Identification of vitamin D2 metabolites and age-related changes in the 1,25-dihydroxyvitamin D3 steroid receptor in male Fischer 344 rats

Nicholas Joseph Koszewski
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Identification of vitamin D$_2$ metabolites and age-related changes in the 1,25-dihydroxyvitamin D$_3$ steroid receptor in male Fischer 344 rats

Koszewski, Nicholas Joseph, Ph.D.

Iowa State University, 1988
Identification of vitamin D₂ metabolites and age-related changes in the 1,25-dihydroxyvitamin D₃ steroid receptor in male Fischer 344 rats

by

Nicholas Joseph Koszewski

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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Iowa State University
Ames, Iowa

1988
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GENERAL INTRODUCTION

Explanation of Dissertation Format

This dissertation is presented in the alternate thesis format and includes two manuscripts. The paper in Section I is concerned with the identification of vitamin D₂ metabolites by FT ¹H NMR, and has been published in *Analytical Biochemistry* (1). The paper in Section II details the identification of a new physiological metabolite of vitamin D₂, 24,26-(OH)₂D₂, and has been published in *Biochemistry* (2). Section III consists of preliminary work studying the effects that age has on the vitamin D steroid receptor prepared from intestine and kidney. Included within each of the three sections is a Literature Cited for references noted within that particular body of work exclusively. A brief review of the literature is included in the General Introduction, and is concerned primarily with firstly, differences in the metabolism of vitamin D₂ and vitamin D₃, and secondly, some characteristics of the 1,25-dihydroxyvitamin D₃ steroid receptor protein. A Summary and Discussion section is included that highlights the major accomplishments of this work, and points out areas for further study. Following the Summary and Discussion, there is a section of Literature Cited for references to both the General Introduction and the Summary and Discussion.

The doctoral candidate, Nicholas Joseph Koszewski, was the principal investigator and author in each of these studies.
Differences in the Metabolism of Vitamin D₂ and Vitamin D₃

Vitamin D₃, contrary to its name, is not truly a vitamin, but rather is now recognized as a pro-steroid hormone (3). The naturally occurring form produced by man, vitamin D₃, is derived from a cholesterol-like precursor found in the skin. The direct action of sunlight on this precursor, 7-dehydrocholesterol, results in cleavage of the B-ring of the steroid structure that upon thermal isomerization yields the characteristic seco-steroid (Figure 1).

Vitamin D₃ circulates in the plasma whereupon it undergoes a series of tissue-specific oxidations that activate the molecule. The first of these occurs in the liver to produce 25-OHD₃,¹ the major circulating form of the vitamin. 25-Hydroxyvitamin D₃ is then specifically hydroxylated at the 1α-position to yield 1,25-(OH)₂D₃, generally considered to be the active hormonal form of the vitamin (3, 4). The simplistic picture outlined here is complicated by the fact that vitamin D₃ can be oxidatively metabolized to a variety of products (5). Most of these many metabolites have no known biological function, and

¹Abbreviations used: 25-OHD, 25-hydroxyvitamin D; 1,25-(OH)₂D, 1,25-dihydroxyvitamin D; 1α-OHD, 1α-hydroxyvitamin D; 24-OHD, 24-hydroxyvitamin D; 1,24-(OH)₂D, 1,24-dihydroxyvitamin D; 1,24,25-(OH)₃D₂, 1,24,25-trihydroxyvitamin D; 1,24,25,26-(OH)₄D, 1,24,25,26-tetrahydroxyvitamin D; 1,24,25,28-(OH)₄D, 1,24,25,28-tetrahydroxyvitamin D.
Figure 1. Structures and numbering schemes of the carbon atoms of vitamin D₃ (upper figure) and vitamin D₂ (lower figure)
Indeed, many have been isolated only from either in vitro work or abnormal physiological conditions. Yet the evidence collected to date indicates that vitamin D₃ is preferentially metabolized at the side chain. In particular, carbon centers C-23, C-24, and C-26 are readily susceptible to further oxidation.

The primary role ascribed to vitamin D₃ is the maintenance of calcium homeostasis (3, 4), although its importance in cell differentiation and immune function has recently been recognized (6-8). The interaction of the activated hormone with target tissues, primarily the intestine, kidney, and bone, results in a net increase in the extracellular calcium concentration. A lack of the vitamin leads to a loss of calcium from the body. The importance of the vitamin is, in retrospect, readily evident as the incidence of rickets, a debilitating disease afflicting mainly children and characterized by poor bone formation, escalated to epidemic proportions during the 19th and early 20th centuries in emerging industrialized nations. During this time vast segments of the population migrated to large industrial cities where often they worked long hours inside factories, and lived in cramped quarters that afforded little sunshine.

It was during the early 20th century that two distinct anti-rachitic factors were isolated and identified (3). The first factor to be fully characterized was designated vitamin D₂ (Figure 1), while the structure of vitamin D₃ became evident some 5 years later. Vitamin D₂ readily undergoes the same hydroxylations in the liver and kidney described for vitamin D₃ leading to the comparable activated
molecule, 1,25-(OH)_{2}D_{2} (9). Vitamin D_{2} evokes the same biological response in man as vitamin D_{3}, and can be readily obtained by the irradiation of ergosterol, an abundant yeast sterol.

Vitamin D_{2} and related metabolites have been shown to contribute significantly to the overall vitamin D status in humans consuming supplemental vitamin D_{2} (10-13). It has also been determined that vitamin D_{2} can readily pass into the milk of lactating mothers and thereby be ingested by the suckling infant (12, 14). In addition, a vitamin D_{2} supplement was observed to significantly raise the 25-OHD plasma concentrations in subjects not previously using a supplement (15, 16). Horst and Littledike measured the concentration of vitamin D_{3} and vitamin D_{2} metabolites in the plasma of various species of both young and aged domestic animals, and found a significant amount of 25-OHD_{2} present in all cases (17). Although the assay used to measure plasma 1,25-(OH)_{2}D could not differentiate between the two vitamin forms, there was a significant decline in circulating 1,25-(OH)_{2}D with age.

Vitamin D_{2} can be metabolized in a similar fashion to produce several metabolites analogous to the vitamin D_{3} system, including the hormonal form of the vitamin. Simple inspection of the side chain, however, would imply that differences between the metabolism of vitamin D_{2} and vitamin D_{3} might exist. The presence of unsaturation at carbon centers C-22/C-23, along with the additional methyl group at C-24, would seem to preclude the existence of the same metabolic pathways for the two vitamins. Indeed, early reports indicated that chickens and New World monkeys were not responsive to the use of
vitamin D$_2$ as an anti-rachitic factor (18-20). Chickens evidently possess the necessary enzymes to convert the vitamin into its active form (21). Hoy et al. have shown that the discrimination against vitamin D$_2$ in chickens occurs because of a faster clearance of 25-OHD$_2$ and 1,25-(OH)$_2$D$_2$ from the plasma in comparison to their vitamin D$_3$ analogs (22). The plasma clearance of the parent vitamins was roughly equivalent.

Horst et al. investigated differences in the metabolism of the two vitamins when administered orally to the pig, rat, and dairy calf (23, 24). This work clearly established that, when presented simultaneously with equal dietary quantities of the two vitamins, pigs and calves discriminated against vitamin D$_2$ and its metabolites. Rats, on the other hand, circulated higher concentrations of vitamin D$_3$, but 25-OHD$_2$ dominated as the major component of the 25-OHD in plasma.

Vitamin D$_2$ has been shown to be less hypercalcemic and less toxic than is vitamin D$_3$ (25, 26). Vitamin D$_2$ was determined to be as effective in the treatment of childhood renal osteodystrophy, and presented fewer hypercalcemic side-effects than did vitamin D$_3$ (27). In addition, 1α-hydroxyvitamin D has proved to be an important therapeutic agent because of its effectiveness in stimulating calcium absorption in the gut and mobilizing calcium from bone (28). The vitamin D$_2$ analog was as effective as the vitamin D$_3$ derivative in standard bioassays. This same study, however, indicated that 1α-OHD$_2$ was 5-15 times less toxic when given to rats than was 1α-OHD$_3$. 
Differences have also been evident in the metabolism of vitamin D$_2$ and vitamin D$_3$ in subcellular fractions from both rat and human liver (29, 30). Vitamin D$_3$ was at least two times more effectively converted to its 25-hydroxylated analog in mitochondrial or microsomal preparations. The authors further suggested that the impaired 25-hydroxylation of vitamin D$_2$, thus preventing its subsequent activation, may account for the sterols lower toxicity.

The 24-position of vitamin D$_2$, in contrast to the similar position in vitamin D$_3$, can be considered to be a highly reactive site. It is both a tertiary carbon as well as an allylic position, and the formation of a reactive intermediate (radical, cation) there would be highly stabilized. The proximity of this reactive center to the 25-position would afford the possibility of hydroxylation at C-24 in vitamin D$_2$. This is indeed the case, as Jones et al. demonstrated, when 24-OHD$_2$ was isolated from the plasma of male rats treated with 100 IU of radio-labelled vitamin D$_2$ (31). Engstrom and Koszewski have determined that production of both isomers of 24-OHD$_2$ occurs in liver homogenates from a variety of species, and actually exceeds the formation of either 25-OHD metabolite (32).

It has recently been shown that 24-OHD$_2$ can undergo 1α-hydroxylation to form 1,24-(OH)$_2$D$_2$, a compound rivalling 1,25-(OH)$_2$D in bio-potency, though the oxidation occurs less efficiently than for either 25-OHD (33). This same study indicated that the concentration of 24-OHD$_2$ in plasma matched that of 25-OHD$_2$ in rats given pharmacological doses of vitamin D$_2$. 24-Hydroxyvitamin D$_2$, however, was shown to
have a much lower affinity for the vitamin D receptor. The limited ability of 24-OH\textsubscript{D}\textsubscript{2} to bind to the receptor, a step necessary for the initiation of a biological response, may aid in explaining the reduced toxicity of vitamin D\textsubscript{2}.

Horst et al. was able to isolate 1,24,25-(OH)\textsubscript{3}D\textsubscript{2} from either bovine or chick kidney homogenates (34). The formation of this metabolite represented an unequivocal deactivation of the vitamin D\textsubscript{2} molecule. Conversely, the comparable vitamin D\textsubscript{3} analog, 1,24,25-(OH)\textsubscript{3}D\textsubscript{3}, maintains significant biological activity, and must undergo further side-chain oxidation to be rendered inactive. Reddy and Tserng isolated and characterized 1,24,25,26-(OH)\textsubscript{4}D\textsubscript{2} and 1,24,25,28-(OH)D\textsubscript{2} from rat kidney perfusions (35). Using 1,25-(OH)\textsubscript{2}D\textsubscript{2} as the substrate, they were able to show that production of these new metabolites proceeded through the initial formation of 1,24,25-(OH)\textsubscript{3}D\textsubscript{2}. The authors suggested that these pathways, which lead to unique vitamin D\textsubscript{2}-specific metabolic products, may in part account for the decreased toxicity observed with vitamin D\textsubscript{2}.

Steroid Receptor Protein for 1,25-Dihydroxyvitamin D\textsubscript{3}

The vitamin D receptor has been shown to be a large, acidic protein that binds 1,25-(OH)\textsubscript{2}D\textsubscript{3} with high affinity. The binding of the hormone is thought to result in conformational changes within the protein, leading to an increased affinity for nuclear components. The receptor-hormone complex is then believed to be able to recognize and bind to specific sequences of DNA (termed response elements; 36, 37),
and alter transcriptional events within the cell. The characteristics of the vitamin D steroid receptor have been reviewed in great detail elsewhere (38-41). What follows is intended to be only a cursory discussion of some of the more salient features of the 1,25-(OH)$_2$D$_3$ steroid receptor protein.

The intestine, a primary target organ for 1,25-(OH)$_2$D$_3$, has been the site that has received the most intense study because of its relatively high concentration of vitamin D receptor. Nevertheless, the receptor constitutes approximately a mere 0.001% of the soluble protein present in the intestinal mucosa (39). Early reports found that the receptor preferentially bound 1,25-(OH)$_2$D$_3$ with high affinity ($K_d = 3.2 \times 10^{-10}$M), and the complex sedimented at 3.7S in high salt sucrose density gradients (42, 43). These values are in generally good agreement with subsequent work on the receptor.

Studies on the steroid receptor have been greatly facilitated by the ability to generate monoclonal antibodies against the protein. Pike et al. were able to prepare a sufficient quantity of purified receptor (ca. 20% pure) from 10 kilograms of chicken intestinal mucosa to successfully develop hybridomas that secreted antibodies directed against the chicken receptor (44-46). One of these monoclonal antibodies, designated 9A7γ, has exhibited good cross-reactivity with vitamin D receptors from other species (47), and is used in the dissertation work presented here. More recently, monoclonal antibodies have been generated against the porcine intestinal receptor as well (48).
The size of vitamin D receptors from various species has been determined using denaturing gel electrophoresis and subsequent immunologic detection of the protein. Mammalian receptors typically range in size from 48-55 kilodaltons (49-51). The chicken vitamin D receptor appears as two bands with molecular weights of 60 and 58 kilodaltons (49, 52, 53). It was suggested that the lower molecular weight protein may arise from cleavage of the larger molecule, although synthesis from multiple receptor transcripts or translation start sites has not been ruled out.

Proteolytic mapping of the chicken intestinal receptor revealed the presence of two distinct functional domains of the protein. Cleavage with trypsin under controlled conditions generated a 20 kilodalton DNA-binding fragment that could be detected immunologically, and a 40-45 kilodalton fragment that retained the hormone (54). Endogenous proteolysis, however, produced a 45-46 kilodalton fragment that was now visible in immunoblots, specifically bound the hormone, but still failed to bind to DNA-cellulose (48). The smaller fragment (ca. 15 kilodaltons) generated from this latter case could not be detected. Endogenous proteolysis evidently cleaves in or near the DNA-binding domain of the receptor so as to render it biologically inactive. Exposure of the chicken receptor to carboxypeptidase A resulted in the immunologic detection of a 56 kilodalton fragment that retained DNA-binding, but was no longer capable of binding hormone (55). The loss of approximately 40 amino acids from the carboxyl terminus produces a protein incapable of binding hormone.
Monoclonal antibodies permitted the screening of cDNA clones encoding the vitamin D receptor from various expression libraries. Clones have been identified that encode the avian (56) and several mammalian intestinal receptors (57, 58). Expression of the clone for the human intestinal receptor has resulted in a functional protein indistinguishable from the native receptor (57). The ability to express intact receptor, or selected domains, from these systems should greatly facilitate a more detailed understanding of the physical properties of the protein.

The complete or partial deduced amino acid sequence for several vitamin D receptors has now been realized, and the degree of homology between several species examined (56-59). The analyses indicated that the vitamin D receptor is highly conserved between species, with the avian protein being the largest at approximately 60 kilodaltons. There is a general decrease in receptor size as one proceeds up the evolutionary ladder with the human receptor being the smallest (ca. 48 K) examined thus far.

A comparison of the sequence of the human vitamin D receptor with other similar hormone receptor systems indicated that several conserved regions existed (60). One of these, a cysteine-rich region near the amino terminus, has been linked to the formation of finger-like structures coordinately bound to zinc. These finger-like projections are believed to be responsible for the binding of the molecule to DNA. Divalent cations, particularly zinc and cadmium, have been shown to stabilize the DNA-binding ability of the vitamin D receptor (38). A
second conserved region has been identified among the various receptors that lies within the hormone-binding domain, but whose exact role has yet to be defined. The highest degree of homology, however, existed between the vitamin D and thyroid receptors. There is a unique region near the carboxyl terminus that is highly homologous between these two proteins exclusively. This finding may indicate that these receptors evolved from a single primordial gene.

The vitamin D receptor is capable of being covalently modified via phosphorylation. Treatment of 3T6 fibroblasts with \( ^{32} \text{P} \)-orthophosphate in the presence and absence of hormone clearly established that the receptor was incorporating the label when exposed to 1,25-(OH)\(_2\)D\(_3\) (61). Receptor isolated from hormone-treated 3T6 cells also displayed a perceptible decrease in its electrophoretic mobility relative to an untreated control, an observation consistent with protein phosphorylation (49, 61).

Hormone treatment has also resulted in significant up-regulation of receptor concentration, both in vivo (62) and in vitro (49, 63-65). In vitro translation of receptor m-RNA isolated from 3T6 cells grown in either the presence or absence of hormone indicated that far more translatable message existed in the treated cells (49). Furthermore, the in vitro translated receptor displayed the same electrophoretic mobility as receptor isolated directly from untreated cells. This was a further indication that in treated cells the receptor undergoes post-translational modification, i.e. phosphorylation (49, 61).
The vitamin D receptor has been detected in a wide variety of tissues (39, 40). Immunocytochemical analyses demonstrated that the protein is often localized to certain cell types within a tissue (47, 66, 67). For example, immunoreactivity was observed in epithelial cells of villi and crypts in the duodenum and in tubular cells of the kidney. Many other tissues where the receptor has been identified are not directly related to the maintenance of calcium homeostasis, and the exact role of the receptor within them remains to be elucidated (6-8).

Several approaches have been taken to ascertain the exact cellular location of the unoccupied receptor. Immunocytochemical staining for the protein has placed it exclusively within the nuclear compartment (66, 67). This result was consistent with earlier observations that indicated the receptor was predominantly localized to the nuclei/chromatin in low-salt extractions of homogenized tissue (68). Alternatively, the cytoplasmic/nuclear ratio of unoccupied receptor was determined to be better than 2:1 when extracted at physiological salt concentrations (69). Walters et al. used cell enucleation with cytochalasin B to demonstrate a similar distribution in two kidney cell lines (70). These contradictory results, sometimes emanating from the same laboratory, indicate the recent turmoil this subject has created (41, 71), but current dogma favors a mostly nuclear localization for the unoccupied vitamin D receptor.

The interaction of 1,25-(OH)$_2$D$_3$ with its receptor protein has been shown to mediate the biological response of the hormone in a
number of systems. Induction of a 25-OHD$_3$-24-hydroxylase enzyme activity has been demonstrated in a dose-dependent fashion for 1,25-(OH)$_2$D$_3$ in several cell culture systems (72, 73). 1,25-Dihydroxy-vitamin D$_3$ was effective in suppressing colony formation by a number of cultured cancer lines, but only in those cell lines where it could be demonstrated that a receptor for the hormone existed (74). In these studies the biological response generated by other vitamin D metabolites was dependent upon that particular molecule's affinity for the receptor. Good correlation was also found between nuclear uptake of 1,25-(OH)$_2$, receptor occupancy, and increased transcription and synthesis of the vitamin D-dependent calcium binding protein in chicken intestine (75).

Vitamin D-dependent rickets type II is a hereditary disease state that provides additional evidence to support the receptor-mediated mode of action for 1,25-(OH)$_2$D$_3$ (76, 77). The disease is characterized by hypocalcemia, secondary hyperparathyroidism, normal serum 25-OHD, and mildly to markedly elevated serum 1,25-(OH)$_2$D$_3$ concentrations. Type II rickets is attributed to a dysfunction of the vitamin D receptor that can manifest itself in many ways. Receptors isolated from cultured skin fibroblasts from one group of patients displaying type II rickets demonstrated a defect in binding to DNA-cellulose (78, 79). Still another case that was recently described indicated that the protein exhibited an abnormally low affinity for the hormone (80). A kidney cell line has been identified that possesses a variant receptor also displaying a low affinity for hormone and may prove useful as
a model system to study this particular abnormality (81). Finally, the disease may present itself in a form that is characterized by normal affinity of the receptor for hormone, and normal or near normal elution of the receptor-hormone complex from DNA-cellulose (79). In these cases the receptor-hormone complexes failed to localize in the nucleus, indicating either an additional domain on the receptor not measured in these assays as defective, or the assays used to measure hormone binding and DNA binding may not accurately reflect cellular conditions.
SECTION I.

USE OF FOURIER TRANSFORM $^1$H NMR IN THE IDENTIFICATION OF
VITAMIN D$_2$ METABOLITES
INTRODUCTION

The identification of vitamin D metabolites has been achieved primarily through the use of uv absorbance and mass spectroscopy (1). The sensitivity of mass spectroscopy (25-200 ng) has been well suited to the study of vitamin D metabolism, particularly because most metabolites are present in biological fluids in microgram to nanogram quantities. The interpretation of mass spectral data, however, often is not straightforward. Specific chemical modifications of the metabolite are often required, followed by additional mass spectral analysis. Routine use of a rapid, sensitive, and nondestructive analytical technique for assigning the structures of vitamin D metabolites is needed.

A method that best meets these criteria is FT $^1$H NMR spectroscopy. The widespread use of $^1$H NMR to aid in the

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1Abbreviations used: FT, Fourier transform; D, deuterium; 24-OHD$_2$, 24-hydroxyvitamin D$_2$; 25-OHD$_2$, 25-hydroxyvitamin D$_2$; 24,25-(OH)$_2$D$_2$, 24,25-dihydroxyvitamin D$_2$; 24,26-(OH)$_2$D$_2$, 24,26-dihydroxyvitamin D$_2$; 1,24,25-(OH)$_3$D$_2$, 1,24,25-trihydroxyvitamin D$_2$; 1,25,26-(OH)$_3$D$_2$, 1,25,26-trihydroxyvitamin D$_2$; 1,25,28-(OH)$_3$D$_2$, 1,25,28-trihydroxyvitamin D$_2$; Δ22-1,25-(OH)$_2$D$_3$; Δ22-1,25-dihydroxyvitamin D$_3$; 1,25-(OH)$_2$D$_3$, 1,25-dihydroxyvitamin D$_3$. 
identification of new vitamin D metabolites has been limited by the relatively large amounts of material and extended data acquisition times needed to obtain a suitable spectrum (1, 2). In spite of these limitations, significant insights concerning the solution conformations of vitamin D$_3$ and its analogs have been described (3-9). It is the advent of FT NMR, coupled with superconducting magnets and their routine use in the laboratory, that will undoubtedly lead to an increased emphasis on $^1$H NMR spectroscopy as a means of identifying vitamin D metabolites on a microgram scale. At the same time, interest in the metabolism and biological properties exhibited by vitamin D$_2$ (Fig. 1) is leading to increased research efforts in this area (10-13). In conjunction with these efforts, we now report our $^1$H NMR findings for a variety of vitamin D$_2$ analogs.
Figure 1. Structure and numbering scheme of the carbon atoms of vitamin D$_2$
MATERIALS AND METHODS

Chemicals and Glassware

Deuterated chloroform was purchased from Aldrich Chemical Co., Milwaukee, WI (99.8 at.% D) and MSD Isotopes, Montreal, Canada (99.96 at.% D). Deuterium oxide was purchased from Aldrich Chemical Co. (99.8 at.% D). Acetone-d₆ was purchased from MSD Isotopes (99.5 at.% D). Glass inserts for the 5-mm NMR tubes were purchased from Wilmad Glass Company, Inc. (Buena, NJ). Crystalline vitamin D₂ was purchased from Sigma Chemical Co. (St. Louis, MO). The compounds 1,25,26-(OH)₃D₂, 1,25,28-(OH)₃D₂, Δ22-1,25-(OH)₂D₃, and 1,25-(OH)₂D₃ were prepared by E. G. Baggiolini and M. R. Uskokovic (unpublished results). Synthetic 24(R),25-(OH)₂D₂ was a gift from Dr. Kobayashi, Department of Hygienic Sciences, Kobe Women's College of Pharmacy, Kobe Japan. 1,24,25-Trihydroxyvitamin D₂ was prepared as described by Horst et al. (14). The remaining vitamin D₂ metabolites were isolated from bovine plasma and purified by HPLC. The identities of these metabolites were confirmed by their comigration on HPLC with known standards, by electron-impact mass spectrometry (data not shown), as well as by their 300-MHZ ¹H NMR spectra (Tables 1 and 2).

¹H NMR Analysis

¹H NMR spectra were recorded on a Nicolet 300-MHZ FT spectrometer equipped with a NMC-1280 computer. A 55° flip-angle was used and
Table 1. 300-MHz FT $^1$H NMR data of vitamin D$_2$ metabolites

<table>
<thead>
<tr>
<th>Proton assignment</th>
<th>Chemical shifts ($\delta$, ppm), signal multiplicities, and coupling constants (J, Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D$_2$</td>
</tr>
<tr>
<td>6</td>
<td>6.22 d 11.4</td>
</tr>
<tr>
<td>7</td>
<td>6.02 d 11.4</td>
</tr>
<tr>
<td>22, 23</td>
<td>5.18 m</td>
</tr>
<tr>
<td>19Z</td>
<td>5.04 brs</td>
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<td>4.81 brs</td>
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<tr>
<td>26, 27</td>
<td>0.83 d 6.6</td>
</tr>
<tr>
<td>18</td>
<td>0.54 s</td>
</tr>
</tbody>
</table>

$^a$Abbreviations used: brs, broad singlet; d, doublet; m, multiplet; s, singlet.
Table 2. 300-MHz FT $^1$H NMR data of 1α-hydroxylated vitamin D$_2$ and vitamin D$_3$ metabolites

<table>
<thead>
<tr>
<th>Proton assignment</th>
<th>Chemical shifts (δ, ppm), signal multiplicities, and coupling constants (J, Hz)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>1,24,25-(OH)$_3$D$_2$</td>
</tr>
<tr>
<td>6</td>
<td>6.37 d 11.1</td>
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<tr>
<td>7</td>
<td>6.00 d 12.0</td>
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<tr>
<td>22, 23</td>
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<td>19E</td>
<td>5.31 brs</td>
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<td>18</td>
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<tr>
<td>3α</td>
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<td>1.27 s</td>
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<tr>
<td>21</td>
<td>1.04 d 6.6</td>
</tr>
<tr>
<td>26, 27</td>
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<td></td>
<td>1.19 s</td>
</tr>
<tr>
<td>18</td>
<td>0.56 s</td>
</tr>
</tbody>
</table>

*Abbreviations used: brs, broad singlet; d, doublet; m, multiplet; s, singlet.
samples received a 6-μs pulse. The sweep width was 4000 Hz. The data acquisition time was 2.05 s, and a delay time of 1.00 s was used. The elapsed time necessary to obtain a suitable spectrum varied from a few minutes to 4 h, depending upon the concentration of metabolite. Spectra were obtained in standard 5-mm NMR tubes at metabolite concentrations of from 0.09 to 20 mM (5 μg-1 mg/130 μl). Chemical shifts were measured relative to chloroform at 7.24 ppm. Coupling constants were generated by the NMC-1280 computer.

To maintain compound purity and to reduce water interference in data collection, the NMR tubes were prepared in the following manner. The tubes were initially rinsed with a 1 M ammonium hydroxide solution followed by distilled water and reagent-grade ethanol. After the tubes were dried under argon, deuterium oxide was added, and the tubes were allowed to stand for 24-48 h at room temperature. When the glass inserts were used, deuterium oxide was added both to the 5-mm tube and to the cavity of the insert, and the pieces were assembled together. After 24-48 h in D₂O, the tubes were rinsed with acetone-d₆ and dried under argon. Vitamin D compounds, stored in ethanol solution, were dried under argon, dissolved in a minimum amount of chloroform-d₅, and dried again before being transferred via chloroform-d into the NMR tube. Chloroform-d₅ also was added to the cavity of the glass insert. The samples were then shielded from light and stored over dry ice prior to their use.
RESULTS AND DISCUSSION

We sought to exploit the recent advances made in FT NMR spectroscopy as an aid to determine the structures of vitamin D₂ metabolites on a microgram scale. The metabolism of vitamin D₂ is particularly well suited to study by $^1$H NMR because the chirality and unsaturation that already exist in the side chain create unique resonances for those methyl groups and olefinic protons. Alterations in the structure of the side chain should be readily observable by a change in chemical shift and/or signal multiplicity of the characteristic resonance. Examination of the values listed in Table 1 reveals that this is indeed the case. The side-chain hydroxylation of vitamin D₂ at C-25 results in distinct singlets for the C-26 and C-27 methyl group resonances in addition to their shift downfield to δ1.16 and δ1.12. At the same time the doublet corresponding to the C-28 methyl group and the multiplet of the side-chain olefinic protons also are shifted slightly downfield.

Crucial to our recent identification of a new vitamin D₂ metabolite, 24,25-(OH)₂D₂ (N. J. Koszewski, J. L. Napoli, T. A. Reinhardt, and R. L. Horst, submitted for publication), was the interpretation of the 300-MHz $^1$H NMR spectrum. We rationalized that the doublet appearing at δ1.05 (J = 6.6 Hz) corresponded to the C-21 methyl group. This was despite the fact that the literature NMR values for vitamin D₂ have placed the position of this group in the vicinity of δ0.90 and the C-28 methyl group near δ1.01 (9, 15). Yet, the overwhelming body
of evidence accumulated in our study indicated that the metabolite had been hydroxylated at the C-24 position, giving rise to a singlet for the C-28 methyl group at δ1.30. To reconcile this difference, we examined the 1H NMR spectra of a number of vitamin D₃ metabolites.

Among the most interesting findings from this work were the assignments of the C-21 and C-28 methyl groups. Both of these signals appear as doublets in vitamin D₃ (J = 6.6 Hz). The previous assignment of the C-21 methyl group to the signal at δ0.90 was most likely made on the basis of a direct comparison with the signals arising from vitamin D₃. In vitamin D₃, the C-21 methyl group signal appears as a doublet at δ0.92. It would therefore follow that the corresponding peaks in vitamin D₂ should arise from the same source. An examination of the data presented in Tables 1 and 2 leads to a different conclusion. That is, the C-21 methyl group gives rise to the doublet at approximately δ1.01, whereas the doublet in the region of δ0.90 actually corresponds to the C-28 methyl group. In the metabolite series consisting of 24-OHD₂, 24,25-(OH)₂D₂, 24,25-(OH)₂D₂, and 1,24,25-(OH)₃D₂, a doublet consistently appears near δ1.03 (J = ca. 6.6 Hz). This signal can only be ascribed to the C-21 methyl group because replacement of the C-24 proton by a hydroxyl group precludes splitting of the C-28 methyl group into a doublet. In all of the C-24 hydroxylated compounds examined in this series, the C-28 methyl group appears as a singlet shifted farther downfield in conjunction with the C-24 hydroxylation. The situation for 25-OHD₂ is similar in that the C-21 methyl group again appears as a doublet at δ1.03. However, the
C-28 methyl resonance now appears as a doublet at 60.99, shifted downfield as a result of its proximity to the C-25 hydroxyl group.

As further confirmation of these findings, we examined (Table 2) the 300-MHz $^1$H NMR spectra of A22-1,25-(OH)$_2$D$_3$ (Fig. 2) and 1,25,28-(OH)$_3$D$_2$. The side chain for this synthetic vitamin D$_3$ metabolite resembles that of 25-OHD$_2$ in every respect except for the replacement of the C-28 methyl group with a proton. Now the C-21 methyl group appears as a doublet at 61.03, in excellent agreement with our observations for the various vitamin D$_2$ metabolites. The position of the C-21 methyl signal in 1,25-(OH)$_2$D$_3$ is located at 60.93. In the $^1$H NMR spectrum of synthetic 1,25,28-(OH)$_3$D$_2$, the C-28 methylene resonances are shifted downfield in accordance with the presence of the C-28 hydroxyl group. The C-21 methyl group again appears as a doublet at 61.04. Clearly then, the C-21 methyl group is significantly deshielded in the presence of C-22/C-23 unsaturation relative to its position in vitamin D$_3$. On the other hand, the upfield position of the C-28 methyl group with respect to the C-21 methyl group in vitamin D$_2$ might be attributable to some shielding effect exerted by the C-26 and C-27 methyl groups.

Although the aforementioned uniqueness of the vitamin D$_2$ side chain lends itself to the monitoring of its metabolism by $^1$H NMR, previous limits on the amount of metabolite required to obtain a suitable spectrum have been prohibitive (1). To be of any practical
Figure 2. 300-MHz $^1$H NMR of 1 mg of $\Delta 22$-1,25-(OH)$_2$D$_3$. The position of the C-21 methyl signal appears at 61.03, shifted 0.1 ppm downfield from its position in 1,25(OH)$_2$D$_3$, and is in good agreement with our vitamin D$_2$ $^1$H NMR data.
use in the study of vitamin D₂ metabolism, the amount of material required to obtain a spectrum needed to be reduced. Using a 300-MHz $^1$H NMR spectrometer and computer, we have successfully worked toward meeting this requirement. To date, our best results have been achieved using a 55° flip-angle and a 6-μs pulse with a sweep width of 4000 Hz. The availability of isotopically enriched chloroform-d solvent (99.96 at.% D) and pretreatment of the NMR tubes with D₂O together greatly reduced the size of extraneous peaks and resulted in better baseline resolution. The use of a glass insert also allowed us to increase the concentration of the sample by reducing the volume to 130 μl of chloroform-d/metabolite in a standard 5-mm FT NMR tube. This effectively places more of the sample within the receiving coils of the NMR instrument. Presented in Fig. 3 is a 300-MHz $^1$H NMR spectrum of 5 μg of 24(R),25-(OH)₂D₂ isolated from bovine plasma. Taken in slightly less than 4 h (ca. 4800 scans), this spectrum permits detailed examination of the regions of particular interest, namely, from 7.00 to 3.50 ppm and from 1.35 to 0.25 ppm. These areas account for the olefinic, hydroxylic, and methyl group protons and, as such, provide a wealth of information concerning the structure of a metabolite. In addition, the nondestructive nature of NMR work permits the ready recovery of the compound in question.

Regarding 24,25-(OH)₂D₂, there are several discrepancies in the literature concerning the $^1$H NMR assignments made for the C-26, C-27, and C-28 methyl groups of this compound (16, 17). We unequivocally established the identity of our biologically prepared 24(R),25-(OH)₂D₂
Figure 3. 300-MHz $^1$H NMR of 5 µg of 24(R),25-(OH)$_2$D$_2$. Data collection required slightly less than 4 h. This spectrum permits detailed examination of areas of particular interest, namely, the olefinic, hydroxylic, and side-chain methyl group resonances.
on two different HPLC solvent systems. In our study, the C-26 and C-27 methyl groups appear as distinct singlets at 61.18 and 61.21. This chemical shift in equivalence can be attributed to their proximity to the C-24 chiral center. In vitamin D\textsubscript{2} itself, the observed resonance of the C-28 methyl protons is 0.07 and 0.09 ppm farther downfield than the C-26 and C-27 methyl group protons (Table 1). Hydroxylation of both C-24 and C-25 would be expected to produce downfield shifts of nearly the same magnitude for these three methyl groups from their relative positions in vitamin D\textsubscript{2}. This is in agreement with our assignment of the C-28 methyl protons to the signal at 61.27, 0.06 and 0.09 ppm downfield from the assigned C-26 and C-27 methyl resonances. In fact, using our data for vitamin D\textsubscript{2}, 24-OHD\textsubscript{2}, and 25-OHD\textsubscript{2}, one can accurately predict the additive effects seen for dihydroxylation in the chemical shifts of these methyl groups for 24,25-(OH)\textsubscript{2}D\textsubscript{2} and 1,24,25-(OH)\textsubscript{3}D\textsubscript{2} (Tables 1 and 2).

Also of some interest in characterizing vitamin D\textsubscript{2} metabolites is the relative position of the complex multiplet corresponding to the C-22/C-23 olefinic protons. This position is dependent upon the number and sites of hydroxylation in the side chain. A downfield shift of 0.25 ppm is evident for hydroxylation at C-24 alone, whereas the same type of shift occurs to a lesser extent (0.14 ppm) for C-25 hydroxylation alone. The effect of dihydroxylation can be seen to be nearly additive in the 0.38-ppm downfield shift observed for this multiplet in the spectra of 24,25-(OH)\textsubscript{2}D\textsubscript{2} and 1,24,25-(OH)\textsubscript{3}D\textsubscript{2}.
There seems to be a general misconception concerning the assignments of the 19Z and 19E protons in 1α-hydroxylated vitamin D metabolites. When a vitamin D metabolite becomes hydroxylated at the 1α-position, the normal assignment of 19Z and 19E protons becomes reversed. This can be attributed to a change in the assigned priority of the C-10 substituent groups in conjunction with the oxygen atom now present at the 1-position. As such, what was 19Z in the unsubstituted case now becomes 19E in the 1α-hydroxylated compound. We have reflected this view in our assignments made in Tables 1 and 2.

We have successfully obtained high-resolution $^1$H NMR spectra of as little as 5 µg of a vitamin D$_2$ metabolite. This increase in sensitivity can be attributed to superconducting magnets for FT NMR instruments, advances in computer software, and careful sample preparation. Although high-resolution FT $^1$H NMR is not yet as sensitive a method as mass spectral analysis, we have demonstrated that it is a viable analytical tool in the determination of microgram quantities of vitamin D metabolite structures.
LITERATURE CITED


SECTION II.

24,26-DIHYDROXYVITAMIN D₂: A UNIQUE PHYSIOLOGICAL METABOLITE OF VITAMIN D₂
INTRODUCTION

The metabolism of vitamin D$_3$ has been extensively characterized and largely consists of oxidative modifications occurring in the side chain (1-8). The metabolism and biological activity of vitamin D$_2$ are generally thought to parallel those of vitamin D$_3$, and as such, it is freely administered to both humans and commercially important mammals (9, 10). As a result, vitamin D$_2$ is prescribed for patients with a wide variety of vitamin D and calcium-related diseases.

Inherent differences in vitamin D$_2$ metabolism from that of vitamin D$_3$ would appear to be inevitable on the basis of its altered side chain structure. The presence of the C-22 alkene, coupled with the methylation of C-24, can be viewed as a barrier to normal oxidative processes known to occur in vitamin D$_3$ (11, 12). In human patients treated with phenobarbitone/phenytoin, the actions of vitamin D$_2$ and vitamin D$_3$ were seen to be quite different with respect to bone metabolism (13). In addition, the 25-hydroxylase activity in human liver exhibited different rates of mitochondrial hydroxylation for vitamin D$_2$ and vitamin D$_3$ (14, 15). Consistent with these findings was the recent observation that other mammals such as the pig and rat discriminated between vitamin D$_2$ and vitamin D$_3$ (11).

In conjunction with an ongoing study concerning the toxicity of vitamin D compounds, we now report the isolation and unequivocal identification of a unique vitamin D$_2$ metabolite, 24,26-((OH)$_2$)D$_2$.\footnote{1}
This represents the first significant deviation of vitamin D₂ metabolism from the more extensively characterized metabolism of vitamin D₃.

Abbreviations: 24,26-(OH)₂D₂, 24,26-dihydroxyvitamin D₂; 24-OHD₂, 24-hydroxyvitamin D₂; 25-OHD₂, 25-hydroxyvitamin D₂; 24,25-(OH)₂D₂, 24,25-dihydroxyvitamin D₂; 25,26-(OH)₂D₃, 25,26-dihydroxyvitamin D₃; 25,26-(OH)₂D₂, 25,26-dihydroxyvitamin D₂; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; GC-MS, gas chromatography-mass spectroscopy; NMR, nuclear magnetic resonance; PCC, pyridinium chlorochromate; ICT, intestinal calcium transport; BCR, bone calcium resorption.
MATERIALS AND METHODS

General

High-performance liquid chromatography (HPLC) was performed with Waters Associates ALC/GPC 204 liquid chromatographic equipment. HPLC columns were purchased from DuPont Instruments (Wilmington, DE). Zorbax-Sil refers to microparticulate silica gel columns. Zorbax-ODS refers to the octadecasilane derivative of the microparticulate silica gel columns. Zorbax-NH₂ refers to the 3-aminopropyl triethoxy derivative of the microparticulate silica gel columns. HPLC solvents were purchased from Burdick and Jackson Laboratories (Muskegon, MI) or Fisher Scientific Company (Fair Lawn, NJ). Florisil was purchased from Fisher Scientific Company. UV spectra were obtained in ethanol with a Beckman Model 25 recording spectrometer. Low-resolution mass spectra were obtained with solid probes by using a Finnigan Model 4000 Quadrupole GC-MS. The analyses were carried out at 70 eV with an ionizer temperature of 150°C while the probe was heated from ambient temperature to 350°C. High-resolution mass spectra were obtained with solid probes using a Kratos MS 50 GC-MS mass spectrometer. The 300-MHz proton NMR spectra were taken in deuterated chloroform on a Nicolet NT-300 FT NMR spectrometer. Gas-liquid chromatographic analysis with flame ionization detection was carried out on a Hewlett Packard Model 5840A gas-liquid chromatograph.
Compounds

Vitamin D<sub>2</sub> was purchased from Sigma Chemical Co. (St. Louis, MO). Inhoffen-Lythgoe diol was a gift from Dr. M. Uskokovic (Hoffmann-La Roche, Nutley, NJ). Standard 25(R),26-(OH)<sub>2</sub>D<sub>2</sub> and 25(S),26-(OH)<sub>2</sub>D<sub>2</sub> were gifts from Dr. D. Williams (University Chemical Laboratory, Cambridge, England). Deuterated chloroform (99.8 atom % D) and pyridinium chlorochromate were purchased from Aldrich Chemical Company (Milwaukee, WI).

Isolation of Vitamin D<sub>2</sub> Metabolites from Cow Plasma

A Jersey cow was given weekly intramuscular injections of 1 g (40 x 10<sup>6</sup> units) of vitamin D<sub>2</sub>. After 4 weeks of treatment, blood was collected in heparinized containers. Plasma was prepared by centrifugation and stored at -20°C.

Vitamin D metabolites were extracted by using the following procedure. To 7 L of plasma was added 1400 g of ammonium sulfate. After shaking for 1 h, 8 L of acetonitrile was added, and the mixture was shaken until an emulsion formed. The phases were separated by adding 2 L of methanol. The resulting mixture was filtered to remove the precipitated proteins, and the remaining pellet was reextracted with 5.4 L of acetonitrile. Water (3 volumes) was added to the combined organic phases, and the mixture was applied to an 18 cm x 18 cm ODS (Waters Associates, Milford, MA) equilibrated in 25% water in acetonitrile. After the sample was applied to the column bed, 5 L of 40% water in methanol was applied to elute the more polar lipids.
vitamin D metabolites were then eluted by adding 8 L of acetonitrile. The acetonitrile was removed by vacuum, and the resultant lipids were dissolved in 2 mL of 2-propanol/methylene chloride/hexane (1:10:89). The dissolved lipids were applied to a Zorbax-Sil HPLC prep column (2.2 cm x 25 cm) and the vitamin D metabolites were eluted by use of a gradient of 2-propanol/methylene chloride/hexane (1:10:89 to 30:10:60).

Metabolism and Biological Evaluation Studies

Metabolites, including 24-OHD$_2$ and 25-OHD$_2$ for use in evaluation studies, were purified further using methods previously described (16). Initial experiments to study in vivo metabolism of various monohydroxylated vitamin D$_2$ substrates were conducted using male weanling rats (Holtzman, Madison, WI) divided into four groups. Three of the groups were maintained on a vitamin D-deficient diet for 4 weeks and then received daily oral supplements of 100 IU of either vitamin D$_2$ (group I) or vitamin D$_3$ (other two groups) for 3 weeks. During the last week of supplementation with vitamin D$_3$ one group of rats received daily intraperitoneal injections of 5 µg of 24-OHD$_2$ (group II) in 100 ml of propylene glycol carrier for 5 days, while the other group was similarly injected with 25-OHD$_2$ (group III). The fourth group of rats (group IV) was maintained on a low-calcium (0.005%), vitamin D-deficient diet for 4 weeks, and then given intraperitoneal injections with 24-OHD$_2$ as described. At the end of the treatment periods, blood was collected by cardiac puncture in heparinized syringes and the plasma was frozen at -20°C.
Extraction of the plasma and isolation of the vitamin D₂ metabolites was carried out as described previously (16, 17).

Biological activity of vitamin D₂ metabolites was determined with male weanling rats (Holtzman, Madison, WI) as described previously (18).

Silylation

24,26-Dihydroxyvitamin D₂ (500 ng) was allowed to react with N-methyl-N-(trimethylsilyl)trifluoroacetamide (50 µL) at 90°C for 2 h to effect persilylation. The mixture was dried under a stream of N₂ and purified by reverse-phase HPLC. The silylated product was eluted with 0.1% methylene chloride in methanol and analyzed by low-resolution mass spectroscopy.

Ozonolysis

A solution of methylene chloride at -90°C was saturated with ozone, and the resultant blue solution was stored on dry ice. To the metabolite (2 µg) in 100 µL of pyridine at -90°C was added 100 µL of the methylene chloride/ozone solution. The mixture was kept at -90°C for 30 s and then dried at room temperature under a stream of N₂. The residue was dissolved in 10 µL of ethanol and analyzed by GLC using a 2 mm x 10 ft 3% SP 2100 column employing N₂ as carrier gas at a flow rate of 25 mL/min. Temperature programming was as follows: 100°C/1 min.; 10°C/min.; 240°C/30 min. The major product peak eluted at 12.75
min. at a temperature of 217°C. The low-resolution mass spectrum was obtained by GC-MS.

Oxidation of Inhoffen-Lythgoe Diol

The diol (100 µg) was dissolved in 200 µL of methylene chloride to which 2 mg of FCC was added. The suspension was kept at room temperature for 20 min with intermittent vortexing, and then 100 µL of 2-propanol was added. The mixture was dried under a stream of N₂. The brown residue was dissolved in methylene chloride and then filtered through a short column of Florisil, and the column was washed with ether. The eluent was dried under a stream of N₂ to ca. 50 µL and analyzed by low-resolution GC-MS as described.

Radioligand Binding Assay

The comparison of the relative binding affinity of 24,26-(OH)₂D₂ to other vitamin D metabolites for the rat plasma vitamin D binding protein was carried out as previously described (19).

Plasma Assays

Vitamin D₂ and vitamin D₃ metabolite concentrations were assayed by HPLC or competitive protein binding as described by Horst et al. (17), or by radioimmunoassay as described by Hollis and Napoli (20).
RESULTS

Because of the lack of commercially available vitamin D₂ metabolites, plasma from a cow intoxicated with vitamin D₂ was used in anticipation of isolating large quantities of 25-OHD₂, 24,25-(OH)₂D₂, and 25,26-(OH)₂D₂ for use as laboratory standards. During the isolation and identification procedures, we observed that the metabolite tentatively identified (because of its HPLC migration character; 16) as 25,26-(OH)₂D₂ reacted unpredictably in several independent identification procedures. Most notable were the insensitivity of the metabolite to periodate (data not shown) and an uncharacteristic mass spectrum. Subsequently, more extensive identification techniques were employed to positively identify the new metabolite.

The metabolite, purified from cow plasma, displayed a UV absorbance spectrum characteristic of a 5(E),7,10(19)-triene chromophore (not shown). The absorbance maximum was at 265 nm, and the absorbance minimum was at 229 nm. On the basis of the absorbance spectrum, approximately 80 µg of the metabolite was isolated from the bovine source.

An insufficient amount of metabolite was isolated from rat plasma for identification. However, the metabolite isolated from the rat plasma comigrated with the bovine compound on two different HPLC systems. Comigration was observed on a Zorbax-Sil column developed in methanol/2-propanol/methylene chloride/hexane (0.75:2.5:25:72, peak elutes at 24-28 mL), and a Zorbax-NH₂ column developed in methanol/2-
propanol/hexane (1:3:96, peak elutes at 40-44 mL). The comigration on two chemically distinct HPLC systems verified that the material isolated from the bovine and rat sources was identical (16). The mobility of this new metabolite relative to several known vitamin D compounds on a Zorbax-Sil column developed in methanol/acetonitrile/methylene chloride (1:20:180) is shown in Figure 1.

The high resolution mass spectrum of the metabolite (Figure 2) had a molecular ion at m/z 428.3286 indicating an elemental composition of $C_{28}H_{44}O_3$ (calculated value of 428.3290). This molecular formula was consistent with a dihydroxylated vitamin D$_2$ metabolite. The peaks at m/z 410 (M-18) and 395 (M-33) can be attributed to sequential losses of water and a methyl group. The characteristically large peaks at m/z 136 and 118 demonstrated that the triene system remained intact and ruled out A-ring substitutions. The peaks at m/z 271 and 253 arise from the cleavage of the steroid side chain and subsequent loss of water. This indicated that the two remaining hydroxyl groups were a part of the side chain. Absence of either a significant M-58 peak or a peak at 59 amu, often seen in 25-hydroxylated vitamin D$_2$ compounds, discounted the presence of a 25-hydroxyl group in the metabolite (16). The peak at m/z 380.3075 (M-48) had an elemental composition of $C_{27}H_{40}O$ (calculated value of 380.3079) representing an unusual loss of $CH_4O_2$ from the molecular ion. An even-numbered loss of this type strongly suggests a rearrangement. The elimination of 48 amu from the parent ion may arise from an
Figure 1. Comparison of the relative mobility of 24,26-(OH)$_2$D$_2$ with various vitamin D metabolites on a Zorbax-Sil column developed in methanol/acetonitrile/methylene chloride (1:20:180)
Figure 2. High-resolution mass spectrum of 24,26-(OH)$_2$D$_2$ isolated from bovine plasma
intramolecular proton transfer involving both the C-24 and C-26 hydroxyl functions. This would result in the elimination of water and formaldehyde molecules (C-26) from the molecular ion.

Persilylation of the metabolite produced a compound with a molecular ion at m/z 644, which established that three alcohol groups were present (Figure 3). The peaks at m/z 554 and 464 represented the successive losses of (CH₃)$_3$SiOH from the parent ion. More significant was the peak at m/z 513. This corresponded to a loss of CH$_3$CHCH$_2$OSi(CH$_3$)$_3$ from the terminus of the side chain as a result of α-cleavage of the silyl ether present at C-24. This also accounted for the peak seen at m/z 131. Cleavage of the side chain resulted in the peak observed at m/z 343. The peak at m/z 333 represented the combined losses of two (CH$_3$)$_3$SiOH functions in addition to the previously discussed loss of 131 amu from the molecular ion.

The major product obtained from ozonolysis of the metabolite was characterized by GC-MS (Figure 4). The low resolution mass spectrum indicated a parent ion at m/z 208. This corresponded to cleavage of the C-7/C-8 and C-22/C-23 olefin bonds. Losses of water (M-18) and a methyl group (M-15) were readily evident. In addition, cleavage of
Figure 3. Mass spectrum of the trimethylsilyl ether derivative of 24,26-(OH)$_2$D$_2$
Figure 4. Comparison of GC-MS of the major product obtained from the ozonolysis of 24,26-(OH)2D3 (upper spectrum) and the product obtained from the oxidation of Lythgoe-Inhoffen diol (lower spectrum)
the side chain accounts for the peak at m/z 151. The mass spectrum of
the ozonolysis product was in excellent agreement with the product
obtained from oxidation of Inhoffen-Lythgoe diol. This placed the
alcohol groups in the terminal portion of the side chain.

A 300-MHz $^1$H NMR spectrum was taken of 40 µg of the metabolite
(Figure 5). The signals at δ6.31, 6.02, 5.03, and 4.81 indicated
that the characteristic triene system was intact. These peak posi­
tions also confirmed the lack of 1α-hydroxylation in the metabolite.
The multiplet corresponding to the C-22/C-23 olefinic protons was
located at δ5.48. The downfield shift of this multiplet, relative to
that observed in vitamin D$_2$, was taken as further evidence of hydroxy­
lation at C-24. The signal at δ3.94 (1 H) represented the 3α-proton
and confirmed the presence of the 3β-hydroxyl group. The multiplet at
δ3.66 (2 H) can be attributed to the presence of the C-26 hydroxyl
group. The singlet at δ0.56 (3 H) and the doublet at δ1.05 (3 H, J =
6.6 Hz) were consistent for the C-18 and C-21 methyl groups. A lone
doublet located at δ0.81 (3 H, J = 6.9 Hz) corresponded to the C-27
methyl group. The observed splitting pattern further confirmed the
absence of substitution at C-25. The C-28 methyl group appeared as a
singlet at δ1.30 and is consistent for C-24 hydroxylation in vitamin
D$_2$ compounds (21).

The affinity of 24,26-(OH)$_2$D$_2$ for the rat plasma vitamin D
binding protein relative to other vitamin D metabolites is presented
in Table 1. The metabolites were assessed for their ability to
displace [$^3$H]-25-OH$_2$D$_3$ from the 4.2S rat plasma vitamin D binding
Figure 5. The 300-MHz $^1$H NMR spectrum of 40 µg of 24,26-(OH)$_2$D$_2$. The insert is an expanded view of the upfield portion of the spectrum.
Table 1. Ability of vitamin D metabolites to displace[^H]-25-OHD₃ from the rat plasma vitamin D binding protein

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Amount that produces 50% displacement of[^H]-25-OHD₃ (ng)</th>
<th>Competitive index relative to 25-OHD₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-OHD₂</td>
<td>0.6</td>
<td>100</td>
</tr>
<tr>
<td>25-OHD₃</td>
<td>0.6</td>
<td>100</td>
</tr>
<tr>
<td>24(R)-25-(OH)₂D₃</td>
<td>0.6</td>
<td>100</td>
</tr>
<tr>
<td>24(R)-25-(OH)₂D₂</td>
<td>1.2</td>
<td>50</td>
</tr>
<tr>
<td>24,26-(OH)₂D₂</td>
<td>2.0</td>
<td>30</td>
</tr>
</tbody>
</table>

[^H]: The amount of 25-OHD₃ that displaces 50% of the[^H]-25-OHD₃ divided by the amount of metabolite that will displace 50% of the[^H]-25-OHD₃ times 100.
protein. Approximately 3.3 times more 24,26-(OH)_{2}D_{2} was needed to achieve 50% displacement than either 25-OHD or 24(R),25-(OH)_{2}D_{3}. The 24(R),25-(OH)_{2}D_{2} exhibited a higher affinity for the binding protein than 24,26-(OH)_{2}D_{2} in this assay, but was twofold less potent than its vitamin D_{3} analog.

The ICT and BCR responses of rats dosed with various amounts of test compound are presented in Table 2. The data indicated that 24,26-(OH)_{2}D_{2} has little, if any, effect on ICT and BCR despite increasing the dose up to 200 ng/rat. In contrast, the administration of a minimal dose (50 ng/rat) of 25-OHD_{2} was seen to significantly increase both ICT and BCR.

Significant quantities of 24,26-(OH)_{2}D_{2} were detected in the plasma of both rats given vitamin D_{2} as their sole source of vitamin D and a cow treated with a large amount of vitamin D_{2} (Table 3). When rats received 24-OHD_{2}, in addition to a vitamin D_{3} supplement, 24,26-(OH)_{2}D_{2} was present in a 4-fold higher concentration than 24,25-(OH)_{2}D_{2} (group II). The concentration of 24,26-(OH)_{2}D_{2} was diminished and only slightly higher than that of 24,25(OH)_{2}D_{2} when 24-OHD_{2} was administered to rats maintained on a low calcium, vitamin D-deficient diet (group IV). No metabolites comigrating with 24,26-(OH)_{2}D_{2} were detected in vitamin D_{3}-supplemented rats given 25-OHD_{2} (group III). Rats supplemented with 25-OHD_{2}, however, possessed a greatly elevated concentration of 24,25-(OH)_{2}D_{2} in their plasma.
Table 2. Biological evaluation of 24,26-(OH)₂D₂ using the rat bioassay

<table>
<thead>
<tr>
<th>Compounds given</th>
<th>Dose (ng/rat)</th>
<th>$^{45}$Ca(serosal)/$^{45}$Ca(mucosal)</th>
<th>Plasma Ca (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24,26-(OH)₂D₂</td>
<td>50</td>
<td>1.3 ± 0.1</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>24,26-(OH)₂D₂</td>
<td>100</td>
<td>1.5 ± 0.1</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td>24,26-(OH)₂D₂</td>
<td>200</td>
<td>1.3 ± 0.1</td>
<td>5.3 ± 0.1</td>
</tr>
<tr>
<td>25-OHD₂</td>
<td>50</td>
<td>2.4 ± 0.1⁵</td>
<td>6.0 ± 0.2⁵</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>1.4 ± 0.1</td>
<td>5.4 ± 0.2</td>
</tr>
</tbody>
</table>

⁵ICT, intestinal calcium transport.

⁶BCR, bone calcium resorption.

⁷Differs from control at p < 0.05.
Table 3. Plasma concentrations of vitamin D metabolites in treated rats

<table>
<thead>
<tr>
<th>Treatment (group)</th>
<th>24,25-(OH)₂D₃ (ng/mL)</th>
<th>24,25-(OH)₂D₂ (ng/mL)</th>
<th>24,26-(OH)₂D₂ (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D₂ᵃ (I)</td>
<td>NDᵇ</td>
<td>4.1 ± 2.3</td>
<td>1.7 ± 1.0</td>
</tr>
<tr>
<td>D₃ᵃ +24-OHD₂ᶜ (II)</td>
<td>3.3 ± 0.1</td>
<td>3.3 ± 0.3</td>
<td>14.3 ± 6.9</td>
</tr>
<tr>
<td>D₃ᵃ +25-OHD₂ᶜ (III)</td>
<td>2.8 ± 0.8</td>
<td>93 ± 18</td>
<td>ND</td>
</tr>
<tr>
<td>-Ca, -D, +24-OHD₂ᶜ (IV)</td>
<td>ND</td>
<td>4.9 ± 0.8</td>
<td>5.9 ± 0.4</td>
</tr>
<tr>
<td>Excess vitamin D₂ (cow)</td>
<td>3.5 ± 1.1</td>
<td>106 ± 36</td>
<td>14.7 ± 8.8</td>
</tr>
</tbody>
</table>

ᵃOral supplement of 100 IU/day.
ᵇNot detected.
ᶜIntraperitoneal injection of 5 μg/day for 5 days.
DISCUSSION

This paper reports the identification of a new vitamin D₂ metabolite as 24,26-(OH)₂D₂. Several independent techniques were employed to characterize the metabolite; however, the mass spectrum was remarkable for the presence of a peak at m/z 380 indicative of a rearrangement of the parent ion. Similar types of rearrangements can be observed in the mass spectra of other 1,3-diols of vitamin D metabolites (4, 7). The identification of the metabolite was greatly facilitated by our ability to obtain a high-resolution ¹H NMR spectrum of the material. A conventional interpretation of the NMR data, whereby the C-21 and C-24 methyl group protons have been assigned to the signals at 60.90 and 1.01, would have suggested the metabolite's structure as 20,26-(OH)₂D₂. However, from the ozonolysis work already discussed, as well as a recent NMR study on vitamin D₂ metabolites by Koszewski et al. (21), the C-24 and not the C-20 position has been hydroxylated.

The 24,26-(OH)₂D₂ isolated in this study exhibited a lower affinity for the rat plasma vitamin D binding protein than 25-OHD. Hydroxylation at C-26 evidently results in yet a further decrease of a 24-hydroxylated vitamin D₂ metabolite's affinity for the binding protein as seen in the lower competitive index of 24,26-(OH)₂D₂ relative to 24(R),25-(OH)₂D₂.

The ability of 24,26-(OH)₂D₂ to stimulate intestinal calcium transport or bone calcium resorption, as measured in our assays, was
insignificant. Horst et al. have previously demonstrated that 24-OHD$_2$ possesses significant biological activity, most likely as a result of its ability to be $\alpha$-hydroxylated (22). Evidently, the formation of 24,26-(OH)$_2$D$_2$ represents a pathway for the deactivation of 24-OHD$_2$. A comparable situation exists with regard to vitamin D$_3$ metabolism, in that C-26 hydroxylation seemingly represents a means of rendering 25-OHD$_3$ biologically inactive (23).

Collectively, the data from our controlled rat studies demonstrated that the preferred metabolic pathway for the metabolism of 24-OHD$_2$ under physiologic conditions is the biosynthesis of 24,26-(OH)$_2$D$_2$ (Table 3). Our data further show that 24-OHD$_2$ is a poor substrate for the production of 24,25-(OH)$_2$D$_2$, with 25-OHD$_2$ being the preferred precursor. The low plasma concentration of 24,26-(OH)$_2$D$_2$ under conditions of vitamin D deficiency and low calcium probably is a result of the channeling of 24-OHD$_2$, the sole dietary source of vitamin D in this experiment, to its biologically active $\alpha$-hydroxylated derivative (22). C-26 hydroxylation would be predicted to be attenuated considerably under these dietary conditions.

Also noteworthy is the observed lack of 25,26-(OH)$_2$D$_2$ production in rats given 25-OHD$_2$ (data not shown). Consistent with this observation was our demonstration that 25,26-(OH)$_2$D$_2$ was not detectable in the plasma of a vitamin D$_2$ intoxicated cow. The only vitamin D$_2$ metabolite migrating in the 25,26-(OH)$_2$D$_2$ region of the prep HPLC column used in these experiments was 24,26-(OH)$_2$D$_2$. 26-Hydroxylation
of 25-OHD$_2$ evidently represents a relatively inefficient process relative to the 26-hydroxylation of 24-OHD$_2$ or 25-OHD$_3$.

Figure 6 summarizes the metabolic pathways involved in the side chain hydroxylations of vitamin D$_2$ in animals receiving normal dietary calcium and vitamin D$_2$. Vitamin D$_2$ is preferentially hydroxylated at the 25-position with C-24 oxidation present as a minor pathway. A major route of 25-OHD$_2$ metabolism results in the production of 24,25-(OH)$_2$D$_2$. On the other hand, only a small amount of 24-OHD$_2$ is metabolized to 24,25-(OH)$_2$D$_2$ with hydroxylation at C-26 to yield 24,26-(OH)$_2$D$_2$ being the major metabolic pathway. This data may aid in explaining differences in biological activity and toxicity known to exist between vitamin D$_2$ and vitamin D$_3$ and represent the first significant deviation of vitamin D$_2$ metabolism from that of vitamin D$_3$. 
Figure 6. Preferred pathways of side-chain oxidations in the metabolism of vitamin D$_2$ under conditions of normal vitamin supplementation
LITERATURE CITED


SECTION III.

THE EFFECT OF AGE ON THE 1,25-DIHYDROXYVITAMIN D₃ STEROID RECEPTOR IN MALE FISCHER 344 RATS
INTRODUCTION

Vitamin D₃, a seco-steroid, is produced in the skin by a process initiated by the direct action of sunlight on a cholesterol precursor. The vitamin then undergoes a series of tissue-specific hydroxylations resulting in production of what is generally regarded as the active hormonal form of the vitamin, 1,25-(OH)₂D₃¹ (1, 2). The primary role of 1,25-(OH)₂D₃ has been characterized as the maintenance of calcium homeostasis with primary target tissues being the intestine, kidney, and bone. Typical of other steroid hormones, 1,25-(OH)₂D₃ interacts with an intracellular receptor protein in target tissues (3-5). This complex is then thought to bind to specific sequences of DNA (termed vitamin D response elements, VDRE) resulting in the alteration of transcriptional events. One such event that has been characterized has been an increase in the synthesis of an intracellular calcium-binding protein in intestinal tissue (6, 7).

Age-related differences in steroid receptors have generated increased research interest. Sharma and Timiras, in examining the glucocorticoid receptor in the livers of Long-Evans male rats, determined that weanling rats possessed a significantly higher number of

¹Abbreviations used: 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃; 24,25-(OH)₂D₃, 24,25-dihydroxyvitamin D₃; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DCC, dextran-coated charcoal; CIM, Carnation Instant nonfat dry milk.
specific binding sites relative to mature (26 weeks) animals (8). Lu et al. examined developmental changes of glucocorticoid receptors in the pancreata of Sprague-Dawley rats. Binding capacities were found to be low in 1-day-old pups accompanied by an increase to maximal levels at weaning, followed by a decline in specific binding as the animals aged (9). A recent report indicated that the ability of uterine estrogen receptors from aged mice to bind non-specifically to DNA-cellulose was only one-half that of young animals (10). This work implied the presence of an endogenous inhibitor that prevented binding to nuclear acceptor sites, particularly in aged animals.

A number of studies have examined the appearance of the vitamin D receptor in embryonic tissues and neonatal animals (11-13), but far less is known concerning the characteristics of the receptor as an animal reaches maturity and progresses into old age. Changes in either receptor number or in the protein itself may have profound consequences in the ability of an animal to respond to the active hormone. The ability to absorb calcium from the intestine is known to decrease with age in various species (14-17). Much of this work has focused on the impaired ability of aged animals to convert 25-OHD$_3$ to 1,25-(OH)$_2$D$_3$ in the kidney (18-22). The lower circulating hormone concentrations present in older animals would imply diminished interaction with receptors in target tissues. Yet, exogenously administered 1,25-(OH)$_2$D$_3$ to young and aged rats has, at least in one instance, failed to exhibit an increase of comparable magnitude in calcium absorption in the aged animals (23). In addition, several reports have recently appeared
concerning the differences in the vitamin D receptor number and occupancy of young and aged male rats (24–26). These preliminary studies described a marked decrease in receptor number in mature animals both in intestinal and bone tissue when measured against weanlings. These latter observations provide a basis for further research to determine if a relationship exists between receptor number and the known impaired ability of an aged animal to absorb calcium from the intestine.

The purpose of the present study was to examine both the intestinal and kidney receptor for vitamin D at two different stages of development in male Fischer 344 rats. This work quantified total unoccupied receptor numbers, and the ability of the various receptor preparations to bind non-specifically to DNA-cellulose. Immunoblots of receptor preparations were also examined to detect possible changes in protein size. In addition, plasma concentrations of 1,25-(OH)₂D₃ and calcium were determined for differences in the two age groups.
MATERIALS AND METHODS

General

Hydroxyapatite was purchased from Bio-Rad Laboratories (Richmond, CA). Soybean trypsin inhibitor was obtained from Sigma Chemical Corp. (St. Louis, MO). Trayslol was purchased from Mobay Chemical Corp. (New York, NY). DNA-Cellulose was prepared according to the procedure of Littman (27) using calf thymus DNA (Sigma) and Whatman CF-11 cellulose (W & R Balston, Ltd., England). 1,25-(OH)$_2$D$_3$ and 24,25-(OH)$_2$D$_3$ were gifts from Dr. M. R. Uskokovic of Hoffmann-La Roche (Nutley, NJ). $[^3H]$.1,25-(OH)$_2$D$_3$ (90 Ci/mmol) was synthesized as previously described (28). High-performance liquid chromatography was carried out with Waters Model ALC/GPC 204 liquid chromatography equipment (Waters Associates, Milford, MA). Gel filtration HPLC analysis was performed by using a Spherogel TSK 3000 SW column (0.75 x 60 cm, Beckman Instruments Inc., Berkeley, CA). The 9A7γ monoclonal antibody directed against the vitamin D receptor was a gift from Dr. J. W. Pike, Baylor College of Medicine (Houston, TX). Rabbit-anti-rat IgG (H+L, mouse serum absorbed) was obtained from Zymed Laboratories (So. San Francisco, CA). Proteins were electrophoretically transferred to Gene Screen hybridization membranes (NEN Research Products, Boston, MA). Electrophoretically pure reagents, Protein A gold colloid, and silver enhancement kit were obtained from Bio-Rad Laboratories. The KTD buffer utilized throughout these studies contained 10 mM Tris-HCl, pH 7.4 (at 4°C), 5 mM dithiothreitol, and the indicated concentration of
potassium chloride (i.e. KTD-500 contains 0.5 M KCl). Protein was assayed by the method of Bradford (29). Concentrations of potassium and plasma calcium were determined using a Perkin Elmer Model 5000 AA spectrophotometer at wavelengths of 404.7 nm and 422.7 nm, respectively.

Animals

Barrier-derived young (1 mo) and aged (18 mo) male Fischer 344 rats were obtained from the National Institute on Aging. The animals were maintained on a normal rat chow diet (Teklad 4%), and were allowed to acclimate to their environment for at least 1 week prior to their use.

Receptor Preparation

The first 20 cm of small intestine were removed and flushed with ice-cold Tris-buffered saline containing Trayslol (50 KIU/ml). All subsequent steps were performed at ca. 4°C. The mucosa was scraped free and a 20% homogenate prepared in KTD-500/sbti (100 µg/ml) using a Polytron tissue homogenizer. The homogenate was spun in an ultracentrifuge at 230,000 g for 30 min to yield a supernatant containing buffer-soluble cellular components (termed cytosol). Kidney preparations were similar to that described above.

Receptor Assays

Unoccupied receptor concentrations were obtained by labelling subsamples of the cytosol in 24 nM [³H]-1,25-(OH)₂D₃ ± a 200-fold
excess of cold steroid at 4°C for 2-3 h. A 4-fold excess of 24,25-(OH)_2D_3 was also added to all tubes to minimize interference from the vitamin D binding protein. The samples were stripped with 1% dextran-coated charcoal and the receptor-hormone complexes were quantitated by hydroxyapatite in triplicate (30). The percentage of binding to DNA was determined by using aliquots of the labelled cytosol samples described above, also in triplicate. Following the charcoal strip, the hormone-receptor complexes were incubated with 500 μl of 50:50 slurry of DNA-cellulose in TD buffer in a final volume of 1 ml at a [K^+] approximating 150 mM. After 45 min with occasional gentle vortexing, the cellulose was pelleted and washed 2x with TED buffer (1 mM EDTA) containing 0.5% Triton X-100. The pellets were then extracted 2x with 1.5 ml of absolute EtOH; the samples were dried and counted for radioactivity.

**DNA-Cellulose Chromatography**

Cytosol was labelled in 5-7 nM [³H]-1,25-(OH)_2D_3 with a 4-fold excess of 24,25-(OH)_2D_3 as described above. A 50:50 slurry of DNA-cellulose in TD buffer was added to the cytosol to yield an approximate [K^+] of 150 mM. After mixing intermittently for 1 h, the slurry was poured into a 1.5 cm diameter column. The column was washed at a flow rate of ca. 40 ml/h with 20-30 volumes of KTD-100 buffer, followed by a linear gradient from TED to KTED-500 (1 mM EDTA). Then 100 μl aliquots were removed from the collected fractions (ca. 3 ml/fraction).
and counted for radioactivity to trace hormone-receptor binding. Samples of receptor-hormone complex were precipitated with either 40% or 70% saturated ammonium sulfate and analyzed in immunoblots. Alternatively, receptor could be precipitated with 8% trichloroacetic acid.

Receptor-Antibody Complexes

Cytosol was incubated in 5-7 mM labelled steroid as above. Following a 1% DCC strip, 25 ng of receptor (as determined by hydroxyapatite assay) was mixed with 100 µl of diluted 9A7γ monoclonal antibody for 6 h at 4°C in a final volume of 400 µl at a [K+] of 200 mM. Preparations were then analyzed via HPLC chromatography.

HPLC Analysis

The chromatography was carried out as previously described with the following modifications (31). The analysis was determined at 4°C. The Spherogel-TSK 3000 SW column was equilibrated in a buffer comprised of 20 mM sodium phosphate (pH 7.3), 1 mM EDTA, 2 mM DTT, 200 mM KCl, 1% glycerol, and 0.1% Tween 20. A flow rate of 1 ml/min was employed and fractions were collected every minute. Collected fractions were counted for radioactivity to trace the elution profile of the hormone-receptor complex.

Immunoblot Analysis

Protein samples were obtained from DNA-cellulose chromatography that had been either precipitated with ammonium sulfate (40% or 70%),
or with trichloroacetic acid (8%). The pelleted proteins in all cases were denatured in SDS-PAGE reducing buffer and resolved on 12% SDS-polyacrylamide gels according to the method of Laemmli (32). Proteins were electrophoretically transferred to Gene Screen hybridization membrane. Transfer was carried out at 100 V for 1 h by using a transfer buffer (0.025 M Tris-HCl, 0.19 M glycine) containing 0.1% SDS. Following the transfer, the membranes were subjected to immunoblot analysis as follows. The membranes were suspended for 1 h in phosphate-buffered saline (PBS, 10 mM sodium phosphate, pH 7.2, 0.9% NaCl) containing 10% CIM. The membranes were then transferred to a solution of PBS/10% CIM containing a 1:2000 dilution of 9A7γ monoclonal antibody and incubated overnite at 4°C. The membrane was washed for 30 min in PBS containing 0.3% Tween 20 with two changes of buffer. After a 1-h incubation of the membrane in PBS/10% CIM, the membrane was suspended overnite at 4°C in a solution of PBS/10% CIM containing a 1:5000 dilution of rabbit-anti-rat antiserum. Following the previously described wash procedure, the membrane was incubated for 4 h in Protein A gold colloid. The membrane was washed for 30 min in PBS, 0.3% Tween 20 with two buffer changes. Color enhancement of immunoblotted proteins was accomplished with silver lactate. Non-specific bands were accounted for by the omission of the monoclonal antibody incubation in the above steps.
1,25-(OH)$_2$D$_3$ Assay

Plasma concentrations of 1,25-(OH)$_2$D$_3$ were determined as previously described (33).

Statistics

The tabularized data from these experiments are reported as the mean ± SEM along with the number of animals in the determination. Statistical analyses were performed by using Student's $t$ test and a confidence level of 95% or greater was considered significant.
RESULTS

Plasma was collected from both young and aged rats, and assayed for calcium and 1,25-(OH)_2D_3 concentrations (Table 1). In both cases the aged Fischer rats possessed significantly lower plasma concentrations than their younger counterparts.

The results from an assessment of unoccupied intestinal receptor status for young and aged animals are presented in Table 2. An initial inspection of the data indicated that there were no differences in the receptor concentrations for young and aged animals. When the data, however, were examined as a function of time following the arrival of the animals to the facility, the aged animals could be placed into two categories: 1) those experiments conducted within the first 28 days of arrival and 2) those that were carried out 29+ days after arrival. Aged animals within the first period possessed receptor concentrations in excess of 500 fmol/mg protein, whereas those from the second time period had only one-half the concentration as the former group. Young animals exhibited no time-dependent difference in receptor concentrations and remained elevated throughout the experiments. In addition, the young rats possessed significantly higher receptor concentrations than did the aged animals during the second time period.

The percent DNA binding was not significantly different for the young and aged intestinal receptor preparations (Table 2). Aged
Table 1. Concentrations of calcium and 1,25-(OH)$_2$D$_3$ in the plasma of young and aged Fischer 344 rats

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$</td>
<td>$10.6 \pm 0.1$ (12)</td>
<td>$9.7 \pm 0.2$ (13)</td>
</tr>
<tr>
<td>1,25-(OH)$_2$D$_3$</td>
<td>$102.2 \pm 14.1$ (12)</td>
<td>$33.1 \pm 5.7$ (13)</td>
</tr>
</tbody>
</table>

$^a$Concentration as mg/dl.

$^b$Concentration as pg/ml.

$^c$The number in parentheses refers to the number of animals examined in determining the average value ± SEM.
Table 2. Properties of intestinal 1,25-(OH)_{2}D_{3} receptor prepared from young and aged Fischer 344 rats

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unoccupied^a</td>
<td>446 ± 26 (14)</td>
<td>368 ± 53 (12)</td>
</tr>
<tr>
<td>7-28 days^b</td>
<td>435 ± 33 (8)</td>
<td>525 ± 52 (5)</td>
</tr>
<tr>
<td>29+ days</td>
<td>461 ± 44 (6)</td>
<td>254 ± 39 (7)</td>
</tr>
<tr>
<td>% DNA binding</td>
<td>55.8 ± 3.0 (14)</td>
<td>51.2 ± 2.9 (12)</td>
</tr>
</tbody>
</table>

^aExpressed as fmol/mg protein.

^bDays following arrival to the laboratory.

^cThe number in parentheses refers to the number of animals examined in determining the average value ± SEM.
animals exhibited no differences during the two projected time periods despite a significant decline in receptor concentration.

Kidney receptor concentrations were lower relative to intestine for both young and aged rats (Table 3). There was, however, no measurable difference in receptor concentration between the two groups. An insufficient number of aged rats were analyzed during the early period described above to ascertain if a time-dependent decline occurred in kidney receptor concentrations. Receptor cytosols from aged rats, in contrast, displayed a marked decrease in their ability to bind to DNA-cellulose relative to samples from young rats (ca. 24% versus 44%).

Figure 1 shows typical DNA-cellulose column chromatography profiles for both young and aged intestinal preparations. In both cases the receptor-hormone complex was eluted from the column at approximately 240 mM potassium concentration. DNA-cellulose chromatography profiles of young and aged kidney preparations are presented in Figure 2. There is a decline in the amount of specifically bound receptor relative to that seen in the intestinal preparations, and the peak breadth was observed to be greater in the kidney preparations. Indeed, kidney preparations from particularly young animals occasionally appeared as a poorly resolved doublet (Figure 2).

When intestinal receptor fractions were pooled following DNA-cellulose chromatography and fractionated with 40% ammonium sulfate, normally 70-80% of the radiolabelled material would precipitate in the case of receptor prepared from a young rat. Employing the same
Table 3. Properties of kidney \(1,25-(OH)_2D_3\) receptors prepared from young and aged Fischer 344 rats

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Aged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unoccupied(^a)</td>
<td>149 ± 8 (8)(^b)</td>
<td>155 ± 8 (8)</td>
</tr>
<tr>
<td>% DNA binding</td>
<td>43.6 ± 3.9 (8)</td>
<td>24.1 ± 2.3 (8)</td>
</tr>
</tbody>
</table>

\(^a\)Expressed fmol/mg protein.

\(^b\)The number in parentheses refers to the number of animals examined in determining the average value ± SEM.
Figure 1. Representative DNA-cellulose column chromatography profiles of intestinal receptor preparations from young (upper panel) and aged (lower panel) rats. Receptor was eluted by using a KCl gradient. Aliquots (100 μl) were removed from collected fractions (3 ml) and counted to trace the hormone-receptor complex.
Figure 2. Typical DNA-cellulose chromatography profiles of kidney receptor prepared from young and aged rats. Receptor from the young rats was eluted as a poorly resolved doublet, implying receptor forms with different affinities for DNA.
conditions with intestinal receptor from aged rats typically resulted in the precipitation of only 20-30% of the radiolabelled material. Thus, 70% saturation with ammonium sulfate was used to insure that the majority of the radiolabel (i.e., hormone-receptor complex) was precipitated.

Representative immunoblots of young and aged receptor preparations following DNA-cellulose column chromatography and ammonium sulfate precipitation (70%) are presented in Figure 3. Young intestinal receptor is visible as a single band at approximately 52K (lane A). The aged intestinal receptor preparation was not detected in an appreciable manner in the immunoblot analysis (lane B), despite loading an equivalent amount of receptor (based on pooled column collections). A lower molecular weight band (ca. 24K) was present in the aged intestinal preparation which was absent in the young sample (lane B versus lane A), and most likely represents a proteolysis product. The mixing of young and aged intestinal receptor resulted in the diminishment of the 52K receptor band, and concomitant appearance of several lower molecular weight bands (lane D). Receptor from the young rat alone was still clearly visible at 52K, together with a single low molecular weight band. Note that lane D contains an equivalent amount of young receptor from the same preparation as in lane C, but which had been mixed and incubated overnight with post-column receptor obtained from an aged animal. The aged and young kidney receptors appear as a pair of bands at approximately 52K and 50K (lanes E and F). The higher
Figure 3. Immunoblot analysis of vitamin D receptor preparations following DNA-cellulose column chromatography. Lane A, young intestine; lane B, aged intestine; lane C, young intestine control for the mixed receptor experiment; lane D, mixed young and aged intestine; lane E, young kidney; lane F, aged kidney.
molecular weight band from kidney preparations co-migrates with the intestinal receptor preparations in this system (data not shown).

Gel filtration HPLC analysis was performed on both young and aged intestinal cytosol preparations (Figure 4). Equivalent amounts of receptor (25 ng) were allowed to interact with varying dilutions of 9A7γ monoclonal antibody. The analysis indicated that the receptor from both the young and aged intestine was shifted to a higher molecular weight species in an identical fashion at equivalent dilutions of the antibody.
Figure 4. Gel filtration HPLC analysis of the affinity of monoclonal 9A7γ for young and aged receptor in cytosol preparations. Hormone-labelled receptor (25 ng) was incubated with the appropriate dilutions of antibody for 6 h at 4°C prior to chromatography.
DISCUSSION

The evaluation of unoccupied intestinal receptor concentrations from young and aged rats indicated that there is an apparent time-dependent decline in the receptor concentration of aged animals, whereas no such event was detected in receptor prepared from young rats. There are a number of possibilities that may account for this phenomenon. The stress associated with cross-country shipment (Indianapolis, IN to Ames, IA) and acclimation to a new environment, resulting in increased plasma concentrations of glucocorticoids, may have a greater impact on aged rats. Glucocorticoids are known up-regulators of the 1,25-(OH)$_2$D$_3$ receptor (34, 35). As a corollary to the explanation given above, these animals were maintained under barrier conditions and were pathogen-free prior to shipment. Some of the aged rats, particularly with regards to the first group studied in our laboratory, displayed signs of illness, including red mattery eyes and a listless behavior when approached. Attempts to identify a pathogen from cultures of lung tissue were inconclusive. Cross-country shipment may have made the aged rats more susceptible to opportunistic infection, resulting in, again, an increase in circulating plasma glucocorticoids with a subsequent increase in 1,25-(OH)$_2$D$_3$ steroid receptor concentrations. Assaying the plasma for the concentration of corticosterone would be a means of testing these possibilities. None of the younger animals developed any clinical signs of illness throughout the time periods involved.
A comparison of the diet administered prior to shipment (Teklad NIH31) and that fed in these experiments (Teklad 4%) reveals the following. There was ample calcium, phosphorus and vitamin D in both diets. The Teklad 4% diet, however, had a higher percentage of calcium in the diet (2% versus 1%) with a concomitant increase in the calcium-to-phosphorus ratio. The relatively high calcium content of the diet may have lessened the need for active transport of calcium from the gut, resulting in the decline in intestinal receptor numbers with time in aged rats. No perceptible decrease in intestinal receptor number for young rats was detected, but these were growing animals with a large demand for calcium and receptor numbers may remain elevated. In either diet, however, the necessary requirements for growth and maintenance are more than met (36). Thus, it is not entirely clear why the intestinal receptor numbers from aged rats would initially appear so high.

Finally, it has been previously noted that Fischer 344 rats undergo marked physiological changes at approximately 18 months of age, including an increase in number of observed lesions and neoplasms (37). It is possible that our studies may have simply coincided with these physiological changes, and that receptor concentrations remain ostensibly elevated up to this point. An examination of animals from earlier ages would be useful to answer this issue.

At this time, it would seem most appropriate to limit the discussion to animals that had the 4-week period to acclimate to their surroundings. In this case, there is a significant decrease in the
number of unoccupied receptors. Similar observations have been made in the Holtzman rat with regard to the 1,25-(OH)₂D₃ steroid receptor (24, 25). The decrease in receptor number combined with the observed lower plasma concentrations of 1,25-(OH)₂D₃ is consistent with the known impaired ability of aged rats to absorb calcium in the intestine (15, 23). Decreased receptor numbers as a function of increasing age have also been reported for other steroid receptor systems (8, 9).

There was no apparent difference in the percentage of intestinal receptor that was able to bind to DNA-cellulose for either young or aged rats, irrespective of the time period involved. This observation would seem to discount the presence of an endogenous inhibitor of DNA binding in the cytosol of aged animals, as has been reported in other steroid receptor systems (10, 38-40).

Immunoblot analysis either failed to detect, or only faintly revealed the 52K receptor protein band of aged intestinal receptor preparations (lane B, Figure 3). Efforts were made to limit potential proteolytic degradation (data not shown) which included the use of various proteolytic inhibitors, EDTA, and elution of bound receptor from DNA-cellulose by sodium molybdate. None of the manipulations appreciably altered the inability to detect the 52K receptor protein band of aged intestinal receptor.

An alternative hypothesis for the failure to detect this band was also viable. Namely, that the epitope the monoclonal antibody recognizes in the aged intestinal receptor had been altered or deleted in the protein. Because the antibody has been shown to bind to a region
near the DNA-binding domain of the chick intestinal receptor (41, 42), an altered epitope in this area of the protein in the aged animals may influence transcriptional activity. This hypothesis was tested and eventually dismissed by using gel filtration HPLC. Both young and aged intestinal cytosol preparations produced the same characteristic shifts in molecular weight at the equivalent concentrations of antibody. This implied that changes in the aged receptor were occurring either on the DNA-cellulose column or in the post-column elutions.

This latter possibility was investigated by preparing young and aged receptor and then mixing pooled young and aged intestinal receptor preparations eluted from DNA-cellulose columns (lane D, Figure 3). An equivalent amount of pooled young receptor solution was withheld to act as the control (lane C, Figure 3). Immunoblots clearly showed that the mixed receptor solution resulted in the diminishment of the 52K receptor band that was still present in the young receptor control blot. Several additional low molecular weight bands also appeared in the mixed receptor experiment undoubtedly arising from more extensive proteolysis of the protein. It would appear that these observations are consistent with the presence of some protease activity, perhaps at a much higher activity in aged rats than in young animals, that copurifies with the aged intestinal receptor isolated from DNA-cellulose chromatography. The inability to appreciably precipitate intestinal receptor from aged rats with 40% ammonium sulfate would further support this hypothesis. Further saturation to 70%, while precipitating the majority of the radiolabelled material, did not increase the
immunological detection of receptor from aged rats on Western blots. It remains to be seen if this protease activity is specific for the vitamin D receptor or is simply an age-related endogenous enzyme that co-purifies on DNA-cellulose chromatography.

The concentration of kidney receptor is significantly lower than the intestine, but there is no apparent difference between young and aged kidney receptor concentrations (Table 3). There is, however, a marked decrease in the ability of aged receptor to bind to DNA-cellulose, despite the equivalence of receptor concentrations with the young rats. This may imply the presence of an endogenous inhibitor in the kidney cytosols of aged rats (10, 38-40).

Immunoblots from kidney preparations surprisingly revealed two receptor protein bands at approximately 52K and 50K. This would appear to explain the broad nature of the receptor peaks eluted from either young or aged preparations by DNA-cellulose chromatography. Evidently, the two forms have slightly different affinities for DNA. It is possible the lower molecular weight form (50K) is the result of proteolysis of the larger (52K) receptor species (43), although multiple transcripts may also be possible (44). A similar type of observation has been made from receptor prepared from chicken intestine (43, 44), but this is the first report of such a phenomenon in a mammalian system.

The results of the preliminary study presented here indicated marked age-related differences in both the intestinal and kidney receptor for vitamin D. There is a lower concentration of intestinal
receptor with age, which, together with a decreased plasma $1,25-(OH)_2D_3$
concentration, may work in tandem to limit the ability to absorb calci-
num. The percentage of binding to DNA, however, does not seem sig-
ificantly different in the intestine. The work presented from the
immunoblots and the gel filtration HPLC suggests that a proteolytic
enzyme with increased activity is present in aged intestinal prepara-
tions. Both the receptor concentration and the percent DNA binding
are lower in the kidney preparations, but receptor prepared from aged
kidney evidently is blocked from binding to DNA to a greater extent
than that from young rats. The presence of an inhibitor to DNA bind-
ing would effectively reduce the ability of the aged kidney to respond
to hormone. In addition, two forms of kidney receptor are visible in
the immunoblots of either young or aged rats. The two forms are not
well resolved on DNA-cellulose chromatography, but do display a slight
difference in their affinity for DNA. Collectively, these data may be
an aid to explaining the physiological changes that occur to calcium
homeostasis as a function of age.
LITERATURE CITED


SUMMARY AND DISCUSSION

The identification of vitamin D metabolites has, in the past, relied primarily upon ultraviolet absorbance, mass spectroscopy, and chemical derivatives. The paper in Section I details efforts to utilize ¹H NMR spectroscopy to aid in the identification of vitamin D₂ metabolites. The uniqueness of the vitamin D₂ side chain, which includes a double bond and an additional methyl group, creates distinct resonance signals for the side chain moieties. It is certainly reasonable, however, to expect that the methodology developed here could prove useful in the identification of vitamin D₃ metabolites as well. High-resolution spectra were obtained on as little as 5 μg of metabolite (ca. 90 μM). This represents a significant improvement over previous reports, yet the method is still less sensitive than mass spectroscopy. It does offer, however, the advantage that precious metabolite may be recovered intact. The steady improvement in magnet design will undoubtedly result in the capability to obtain spectra of nanogram quantities of vitamin D metabolites.

Equally important was the reassignment of the C-21 and C-28 methyl group signals in the NMR spectrum of vitamin D₂. An examination of a variety of vitamin D₂ and selected vitamin D₃ metabolites clearly indicated that previous reports had erroneously misassigned the two signals. It is evident from this work that the C-21 methyl group gives rise to the doublet at 61.03, while the C-28 methyl group appears as the doublet at 60.90.
The ability to obtain high-resolution $^1$H NMR spectra of microgram quantities of vitamin D metabolites was of critical importance in ascertaining the structure of 24,26-(OH)$_2$D$_2$, a new physiological metabolite of vitamin D$_2$. Conventional means of identifying the metabolite had been unsuccessful, but the $^1$H NMR spectrum readily placed the position of the hydroxyl groups in the side chain. The mass spectrum was unique for the even-numbered loss from the molecular ion, and was attributed to an intramolecular rearrangement of the 1,3-diol. As discussed in Section II, this metabolite was unresponsive when tested in traditional bioassays, displayed a reduced affinity for the plasma vitamin D-binding protein, and most likely represents a means of inactivating 24-OHD$_2$. The pathway from vitamin D$_2 \rightarrow$ 24-OHD$_2 \rightarrow$ 24,26-(OH)$_2$D$_2$ is significant because it represents an important deviation from the documented metabolic pathways of vitamin D$_3$. The exact location and specificity of the 26-hydroxylase remains to be determined. Because of the limited amount of material isolated it was also not possible to account for the stereochemistry of the molecule either at C-24 or C-25. There was no indication that multiple isomers were isolated in this study.

Perhaps overlooked in this work was the inability to identify the presence of 25,26-(OH)$_2$D$_2$. On the other hand, 25,26-(OH)$_2$D$_3$ has been determined to be a physiological derivative in the metabolism of vitamin D$_3$ (82, 83). The failure to detect the formation of 25,26-(OH)$_2$D$_2$
in the present study may be an additional divergence of vitamin D$_2$ and vitamin D$_3$ metabolic pathways.

The preliminary study examining the effects that age has on the 1,25-(OH)$_2$D$_3$ steroid receptor protein in rats should provide a good basis for further research. There are several aspects of this work that will require additional experiments, the first of which would be to reexamine the time-dependent decline in aged intestinal receptor concentration. The feeling of this author is that the decline may, in fact, be related to the general health of the animals upon arrival. Many of the aged animals that initially arrived in the laboratory appeared ill, and numbers obtained from these rats may have skewed the overall distribution. Given adequate time those sick animals eventually recovered (or died as in two cases), and receptor numbers reflected their "true" status; i.e., significantly lower relative to the young rats. It is interesting to note, however, that none of the young animals developed any signs of sickness.

Diet may be a further consideration that needs to be addressed; however, a comparison of the two diets revealed only subtle differences. Both diets more than adequately meet the requirements needed for a young, growing rat, or for the maintenance of an aged rat. Nevertheless, it is an area that should be considered in future work.

The observation that immunoblots failed to appreciably demonstrate intestinal receptor from aged rats deserves closer inspection. The results from this study would seem to indicate the presence of a protease in the aged cytosols that co-purifies with the receptor on
DNA-cellulose. It would be interesting to determine if this activity was specific for the vitamin D receptor, or was somehow related to the altered metabolism of vitamin D with age. The activity may also be present in the cytosols of young rats, but at a lower degree of activity that then permits the detection of the 52K band on the immunoblots.

Inclusion of the kidney receptor for vitamin D in this work yielded two surprising observations. The first of these pertained to the inability of the aged receptor to bind to DNA-cellulose as avidly as that displayed by similar preparations from young rats. This would imply that an inhibitor to DNA binding is present in the cytosol of aged rats. Alternatively, one cannot dismiss the possibility that an enhancer to binding exists in the young cytosols (38, 84). A series of mixing experiments examining whether or not this putative inhibitor could negatively influence DNA binding in other vitamin D receptor preparations would be useful.

Secondly, the detection of two protein bands at 52K and 50K specific for the kidney receptor was unexpected. The broad nature of the DNA-binding peaks from the kidney preparations indicates the two forms possess slightly different affinities for binding. Whether or not the two forms arise as the result of proteolysis during the cytosol preparation or actually represent the existence of multiple transcripts of the receptor in kidney needs to be answered.

In summary, the work included in this dissertation has exploited the recent advances in $^1$H NMR spectroscopy to aid in the identification of vitamin D$_2$ metabolites. It has also resulted in the
identification of a new, physiological vitamin D$_2$ metabolite, 24,26-(OH)$_2$D$_2$. The isolation and characterization of this compound firmly establishes an alternative pathway in the metabolism of vitamin D$_2$ not available in the vitamin D$_3$ system. The age-related study on the vitamin D receptor indicated that aged animals evidently possess lower concentrations of intestinal receptor. This work also suggested that a protease activity, the nature of which is unknown, co-purifies particularly with intestinal receptor from aged rats when chromatographed on DNA-cellulose. Multiple forms of the kidney receptor appeared in immunoblots of both young and aged rats. In addition, cytosolic receptor extracts from the kidneys of aged rats displayed a much lower percent binding to DNA-cellulose than similar preparations from young rats. These results may serve to aid in further understanding the differences in calcium homeostasis between young and aged animals.
LITERATURE CITED


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