THE ISOLATION AND QUANTITATION OF
1α,24R,25-TRIHYDROXYVITAMIN D FROM PLASMA

by

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STATEMENT BY AUTHOR

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ABSTRACT

The experiments described in this monograph involve the development of a procedure for the isolation of a recently characterized vitamin D metabolite, \( \text{Ia}, 24R, 25 \)-trihydroxyvitamin D, from biological fluids. The application of a modified competitive ligand binding (radioreceptor) assay originally designed for \( \text{Ia}, 25 \)-dihydroxyvitamin D\(_3\) has been successful in the quantitation of \( \text{Ia}, 24R, 25 \)-trihydroxyvitamin D concentrations. Purification of the trihydroxy-metabolite from the biological sample is required prior to assay. The scheme devised consists of Sephadex LH-20 chromatography and high pressure liquid chromatography. It is feasible for human studies in that a minimum of 10 milliliters of plasma is required. With the use of this procedure, plasma levels of \( \text{Ia}, 24R, 25 \)-trihydroxyvitamin D have been measured for the first time in humans, cows, rats and chickens. This metabolite has been previously detected in humans and rats by using injections of radioactive precursors, but the total plasma levels were not quantitated. In a published report, tracer studies failed to detect \( \text{Ia}, 24R-25 \)-trihydroxyvitamin D\(_3\) in the chicken, but the present radioreceptor assay indicated that the chick possesses significant circulating levels of the trihydroxy-metabolite. In rats the circulating level of this metabolite is equal to the \( \text{Ia}, 25 \)-dihydroxyvitamin D level, and although this level is lower than the dihydroxy-metabolite level in
humans, it is present in significant quantities. In conclusion, 1α, 24R, 25-trihydroxyvitamin D is a quantitatively significant vitamin D metabolite. However, it is not clear whether it represents the initial sterol in the degradation pathway of the 1α, 25-dihydroxyvitamin D hormone or is itself an important physiological mediator of mineral metabolism. The assay described here will be useful in defining the role of 1α, 24R, 25-trihydroxyvitamin D in health and disease.
CHAPTER 1

INTRODUCTION

Vitamin D

The field of vitamin D has evolved from several related disciplines including nutrition, physiology, and biochemistry. The contributions of each field are highly interrelated, and the information presented here summarizes the contributions of each. In 1906 it was known that the disease rickets, which is a condition characterized by abnormal bone ossification, was caused by the lack of some dietary agent (1). By 1924 (2, 3, 4, 5, 6) it had been established that vitamin D was responsible for the prevention of rickets. It has since been established that vitamin D accomplishes this by stimulating intestinal calcium transport, by increasing bone calcium mobilization or possibly by a direct action of the calcification process, and by stimulating a phosphorus-transporting mechanism independent from that of calcium.

An organism can obtain vitamin D from the diet (vitamin D$_2$) or from the irradiation of the precursor, 7-dehydrocholesterol (yielding vitamin D$_3$), which is present in the skin. The recommended daily requirement of vitamin D$_3$ in humans is 100 I.U. to 400 I.U. One I.U., an international unit, of vitamin D$_3$ is $0.025 \mu g$ or $65 \times 10^{-12}$ moles (65 pmoles). The chick and the rat require 10 I.U. daily (7).
The Metabolism of Vitamin D

Vitamin D must be metabolically activated before functioning biochemically in its target tissues (8, 9). The metabolism of vitamin D is characterized by a series of hydroxylations. The first step in the metabolism of vitamin D as shown in figure 1, is the hydroxylation, on carbon 25, by a hydroxylase that is found in the liver, kidney and intestine (10). Hydroxylation on carbon 1, which occurs solely in the kidney (11), produces 1α, 25-dihydroxyvitamin D, a novel sterol hormone, which is responsible for alleviating the physiological demands of calcium and phosphate need. This second hydroxylation produces the most active form of vitamin D; i.e., this metabolite is more potent than either of its precursors with regard to eliminating rickets. Because 1α, 25-dihydroxyvitamin D is the most biologically active vitamin D metabolite, is produced in the kidney with the intestine and bone as target sites, and is present in low circulating concentrations, it may be classified as a hormone.

This hormone, 1, 25-(OH)\(_2\)D\(_3\), (see Appendix 1 for the explanation of the abbreviations used) is not the only product of 25-(OH)D\(_3\). Other compounds are 24, 25-dihydroxyvitamin D\(_3\) as is shown in figure 1 and 25, 26-dihydroxyvitamin D\(_3\) (not shown). Further metabolism of 24,25-(OH)\(_2\)-D\(_3\) produces 1α, 24, 25-trihydroxyvitamin D\(_3\), which is presently the only reported trihydroxylated vitamin D metabolite. The roles of 24, 25-(OH)\(_2\)-D, 25, 26-(OH)\(_2\)D or 1, 24, 25-(OH)\(_3\)D are as yet unclear.

1. Whenever there is no designation of vitamin D as D\(_2\) or D\(_3\), then either form is implied.
Figure 1. The Metabolism of Vitamin D₃.
The molecular mechanism of action of 1, 25-(OH)₂D at the intestinal target cell is shown in figure 2. The model proposed here is partially hypothetical and represents basic features of all steroid hormone actions on their respective target sites (12). In the vitamin D system it is known that the hormone 1, 25-(OH)₂D enters the target cell and interacts reversibly but with high association with an intracellular protein which is located only in the target organ. This association of the hormone with the cytoplasmic receptor is followed by a redistribution of the steroid from the cytoplasm to the nucleus, where it associates with chromatin (13, 14, 15). This cytosol receptor has been characterized as protein with molecular weight of 45,000, which has a high affinity for 1, 25-(OH)₂D₃ ($K_d=2.2\times10^{-9}\text{M}$) and specifically binds the hormonal form the vitamin. Only larger concentrations (>100x) of closely related metabolites and analogs such as 25-(OH)D₃, 1α-hydroxyvitamin D₃, and 5,6 trans-25-hydroxyvitamin D₃ can displace the hormone.

The steroid hormone model dictates that subsequent to the migration of the steroid hormone complex to the nucleus, there is induction of new protein synthesis. The idea is that the hormone-receptor complex interacts with the chromatin in such a way as to increase transcription and translation of the messenger RNA's (mRNA) for these new proteins. Every steroid hormone system would be characterized by its own target cell, its own specific protein, and its own physiological response.
Figure 2. Proposed Molecular Mechanism of Action of 1,25-(OH)$_2$D$_3$ in the Intestinal Mucosa Cell. (R represents receptor protein.)
Preliminary in vivo work has intimated that 1, 25-(OH)$_2$D is effecting transcriptional alterations in the target intestine. Tsai and Norman (16) observed that tritiated uridine incorporation into rapidly labeled intestinal RNA is enhanced by administration of 1, 25-(OH)$_2$D in vivo. Zerwekh et al. (17) have found that the activity of DNA dependent RNA polymerase II, the nucleoplasmic enzyme which catalyzes the synthesis of mRNA, is enhanced in activity after treatment of chicks with 1, 25-(OH)$_2$D$_3$. Furthermore, Zerwekh et al. (18) have shown that intestinal chromatin has increased template efficiency following the administration of 1, 25-(OH)$_2$D$_3$ to rachitic chickens. This shows that the chromatin has an increased capacity for the biosynthesis of RNA.

As is depicted in figure 2, one of the suspected induced proteins is calcium binding protein, CaBP. This protein, CaBP, originally described in the chick by Wasserman et al. (19), binds calcium ($K_d=10^{-5}$M) and the temporal appearance of CaBP and calcium absorption after a dose of 1, 25-(OH)$_2$D is in close agreement. However, the correlation breaks down at later times because the decay of calcium transport occurs more rapidly than the disappearance of CaBP. There is a possibility that there are other proteins involved in calcium transport (20). Lawson has identified two brush border membrane proteins which could possibly function as calcium transport proteins. However, the function of these two proteins is unknown. Another protein that has been found possesses two functions— a calcium ATPase and an alkaline phosphatase enzymatic activities (21, 22).
The Nature of the Vitamin D-Related Hydroxylases

There are three enzymes responsible for the metabolism of vitamin D. The first is the 25-hydroxylase, which, in mammals, is found predominantly in the liver (10). The second enzyme, the renal 25-hydroxyvitamin D -la-hydroxylase (1α-OHase), catalyzes the crucial reaction, the production of the hormone. This rate-limiting enzyme is found exclusively in the mitochondria of kidney tissue (11) and is made up of a flavoprotein, renal ferredoxin and cytochrome P450 (23) which together mediate molecular oxygen and NADPH. The hydroxylation of 25-(OH)D_3 by the activity of this enzyme is stringently controlled and is regulated by many factors, such a parathyroid hormone, low phosphate, by 1, 25-(OH)_{2}D_3 itself, and possibly other hormones such as prolactin, estrogen, cortisol, and growth hormone.

Figure 3 shows the complex interplay of hormones and other factors which are postulated to regulate the renal production of 1,25-(OH)_{2}D. The primary stimuli appear to be low serum phosphate and low serum calcium. Low phosphate may act directly at the kidney, whereas low calcium signals the output of parathyroid hormone (PTH), which then acts at the kidney. Once, 1, 25-(OH)_{2}D levels are enhanced in the blood, the hormone acts on target tissues such as intestine and bone to mobilize phosphate and calcium. This demonstrates a classic endocrine feedback system.

The third enzyme is the 25-hydroxyvitamin D_{3}-24 hydroxylase (24-OHase). It is located in kidney mitochondria. This enzyme utilizes
Figure 3. Model for Regulation of $1,25-(OH)_2D_3$ Biosynthesis.

Solid arrows indicate a positive effect; dashed arrows refer to negative feedback.
molecular oxygen, is blocked by inhibitors of oxidative phosphorylation, is not sensitive to carbon monoxide, and therefore does not appear to be dependent upon cytochrome P450 (24).

There is also evidence for the extrarenal location of this enzyme in rats (25) and man (26). Although the role of 24-hydroxylated vitamin D metabolites is not known, it has been shown (27) that 1, 25-(OH)₂D induces the 24-hydroxylase, whereas PTH suppresses it, and that, in the rat, 24, 25-(OH)₂D₃ becomes a major circulating dihydroxylated metabolite under conditions of normal calcium and phosphate levels (28). The role of the 24-hydroxylated D vitamins could possibly range from degradative intermediates to operative compounds.

The 24-hydroxyvitamin D Compounds

Since much attention has been focused on 1, 25-(OH)₂D, the other vitamin D metabolites that were found to exist in plasma have consequently received secondary attention. However, a thorough understanding of the functions of vitamin D cannot be elucidated from studies of 1, 25-(OH)₂D alone.

In 1971, Boyle et al. (24) observed that when the biosynthesis of 1, 25-(OH)₂D was suppressed by high calcium diets and administration of vitamin D, there was the appearance of another dihydroxy-metabolite on LH-20 Sephadex chromatograms. This metabolite was later identified as 24, 25-(OH)₂D₃ (25). To date, the role of this compound has not been firmly established.
This compound has been found in the plasma of normal animals (rats, chickens, pigs) and in man (24, 26, 27). Its biosynthesis is stimulated at a time when the 1-hydroxylation of 25-(OH)D is suppressed (28). It has significant biological activity in rats (29, 30), but is less effective in the chick (31). Nephrectomy prevents expression of biological activity of 24, 25-(OH)_{2}D (29), which led to the discovery that 24, 25-(OH)_{2}D could serve as a substrate for the kidney 1-hydroxylase. The new polar metabolite was isolated and identified as 1, 24,25-(OH)_{3}D (32).

Subsequent to the discovery of 1, 24, 25-(OH)_{3}D, the studies which have followed are of three types. The first type has been concerned with the route of synthesis of 1, 24, 25-(OH)_{3}D. Another major quest has been to verify the presence of this metabolite in plasma, and finally another major consideration has been to elucidate the biological role of the 24-hydroxylated metabolites, 24, 25-(OH)_{2}D and 1, 24, 25-(OH)_{3}D.

The route of in vivo synthesis of 1, 24, 25-(OH)_{3}D depends upon the substrate involved and the appropriate enzymes. It has been shown that 1α-(OH)D, 24-(OH)D_{3}, 25(OH)D_{3}, 1, 25-(OH)_{2}D_{3} or 24,25-(OH)_{2}D_{3} can be metabolized by D-deficient animals to 1, 24, 25-(OH)_{3}D (29, 33, 34). These conversions can occur in rats on a high calcium, low phosphorus diet, or a low calcium, normal phosphorus diet (33). Early studies seemed to indicate that 25-(OH)D was first 24-hydroxylated in the kidney, which was subsequently 1-hydroxylated (31, 32). However, whenever there was sufficient 24-hydroxylation, the 1-hydroxylase activity was absent.
Therefore, the logical sequence would be the 1-hydroxylation of 25-(OH)D to form 1, 25-(OH)₂D. It was shown by Tanaka and Deluca (35,36) that 1, 25-(OH)₂D induces the 24-hydroxylase and therefore 1, 24, 25-(OH)₃D is normally produced by the 24-hydroxylation of 1, 25-(OH)₂D. Nephrectomy failed to eliminate the conversion of 1, 25-(OH)₂D to 1, 24, 25-(OH)₃D, which implied that there was an extrarenal source of the 24-hydroxylase. An extrarenal 24-hydroxylase has finally been located, found in chick and rat cartilage by Garabedian et al. (37), and it has also been reported in rat intestine (38).

*In vitro* studies of these hydroxylases show that in chick kidney homogenates or chick mitochondrial preparations, the 24-hydroxylase has a requirement for a hydroxyl group at position 25 before either 1- or 24-hydroxylation (33, 39). Studies by Friedlander and Norman (40) corroborate those studies which show the *in vitro* synthesis of 1, 24, 25-(OH)₃D from chick kidney. However, they were unable to demonstrate the presence of 1, 24, 25-(OH)₃D in vivo in such tissues as intestine, liver, or blood, regardless of high (3%) or low (0.05%) of dietary calcium conditions in chickens dosed with physiological amounts of radioactive vitamin D₃ for 8 days.

It is important to note, at this point, the detection method used in the studies mentioned in this chapter. These studies were all "tracer" experiments. Therefore, to detect 1, 24 25-(OH)₃D₃, regardless of the tissue in question, a radioactive vitamin D₃ precursor was given to the animal. After a designated time, lipid was extracted from the tissue,
and the purified lipid extract was resolved via LH-20 Sephadex chromatography. The more recent studies (30, 33, 36) employ high pressure liquid chromatography as a final chromatographic step. Once purified, identification of 1, 24, 25-(OH)_{3}D_{3} eluted from these columns entailed either comigration with a biosynthesized radioactive 1, 24, 25-(OH)_{3}D_{3} or the use of physical and chemical means such as ultraviolet absorption spectroscopy ($\lambda_{\text{max}}=265$ nm), sensitivity to periodate treatment, and mass spectrometry. The configuration of the 24-hydroxyl group was identified as the R-epimer by high pressure liquid chromatography and was verified using R and S epimers of chemically synthesized 1, 24, 25-(OH)_{3}D_{3}. A major disadvantage of these "tracer" studies is that there is no way of knowing the endogenous circulating levels of the particular metabolite.

What is the biological role of 1,24,25-(OH)_{3}D? The answer to this question must also consider the role of 24,25-(OH)_{2}D. Early studies of 24,25-(OH)_{2}D by Boyle et al. (29) reported that 24,25-(OH)_{2}D was capable of supporting growth, elevating serum calcium and calcifying bones of rats on a normal calcium, normal phosphorus diet. Furthermore, at dose levels comparable to 1,25-(OH)_{2}D, 24,25-(OH)_{2}D was capable of inducing intestinal calcium transport, but had little activity to mobilize calcium from bone. This biological response was eliminated by nephrectomy. The implication, therefore, was that 1,24,25-(OH)_{3}D was responsible for the induction of calcium transport to the intestine.

Further studies probing into the function of 1,24,25-(OH)_{3}D (32) have shown that in terms of curing rickets, 1,24,25-(OH)_{3}D was 60% as active as vitamin D and 6.0% as active as 1,25-(OH)_{2}D. Also in agreement with Boyle et al. (29), Holick et al. (32) demonstrated that
on a weight basis, 1,24,25-(OH)\textsubscript{3}D is less active than 1,25-(OH)\textsubscript{2}D in stimulating and sustaining intestinal calcium transport and bone calcium mobilization. However, it appears to have a preferential action on the intestine.

The reports of the effect of 1,24,25-(OH)\textsubscript{3}D on intestinal phosphate absorption have been conflicting and somewhat incomplete. Walling, et al. (41) reported that 1,24,25-(OH)\textsubscript{3}D stimulates active intestinal phosphate adsorption using everted gut sacs of vitamin D-deficient rat duodenum. However, Chin et al. (42) reported that 24,25-(OH)\textsubscript{2}D had a negative effect in stimulating intestinal phosphate transport using everted gut sacs of vitamin D-deficient rat jejunum. Thus the effects of 24,25-(OH)\textsubscript{2}D and 1,24,25-(OH)\textsubscript{3}D seem to oppose each other. Further studies are needed to correlate these reports. Note also that different sections of the intestine were studied.

In light of these studies, much remains to be elucidated before the significance of 24,25-(OH)\textsubscript{2}D and 1,24,25-(OH)\textsubscript{3}D is realized. There are several questions that should be addressed. Is 1,24,25-(OH)\textsubscript{3}D present in all organisms? Is 1,24,25-(OH)\textsubscript{3}D responsible for any of the functions currently thought to be due to 1,25-(OH)\textsubscript{2}D? Is 1,24,25-(OH)\textsubscript{3}D merely a pre-excretion product? Does 1,24,25-(OH)\textsubscript{3}D have any regulatory roles, i.e., feedback regulation of the 1-OHase?

The purpose of this thesis is to provide a method to initiate further studies concerning 1,24,25-(OH)\textsubscript{3}D. The method developed here allows for the isolation, purification, and quantitation of the endogenous circulating plasma concentration of 1,24,25-(OH)\textsubscript{3}D. This assay
has been used to determine that 1,24,25-(OH)\textsubscript{3}D is present in cows, chickens, rats and humans. Some preliminary findings are also included which monitor the changes in 1,24,25-(OH)\textsubscript{3}D concentration as a function of dietary, ionic, or hormonal variations in the rat and chick. This is the first time that high pressure liquid chromatography (HPLC) has been routinely used in our laboratory. The use of HPLC is becoming a major tool in the field of vitamin D (43, 44, 45, 46). The unique combination of HPLC and highly specific and sensitive radioreceptor assay provides the basis for the measurements of 1,24,25-(OH)\textsubscript{3}D detailed in this monograph.
CHAPTER 2

MATERIALS AND METHODS

Animals and Diets

Chickens

White Leghorn cockerels were obtained as one-day old chicks from Demler Farms, Anaheim, California. They were raised in a room free from ultraviolet light for three to six weeks. They were grown on one of the following diet regimens.

1. **Standard Rachitogenic Diet.** This diet has been described (45). The calcium content was 0.67%. Animals were termed rachitic when their growth plateaued as a weight of about 150 g. Chicks grown on this same vitamin D-deficient diet but supplemented with 100 I.U. of vitamin D_3 per week exhibited normal increased growth to about 250 grams during this period. A rachitic chick usually has a plasma calcium concentration of 6 mg per 100 ml and phosphorus concentrations of 5 mg per 100 ml. Vitamin D supplemented chickens had normal plasma calcium concentrations of 10.4-11.0 mg per 100 ml plasma and normal phosphorus concentrations of 6.3 mg per 100 ml. (47).

2. **Vitamin D containing diet.** Chicks at two weeks of diet No. 1 were fed 2 I.U. of 1α,25-(OH)_2D_3 per gram of diet for 4 weeks.

3. **Diethylstilbestrol (DES).** The estrogen analogue, DES, was administered to chicks that had been raised on diet regimen No. 2.
At 4 weeks, 5 mg/dose in 0.1 ml ethanol of DES was injected subcutaneously for 5 days.

Rats

Weanling male Holtzman rats were fed a semi-synthetic diet containing 24% casein, 24% corn starch, 40% sucrose, 5% corn oil, 5% salts, and 2% cellulose fiber. The basic salt mixture has been described (48) and was varied in order to achieve the desired levels of calcium and phosphorus. The variations were as follows:

1. Normal diet. 0.6% Ca, 0.6% P, 2 IU/g vitamin D₃.
2. Low Ca. 0.01% Ca, 0.6% P, 2 IU/g vitamin D₃.
3. Low P. 0.6% Ca, 0.04% P, 2 IU/g vitamin D₃.

Cows

Bovine animal plasma was obtained courtesy of Jones' Slaughterhouse, Tucson, Arizona.

Patients

Blood was drawn from healthy volunteers (ages: 1 day, 16 years, and 20-50 years old) in Phoenix and Tucson, Arizona.

Radiochemicals

25-Hydroxy[³H] vitamin D₃ (6.7, 11.2, or 94 Ci/mmmole) was purchased from the Amersham Corporation, Chicago, Illinois. The radiochemical purity of 25-OH[³H]D₃ was determined following purification on Celite liquid-liquid partition chromatographic columns or
high pressure liquid chromatography, and its specific activity was
determined by ultraviolet absorbance spectrophotometry at 265 nm.
$\text{1a,25-(OH)}_2^\text{[3}^\text{H}]\text{D}_3$ was biosynthesized \textit{in vitro} by a modification of a
method of Fraser and Kodicek (49). \text{1a-24R,25-(OH)}_3[(26,27), methyl-$^\text{3}^\text{H}$]
vitamin D$_3$ (7Ci/m mole) was kindly supplied by S. Ishizuka, Department
of Biochemistry, Teijin Institute for Biomedical Research, Tokyo, Japan.

\textbf{Sterols and Other Chemicals}

Crystalline $\text{1a,25-(OH)}_2\text{D}_3$ and $\text{1,24,25-(OH)}_2\text{D}_3$ were obtained from
Hoffmann-La Roche.

Chemicals used in the enzymatic generation of $\text{1,25-(OH)}_2^\text{[3}^\text{H}]\text{D}_3$
from $\text{25-OH}\text{D}_3$ were obtained from Sigma Chemical Co., St. Louis, Missouri,
and included: L-malic acid, monosodium salt; D-glucose-6-phosphate,
monosodium salt; nicotinamide adenine dinucleotide phosphate, mono­
sodium salt (NADP+); and the enzyme glucose-6-phosphate dehydrogenase
(Type VII, baker's yeast).

Triton X-100 was obtained from Rohm and Haas Co., Philadelphia,
Pennsylvania. Liquiflour, a concentrated liquid scintillation solution
consisting of 100 g of 2,5-diphenyloxazole and 1.20 g of p-bis-[2-(5­
phenyloxazoyl)] benzene per liter toluene, was purchased from New
England Nuclear Corp., Boston, Massachusetts.

Tris-(hydroxymethyl) aminoethane (tris, Sigma 7-9) and ethylene-
diaminetetraacetic acid (EDTA, tetrasodium salt) were obtained from
Sigma Chemical Co., St. Louis, Missouri. Glass fiber filters (Type AE)
were obtained from Gelman, Ann Arbor, Michigan.
All solvents were reagent grade, and those employed in chromatographic procedures were glass distilled before use. However, hexane used for high pressure liquid chromatography was nanograde (glass distilled) and was not redistilled.

**Chromatography Materials**

Silicic acid (Bio-Sil HA, minus 325 mesh) was obtained from Bio-Rad Laboratories, Richmond, California. Sephadex LH-20 (Lipophilic, Particle size 25-100 μ) was purchased from Sigma Chemical Co., St. Louis, Missouri. Celite, which served as a support medium for liquid-liquid partition chromatography, was supplied by Johns Manville Co., Lompoc, California. The columns used for high pressure liquid chromatography were two pre-packed Zorbax-Sil Adsorption columns 25 cm x 2.1 mm, in tandem. The HPLC apparatus used was a Dupont 830 Liquid Chromatograph with a Rheodyne Model 7-10 sample injection port equipped with a model 70-11 Loop filler port and a 70-24 100 μl injection loop.

**Buffers**

The buffers used were:

Buffer 1. Sucrose-TKM = 0.25M Sucrose, 0.05M Tris-HCl, pH 7.4, 0.025M KCl, 5mM MgCl₂, 12mM Thioglycerol and 1mM EDTA.

Buffer 2. EDTA = 0.008M EDTA, 25 mM NaCl, pH 8.

Buffer 3. Triton-Tris = 1% Triton X-100, 0.01 M Tris HCl, pH 7.5.

Buffer 4. Tris-Buffer = 0.01M Tris-HCl, pH 7.5.
Preparation of Radioactive 1,25-Dihydroxyvitamin D₃

1,25-(OH)₂[³H]D₃ was produced in vitro by a modification of Lawson et al. (50, 51). The kidneys of 5 rachitic chicks (7.5 g) were excised and homogenized with a Potter-Elvenjem homogenizer at 1-3°C in Buffer 1. The homogenate (75 ml) was mixed with a phosphate buffer, pH 7.4, containing Mg²⁺ and a NADPH generating system. In a final volume of 375 ml the mixture contained: 0.16 M KH₂PO₄, 3.2 mM MgCl₂, 7.8 mM L-malate 3.7 mM glucose-6-phosphate, 0.3 mM NADP⁺, and 150 units of glucose-6 phosphate dehydrogenase. Five hundred international units of 25-hydroxy[25(27)-methyl-³H] vitamin D₃ (94 Ci/m mole) were added to this reaction mixture in 1 ml of ethanol. Incubation was carried out in 5 separate flasks with gentle shaking under air at 37°C for 1 hour. Termination of the reaction occurred with the addition of 3.2 volumes methanol-chloroform (2:1, v/v) and stirring for 10 minutes. After standing for 15 minutes, the solution was filtered to remove the residual protein and the sterols extracted according to the method of Bligh and Dyer (52). One volume of chloroform and one-half volume of water were added to the extracts to cause a phase separation into an aqueous methanol phase and a lower chloroform phase which contained 85-95% of the sterols. This chloroform phase was flash evaporated to dryness and the resulting lipids solubilized in diethyl ether. After centrifugation at 12,000 x g for 10 minutes to clarify the solution, the diethyl ether was evaporated under a stream of nitrogen and the
1,25-(OH)$_2$$[^{3}H]$D$_3$ was then purified by column chromatography as described below.

**Chromatographic Methods**

**Silicic Acid Column Chromatography**

Silicic acid was activated by heating in a vacuum oven to 120°C for 24 hours just prior to use. Twenty-five grams of activated silicic acid were then suspended in diethyl ether and poured into a column (1.8 x 18 cm) with the aid of 3 psi nitrogen pressure for packing. Samples for purification were applied in 10 ml of diethyl ether. Ten fractions were collected from columns which were batch eluted with 5% acetone in diethyl ether as follows: fraction numbers 1, 2, and 6-10, 50 ml; fractions 3-5, 20 ml. 1,25-(OH)$_2$$[^{3}H]$D$_3$ eluted in fractions 5 through 7. Each column was eluted under 3 psi of nitrogen pressure and at a flow rate of 7 ml per minute. Fractions 5-7 were flash evaporated, solubilized in ether, dried under nitrogen, and resolubilized in 65% chloroform in hexane.

**Sephadex LH-20 Column Chromatography**

Liquid-gel partition chromatography on Sephadex LH-20 was performed by the method of Holick and DeLuca (53). Five grams of Sephadex LH-20 were suspended in 65% chloroform in hexane and formed a 1 x 15 cm column with gravity flow. Two fractions were collected. The first was 40 ml and the second, which contained 1,25-(OH)$_2$D$_3$, was 75 ml. Columns were eluted with 65% chloroform in hexane.
Celite Column Chromatography

Celite was washed with concentrated HCl and organic solvents, and the fine particles were removed prior to use (54). Column chromatography for the resolution of dihydroxy-D-vitamins was according to the procedure of Haussler and Rasmussen (55). Five volumes of 10% ethyl acetate in hexane were equilibrated with 1 volume of 45% water in ethanol. The lower water-ethanol phase served as a stationary phase, and 11 ml was mixed with 11 g Celite (1:1, v/v). The Celite was then suspended in excess ethyl acetate-hexane (upper phase, mobile) and "fluffed" for 15-30 minutes in a beaker with magnetic stirrer. This step was essential for uniform coating of the stationary phase on the inert Celite particles and for proper equilibration of the two organic phases with the packing material prior to column construction. The "fluffed" Celite preparation was then packed with a glass rod into a homogenous column (27.5 cm³). Proper packing was achieved by building the column in 3-4 stages; the entire 11 g of Celite material, when packed, occupied 35 cm. Columns were run under 3 lbs nitrogen pressure to achieve flow rates of about 1.5 ml/min. Samples were applied to all Celite columns in 0.4 ml of the mobile phase and 5 ml fractions were collected.

Preparation of Radioactive 1,24,25-trihydroxyvitamin D₃

[26,27-methyl-³H] ¹α, 24 R, 25-(OH)₃D₃ was synthesized by incubating [26-27-methyl-³H] 25-OH-D₃ with vitamin D-deficient chick kidney homogenates. The product formed here, [26-27-methyl-³H] 1,25(OH)₂D₃ was then incubated with chick kidney homogenates from chicks that had
been dosed with $1\alpha$-OH-D$_3$. The product formed after the second incubation, $[^3H]1\alpha,24$R, 25-(OH)$_3$D$_3$, was purified and identified using Sephadex LH-20 column chromatography, high pressure liquid chromatography using a Zorbax Sil column (Dupont) and periodate oxidation cleavage according to S. Ishizuka, personal communication. 

\textit{(56)}

\textbf{Sample Storage}

The post-Celite sample showed a negligible visible residue when the total sample was taken to dryness prior to solubilization for storage. Nonradioactive 1,25-(OH)$_2$D$_3$ was analyzed spectrophotometrically (see "Spectrophotometric Determinations"), and the concentration was adjusted to 0.2 ng/ml (0.48 pmoles/ml) with redistilled 100% ethanol. Radioactive 1,25-(OH)$_2$D$_3$ was taken to a concentration of 1 ng/ml (2.4 pmoles/ml) in a similar manner. Radioactive 1,24,25-(OH)$_3$D$_3$ was taken to a concentration of 16.8 ng/ml (39 pmoles/ml). All sterols were stored at -20°C.

\textbf{The Assay for 1,25-dihydroxyvitamin D and 1,24,25-trihydroxyvitamin D}

\textbf{Sterol Extraction from Plasma}

Ten milliliters of plasma were routinely required (except where otherwise noted) for triplicate assay. Two methods were used for the sterol extraction. The first was a modification of the procedure of Bligh and Dyer; it has been explained in the section dealing with the generation of the vitamin D$_3$ hormone and will not be repeated here. When this method was used, 100-200 ml of plasma or 700 mls of sera
were extracted. The second method was a modification of the method described by Hughes (57). Plasma volumes were measured and transferred to a 250 ml centrifuge bottle. Tracer sterols (approximately 1000/cpm of 1,25-(OH)$_2$-[${}^3$H]D$_3$ and 1500 cpm of 1,24,25-(OH)$_2$-[${}^3$H]D$_3$ were added directly to the plasma. Five volumes of acetone were added to each container followed by vigorous mixing for 30 minutes at room temperature. The centrifuge bottles were then spun at 5000 rpm (Rotor type GSA and centrifuge RC-2B; Sorvall Instruments Division of E.I. Du Pont, Inc.) for 10 minutes to pellet proteins. Supernatants were flash evaporated to dryness. One ml of water was added and the material was transferred to a 20 ml glass (scintillation type) vial with diethyl ether. Two phases were present, and the lower phase was carefully pipetted away and discarded. The diethyl ether was subsequently dried under nitrogen before processing via chromatography.

**Chromatography**

Silicic acid (activated by heating at 120°C for 24 hours in a vacuum oven) chromatography was used for removing lipids from the plasma samples with the larger volumes (100-200 mls). Ten ml glass pipettes, fitted with glass wool plugs, served as columns. After sample application, one 5 ml column was washed with 10% acetone in ether (15 mls), and the use of 100% acetone eluted both 1,25-(OH)$_2$D$_3$ and 1,24,25-(OH)$_3$D$_3$ within 15-30 mls. The silicic acid fraction containing both sterols was dried under nitrogen resolubilized in the solvent to be used for the next chromatographic step. Sephadex LH-20 column chromatography was performed using a modification of the technique of Holick and
DeLuca (53). The solvent system used was 75% chloroform, 23% hexane and 2% methanol. One 1 x 20 cm (5 g) column resulted in the separation of 1,25-(OH)$_2$D$_3$ from 1,24,25-(OH)$_3$D$_3$. 1,25-(OH)$_2$D$_3$ eluted between 24-28 mls and 1,24,25-(OH)$_3$D$_3$ eluted between 54-72 mls. Throughout the remainder of the purification scheme the sterols were treated separately. The next stage of purification utilized high pressure liquid chromatography (which will be described below).

An alternative column chromatographic treatment of smaller plasma volumes (10 mls) was as follows. After sterol extraction the sample was processed by LH-20 Sephadex chromatography. The method was a modification of Brumbaugh et al. (58). Six ml columns were built using the 10 ml glass pipettes previously mentioned for Silicic acid chromatography. The solvent system was 65% chloroform in hexane. 1,25-(OH)$_2$D$_3$ emerges from 50-100 mls. Throughout the remainder of the purification scheme the sterols were processed separately.

Regardless of the initial plasma chromatographic steps, the final chromatographic step prior to assay was the use of high pressure liquid chromatography.

1,25-(OH)$_2$D$_3$ or 1,24,25-(OH)$_3$D$_3$ LH-20 Sephadex fractions were evaporated under nitrogen and resolubilized in 50 µl of 20% isopropanol in hexane, except where otherwise noted. The sample was loaded into the sample loop with a 50 µl Hamilton syringe followed by a 30 µl wash of the HPLC sample cup. The samples were eluted with 20% isopropanol in hexane through the Zorbax-Sil columns at 3000 psi. 1,25-(OH)$_2$D$_3$ emerges at 6 minutes (0.73 ml/minute). 1,24,25-(OH)$_2$D$_3$ emerges between
9-10 minutes. All samples for sterol assay were evaporated under nitrogen and solubilized in 400 µl ethanol (except where otherwise noted). Determination of yield was accomplished by counting an aliquot of each sample for tritium (final yields ranged from 50-75% for 1,25-(OH)₂D₃). An alternative solvent system was used in HPLC for the purification of 1,25-(OH)₂D₃ was 10% isopropanol in hexane. This solvent system resulted in a longer retention time of 1,25-(OH)₂D₃. The elution time in this system was 8-14 minutes as compared to 5-6 minutes from the system using 20% isopropanol in hexane. The advantage in using the slower system was to minimize the presence of contaminating lipid in the purified 1,25-(OH)₂D₃.

Preparation of Reconstituted Cytosol and Chromatin

The following is a description of the procedure for preparing rachitic chick intestinal cytosol and chromatin prior to reconstruction for use in the competitive receptor binding assay. The revised assay procedure is shown in figure 4. Intestine was split lengthwise, washed free of debris in isotonic buffer (Buffer 1, Sucrose-TKM, then scraped free from the serosa on an inverted Petri dish with a microscope slide. Two grams of the mucosa were then homogenized in 25 mls of Buffer 1. During homogenization and subsequent steps in the tissue preparation, all tubes, flasks, pipettes, rotors, and centrifuges were rigorously kept at 0-4°C. Homogenates were centrifuged at 1200 x g and then the supernatant was spun at 100,000 x g (Spinco 35 rotor, L5-50 Beckman Ultracentrifuge). Chromatin was prepared from crude nuclei (isolated from the original 1200 x g centrifugation) by homogenizing successively
Radioligand receptor assay for $1,25-(\text{OH})_2\text{D}_3$

**Preparation**

- Thaw
- Stir at 2°C while frozen (liquid nitrogen)
- Reconstitute chick intestinal cytosol-chromatin receptor system at 2°C

**Incubation**

- [³H] $1,25-(\text{OH})_2\text{D}_3$ (5000 CPM, 94 Ci/m mole)
- $1,25-(\text{OH})_2\text{D}_3$ (Nonradioactive std. or purified from plasma)
- 10% distilled ethanol
- 100X reconstituted receptor (12.5µg DNA and 50µg protein)

- 105 min, add 1ml cold 1% triton X-100 (in 0.01M tris-HCl, pH 7.5)

**Assay**

- Filter to trap bound hormone and wash with 2ml of 1% triton X-100
- Extract filters with acetone, dry, and count

Figure 4. Radioligand Receptor Assay for $1,25-(\text{OH})_2\text{D}_3$. 
in 25 ml portions of Buffer 2, Buffer 3, and Buffer 4. The chromatin was harvested by sedimentation at 3000 x g for 10 minutes after each wash. The entire 2 g of mucosa chromatin was then combined with half the cytosol fraction (approximately 11 ml) by homogenation to create a "reconstituted cytosol chromatin" receptor system for the competitive binding assay. The homogenate was then forced through a 22G needle. The material was used fresh or frozen in liquid nitrogen and stored at -80°C for later use.

**Incubation and Filtration Procedure**

New glass culture tubes (borosilicate; 13 x 100 mm) were used for individual assay incubations. To each tube, 20 μl of radioactive 1,25-(OH)₂-[³H]D₃ (1000 pg per ml ethanol) was added. The standard curve (figure 5) was constructed by adding various quantities (0-100 μl) of nonradioactive 1,25-(OH)₂D₃ (200 pg per ml ethanol). For plasma samples, 5-100 μl of the sample was used. (This volume required adjustment in subsequent assays if the competition was not on the standard curve).

To each assay tube containing 1,25-(OH)₂-[³H]D₃ and unlabeled sterol (dried together with a stream of nitrogen) was added 10 μl of distilled ethanol and 100 μl of reconstituted cytosol chromatin system (diluted 1:8 so that there was 10-12 μg DNA and 50 μg protein). The ethanol which was added just prior to the receptor system aided in solubilizing the sterols to achieve a higher binding efficiency and reduced non-specific binding. The final concentration of 1,25-(OH)₂[³H]D₃ was 0.4 nM. After incubation for 105 minutes at 25°C with
shaking in water bath, the quantity of labeled sterol bound to chromatin was determined by filtration. To each assay tube, 1 ml of cold Buffer 3 was added and the entire mixture applied to a Gelman Type AE glass fiber filter (presoaked for 30 minutes in deionized water; prerinsed on the manifold with 1-2 ml Buffer 3) at a gravity flow or at a very low vacuum. Each of the filters was washed with 2 ml of Buffer 3. Following filtration, the filters were placed in liquid scintillation vials with 5 ml of reagent grade acetone. After 5 minutes the acetone was evaporated on a hot plate under a stream of air. Without removal of the glass filter, sterols were solubilized in 5 ml non-aqueous based counting solution. The samples were counted, and routinely 1100-1600 cpm of the approximately 5000 cpm of 1,25-(OH)$_2$[H]D$_3$ present in the incubation was recovered in the chromatin in the absence of competing nonradioactive sterol (26% binding efficiency). Interassay variation was 10-15%.

**Liquid Scintillation Counting**

Liquid scintillation counting of all radioactive sterols was carried out after the addition of 5 ml liquid scintillation counting solution. This solution consisted of 4 g 2,5-diphenyl-oxazole and 50 mg 1,4-bis[2-(5-phenyloxazoyl)] benzene per liter of toluene. Samples were counted in a Beckman LS-250 ambient temperature or a Beckman LS-230 refrigerated liquid scintillation spectrometer. Samples were counted to a 2-10% error.
CHAPTER 3

RESULTS

In order to utilize the most expedient route to develop an assay for quantitation of 1,24,25-(OH)$_3$D$_3$, it was desirable to modify the competitive binding radioreceptor assay that has been used for 1,25-(OH)$_2$D$_3$. This assay (figure 4) measures the level of 1,25-(OH)$_2$D$_3$ in a plasma sample based on the competition of labeled and unlabeled 1,25-(OH)$_2$D$_3$ for its cytoplasmic receptor. The assay utilizes a vitamin D-deficient chick intestinal system. The 1,25-(OH)$_2$D$_3$ receptors in a vitamin D chicken are unoccupied by sterol and thus are available to be used for a competitive receptor binding assay. The quantitation method is based on the receptor mechanism of action of 1,25-(OH)$_2$D$_3$ at its target tissue, the intestine (figure 2). A cytoplasmic fraction of intestinal tissue, containing the 1,25-(OH)$_2$D$_3$ receptor, is incubated with the chromatin isolated from the same tissue. The addition of sterol, which binds to the receptor, results in subsequent binding of the sterol-receptor complex to the chromatin. A glass fiber filter allows trapping of these complexes while unbound sterol can be removed by washing. The filters can then be counted by liquid scintillation counting. The amount of sterol bound to chromatin is monitored by competition of radioactive and nonradioactive sterols present in the medium. A standard curve is generated by adding identical amounts of 94 Ci/mmmole radioactive (hot) sterol to each of several culture tubes.
followed by the addition of various amounts of nonradioactive (cold) sterol. A typical standard curve is shown in figure 5. Each point represents a 20 pg of radioactive 1,25-(OH)$_2$D$_3$ and 0-20 pg of nonradioactive 1,25-(OH)$_2$D$_3$. The sensitivity of this assay is such that 2 pg of 1,25-(OH)$_2$D$_3$ can be detected.

The inset represents a saturation curve generated by adding increasing amounts of 1,25-(OH)$_2$[^3]H]D$_3$ to a constant amount of the cytosol-chromatin preparation. The concentration of sterol necessary to saturate the receptors was determined from the region of the curve that flattens out as the sterol concentration is raised. This saturation curve was used to determine the amount of tritiated 1,25-(OH)$_2$D$_3$ (20 pg) to be used for generating the standard curve.

In order to utilize this assay for the quantitation of 1,24,25-(OH)$_3$D$_3$, it was necessary to determine the affinity of this compound for the 1,25-(OH)$_2$D$_3$ receptor. The determination of the concentration of 1,24,25-(OH)$_3$D$_3$ is dependent on its relative binding affinity for the 1,25-(OH)$_2$D$_3$ receptor. If 1,24,24-(OH)$_3$D binds equally well to this receptor as 1,25-(OH)$_2$D$_3$ does, then the same assay could be used, so that according to the standard curve, the pg of nonradioactive 1,25-(OH)$_2$D$_3$ added per tube would be equivalent to the pg of 1,24,25-(OH)$_3$D present in the biological sample.

To determine this binding affinity, the percentage of binding of tritiated 1,25-(OH)$_2$D$_3$ was monitored as a function of the amount of unlabeled sterol present. Therefore, 2 curves were generated; in one case varying amounts of unlabeled 1,25-(OH)$_2$D$_3$ were added, whereas the second curve was generated by adding various amounts of unlabeled
Figure 5. Typical 1,25-(OH)$_2$D$_3$ Assay Standard Curve.
Figure 6 reveals that the concentration of 1,24,25-(OH)$_3$D$_3$ necessary to give 50% binding is equal to 2.6 times more than the concentration of 1,25-(OH)$_2$D$_3$. Therefore the relative binding affinity of 1,24,25-(OH)$_3$D$_3$ for the 1,25-(OH)$_2$D$_3$ intestinal receptor is 1/2.6, or 0.38 that of 1,25-(OH)$_2$D$_3$. Therefore, in order to use the 1,25-(OH)$_2$D$_3$ competitive receptor binding assay for the quantitation of 1,24,25-(OH)$_3$D$_3$ from biological fluid, the protocol described in figure 4 can be used, and only the standard curve is modified, so that 2 pg cold 1,25-(OH)$_2$D$_3$ corresponds to 2.6 times 2 pg or 5.2 pg of 1,24,25-(OH)$_3$D$_3$.

Prior to the utilization of the assay for the determination of 1,24,25-(OH)$_3$D$_3$ levels in plasma, it was necessary to establish a purification scheme. The initial scheme used is shown in figure 7, and consists primarily of three parts, the first being the lipid extraction of the sterols from sera or plasma, which was a modification of the Bligh and Dyer technique already described (Chapter 2). The second part of this initial scheme is the purification of sterols by silicic acid, Sephadex LH-20, and high pressure liquid chromatography. This purification scheme removes contaminating lipids. The elution profiles of crystalline 1,25-(OH)$_2$[${}^3$H]D$_3$ and 1,24,25-(OH)$_3$[${}^3$H]D$_3$ for each column are shown in figures 8-10. Figure 8 describes the silicic acid column system. This column uses two solvent systems, 10% acetone in ether to remove neutral lipids, and 100% acetone, which strips both sterols off within the same fractions (15-20 mls). The next column, Sephadex LH-20, effects a separation between the two sterols. Figure 9 shows that using a solvent system of 75% chloroform, 23% hexane, and 2% methanol,
100-200 mls \( \text{PLASMA} \)

Tracer Addition

1,25(OH)\(_2\)\([^3]H\)D\(_3\) – 3000 cpm; 11 Ci/mmole
1,24,25(OH)\(_3\)\([^3]H\)D\(_3\) – 3000 cpm; 7 Ci/mmole

Extraction

Add 2:1 METHANOL: CHLOROFORM 3.2 volumes
Shake: ½ hour
Spin: 4000 xg for 10 minutes
Add to supe: 1 volume CHCl\(_3\), ½ volume H\(_2\)O
Allow to phase split
Remove lower phase; Rotovac
Add ether (15 mls)

Chromatography (3 steps)

Step 1 Silicic acid chromatography
Step 2 LH-20 Sephadex chromatography
Step 3 High pressure liquid chromatography

Yield and Assay

Figure 7. Initial Purification Scheme for 1,24,25-(OH)\(_3\)D\(_3\).

In this scheme 100-200 mls plasma were required. The routine column yields for crystalline 1,24,25-(OH)\(_3\)D\(_3\) were 83% for silicic acid, 73% for LH-20 Sephadex, and 60% for HPLC. However, when actual plasma samples were processed the combined (or aggregate) 1,25-(OH)\(_2\)D yields were (on the average) 25% and the 1,24,25-(OH)\(_3\)D yields were 10%.
Figure 8. Silicic Acid Column Chromatography.

The initial purification step to remove contaminating lipids from the sample. A 5.0 ml column was run using a solvent system of 10% acetone in ether to wash lipids into the first fraction (1-15 mls). The arrow represents where 100% acetone is used to elute 1,25-(OH)2D and 1,24,25-(OH)3D from column. Fractions 2 and 3 were pooled and applied to the next column. Yield = 82% ± 5.0%.
Figure 9. Initial LH-20 Sephadex Column Chromatography.
1,25-(OH)₂D₃ elutes between 24-45 mls, and 1,24,25-(OH)₃D₃ elutes between 51-69 mls. The final chromatographic step utilized was high pressure liquid chromatography (HPLC). The appropriate fraction from the LH-20 Sephadex step was applied to HPLC for further purification. Using a solvent system of 20% isopropanol in hexane, 1,25-(OH)₂[³H]D₃ elutes at 6 minutes (figure 10), and 1,24,25-(OH)₃[³H]D₃ elutes between 9-10 minutes (figure 11). Also shown in figure 10 is the elution pattern of tritiated 1,25-(OH)₂D₃ when a solvent system of 10% isopropanol in hexane is used. Using this solvent system, 1,25-(OH)₂D₃ is retained on the column longer, thus causing a better separation of the sterol from contaminating lipid. Figure 10 demonstrates that 1,25-(OH)₂D₃ elutes between 8-12 minutes.

The purification scheme just described, in figure 7, was abandoned in favor of a revised scheme, because after purification the average final yields of 1,24,25-(OH)₃D and 1,25-(OH)₂D were too low. Simultaneously there was the availability of a higher specific activity (94 Ci/mmmole) 1,25-(OH)₂[³H]D₃ radioligand receptor assay. Due to the increased sensitivity of this assay over the previous 11.2 Ci/mmmole assay, the amount of plasma necessary was reduced from 100-200 mls to 10 mls. The new scheme, therefore, was desirable due to its consistently higher yields and its feasibility for small plasma volumes. Without this assay, routine studies in humans would have been impossible.

The revised purification scheme is described in figure 12. This scheme also consists of three parts: a lipid extraction, LH-20 Sephadex and high pressure liquid chromatography, and the assay. The lipid extraction was developed by Pike (59), to be used for small amounts of
Figure 10. High Pressure Liquid Chromatography of 1,25-(OH)₂[³H]D₃.
Figure 11. High Pressure Liquid Chromatography of 1,24,25-(OH)₃D₃.

When crystalline 1α,24,25(OH)₃[³H]D₃ is run through a Zorbax-Sil adsorption column with 20% isopropanol in hexane, it elutes between 9-10 minutes. Yield = 60%.
10 mls PLASMA

Tracer Addition

1,25(OH)₂ [³H] D₃ — 1000 cpm; 94 Ci/mmol
1,24,25(OH)₃ [³H] D₃ — 1600 cpm; 7 Ci/mmol

Extraction

Add: ACETONE — 5 volumes
Shake: ½ hour
Spin: 4000 xg for 10 minutes
Rotovac supe to dryness
Add: ether (15 mls) + H₂O (1 ml)
Allow phases to split
Remove & discard lower phase

Chromatography (2 steps)

Step 1
LH-20 Sephadex chromatography
6 cm column; 65% CHCl₃ in Hexane

<table>
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<th>cpm</th>
<th>1,25</th>
<th>1,24,25</th>
<th>COLLECT: 20-40 mls</th>
<th>50-100 mls</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>20-40 mls</td>
<td>50-100 mls</td>
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</tbody>
</table>

Step 2
High pressure liquid chromatography

Yield and Assay

Figure 12. Revised 1,24,25-(OH)₃D₃ Purification Scheme.
plasma. This extraction is easier than the Bligh and Dyer method. Briefly, the procedure involves adding five volumes of acetone to the plasma sample to which the radioactive tracer sterols have been added. This mixture is agitated by shaking for at least one half hour, which is followed by centrifuging to remove the plasma proteins. The acetone is removed from the supernatant by roto-evaporation, and the nonpolar lipids are isolated by dissolving the roto-evaporated residue in diethyl ether and water. Once the water phase is removed, the lipids present in the diethyl ether are purified by column chromatography.

The column chromatographic purification steps in the revised procedure are somewhat different from the original scheme. There are two major differences; the first is the elimination of the silicic acid column chromatography, the second difference is in the LH-20 Sephadex column system. The "new" LH-20 Sephadex column is a modification of the LH-20 Sephadex column used routinely in our laboratory. The solvent system used is 65% chloroform in hexane, and the columns have been miniaturized to 6 ml columns (using 10 ml pipettes). Figure 13 demonstrates that the elution volume of $1,25-(OH)_2[^3H]D_3$ is 20-40 mls, and that of $1,24,25-(OH)_3[^3H]D_3$ is 50-100 mls. After LH-20 the remaining steps are similar to the other scheme, i.e., HPLC as the final purification step, followed by the determination of the yield and the quantitation of the desired sterols by the radioligand assay.

One of the tests of a good purification scheme is to show that the material collected from the columns was in fact identical to the radioactive sterol that was added as tracer. The experiments designed to prove this entailed purifying a 10 ml human plasma sample to which
Figure 13. Revised LH-20 Sephadex Column Chromatography.

The first column chromatographic step in the revised purification scheme after lipid extraction. Column length = 6 ml of a 10 ml glass pipette. The solvent system used = 65% chloroform in hexane. Elution volume of $1_{25}(OH)_2D_3 = 20-40$ mls, of $1_{24,25}(OH)_3D_3 = 50-100$ mls.
had been added tritiated tracer 1,25-(OH)$_2$D$_3$ and 1,24,25-(OH)$_3$D$_3$. At the HPLC step, one minute fractions were collected and the tritiated sterol was detected by liquid scintillation counting. Aliquots of these same one minute fractions were assayed by the radioligand competitive sterol binding assay. Figures 14 and 15 show the identity of endogenous cold sterol detected by the assay and the tritiated tracer sterol. In figure 14 this comigration experiment shows that 1,25-(OH)$_2$[${}^3$H]D$_3$ and 1,25-(OH)$_2$D$_3$ are detected in fraction 6. Similarly, figure 15 shows that 1,24,25-(OH)$_3$[${}^3$H]D$_3$ and 1,24,25-(OH)$_3$D$_3$ are detected in fractions 9 and 10, although there is much more "noise" in the 1,24,25-(OH)$_3$D$_3$ assay. These comigration experiments provide evidence that the hot tracer is identical to the cold endogenous sterol with respect to their migration properties throughout the purification procedure.

Possessing a consistent and reliable purification scheme and a well-established and highly sensitive assay procedure, it was then possible to determine 1,24,25-(OH)$_3$D$_3$ concentrations from various biological samples. Table 1 lists 1,24,25-(OH)$_3$D concentrations from a variety of human plasma donors. The control used was a vitamin D-deficient chick plasma sample that was processed using the same isolation and quantitation procedure as the human samples. A vitamin D-deficient chick should have no 1,25-(OH)$_2$D$_3$ or 1,24,25-(OH)$_3$D. Therefore, this control represents the lower limits of the assay background caused primarily by the lipid residue in the sample. Another correction necessary for the 1,24,25-(OH)$_3$D values was for the contribution of the tracer. Due to the low specific activity of the tritiated
Figure 14. Comigration Check of $1,25$-(OH)$_2[3H]D_3$ and $1,25$-(OH)$_2D_3$.

A 10 ml human plasma sample was purified using the revised purification scheme shown in figure 12. At the HPLC step one-minute fractions were collected and 100 µl aliquots counted for tritium for detection of the added tracer sterol. In order to detect the endogenous ''cold'' sterol, 50-100 µl aliquots were assayed in triplicate by the assay procedure described in figure 4, using the 94 Ci/m mole $1,25$-(OH)$_2[3H]D_3$. This figure shows that the tracer is an indicator of the endogenous sterol. The arrows represent the fractions routinely collected for analysis in future experiments.
Figure 15. Comigration Check of 1,24,25-(OH)\textsubscript{3}[\textsuperscript{3}H]D\textsubscript{3} and 1,24,25-(OH)\textsubscript{3}D\textsubscript{3}.

A 10 ml human plasma sample spiked with tritiated sterols and purified using the revised purification scheme shown in figure 12. The 1,24,25-(OH)\textsubscript{3}D\textsubscript{3} fraction of the LH-20 Sephadex column was applied to HPLC and one minute fractions were collected. 100 μl aliquots were counted for tritium for detection of the added tracer sterol. In order to detect the endogenous "cold" sterol, 50-100 μl aliquots were assayed in triplicate by the assay procedure described in figure 4, using the 94 Ci/m mole assay. This figure shows that 1,24,25-(OH)\textsubscript{3}D\textsubscript{3} elutes at 9-10 minutes from HPLC and that the tracer is an indicator of the endogenous sterol. The arrows represent the fractions that were routinely collected for analysis in future experiments.
Concentration (ng/ml : SEM)

<table>
<thead>
<tr>
<th>Source</th>
<th>1,25 dihydroxy-vitamin D</th>
<th>1,24,25 trihydroxy-vitamin D*</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Control)</td>
<td>1.2 ± 0.92</td>
<td>0.93 ± 0.79</td>
<td>0.29</td>
</tr>
<tr>
<td>L1</td>
<td>30.9 ± 1.9</td>
<td>5.8 ± 2.1</td>
<td>0.19</td>
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<tr>
<td>L2</td>
<td>30.3 ± 2.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>L3</td>
<td>25.3 ± 1.5</td>
<td>3.7 ± 0.32</td>
<td>0.15</td>
</tr>
<tr>
<td>L4</td>
<td>69.5 ± 2.8*</td>
<td>5.5 ± 1.8*</td>
<td>0.79</td>
</tr>
<tr>
<td>L5</td>
<td>30.2 ± 2.7</td>
<td>4.5 ± 0.7</td>
<td>0.15</td>
</tr>
<tr>
<td>L6</td>
<td>25.5 ± 1.2</td>
<td>6.3 ± 2.1</td>
<td>0.25</td>
</tr>
<tr>
<td>P1</td>
<td>35.6 ± 6.4</td>
<td>18.9 ± 2.2</td>
<td>0.53</td>
</tr>
<tr>
<td>P2</td>
<td>28.2 ± 5.4</td>
<td>7.6 ± 2.0</td>
<td>0.29</td>
</tr>
<tr>
<td>P3</td>
<td>40.7 ± 4.1</td>
<td>3.5 ± 0.16</td>
<td>0.09</td>
</tr>
<tr>
<td>T0</td>
<td>30.9 ± 1.9</td>
<td>8.6 ± 1.9</td>
<td>0.23</td>
</tr>
<tr>
<td>T1</td>
<td>48.0 ± 3.0</td>
<td>9.67 ± 2.1</td>
<td>0.36</td>
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<tr>
<td>T2</td>
<td>44.1 ± 2.2</td>
<td>12.8 ± 5.5</td>
<td>15.9 ± 4.7</td>
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<tr>
<td>T3</td>
<td>39.2 ± 3.1</td>
<td>25.1 ± 2.9</td>
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</tr>
<tr>
<td>T4a</td>
<td>38.7 ± 1.8</td>
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<tr>
<td>T4b</td>
<td>29.3 ± 1.2</td>
<td>22.7 ± 5.4</td>
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</tr>
<tr>
<td>T4c</td>
<td>30.8 ± 2.4</td>
<td>14.1 ± 3.9</td>
<td>19.0 ± 1.8</td>
</tr>
<tr>
<td>T4d</td>
<td>35.5 ± 2.4</td>
<td>20.1 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>AVERAGE</td>
<td>34.6 ± 3.0</td>
<td>8.5 ± 2.0</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*Corrected for control and tracer (24,25(OH),1-HOD remaining when yield was determined.
*Not included in the average of normal individuals because donor was taking oral contraceptives.

Table I. Human Plasma Levels of 1,25-(OH)2D and 1,24,25-(OH)3D.

The sources of plasma were:
L1-L6 = Tucson normal adults (approx. 10 mls).
P1-P3 = Phoenix normal adults (approx. 100 mls).
T0 = Tucson newborn umbilical cord blood, 75 mls.
T1-T3 = Tucson adolescent, three separate determinations (16 years old, approx. 100 mls).
T4a-d = Tucson adolescent, 16 years old (10 mls).

P1, P2, T0 and T2 were purified using the initial purification scheme. In all cases (except T4a-d) the 20% isopropanol in hexane HPLC solvent system was used.

The corrected human levels here are represented in pg/ml ± standard error of the mean (SEM). All 1,24,25-(OH)3D values were corrected for the control and the amount of tracer present. The control was a vitamin D deficient chicken, since there should be no detectable vitamin D metabolites, the values represented here are due to assay background primarily due to the lipid residue present. The amount of trihydroxy-tracer present at the time of assay was determined by accounting for the specific activity of the sterol, molecular weight of the sterol, yield of the purified sample, and the aliquot being assayed.

The ratio of the trihydroxy-metabolite to the dihydroxy-metabolite is shown.

The effect of oral contraceptives on 1,25-(OH)2D3 levels is explained in the text.
1,24,25-(OH)\textsubscript{3}D\textsubscript{3} added, there was a significant amount of this tracer sterol remaining after purification of the sample. This amount was calculated and the level of 1,24,25-(OH)\textsubscript{3}D\textsubscript{3} in each human sample was corrected for this. The range of 1,24,25-(OH)\textsubscript{3}D\textsubscript{3} values is from 0.0 to 19.0 pg/ml, and the average value is 8.5 ± 2.0 pg/ml. The 1,25-(OH)\textsubscript{2}D\textsubscript{3} values have also been included and are in agreement with what has been previously reported (60,61,62,63). The average value of 1,25-(OH)\textsubscript{2}D\textsubscript{3} is 34.5 ± 3.0 pg/ml (± SEM). The ratio of the trihydroxy-vitamin to the dihydroxy-vitamin is 0.25 (average), i.e. 1,25-(OH)\textsubscript{2}D\textsubscript{3} is present as four times the 1,24,25-(OH)\textsubscript{3}D\textsubscript{3} concentration. Notice that in certain individuals the ratio was much higher; approximately 0.5. Although the presence of 1,24,25-(OH)\textsubscript{3}D\textsubscript{3} has been previously reported in man using the tracer detection studies described in Chapter 1, this monograph represents the first report of the circulating steady state plasma 1,24,25-(OH)\textsubscript{3}D\textsubscript{3} levels.

The sources of the human plasma were as follows: L1-L6 and P1-P3 were normal Tucson and Phoenix adults respectively; TO represents plasma taken from the umbilical cord of a Tucson newborn; T1-T3 represent an adolescent female, whose 3 plasma samples were processed as individual separate experiments; and T4a-T4d was plasma from the same Tucson adolescent that were processed as separate aliquots in the same experiment to determine interassay variation. The interassay variation for 1,24,25-(OH)\textsubscript{3}D\textsubscript{3} was determined by dividing one plasma sample into 4 equal parts and then purifying and assaying them. The range of values for 1,24,25-(OH)\textsubscript{3}D\textsubscript{3} was 14.1 to 22.7 pg/ml, with the average of 19.0 ± 1.8 pg/ml. The range of values for 1,25-(OH)\textsubscript{2}D\textsubscript{3} was 29.3 to 39.7 pg/ml, with
the average of $33.6 \pm 2.2$ pg/ml. Therefore, the interassay variation for $1,24,25-(OH)_3D$ and $1,25-(OH)_2D$ was 18.8% and 13.1% respectively.

One further aspect of the interassay variation experiment was that the $1,25-(OH)_2D$ samples were purified using the HPLC solvent system of 10% isopropanol in hexane. All the other human samples were purified using the 20% isopropanol in hexane solvent system (see Figure 10 for the elution pattern of $1,25-(OH)_2D$ using these two solvent systems). Table I provides evidence that the level of $1,25-(OH)_2D$ appeared greater when the 20% isopropanol system was used; $43.8 \pm 2.5$ versus $33.6 \pm 2.2$ pg/ml.

There are several interesting findings represented in Table I. There were two donors with supranormal values of $1,25-(OH)_2D_3$ (L2 = 58.3 and L4 = 69.5). Notice that L2 had no detectable $1,24,25-(OH)_3D$. There is no known explanation for this individual to have such high $1,25-(OH)_2D_3$ levels. However, L4 was taking oral contraceptives.

Oral contraceptives containing estrogen, progesterone or their analogues are known to elevate $1,25-(OH)_2D_3$ levels (64,65,66,67). It is interesting to note that the $1,24,25-(OH)_3D$ level of this individual is not correspondingly elevated. Further studies would have to be made to explain this lack of elevation.

Further applications of this new procedure for determining $1,24,25-(OH)_3D$ levels are shown in Table II. The sources of plasma studied here were cows, chickens, and rats. The data represent measurements made using normal animals and animals whose vitamin D metabolites have been altered by dietary or hormonal variations. These variations are known to modify $1,25-(OH)_2D_3$ levels as illustrated in figure 2.
Table II: Plasma Levels of 1,25-(OH)$_2$D$_3$ and 1,24,25-(OH)$_3$D$_3$ in Various Animals.

The levels in normal cows, chickens, and rats are shown; along with the levels in chickens and rats with physiological variations.
These data demonstrate that there is definitely a species variation in the levels of $1,25-(OH)_{2}D$ and $1,24,25-(OH)_{3}D$; this variation is also seen in the ratios of the two metabolites. In the normal animals the rat has approximately equal amounts of $1,25-(OH)_{2}D_3$ and $1,24,25-(OH)_{3}D_3$, whereas the cow $1,24,25-(OH)_{3}D_3$ level is approximately one-half of $1,25-(OH)_{2}D$, and in the chicken $1,24,25-(OH)_{3}D_3$ is 33 fold lower than $1,25-(OH)_{2}D$. These data are preliminary in that the chicken and rat levels were determined from a single plasma pool from 5-10 animals. Finally, the $1,25-(OH)_{2}D$ levels determined by this new procedure are comparable to the levels reported by other workers using other methods (61,62,63).

The physiological variations in the chick are a result of a dietary and hormonal alteration. The dietary modification involved was a vitamin D-deficient regime. It is to be expected that a rachitic chicken has no $1,25-(OH)_{2}D_3$ or $1,24,25-(OH)_{3}D_3$, since there is no source of vitamin D in its diet. However, there was a low level of $1,25-(OH)_{2}D$ and $1,24,25-(OH)_{3}D$ detectable. The apparent presence of these metabolites may be because of slight contamination of the diet, but it is probably nonspecific background detected in the assay.

The second physiological modification studied in the chicken was the effect of an estrogen hormonal analogue, diethylstilbestrol (DES), on the $1,25-(OH)_{2}D$ and $1,24,25-(OH)_{3}D$ levels in normal chicken. It is interesting to note that the $1,25-(OH)_{2}D_3$ level in the DES-dosed chick increases 1.5 fold, whereas the $1,24,25-(OH)_{3}D$ level increases 6.3 fold. This could imply that the two hydroxylases (i.e. the 1-hydroxylase and the 24-hydroxylase) are activated at different rates,
or that the rate of degradation of 1,24,25-(OH)₃D has been affected. These results certainly merit further investigation.

With respect to the study of the physiological variations in the rat, the parameters studied were the effects of dietary modifications for various lengths of time on the 1,25-(OH)₂D and the 1,24,25-(OH)₃D levels. The modification, a low phosphate (0.4%P) or low calcium (0.01% Ca) diet, are known regulators of 1,25-(OH)₂D₃ biosynthesis (68, 69, 70). In the low phosphate rat the 1,24,25-(OH)₃D level increases and parallels the increase in the 1,25-(OH)₂D does not rise to the same extent. (A 2.3-2.7 fold increase for 1,24,25-(OH)₃D versus a 3.9-4.5 fold increase for 1,25-(OH)₂D). Surprisingly, the low calcium rat exemplifies a different situation. By days 20 and 32, the 1,25-(OH)₂D level has risen 5.2-6.5 fold; however, the 1,24,25-(OH)₃D level has decreased 5.7-3.2 fold. This is certainly interesting and will provide the basis for a model to be presented in the next chapter.
CHAPTER 4

DISCUSSION

The experiments reported in this thesis have been designed for the development of a procedure to quantitate \(1,24,25-(\text{OH})_3\text{D}\) isolated from biological fluid. The procedure described here could play a major role in determining the status of \(1,24,25-(\text{OH})_3\text{D}\) as an important metabolite or as an insignificant by-product of the hormonal form of vitamin D, \(1,25-(\text{OH})_2\text{D}_3\). Evidence is presented here that demonstrates that \(1,24,25-(\text{OH})_3\text{D}\) is present in significant quantities in man, rats, and cows, and that in rats and chicks \(1,24,25-(\text{OH})_3\text{D}_3\) levels are regulated by physiological variations.

The controversy that exists regarding the significance of \(1,24,25-(\text{OH})_3\text{D}\) as an important metabolite of vitamin D can be represented by the viewpoints of two groups of workers. DeLuca et al. argue in favor of the role of this metabolite on the basis that it exists in high concentrations, that it persists for a long time in plasma (48 hours), and that \(1,24,25-(\text{OH})_3\text{D}\) can be synthesized \textit{in vivo} under normal circumstances. This viewpoint is contrary to that of Norman and Friedlander. Since they could not detect \(1,24,25-(\text{OH})_3\text{D}\) in the chicken, they maintain that if it is present, it is not present in significant levels to be important. They also point out that the 24-hydroxylated metabolites are always less biologically active, and,
therefore, they believe that the role of 1,24,25-(OH)₃D is primarily the first step in the excretion process of 1,25-(OH)₂D.

The results presented in this thesis corroborate the idea of DeLuca et al. Some of the data that support this is that 1,24,25-(OH)₃D has a higher affinity for the 1,25-(OH)₂D intestinal receptor than all other vitamin D metabolites tested to date (except for 1,25-(OH)₂D₃ itself). The relative affinity reported here is 1/2.6; i.e., 1,24,25-(OH)₃D binds to the 1,25-(OH)₂D receptor 2.6 times less effectively than does 1,25-(OH)₂D. Vitamin D binds to this receptor 1/10,000 as well as 1,25-(OH)₂D, and 25(OH)D binds 1/500 as well. The affinity of this receptor protein for 1,25-(OH)₂D and several analogs has been reported to correlate well with their respective biological activities (13,71). Therefore, it is tempting to speculate that 1,24,25-(OH)₃D has a high biological activity on the basis of its high affinity for the 1,25-(OH)₂D intestinal receptor.

Further evidence that supports an important role for 1,24,25-(OH)₃D is the concentration of this compound in the plasma of the various species examined. Then, the rat it is comparable to 1,25-(OH)₂D the levels of both are approximately 224 pg/ml. In man and cows the level of 1,24,25-(OH)₃D is one-half to one-quarter that of 1,25-(OH)₂D. The chicken was the only species where 1,24,25-(OH)₃D was much less than 1,25-(OH)₂D—approximately 33 fold less. However, the detection of 1,24,25-(OH)₃D in plasma from normal animals is not sufficient to establish its biological role.

The most demonstrative experiments for establishing the role of 1,24,25-(OH)₃D would be in vivo and in vitro studies of the factors
that effect the biosynthesis of 1,24,25-(OH)₃D. Preliminary studies, reported within this monograph, show that the level of 1,24,25-(OH)₃D is regulated by dietary status in the rat and by hormonal status in the chick. In the chicken the estrogen analogue, diethylstilbestrol (DES), raises the level of 1,24,25-(OH)₃D to a greater extent than it raises 1,25-(OH)₂D—a 6.3 fold increase for 1,24,25-(OH)₃D but only a 1.5 fold increase of 1,25-(OH)₂D.

The implications of this pertain to the regulation of the two enzymes, the 1-hydroxylase and the 24-hydroxylase; and the consequence of this could be that although 1,24,25-(OH)₃D is supposedly not functionally active in the normal chicken, this metabolite may play a larger role during hormonally altered physiological states.

The rat studies presented here provide stronger evidence for the regulation of 1,24,25-(OH)₃D biosynthesis possibly through the regulation of the two hydroxylases. A low phosphate diet seems to have an opposing effect on the level of 1,24,25-(OH)₃D than does a low calcium diet. A low phosphate diet causes 3.9-4.5 fold increase in the circulating concentration of 1,25-(OH)₂D and a 2.3-2.7 fold increase in the circulating concentration of 1,24,25-(OH)₃D. This rise in 1,24,25-(OH)₃D levels could have been expected due to reports by MacIntyre et al. (72) and Tanaka et al., where 1,25-(OH)₂D was shown to induce the 24-hydroxylase in the chick kidney (35). However, although 1,25-(OH)₂D levels here were elevated due to the low calcium diet (5.3-6.5 fold), the circulating concentration of 1,24,25-(OH)₃D was decreased, (3.5-5.9 fold). It is apparent from these data that
1,24,25-(OH)3D levels are not always raised due to a high circulating 1,25-(OH)3 levels. A careful examination of the factors involved in the biosynthesis of 1,25-(OH)2D brings to mind the involvement of parathyroid hormone (PTH) (see figure 3). Both low calcium and low phosphate are signals to turn on the synthesis of 1,25-(OH)2D but low calcium does this indirectly, through the mediation of PTH. Although it has been established that PTH induces the 1-hydroxylase, it may through an unknown mechanism suppress the 24-hydroxylase. A model representing the effect of low calcium and low phosphate on the levels of vitamin D metabolites in rats is:

\[
\text{Low Phosphate: } 25-(OH)D \xrightarrow{(+)} 1,25-(OH)2D \xrightarrow{(+)} 1,24,25-(OH)3D \xrightarrow{(-)} PTH
\]

\[
\text{Low Calcium: } 25-(OH)D \xrightarrow{(+)} 1,25-(OH)2D \xrightarrow{(-)} 1,24,25-(OH)3D
\]

In this model low phosphate directly raises 1,25-(OH)2D levels, and 1,25-(OH)2D induces the 24-hydroxylase to raise the 1,24,25-(OH)3D level. This can have several interpretations. The first is that the increase in the 24-hydroxylase may be to quickly remove 1,25-(OH)2D from the system, whereas another interpretation could be that both sterols are necessary to relieve the low phosphate stress. And finally, it could be argued that 1,24,25-(OH)3D alone is responsible for relieving the low phosphate stress, so that 1,25-(OH)2D would be only a precursor for this purpose while not possessing any biological activity of its own.

The effect of low calcium on 1,24,25-(OH)3D levels could be to cause the suppression of the 24-hydroxylase activity (possibly by cyclic AMP) or to suppress the biosynthesis of the enzyme itself. However,
calcium itself may be playing a direct role here in the suppression of
the activity of the enzyme.

This model could be tested by in vivo experiments which would
include testing the effect of 1,24,25-(OH)\textsubscript{3}D in relieving low phosphate
stress. (Studies by Walling et al. using everted gut sacs have shown
that 1,24,25-(OH)\textsubscript{3}D alone can cause increased phosphate absorption in
the intestine (41).) Further experiments especially designed for the
study of the low calcium model would be to remove the effect of PTH
by parathyroidectomy followed by dosing the animal with 1,25-(OH)\textsubscript{2}D
and then measuring the circulating concentration of 1,24,25-(OH)\textsubscript{3}D.

It appears that a major portion of the studies of 1,24,25-(OH)\textsubscript{3}D will
focus around the study of the control of the two hydroxylases. An
in vivo approach would be to use strontium. This metal has been shown
to induce rickets by a selective block of the renal 1-hydroxylase (73).
The use of strontium could possibly allow studies of the 24-hydroxylase
without the effect of the 1-hydroxylase. If the two hydroxylases
differ in their responses to strontium, then the procedure for isolating
and quantitating 1,24,25-(OH)\textsubscript{3}D as described in this thesis could then
be used to study the production of 1,24,25-(OH)\textsubscript{3}D as a function of
putative 24-hydroxylase regulators. It is also interesting to note that
agents that are known to block transcription also block the turn on
of the 24-hydroxylase by 1,25-(OH)\textsubscript{2}D. This could possibly be an effect
on the biosynthesis of the enzyme itself (36). Therefore, transcriptional
inhibitors, such as Actinomycin D, would be useful tools in future
studies.
In addition to in vivo studies, in vitro studies are also plausible. A good system to study this is the primary culture of renal cells that retains its suppressibility of the 1-hydroxylase by 1,25-(OH)₂D₃ (74). If the 24-hydroxylase is present there, as it has been found in tissue culture of chondrocytes (30), and these systems retain in vivo characteristics, then these would be good systems to study the effect of ionic and hormonal manipulations and thus the interrelationships involved in the functions and biosynthesis of 1,24,25-(OH)₃D.

As it stands to date, there is still much to be done to establish the roles of the 24-hydroxylated vitamin D metabolites. The study presented here describes a procedure for the chromatographic isolation and quantitation by a radioreceptor competitive binding assay of 1,24,25-(OH)₃D. The level of 1,24,25-(OH)₃D in the human as determined by this procedure is 8.5 ± 2.0 pg/ml. The normal values for cow, chicken, and rat 1,24,25-(OH)₃D levels are 29.6 ± 5.7, 4.3 ± 1.6, and 243.5 ± 4.3, respectively. Preliminary results indicate that the levels of this sterol are regulated by the physiological status of the animal. Figure 16 summarizes the possible roles of 1,24,25-(OH)₃D, which could by degradation, regulation, or target site actions. Much of the discussion here has focused on the enzyme, hormonal, and mineral aspects of the regulation of 1,24,25-(OH)₃D biosynthesis. It could be that 1,24,25-(OH)₃D is just a pre-excretory product, and the possible end products could be glucuronides of sulfates produced by the liver and excreted in the feces or urine.
Degradation:

\[
\text{Vitamin D} \rightarrow 25(\text{OH})D \rightarrow 1,25(\text{OH})_2D \rightarrow 1,24,25(\text{OH})_3D
\]

? = Side chain cleavage, = GLUCURONIDATION, SULFONATION

Regulation

- Enzyme: \(1\alpha\) hydroxylase vs 24 hydroxylase
- Hormonal: \(1,25(\text{OH})_2D\); PTH; estrogen; others??
- Mineral: Calcium and/or phosphate

Target site action:

- Gut
- Kidney
- Bone
- Parathyroid glands
- Pituitary
- ??? Liver

Figure 16. Possible Roles for \(1,24,25-(\text{OH})_3D_3\).
If it has an important physiological role, 1,24,25-(OH)₃D will be found to act upon certain organs for its particular effects. There may be sites of action in the known target organs for 1,25-(OH)₂D, i.e., intestine, kidney, bone and parathyroid glands. However, there may be new and as yet unknown targets.
APPENDIX I

ABBREVIATIONS USED

1. $1\alpha, 25-(OH)_2D_3$  
   $1,25-(OH)_2D_3$  
   1-alpha, 25 dihydroxycholecalciferol  
   (usually the $1,25-(OH)_2D_3$ used was $1\alpha, 25-(OH)_2D_3$)

2. $25-(OH)D_3$  
   25 hydroxycholecalciferol

3. $24,25-(OH)_2D_3$  
   24,25 dihydroxycholecalciferol

4. $25,26-(OH)_2D_3$  
   25,26 dihydroxycholecalciferol

5. $1\alpha,24,25-(OH)_3D_3$  
   $1,24,25-(OH)_3D_3$  
   1-alpha,24,25 trihydroxycholecalciferol  
   (usually the $1,24,25-(OH)_3D_3$ used was $1\alpha,24,25-(OH)_3D_3$)

6. $1\alpha,25-(OH)_2D_2^*$  
   1-alpha, 25 hydroxyergosterol

7. $1\alpha,25-(OH)_2D$  
   1-alpha, 25 hydroxycholecalciferol and/or ergosterol

8. $1\alpha,(OH)D$  
   1-alpha hydroxycholecalciferol and/or ergosterol

*D$_2$ in the above compounds would indicate ergosterol rather than cholecalciferol.
LIST OF REFERENCES


56. Ishizuka, S., (February 28, 1977), Department of Biochemistry, Teijin Institute for Biomedical Research, Tokyo, Japan.


