Role of kidney tissue in the production of 25-hydroxyvitamin D₃-26,23-lactone and 1α,25-dihydroxyvitamin D₃-26,23-lactone
(vitamin D/kidney/25-hydroxyvitamin D/1,25-dihydroxyvitamin D)

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MATERIALS AND METHODS

Vitamin D Metabolites. [3α,3H]-25-OH-D₃, [23,24-3H]-25-OH-D₃ (10), and [26,27-3H]-25-OH-D₃ (11) were synthesized in our laboratories. Crystalline 24R,25-(OH)₂D₃, 1,24R,25-trihydroxyvitamin D₃ [1,24R,25-(OH)₂D₃] and 1,25-(OH)₂D₃ were gifts from Hoffman-LaRoche. 25,26-(OH)₂D₃ was synthesized by the method of Lam et al. (12). 25-OH-D₃-26,23-lactone was isolated from chicken plasma by the method of Wichmann et al. (8). 25-OH-D₃ was a gift from Upjohn.

Animals. For in vivo production of 25-OH-D₃-26,23-lactone, male weaning rats were purchased from Holtzman, Madison, WI, and fed an adequate calcium, adequate phosphorus diet (13) supplemented with 25 international units of vitamin D₃ per day for 6 days. Two days before the conclusion of the experiment, the rats were given 25-OH-D₃ (5 μg in 0.1 ml of 1.2-propandiol per day) subcutaneously. Twenty hours before sacrifice, the rats were either nephrectomized or sham operated and then given intrajugularly 10 μCi [3α,3H]-25-OH-D₃ (28 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) in 0.05 ml of 95% ethanol.

For in vitro production of 25-OH-D₃-26,23-lactone and 1α,25-(OH)₂D₃-26,23-lactone, single-comb White Leghorn chickens (Northern Hatcheries, Beaver Dam, WI) were fed a normal diet for 10 weeks and given 10⁶ international units of vitamin D₃/day for 3 days, followed by 1.5 × 10⁷ international units for 1 day, 4 days before sacrifice.

For in vitro production of 1α,25-(OH)₂D₃-26,23-lactone from 25-OH-D₃-26,23-lactone, one-day old male White Leghorn chickens (Northern Hatcheries) were fed a rachitogenic diet (14) for 4 weeks.

Extraction of Rat Serum. Twenty-four hours after the administration of [3α,3H]-25-OH-D₃, rats were killed and their blood was collected. The blood was centrifuged to yield serum. Seven milliliters of serum was pooled from three rats in each of the nephrectomized or the sham-operated group. The serum was mixed with authentic 1α,25-(OH)₂D₃, 25,26-(OH)₂D₃, and


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24R,25-dihydroxyvitamin D3 [24R,25-(OH)2D3] (200 ng each) and 100 ng of 25-OH-D26,23-lactone dissolved in 50 µl of ethanol. After 1-hr incubation at room temperature, extractions of the sera were carried out (15).

**In Vitro Incubation of Chicken Kidney or Liver Homogenate with Labeled Substrate.** Chickens were killed by decapitation, and their kidneys were placed in ice-cold 0.25 M sucrose. A 20% homogenate of kidney was prepared in 0.25 M sucrose. A liver homogenate was prepared in the same way. Incubation was carried out in a 25-ml erlenmeyer flask that contained, in a 2-ml total volume, 200 mg of tissue, 50 mM phosphate buffer, pH 7.4/2.24 mM glucose 6-phosphate/20 mM ATP/160 mM nicotinamide/0.4 mM NADP/5 mM MgCl2/0.1 M KCl/glucose 6-phosphate dehydrogenase (0.5 units) (Sigma). The reaction was initiated by addition of 0.4 µCi of either [3α-3H]-25-OH-D3, [23,24-3H]-25-OH-D3, or [26,27,3H]-25-OH-D3 in 10 µl of 95% ethanol. The mixtures were incubated at 37°C with shaking at 100 oscillations per min for 1 hr under an atmosphere of air. The reaction was stopped by addition of methanol/chloroform (2:1). One hundred nanograms each of authentic 24R,25-(OH)2D3 and 25-OH-D26,23-lactone was added to each flask. After 1 hr of incubation at room temperature, the incubation mixture was extracted (15).

**In Vitro Incubation of Chicken Kidney Homogenate with Unlabeled 25-OH-D3 or 1,25-(OH)2D3.** Incubation conditions were as described above except that incubation was carried out in 4-ml reaction volume in a 125-ml erlenmeyer flask containing 400 mg of kidney tissue. In some experiments, the glucose 6-phosphate and glucose 6-phosphate dehydrogenase was replaced by 25 mM sodium succinate (Table 1). The reaction was initiated by addition of either 400 µg of 25-OH-D3 or 20 µg of 1,25-(OH)2D3 in 100 µl of 95% ethanol. The mixtures were incubated for 1 hr at 37°C with shaking at 100 oscillations per min. The reaction was stopped by addition of methanol/chloroform (2:1) and extracted as described (15).

**Production of 1,25-(OH)2D3-26,23-Lactone from 25-OH-D3-26,23-Lactone.** Kidney homogenate (20% wt/vol) from rachitic chickens was prepared as described above in 0.19 M sucrose/15 mM Trisacetate/1.9 mM MgCl2. Homogenate (600 mg) was incubated (total volume 4.5 ml) in 0.19 M sucrose/15 mM Trisacetate, pH 7.4/1.9 mM MgCl2/25 mM sodium succinate in a 125-ml erlenmeyer flask using an air atmosphere and shaking at 100 oscillations per min. To initiate the reaction, 50 µl of ethanol containing 10 µg of 25-OH-D3-26,23-lactone was added. The incubation was continued for 2.5 hr and terminated by the addition of methanol/chloroform (2:1). Extraction was carried out as described (15).

**Purification of Extract by Sephadex LH-20 Chromatography.** To purify the 25-OH-D3-26,23-lactone, the lipid extract of either serum or incubation medium was applied to a Sephadex LH-20 column (0.7 x 14 cm) packed in 65% CHCl3/hexane as described (16). The column was eluted with the same solvent; the first 11 ml of eluant was discarded, and the next 25 ml was collected. The elution positions of the metabolites were established with the authentic compounds. The initial purification of 1,25-(OH)2D3-26,23-lactone produced by chicken kidney homogenates involved chromatography of the extracts on Sephadex LH-20 (1 x 16 cm column) and elution with CHCl3/hexane/MEOH (97:2:3). The first 45 ml of eluant was discarded, and the next 80 ml (representing the elution volume of 1,25R,25-(OH)2D3 used as calibration standard) was pooled for subsequent high-performance liquid chromatography (HPLC) purification.

**HPLC of Rat Serum Extract.** The purified extract of serum from either nephrectomized or sham-operated rats was dried under nitrogen, dissolved in 8% 2-ProOH/hexane and subjected to HPLC using a Zorbax-Sil column (4.6 mm x 25 cm). The HPLC was performed at a pressure of 1000 lbf/in2 (1 lbf/in2 = 6895 Pa) and a flow rate of 2 ml/min; the solvent was 8% 2-ProOH/hexane. Fractions of 0.8 ml were collected, and aliquots were counted in toluene counting solution (17). UV absorbance was monitored at 254 nm by a model 440 absorbance detector (data not shown). The fractions containing 25-OH-D3-26,23-lactone (16-18 ml) were combined and applied to the reversed-phase HPLC using a Lichrosorb RP-18 column (4.6 mm x 25 cm) (E. Merk, Darmstadt, West Germany); this was eluted with 22% H2O/MEOH at a pressure of 1300 lbf/in2 and a flow rate of 2 ml/min (Fig. 1).

**HPLC of Extracts from Incubations.** To further purify the 25-OH-D3-26,23-lactone, the extracts were treated as described above, except that the straight-phase HPLC was eluted with 6% 2-ProOH/hexane (Fig. 2A). Fractions of 0.8 ml were collected, and an aliquot of each fraction was counted as above. The fractions containing 25-OH-D3-26,23-lactone (Fig. 2A) were combined and applied to the reversed-phase HPLC as described above except that the elution solvent was 25% H2O/MEOH. Fractions of 0.8 ml each were collected and counted (Fig. 2B).

The extracts of tissue incubated with unlabeled 25-OH-D3 were subjected to straight-phase HPLC as described above, and the 25-(OH)2D3-26,23-lactone fraction (16-19 ml) was collected. The putative 25-OH-D3-26,23-lactone was then subjected to reversed-phase HPLC as described above, and the lactone fraction (22-27 ml) was collected.

For the experiments dealing with 1,25-(OH)2D3-26,23-lact-
When vitamin D-repleted rats with intact kidneys were given \([3\alpha-^3\text{H}]25\text{-OH-D}_3\) the main metabolite observed was 24,25-(OH)_2D_3 with much less 25,26-(OH)_2D_3, 1,25-(OH)_2D_3, and a substance that comigrated with authentic 25-OH-D_3-26,23-lactone. In contrast, nephrectomized rats produced almost none of these metabolites. The fraction containing the putative metabolite was subjected to reverse-phase HPLC using 15% 2-PrOH/hexane after purification on Sephadex LH-20. The putative 1,25-(OH)_2D_3-26,23-lactone from the two substrates used was collected and rechromatographed on a reverse-phase system (Zorbax-ODS) at 25% H_2O/MeOH as eluting solvent (Fig. 3). The product eluting at 17 ml was collected and applied to a straight-phase silica gel column (Zorbax-Sil). This was eluted with 15% 2-PrOH/hexane. The resulting product (eluting at 20 ml) was then subjected to physical characterization.

Rat Plasma Protein Competitive Binding Assay. The fractions containing 25-OH-D_3-26,23-lactone collected from the reverse phase HPLC were dried under N_2 and dissolved in 100 μl of ethanol. Duplicate 50 μl portions of each were used for measurement of 25-OH-D_3-26,23-lactone by a rat plasma protein competitive assay (9).

General Procedures. All HPLC was carried out on a Waters Associates ALC/GPC 204 instrument equipped with either a fixed-wavelength UV detector (at 254 nm) or a variable-wavelength detector (at 265 nm). Scintillation counting was performed by using a Packard Instruments (Downers Grove, IL) 3255 spectrometer and mass spectrometry was performed by using an AEI MS-9 instrument (Associated Electrical Industries, Manchester, England) having a direct probe inlet and operated at 70 eV and a source temperature of 140°C above ambient.

RESULTS

When vitamin D-repleted rats with intact kidneys were given \([3\alpha-^3\text{H}]25\text{-OH-D}_3\), the main metabolite observed was 24,25-(OH)_2D_3 with much less 25,26-(OH)_2D_3, 1,25-(OH)_2D_3, and a substance that comigrated with authentic 25-OH-D_3-26,23-lactone. In contrast, nephrectomized rats produced almost none of these metabolites. The fraction containing the putative metabolite was subjected to reverse-phase HPLC using 15% 2-PrOH/hexane after purification on Sephadex LH-20. The putative 1,25-(OH)_2D_3-26,23-lactone from the two substrates used was collected and rechromatographed on a reverse-phase system (Zorbax-ODS) at 25% H_2O/MeOH as eluting solvent (Fig. 3). The product eluting at 17 ml was collected and applied to a straight-phase silica gel column (Zorbax-Sil), this was eluted with 15% 2-PrOH/hexane. The resulting product (eluting at 20 ml) was then subjected to physical characterization.

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and 408 (M+ – 2 × H2O). In addition, the spectrum showed characteristic fragment peaks at m/e = 269 (M+ – sidechain – H2O) and 251 (269 – H2O), as well as the ring A fragments at m/e = 152 and 134 diagnostic for 1-hydroxylated vitamin D derivatives. The characteristic intensity ratio of the peaks at m/e = 152 and 134 (40:100) further established the α-configuration for the 1-hydroxy function, because it is known (18) that a 1,25-hydroxy substituent leads to a 152:134 peak ratio of 100:90. Given the formation of this metabolite from 25-OH-D3-26,23-lactone, these data prove the compound to be 1,25-(OH)2D3-26,23-lactone.

In view of this result, it was of interest to determine whether chicken kidney preparations capable of converting 25-OH-D3 to its lactone could also use 1α,25-(OH)2D3 as substrate for conversion to the 1α-hydroxylactone analog. Incubation of 1α,25-(OH)2D3 with kidney homogenates from chickens given large doses of vitamin D3 gave (as expected) a product that was chromatographically identical (on two HPLC systems) with 1,24,25-(OH)3D3, but only a trace of material corresponding in elution position to 1α,25-(OH)2D3-26,23-lactone.

DISCUSSION

The isolation and characterization of 25-OH-D3-26,23-lactone (8) and the fact that this compound is a major vitamin D metabolite under conditions of high intake raise questions concerning its possible in vivo function, mode of biosynthesis, and site of production.

The involvement of kidney in lactone formation is evident from initial in vivo experiments with rats on a normal vitamin D diet. The results show that nephrectomy prevents the conversion of 25-OH-D3 to the lactone, whereas sham animals carry out the conversion. As anticipated, the production of 1,25-(OH)2D3 and of 24,25-(OH)2D3 was either eliminated or greatly reduced by nephrectomy (19, 20). These results, therefore, suggest that the kidney plays an important role in production of the lactone. Kidney homogenates from chickens given high doses of vitamin D readily produced a compound that comigrated on straight-phase HPLC with authentic 25-OH-D3-26,23-lactone. The putative lactone also comigrated with authentic lactone on reversed-phase HPLC, a system known to be a very effective method for the resolution of the lactone and 24,25-(OH)2D3 compounds (5). It is, therefore, evident from both in vivo and in vitro experiments that production of the 25-OH-D3-26,23-lactone takes place largely in the kidney (although biogenesis in other tissues cannot be excluded at this time).

Because the kidney can produce the lactone from 25-OH-D3, it was of interest to determine whether that organ could also produce a lactone from 1,25-(OH)2D3. The results show that kidney preparations from chickens given large doses of vitamin D readily convert 1,25-(OH)2D3 to 1,24,25-(OH)3D3 but produce only trace amounts of 1,25-(OH)2D3-26,23-lactone. Evidently 1,25-(OH)2D3 is a poor substrate analog for the system effecting the conversion of 25-OH-D3 to the lactone. 1,25-(OH)3D3-26,23-Lactone could be produced, however, by C-1-hydroxylation of 25-OH-D3-26,23-lactone. Incubation of 25-OH-D3-26,23-lactone with homogenates obtained from rachitic chickens known to be rich in 1-hydroxylase activity and deficient in other vitamin D hydroxylases (1, 2) converted the lactone to a more polar compound that eluted on HPLC somewhat before 1,24R,25-(OH)3D3 (Fig. 3A). This compound was isolated and identified as 1,25-(OH)2D3-26,23-lactone by mass spectrometry. Presumably, 1-hydroxylation of 25-OH-D3-26,23-lactone involves the enzyme that normally catalyzes the conversion of 25-OH-D3 to 1,25-(OH)2D3. That system appears specific for a 25-hydroxy group but can accommodate other side-chain modifications in the substrate, as indicated, for example, by the conversion of both 24,25-(OH)2D3 and 25,26-(OH)2D3 to the corresponding 1α-hydroxy analogs by chicken kidney 1α-hydroxylase. Because 1α-hydroxylase activity is generally low under the conditions in which the lactone is formed (9), it is unlikely that much 1,25-(OH)2D3-26,23-lactone can be formed by 1-hydroxylation in vivo, and the small amounts of 1,25-(OH)2D3-26,23-lactone formed from 1,25-(OH)2D3 in vitro by the lactonizing system suggests that this pathway is also minimal in vivo. In support of this, only small amounts of 1,25-(OH)2D3-26,23-lactone (15 ng/6 liters) could be detected in the plasma of chickens containing substantial amounts of 25-OH-D3-26,23-lactone (300 µg/6 liters).

The biological importance of 25-OH-D3-26,23-lactone and its 1α-hydroxy analog remains unknown. The 25-OH-D3-26,23-lactone has a weak effect on intestinal calcium transport and bone calcium mobilization, and it is likely that the effect of the 1α-hydroxylated compound will be similar. However, it is possible that these compounds have an important role in other aspects of vitamin D action, a possibility that can be ascertained only by further investigation.

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