Intestinal Synthesis of 24-Keto-1,25-dihydroxyvitamin D₃
A METABOLITE FORMED IN VIVO WITH HIGH AFFINITY FOR THE VITAMIN D CYTOSOLIC RECEPTOR*

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24-Keto-1,25-dihydroxyvitamin D₃ has been identified as an intestinal metabolite of 1,25-dihydroxyvitamin D₃ by ultraviolet absorbance, mass spectroscopy, and chemical reactivity. The metabolite was produced from 1,25-dihydroxyvitamin D₂ and 1,24R,25-trihydroxyvitamin D₃ in rat intestinal mucosa homogenates. 24-Keto-1,25-dihydroxyvitamin D₃ is present in vivo in the plasma and small intestinal mucosa of rats fed a stock diet, receiving no exogenous 1,25-dihydroxyvitamin D₃, and in the plasma and small intestinal mucosa of rats dosed chronically with 1,25-dihydroxyvitamin D₃, 24-Keto-1,25-dihydroxyvitamin D₃ has affinity equivalent to 1,24R,25-trihydroxyvitamin D₃ for the 3.7 S cytosolic receptor specific for 1,25-dihydroxyvitamin D₃ in the intestine and thymus. In cytosolic preparations contaminated with the 5 S vitamin D-binding protein, both metabolites are about 7-fold less potent than 1,25-dihydroxyvitamin D₃. 24-Keto-1,25-dihydroxyvitamin D₃ is present in vivo in uivo; nor was the latter compound generated in detectable amounts from 1,25-dihydroxyvitamin D₃ by intestinal homogenates. The C-24 oxidation is a significant pathway of intestinal 1,25-dihydroxyvitamin D₃ metabolism that produces metabolites with high affinity for the cytosolic receptor which mediates vitamin D action.

The most abundant vitamin D₃ metabolite in plasma is 25-OHD₃ (1, 2). 25-OHD₃, itself undergoes extensive metabolism (3, 4), which is closely regulated by parathyroid hormone (5, 6) and by several 25-OHD₃ metabolites (7). During hypocalcemia, and/or hypovitaminosis D, 25-OHD₃ is converted into 1,25-(OH)₂D₃, primarily in kidney. 1,25-(OH)₂D₃ is a hormone considered to be a primary mediator of calcium and phosphorus metabolism (8-12). Indeed, 1,25-(OH)₂D₃ is apparently the major vitamin D₃ metabolite in at least one vitamin D target tissue, intestine (13, 14). However, compounds more polar than 1,25-(OH)₂D₃, such as 24,25-(OH)₂D₃ (15, 16) and 25,26-(OH)₂D₃ (17, 18) have been identified as circulating vitamin D₃ metabolites. The functions of these metabolites are unclear, and further investigations of 1,25-(OH)₂D₃ metabolism are necessary to determine their role, relative to 1,25-(OH)₂D₃ in maintaining calcium homeostasis.

Under physiological conditions, 5,6-trans-25-OHD₃ (19) and 24R,25-(OH)₂D₃ (1, 20, 21) are the 25-OHD₃ metabolites of highest concentration in plasma. 5,6-trans-25-OHD₃ probably mimics the actions of 1,25-(OH)₂D₃ because of its pseudo-1α-hydroxyl group. 24R,25-OHD₃, or perhaps its metabolite 24-keto-25-OHD₃ (22-24) may promote bone mineralization (25). An alternative pathway of 25-OHD₃ metabolism, especially during hypervitaminosis D₃ provides 25-OHD₃-26,23-lactone (26, 27). (23S,25R)-25-OHD₃-26,23-lactone binds with 5-fold higher affinity than 25-OHD₃ to the plasma vitamin D-binding protein (28, 29). Thus, it may indirectly exaggerate vitamin D toxicity by displacing biologically active metabolites, causing their concentrations in tissues to increase. 25-OHD₃-26,23-lactone is formed in kidney and in extrarenal tissues (exclusively from 25S,26-(OH)₂D₃, a circulating 25-OHD₃ metabolite (30, 31). The pathway proceeds through 25,26-(OH)₂D₃ (32). 25-OHD₃-26,23-lactone cannot be produced in uivo nor in vivo from 25S,26-(OH)₂D₃ (33, 34), a kidney microsomal metabolite of 25-OHD₃ (35).

During the course of investigating the pathways of 25-OHD₃-26,23-lactone biosynthesis, a unique metabolite was produced from 23S,25-(OH)₂D₃, namely 23-keto-25-OHD₃ (36, 37). 23-Keto-25-OHD₃ has 4-fold higher affinity than 25-OHD₃ for the bovine thymus cytosolic 1,25-(OH)₂D₃ receptor. 23-Ketonization is, therefore, the first modification, besides 1α-hydroxylation, that appears to enhance affinity for the 1,25-(OH)₂D₃-specific receptor. To answer the obvious question of whether a 1α-hydroxylated 23-keto-25-OHD₃ would have higher affinity than 1,25-(OH)₂D₃ for the cytosolic receptor, 23-keto-1,25-(OH)₂D₃ was prepared in vitro from 23-keto-25-OHD₃. 23-Keto-1,25-(OH)₂D₃ had 2-fold lower affinity than 1,25-(OH)₂D₃ for the bovine thymus cytosolic receptor, but appeared to be present in blood and intestine of 1,25-(OH)₂D₃-dosed animals, upon preliminary examination (36, 37). Because of their ability to bind with tissue 1,25-(OH)₂D₃ receptors, the in vivo presence of 23-keto derivatives of 25-OHD₃ and 1,25-(OH)₂D₃ might be significant to vitamin D toxicity.
These studies were undertaken to further examine 1,25-(OH)\textsubscript{2}D\textsubscript{3} metabolism and to rigorously determine whether 23-keto-1,25-(OH)\textsubscript{2}D\textsubscript{3} is a detectable metabolite of 1,25-(OH)\textsubscript{2}D\textsubscript{3} in vivo and in vitro. This paper also reports the identification of a new 1,25-(OH)\textsubscript{2}D\textsubscript{3} metabolite as 24-keto-1,25-(OH)\textsubscript{2}D\textsubscript{3}. While 24-keto-1,25-(OH)\textsubscript{2}D\textsubscript{3} is produced by intestine in vitro and is present in vivo in the plasma and intestinal mucosa of rats, it is not detected in the urine. The 23-keto-1,25-(OH)\textsubscript{2}D\textsubscript{3} receptor, 23-Keto-1,25-(OH)\textsubscript{2}D\textsubscript{3} in contrast, does not appear to be a quantitatively significant intestinal metabolite of 1,25-(OH)\textsubscript{2}D\textsubscript{3} in vivo or in vitro.

**MATERIALS AND METHODS**

**General**—Ultraviolet absorbance spectra were taken in 2-propanol with a Beckman Model 25 recording spectrophotometer. A molar absorptivity (ε) of 18,200 liters mol\textsuperscript{-1} cm\textsuperscript{-1} was used for all vitamin D compounds. HPLC was performed with Waters Associates ALC/GPC 204 liquid chromatographic equipment. Calibrators were detected at 254 nm. The normal phase HPLC columns used were DuPont Zorbax-Sil. All solvents were distilled in glass and were filtered through a 0.45-μm filter. Silica gel Sep-Paks were purchased from Waters Associates, Inc. (Milford, MA). Radioactivity was measured in a Beckman LS3000 liquid scintillation counter. Mass spectra were obtained at 70 eV from the solids probe of a Finnigan Model 4021 EI/CI GC/MS coupled with an INCOS 2000 Data System. To obtain spectra, the probe was heated from ambient to 320 °C at an ionizer temperature of 250 °C. CI-NCI mass spectra were obtained with dichlorodifluoromethane as the reagent gas.

**Compounds**—Synthetic 1,25-(OH)\textsubscript{2}D\textsubscript{3}, 1,23S,25-(OH)\textsubscript{3}D\textsubscript{3}, 1,24R,25-(OH)\textsubscript{3}D\textsubscript{3}, 1,25S,26-(OH)\textsubscript{3}D\textsubscript{3}, and 1,25-(OH)\textsubscript{2}D\textsubscript{3}-26,23-lactone were gifts from Dr. Milan R. Uskokovic and Dr. John J. Partridge, Hoffmann-LaRoche, Nutley, NJ. The compounds were synthesized in their laboratory by the methods described (38-40). 23-Keto-1,25-(OH)\textsubscript{2}D\textsubscript{3} was prepared from 23-keto-25-OHD\textsubscript{3} with kidney homogenate. Mass spectra were obtained at 70 eV from the solvents of a Finnigan Model 4021 EI/CI GC/MS coupled with an INCOS 2006 Data System. To obtain spectra, the probes were heated from ambient to 320 °C at an ionizer temperature of 250 °C. CI-NCI mass spectra were obtained with dichlorodifluoromethane as the reagent gas.

**Production of 24-Keto-1,25-(OH)\textsubscript{2}D\textsubscript{3}**—Twenty-three-month-old male Sprague-Dawley rats (Harlan, Indianapolis, IN) were treated intraperitoneally with 1,25-(OH)\textsubscript{2}D\textsubscript{3} (250 ng in 0.1 ml of propylene glycol). Five h after the last dose, the rats were decapitated, and their entire small intestines were collected, rinsed with 0.9% sodium chloride, split apart, and the 1,25-(OH)\textsubscript{2}D\textsubscript{3} (25 ng in 0.1 ml of propylene glycol). Five h after the last dose, the rats were decapitated, and their entire small intestines were collected, rinsed with 0.9% sodium chloride, split longitudinally, and scraped free of mucosa. A 20% (w/v) mucosal homogenate was prepared with a solution of 50 mM sodium phosphate buffer, pH 7.4, 250 mM sucrose, and 1 mM phenylmethylsulfonyl fluoride. To each of 103 Erlenmeyer flasks (125 ml) were added: homogenate (5 ml); 100 mM sodium phosphate buffer (10 ml), pH 7.4; 2.5 ml of a solution of sodium succinate (8 mM), nicotinamide (160 mM), MgCl\textsubscript{2} (1.6 mM), and KCl (100 mM); 2.5 ml of a solution of ATP (20 mM), NADP (1.8 mM), and sodium isocitrate (20 mM); isocitrate dehydrogenase (4.5 units); and alcohol dehydrogenase (8 units). After a 5-min preincubation, the reactions were initiated by adding to each flask 1,25-(OH)\textsubscript{2}D\textsubscript{3} (2 μg) in ethanol (20 μl). The mixtures, open to the atmosphere, were incubated at 37 °C for 15 min with shaking. The reactions were quenched with 1.5 volumes of methanol/dichloromethane (2:1). To effect phase separation, an additional volume of dichloromethane was added. The dichloromethane phase was removed, and the aqueous phase was extracted with an additional 1.5 volumes of dichloromethane. The solvent was evaporated from the combined dichloromethane phases under reduced pressure.

The residue from the organic extract was placed on a Sephadex LH-20 column (2 × 26 cm) and eluted with chloroform/hexane/methanol (75:23:2) (17). The material eluting between 105 and 250 ml was collected. In this column, 1,25-(OH)\textsubscript{2}D\textsubscript{3} had eluted between 125 and 225 ml. The residue was evaporated under vacuum and resuspended in Sep-Pak with 10 ml of ethyl acetate. The recovered residue was injected onto a normal phase HPLC column (0.62 × 25 cm) equilibrated with 2-propanol/hexane (1:9). The peak that eluted between 64 and 68 ml was collected. The 1,25-(OH)\textsubscript{2}D\textsubscript{3} eluted at 54 ml. The material was placed on a Sephadex LH-20 column (0.46 × 25 cm) and eluted with 2-propanol/chloroform/methanol (6:94) (34). The metabolite eluted at 16 ml, compared to 21 ml for 1,25-(OH)\textsubscript{2}D\textsubscript{3}. Finally, the metabolite was placed on a normal phase HPLC column (0.46 × 25 cm) equilibrated with hexane/dichloromethane/2-propanol (8:1:1.2). The metabolite eluted at 18 ml.

**Metabolism of 1,25-(OH)\textsubscript{2}D\textsubscript{3} in Intestinal Homogenates**—The procedure described above was used except 1,24R,25-(OH)\textsubscript{2}D\textsubscript{3} (2 μg) was introduced as substrate into each of four Erlenmeyer flasks.

**Silylation**—To 300 ng of 24-keto-1,25-(OH)\textsubscript{2}D\textsubscript{3} in methanol (30 μl) were added several small crystals of sodium borohydride. After 6 min, the reaction was quenched with dilute hydrochloric acid. The solvent was evaporated under a stream of nitrogen. The residue was extracted with chloroform (8 × 0.1 ml). The chloroform was evaporated and the residue was analyzed by HPLC. The material recovered from HPLC was analyzed by CI-mass spectrometry.

**Results**—Unlabeled 1,25-(OH)\textsubscript{2}D\textsubscript{3} was incubated with intestinal homogenates prepared from 1,25-(OH)\textsubscript{2}D\textsubscript{3}-treated rats. The organic extract was partially purified by Sephadex LH-20 chromatography, and a metabolite that eluted in the 1,25-(OH)\textsubscript{2}D\textsubscript{3} area was isolated through three different HPLC steps. The last HPLC system yielded a single major component (Fig. 1A). The new metabolite migrated near 1,25-(OH)\textsubscript{2}D\textsubscript{3} on
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In this case, however, the base peak was m/z 59, which is produced by C-24/C-25 bond lysis. Although this peak is common in the mass spectra of 25-hydroxylated vitamin D compounds, it is usually not the base peak. The intensity of m/z 59 in this case suggests labilization of the C-24/C-25 bond. The molecular weight of 430, the side chain functionalization, and the labilization of the C-24/C-25 bond are consistent with a ketone at C-24, namely 24-keto-1,25-(OH)₂D₃.

An electron impact mass spectrum of the silylated metabolite had a molecular ion at m/z 646, and therefore indicated that only three hydroxyl groups were present (Fig. 2A). The peaks at m/z 556 and 466 represent loss of one and two (CH₃)₂OH groups from m/z 646, respectively. Peaks at m/z 631, 541, and 451 indicate loss of methyl groups from m/z 646, 556, and 466, respectively. The base peak at m/z 206 is the trimethylsilyl counterpart to m/z 134. The peak at m/z 73 represents (CH₃)₃Si⁺. Finally, the peaks at m/z 131 and 515

the ternary system depicted, but was distinct from other 1,25-(OH)₂D₃ derivatives. Parenthetically, this ternary system of hexane, dichloromethane, and 2-propanol resolves 1,25-(OH)₂D₃, 1,25-(OH)₂D₃-26,23-lactone, and 1,24R,25-(OH)₃D₃, a result not readily attainable with binary systems composed of either hexane or dichloromethane. The metabolite had a λ_max at 265 nm, which is consistent with a vitamin D-like cis-5,7,10(19)-triene chromophore. Based on its UV absorbance, approximately 2 μg were isolated.

Cl⁻-NCI mass spectroscopy of the metabolite confirmed its molecular weight as 430. An electron impact mass spectrum also indicated a molecular weight of 430 (Fig. 2A). Peaks at m/z 412 and 394 indicated loss of one and two molecules of water, respectively, from the molecular ion. The peak at m/z 371 was produced by loss of 59 atomic mass units from the molecular ion. The peak at m/z 269 resulted from loss of the side chain and one water molecule. Loss of water from m/z 269 produced m/z 251. The latter two peaks indicated that the functionalization of 1,25-(OH)₂D₃ had occurred on the side chain. Peaks at m/z 152 and 134 are typical of the 5,7,10(19)-triene system of 1α-hydroxylated vitamin D compounds. They result from C-7/C-8 schism to give m/z 152. Loss of water from m/z 152 yields m/z 134, which is usually

Cl⁻-NCI mass spectroscopy provides chemical ionization spectra in which chloride ion addition to the molecular ion of the sample has occurred. The addition of chloride ion is readily determined by double peaks 2 mass units apart due to the two isotopes of chloride, ³⁵Cl and ³⁷Cl. Fragmentation is minimal (J. L. Napoli and B. C. Pramanik, unpublished observations).

Fig. 2. Electron impact mass spectra of 24-keto-1,25-(OH)₂D₃ produced from 1,25-(OH)₂D₃ (A), and 1,24R,25-(OH)₃D₃ (B) and electron impact mass spectrum of trisilyl-24-keto-1,25-(OH)₂D₃ (C).

Fig. 1. Final purification of 24-keto-1,25-(OH)₂D₃. A, metabolite produced from 1,25-(OH)₂D₃; B, metabolite produced from 1,24R,25-(OH)₃D₃. A normal phase HPLC column (0.46 x 25 cm) was eluted with hexane/dichloromethane/2-propanol (8:1.2). The elution positions of standards are indicated: 1, 1,25-(OH)₂D₃; 4, 1,23S,25-(OH)₂D₃ and 1,25-(OH)₂D₃-26,23-lactone. 1,24R,25-(OH)₃D₃ elutes in 33 ml in this system.
result from C-24/C-25 bond schism. In other words, m/z 131 is the silylated counterpart of m/z 59.

The structural assignment as 24-keto-1,25-(OH)D$_3$ was supported by chemical reduction of the metabolite. Upon treatment with sodium borohydride, the metabolite was converted into a more polar compound which migrated with authentic 1,24,25-(OH)$_3$D$_3$ in an HPLC system that separates 1,25-(OH)$_2$D$_3$ from 1,23S,25-(OH)$_2$D$_3$, 1,24R,25-(OH)$_3$D$_3$ and 1,25S,26-(OH)$_2$D$_3$ by wide margins (Fig. 3). A CI-NCI mass spectrum of the material recovered from the HPLC column indicated a molecular ion at m/z 432, i.e. 1,24,25-(OH)$_3$D$_3$.

Further support for the structure as 24-keto-1,25-(OH)$_2$D$_3$ was provided by production of the metabolite from 1,24R,25-(OH)$_2$D$_3$. Incubation of 1,24R,25-(OH)$_2$D$_3$ with an intestinal mucosal homogenate from rats treated with 1,25-(OH)$_2$D$_3$ produced about 200 ng of a compound (estimated by HPLC peak height; Fig. 1B) that eluted in three HPLC systems like the metabolite produced from 1,25-(OH)$_2$D$_3$. It also had an essentially identical mass spectrum (Fig. 2B).

To distinguish 24-keto-1,25-(OH)$_2$D$_3$ and another recently characterized vitamin D$_3$ metabolite, 23-keto-1,25-(OH)$_2$D$_3$ (36), their mass spectra were compared (Table I). The 23-keto compound had a molecular ion of very low intensity, most likely the result of a McLafferty rearrangement between the C-23-ketone and the C-25-hydroxyl group, which are able to align in a pseudo-six-member ring. The resultant proton transfer and loss of (CH$_3$)$_2$CO (m/z 58) produced peaks at m/z 372, 354 (loss of H$_2$O from 372), and 356 (loss of H$_2$O from 354). These were absent or of relatively low intensity in the mass spectrum of 24-keto-1,25-(OH)$_2$D$_3$. Instead, the spectrum of the latter compound had a more intense molecular ion and peaks resulting from dehydration of the molecular ions. It also had a relatively intense peak (compared to the molecular ion) at m/z 371. Thus, these two vitamin D$_3$ side chains are distinguishable by mass spectroscopy.

The relationship of the newly identified metabolite to the

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**Fig. 3.** HPLC migration of the 24-keto-1,25-(OH)$_2$D$_3$ sodium borohydride reduction product. A normal phase HPLC column (0.46 x 25 cm) was eluted with hexane/dichloromethane/2-propanol (81:1.6). The elution positions of synthetic standards are indicated: 1, 1,25-(OH)$_2$D$_3$; 4, 1,23S,25-(OH)$_2$D$_3$; 6, 1,24R,25-(OH)$_3$D$_3$; 7, 1,23S,26-(OH)$_2$D$_3$.

**Fig. 4.** HPLC analysis of 1,25-(OH)$_2$[3H]D$_3$ metabolism (HPLC system 1). Metabolites were extracted from intestinal homogenates incubated with 1,25-(OH)$_2$[3H]D$_3$ (A) and plasma (B) and intestinal mucosa (C) of 1,25-(OH)$_2$[3H]D$_3$-dosed rats. Animals in these experiments were treated with unlabeled 1,25-(OH)$_2$D$_3$ as described under “Materials and Methods.” A normal phase HPLC column (0.46 x 25 cm) was eluted with hexane/2-propanol (9:1). The elution positions of standards are indicated: 1, 1,25-(OH)$_2$D$_3$; 2, 24-keto-1,25-(OH)$_2$D$_3$; 3, 23-keto-1,25-(OH)$_2$D$_3$; 4, 1,23S,25-(OH)$_2$D$_3$; 5, 1,25-(OH)$_2$D$_3$-26,23-lactone; 6, 1,24R,25-(OH)$_3$D$_3$. Aliquots were counted for radioactivity, and the remaining material was analyzed in HPLC system 2. The data is plotted as counts/min/fraction. The broken lines represent amplification of part of the graphs. The scale (y axis) of the solid lines is placed to the left of each section. The scale of the broken lines is placed to the right of each section. The two y axis legends between A and B, and B and C refer to the sections on either side. Fractions 1-11 were 2 ml each; fractions 12-80 were 1 ml each.
other intestinal homogenate-produced metabolites with 1,25-(OH)$_2$[H]D$_3$ as substrate was studied (HPLC system 1; Fig. 4A). Most of the radioactivity (90 and 87%, in the duplicates) was recovered as unreacted substrate. However, 24-keto-1,25-(OH)$_3$[H]D$_3$ represented 4.0 and 4.2% of the total recovered tritium in each analysis. A second peak, migrating closely with 1,23S,25-(OH)$_3$D$_3$ and 23-keto-1,25-(OH)$_3$D$_3$, accounted for 4.2 and 5.3% of the recovered radioactivity. Finally, a peak that co-migrated with 1,24R,25-(OH)$_3$D$_3$ accounted for 2.4 and 3.7% of the recovered radioactivity.

The occurrence of these peaks in vivo was investigated. Rats not supplemented with exogenous 1,25-(OH)$_2$D$_3$ were dosed with 1,25-(OH)$_2$[H]D$_3$. 24-Keto-1,25-(OH)$_3$[H]D$_3$ was observed (HPLC system 1) in both plasma (2.3% of recovered tritium relative to 89% for 1,25-(OH)$_2$[H]D$_3$) and intestinal mucosa (1.4% of recovered tritium relative to 88% for 1,25-(OH)$_2$[H]D$_3$). In both plasma and intestine, radioactive eluting in the 23-keto-1,25-(OH)$_2$D$_3$/1,23S,25-(OH)$_2$D$_3$ area was less than 0.5% of the recovered radioactivity. 1,25-(OH)$_2$[H]D$_3$-26,23-lactone represented 1% and less than 0.2% of the recovered tritium in plasma and intestine, respectively, compared to 7.5 and 7.0%, respectively, for 1,24,25-(OH)$_2$[H]D$_3$ (graphs not shown). In a second experiment, rats treated chronically with unlabeled 1,25-(OH)$_2$D$_3$ were dosed with 1,25-(OH)$_2$[H]D$_3$. The plasma of the 1,25-(OH)$_2$D$_3$-dosed animals also had radioactive peaks co-migrating with each of 1,25-(OH)$_2$D$_3$, 24-keto-1,25-(OH)$_2$D$_3$, and 1,24,25-(OH)$_2$D$_3$. An additional large peak corresponding to 1,25-(OH)$_2$D$_3$, 26,23-lactone was observed. Intestinal mucosa of 1,25-(OH)$_2$D$_3$-dosed rats had a profile of 1,25-(OH)$_2$D$_3$ metabolites qualitatively similar to plasma (Fig. 4C). However, there were differences in the proportions of the metabolites. Notably, there seemed to be more 24-keto-1,25-(OH)$_2$[H]D$_3$ and the peak that migrated with 23-keto-1,25-(OH)$_2$D$_3$ and 1,24R,25-(OH)$_3$D$_3$ in intestine, relative to both recovered substrate and to 1,25-(OH)$_2$[H]D$_3$-26,23-lactone and 1,24R,25-(OH)$_3$[H]D$_3$. These data show that 1,25-(OH)$_2$D$_3$ dosing changes the relative abundance of 1,24R,25-(OH)$_3$D$_3$ and 1,25-(OH)$_2$D$_3$ in vivo, so that the latter becomes predominant. 1,25-(OH)$_2$D$_3$ treatment also increases the relative abundance of the peak in the 23-keto-1,25-(OH)$_2$D$_3$/1,23S,25-(OH)$_2$D$_3$ elution region.

The compositions of the peaks from 1,25-(OH)$_2$D$_3$-treated animals recovered from the first HPLC system (HPLC system 1; Fig. 4) were examined on a second HPLC system (HPLC system 2), which had different selectivity (2). Note that the elution order of standards in HPLC system 2 was different from HPLC system 1. Re-chromatography of the 24-keto-1,25-(OH)$_2$[H]D$_3$ peak recovered from the intestinal homogenates showed a major peak that co-chromatographed with unlabeled 24-keto-1,25-(OH)$_2$D$_3$ and some carry over of 1,25-(OH)$_2$[H]D$_3$. The latter becomes predominant. 1,25-(OH)$_2$D$_3$ treatment also increases the relative abundance of the peak in the 23-keto-1,25-(OH)$_2$D$_3$/1,23S,25-(OH)$_2$D$_3$ elution region.

Further analysis of 24-keto-1,25-(OH)$_3$[H]D$_3$ from HPLC system 1 (Fig. 4). The materials in fractions 20-24 were collected and re-analyzed on a normal phase HPLC column eluted with dichloromethane/2-propanol (94:6; HPLC system 2). A, intestinal homogenates; B, plasma (dashed line) and intestinal mucosa (solid line). The elution positions of standards are indicated: 2, 24-keto-1,25-(OH)$_2$D$_3$; 3, 23-keto-1,25-(OH)$_2$D$_3$; 4, 1,23S,25-(OH)$_3$D$_3$; 6, 1,24R,25-(OH)$_3$D$_3$. The data are plotted as counts/min/fraction. Fractions were 1 ml each.

Further analysis of the 23-keto-1,25- (OH)$_2$D$_3$/1,23S,25- (OH)$_2$D$_3$ fractions of system 1 (Fig. 4). The materials in fractions 28-34 were collected and re-analyzed in HPLC system 2 as described in the legend of Fig. 4. A, intestinal homogenates; B, plasma (dashed line) and intestinal mucosa (solid line). The data are plotted as counts/min/fraction. The fractions were 1 ml each.
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Fig. 7. Competitive binding protein assays of 1,25-(OH)₂D₃ and derivatives. Either crude chick intestinal cytosol (A) or calf thymus cytosol substantially free of the 5 S vitamin D-binding protein (B) was used as the source of receptor. Points are the mean of triplicates. Individual values deviated ±10% from the mean. The compounds tested were 1,25-(OH)₂D₃ (●); 24-keto-1,25-(OH)₂D₃ (○); 1,24R,25-(OH)₃D₃ (△); 23-keto-1,25-(OH)₂D₃ (▲); and 1,23S,25-(OH)₂D₃ (■).

TABLE II

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chick cytosol</th>
<th>Thymus cytosol</th>
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<tr>
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<td>50% Displacement *</td>
<td>Relative affinity *</td>
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<td>1,25-(OH)₂D₃</td>
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<td>1,23S,25-(OH)₂D₃</td>
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* Amount/tube to produce 50% displacement of 1,25-(OH)₂[2,6,27-³H]D₃

** Binding potency relative to 1,25-(OH)₂D₃.

A new metabolite of 1,25-(OH)₂D₃ has been unequivocally identified as 24-keto-1,25-(OH)₂D₃. The structural assignment is supported by diverse evidence. The ultraviolet absorbance and mass spectra of the metabolite and its trisilyl derivative showed that it is a side chain ketone derivative of 1,25-(OH)₂D₃. The mass spectra also suggest that the ketone function is at C-24 and exclude a C-23-ketone from consideration. Chemical reduction of the metabolite produced a compound that coeluted with 1,24,25-(OH)₃D₃ on an HPLC system that readily separates 1,25-(OH)₂D₃, 1,23,25-(OH)₃D₃, 1,24,25-(OH)₃D₃, and 1,25,26-(OH)₃D₃. Furthermore, CI-NCI mass spectroscopy of the reduction product indicated a molecular weight consistent with 1,24,25-(OH)₃D₃. Synthesis of a metabolite in vitro from 1,24,25-(OH)₂D₃, with chromatographic and mass spectral characteristics identical with the metabolite produced from 1,25-(OH)₂D₃, provided the final demonstration that the structural assignment as 24-keto-1,25-(OH)₂D₃ is correct.

24-Keto-1,25-(OH)₂D₃ is produced in small intestine in vitro from 1,25-(OH)₂D₃ at low substrate concentrations and is present in the plasma and small intestinal mucosa of rats maintained on a stock diet. It is also present in the plasma and small intestinal mucosa of rats dosed chronically with 1,25-(OH)₂D₃. These data indicate that 24-keto-1,25-(OH)₂D₃ is on the pathway of 1,25-(OH)₂D₃ metabolism under physiological circumstances and, during states of 1,25-(OH)₂D₃ excess, in at least one vitamin D target tissue (intestine). Under the experimental conditions reported here, however, there was about 60-fold less 24-keto-1,25-(OH)₂D₃ than 1,25-(OH)₂D₃ in intestinal mucosa under physiological conditions. 24-Keto-1,25-(OH)₂D₃ binds equicotently with 1,24R,25-(OH)₃D₃ to the 3.7 S 1,25-(OH)₂D₃ cytosolic receptor in calf thymus. The 24-keto compound has higher affinity than 23-keto-1,25-(OH)₂D₃, a metabolite produced from 1,23,25-(OH)₃D₃ and/or 23-keto-25-OHD₃ in chick kidney homogenates (36, 37). With crude chick intestinal cytosol, 24-keto-1,25-(OH)₂D₃ is about 7-fold less potent than unlabeled 1,25-(OH)₂D₃ in displacing 1,25-(OH)₂[³H]D₃ from the 1,25-(OH)₂D₃ receptor. One key difference between these two preparations is the diminished concentration in the latter preparation of a second protein, which sediments at 5 S (41). The 5 S protein, an artifact of the homogenization procedure, also binds vitamin D metabolites and probably discriminates differently than the 3.7 S protein. Therefore, data generated with cytosolic preparations substantially free of the 5 S con-
Intestinal Synthesis of 24-Keto-1,25-dihydroxyvitamin D3

taminant are likely to reflect affinity of ligand for receptor more accurately than those obtained with crude preparations. On the other hand, differences in receptor structure have not been studied.

1,25-(OH)2D3 was reported to undergo side chain metabolism in vivo by Harnden et al. (44) and Kumar et al. (45). They demonstrated that the intestine was one site of conversion (46) and rigorously identified 1,24,25-(OH)3D3 as a rat intestinal 1,25-(OH)2D3 metabolite in vitro (47). In contrast, Ohnuma and Norman (48) were unable to detect 1,24,25-(OH)3D3 as an in vitro product of 1,25-(OH)2D3 metabolism with rat intestinal mucosal homogenates. Instead, they found a product which co-migrated with a radioactive peak extracted from the plasma of rats dosed with 1,25-(OH)2[26,27-3H]D3. This peak had been tentatively identified as 1,25-(OH)2D3-26,23-lactone by Harnden et al. (52), but showed a fragmentation pattern similar to that of 23-keto-25-OHD3. Furthermore, it was reported that the putative 23-keto-1,25-(OH)2D3 is the sole precursor of the putative 1,25,26-23-oxo-D3 (53). Consequently, the present work was consistent with the results of Kumar et al. (47). However, we also detected a product that co-migrated with authentic synthetic 1,25-(OH)2D3-26,23-lactone in two HPLC systems with different selectivities. About 10-fold less 1,25-(OH)2D3-26,23-lactone was detected in the plasma and intestine of rats not treated with exogenous 1,25-(OH)2D3.

Larger amounts of 1,24,25-(OH)3[3H]D3 than 1,25-(OH)2[3H]D3-26,23-lactone were detected in the plasma and intestine of rats treated with exogenous 1,25-(OH)2D3. This is consistent with the results of Ramberg et al. (50) who showed that about 4-fold more 1,25-(OH)2D3 was converted into 1,24,25-(OH)3D3 than into 1,25-(OH)2D3-26,23-lactone in normal bovine. On the other hand, the present work also showed that chronic 1,25-(OH)2D3 dosing produces a significant change in the relative amounts of 1,24,25-(OH)3[3H]D3 and 1,25-(OH)2[3H]D3-26,23-lactone in vivo, such that the latter metabolite predominates. By analogy to 25-OHD3-26,23-lactone biosynthesis, which increases during chronic vitamin D3 treatment (26-28), it is reasonable to expect that one cause of the change in the relative amounts of 1,24,25-(OH)3D3 and 1,25-(OH)2[3H]D3-26,23-lactone is in vitro production of 25-OHD3 and 1,25-(OH)2D3 into their respective 26,23-lactones. In the present work, no evidence of significant production of 23-keto-1,25-(OH)2D3 by intestinal homogenates was obtained. Nor was a substantial presence of this putative metabolite observed in vivo relative to 24-keto-1,25-(OH)2D3. Our conclusions are based on HPLC comparisons of radiolabeled 1,25-(OH)2[3H]D3 metabolic products in vitro and in vivo with standards. Notably, our standard 23-keto-1,25-(OH)2D3 was produced from two different substrates with known side chains, namely 1,23S,25-(OH)2D3 and 23-keto-25-OHD3 (36, 37). Thus, the key functionality was already in place in our starting materials. Moreover, the mass spectral data obtained from 23-keto-1,25-(OH)2D3 produced in our laboratory, were distinct from those of 24-keto-1,25-(OH)2D3 and the metabolite isolated by Ohnuma et al. (52).

In summary, this report describes the identification of a physiological 1,25-(OH)2D3 metabolite produced by intestine as 24-keto-1,25-(OH)2D3. 24-Keto-1,25-(OH)2D3 is rapidly generated from 1,25-(OH)2D3 and is present in a vitamin D3 target tissue. It has high affinity for the cytosolic 1,25-(OH)2D3 receptor. Its physiological function, if any, is not certain, but it is intriguing to consider that the metabolite acts in situ as a mediator of calcium homeostasis. Alternatively, it may be part of an inactivation pathway. Further research will determine which of these is the more viable hypothesis.

REFERENCES

Intestinal Synthesis of 24-Keto-1,25-dihydroxyvitamin D$_3$
Intestinal synthesis of 24-keto-1,25-dihydroxyvitamin D3. A metabolite formed in vivo with high affinity for the vitamin D cytosolic receptor.

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