of plasma is not detected by UV and therefore must be collected by harvesting the appropriate fractions determined by a calculation of its elution time with a radioactive standard. A typical UV profile is seen in Figure 3-6. The hormone is then quantitated by competitive binding assay (Figure 3-6). An example of the newly developed binding assay for 1,25-(OH)$_2$D$_3$ is seen in Figure 3-7. When cytosol-chromatin mixture (diluted with 7 volumes of STKM buffer) is incubated with increasing amounts of 1,25-(OH)$_2$[${}^3$H]D$_3$, 20 pg (6000 cpn) of hormone is sufficient to saturate the cytosolic receptor component in the presence of excess chromatin. Therefore, a typical standard curve can be generated using the receptor-chromatin combination, 20 pg of 1,25-(OH)$_2$[${}^3$H]D$_3$, and increasing concentrations of competing 1,25(OH)$_2$D$_3$ from 2-20 pg hormone. The total binding efficiency in this assay is about 25%, as determined by the recovery of 1,25-(OH)$_2$[${}^3$H]D$_3$ on filters in the absence of competing nonradioactive sterol. Thus 1,25-(OH)$_2$D is generally purified from 2 ml of plasma and then 3 separate aliquots assayed for 1,25-(OH)$_2$D (interassay variation is 10-15%). Serum or plasma 1,25-(OH)$_2$D concentration is described in pg/ml, ng/dl (pg/ml ÷10), or pmol/l (pg/ml x 2.4).
Figure 3-6. Purification of 1,25-(OH)₂D₃ on High Pressure Liquid Chromatography.

A 2 ml plasma sample, purified by silicic acid and mini-Sephadex LH-20 chromatography, was dried under a stream of nitrogen, resolubilized in 0.1 ml 15% isopropanol in hexane (v/v), and applied to a 25 cm x 4.6 mm Dupont Zorbax-Sil-850 column. The column was eluted with the same solvent at a flow rate of 1 ml/minute, and the 1,25-(OH)₂D emerged between 14-17 minutes from time of injection [as calibrated with 1,25-(OH)₂[³H]D₃ standard (O—O)]. The UV₂₅₄ profile was monitored (——) and two fractions during the elution of 1,25-(OH)₂D were measured by competitive binding assay for 1,25-(OH)₂D (vertical bars).
1,25-(OH)$_2$D$_3$ (20 pg, 110 Ci/mmol) was incubated with increasing amounts of non-radioactive 1,25-(OH)$_2$D$_3$ (0-20 pg) in 0.1 ml of cytosol-chromatin mixture (diluted from 6.7 Ci/mmol receptor assay 8-fold with STKM). Bound hormone was separated from free ligand and plotted as a function of added unlabeled 1,25-(OH)$_2$D$_3$. These data represent a typical standard curve for the quantification of 1,25-(OH)$_2$D from 2 ml of plasma or serum. Points represent the average ± SEM for a triplicate assay.
Circulating 1,25-Dihydroxyvitamin D\textsubscript{3} during Physiologic States of Calcium Stress

The physiologic states of growth and reproduction pose interesting challenges to the normal calcium and phosphorus regulating mechanisms in higher organisms. Indeed, the intestinal absorption of calcium is known to be highly elevated under each of these circumstances to meet the additional requirements of mineral. Since the bulk of current evidence supports the contention that 1,25-(OH)\textsubscript{2}D\textsubscript{3} is the hormone which mediates this action (review 23,24), the first experiments were designed to measure 1,25-(OH)\textsubscript{2}D\textsubscript{3} during these calcium stress situations.

**Growth.** Serum was collected from adult humans (> 18 yrs), rats (> 250 g), and chickens (egglaying hens) and from young humans (5-10 yrs), rats (< 100 g), and chicks (females, 8 weeks). The samples were purified by Sephadex LH-20, micro-silicic acid, and micro-Celite chromatography, and then assayed in triplicate for the 1,25-(OH)\textsubscript{2}D\textsubscript{3} metabolite. Table 3-1 summarizes the results of this experiment which suggests that when young animals and humans are experiencing growth, the 1,25-(OH)\textsubscript{2}D\textsubscript{3} hormone is elevated 2-3 fold.

**Pregnancy.** Serum was collected from control (5, mean age - 25 yrs; not on birth control pills) and pregnant, (5, mean age - 26 yrs; 38 weeks pregnant) human female volunteers. The samples were purified and the level of circulating 1,25-(OH)\textsubscript{2}D\textsubscript{3} determined. In addition, healthy non-pregnant and pregnant rats (20 days) were also sacrificed, serum collected and purified by micro-silicic acid, micro-LH-20, and HPLC, and the samples assayed for 1,25-(OH)\textsubscript{2}D\textsubscript{3}. The data (Table 3-2)
Table 3-1. Enhancement of 1,25-(OH)₂D during Growth

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of sets</th>
<th>Individuals per set</th>
<th>Serum 1α,25-(OH)₂D (pg/ml ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Hens</td>
<td>4</td>
<td>3</td>
<td>20 ± 8</td>
</tr>
<tr>
<td>Young Hens</td>
<td>4</td>
<td>6</td>
<td>42 ± 4*</td>
</tr>
<tr>
<td>Adult Rats</td>
<td>6</td>
<td>3</td>
<td>64 ± 12</td>
</tr>
<tr>
<td>Weanling Rats</td>
<td>4</td>
<td>7</td>
<td>173 ± 14*</td>
</tr>
<tr>
<td>Humans &gt;18 yrs.</td>
<td>58</td>
<td>1</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>Humans 5-10 yrs.</td>
<td>4</td>
<td>1</td>
<td>64 ± 9*</td>
</tr>
</tbody>
</table>

*Significantly different from control adults P < 0.05
Table 3-2. Enhancement of \(1,25-(OH)_2D\) during Pregnancy

<table>
<thead>
<tr>
<th>Group</th>
<th>Individuals</th>
<th>Serum (1,25-(OH)_2D) (pg/ml (+) S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Human Females</td>
<td>5</td>
<td>39 (+) 17</td>
</tr>
<tr>
<td>Pregnant (38 wk) Females</td>
<td>5</td>
<td>73 (+) 19*</td>
</tr>
<tr>
<td>Control Female Rats</td>
<td>7</td>
<td>100 (+) 7</td>
</tr>
<tr>
<td>Pregnant Rats (21 days)</td>
<td>7</td>
<td>179 (+) 9*</td>
</tr>
</tbody>
</table>

*Significantly different from appropriate controls \(P < 0.05\)
indicate that circulating 1,25-(OH)$_2$D is strikingly elevated during pregnancy, probably in response to the increased calcium and phosphate needs observed during this state.

**Lactation.** In rats, lactation is characterized by elevated intestinal calcium absorption and a lactation associated calcium drain (139). Thus, the dietary vitamin D$_3$ levels in lactating rats would appear to be extremely important for maintaining proper serum calcium and phosphorus levels. To test this assumption, we performed the following experiment: rats with or without litters of 10-6 day old pups (lactating) were randomly assigned to one of two dietary regimes. One group (controls and lactating rats) received a basal diet (see Methods) without the inclusion of vitamin D$_3$ (-D). The other group (controls and lactating rats) was fed the same diet, but supplemented with 5 IU vitamin D$_3$/g of diet (+D). Initially, and at weekly intervals during the experiment, non-fasted adults were bled from the tail or by heart puncture, and the adults and their litters weighed. If the size of the litter fell below seven, the mother and pups were excluded from the experiment. During day 9-13, food intake was measured and feces collected to determine daily intake and rate of net calcium absorption for each group. Finally, serum was analyzed for Ca, P, and 1,25-(OH)$_2$D$_3$.

The results are expressed in Table 3-3 and illustrate several important features of calcium metabolism during lactation. First, in non-lactating control rats, the presence (+D) or acute absence (-D) of vitamin D$_3$ has insignificant effects on 1,25-(OH)$_2$D (+D, 56 pg/ml; -D, 46 pg/ml) and no effects on calcium or phosphorus. Presumably, these results are due to the absence of a lactational drain on calcium,
Table 3-3. Serum Calcium Phosphate and \(\text{1,25-(OH)}_2\text{D}\) Levels in Lactating and Non-lactating Rats

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Diet group</th>
<th>7th day of lactation</th>
<th>20th day of lactation</th>
<th>1(\alpha,25-(OH)_2\text{D}_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ca (\text{mg dl}^{-1})</td>
<td>Ca (\text{mg dl}^{-1})</td>
<td>1(\alpha,25-(OH)_2\text{D}_3) (\text{ng dl}^{-1})</td>
</tr>
<tr>
<td>1</td>
<td>Lactating</td>
<td>-D</td>
<td>9.8 ± 0.2</td>
<td>7.8 ± 0.4*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+D</td>
<td>9.6 ± 0.3</td>
<td>9.4 ± 0.5</td>
</tr>
<tr>
<td>2</td>
<td>Lactating</td>
<td>-D</td>
<td>9.4 ± 0.1</td>
<td>7.6 ± 0.2*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+D</td>
<td>9.1 ± 0.1†</td>
<td>9.4 ± 0.2†</td>
</tr>
<tr>
<td></td>
<td>Non-lactating</td>
<td>+D</td>
<td>9.8 ± 0.1</td>
<td>10.1 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>Lactating</td>
<td>-D</td>
<td>8.8 ± 0.1†</td>
<td>6.9 ± 0.3*‡</td>
</tr>
<tr>
<td></td>
<td>Non-lactating</td>
<td>-D</td>
<td>10.3 ± 0.1</td>
<td>10.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+D</td>
<td>10.5 ± 0.1</td>
<td>10.2 ± 0.3</td>
</tr>
</tbody>
</table>

Each Ca and P value is the mean ± s.e. of 7-11 rats. Each 1\(\alpha,25-(OH)_2\text{D}_3\) value is the mean ± s.e. of three or four pools of blood, each from three rats (except for lactating -D rats in experiment 1, two pools from five rats); determinations were in triplicate.

*Different from initial (as paired differences) and from lactating +D where appropriate, P < 0.01.
†Different from non-lactating P < 0.01.
‡Different from non-lactating P < 0.001.
§Different from lactating -D, P < 0.025.
which in turn imposes only a minor stress on the metabolism of vitamin D3 during the short term duration of the experiment. However, if lactating rats fed +D diets are analyzed for Ca, P, and 1,25-(OH)2D, a moderate hypocalcemia, hyperphosphatemia, and a highly elevated (2–4-fold) 1,25-(OH)2D hormone level are observed. These effects are lactation associated, and are explained by the fact that loss of calcium to milk leads indirectly to an elevation in 1,25-(OH)2D. This elevation probably acts to increase the resorption of mineral from bone and/or stimulate the intestinal absorption of calcium and phosphorus. In fact, intestinal absorption of this ion is enhanced 4-fold in lactating rats over non-lactating controls (data not shown). In contrast, if lactating rats are placed on −D diets and then analyzed for serum minerals, a striking hypocalcemia and hypophosphatemia become evident. Significantly, 1,25-(OH)2D levels are also strongly depressed. These results indicate that the need for increased absorption of mineral is not being met during lactation, probably due to the lack of precursors for conversion to 1,25-(OH)2D, and the depression in absorption leads to a deficiency in circulating calcium and phosphorus. Thus, the control of serum minerals by 1,25-(OH)2D is strongly supported by these observations using the lactational state as a model for physiological calcium stress.

Egg laying. Serum was collected by wing puncture from normal (12 months old, non-laying) and laying (12 months old, egg in uterus) hens obtained from the University of Arizona Poultry Farm. 1,25-(OH)2D was purified and analyzed by radioreceptor assay. Both extreme hypercalcemia (20–30 mg%) and elevated intestinal calcium absorption are well
documented in laying hens (140) and were therefore assumed. The results (Figure 3-8) indicate that a dramatic elevation in 1,25-(OH)_2D occurs in hens during the active egglaying phenomenon.

Influence of Hormonal Injections on Circulating 1,25-Dihydroxyvitamin D and 25-Hydroxyvitamin D-1-hydroxylase

Normal factors which are known to regulate 1,25-(OH)_2D biosynthesis apparently are not entirely adequate to account for the striking elevations in 1,25-(OH)_2D seen in the previous investigations. Accordingly, several hormonal factors known to be involved in calcium metabolism were investigated for potential effects on the synthesis of 1,25-(OH)_2D. They include growth hormone, prolactin and placental lactogens, and sex hormones such as estrogen. The experiments were designed to evaluate the effects of these potential modulators on both renal vitamin D metabolizing enzymes and on the circulating level of 1,25-(OH)_2D. However, it must be remembered that the conclusion to these experiments can only be considered in light of the complex physiologic events which accompany a study involving intact animals.

Growth Hormone. Growth hormone has previously been demonstrated to exhibit effects on vitamin D metabolism (141). Further, the similarity of amino acid sequence between prolactin and growth hormone suggest that these hormones might have a similar action (142). To investigate the potential effects of growth hormone, rats were maintained on a basal diet containing 1% calcium and 0.9% phosphorus, and were supplemented with 2 IU vitamin D_3/g diet. After eight days of equilibration, half the rats were injected with 2-50 μg doses/day of human growth hormone (HGH) for 5 days. The remaining rats were injected with vehicle alone.
Figure 3-8. Effect of Egglaying on Circulating 1,25-(OH)$_2$D.

Serum from laying (10) and non-laying (12) hens was drawn via brachial vein, pooled (laying = 5 pools, non-laying = 4 pools), and assayed for 1,25-(OH)$_2$D. Values represent the average ± SEM. Laying hen levels are significantly different from non-laying levels, p <0.005.
The results are seen in Table 3-4, and seem to indicate that HGH has no effect on rats when administered as described. However, these observations, coupled with the results of an experiment to be considered later, reveal that in mammals, the action of growth hormone and prolactin are highly dependent upon the normal physiologic setting under which they exert their effects. Thus, the absence of an active growth phase in the present experiment probably prevented the potential effect of growth hormone on vitamin D metabolism.

**Prolactin.** Prolactin is known to be elevated during pregnancy, lactation and avian egglaying (143-145). Thus, to test prolactin, the hormone (20 μg, 50 μg, and/or 100 μg) was administered to chicks and rats as described in Methods. In the case of chicks, kidney homogenates and serum were assayed for hydroxylase activity and 1,25-(OH)₂D levels respectively; in rats, only 1,25-(OH)₂D was measured. The results in Figure 3-9 represent a comparison of kidney homogenate activity on vitamin D metabolism between control and prolactin treated (50 μg) chicks. The profiles are generated by Sephadex LH-20 resolution of metabolites produced in vitro by the action of renal enzymes on 25-(OH)[³H]D₃ production. Further, administration of prolactin to both rats and chicks does result in an elevation in circulating 1,25-(OH)₂D (Table 3-5). However, although chicks injected with 100 ug of prolactin (2-50 μg doses/day) respond with a doubling of the sterol hormone, rats only demonstrate a 50% increase. Collectively, these observations suggest that administration of prolactin can influence the metabolism of vitamin D in vivo. Since hormones such as prolactin exhibit contrasting effects in birds versus mammals, it is possible that the minor
Table 3-4. Effects of Growth Hormone Administration in Rats on 1,25-(OH)₂D₃

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Rats</th>
<th>1,25-(OH)₂D₃ (pg/ml ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>2</td>
<td>122 ± 4</td>
</tr>
<tr>
<td>50 µg Growth Hormone x 2/day</td>
<td>4</td>
<td>124 ± 7</td>
</tr>
</tbody>
</table>

No significant difference between groups, P <0.1.
Figure 3-9. Effect of Prolactin on Chick Kidney Enzyme Activity.

Sephadex LH-20 profiles of kidney enzyme activity generated after one hour from control (A) and prolactin treated (50 µg) (B) 15-day old chicks. Kidney enzyme assays were performed as described in Methods.
Table 3-5. Circulating 1,25-(OH)₂D in Prolactin Treated Chicks and Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Sets</th>
<th>No. of Animals Per Set</th>
<th>Plasma 1α,25-(OH)₂D (pg/ml ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, Chicks</td>
<td>4</td>
<td>10</td>
<td>159 ± 11   (^a)</td>
</tr>
<tr>
<td>20 μg Prolactin/day, Chicks</td>
<td>4</td>
<td>10</td>
<td>148 ± 13  ()</td>
</tr>
<tr>
<td>100 μg Prolactin/day, Chicks</td>
<td>4</td>
<td>10</td>
<td>286 ± 45  (^b)</td>
</tr>
<tr>
<td>Control, Rats</td>
<td>2</td>
<td>1</td>
<td>122 ± 4   (^c)</td>
</tr>
<tr>
<td>100 μg Prolactin/day, Rats</td>
<td>4</td>
<td>1</td>
<td>176 ± 22  (^d)</td>
</tr>
</tbody>
</table>

\(^a\)Significantly different from \(^b\), \(P < 0.005.\)
\(^b\)Significantly different from \(^a\), \(P < 0.005.\)
\(^c\)Significantly different from \(^b\), \(P < 0.05.\)
effects of prolactin on 1,25-(OH)\textsubscript{2}D synthesis in rats as compared to chicks can be attributed to the physiologic setting under which these experiments were carried out. Nevertheless, the hormone does appear to modulate, either directly or in conjunction with other hormones, the elevation in 1,25-(OH)\textsubscript{2}D.

**Steroids.** An abundance of evidence suggests that the metabolism of vitamin D is enhanced during situations typified by high circulating levels of estrogen, especially mammalian pregnancy and avian egglaying. As a result, experiments were designed to investigate the effects of estrogen, testosterone, and/or progesterone administration on vitamin D\textsubscript{3} metabolism in chicks. Although previous investigators have documented an effect of estrogen on 25-hydroxyvitamin D\textsubscript{3}-1-hydroxylase activity (89,146-148), these observations were not correlated with an enhancement of circulating 1,25-(OH)\textsubscript{2}D. Table 3-6 summarizes the vitamin D metabolizing effects of a single 5 mg injection of specific steroid in 18 day old chicks fed a rachitogenic diet supplemented orally with 40 IU of vitamin D\textsubscript{3} (see Methods). Diethylstilbestrol was used as a synthetic estrogen since it has been shown to produce effects equivalent to that seen with estradiol-17β. The results suggest that only DES, or DES in combination with testosterone or progesterone, is capable of stimulating the 1-OHase enzyme, although all steroids appear to depress 24-OHase activity. Further, DES, or DES and progesterone, stimulate hypercalcemia which is a characteristic effect of estrogen in birds (140). However, neither the presence of testosterone, testosterone and estrogen, testosterone and progesterone, nor progesterone demonstrate any such hypercalcemic effect. A dose response curve for renal vitamin
<table>
<thead>
<tr>
<th>Group</th>
<th>Renal Enzyme</th>
<th>Plasma Ca</th>
<th>Plasma P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24-OHase</td>
<td>l-OHase</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>99 ± 15</td>
<td>23 ± 6</td>
<td>10.9 ± 0.6</td>
</tr>
<tr>
<td>Testosterone</td>
<td>62 ± 14</td>
<td>59 ± 15</td>
<td>10.5 ± 0.5</td>
</tr>
<tr>
<td>Progesterone</td>
<td>23 ± 11</td>
<td>26 ± 9</td>
<td>9.5 ± 0.7</td>
</tr>
<tr>
<td>DES</td>
<td>13 ± 8</td>
<td>634 ± 78</td>
<td>14.3 ± 2.3</td>
</tr>
<tr>
<td>Testosterone + Progesterone</td>
<td>32 ± 12</td>
<td>94 ± 27</td>
<td>9.3 ± 1.7</td>
</tr>
<tr>
<td>DES + Testosterone</td>
<td>4 ± 3</td>
<td>555 ± 88</td>
<td>9.1 ± 0.3</td>
</tr>
<tr>
<td>DES + Progesterone</td>
<td>0</td>
<td>503 ± 117</td>
<td>12.0 ± 1.3</td>
</tr>
</tbody>
</table>

Values are means ± SD. Chicks were kept 18 days on D-deficient diet (see Methods) supplemented with 40 IU vitamin D 3 times/week. Chicks were then injected with 5 mg of hormone or vehicle and, after 24 h, bled by cardiac puncture, and decapitated; kidneys were excised for renal hydroxylase assays, and serum was collected for Ca and P measurement. *Three chicks per group, duplicate estimation.
D metabolizing enzymes is also seen (Figure 3-10), suggesting that 500 μg of DES are required to stimulate the renal synthesis of $1,25-(\text{OH})_2\text{D}_3$.

The effects of these hormones on vitamin D metabolism was also corroborated by measuring the circulating concentration of $1,25-(\text{OH})_2\text{D}$, after administration of these hormones in vivo. This was performed because the potential modulation of an endocrine reaction must reflect a change in the circulating level of that hormone for it to be physiologically relevant. Five mg/day of DES, testosterone, or DES and testosterone were administered for five days by subcutaneous injection into four week old male chicks fed a rachitogenic diet supplemented with vitamin D$_3$ (2 IU/g diet). The results (Table 3-7) suggest that administration of an estrogen-like compound can dramatically elevate circulating $1,25-(\text{OH})_2\text{D}$ either alone, or in combination with testosterone. Importantly, testosterone alone has no effect, suggesting a specificity to this stimulation as shown previously (Table 3-6). Furthermore, the time course of enhancement of $1,25-(\text{OH})_2\text{D}$ in the circulation is rapid (Figure 3-11), in accordance with the stimulation of this metabolite by other known modulators such as PTH and phosphate (149). $1,25-(\text{OH})_2\text{D}$ is increased 2-fold, from 68 to 121 pg/ml, within 12 hours after a single injection of 5 mg DES, and then continues to rise and plateau between 48-72 hours. Finally, Figure 3-12 summarizes the effect of increasing doses of DES on vitamin D metabolizing enzymes, serum Ca, and serum $1,25-(\text{OH})_2\text{D}$. Although the stimulation of the 1-OHase requires a single 500 μg injection of DES (see also Figure 3-10), it requires at least 1 mg of DES to influence the circulating level of $1,25-(\text{OH})_2\text{D}$. These observations, taken cummulativey, imply an action by estrogen on
Figure 3-10. Effect of Increasing Dose of Diethylstilbestrol on Chick Kidney Enzyme Activity.

Male chicks (6 per point) were injected with the indicated amount of DES (see Methods). After 24 hours, renal enzyme activity was measured and expressed as fmoles of either 1,25-(OH)_{2}D_{3} or 24,25-(OH)_{2}D_{3} formed per minute per mg protein. Data represent the average ± SD.
Table 3-7. Enhancement of Circulating 1,25-(OH)2D by Sex Hormone Administration*  

<table>
<thead>
<tr>
<th>Group</th>
<th>Sets</th>
<th>Birds per Set</th>
<th>1α,25-(OH)2D3, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>3</td>
<td>98 ± 24</td>
</tr>
<tr>
<td>Testosterone</td>
<td>4</td>
<td>3</td>
<td>96 ± 17</td>
</tr>
<tr>
<td>DES</td>
<td>5</td>
<td>3</td>
<td>254 ± 26†</td>
</tr>
<tr>
<td>Testosterone + DES</td>
<td>4</td>
<td>3</td>
<td>298 ± 19‡</td>
</tr>
</tbody>
</table>

Values are means ± SD. *Hormone(s) or vehicle injection 5 mg/day in 0.1 ml ethanol for 5 days (see Methods). Animals were killed 1 h after final injection and bled; serum was prepared and frozen at -20°C until used. †Significantly different from control; P < 0.01. ‡Significantly different from controls and DES treatment; P < 0.05.
Figure 3-11. Time Course of Enhancement of 1,25-(OH)_2D by DES in Chicks.

Injections of 5 mg of DES were given at 0 hours, and chicks were killed and serum was collected for hormone assay 12 or 24 hours later. Animals killed after 48 and 72 hours received injections at 0, 24, and 0, 24, and 48 hours respectively. Controls received vehicle at 0 hours and were killed after 12 hours. Each point represents 2-4 samples, 2 chicks per sample ± SD.
Figure 3-12. Effect of Increasing Dose of DES on Chick Kidney Enzyme Activity, Serum Calcium, and 1,25-(OH)$_2$D.

Dose response of kidney vitamin D hydroxylase, serum 1,25-(OH)$_2$D, and calcium to DES. For hydroxylase assay, chicks were maintained 18 days prior to a single injection of hormone. Chicks were killed 26 hours after injection and kidneys prepared and assayed in duplicate (6 chicks per point). For 1,25-(OH)$_2$D, 4-6 week old chicks were dosed at appropriate levels of DES in 0.1 ml ethanol for 5 days and killed 1 hour after 5th injection. Dose levels are plotted on log scale. Each point is average of 3-4 chicks.
vitamin D metabolism in the chick. However, they must be regarded as a specific observation peculiar to birds until results of this nature are also observed in mammals. Nevertheless, they strongly support the current concept that estrogen, along with growth hormone and prolactin, are potential physiologic modulators of $1,25-(\text{OH})_2\text{D}$ biosynthesis.

Influence of Tissue Ablation and Hormone Inhibitors on Serum Calcium, Phosphorus, and $1,25$-Dihydroxyvitamin D

To further substantiate the roles of growth hormone and prolactin as modulators of $1,25-(\text{OH})_2\text{D}$ biosynthesis, several distinct experiments were undertaken. In view of the potentially selective physiologic settings under which these polypeptide hormones influence vitamin D metabolism, the state of growth was used as a model in which to study growth hormone, and lactation was employed to investigate prolactin. Further, the interrelationship between PTH and these two hormones was also considered since the influence of PTH on vitamin D metabolism is a well investigated phenomenon. The primary approach to these experiments was to investigate the level of $1,25-(\text{OH})_2\text{D}$ in response to the selective removal of these polypeptide hormones during the appropriate state of either growth (GH) or lactation (PRL and PTH).

**Hypophysectomy in Growing Rats.** Growing male Wistar rats (mean weight 212 g before surgery) were maintained on a basal rachitogenic diet with 1% calcium, 0.9% phosphorus and supplemented with 2 IU/g diet of vitamin D$_3$. The rats were randomly assigned to five groups as depicted in Figure 3-13: four groups were hypophysectomized (HPX) by the transsphenoidal route and one group was sham-operated. After eight days of equilibration, during which time all HPX rats were allowed drinking
Figure 3-13. Design of Experiment Involving Hypophysectomy in Rats.
water with 5% glucose included, the groups were treated for seven days in the following manner. The Sham-Operated-Group One received vehicle alone, the HPX-Group Two received vehicle alone, the HPX-Group Three received 50 μg of human growth hormone (HGH) twice daily, the HPX-Group Four received 250 μg of HGH twice daily, and the HPX-Group Five received 50 μg of ovine prolactin (oPRL) twice daily. At the termination of treatment, plasma was collected and calcium, phosphorus, $1,25-(\text{OH})_2\text{D}$, and PTH were measured.

The results of this experiment are seen in Figure 3-14. Interestingly, hypophysectomy in growing rats causes a dramatic fall in both plasma $1,25-(\text{OH})_2\text{D}$ and PTH. Significantly, the administration of HGH (2-50 μg doses/day) serves to restore the level of circulating $1,25-(\text{OH})_2\text{D}$ to normal, without an accompanying increase in PTH. These observations suggest that indeed growth hormone is capable of influencing the biosynthesis of $1,25-(\text{OH})_2\text{D}$, and it appears to do so independently of PTH. The higher dose of HGH also restores $1,25-(\text{OH})_2\text{D}$ to normal levels, but concomitantly elevates PTH to levels equivalent to the sham-operated group. Finally, oPRL has no effect under the physiologic setting of active growth. The results of this experiment on other parameters are summarized in Table 3-8. Important observations here are that indeed HPX results in a decrease in growth, and that both dosage levels of HGH serve to return the rate of growth to normal. Ovine PRL, however, cannot substitute. Thus, these data suggest that during growth, the elimination of growth hormone by HPX adversely affects both the production of $1,25-(\text{OH})_2\text{D}$ and the generalized affects of growth previously recorded (150).
Male Wistar rats (mean weight 212 g before hypophysectomy) were used and fed a diet containing 1% calcium, 0.9% phosphorus and 2 IU vitamin D₃ per g for 4 weeks after weaning. After hypophysectomy, each group was allowed to equilibrate for 8 d with 5% glucose as drinking water for the hypophysectomised groups. The sham-operated group(s) received solvent, but for a further 7 d the other (hypophysectomised) groups received solvent alone (CHPX), human growth hormone 50 µg twice daily (HGH1), human growth hormone 250 µg twice daily (HGH2), or 50 µg twice daily of ovine prolactin. Values are mean ± s.e.m.; the number of samples in each group is shown above the columns. To provide adequate volumes for replicate analyses of 1,25-(OH)₂D₃, plasma was pooled from subgroups of animals to provide four to six samples in each group; triplicate assays for 1,25-(OH)₂D₃ were made by the radio-receptor technique. The effects of hypophysectomy and of both doses of growth hormone are highly significant (P <0.01). PTH was measured by radioimmunossay on plasma samples from individual rats except where insufficient plasma was available. Extracts of rat parathyroid tissue produced displacement curves indistinguishable from those due to human PTH. The results are expressed in terms of MRC human parathyroid hormone standard (reference 75/549). The changes in PTH due to hypophysectomy (CHPX) and to 250 µg HGH twice daily are highly significant (P <0.01). 50 µg HGH twice daily produced marked elevation in 1,25-(OH)₂D₃ without any change in PTH levels.
Figure 3-14. Effect of Hypophysectomy on PTH and 1,25-(OH)₂D.
Table 3-8. Effect of Growth Hormone in Hypophysectomized Rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma calcium (mmol 1⁻¹)</th>
<th>Plasma phosphate (mmol 1⁻¹)</th>
<th>Mean weight gain (g)</th>
<th>Plasma 1,25-(OH)₂D₃ (pmol 1⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>2.91 ± 0.03 (6)</td>
<td>2.80 ± 0.17 (6)</td>
<td>27.8 ± 1.6 (20)</td>
<td>160 ± 31 (6)</td>
</tr>
<tr>
<td>Control hypophysectomised</td>
<td>2.73 ± 0.05 (4)</td>
<td>2.03 ± 0.06 (4)</td>
<td>-2.0 ± 0.9 (21)</td>
<td>72 ± 12 (4)</td>
</tr>
<tr>
<td>Human growth hormone 50 μgx2 per d</td>
<td>2.83 ± 0.09 (5)</td>
<td>2.34 ± 0.13 (5)</td>
<td>30.0 ± 1.0 (21)</td>
<td>148 ± 17 (5)</td>
</tr>
<tr>
<td>Human growth hormone 250 μgx2 per d</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovine prolactin 50 μgx2 per d</td>
<td>2.71 ± 0.06 (4)</td>
<td>1.89 ± 0.17 (4)</td>
<td>-0.3 ± 1.1 (20)</td>
<td>81 ± 21 (4)</td>
</tr>
</tbody>
</table>

Plasma calcium and phosphate values compared with weight gains and 1α,25-(OH)₂D₃ levels. Plasma samples were from pooled subgroups of each experimental group. All values are mean ± s.e.m. (number of samples). The changes in plasma 1α,25-(OH)₂D₃ levels produced by hypophysectomy and by administration of growth hormone are both highly significant (P < 0.01).

*Calcium and phosphate were not measured in this group to conserve plasma.
Parathyroidectomy in Lactating Rats. Overwhelming evidence supports the contention that PTH is a primary positive modulator of 1,25-(OH)\textsubscript{2}D biosynthesis, with the parathyroid glands acting as an intermediary to sense low serum calcium and elaborate PTH (151). This hormone stimulates the 1-OHase enzyme to increase the hydroxylation of 25-OHD to the active metabolite 1,25-(OH)\textsubscript{2}D (152). Since in rats, PTH is elevated along with prolactin during lactation, it may play a significant role in the regulation of 1,25-(OH)\textsubscript{2}D biosynthesis, either independently or synergistically with prolactin. In an attempt to dissect the specific effects of PTH and prolactin on circulating 1,25-(OH)\textsubscript{2}D during lactation, an experiment involving parathyroidectomy during this state was performed. Parathyroidectomy (PTX) was performed by excision on 12 rats suckling ten 12-or-13 day old pups—sham operations were performed on a similar group of lactating and non-lactating rats. Completeness of PTX was assumed when a post-operative (48 hour) decrease in serum calcium of at least 3 mg/100 ml plasma was observed. At this time, serum was collected and analyzed, by appropriate pooling, for calcium, phosphorus, and 1,25-(OH)\textsubscript{2}D. The results are expressed in Table 3-9 and illustrate a dramatic effect on mineral and vitamin D metabolism. Typical hypocalcemia and hyperphosphatemia is observed during lactation, an effect seen previously (Table 3-3) and strongly exaggerated by PTX. Lactation also causes a striking 5-fold increase in 1,25-(OH)\textsubscript{2}D (see also Table 3-3), supporting the important role of this hormone in mineral provision as already discussed. Interestingly, PTX serves to markedly inhibit the lactation effect on 1,25-(OH)\textsubscript{2}D by reducing the hormone levels to only 2-fold above the control non-lactating rats. These
Table 3-9. Effect of Parathyroidectomy on Serum $1,25-(OH)_2D$

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Rats</th>
<th>Serum calcium (mg/dl)</th>
<th>Serum phosphorus (mg/dl)</th>
<th>Body weights (g)</th>
<th>Serum $1,25-(OH)_2D^*$ (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before Surgery</td>
<td>48 h Later</td>
<td>Before Surgery</td>
<td>48 h Later</td>
</tr>
<tr>
<td>Nonlactating</td>
<td>10</td>
<td>10.2 ± 0.1</td>
<td>9.9 ± 0.2</td>
<td>5.6 ± 0.4</td>
<td>4.4 ± 0.3</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>12</td>
<td>9.6 ± 0.1†</td>
<td>9.4 ± 0.2†</td>
<td>6.4 ± 0.3†</td>
<td>5.8 ± 0.3</td>
</tr>
<tr>
<td>Lactating</td>
<td>8</td>
<td>9.8 ± 0.1</td>
<td>4.5 ± 0.3</td>
<td>6.7 ± 0.4</td>
<td>8.4 ± 0.3</td>
</tr>
<tr>
<td>Parathyroid-de-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Average value from triplicate assays of 4-6 pools of serum from 2-3 rats each.
†Significantly different (P <0.01) from value for non-lactating sham-operated group.
(Values before and after surgery were combined).
‡Significantly different (P <0.01) from value for non-lactating sham-operated group.
§Significantly different (P <0.01) from value for lactating sham-operated group.
results suggest that, at least at 12-13 days of lactation, PTH may indeed strongly influence the circulating titers of 1,25-(OH)$_2$D hormone. They do not, however, rule out an influence of prolactin during lactation, since the PTX lactating rats continue to maintain a 1,25-(OH)$_2$D level twice that of normal. Cumulatively, these data suggest that PTH, and most likely prolactin, are important in maintaining the characteristic increase in 1,25-(OH)$_2$D routinely observed in lactation. They may act independently or synergistically, although the mechanism of action for both these hormones on 1,25-(OH)$_2$D elevation remains to be defined.

Inhibition of Prolactin Secretion in Lactating Rats. The results of the last experiment suggested that prolactin might play a role in the maintenance of high circulating 1,25-(OH)$_2$D during lactation. In order to evaluate this contribution, a study was initiated in which circulating prolactin titers were abolished in rats with bromocriptine (CB-154, prolactin secretion inhibitor) (152). The design of the experiment is depicted in Figure 3-15. Age-matched pregnant and non-pregnant female Wistar rats were fed a general diet containing 1.1% calcium and 0.9% phosphorus supplemented with 2 IU/g diet vitamin D$_3$. Upon parturition in the pregnant group, the rats were divided into five groups. After four days they were treated as follows for 1.5 days: NL-Group One, vehicle; NL-Group Two, 500 μg CB-154 twice daily, L-Group Three, vehicle; L-Group Four, 500 μg CB-154 twice daily; L-Group Five, 500 μg CB-154 and 500 μg PRL twice daily. Treatment was administered via subcutaneous injection. After administration, plasma was taken for the measurement of 1,25-(OH)$_2$D$_3$ and PTH, and the results are summarized in
Figure 3-15. Design of Experiment Involving Inhibition of Prolactin Secretion in Lactating Rats.
Table 3-10. The previously described effect of lactation of $1,25-(OH)_2D$ levels (see above) is demonstrated in this experiment (26 pg/ml to 40 pg/ml) although the results are not as striking. This is possibly due to the higher percentage of calcium in the diet during this experiment. Interestingly, bromocriptine has little or no effect on $1,25-(OH)_2D$ concentrations in non-lactating rats, but demonstrates a significant depressing effect on the hormone when administered to lactating animals. Although prolactin therapy in lactating rats injected with bromocriptine does not result in a repair of the observed reduction in $1,25-(OH)_2D$, this phenomenon may require longer term administration of prolactin. Interestingly, an increase in PTH is also observed in lactation, as was alluded to in the previous section. However, inasmuch as CB-154 administration evidences no effect on PTH levels (in either lactators or non-lactators), but blocks prolactin secretion, the reduction in circulating sterol hormone in lactation could be attributed to the absence of a prolactin influence, although alternative explanations are possible. Thus, these data support an action by prolactin on $1,25-(OH)_2D$ biosynthesis, although it is clear that the identification of specific effects on vitamin D metabolism are not entirely possible with this type of an experiment. Further, the results stress the complexity of interactions which occur in $1,25-(OH)_2D$ synthesis during lactation.

The Dynamic Nature of $1,25$-Dihydroxyvitamin D

The concentrations of all hormones, whether polypeptide or steroid, are capable of being rapidly and dynamically modulated in response to sudden changes in the specific needs of an organism. Further,
Table 3-10. Effects of Inhibition of Prolactin Secretion on Lactating and Non-lactating Rats

<table>
<thead>
<tr>
<th></th>
<th>Non-lactating</th>
<th></th>
<th></th>
<th>Lactating</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CB154</td>
<td>Control</td>
<td>CB154</td>
<td>CB154 + PRL</td>
<td></td>
</tr>
<tr>
<td>Average Weight</td>
<td>218 ± 4</td>
<td>216 ± 11</td>
<td>234 ± 6</td>
<td>228 ± 7</td>
<td>231 ± 5</td>
<td></td>
</tr>
<tr>
<td>(g ± S.E.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,25-(OH)2D</td>
<td>26 ± 2</td>
<td>22 ± 3</td>
<td>40 ± 3a</td>
<td>25 ± 6b</td>
<td>29 ± 6c</td>
<td></td>
</tr>
<tr>
<td>(pg/ml ± S.E.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH</td>
<td>0.21 ± 0.03</td>
<td>0.19 ± 0.07</td>
<td>0.38 ± 0.05a</td>
<td>0.37 ± 0.06a</td>
<td>0.48 ± 0.07b</td>
<td></td>
</tr>
<tr>
<td>(μg Eq./l)</td>
<td>(10)</td>
<td>(12)</td>
<td>(9)</td>
<td>(9)</td>
<td>(10)</td>
<td></td>
</tr>
</tbody>
</table>

*a Different from non-lactating control, P <0.01.
*b Different from lactating control P <0.05.
*c Not significantly different from lactating control (P <0.1)
    or from non-lactating control or from lactating +CB154.
these rapid change in hormone levels are generally ascribed to altered rates of synthesis and not degradation. With respect to vitamin D, the bulk of evidence suggests that 1,25-(OH)\(_2\)D is an active novel sterol hormone whose level is tightly regulated by both the homeostatic and physiological demands of the individual. Certainly the data presented so far in this dissertation support this concept. However, in order to investigate 1,25-(OH)\(_2\)D in a more dynamic sense, two experiments were designed to measure this hormone sequentially during pregnancy in humans and during reproduction in rats. The results dramatically support the dynamic nature of 1,25-(OH)\(_2\)D in response to the organism's need for calcium.

**Human Pregnancy.** An investigation of potential hormonal effects in pregnancy on vitamin D metabolism was not performed. Nevertheless, 1,25-(OH)\(_2\)D was measured in two age-matched human females, one pregnant, and the other normal and non-pregnant. Serum was drawn from each subject at one month intervals from the fifth to the eighth month, and analyzed for 1,25-(OH)\(_2\)D. Since the ninth month (38 weeks) had been previously measured in several subjects (Table 3-2), this time period was not sampled in the current study. The results are illustrated in Figure 3-16, and demonstrate that although 1,25-(OH)\(_2\)D remains relatively unchanged during the test period in the control subject, the hormone was elevated over 2-fold during the course of pregnancy.

**Reproduction in Rats.** 1,25-Dihydroxyvitamin D, calcium, and phosphorus were determined in the serum of rats at different reproductive stages (6-8 animal per group). These stages included: non-lactating controls (lactation was terminated for at least two weeks),
Figure 3-16. Time Course of the Elevation of 1,25-(OH)₂D during Pregnancy.

Serum was collected from subjects (control and pregnant) during the 5-8th month of pregnancy, and then assayed for 1,25-(OH)₂D. Circulating 1,25-(OH)₂D is plotted against time (months). Each point represents the triplicate estimation of a single serum sample.
pregnancy (21 days), shortly after parturition (1-2 days), lactation (7-8 days), lactation (13-16 days), and post-lactation (2 days post-lactation, 7 days post-weaning). The individual groups of rats were maintained on a basal diet (see Methods) containing 0.37% calcium, 0.32% phosphorus, and 5 IU/g diet of vitamin D₃ for at least six days prior to the experiment. At the appropriate times, the animals were sacrificed, and serum collected. Control animals were killed at times ranging from 6-20 days after receipt in the laboratory, corresponding to the times the pregnant and lactating rats were sacrificed. The results of this experiment are depicted in Figure 3-17. Pregnancy (21 days) in rats, as in humans, leads to an appropriate 2-fold increase in 1,25-(OH)₂D most likely in response to the increased mineral demands of the fetus. Parturition, which temporarily relieves the calcium drain imposed by the fetus, leads to a decrease in the hormone to control levels. However, by 7-8 days of lactation, 1,25-(OH)₂D has increased again to a level equivalent to that seen in pregnancy. The hormone then remains high throughout lactation only to fall again precipitously 1-2 days after weaning. A small but significant reduction in serum calcium is observed during later stages of lactation (described earlier) but significant hypercalcemia and hyperphosphatemia occurred after weaning, apparently in response to the sudden absence of lactation-associated calcium drain into milk. Finally, by seven days, 1,25-(OH)₂D and calcium have returned to normal. Although these data do not reveal the modulating influences which control 1,25-(OH)₂D they do illustrate the fluctuation in the sterol hormone which occur during the
Figure 3-17. The Dynamics of Circulating 1,25-(OH)₂D During Reproduction in Rats.

Control rats were non-lactating, non-pregnant, age-matched female animals, 3 of which were bled on the same day as the 7-8 day lactating group while the remainder (5 rats) were bled on the same day as the 13-16 day lactating group. All numbers were the average of triplicate determinations on each of 7-8 individual rat serum samples ± SEM. Values designated with an asterisk are significantly different from the control, p < 0.01.
physiological changes of reproduction. The results emphasize the tight control exerted over this hormone during states of enhanced mineral need.

Discussion

The experiments reported in this chapter were designed to examine several of the events which surround the mineral regulating hormone 1,25-(OH)₂D, during phases of growth and reproduction. Certainly, the development and utilization of a sensitive, radioreceptor assay for 1,25-(OH)₂D (65,35) has been instrumental in the elucidation of a number of new aspects of the ionic and hormonal regulation of 1,25-(OH)₂D biosynthesis. This technique and the recent improvements incorporated into both the purification (Figures 3-5,3-6) and assay (Figures 3-4, 3-7) methodologies, have been used in the studies reported here to implicate 1,25-(OH)₂D as the primary effector of intestinal calcium and phosphate transport during growth and reproduction. In addition, this assay was also utilized as the primary tool in an investigation of the effects and properties of new hormonal regulators of vitamin D metabolism, purported to be influential during the above physiological states. It should be remembered, however, that the conclusion to these studies must be qualified by the realization that the various hormonal and metabolic networks which surround growth and reproduction are so extensive, that a consideration of individual events is extremely difficult. Further, cross-sectional studies of induced endocrine events are often misleading, since observations before and after sampling are unknown. Nevertheless, an attempt is made here, in conjunction with previous
observations, to develop a general outline for the role and biosynthetic regulation of $1,25-(\text{OH})_2D$.

Circulating $1,25$-Dihydroxyvitamin D During Growth and Reproduction

It is now known that vitamin D, through the agency of its active metabolite $1,25-(\text{OH})_2D$, is the component which directs the intestinal absorption of calcium and phosphorus, contributes to the regulation of serum ion levels through bone mineral resorption, and participates in the regulation of tubular reabsorption of ions (see review 23,24). Interestingly, the level of $1,25-(\text{OH})_2D$ has been monitored in a number of pathological situations characterized by abnormal mineral uptake (67,68), and the results have generally supported a positive correlation between $1,25-(\text{OH})_2D$ levels and the extent of intestinal calcium and phosphorus absorption. The data reported in this chapter represent the first measurements of $1,25-(\text{OH})_2D$ during physiologic states of health characterized by increased absorption of calcium and phosphorus (139,140,153-159). Thus, in positive fashion, $1,25-(\text{OH})_2D$ is elevated in growth (Table 301), pregnancy (Table 3-2; Figures 3-6,3-7), lactation (Tables 3-3, 3-9; Figure 3-7), and egglaying (Figure 3-8). The increased utilization of mineral in these situations is primarily due to the additional requirements imposed by skeletal and soft tissue development during growth (150), fetal development during pregnancy (91), milk secretion during lactation (139), and the formation of egg content and shell formation in laying avian species (140).
Hormonal Regulation

The modulation of $1,25-(OH)_2D$ biosynthesis during periods of normal calcium and phosphate balance is accomplished by serum calcium, phosphorus, and parathyroid hormone. However, as important as these factors are, they do not appear to entirely account for the changes in mineral absorption observed in situations such as growth and reproduction. In fact, several other hormones have been implicated as playing a role in the metabolism of calcium and phosphorus. They include growth hormone, prolactin (placental lactogen), and estrogens, each elevated during the appropriate physiological state(s). By necessity, this discussion is organized around the actions of the individual hormone, although an attempt is made to use the appropriate physiologic situation as a setting in which to discuss the influence of these potential regulators. In fact, evidence considered below suggests that the action of these hormones on vitamin D metabolism may be situation dependent, i.e., they influence the biosynthesis of $1,25-(OH)_2D$ only during specific hormonal and physiologic settings.

Growth Hormone. The general effects of growth hormone (GH) on the development of an organism are extensive (150). However, in contrast to hormones such as prolactin, placental lactogens, and estrogen (to be discussed), the absolute level of GH in growing animals can not be directly correlated with its observed effects. GH levels fluctuate widely over short periods of time in response to an extreme variety of stimuli (150), and may range from undetectable in resting states to highly elevated bursts during the onset of sleeping. The most likely reason for the lack of correlation between GH and growth activity
is that this hormone stimulates the release of somatomedins (SM) (160). Thus, these factors most likely influence growth. Indeed, in a wide variety of growth disorders, growth rates of humans correlate much better with SM concentration, suggesting that potential relationships between GH and growth activity should be viewed through the circulating level of SM.

The influence of GH on calcium metabolism was first observed by Beck et al. (141) who demonstrated that GH stimulated the intestinal absorption of calcium in a GH deficient dwarf. Subsequently, additional similarities of action between vitamin D and growth hormone were documented (161). Recently, Spencer and Tobiassen (162) demonstrated that hypophysectomy in rats increase the normal ratio of 25-OHD$_3$ to 1,25-(OH)$_2$D$_3$ as measured by the extraction of metabolites from both serum and intestinal tissue. These observations were followed by evidence which suggested that an elevation in the degradation rate of 1,25-(OH)$_2$D was not the cause of these effects (163). Cumulatively, these data inferred an action by GH on intestinal calcium absorption via the stimulation of 1,25-(OH)$_2$D biosynthesis.

The results described here extend the observations made by Spencer and Tobiassen (162) and Spencer and coworkers (163). First of all, 1,25-(OH)$_2$D is elevated during growth (Table 3-1) and most likely mediates the increased absorption of mineral seen in this state. This elevation in 1,25-(OH)$_2$D can be dramatically reduced by hypophysectomy in growing rats (Figure 3-14, Table 3-8), an action which can be reversed by the administration of GH (100 µg/day). Further, the specificity of these events is illustrated by the fact that prolactin
administration to HPX rats is incapable of restoring $1,25-(OH)_2D$ levels to normal (Figure 3-14) and GH administration to adult (non-growing) rats also has no effect on circulating $1,25-(OH)_2D$ (Table 3-4). A direct effect of growth hormone on vitamin D metabolism, nevertheless, cannot be unequivocally defined by these results. PTH was concomitantly depressed upon HPX (Figure 3-14). Thus, although at the lower dose of GH (100 µg/day) the rise in $1,25-(OH)_2D$ was not accompanied by a restoration of PTH levels, it is conceivable that early transient changes in PTH might have led to a persistent change in $1,25-(OH)_2D$. This seems unlikely in view of the observation that the degradation rate of $1,25-(OH)_2D$ under GH administration remains unaltered (163). Further, the higher dosage of GH (250 µg/day), in contrast to the lower dose, does raise PTH along with $1,25-(OH)_2D$, suggests that the rise in PTH is secondary to the primary effect of GH on $1,25-(OH)_2D$ levels. It also seems unlikely that calcium or phosphorus changes were primary to the observed effects on $1,25-(OH)_2D$. These plasma variables were slightly elevated (Table 3-4) upon GH administration, an effect which would serve to depress the sterol hormone level. Thus these results support the contention that GH is capable of modulating the synthesis of $1,25-(OH)_2D$ during times of growth, either independently, or in combination with other factors. The interesting observation that there are apparent cytoplasmic receptors in the pituitary for $1,25-(OH)_2D$, as discussed in Chapter 4, lend added credence to an association between GH and vitamin D metabolism.

A direct correlation between plasma GH and plasma $1,25-(OH)_2D$ cannot be demonstrated. As previously considered, since somatomedins
are induced in response to GH, it is conceivable that these hormonal compounds are the actual effectors of \(1,25-(OH)_2D\) synthesis. Interestingly, if this is true, it most likely explains the lack of effect of GH on isolated kidney tubules to produce \(1,25-(OH)_2D\) (164). Naturally, it would be of interest to investigate the effects of SM both in vivo and in vitro on the metabolism of vitamin D. However, further elucidation of this aspect of vitamin D regulation must await further research.

In conclusion, injection of GH in rats results in an increase in conversion of 25-OHD\(_3\) to \(1,25-(OH)_2D\) (163); hypophysectomy leads to the opposite effect (162). Hypophysectomy during growth also depresses plasma \(1,25-(OH)_2D\), an action which can be reversed by the administration of growth hormone but not prolactin. Finally, the potential action of GH on vitamin D metabolism is possibly mediated by SM.

**Prolactin.** The effects of prolactin on different animal species are probably the most extensive and variable of any single polypeptide hormone (see review, 165). Nevertheless, in contrast to GH, the activity of prolactin in mammals and birds can be positively correlated with its circulating plasma concentration. Thus, the hormone is increasingly elevated during pregnancy (143) and remains high in humans after birth and during lactation, with major bursts of release occurring at suckling (153). In rats, prolactin falls precipitously at parturition, but then is rapidly elevated within several days where it continues to remain until late in lactation (144). Prolactin is also elevated in mature hens at the onset of laying (145). As a result, the potential for prolactin action in the regulation of calcium and phosphorus in each of these situations is very high. However, its elevation is accompanied by high
circulating levels of a host of other hormones involved in calcium metabolism, making it difficult to assess the action of prolactin independently of all other variables.

Prolactin has long been known to have marked effects in enhancing plasma calcium both in lower (166,167) and higher (168,169) vertebrates. In 1963, Finkelstein and Schacter (170) demonstrated that hypophysectomy led to the defective duodenal absorption of calcium. Importantly, this reduction in mineral absorption was normalized by either prolactin or GH administration. Further, the administration of prolactin to rats is capable of causing a hypercalcemia and hypercalciiuria, an affect which is still manifested even if parathyroidectomy is performed prior to the prolactin treatment (171). Since both prolactin and placental lactogens have a strong amino acid sequence homology with GH (142), their actions might be expected to be similar in nature. In fact, placental lactogens promote many of the physiological responses elicited by GH (172,173), including a similar impact on skeletal growth and remodeling (173). Thus abundant evidence supports a calcemic action by these hormones.

The results of experiments described here suggest that prolactin exerts its calcemic action in rats through the stimulation of 1,25-(OH)$_2$D synthesis. First, during situations of high serum prolactin levels, 1,25-(OH)$_2$D is correspondingly increased (pregnancy, Table 3-2; lactation, Table 3-3; and egglaying, Figure 3-8). Secondly, physiological injections of prolactin in vivo lead to elevated levels of renal 1-OHase activity, as measured in crude chick kidney homogenates. This enhancement of enzyme activity results in an elevation in the
circulating level of 1,25-(OH)$_2$D in both chicks and rats (Table 3-5). Finally, the administration of an inhibitor of prolactin secretion such as bromocriptine is capable of depressing 1,25-(OH)$_2$D levels in lactating rats (Table 3-9), although it has no effect on control (non-lactating) rats. These results, taken in conjunction with the presence of high affinity binding components for 1,25-(OH)$_2$D in the pituitary gland (Chapter 4), support a potential action and interrelationship between prolactin and 1,25-(OH)$_2$D biosynthesis.

These data do not rule out a potential coordinated effect of both PTH and prolactin on 1,25-(OH)$_2$D biosynthesis, during lactation. Certainly PTH is elevated (Table 3-10) along with prolactin (144) in lactating rats, and although parathyroidectomy dramatically reduces the circulating concentration of 1,25-(OH)$_2$D (Table 3-9), this depression is not complete. With regard to prolactin, its inhibition (through bromocriptine) causes a small but significant depression in 1,25-(OH)$_2$D in lactating animals (Table 3-10), an effect that is not accompanied by a decrease in PTH. Further, prolactin administration to bromocriptine-treated lactating rats results in striking changes in PTH accompanied by insignificant changes in 1,25-(OH)$_2$D. Whether these are cause and effect relationships will eventually be determined by sequentially sampling after hormone administration. These observations make it difficult to assign a definitive independent action on 1,25-(OH)$_2$D biosynthesis to either prolactin or PTH. Interestingly, the same consideration of a PTH-prolactin interrelationship most likely holds true during pregnancy since both of these hormones are similarly elevated (152,172). Thus,
it is likely that the overall regulation of 1,25-(OH)\(_2\)D biosynthesis is due to both hormones, with one or the other dominating under specific conditions of mineral stress.

MacIntyre and coworkers (174) contend that metabolic and hormonal conditions surrounding a physiologic setting are of primary importance to the potential action of certain hormones in vitamin D metabolism. This hypothesis has support in the observation that bromocriptine has no effect on 1,25-(OH)\(_2\)D in non-lactating rats (Table 3-10) and likewise, prolactin administration to HPX rats (Figure 3-14), also has no effect. Thus, this hormone may exert effects only under certain real situations. Interestingly, this sensitivity may be due to the presence or absence of receptors for hormones such as prolactin, since the induction of specific binding proteins which mediate hormone action are highly dependent upon the endocrine state of the animal (144,175).

In summary, a cause and effect relationship probably exists between prolactin and 1,25-(OH)\(_2\)D, since both are increased during situations requiring increased calcium and phosphorus uptake. Injections of prolactin lead to an activation of the 1-OHase enzyme and a subsequent elevation of the 1,25-(OH)\(_2\)D hormone. In contrast, elimination of prolactin from the circulation correspondingly produces a depression in the sterol hormone. These data suggest that prolactin should be included with calcium, phosphorus, PTH, and GH as primary modulators of vitamin D metabolism.

Estrogens. There is abundant clinical evidence that sex hormones such as estrogen play a significant role in the metabolism of calcium (176,177) in addition to their general sex hormone effects.
Although a mechanism for the effects of this hormone has not been defined, it is known that the steroid is involved in bone mineral resorption and remodeling in mammals (177). However, the most pronounced effects of estrogens (and androgens) come from studies on birds. These animals display a highly active mineral metabolism, predominantly at the onset and during laying (140). Thus, high levels of estrogen at this time (140) lead to a dramatic elevation in total serum calcium, an effect which can only be duplicated in male birds by the injection of estrogens (178). This elevation in females is primarily due to increased hepatic synthesis of yolk proteins capable of binding and transporting significant amounts of calcium (157) in the serum. The retention of mineral in birds requires the coordinated action of both estrogens and androgens. Interestingly, birds store calcium and phosphorus in a highly mobilizable formation called medullary bone (179), capable of rapid osteolysis and osteogenesis in response to sudden changes in mineral demand during laying. Importantly, the provision for calcium and phosphorus needs are met through intestinal uptake, a process mediated by vitamin D.

The first link between the physiologic state of egg laying and the metabolism of vitamin D was provided by Kenny (90) who observed that elevated 1-OHase activity was a characteristic of this state. It was soon shown that ovariectomy in quail led to a depression in 1-OHase activity (146), and subsequently, that the injection of estrogens could stimulate the 1-OHase enzyme and correspondingly depress the 24-OHase (89,147). Thus, at least in birds, estrogen demonstrates an action, whether direct or not, on the synthesis of $1,25-(OH)_2D$. The final
studies of the regulation of this sterol hormone were designed to demon-
strate that the stimulation of renal enzymes by estrogen, in vivo re-
sulted in an elevation in circulating $1,25-(\text{OH})_2 \text{D}$.

Injections of DES (a non-steroidal estrogen) indeed cause an
elevation in 1-OHase activity and a concomitant depression in the 24-
OHase (Table 3-6), results which are in accord with those of other
investigators (89,147,148). Testosterone and other steroids are in-
capable of this action unless accompanied by DES. Importantly, an
injection of DES or DES and testosterone leads to a dramatic elevation
in $1,25-(\text{OH})_2 \text{D}$ (Table 3-7), an effect which is rapid (Figure 3-11) and
similar in nature to the stimulation of $1,25-(\text{OH})_2 \text{D}$ by low phosphate
(149). In contrast to other investigators (89,147), the data do not
indicate that testosterone is necessary for $1,25-(\text{OH})_2 \text{D}$ biosynthesis.
However, this observation must be tempered by the fact that young male
chicks were used which could conceivably have contributed low levels of
endogenous androgens. Finally, although large doses of DES were required
for both 1-OHase and $1,25-(\text{OH})_2 \text{D}$ enhancement (Figure 3-12), these effects
could represent the long term effects of estrogen on both birds and
mammals during reproduction.

The current observations suggest that estrogen is a powerful
modulator of vitamin D metabolism in birds. Whether these results can
be extended to humans, and relate to the high estrogen levels observed
in pregnancy (180) remain to be shown. However, if estrogen is influen-
tial during this period, and during egglaying, it seems likely that the
steroid acts on $1,25-(\text{OH})_2 \text{D}$ biosynthesis in conjunction with other hor-
monal regulators such as those previously considered (PTH and
prolactin). Thus, the exact role of estrogens in vitamin D metabolism is not defined here. However, what is clear from these studies is that the endocrine control of this mineral regulating system is much more involved than originally postulated, and this complexity is highly dependent upon the physiologic state of the animal.

Modes of Stimulation

The specific mechanism of action of these hormones on the metabolism of vitamin D remains relatively unknown, even for modulators such as PTH. The possibilities are considerable and include 1) a direct action on the renal enzymes, 2) an indirect action on known modulators such as PTH, calcium, and phosphorus, and 3) an indirect action via unknown hormonal or metabolic intermediates. Nevertheless, several important observations have recently been made. GH administration in vivo appears to affect tissue uptake of $1,25-(OH)_2$D and stimulates $2$-OHase activity independent of PTH (161), although GH is incapable of stimulating this activity in isolated renal tubules (162). Prolactin has been demonstrated to have direct effects on the renal enzymes, since it is capable of stimulating the 1-OHase and depressing the 24-OHase in isolated renal tubules (162). Thus, the mode of action of both prolactin and GH on $1,25-(OH)_2$D biosynthesis may be similar in nature to that of PTH (152). The effects of estrogen are more complex. Castillo et al. (89), suggested very early that estrogen and PTH effects were independent, since administration of these hormones resulted in additive stimulation of the 1-OHase. However recently, most research has shown that estrogen effects are indirect. The hormone, unlike PTH,
has no effect on primary kidney cell cultures (181), although the results are generally inconclusive (182). Further, injections of ethylene dibromide, a hepatotoxic agent, to normal chicks prevents the standard stimulation of the 1-OHase in vivo, suggesting an intermediate effect on liver (183). Other possibilities have been suggested. Since estrogen is a modulator of prolactin secretion (184), this steroid could influence vitamin D metabolism through the presumed direct effects of prolactin on the kidney. Further, at least in birds, it has been suggested (185) that estrogenic effects, i.e., medullary bone formation, lead to a functional ionic serum calcium depletion which in turn stimulates renal enzymes via PTH. Regardless of the mechanism, these hormones may act independently, coordinately, or synergistically under a specific set of physiological conditions, to alter the metabolism of vitamin D. Extensive research will be necessary to adequately dissect these potentially complex events. Although some of this work will be made possible with improved kidney cell culture systems currently in use (182), a thorough in vivo investigation of physiological states such as growth and reproduction will also be of primary importance. A general interpretation of the effects of potential modulators on vitamin D metabolism is summarized in Figure 3-18.

The Dynamic Nature of the Vitamin D Hormone

The final studies demonstrate that $1,25-(OH)_2D$ is progressively elevated in response to calcium stress situations such as pregnancy and lactation. During the last trimester of human pregnancy, when calcium retention in the form of fetal bone is the most extensive (91),
25-OHD is converted by the 25-hydroxylase enzyme to 1,25-(OH)$_2$D. This reaction is regulated either directly or indirectly by the modulators indicated. Those arrows accompanied by a (+) indicate positive effects on the 1-OHase whereas arrows accompanied by (−) indicate negative effects of the kidney enzyme.
1,25-(OH)$_2$D is increasingly elevated (Figure 3-16). These results are in apparent contrast to recent measurements of 1,25-(OH)$_2$D during pregnancy which record an elevation in sterol hormone as early as four months (186). Nevertheless, it does demonstrate that the animal responds to its calcium needs by calling for an increase in 1,25-(OH)$_2$D, probably by way of prolactin, estrogens, or PTH, or some combination of hormones. In rats, serum 1,25-(OH)$_2$D is elevated during both pregnancy and lactation (Figure 3-17). Most important however, is the observation that 1,25-(OH)$_2$D returns to control levels immediately postpartum and is also dramatically depressed 1-2 days post-weaning. These results imply a highly regulated control of 1,25-(OH)$_2$D biosynthesis which is capable of rapidly inducing circulating changes in the calcemic hormone in response to the mineral needs of the organism.

**Summary**

In summary of this chapter, the use of radioreceptor assay has allowed the quantitation of the active metabolite of vitamin D during physiologic states characterized, in part, by an excess metabolism of calcium and phosphorus. Combined with the observations of others, the results suggest:

1) 1,25-(OH)$_2$D is elevated during growth, pregnancy, lactation, and avian egglaying probably to mediate the enhanced absorption of mineral.

2) GH, prolactin, and estrogen (DES), as well as PTH, are capable of modulating the extent of renal 1-OHase activity, and thus the level of circulating 1,25-(OH)$_2$D.
3) The circulating level of $1,25-(OH)_2D$ fluctuates dynamically in indirect response to the calcium and phosphorus needs of the organism.

The observations made here represent an increase in the extent of our knowledge about vitamin D metabolism. Hopefully this new information can be utilized in the understanding and treatment of a number of human maladies which are known to be caused by hormone directed imbalances in calcium metabolism. Although it is clear that these results add additional complexity to the regulation of vitamin D metabolism, a subsequent thorough investigation will be necessary to precisely define the exact effects and mechanism of these hormones on vitamin D.
Chapter 4 outlines the results of recent experiments which were designed to further investigate properties of the receptor for 1,25-(OH)_2D. It begins by describing several characteristics of the chick intestinal 1,25-(OH)_2D receptor, elucidated through the use of binding techniques and sucrose gradient, chromatographic, and electrophoretic analyses. These procedures are then applied to an investigation of potential 1,25-(OH)_2D receptors in other tissues, including the parathyroid gland, pancreas, pituitary, and placenta. The results suggest a more ubiquitous distribution of this specific protein than was originally ascribed. General hypotheses for the presence of receptors in these tissues are then discussed.

Results

General Properties of the Intestinal 1,25-Dihydroxyvitamin D Receptor

The receptor for 1,25-(OH)_2D was originally described in the cytoplasmic fraction of rachitic chick intestinal mucosa by Brumbaugh and Haussler (102,103). This important macromolecule demonstrated a molecular weight of approximately 47,000 and a sedimentation value of 3.7S. Further, it displayed an affinity for 1,25-(OH)_2D_3 of 2.2 \times 10^{-9} \text{M} and a 500-fold greater specificity for this sterol than that for
25-OHD. Of primary importance were the observations that the 1,25-(OH)D-receptor complex was capable of accumulating within the nucleus bound to the chromatin fraction, both in vivo and in vitro. Further, the binding of the 1,25-(OH)D-receptor to chromatin in vitro was demonstrated to occur only at 25°, suggesting a temperature dependent activation as described for other receptors. The first series of experiments were designed to investigate several of these properties, in light of recent conflicting results (187).

Cytoplasmic Receptor Binding of 1,25-Dihydroxyvitamin D. Hormone-free receptors for 1,25-(OH)D are found exclusively in the cytoplasmic (cytosolic) fraction of rachitic chick intestinal mucosa. Thus, Figure 4-1 illustrates the results of sucrose gradient analysis (SDG) of intestinal mucosal cytosol incubated with either 4nM 1,25-(OH)D³ (110 Ci/mmol) or similarly with the inclusion of 0.4 µM nonradioactive 1,25-(OH)D³, for two hours at 0°. The results reveal the presence of a peak of macromolecular-bound hormone which sediments at 3.3S and can be displaced with the inclusion of unlabeled 1,25-(OH)D³. These results suggest that 1,25-(OH)D associates in vitro with a specific macromolecule in the intestine which sediments between chymotrypsinogen and ovalbumin at 3.3S, similar to the originally described 3.7S property (102). The demonstration that an excess of nonradioactive hormone shifts the radioactive hormone peak to a smaller, nonspecific binding component(s) confirms original findings that this macromolecule is both saturable and of high affinity. Control tissues such as serum, liver, and spleen show no such binding when similarly incubated with 1,25-(OH)D.
Figure 4-1. Sucrose Gradient Analysis of the Intestinal Cytosolic 1,25-(OH)₂D Receptor.

Aliquots of receptor material (0.4 ml) were incubated with either 4nM 1,25(OH)₂[³H]D₃ (110 Ci/mmol) for 2 hours at 0° (O—O), or similarly with the inclusion of 0.4 μM unlabeled 1,25-(OH)₂D₃ (□—□). The aliquots were layered onto 5-20% sucrose gradients prepared in KETT-0.3, and centrifuged at 220,000 x g for 22.5 hours. The gradients were then fractionated from the top (5 drop fractions) and counted for tritium. External markers are: chymotrypsinogen, C (2.5S) and ovalbumin, O (3.7S).
Ammonium Sulfate Precipitation of the Receptor. The 1,25-(OH)₂D receptor can be precipitated from the cytosolic fraction obtained from intestine with ammonium sulfate (AmS) at 40% of saturation (125). If the precipitated sediment is resolubilized in KETT-0 (called an AmS-R preparation), incubated with 1,25-(OH)₂D₃ and analyzed exactly as described above, the results are seen in Figure 4-2. The 1,25-(OH)₂D₃ hormone again associates with a 3.3S macromolecule which demonstrates both saturability and high affinity. Further, there is no change in the sedimentation property under the high salt conditions of sucrose gradient centrifugation which is unlike that observed for other steroid hormone receptors (188-189). These findings show that AmS precipitation is an effective procedure for isolating the 1,25-(OH)₂D receptor from cytosol in a physically unchanged form (as characterized by SDG analysis). Thus, cytosol is routinely treated in this way, to provide minor purification of the receptor and to concentrate the protein in storable form.

Hormone Binding Affinity of the Receptor. The binding affinity of the 3.3S macromolecule was next investigated. AmS-R was incubated with increasing concentrations of 1,25-(OH)₂[³H]D₃ (total binding) or similarly with the inclusion of 100-fold excess of nonradioactive hormone (nonspecific binding) for 20 minutes at 25° to achieve equilibrium. Bound ligand was separated from free hormone by the DEAE-filter method of Santi et al. (127) (see Methods), and the results depicted in Figure 4-3. They demonstrate saturation of the receptor at very low concentrations of hormone (<10⁻⁹M), suggesting a limited number of binding sites (Figure 4-3A). In this range of hormone concentration, most binding of sterol was specific (total minus nonspecific) for 1,25-(OH)₂D₃ since
Figure 4-2. Sucrose Gradient Analysis of the Intestinal Ammonium Sulfate Precipitated 1,25-(OH)₂D Receptor.

Aliquots of receptor material (0.4 ml) were incubated with either 4nM 1,25-(OH)₂[³H]D₃ (110 Ci/mmol) for 2 hours at 0°C (O—O), or similarly with the inclusion of 0.4 μM unlabeled 1,25-(OH)₂D₃ (□—□). The aliquots were layered onto 5-20% sucrose gradients prepared in KETT-0.3, and centrifuged at 220,000 x g for 22.5 hours. The gradients were then fractionated from the top (5 drop fractions) and counted for tritium. External standards are as in Figure 4-1.
Specific binding of $1,25-(OH)_2[^3H]D_3$ (7 Ci/mmol) by intestinal ammonium sulfate precipitated cytosol. Aliquots of resolubilized material (0.5 ml, 200 µg protein) were incubated with increasing amounts of $1,25-(OH)_2[^3H]D_3$ in the presence (nonspecific, □—□) or absence (total, ○—○) of a 100-fold excess of nonradioactive $1,25-(OH)_2D_3$ for 20 minutes at 25°. Bound and free sterol were determined by DEAE-filter techniques. (B) Scatchard analysis of specific binding (Δ—Δ) in A. Regression analysis was utilized to calculate the slope of the line, $R = -0.93$. 

Figure 4-3. Determination of Dissociation Constant for Intestinal $1,25-(OH)_2D$-Receptor Interaction.
nonspecific binding was relatively small. Scatchard analysis of specific receptor binding was linear (Figure 4-3B) suggesting a single class of receptor sites as previously observed (103). However, the dissociation constant for the macromolecular-hormone complex at $25^\circ$ was $6.3 \times 10^{-10}$ M, slightly lower than that previously reported (103).

**Chromatin Binding of the Receptor.** The receptor-$1,25-(OH)_2D$ complex was originally demonstrated to bind to chromatin only at $25^\circ$ (not at $0^\circ$), suggesting a "temperature dependent" translocation to the nucleus (103). More recent work (187) has shown that, in contrast, the complex does appear capable of binding to chromatin at the lowered temperatures. In the interest of resolving this controversy, reconstituted intestinal cytosol-chromatin (see Methods) was incubated with 4nM $1,25-(OH)_2[^3H]D_3$ for increasing periods of time at both $0^\circ$ and $25^\circ$, and the ternary complex ($1,25-(OH)_2D$-receptor-chromatin) separated from unbound hormone by filtration and trapping on glass fiber filters (see Methods). The results are illustrated in Figure 4-4, and seem to indicate that the extent of binding of the $1,25-(OH)_2D$-receptor to chromatin is not strictly dependent upon temperature. In fact, equivalent maximum binding is achieved at both $0^\circ$ and $25^\circ$, although it requires over eight hours at $0^\circ$ to reach the level attained in 30 minutes at $25^\circ$. Thus, this observation is in agreement with data recently offered by Wecksler (187).

**Chromatography of the Intestinal 1,25-Dihydroxyvitamin D-Receptor**

In order to further characterize the intestinal $1,25-(OH)_2D$ receptor, its chromatographic behavior on a series of ion exchange,
Figure 4-4. Time Dependent Binding of the 1,25-(OH)2D-Receptor Complex to Chromatin at Different Temperatures.

Aliquots of a cytosol-chromatin mixture (0.1 ml) were incubated with 4 nM 1,25-(OH)2 [3H]D3 (6.7 Ci/mmol) for increasing amounts of time at both 0°C (□ — □) or 25°C (○ — ○). The ternary complex was separated by filtration, dried, and counted for tritium. Each point represents a triplicate assay for chromatin binding activity. The data are plotted as cpm of bound 1,25-(OH)2 [3H]D3 versus time (hours).
adsorption, group selective affinity, and filtration gels was investigated. These experiments were necessary in order to analyze the binding properties of the 1,25-(OH)₂D receptor to these resins, and to compare its properties to that observed for other receptor proteins. Further, the results allowed an initial evaluation of each resin for its potential effectiveness in a comprehensive receptor isolation scheme.

**Diethylaminoethyl and Phosphate Group Resins.** The chromatography of the 1,25-(OH)₂D receptor was investigated on diethylaminoethyl (DEAE)-cellulose, DEAE-Sephadex, and phosphocellulose (equilibrated in KETT-0.05) using an AmS-R preparation as receptor source. The sample was incubated with 2nM 1,25-(OH)₂[³H]D₃ (7 Ci/mmol) for 1-2 hours at 0°C (in 5% ethanol v/v). The results are depicted in Figure 4-5, and show that in each case the receptor binds to the resin under low KCl conditions, and elutes as a single peak of macromolecular-bound hormone during a linear KCl gradient. The receptor elutes at approximately 0.2M KCl with the DEAE functional group (Figure 4-5A, 4-5B) and 0.3M KCl with a phosphate group (Figure 4-5C). Specific activity of the receptor was enhanced 4-, 6-, and 15-fold with DEAE-cellulose, DEAE-Sephadex, and phosphocellulose respectively. Sucrose gradient analysis of each of the specific binding peaks (not shown) revealed a 3.3S macromolecule.

**Hydroxylapatite.** An AmS-R preparation was incubated exactly as above, and then chromatographed on hydroxylapatite (equilibrated in HAP-0). The results are depicted in Figure 4-5D. They reveal that the receptor adsorbs to hydroxyapatite crystals under conditions of low phosphate (<0.01M K₂HPO₄), and again, can be eluted as a single peak
Figure 4-5. Chromatography of the Intestinal 1,25-(OH)$_2$D-Receptor Complex on DEAE-cellulose, DEAE-Sephadex, Phosphocellulose, and Hydroxylapatite.

Individual preparations of cytosol from 3-6 grams of mucosa were ammonium sulfate precipitated, resolubilized in KETT-0, and then labeled with 2 nM 1,25-(OH)$_2$[H]$^3$D$_3$ (7 Ci/mmol) for 1 hour at 0°. They were then applied to the indicated resin, washed extensively with KETT-0.05, and eluted with a linear KCL gradient (— — —). Fractions (2-4 ml) were collected and aliquots counted for tritium (O—O).
Figure 4-5. Chromatography of the Intestinal 1,25-(OH)₂D-Receptor Complex
of bound tritium during a linear $K_2KPO_4$ gradient at 0.08M. Purification of the receptor is approximately 3-fold.

**DNA-Cellulose.** The binding of the 1,25-($\text{OH})_2D$ receptor to DNA-cellulose, a group selective affinity resin, was next investigated. Since the receptor's apparent mode of action is to influence the expression of genetic information, it was thought that this protein might interact with the DNA affinity ligand. An AmS-R preparation was incubated with 4mM 1,25-($\text{OH})_2[\text{H}]_3 (7 \text{ Ci/mmol}) at 0^\circ$ for two hours, and then chromatographed on DNA-cellulose. The results (Figure 4-6) illustrate that the 1,25-($\text{OH})_2D$-binding macromolecule is quantitatively retained by this resin under low salt conditions and can be subsequently eluted as a single peak of bound tritium during a linear salt gradient at 0.28M KCl. Relatively few proteins bind to this resin, and most which do elute prior to receptor elution. Further, washing of this resin with KETT-buffer containing 1 M KCl fails to elute any additional radioactivity, although organic solvent extraction of an aliquot of DNA-cellulose reveals an abundance of tritium, which probably represents free 1,25-($\text{OH})_2D_3$. Sucrose gradient analysis of the concentrated macromolecular-bound hormone peak (not shown) substantiates the presence of the 3.3S 1,25-($\text{OH})_2D$ receptor indistinguishable from the less pure material. Most importantly, a comparison of receptor specific activity before and after chromatography reveals a dramatic 200-fold purification in this protein, verifying the OD 280 profile observation that few proteins from intestinal cytosol bind to DNA.
An 8 gram preparation of intestinal mucosal cytosol was ammonium sulfate precipitated, resolubilized in KETT-0, and labeled with 4 nM 1,25-(OH)$_2$D$_3$ (7 Ci/mmol) for 2 hours at 0°C. The sample was applied to a 2 x 3 DNA-cellulose column, the resin washed with KETT-0.1 and the receptor eluted during a 0.1-0.5 M KCL gradient (---). Fractions of 3 ml were collected and aliquots counted for tritium (O-O). Relative protein profile (——) was monitored at OD$_{280}$. 

Figure 4-6. Chromatography of the Intestinal 1,25-(OH)$_2$D-Receptor Complex on DNA-cellulose.
Blue Dextran-Sepharose. The group selective affinity resin blue dextran-Sepharose has been used to purify a number of dehydrogenases and nuclear proteins (see discussion), and also has been shown to bind the 1,25-(OH)$_2$D receptor (125). It was thus tested for its capacity to purify the 1,25-(OH)$_2$D receptor. An AmS-R preparation was labeled with hormone as above, and then chromatographed on blue dextran-Sepharose. The results in Figure 4-7 indicate that the 1,25-(OH)$_2$D receptor binds to this resin, since a macromolecular-bound peak of tritium was eluted from the resin during an increasing salt gradient at 0.40M KCl. Again, the protein profile suggests that few proteins interact with this resin as strongly as the 1,25-(OH)$_2$D-binding component. Sucrose gradient analysis of the concentrated 0.4M KCl eluant (not shown) confirms that this material contains the 1,25-(OH)$_2$D receptor. Purification attained through the use of this affinity resin is estimated to be approximately 90-100 fold, slightly less than with DNA-cellulose.

Heparin-Sepharose. A final resin, heparin-Sepharose (the exact mechanism of interaction between this resin and protein is unknown), was tested for its capacity to retain the 1,25-(OH)$_2$D receptor. As can be seen in Figure 4-8, the chromatography of a 1,25-(OH)$_2$[$^3$H]D incubated (4nM), AmS-R preparation reveals that the 1,25-(OH)$_2$D-receptor complex is retained by heparin, and is similarly eluted during a salt gradient at 0.20M KCl. Purification of the 1,25-(OH)$_2$D receptor is approximately 20-40 fold, considerably less than DNA-cellulose or blue dextran-Sepharose. However, sucrose gradient analysis again bears out the presence of a 3.3S component bound to 1,25-(OH)$_2$D (data not shown).
Figure 4-7. Chromatography of the Intestinal 1,25-(OH)₂D-Receptor Complex on Blue Dextran-Sepharose.

A 6 gram preparation of intestinal mucosal cytosol was ammonium sulfate precipitated, resolubilized in KETT-0, and labeled with 4 nM 1,25-(OH)₂[³H]D₃ (7 Ci/mmol) for 1 hour at 0°. The sample was applied to a 2.5 x 3 cm blue dextran-Sepharose column, and eluted during a 0.1 - 0.6 M KCL gradient (——). Fractions of 5 ml were collected and aliquots counted for tritium (O——O). Relative protein profile (——) was generated at OD₂₈₀.
Figure 4-8. Chromatography of the Intestinal 1,25-(OH)₂D-Receptor Complex on Heparin-Sepharose.

A 6 gram preparation of intestinal mucosal cytosol was precipitated with ammonium sulfate, resolublized in KETT-0, and labeled with 4 nM 1,25-(OH)₂[^3]H]D₃ (7 Ci/mmol) for 1 hour at 0°C. The sample was applied to a 2.5 x 2 cm heparin-Sepharose column, and eluted during a 0.05 - 0.4 M KCl gradient (---). Fractions of 3 ml were collected and aliquots counted for tritium (○—○).
Sephacryl S-200. Agarose has been the primary gel filtration media used for 1,25-(OH)₂D receptor chromatography. However, due to rapid dissociation of the receptor-hormone complex during lengthy experiments, this resin has been suitable mostly for analytical studies. Sephacryl S-200, however, provides a rigid medium (allyl dextran covalently cross-linked with N,N¹-methylene bisacrylamide) for the rapid chromatography of 1,25-(OH)₂D receptors. The results are illustrated in Figure 4-9. The receptor appears to comigrate in this medium with bovine serum albumin and elutes immediately after the major large proteins emerge from the column allowing a purification of the 1,25-(OH)₂D-receptor complex of about 10-12 fold. However, the utility of this resin is its use as a successful sizing technique with high receptor yields (60-80%).

Gel Electrophoresis of the Intestinal 1,25-Dihydroxyvitamin D Receptor

Electrophoresis of receptor proteins under nondenaturing conditions is a viable technique for identification, characterization, and purification. The receptor for 1,25-(OH)₂D can also be subjected to electrophoresis, and identified through its tritiated hormonal ligand. The results in Figure 4-10 demonstrate the electrophoresis of aliquots of a crude cytosolic preparation of intestinal mucosa incubated with 4nM 1,25-(OH)₂[³H]D₃ (110 Ci/mmol) or similarly with the inclusion of 100-fold excess of nonradiolabeled hormone for two hours at 0⁰. Figure 4-10 illustrates the tritium profile along the gel after electrophoresis, and indicates that macromolecular-bound hormone migrates approximately two-thirds of the way into the gel under these conditions.
Figure 4-9. Chromatography of the Intestinal 1,25-(OH)₂D- Receptor Complex on Sephacryl.

Cytosol from 4 grams of intestinal mucosa was salt precipitated, resolubilized in 1 ml of KETT-0.3 (Sephacryl buffer), and labeled with 4 nM 1,25-(OH)₂[³H]D₃ (7 Ci/mmol) for 1 hour at 0°. It was then chromatographed on a 1.6 x 60 cm Sephacryl column in Sephacryl buffer with an ascending flow rate of 60 ml/hour (O—O). Void volume, V₀, indicated by blue dextran; numbers indicate peak elution of 1, BSA; 2, ovalbumin; 3, chymotrypsinogen; 4, ribonuclease. Relative protein profile was monitored at OD₂₈₀(—).
Intestinal mucosal cytosol was prepared as described in Methods. Aliquots were incubated with 4 nM 1,25-(OH)\textsubscript{2}\textsuperscript{[3H]}D\textsubscript{3} (110 Ci/mmol) or similarly with the inclusion of 0.4 μM 1,25-(OH)\textsubscript{2}D\textsubscript{3} for 1 hour at 0°C. The samples were then treated and electrophoresed as in Methods. Gels were sliced into 2 mm fractions and the fractions extracted and counted for tritium. One gel was stained with Coomassie blue. Samples are: 0.1 ml cytosol incubated with 4 nM 1,25-(OH)\textsubscript{2}\textsuperscript{[3H]}D\textsubscript{3}, (——); 0.1 ml cytosol incubated with 4 nM 1,25-(OH)\textsubscript{2}\textsuperscript{[3H]}D\textsubscript{3} and 0.4 μM 1,25-(OH)\textsubscript{2}D\textsubscript{3} (— — —); 0.05 ml cytosol incubated with 4 nM labeled hormone (---------).
Significantly, the inclusion of excess unlabeled hormone leads to an obliteration of the peak, supporting the contention that this bound-tritium peak represents the $1,25-(\text{OH})_2\text{D}$-receptor complex. The staining of an equivalent gel shows the extensive number of proteins present in this crude preparation. The electrophoresis of labeled AmS-R preparation can also be identically treated (data not shown) with equivalent results.

Distribution of Radiolabeled $1,25$-Dihydroxyvitamin D in Rachitic Chick Tissue

An investigation of receptors in tissues other than the intestine was preceded by a study designed to show which tissues were capable of accumulating $1,25-(\text{OH})_2^3\text{H}]\text{D}_3$ after the administration intracardially of 2 IU of $1,25-(\text{OH})_2^3\text{H}]\text{D}_3$ (7 Ci/mmol) per rachitic chick. Two hours after injection the pituitary (P), pancreas (PA), parathyroid glands (PTG), intestine, liver, brain, and muscle were excised, radioactivity extracted with chloroform methanol(2:1 v/v), and counted via scintillation. The data are summarized in Table 4-1, and indicate an abundance of tritium in intestine, PTG, P, and PA, and then less in the remaining tissues. Liver generally retains high hormone levels due to the method of injection. These observations support the idea that intestine and PTG are sites of $1,25-(\text{OH})_2\text{D}$ action, and offer evidence of other target tissues, including pancreas and pituitary.

Identification of $1,25$-Dihydroxyvitamin D Receptors in Other Tissues

The presence of $1,25-(\text{OH})_2\text{D}$-binding components were investigated by utilizing group selective affinity chromatography as previously
Table 4-1. Tissue Distribution of 1,25-(OH)₂D₃ After Injection

<table>
<thead>
<tr>
<th>Tissue</th>
<th>1,25-(OH)₂³H D₃ (dpm)/g-tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parathyroid gland</td>
<td>17,066</td>
</tr>
<tr>
<td>Pancreas</td>
<td>19,197</td>
</tr>
<tr>
<td>Intestine</td>
<td>41,752</td>
</tr>
<tr>
<td>Liver</td>
<td>15,689</td>
</tr>
<tr>
<td>Pituitary</td>
<td>8,461</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>4,758</td>
</tr>
<tr>
<td>Muscle</td>
<td>2,516</td>
</tr>
</tbody>
</table>

2 IU 1,25-(OH)₂³H D₃ was injected/chick.
Tissue was extracted by the technique of Bligh and Dyer (119), the extract dried, and counted for tritium. Data are expressed as dpm/g-tissue.
described (DNA-cellulose and/or blue dextran-Sepharose) and sucrose gradient analysis. Chromatographic techniques were advantageous for two reasons. First, they provided a means of identification of receptors, by analogy with the characteristics demonstrated on these resins by the intestinal $1,25-(OH)_2D$ receptor. Secondly, since sucrose gradient analysis was often insufficient to investigate adequately potential receptors in some tissues (due to scarcity of receptors, high nonspecific binding, volume limitations of the technique, etc.), prior chromatography served to purify the $1,25-(OH)_2D$-binding component (if present) from larger amounts of tissue, thus concentrating potential receptors and reducing nonspecific binding. Subsequent sucrose gradient analysis then became an effective tool in identification and the determination of sedimentation property. These techniques, in conjunction with the use of high specific activity $1,25-(OH)_2[3^H]D_3$ (110 Ci/mmol), increased the sensitivity of detection of $1,25-(OH)_2D$-binding components ("receptors") 10-100 fold.

**Parathyroid Glands.** The parathyroid glands (PTG) were first investigated. Since a $1,25-(OH)_2D$ receptor was previously demonstrated in the PTG by Brumbaugh, Hughes, and Haussler (109) and Hughes and Haussler (110) using sucrose gradient centrifugation, the similarity of its properties on DNA-cellulose and blue dextran-Sepharose were of particular interest. Further, a question has existed as to whether the sedimentation value of this receptor was similar to that of the intestinal $1,25-(OH)_2D$ receptor (see 102,110). PTG cytosols were precipitated with AmS at 40% of saturation, resolubilized in KETT-0, and labeled with 10 nM $1,25-(OH)_2[3^H]D_3$ (110 Ci/mmol) for two hours at 0°. They
were then chromatographic on DNA-cellulose and blue dextran-Sepharose. The binding of the PTG 1,25-(OH)_2D receptor to DNA-cellulose is depicted in Figure 4-11A, and the results suggest a behavior on this resin similar to that of the intestinal receptor. Sucrose gradient analysis (Figure 4-11B) verifies that the sedimentation value of this receptor is identical to the intestinal receptor. Figure 4-12A illustrates PTG 1,25-(OH)_2D receptor chromatography on blue dextran-Sepharose. The elution of this protein is again similar to that found in intestine, and sucrose gradient analysis verifies its 3.3S sedimentation property (Figure 2-12B). The results support a 3.3S receptor for 1,25-(OH)_2D in PTG and demonstrate that its properties, at least on two affinity resins and sucrose gradients, are identical to the intestinal receptor.

Pancreas. Morrisey et al. (190) demonstrated in 1969 that CaBP, the gene product induced by 1,25-(OH)_2D, was present in pancreatic tissue. This observation led to an investigation of 1,25-(OH)_2D receptors in the pancreas of the chick utilizing the techniques described above. Pancreatic cytosols were treated with AMs at 40% of saturation, the precipitates resolubilized, and then labeled with either 2nM 1,25-(OH)_2[^3]H]D_3 (110 Ci/mmol) or similarly with 0.4 μM 1,25-(OH)_2D for two hours at 0°C. Aliquots were analyzed directly by sucrose gradient centrifugation and demonstrate (data not shown) a possible binding component for 1,25-(OH)_2D in pancreas which is obliterated by nonradioactive hormone. However, the results were not reproducible. Therefore, labeled material (as above) was examined by chromatography on DNA-cellulose, as seen in Figure 4-13A. The results of this experiment support the presence of a binding component for 1,25-(OH)_2D in the pancreas, which
Figure 4-11. Chromatography of the Chick Parathyroid Gland 1,25-(OH)₂D₃-Receptor on DNA-cellulose and Subsequent Sucrose Gradient Analysis.

(A) Cytosol was prepared from 1 gram of parathyroid glands, precipitated with ammonium sulfate, resolubilized in KETT-0, and incubated with 1 nM 1,25-(OH)₂[³H]D₃ (110 Ci/mmol) for 1 hour at 0°. It was then chromatographed as described in Methods on a 2 x 3 cm DNA-cellulose column. Fractions were collected (3 ml) and aliquots counted for tritium (O—O). KCl gradient was monitored by conductivity (— — —). (B) An aliquot of the macromolecular-bound tritium peak was centrifuged on a 5-20% sucrose gradient prepared in KETT-0.3 as described in Methods. The gradient was fractionated and counted for tritium (O—O). External markers were as in Figure 4-1.
Figure 4-12. Chromatography of the Chick Parathyroid Gland 1,25-(OH)₂D-Receptor on Blue Dextran-Sepharose and Subsequent Sucrose Gradient Analysis.

(A) Cytosol was prepared from 1 gram of parathyroid glands, precipitated with ammonium sulfate, resolubilized in KETT-0, and incubated with 1 nM 1,25-(OH)₂[³H]D₃ (110 Ci/mmol) for 1 hour at 0°C. It was then chromatographed as described in Methods on a 2.5 x 3 cm blue dextran-Sepharose column. Fractions (3 ml) were collected and aliquots counted for tritium (O—O). KCL gradients were monitored by conductivity (— — —). (B) An aliquot of the macromolecular-bound tritium peak was analyzed via sucrose gradient centrifugation, fractionated, and counted for tritium (O—6). External markers are as in Figure 4-1.
Figure 4-12. Chromatography of the Chick Parathyroid Gland 1,25-(OH)$_2$D-Receptor
interacts with DNA and is eluted at 0.28M KCl, similarly to both the intestine and parathyroid receptor forms. Figure 4-13B confirms that this binding component is a 3.3S macromolecule on 5-20% sucrose gradients. In addition, DEAE filter assay for receptor in the pancreas (data not shown) suggests that it exists in approximately 25% of the titers present in intestine.

**Pituitary.** In view of the potential interaction between pituitary hormones and vitamin D metabolism (Chapter 3), the presence of 1,25-(OH)\(_2\)D receptors were investigated in this tissue. Sucrose gradient analysis of chick pituitary cytosol reveals the presence of a binding component smaller than the 3.3S receptor in other tissues (Figure 4-14), although similar analysis of bovine pituitary suggests a 3.7S macromolecule which binds 1,25-(OH)\(_2\)D and is displaceable with excess unlabeled hormone (Figure 4-15). In order to solve this discrepancy, the pituitaries from 400 chicks were excised and cytosol prepared. The high speed supernatant was then incubated with 20nM 1,25-(OH)\(_2\)\[^3\text{H}]\)D (110 Ci/mmol) for two hours at 0°C, and then chromatographed on DNA-cellulose. A macromolecular-bound peak of tritium eluted at 0.28M KCl, again like the intestinal, PTG, and pancreatic receptors (Figure 4-16A). Importantly, sucrose gradient analysis of the concentrated tritium peak from this chromatographic procedure also revealed a 3.3S macromolecule (Figure 4-16B), suggesting that the pituitary be included as a target tissue for 1,25-(OH)\(_2\)D.

**Placenta.** Mammalian placental tissue actively transports calcium against an electrochemical gradient (191,192) much like the intestine. Further, CaBP has also been identified in this tissue, suggesting
Figure 4-13. Chromatography of the Chick Pancreatic 1,25-(OH)$_2$D$_3$-Binding Component on DNA-cellulose and Subsequent Sucrose Gradient Analysis.

(A) Cytosol was prepared from 8 grams of chick pancreas, precipitated with ammonium sulfate, resolubilized in KETT-0, and incubated with 1 nM 1,25-(OH)$_2$[${}^3$H]D$_3$ (110 Ci/mmol) for 1 hour at 0°C. It was then chromatographed as described in Methods on a 2.5 x 6 cm DNA-cellulose column. Fractions (5 ml) were collected and aliquots counted for tritium (O—O). KCL gradients were monitored by conductivity (— — —). (B) An aliquot of the bound tritium peak was analyzed via sucrose gradient centrifugation, fractionated, and counted for tritium (O—O). External markers are as in Figure 4-1.
Figure 4-14. Sucrose Gradient Analysis of Chick Pituitary Cytosol.

Cytosols were prepared from chick hypothalamus and pituitary and incubated with 4 nM 1,25-(OH)₂[¹³H]D₃ (110 Ci/mmol) for 2 hours at 0°C. Aliquots (0.2 ml) were layered onto 5-20% sucrose gradients prepared in KETT-0.3, and centrifuged as described in Methods. The gradients were fractionated and counted for tritium. Aliquots are pituitary cytosol (O——O) and hypothalamus (□——□). An aliquot of chick pituitary cytosol (Δ——Δ) was also analyzed after incubation with labeled hormone and a 100-fold excess of unlabeled hormone. External markers are: chymotrypsinogen, C (2.5S); ribonuclease, R (1.8S).
Figure 4-15. Sucrose Gradient Analysis of Bovine Pituitary Cytosol.

Cytosol from 1 gram of bovine pituitary was prepared and incubated with either 4 nM 1,25-(OH)\(_2\)[\(^3\)H]D\(_3\) (6.7 Ci/mmol) (\(\circ\)---\(\circ\)) or similarly with the inclusion of 0.4 \(\mu\)M unlabeled hormone (\(\square\)---\(\square\)), for 1 hour at 0\(^\circ\). Aliquots (0.2 ml) were layered onto 5-20\% sucrose gradients prepared in KETT-0.3, and centrifuged as in Methods. The gradients were fractionated and counted for tritium. An aliquot (\(\Delta\)---\(\Delta\)) was also similarly incubated with 25-OH[\(^3\)H]D\(_3\) (6.7 Ci/mmol) and analyzed via sucrose gradients. External markers are as in Figure 4-1.
Figure 4-16. Chromatography of Chick Pituitary 1,25-(OH)\textsubscript{2}D-Binding Component on DNA-cellulose and Subsequent Sucrose Gradient Analysis.

(A) Cytosol was prepared from the pituitaries of 400 chicks (650 mg) and incubated with 20 nM 1,25-(OH)\textsubscript{2}\textsuperscript{[\textsuperscript{3}H]D\textsubscript{3} (110 Ci/mmol) for 1 hour at 0\textdegree. It was then chromatographed on a 2 x 3 cm DNA-cellulose column as described previously, fractionated and aliquots counted for tritium (O—O). KCL gradient was measured by conductivity (——). (B) An aliquot of the macromolecular-bound tritium peak was analyzed via sucrose gradient centrifugation. Gradients were fractionated and counted for tritium (O—O). External markers are as in Figure 4-1.
a potential target for $1,25-(OH)_2D_3$ (193). As a consequence, rat placental cytosol was prepared from placenta taken from one female rat at term, and treated with AmS in the standard way. The resolubilized precipitate was labeled with 10nM $1,25-(OH)_2[\text{H}]D_3$ (110 Ci/mmol) for two hours at $0^\circ$, and then chromatographed on DNA-cellulose. Figure 4-17 reveals that again a $1,25-(OH)_2D$-binding component interacts with DNA under low salt conditions, and elutes during a linear KCl gradient at 0.28M. DEAE filter assay would suggest (not shown) that the concentration of receptors in this tissue represent 5-10% of that seen in intestine. These findings indicate that most likely placental tissue also contains receptors for $1,25-(OH)_2D$.

**Discussion**

The receptor for $1,25-(OH)_2D$ was originally identified by sucrose gradient analysis in chick intestine in 1974 (102), although indirect evidence for this component has been accumulating steadily for several years (101). The $1,25-(OH)_2D$ receptor was subsequently shown to retain a number of properties typical of steroid hormone receptors including: 1) high affinity for the hormone, 2) specificity for the hormone, and 3) saturability at low hormone concentrations. Furthermore, at the time, it was only present in the intestine suggesting tissue specificity. Subsequently, $1,25-(OH)_2D$ receptors were also identified in rat intestine (107), PTG (110), and bone (108). The results found in this chapter reveal new properties about the chick intestinal receptor, and also show that the receptor protein shares a wider tissue distribution than was previously thought.
Figure 4-17. Chromatography of Rat Placental 1,25-(OH)₂D-Binding Component on DNA-Cellulose.

Cytosol was prepared from 4 grams of rat placenta, ammonium sulfate precipitated, resolubilized in KETT-0, and labeled with 2 nM 1,25-(OH)₂[³H]D₃ (110 Ci/mmol) for 1 hour at 0°. The sample was applied to a 2 x 2 cm DNA-cellulose column, and eluted from the resin during a linear KCL gradient (---). Fractions (3 ml) were collected and aliquots counted for tritium (O—O).
The Intestinal Receptor for 1,25-Dihydroxyvitamin D

Several properties of the 1,25-(OH)$_2$D receptor were evaluated using higher specific activity 1,25-(OH)$_2$[³H]D$_3$ (110 Ci/mmol). The results of sucrose gradient analysis indicate a protein migrating at 3.3S, a value similar to previous reports (102), and observed in cytosol (Figure 4-1), ammonium sulfate precipitates (Figure 4-2), and after chromatography on DNA-cellulose and blue dextran-Sepharose. Although this molecule may represent a fragment or subunit of a larger form of the 1,25-(OH)$_2$D receptor, as observed in other systems (97), this remains to be demonstrated. Also, high salt (AM$S$) has no effect on the sedimentation value of the receptor, in contrast to that seen for other receptors (189). Further, the sedimentation value of 3.3S holds true for all the tissue binding components identified for 1,25-(OH)$_2$D, as would be expected for a homologous receptor molecule. In addition, the dissociation constant was determined (Figure 4-3B) under conditions more favorable towards equilibrium (25°C for 20 minutes). These results ($K_d = 6 \times 10^{-10}$ M) are in accord with the general observations made by others. However, they must be tempered with the fact that these data were determined on unpurified cytosolic receptor, in which hormone free receptor sites are being lost during the course of incubation (125). Thus, until stabilized, purified receptor is available, these results must be considered only approximate.

A controversy has existed as to the nature of 1,25-(OH)$_2$D receptor binding to chromatin. It was originally reported that ternary complex formation occurred only upon incubation of 1,25-(OH)$_2$D receptor and chromatin at 25°C, not at 0°C (103). These data gave rise to the
idea of "temperature-dependent activation" of the receptor to a form capable of binding chromatin (the receptor does bind 1,25-(OH)$_2$D at 0°). The results of Figure 4-4 suggest that the dependency of binding is upon both time and temperature. Indeed, the ternary complex does form at 0° as well as 25°, although it takes considerably longer, an observation also made by Wecksler (187). This may be due to sterol solubility at this temperature, diffusion, or an activation phenomenon. Whether the receptor is capable of binding chromatin without benefit of hormonal ligand has not yet been determined. Thus, in a strict sense, the activation phenomenon observed for the glucocorticoid receptor (194) does not appear to be a property typical of the 1,25-(OH)$_2$D receptor. In contrast, progesterone (195) and estrogen (189) receptors do undergo physical transformations during the course of nuclear binding.

Analysis of the intestinal 1,25-(OH)$_2$D receptor on the chromatographic gels defined in this dissertation was accomplished for several purposes: 1) to determine which functional groups or ligands will interact with the 1,25-(OH)$_2$D-receptor complex, and then to characterize the behavior of this receptor during its subsequent elution from these resins, 2) to use the most functional resins to chromatograph and identify new target tissues for 1,25-(OH)$_2$D, i.e., identify new 1,25-(OH)$_2$D receptors, and 3) to survey and assess the capability of a number of resins for their possible utility in an isolation scheme designed to purify the intestinal form of the 1,25-(OH)$_2$D receptor.

An extensive number of chromatographic ion exchange resins have been used to study steroid hormone receptor molecules. For example, Schrader and O'Malley (196), and Schrader and coworkers (197) utilized
DEAE-cellulose, phosphocellulose, and hydroxylapatite, in combination with gel filtration, to investigate the progesterone receptor. In the case of DEAE-cellulose and phosphocellulose, there are two $[^3H]$-progesterone bound peaks associated with the chromatography of chick oviduct prepared exactly as the intestinal tissue is prepared here. They have been designated subunits A and B of the progesterone receptor, and demonstrate different nuclear binding properties (198,199). In the case of the $1,25-(\text{OH})_2\text{D}$ receptor, it binds to each of these exchange resins, but in contrast, apparently elutes as a single peak of associated $1,25-(\text{OH})_2[^3H]\text{D}_3$, suggesting a single protein species. Interestingly, the $1,25-(\text{OH})_2\text{D}$ receptor elutes at similar salt concentrations on both DEAE- and phosphocellulose as the progesterone receptor (197). Unfortunately, although these resins have been utilized in the purification of both the progesterone and estrogen receptors, they provide insufficient power, both in degree of purification and in binding capacity, to be successfully used to isolate the $1,25-(\text{OH})_2\text{D}$ receptor.

An alternative technique for the study of steroid hormone receptors is the use of group selective affinity resins. For example, native DNA linked to an insoluble matrix such as cellulose offers a method of extracting predominantly DNA binding proteins from crude extracts of tissue. Indeed, the observation that a particular protein binds to this type of resin is at least suggestive of a potential interaction in vivo between that protein and the cellular genome, although caution must be taken in relying heavily upon this interpretation. The results in Figure 4-6 indicate that the $1,25-(\text{OH})_2\text{D}$ receptor binds to DNA-cellulose, and elutes as a single binding species. Purification of
the receptor on this gel is the highest so far observed, which implies a specificity to the interaction which is not observed in other resins. Further, it seems likely that the interaction does not merely represent binding to phosphate groups, since the presence of a phosphate function on phosphocellulose leads to far less purification (200-fold vs. 15-fold). The interaction of the 1,25-(OH)\(_2\)D receptor with DNA certainly supports the current concept that this protein functions to alter the transcription of specific genes.

The active chromophore ligand, Cibacron Blue-F3GA, has recently been used to study and purify a number of nuclear proteins (200,201). The blue dye apparently has a biospecific affinity for nucleotide requiring enzymes, probably because of the structural similarity between nucleotide cofactors and the dye. Further, it is postulated that those proteins which bind to Cibacron Blue may have a dinucleotide fold domain as a part of the molecule (202). Figure 4-7 demonstrates that indeed the 1,25-(OH)\(_2\)D receptor interacts quite strongly with the blue dye as described previously (125). Again, it elutes after most other proteins as a single binding species, and purification is considerable, although less than DNA-cellulose. Thus, blue dextran-Sepharose may have utility in the isolation of this sterol hormone receptor. Interestingly, this resin has recently been used to investigate potential genomic binding sites on the estrogen receptor (203). This observation, and the fact that blue dextran-Sepharose is used to purify proteins that bind nucleic acids (200,201), again imply that the 1,25-(OH)\(_2\)D receptor may function within the cell nucleus by interacting with and modifying DNA.
The use of heparin-Sepharose in the study of steroid hormone receptors is considerable (132,204). Heparin is a polysulfate-containing mucopolysaccharide thought to bind cellular proteins on the basis of either charge ($SO_4^{3-}$) or by hydrophobic interaction. Since the $1,25-(OH)_2D$ receptor binds to both hydrophobic resins (data not described in this dissertation) and ion exchange resins (Figure 4-5), the observation that it binds to heparin is not surprising (Figure 4-8). Nevertheless, although the interaction is unknown, it does afford moderate purification of the $1,25-(OH)_2D$ receptor and therefore could be used in a purification scheme (see Chapter 5).

These series of observations suggest that the receptor for $1,25-(OH)_2D$ displays a number of interesting and unique properties as demonstrated by binding techniques, and on ion exchange and pseudo-affinity chromatographic gels. However, it is clear from this data, that the power to purify the intestinal $1,25-(OH)_2D$ receptor potentially resides in resins such as DNA-cellulose, blue dextran-Sepharose, and possibly heparin-Sepharose. A test of these resins in sequence will be described in the next chapter.

Gel electrophoresis is also capable of providing much information about the physical characteristics of protein molecules, including receptors (205,206). Its application to steroid hormone receptors has met with mixed success, primarily because of elevated rates of receptor-hormone complex dissociation under conditions of nonequilibrium and electrophoresis. This technique has been used to investigate, purify, and identify receptors such as the progesterone B subunit (197), the estrogen receptor (207) and the androgen receptor (208), although it
has been unsuccessful in the identification of the progesterone A subunit (209). Crude \(1,25-(\text{OH})_2\ D\) receptors (cytosolic or AmS-R preparations), however, can be electrophoresed under non-denaturing conditions with the retention of enough hormone to fractionate and follow binding (Figure 4-10). Interestingly, comparisons of the \([\text{H}]\)-hormone profile with a stained gel indicate the receptor rapidly migrates within the electrical field at a rate greater than expected for its molecular weight. This observation suggests that the \(1,25-(\text{OH})_2\ D\)-receptor may be a highly charged molecule, an idea supported by previous results on chromatographic resins. Unfortunately, yields of \(1,25-(\text{OH})_2\ D\)-receptor complexes with this procedure are 10-25%. Thus, the technique has yet to be applied towards purification. In addition, its primary application in identifying a protein species as the \(1,25-(\text{OH})_2\ D\) receptor also remains to be accomplished. Nevertheless, the present results indicate that electrophoresis can be performed with the sterol receptor, and therefore its potential in investigations of this protein are possible.

New Receptors for 1,25-Dihydroxyvitamin D

The presence of specific intracellular receptors within a tissue implies that that organ represents a target for the steroid hormone in question. Indeed, intestinal receptors for \(1,25-(\text{OH})_2\ D\) represent the means whereby hormone is conveyed to the nucleus and is capable of initiating events leading to the synthesis of active products. It now appears that receptors for \(1,25-(\text{OH})_2\ D\) are present in several other organs, and thus a unifying hypothesis for their roles in those tissues requires development.
Pancreas (Figure 4-13) appears to contain a 3.3S binding component for \(1,25-(\text{OH})_2\text{D}\) which behaves on DNA-cellulose similarly to both the intestinal and PTG receptors. The identification of this new receptor has recently been confirmed (210). Further, both pituitary (Figure 4-16) and placenta (Figure 4-17) contain similar components, although in less abundance. These results are in keeping, in part, with the distribution of \(1,25-(\text{OH})\text{D}\) observed in Table 4-1. Thus these sterol binding components most likely represent intracellular receptors for \(1,25-(\text{OH})_2\text{D}\). Nevertheless, caution must be exerted in drawing these conclusions since more direct evidence is needed on the action of the macromolecules within these tissues. Importantly, they are not present within liver or serum, although evidence has been offered recently for the presence of these components in both rat and chick kidney (211,212).

The 3.3S component described here is readily distinguishable from the 5.8S intracellular 25-OHD\(_3\)-binding component (DBP) (213). Although not shown, pituitary, placenta, and pancreas also contain this macromolecule. At present a role for the 5.8S ubiquitous component has not been assigned, although it has been determined that it retains a higher affinity for 25-OHD\(_3\) than for \(1,25-(\text{OH})_2\text{D}\). Recent work suggests that the 5.8S cytoplasmic component represents an artificial combination of the 4S plasma carrier protein for 25-OHD\(_3\) and the other 4S intracellular component which has not been shown to bind sterol (214). Importantly, the 5.8S macromolecule does not convey 25-OHD\(_3\) to the nucleus, although a report to the contrary has recently appeared (215). These results suggest that the 5.8S macromolecule is most likely not a steroid hormone receptor. However, since this molecule also binds \(1,25-(\text{OH})_2\text{D},\)
a function in the delivery of this metabolite to the cell or possibly across the plasma membrane is possible, analogously to that seen in the vitamin A system (216).

The 1,25-(OH)$_2$D receptor exists in much less abundant titers in pancreatic, pituitary, and placental cytosols than within intestine. Estimates utilizing DEAE-filter techniques (see Methods) indicate the concentration of receptor in intestine is 3-4 fold greater than in pancreas, and even more so than pituitary and placenta. This lack of abundance can be explained in two ways: 1) the molecule exists equally in all the cells of a particular tissue, but is rare in each or 2) the receptor maintains a limited cellular distribution within tissues. The latter seems the most likely in view of the tremendous number of cell types and the wide variety of functions carried out by pancreas, placenta, and pituitary.

Mechanism of 1,25-Dihydroxyvitamin D Action

A number of aspects of the cellular action of 1,25-(OH)$_2$D in intestinal tissue have been described (see Introduction), and will only be briefly summarized here. 1,25-(OH)$_2$D apparently diffuses into the cell where it binds to the specific receptor considered in this dissertation. The receptor-hormone complex subsequently accumulates within the nucleus where it associates with the chromatin fraction. Although specific mRNA's have not been shown to be induced, de novo, by 1,25-(OH)$_2$D, abundant evidence has been put forth that this is the case. Corradino (217) has shown that two mRNA inhibitors, α-amanitin and actinomycin D, block the accumulation of calcium within the intestinal
cell, when grown in organ culture. More specifically, Tsai et al. (101) demonstrated that $1,25-(\text{OH})_2\text{D}$ caused an elevation in the incorporation of UMP into RNA in rachitic chicks. However, it was Zerwehk et al. (111) who showed that RNA synthesis could be stimulated in vitro by reconstituting receptor containing intestinal cytosol with intestinal chromatin. This effect was entirely dependent upon the presence of receptor. Consequently, this evidence suggests that $1,25-(\text{OH})_2\text{D}$ acts at the level of the cellular genome to induce the transcription of specific genes. Several endpoint proteins for vitamin D action have been described (8,113, 115), although CaBP is the most well studied. This protein was originally described by Wasserman and Taylor (218), and was recently sequenced by Huang, Cohn, and Hamilton (219). It constitutes 1-2% of the total protein in normal chick intestine, but is absent in D-deficient animals. Further, it is found in the intestines of a number of species, and has also been observed in a number of other tissues (23,24). Its appearance correlates well with the onset of intestinal calcium absorption, although other aspects of its relationship with both vitamin D and calcium transport are the subject of current debate (8). In fact, its role as a transport component is in doubt, and it is currently thought that this component may be a homeostatic buffer protein induced to protect the cell against an increasing concentration of calcium. Interestingly, a recently study (115) suggests that action may be induced by $1,25-(\text{OH})_2\text{D}$ within the intestinal microvillus, and that this effect might somehow be responsible for altered transport rates. Nevertheless, the ultimate effect of $1,25-(\text{OH})_2\text{D}$ is to increase the uptake of both calcium and phosphate ion.
The effects of $1,25-(\text{OH})_2\text{D}$ on placental tissue are likely to be similar to that of intestine. Placenta has been shown to transfer significant amounts of calcium from dam to fetus for eventual deposition onto the fetal skeleton (191). Since this transfer is demonstrated to be against an electrochemical gradient, an active transport mechanism may be involved (192). In fact, Shami and Radde (220) have postulated that a Ca-ATPase, a system similar in nature to that found in intestine (221), may be responsible for the transport. However, CaBP is also present (193). These data certainly suggest that $1,25-(\text{OH})_2\text{D}$ might exert effects on this tissue analogously to that seen in intestine. Thus $1,25-(\text{OH})_2\text{D}$, via cytoplasmic receptor, may direct the induction of proteins responsible for placental transfer of calcium to the fetus.

The effects of $1,25-(\text{OH})_2\text{D}$ on tissues such as parathyroid, pancreas, and pituitary glands are highly speculative. Nevertheless, several possibilities are consistent with current evidence. In view of the modulating effects PTH (82,83) and pituitary growth hormone and prolactin (see Chapter 3) exert on the biosynthesis of $1,25-(\text{OH})_2\text{D}$, a negative feedback control by $1,25-(\text{OH})_2\text{D}$ on these tissues can be envisioned. In addition, a relationship between pancreatic insulin and $1,25-(\text{OH})_2\text{D}$ has also been described (222). Thus, $1,25-(\text{OH})_2\text{D}$ may function by complexing with the receptors presently identified, migrating to the nucleus and directing the synthesis of specific gene products. How this action might regulate the secretion of hormones such as GH, prolactin, insulin, or PTH is unknown. Of course, one possibility is that $1,25-(\text{OH})_2\text{D}$ influences the transcription of mRNA for these hormones, although no evidence has been presented for this action. A more
reasonable mechanism might be the induction of the same or similar pro-
teins as in intestine, which might indirectly influence hormone secre-
tion. With respect to the parathyroid gland, Chertow et al. (223) have
shown that $1,25-(\text{OH})_2\text{D}$ suppresses the secretion of PTH both in vivo in
rats and in isolated slices of bovine parathyroid gland. Although no
action of $1,25-(\text{OH})_2\text{D}$ has been demonstrated on pituitary, the sterol is
known to effect exocrine release within the pancreas in a negative
fashion (224). Since CaBP is also present in parathyroid glands (225),
and pancreas (190), it is possible that this protein is capable of alter-
ing the active intracellular calcium content, and thus depressing the
calcium-dependent secretion mechanisms involved in polypeptide hormone
release. An alternative, but no less viable, hypothesis is that the in-
duction or appearance of actin in response to $1,25-(\text{OH})_2\text{D}$ (115) leads to
an increase or complexity in the cellular terminal web. Importantly,
this web is known to regulate the exocytotic release of hormone con-
taining granules, both from pancreas (226) and pituitary (227). Thus,$
1,25-(\text{OH})_2\text{D}$ might feedback-inhibit the biosynthesis or secretion of prod-
ucts which regulate its own formation. Since $1,25-(\text{OH})_2\text{D}$ may control
the secretion of exocrine pancreatic enzymes (193), a more general and
extremely interesting postulate is that $1,25-(\text{OH})_2\text{D}$ is actually involved
in the cellular secretion of proteins, irrespective of feedback influ-
ence. Much research will be necessary to support this exciting
hypothesis.
Summary

To conclude this chapter, the intestinal receptor for 1,25-(OH)₂D is a specific 3.3S macromolecule which binds hormone with extremely high affinity (Kd$^{10} \text{ M})$. Upon binding hormone, the complex is capable of forming a ternary complex with homologous chromatin in a time dependent fashion at either 0° or 25°. Further, the intestinal receptor displays a characteristic binding pattern on a series of specific ion exchange and group selective affinity gels including DNA-cellulose, blue dextran-Sepharose, and heparin-Sepharose. These techniques were subsequently used to identify binding components for 1,25-(OH)₂D in placenta, pancreas, and pituitary. By analogy with the intestinal receptor, these components are probably receptors for the sterol hormone. The presence of receptors in tissues other than intestine suggest a more general distribution of 1,25-(OH)₂D-sensitive cells. Exactly how 1,25-(OH)₂D influences the physiology of these newly defined target tissues remains to be elucidated. Nevertheless, these findings introduce an entirely new avenue of research within the field of vitamin D action.
Chapter 5 will outline the results of experiments designed to purify the intestinal receptor for $1,25-(\text{OH})_2\text{D}$. This task involves the sequential use of several of the chromatographic techniques described in the previous chapter coupled with initial selective precipitation procedures. The section begins by demonstrating that several potentially contaminating 25-OHD-binding proteins are eliminated from the purified preparation of the $1,25-(\text{OH})_2\text{D}$ receptor, due to their lack of interaction with DNA. A general experiment then follows which evaluates the utility of a combination of DNA-cellulose, blue dextran-Sepharose, and Sephacryl chromatography in the purification of the $1,25-(\text{OH})_2\text{D}$-receptor. Finally, Polymin P precipitation of the intestinal receptor is subsequently devised which, in conjunction with the previous chromatographic steps and heparin-Sepharose, lead to an 86,000-fold purification of receptor, approximately 50% of homogeneity. The preparation is shown to contain the $1,25-(\text{OH})_2\text{D}$-receptor and 3-5 contaminating protein species. The impact of this procedure is then considered.
Results

Elimination of Serum and Cytosolic Vitamin D-Binding Proteins

The 4.1S serum 25-OHD-binding protein (serum DBP) and the 5.8S cytoplasmic 25-OHD-binding protein (cytosolic DBP) appear in their respective tissue compartments (physiological or artifactual) in vast excess relative to the 1,25-(OH)$_2$D receptor. With respect to this abundance, and because of the similarity of sedimentation value between the 1,25-(OH)$_2$D receptor and the serum DBP (3.3S vs. 4.1S), it was of interest to investigate the behavior of these binding proteins on a chromatographic resin such as DNA-cellulose. Figure 5-1A (see also Figure 4-6) demonstrates that when intestinal cytosol is incubated with 1,25-(OH)$_2$[H]D$_3$ and then chromatographed on DNA-cellulose, the result is the appearance of a macromolecular-bound peak of tritium which can be shown to be the 3.3S 1,25-(OH)$_2$D-receptor complex (Figure 5-1B). However, if this same cytosol is incubated with 25-OH[H]D$_3$ and then chromatographed on DNA-cellulose (Figure 5-2A), 25-OHD associates predominantly with the fraction which does not interact with DNA (a small fraction of 25-OHD associates with a component which does interact and represents the 1,25-(OH)$_2$D receptor which binds 25-OHD$_3$ approximately 1/500 as well as 1,25-(OH)$_2$D$.^3$). Sucrose gradient analysis of this fall through tritium peak reveals the ubiquitous 5.8S cytosolic binding component for 25-OHD$_3$ (Figure 5-2B). Further, Figure 5-3A depicts the results of chick serum incubated with 1,25-(OH)$_2$[H]D$_3$ and then subsequently chromatographed on DNA-cellulose. This component (which also binds 25-OHD$_3$ similarly does not interact with DNA-cellulose, and
Figure 5-1. DNA-Cellulose Chromatography of Chick Intestinal Cytosol Incubated with 1,25-(OH)$_2$D$_3$ and Subsequent Sucrose Gradient Analysis.

(A) Cytosol from 3 grams of intestinal mucosa was ammonium sulfate precipitated, resolubilized in KETT-0, and incubated with 20 nM 1,25-(OH)$_2$[${}^3$H]D$_3$ (7 Ci/mmol) for 1 hour at 0°. The sample was then chromatographed on a 2 x 3 cm DNA-cellulose column as described in Methods. Fractions (3 ml) were collected and an aliquot counted for tritium (O—O). KCL gradient was monitored by conductivity (— —). (B) An aliquot of the DNA-cellulose bound tritium peak was analyzed via sucrose gradient centrifugation (O—O) as in Methods. External markers are: chymotrypsinogen, C (2.5S); ovalbumin, O (3.7S).
Figure 5-2. DNA-Cellulose Chromatography of Chick Intestinal Cytosol Incubated with 25-OH[3H]D₃ and Subsequent Sucrose Gradient Analysis.

Cytosol from 3 grams of intestinal mucosa was ammonium sulfate precipitated, resolubilized in KETT-0, and incubated with 20 nM 25-OH[3H]D₃ (6.7 Ci/mmol) for 1 hour at 0°. The sample was then chromatographed on a 2 x 3 cm DNA-cellulose column as described in Methods. Fractions (3 ml) were collected and an aliquot counted for tritium (O—O). KCL gradient was monitored by conductivity (— — —). (B) An aliquot of the non-interacting fall through tritium was analyzed via sucrose gradient centrifugation (O—O) as described. External markers are as in Figure 5-1.
Figure 5-3. DNA-Cellulose Chromatography of Chick Serum Incubated with 1,25-(OH)$_2$[H]$\text{D}_3$ and Subsequent Sucrose Gradient Analysis.

(A) Serum (0.5 ml) from a rachitic chick was diluted to 2.1 ml with KETT$_0$, and incubated with 20 nM 1,25-(OH)$_2$[H]$\text{D}_3$ (7 Ci/mmol) for 1 hour at 0°. The sample was then chromatographed on a 2 x 3 cm DNA-cellulose column as described in Methods. Fractions (3 ml) were collected and an aliquot counted for tritium (O—O). KCL gradient was monitored by conductivity (— — ). (B) An aliquot of the non-interacting tritium was analyzed via sucrose gradient centrifugation (O—O). External markers were: ovalbumin, 0 (3.7S); bovine serum albumin, BSA (4.4S).
therefore can also be found in the fall through fraction after chromatography. Sucrose gradient centrifugation of an aliquot of this fraction demonstrates the presence of a 4.1S macromolecule bearing 1,25-(OH)$_2$D (Figure 5-3B). Thus, when DNA-cellulose is used in a chromatographic purification scheme for the intestinal 1,25-(OH)$_2$D receptor, both the 4.1S serum DBP and the 5.8S cytoplasmic DBP are eliminated from the preparation. Since initial ammonium sulfate precipitation of intestinal cytosol also serves to partially eliminate these two potential contaminants from intestinal cytosol, these observations assure a DBF-free purified 1,25-(OH)$_2$D-receptor.

Purification of the Intestinal 1,25-Dihydroxyvitamin D Receptor: Sequence 1

The receptor for 1,25-(OH)$_2$D requires an approximately 200,000-fold purification from intestinal cytosol to achieve homogeneity. Thus, the first step in the purification of the intestinal receptor was to test the combined power of the primary columns, DNA-cellulose, blue dextran-Sepharose, and Sephacryl as described in Chapter 4. Sufficient intestinal tissue was used so that enough protein would be present in the final purified material to permit accurate receptor specific activity assessment. The purification scheme is outlined in Figure 5-4 and summarized in Table 5-1. Cytosol from 250 g of rachitic chick intestinal mucosa (containing 7.457 g of protein) was prepared, an aliquot tested for 1,25-(OH)$_2$D-receptor binding activity, and the remaining solution precipitated with ammonium sulfate at 40% of saturation. The sediment was resolubilized in two batches, each containing 125 g equivalent of intestinal mucosa in 200 ml KETT-0. Total protein at this
SEQUENCE 1

INTESTINAL MUCOSA
(250 g)
\[\begin{align*}
&\text{Cytosol} \quad \downarrow \\
&\text{Ammonium Sulfate} \quad \downarrow \\
&\text{Precipitate} \quad \downarrow \\
&\text{Resolubilize (125 g in 200 ml KETT-0)} \quad \downarrow \\
&\text{DNA-cellulose (5 x 13 cm)} \quad \downarrow \\
&\text{Sephacryl (1.6 x 60 cm)} \quad \downarrow \\
&\text{Blue Dextran-Sepharose (2.5 x 5 cm)} \quad \downarrow \\
&\text{DNA-cellulose (2 x 3 cm)} \quad \downarrow \\
&\text{Hydroxylapatite (Concentration)} \quad \downarrow \\
&\text{Test} 
\end{align*}\]

Figure 5-4. Methodology for the Purification of the Chick Intestinal 1,25-(OH)_2D Receptor: Sequence 1.
Table 5-1. Purification of the Chick Intestinal Receptor for $1,25\text-(OH)_2\text{D}$: Sequence 1

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Receptor (cpm x 10^{-6})</th>
<th>Spec. Act. (cpm x 10^{-3}/mg Prot.)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>7475.0</td>
<td>-</td>
<td>0.58</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ammonium Sulfate (40% sat.)</td>
<td>2217.0</td>
<td>4.10</td>
<td>1.80</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>DNA-Cellulose (0.28 M)</td>
<td>16.7</td>
<td>4.04</td>
<td>239</td>
<td>99</td>
<td>400</td>
</tr>
<tr>
<td>Sephacryl</td>
<td>3.8</td>
<td>2.87</td>
<td>743</td>
<td>71</td>
<td>1,300</td>
</tr>
<tr>
<td>Blue Dextran-Sepharose (0.4 M)</td>
<td>0.44</td>
<td>1.38</td>
<td>3,136</td>
<td>34</td>
<td>5,400</td>
</tr>
<tr>
<td>DNA-Cellulose (0.28 M)</td>
<td>0.072</td>
<td>0.97</td>
<td>26,944</td>
<td>24</td>
<td>23,250</td>
</tr>
</tbody>
</table>

For details to purification, see Results. Initial DNA-cellulose: 180 ml/hr flow rate, 600 ml linear gradient of 0.1-0.8 M KCl, 15 ml fractions; Sephacryl: 60 ml/hr ascending flow rate in KETT-0.30, 3 ml fractions; blue dextran-Sepharose: 60 ml/hr flow rate, 240 ml linear gradient from 0.2-0.6 M KCl, 5 ml fractions; DNA-cellulose: 60 ml/hr flow rate, 120 ml linear gradient from 0.1-0.6 M KCl, 3 ml fractions.
stage was 2.217 g, and a specific binding assay for the receptor revealed a 3-fold enhancement of specific activity. The batches were then separately labeled with a saturating concentration (24nM) of 1,25-(OH)$_2$-[${}^3$H]D$_3$ (7.7 Ci/mmol added in ethanol, 5% v/v) for 12 hours at 0°C, and each subsequently chromatographed on DNA-cellulose (5 x 13 cm). Composite chromatography is seen in Figure 5-5A. Each sample was applied to DNA-cellulose, the resin washed extensively, and then the receptors eluted from the resin during a linear KCl gradient at 0.28M (see also Figure 4-6). The separate pooled receptor peaks from each DNA-cellulose chromatography were salt precipitated, resolubilized in 1 ml KETT-0.3, and each resolved by gel filtration on a 1.6 x 60 cm Sephacryl column (Figure 5-5B). After this step, the two pooled receptor peaks were ammonium sulfate precipitated and collectively resolubilized in KETT-0.2 (6 ml). Measurement of protein at this stage relative to 1,25-(OH)$_2$D binding activity revealed an approximate 1300-fold enhancement of receptor specific activity. The receptor preparation was then subjected to blue dextran-Sepharose chromatography (2.5 x 5 cm) and eluted from this resin during a KCl gradient (Figure 5-5C). Dilution with KETT-0 reduced the KCl concentration in the pooled blue dextran-Sepharose peak to 0.1M, and it was subsequently rechromatographed on DNA-cellulose (Figure 5-5D). Prior to this step, a 5,400-fold purification of the intestinal receptor had been achieved. Final purification of the receptor-1,25-(OH)$_2$D complex was approximately 23,250-fold in 24% yield representing approximately 10% of purity.

In order to verify that the receptor for 1,25-(OH)$_2$D was present during purification, sucrose gradient analysis of an aliquot was
Figure 5-5. Composite of the Chromatography Employed in the Purification of the Chick Intestinal 1,25-(OH)₂D Receptor: Sequence 1.

(A) DNA-cellulose chromatography (5 x 13 cm) of 125 gram equivalents of intestinal mucosal cytosol ammonium sulfate precipitated, resolubilized in KETT-0, and labeled with 24 nM 1,25-(OH)₂[³H]D₃ (7.7 Ci/mmol) for 12 hours at 0°C. (B) Sephacryl chromatography (1.6 x 60 cm) of the concentrated DNA-binding peak depicted in A. (C) Blue dextran-Sepharose chromatography (2.5 x 5 cm) of combined, tritium-containing peak in B. (D) Final DNA-cellulose chromatography (2 x 3 cm) of blue dextran-Sepharose peak in C. For detail, see Methods and text in Chapter 5.
Figure 5-5. Chromatography in the Purification of Chick Intestinal 1,25-(OH)$_2$D Receptor: Sequence 1.
performed after each isolation step. The receptor, purified after the second DNA-cellulose chromatography (23,250-fold), was concentrated on a 0.8 x 1 cm hydroxylapatite column to 1 ml prior to sucrose gradient centrifugation. The data in Figure 5-6 indicate that the 3.3S receptor is present after each procedure, that it retains 1,25-(OH)$_2$D, and that it is not physically altered relative to its sedimentation value.

These data suggest that the 1,25-(OH)$_2$D-receptor complex can be purified significantly through the concerted use of group selective affinity resins DNA-cellulose and blue dextran-Sepharose, and gel filtration on Sephacryl. This purification is over 30-fold greater than that achieved previously by ion exchange and blue dextran-Sepharose chromatography alone (125). However, it is still 10-fold short of isolating the 1,25-(OH)$_2$D-receptor complex in homogeneous form.

Polymin P Precipitation

The above observations indicated that in order to achieve further purification of the 1,25-(OH)$_2$D-receptor complex, an initial more selective precipitation procedure had to be incorporated into the isolation scheme. This was deemed necessary in order to cope with the chromatography of protein derived from at least 1 kg of intestinal tissue. The selective precipitation procedure developed employs Polymin P (a polyethylenimine), a compound used to precipitate negatively charged macromolecules in solution. The results depicted in Figure 5-7A indicate that the 1,25-(OH)$_2$D receptor from intestinal cytosol can be rendered insoluble by the addition of Polymin P (0.08% v/v), and subsequent sedimentation at low centrifugal force leaves the receptor
Figure 5-6. Sucrose Gradient Analysis of the Intestinal 1,25-(OH)₂D-Receptor Complex during Sequence 1 Purification.

Aliquots of 1,25-(OH)₂D-receptor complex were analyzed via sucrose gradient centrifugation (○—○) as described in Methods, after the DNA-cellulose purification step (A), the Sephacryl step (B), the blue dextran-Sepharose step (C), and the final DNA-cellulose step (D). C and D include sucrose gradient analysis of cytosol labeled for 1 hour at 0° with tritiated hormone. External markers are as in Figure 5-1.
Figure 5-6. Analysis of the Intestinal 1,25-(OH)$_2$D-Receptor Complex during Sequence 1 Purification.
Figure 5-7. Polymin P Precipitation of the Intestinal 1,25-(OH)\textsubscript{2}D Receptor and Subsequent Elution with Potassium Chloride.

(A) The indicated volume of 10% Polymin P was added to 1 ml aliquots of prepared cytosol, thoroughly mixed and incubated for 10 minutes. After centrifugation at 16,000 x g for 10 minutes, 0.1 ml of the supernatant was assayed in duplicate for 1,25-(OH)\textsubscript{2}D-binding activity (O—O) by the DEAE-Filter technique described in Methods. (B) 0.24 ml of 10% Polymin P was added with complete mixing to 30 ml of intestinal cytosol. After 10 minutes, the sample was divided into 1 ml aliquots, and each centrifuged 16,000 x g for 10 minutes. The supernatants were then discarded, and the pellets resuspended with a Dounce homogenizer in 1 ml of KETT-buffer containing the indicated KCl concentration. After 10 minutes, the mixture was centrifuged 16,000 x g for 10 minutes, and the resultant supernatant assayed for 1,25-(OH)\textsubscript{2}D-binding activity (O—O) as above. In both cases, an aliquot of the supernatant was assayed at 650 nm for protein (O—O) as in Methods.
in pellet form. Since Polymin P is a positively charged polymer, the receptor can also be selectively released from this macromolecule with salt. Figure 5-7B demonstrates that if the pellet is resolubilized in KETT-0.5, after brief centrifugation, the receptor remains in the supernatant. This procedure, coupled with ammonium sulfate precipitation, purifies the receptor for 1,25-(OH)₂D 10-15 fold in approximately 50% yield. Thus, it allows over 1000 g of intestinal mucosa to be processed in single chromatographic steps (unlike Scheme 1), and potentially eliminates proteins found in the purified material derived from Sequence 1.

Purification of the Intestinal 1,25-Dihydroxyvitamin D Receptor: Sequence 2

The procedures in Sequence 1 achieve a purification of the 1,25-(OH)₂D-receptor approximately 10% of theoretical homogeneity. Thus, Polymin P precipitation of intestinal mucosal cytosol, group selective affinity, gel filtration, and heparin-Sepharose chromatography (as described in Chapter 4) were next tested in an effort to purify this rare intestinal protein. The purification scheme is outlined in Figure 5-8 and the results are summarized in Table 5-2. Approximately 800 g of intestinal mucosa was harvested from 350 rachitic chick intestines, homogenized, and the cytosolic fraction prepared. After an aliquot of cytosol was assayed for receptor specific activity, the remainder of this solution was precipitated with a 0.04-0.08% Polymin P cut. Briefly, cytosol was made 0.04% with Polymin P (v/v) and the precipitate discarded. The supernatant was then made 0.08% with Polymin P (v/v), and the pellet (containing receptor) retained. The pellet was extracted with
SEQUENCE 2

Intestinal Mucosa
(800 g)
↓
Cytosol
↓
Polymin P
(0.04 - 0.08%)
↓
Resolubilize
(100 ml KETT-0)
↓
DNA-cellulose
(5 x 13 cm)
↓
Sephacryl
(1.6 x 60 cm)
↓
Blue Dextran-Sepharose
(2.5 x 5 cm)
↓
DNA-cellulose
(2 x 3 cm)
↓
Heparin-Sepharose
(2.5 x 2 cm)
↓
Hydroxylapatite
(concentration)
↓
Test

Figure 5-8. Methodology for the Purification of the Chick Intestinal 1,25-(OH)₂D Receptor: Sequence 2.
Table 5-2. Purification of the Chick Intestinal Receptor for 1,25-(OH)₂D₃: Sequence 2

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Receptor (cpm x 10⁻⁶)</th>
<th>Spec. Act. (cpm x 10⁻³/mg Prot.)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosol</td>
<td>22,400.0</td>
<td>-</td>
<td>0.58</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Polymin P (Precipitate)</td>
<td>1,170.0</td>
<td>9.10</td>
<td>7.69</td>
<td>100</td>
<td>13</td>
</tr>
<tr>
<td>DNA-Cellulose (0.28M)</td>
<td>19.8</td>
<td>9.00</td>
<td>454.50</td>
<td>99</td>
<td>780</td>
</tr>
<tr>
<td>Sephacryl</td>
<td>-</td>
<td>6.35</td>
<td>-</td>
<td>70</td>
<td>-</td>
</tr>
<tr>
<td>Blue Dextran-Sepharose (0.4M)</td>
<td>0.85</td>
<td>4.50</td>
<td>5,290.00</td>
<td>49</td>
<td>9,100</td>
</tr>
<tr>
<td>DNA-Cellulose (0.28M)</td>
<td>0.216</td>
<td>2.75</td>
<td>12,700.00</td>
<td>30</td>
<td>22,000</td>
</tr>
<tr>
<td>Heparin-Sepharose (0.20M)</td>
<td>0.026</td>
<td>1.30</td>
<td>50,000.00</td>
<td>14</td>
<td>86,000</td>
</tr>
</tbody>
</table>

For details to purification see Results. Initial DNA-cellulose: 180 ml/hr flow rate, 600 ml linear gradient of 0.1-0.8 M KCl, 15 ml fractions; blue dextran-Sepharose: 60 ml/hr flow rate, 240 ml linear gradient from 0.2-0.6 M KCl, 5 ml fractions; DNA-cellulose: 60 ml/hr flow rate, 120 ml linear gradient from 0.1-0.6 M KCl, 3 ml fractions; heparin-Sepharose: 50 ml/hr flow rate, 100 ml linear gradient from 0.05-0.4 M KCl, 3 ml fractions. Gel filtration on Sephacryl was achieved in KETT-0.3 at an ascending flow rate of 60 ml/hr, 3 ml fractions.
KETT-0.5 to resolubilize the receptor, and after brief centrifugation to remove insoluble material, the resultant supernatant was ammonium sulfate precipitated. This precipitate was then resolubilized in 100 ml of KETT-0 and labeled with a saturating concentration (35 nM) of $^{3}\text{H}D$ for 12 hours at $0^\circ$. Protein analysis at this stage revealed a reduction in cytosolic protein from 22.4 g to 1.17 g, and an enhancement of receptor specific activity of 13-fold. Sequential chromatographic steps then followed (see Figure 5-9). After dilution of the sample to 0.1M salt, the preparation was applied to DNA-cellulose (5 x 13 cm), the column washed with KETT-0.1, and then the receptor eluted from the resin during a KCl gradient. Protein was reduced by this procedure to 19.8 mg allowing a cumulative 780-fold purification of the $^{2}$,25-(OH) D-receptor complex. The pooled receptor peak was then salt precipitated, resolubilized in 1 ml KETT-0.3, and resolved by gel filtration on Sephacryl. Next, the receptor was chromatographed on blue dextran-Sepharose. The sample was diluted to 0.15M KCl with KETT-0, and then applied directly to a blue dextran-Sepharose column (2.5 x 4 cm). After washing the resin with KETT-0.2, the receptor was eluted during a linear standard KCl gradient. Purification after this procedure was calculated to be 9,100-fold (Table 5-2). The pooled receptor preparation was then similarly chromatographed again on DNA-cellulose and then finally on heparin-Sepharose (Figure 5-9). Final purification, as determined by enhancement of specific activity over intestinal cytosol, was approximately 86,000-fold in 14% yield. This value suggests that the receptor is nearly 50% homogeneous on the basis of theoretical calculations (see Discussion).
Figure 5-9. Composite of the Chromatography Employed in the Purification of the Chick Intestinal 1,25-(OH)₂D Receptor: Sequence 2.

(A) DNA-cellulose chromatography of 800 gram equivalents of intestinal mucosal cytosol precipitated with Polymin P, resolubilized in KETT-0, and labeled with 35 nM 1,25-(OH)₂[³H]D₃ (7 Ci/mmol) for 12 hours at 0°C. (B) Sephacryl chromatography of purified receptor peak from A. (C) Blue dextran-Sepharose chromatography of purified receptor peak from B. (D) DNA-cellulose rechromatography of purified receptor peak from C. (E) Heparin-Sepharose chromatography of purified receptor peak from D. For details, see text, Chapter 5.
Figure 5-9. Chromatography of Purification of Chick Intestinal 1,25-(OH)$_2$D Receptor: Sequence 2.
The post heparin-Sepharose purified material (concentrated as in Sequence 1 with hydroxylapitate) was then subjected to analysis by SDS-gel electrophoresis to determine the number of protein species present in the preparation (Figure 5-10). In addition, protein from the two previous steps were also similarly analyzed. Aliquots of protein (10 μg) representing approximately 1% of the blue dextran-Sepharose purified sample (Lane 2), 5% of the DNA-cellulose purified sample (Lane 3), and 40% of the heparin-Sepharose purified sample (Lane 4), were denatured with 1% sodium dodecyl sulfate and electrophoresed on an 11% polyacrylamide slab gel. Accompanying the electrophoretic separation of samples were standard proteins (Lane 1,5). The results indicate a purification of proteins during the final three steps in this procedure, with the final heparin-Sepharose sample (Lane 4) containing approximately 8-10 protein bands, four of which represent 95% of the total protein. On the basis of total receptor bound hormone present just prior to electrophoresis, it is likely that one of these major protein species represents the purified receptor protein, although due to the technique, the receptor cannot be identified. It is significant, however, that each of these protein bands is well within the molecular weight anticipated for the 1,25-(OH)₂D receptor on the basis of previous work (102).

Sucrose gradient analysis of the last two purification steps was also performed to confirm the presence of the 1,25-(OH)₂D-receptor complex. As depicted in Figure 5-11, post DNA-cellulose and post heparin-Sepharose purified samples contain a macromolecule which sediments at 3.3S, and binds and retains 1,25-(OH)₂D₃. Importantly, a similar
Protein samples were prepared and subjected to electrophoresis as described in Methods. Flanking lanes (1,5) represent standard proteins of known MW, and are represented by 1: phosphorylase a, 94,000; 2: BSA, 68,000; 3: pyruvate kinase, 57,000; 4: aldolase subunit, 40,000; 5: carbonic anhydrase, 29,000; 6: myoglobin, 18,000. Lane 2 represents 10 μg of protein derived from the blue dextran-Sepharose purified step, 1% of sample. Lane 3 represents 10 μg of protein derived from the second DNA-cellulose purified step, 5% of sample. Lane 4 represents 8-10 μg of protein derived from the final heparin-Sepharose purified step, 40% of sample. The slab gel was stained as described in Methods.
Figure 5-11. Sucrose Gradient Analysis of the Intestinal 1,25-(OH)_2D-Receptor Complex after Sequence 2 Purification.

Aliquots of the 1,25-(OH)_2D-receptor complex were analyzed via sucrose gradient centrifugation (○—○) as described in Methods, after the second DNA-cellulose step (A) and the heparin-Sepharose step (B). B also contains the results of sucrose gradient analysis (□—□) of cytosol labeled for 1 hour at 0°C with 1,25-(OH)_2[^3H]D_3 (110 Ci/mmol). External markers were as in Figure 5-1.
concurrent analysis of crude cytosol (see Figure 5-11) confirms the similarity in sedimentation value between the crude cytosol receptor and the purified 1,25-(OH)$_2$D receptor.

**Discussion**

The importance of purifying the intestinal receptor for 1,25-(OH)$_2$D$_3$ has been briefly considered in both the Introduction and in Chapter 4. This protein apparently functions within the nucleus (102, 103) to direct the synthesis of RNA (101,102), although details of this action are unavailable. Certainly, investigations of the receptor for 1,25-(OH)$_2$D$_3$ have been directed primarily towards the eventual elucidation of its action within the intestinal nucleus. This requires the initial purification of the receptor component, an approach several research groups have taken in defining the action of steroid hormones such as estrogen and progesterone (96,97). Although other components necessary for a functional study of the 1,25-(OH)$_2$D$_3$ receptor remain to be isolated, including both DNA fragments containing 1,25-(OH)$_2$D$_3$-activated genes and mRNA's to 1,25-(OH)$_2$D$_3$-dependent proteins, the purification of a functionally active receptor molecule appears to be the most difficult. Ultimately, the reconstitution of 1,25-(OH)$_2$D$_3$-receptor complexes with intestinal nuclei or chromatin should produce results which may reveal the exact nature of 1,25-(OH)$_2$D to induce protein products active in mineral uptake and homeostasis.

Progress in the purification of the chick intestinal receptor for 1,25-(OH)$_2$D has been slow, generally hampered by its lack of abundance in the intestine and by its extreme lability, particularly as
purification proceeds. The receptor requires approximately a 200,000-fold enhancement of specific activity over cytosol to attain homogeneity. This theoretical calculation is based upon two assumptions: 1) the molecular weight of the receptor (50,000), and 2) a single binding site on the receptor for 1,25-(OH)\(_2\)D. The high degree of purification needed is, of course, due to the paucity of 1,25-(OH)\(_2\)D receptor proteins in intestinal tissue (although tissues such as pancreas, parathyroid glands, and pituitary contain even less receptor). In fact, the receptor exists as approximately 0.001% or less of the total protein in the cytoplasmic fraction (200-250 μg receptor per kilogram of intestinal tissue). Although one can calculate that this represented 1-2 x 10\(^3\) molecules of receptor per cell, this calculation assumes that the receptor exists equivalently in all intestinal cells, an assumption which is probably not the case. Thus, a final yield of receptor-hormone complexes of 5-10% after a chromatographic purification scheme leaves 5-15 μg of receptor per kilogram of starting intestinal material available for analysis. To compound this problem, the cytosolic receptor demonstrates an extreme lability to temperature, and must be maintained routinely at 0-4°. Although the lability of the receptor is much reduced in combination with hormone, free receptor molecules decay rapidly and cannot be subsequently labeled with hormone. Finally, any degree of purification enhances the tendency for the receptor-hormone complex to dissociate, leading to a rapid deterioration of receptor capacity to bind 1,25-(OH)\(_2\)D.

The present effort to purify the 1,25-(OH)\(_2\)D receptor has utilized the intestine from 4-6 week old rachitic chicks as a receptor
source. Preparations derived from rachitic chicks benefit from the primary advantage that titers of endogeneous $1,25-(\text{OH})_2D$ are virtually nonexistent. Thus, the concentration of $1,25-(\text{OH})_2D$ receptors with available sterol binding sites is maximal, providing a unique advantage in assessing the overall extent of purification of the receptor protein. The use of intestinal tissue in the isolation scheme is due to three factors: 1) the extent of tissue available (3-5/chick g), 2) the observation that titers of receptor in intestine are 5-10 fold greater than in other tissues examined, and 3) the fact that more is known about the chick intestinal receptor than its form in tissues such as parathyroid gland, pituitary, and pancreas.

A number of chromatographic approaches have been taken both toward the purification of steroid hormone receptor molecules in general, and the $1,25-(\text{OH})_2D$ receptor in particular.

1) The most selective technique, in theory, is true affinity chromatography, taking advantage of the selective and highly specific interaction between a protein and a ligand or substrate. Unfortunately, in practice, the use of immobilized steroidal ligands for the purification of steroid receptors has generally been unsatisfactory, primarily because of extensive nonspecific binding, increased lability of hormone-free receptors, and the tendency for crude tissue cytosols to metabolize the immobilized ligand. In the case of the $1,25-(\text{OH})_2D$ receptor, initial attempts (228) have been completely unsuccessful.
2) Ion exchange and adsorption chromatography have also been employed in the purification of a number of steroid hormone receptors (199,229). However, the use of DEAE-cellulose, phosphocellulose, and hydroxylapatite have generally proved to be inadequate in providing significant purification of the 1,25-(OH)$_2$D receptor (125).

3) An alternative technique for the purification of a host of proteins which interact with nucleotides and nucleic acids has been the use of DNA-cellulose, blue dextran-Sepharose, and heparin-Sepharose. DNA cellulose has been used to characterize and purify a number of steroid hormone receptors and nuclear proteins including the progesterone subunit A (209) the glucocorticoid receptor (194), and the androgen receptor (208). In fact, the progesterone A receptor which demonstrates an extensive number of characteristics similar to the 1,25-(OH)$_2$D receptor (209), has been shown to function specifically in vitro by binding to purified DNA (229) and to cloned sequences of the ovalbumin gene (M. Hughes, personal communication), the piece of DNA it is known to induce in the oviduct in vivo (230). Blue dextran has also been used to study and purify an extensive number of dehydrogenase enzymes (132,231) and proteins which interact with DNA, including RNA-polymerase (200) and DNA-polymerase (201). Finally, although its mode of interaction is not known, heparin-Sepharose has been successfully used in the purification of the "native" form of the
estrogen receptor (132,204). Certainly these resins have particular utility in achieving a major purification of the intestinal receptor for 1,25-(OH)_2D. Whether they are capable of purifying the receptor to homogeneity, however, remains to be determined. Nevertheless, as considered more thoroughly in the previous chapter, the binding of this receptor to DNA and blue dextran imply certain functional features of the receptor molecule during its presumed action on the intestinal cell genome.

The receptor for 1,25-(OH)_2D is purified by the chromatographic techniques described in Sequence 2 over 86,000-fold, which is approximately 50% pure on theoretical grounds (Table 5-2). It contains one major and three minor protein species on SDS-gel electrophoresis, with molecular weight between 50,000-65,000 (Figure 5-10). These weights are well within the range expected for the 1,25-(OH)_2D receptor (102). The purified sample does contain 1,25-(OH)_2D specifically bound to a 3.3S macromolecule (Figure 5-11), although this protein species cannot be identified on the gel due to denaturation. This will most likely require the application of nondenaturing gel electrophoresis discussed in the previous chapter. Certainly, the fact that the crude 1,25-(OH)_2D receptor can be successfully electrophoresed suggests that this technique is promising. Interestingly, it is conceivable that proteins which contaminate the preparation are receptors for other steroid hormones. A battery of these hormones do impinge on the intestine as a target tissue, and it should be expected that their receptors would be similarly purified on selective affinity resins such as DNA-cellulose
and blue dextran-Sepharose. If this is the case, ultimate purification of the 1,25-(OH)$_2$D receptor with these procedures may prove difficult. Nevertheless, the partial isolation of the 1,25-(OH)$_2$D-receptor complex by the concerted use of these unique and powerful resins is a significant advance in the isolation of this interesting macromolecule.

Although the purification techniques described in this chapter are capable of a major isolation of the intestinal 1,25-(OH)$_2$D receptor, they do not result in homogeneity. Thus, the primary impact of procedures such as Polymin P precipitation, and DNA-cellulose and blue dextran-Sepharose chromatography lie in their power to rapidly enrich the population of 1,25-(OH)$_2$D receptors (10-50% of purity) from large amounts of chick intestinal tissue. Certainly, as seen in Table 5-2, Polymin P precipitation and DNA-cellulose chromatography are capable of reducing nearly a kilogram equivalent of intestinal cytosolic protein (22g) to less than 20 mg. This represents a major advance in providing critically needed first steps for the subsequent homogeneous purification of the intestinal receptor. The capability of these initial steps allows a tremendous latitude in the selection of final isolation steps. For example, techniques such as preparative gel electrophoresis and/or isoelectric focusing might be employed. Alternatively, one could envision the purification of hormone-free receptors by Polymin P and DNA-cellulose (the unliganded receptor does bind to DNA), and then subsequent complete isolation through true ligand affinity chromatography. Certainly prepurification to the extent achievable by these procedures would most likely eliminate the major problems which accompany affinity chromatography. Finally, the partially purified receptor could be used
to prime mice for the development of a monoclonal antibody strain, capable of being utilized not only for final purification but also for subsequent characterization. Thus, although the procedures described here appear to fall short of homogeneous isolation of the 1,25-(OH)$_2$D receptor, their power appears to offer a number of viable alternatives in the final isolation.

**Summary**

The rachitic chick intestinal receptor for 1,25-(OH)$_2$D has been purified to 50% of homogeneity by a combination of techniques which include Polymin P precipitation and group selective affinity chromatography. The final purified preparation contains the receptor and several other contaminating protein species. Although the protein remains to be purified to homogeneity, the present isolation scheme is not only powerful, but the initial steps are amenable to a number of subsequent procedures which might be capable of achieving final purity. With this accomplished, both physical and functional studies of this receptor protein can be initiated. It is expected that these experiments will be fruitful in understanding the means whereby steroid hormones in general and 1,25-(OH)$_2$D in particular act to stimulate the production of certain proteins.
CHAPTER 6

GENERAL SUMMARY

The data described in this dissertation contribute to the elucidation of mechanisms whereby vitamin D acts to regulate the homeostasis of calcium and phosphorus. These mechanisms involve two primary events. The first is the metabolism of vitamin D and the metabolic events which surround the conversion of its intermediate metabolite 25-OHD$_3$ to 1,25-(OH)$_2$D$_3$, the active hormonal form. The second event is the steroid hormone-like action of 1,25-(OH)$_2$D$_3$ on the target cell, where the hormone orchestrates the events which dictate the synthesis of proteins biologically active in mineral uptake and regulation.

Chapter 3 contains the results of experiments which demonstrate that during periods of high mineral metabolism such as growth and reproduction, the level of 1,25-(OH)$_2$D is maintained considerably above normal (Figure 3-8, Tables 3-1,3-2,3-3). It seems likely that these high levels of hormone are responsible for the timely elevation in intestinal absorption of calcium and phosphorus observed during these physiological states. Hormones responsible for increasing the biosynthesis of 1,25-(OH)$_2$D appear to include prolactin (and placental lactogen) (Figures 3-9,3-14, Tables 3-5,3-9), growth hormone (Tables 3-4,3-8), estrogen (Figures 3-11,3-12, Tables 3-6,3-7), and parathyroid hormone (Table 3-10). Whether the action of each of these potential regulators is direct (influences the renal enzymes) or indirect (via intermediates)
remains to be unequivocally demonstrated. However, it is clear that
in the case of prolactin and growth hormone, their effects on vitamin
D metabolism are manifested predominantly during specific physiological
settings (Tables 3-8,3-9). Further, the stringent regulation of $1,25-(OH)_2D$
is exemplified by the highly dynamic nature of this hormone as it
fluctuates coincident with the mineral demands of the organism during
pregnancy (Figures 3-16,3-17) and lactation (Figure 3-17). Thus, the
observations made in this chapter not only emphasize the complexity of
events which mediate control of vitamin D metabolism, but support the
general contention that $1,25-(OH)_2D$ is the active hormonal metabolite of
vitamin D.

$1,25-(OH)_2D$ modifies the general pattern of cellular RNA bio-
synthesis through the agency of a specific, high affinity binding pro-
tein. The receptor-sterol complex is formed in the cytoplasm, and then
accumulates within the nucleus where it dictates a specific alternation
in RNA and protein synthesis. Chapter 4 outlines the results of experi-
ments designed to further describe properties of these $1,25-(OH)_2D$-
binding components. Previously detected in intestine and parathyroid
glands, the intestinal form binds $1,25-(OH)_2D$ with high affinity
($K_d = 6 \times 10^{-10}$ M) (Figure 4-3), sediments at 3.3S (Figures 4-1,4-2),
and binds to homologous chromatin in a time dependent fashion (Figure
4-4). The receptor-hormone complex also interacts with DNA (Figure
4-6), blue dextran (Figure 4-7), and heparin (Figure 4-8) ligands, in
addition to ion exchange (Figure 4-5) and adsorption resins (Figure
4-5). Importantly, its binding to DNA and blue dextran affinity
ligands offers potential insight into the protein's function in the
cell nucleus in vivo. In addition, the receptors characteristic elu-
tion patterns from these gels add to the accumulating body of knowledge
about this protein. The further utility of these techniques in identi-
fying and purifying this component for 1,25-(OH)₂D is described below.

A relationship between the modulation of 1,25-(OH)₂D biosyn-
thesis by pituitary, placental, and pancreatic hormones considered in
part in Chapter 3 is further supported by experiments documented in
Chapter 4. By employing DNA-cellulose affinity chromatography and su-
crose gradient analysis, as outlined for the intestinal receptor, identi-
cal components for 1,25-(OH) D are described in the pituitary (Figure
2 4-16) and pancreas (Figure 3-13). They elute from the DNA affinity li-
gand similarly to the intestinal receptor, demonstrate hormone binding
at very low sterol concentrations, and sediment at 3.3S. By analogy,
these components probably represent receptors for 1,25-(OH)₂D. Their
presence in pituitary and pancreas most likely represents a means of
negative feedback expression, as postulated for the parathyroid glands
(110). Thus, elevated 1,25-(OH)₂D levels serve as a signal, through
specific receptors, to depress the synthesis of hormones responsible for
the original sterol elevation. With respect to the placenta, however,
1,25-(OH)₂D receptors may influence the transepithelial transport of
mineral in addition to their potential influence on placental lactogen
release. Complete elucidation of receptors and the role of 1,25-(OH)₂D
in the functioning of each of these tissues, nevertheless, remains to be
be accomplished. Importantly, the results revealed in this chapter
predict a potentially wider distribution of effects for 1,25-(OH)₂D
than previously believed.
One of the most important current goals of vitamin D research is the isolation and subsequent study of the functional and physical properties of the receptor for 1,25-(OH)₂D. Despite the difficulties encountered in its isolation, Chapter 5 details results of experiments which utilize the chromatographic techniques described in Chapter 4 in combination with Polymin P precipitation to purify the rachitic chick intestinal 1,25-(OH)₂D-receptor. A sequential procedure has been developed which is capable of purifying the receptor to approximately 50% of homogeneity in 10-15% yield (Table 5-2, Figure 5-9). The purified preparation appears to contain both the receptor (Figure 5-11) and several contaminating protein species (Figure 5-10). The present level of receptor isolation should allow 1) further purification of the protein to homogeneity, 2) the isolation of hormone-free receptor, and 3) the study of both physical and functional properties of this receptor protein. An investigation of the latter aspects of the 1,25-(OH)₂D receptor should prove exciting in further defining the molecular action of 1,25-(OH)₂D within the target cell nucleus. More importantly, however, elucidation of receptor function will most likely contribute to significant knowledge of a general nature on the mechanism whereby steroid hormones influence the expression of DNA.

A considerable portion of the data compiled in this dissertation has been published by the author (232-238).
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