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HE 26-HYDROXYLATION OF 25-HYDROXYVITAMIN D_3 IN VITRO BY CHICK RENAL HOMOGENATES[+]

by

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Kidney homogenates from vitamin D supplemented chicks produce a metabolite from 25-OH-[^{3}H]D_3 which comigrates with synthetic 25,26-dihydroxyvitamin D_3 on Sephadex LH-20 and high pressure liquid chromatography. This metabolite has been isolated in pure form from such incubations and positively identified as 25,26-dihydroxyvitamin D_3 by mass spectrometry. Kidney homogenates from Vitamin D deficient chicks do not produce this metabolite. Additionally, parathyroid hormone administration suppresses the 26-hydroxylase activity in vitamin D repleted chickens.

In the search for polar metabolites of 25-hydroxyvitamin D_3[1] (25-OH-D_3) Suda et al (1) isolated from the plasma of pigs a metabolite of vitamin D in pure form and unequivocally demonstrated its structure to be 25,26-dihydroxyvitamin D_3 (25,26-$(OH)_2D_3$). This metabolite possessed some biological activity in the stimulation of intestinal calcium transport but had little activity in the mobilization of calcium from bone and in the mineralization of rachitic epiphyseal plate. The structure of the metabolite was confirmed by the chemical synthesis (2) in which the synthetic material elicited an identical biological activity to that reported by Suda et al (1). Of some interest was the fact that this compound could not stimulate intestinal calcium absorption in nephrectomized rats suggesting that it must be 1-hydroxylated before it functions. Little attention has been paid to this metabolite of vitamin D hence its site of synthesis and possible regulation has remained unknown. Recently

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[1]Abbreviations: 25,hydroxyvitamin D_3, 25-OH-D_3; 25,26-dihydroxyvitamin D_3, 25,26-$(OH)_2D_3$; 24R,25-dihydroxyvitamin D_3, 24R,25-$(OH)_2D_3$; 1,25-dihydroxyvitamin D_3, 1,25-$(OH)_2D_3$; 25-hydroxyvitamin [23,24-^{3}H]D_3, 25-OH-[23,24-^{3}H]D_3.

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we have been able to demonstrate clearly the presence of a 25-OH-D_3 26-hydroxylase in the renal tissue of chicks repleted with vitamin D. This hydroxylase is absent in vitamin D deficiency and is suppressed by parathyroid hormone injections.

Materials and Methods

Crystalline 25-OH-D_3 was a gift of the Philips-Duphar Co. (Weesp, The Netherlands). 24R,25-dihydroxyvitamin D_3 (24R,25-$(OH)_2D_3$) and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) were gifts from Hoffmann-LaRoche, Inc. (Nutley, New Jersey). Both the 25,26-$(OH)_2D_3$ and the 25-hydroxyvitamin [23,24-^{3}H]D_3 (25-OH-[23,24-^{3}H]D_3) were synthesized in this laboratory by the methods of Lam et al. (2) and Yamada et al. (3). Parathyroid extract was a gift from the Eli Lilly Company (Indianapolis, Indiana).

Animals. One-day-old Leghorn chicks were obtained from Northern Hatcheries (Beaver Dam, Wis.) and were fed a high calcium, vitamin D-deficient diet (4) for a period of 4 to 8 weeks. They were either given a single subcutaneous injection of 0.05 ml ethanol or they were given a single subcutaneous injection of 6.5 nmole of vitamin D_3 in 0.05 ml of ethanol 48 hours before they were killed by decapitation for the preparation of renal homogenates. Where indicated, parathyroid extract injections were given at a level of 20 units subcutaneously to the vitamin D repleted chicks 30, 23, and 6 hours prior to the time they were killed for their renal tissue.

In vitro incubation of kidney homogenate. Kidneys from 3-4 chickens were pooled and a 20% (w/v) homogenate was prepared in ice-cold 0.19 M sucrose containing 15 mM Tris-acetate (pH 7.4) and 1.9 mM magensium acetate. The incubations were carried out in 25 ml Erlenmeyer flasks. Each flask contained 200 mg of kidney tissue, 0.19 M sucrose, 15 mM Tris-acetate, (pH 7.4), 1.9 mM magnesium acetate and 25 mM succinate in 1.5 ml final volume. The contents of each flask were flushed for 30 seconds with 100% oxygen and stoppered. The substrate (2.6 nmole of 25-OH-[23,24-^{3}H]D_3 disolved in 0.025 ml of 95% ethanol) was added to each vessel. The reaction mixtures were incubated at 37° for 10 minutes. The reaction was stopped by addition of 10 ml of dichloromethane. Extraction was carried out by the dichloromethane extraction procedure described by Eisman et al. (5).

Sephadex LH-20 chromatography of putative 25,26-$(OH)_2D_3$. The dichloromethane extracts were applied directly to a Sephadex LH-20 column (2 x 34 cm), eluted with hexane/chloroform/methanol (9/1/1) to resolve 25,26-$(OH)_2D_3$ from 1,25-$(OH)_2D_3$ as described by Ribovich and DeLuca (7) (Figure 1).

Purification of extract from the incubation mixtures for high pressure liquid chromatography (Figures 2 and 3) The dichloromethane extracts of the incubation mixtures were evaporated to dryness with a rotary evaporator and dissolved in 10 ml of methanol/water (90/10). This solution was vortexed with 10 ml of hexane and the phases allowed to separate. The hexane phase was discarded and the methanol/water phase was adjusted with chloroform and water to provide a mixture of methanol/chloroform/water (1/1/1). The phases were allowed to separate and the chloroform phase was removed and applied to a Sephadex LH-20 column (.7 x 14 cm) and eluted with 36 ml of chloroform, hexane (65:35) by the method of Holick and DeLuca (6). The last 25 ml of

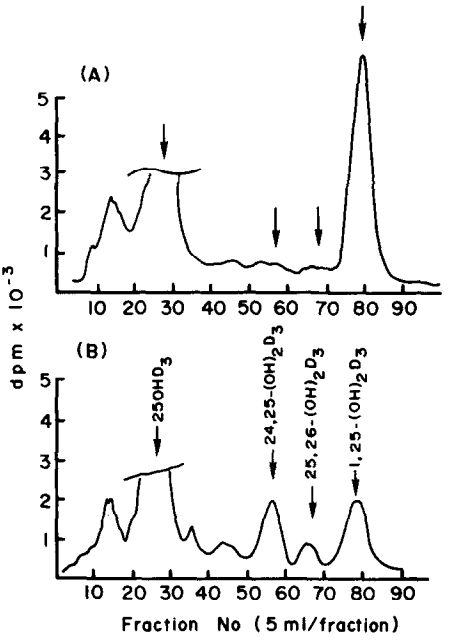

Figure 1. Stimulation of 26-hydroxylation of 25-OH-D_3 by vitamin D_3 administration. Chicks were fed a high calcium-vitamin D-deficient diet for 6 weeks. They were then injected subcutaneously with [A] 0.05 ml 95% ethanol or [B] 6.5 nmole vitamin D_3 in 0.05 ml ethanol 48 hours prior to sacrifice. The kidneys from each group of chicks were pooled, homogenized and incubated with 25-OH-[23,24-^{3}H]D_3 as described in text. The extracts of the incubation mixture were applied to Sephadex LH-20 columns (2 x 34 cm) and eluted with a solvent system of hexane/chloroform/methanol (9/1/1). Five ml fractions were collected for determination of radioactivity. The columns were previously calibrated with the indicated synthetic compounds (arrows).

effluent which contained the metabolite in question was applied to a Sephadex LH-20 column (.7 x 14 cm) which was developed and eluted with 25 ml of hexane/chloroform/methanol (9/1/1) as described by Eisman et al. (5). The last 18 ml of effluent was mixed with authentic 25,26-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ and applied to a high pressure liquid chromatographic system (Figures 2 and 3).

High pressure liquid chromatography. The purified extract from the above described procedure was injected into a Waters Model ALC/GPC 204 high pressure liquid chromatographic system equipped with a DuPont Zorbax Sil column (4.6 mm x 25 cm) developed with a solvent system of 9% isopropanol in hexane at a pressure of 700 psi. The 254 nm ultraviolet monitor was used to detect the added synthetic 25,26-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$. The flow rate was 1.5 ml/min and 0.5 ml fractions were collected for tritium counting as shown in Figure 2. In the case of the experiments shown in Figure 3, 1 ml fractions were collected.

Mass spectrometry. Biologically generated 25,26-$(OH)_2D_3$ which was purified by high pressure liquid chromatography described above was subjected

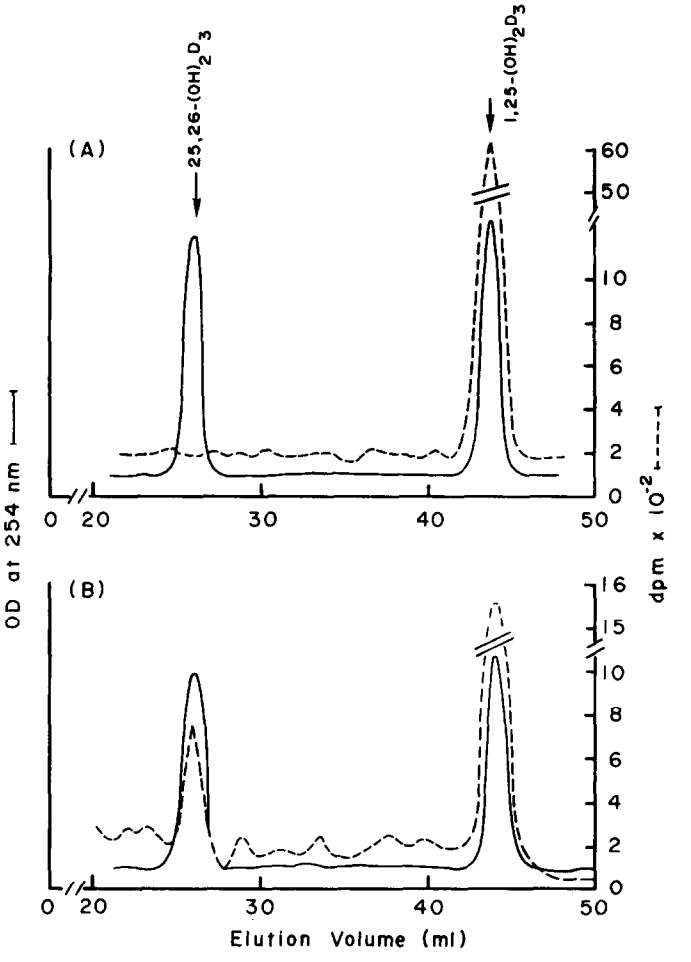

Figure 2. Cochromatography of synthetic 25,26-$(OH)_2D_3$ and 25,26-$(OH)_2$-[23,24-^{3}H]D_3 produced by kidney homogenate in vitro. Chicks were fed high calcium vitamin D-deficient diet for 8 weeks. One group of chicks was given subcutaneously 0.05 ml ethanol [A] or 6.5 nmole of vitamin D_3 in 0.05 ml of ethanol [B] 45 hours prior to sacrifice. The extracts of the reaction mixture were purified as described in the text and were injected together with authentic 25,26-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ into the high pressure liquid chromatography system. One half ml fractions were collected and radioactivity of each fraction was determined while 254 nm absorbance was monitored with the ultraviolet detector.

to mass spectrometry using an A.E.I. MS-9 mass spectrometer at 70 e.v. and a direct probe inlet at a temperature of ca. 120° above ambient.

Radioactive determinations. These were performed with a Packard Tricarb Scintillation Counter Model 3375 equipped with automatic standarization. The Scintillation mixture used has been described previously (1).

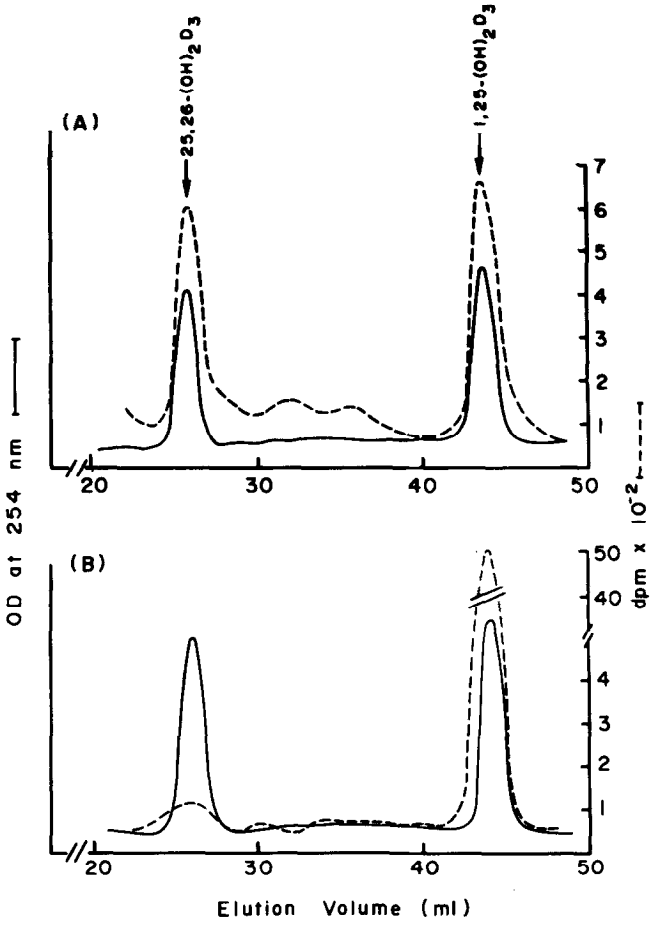

Figure 3. Suppression of 26-hydroxylation of 25-OH-D_3 by parathyroid extract. Chicks were fed the high calcium-vitamin D deficient diet for 4 weeks and were given 6.5 nmole vitamin D_3 in 0.05 ml ethanol subcutaneously 36 hours prior to sacrifice. Chickens in group [B] were given 20 units of parathyroid extract 30, 23, and 6 hours prior to sacrifice while chicks in group [A] received no parathyroid extract. The dichloromethane extracts of the incubation mixtures were purified as described in text and injected into the high pressure liquid chromatographic apparatus with synthetic 25,26-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$. One ml fractions were collected and radioactivity of each fraction was determined while absorbance at 254 nm was recorded.

Results

Figure 1 illustrates the radioactive profiles observed from the incubation mixtures upon chromatography on a 2 x 34 cm Sephadex LH-20 column developed with a solvent system of hexane/chloroform/methanol (9/1/1). As expected,

vitamin D deficient chick homogenates (Figure 1A) produce only 1,25-$(OH)_2D_3$ from 25-OH-D_3. On the other hand, chicks repleted with vitamin D produce not only 1,25-$(OH)_2D_3$ but also 24,25-$(OH)_2D_3$ and a compound which is eluted in the same position as standard synthetic 25,26-$(OH)_2D_3$. Similar extracts were then subjected to co-chromatography with synthetic 1,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ on high pressure liquid chromatography (Figure 2). The results show that vitamin D deficient preparations yielded only 1,25-$(OH)_2$[23,24-^{3}H]D_3 (Figure 2A) whereas the vitamin D replete chick preparations yielded in addition a ^{3}H labeled compound which exactly co-chromatographs with synthetic 25,26-$(OH)_2D_3$. A sufficient quantity of this metabolite peak was then isolated from several incubations and purified through chromatography on the Sephadex LH-20 (2 x 34 cm) with a solvent system of hexane/methanol/chloroform (9/1/1) followed by chromatography on a high pressure liquid system using the Zorbax Sil column as shown in Figure 2. The pure material was then used for mass spectrometry. The mass spectrum of the isolated metabolite confirmed its strucutral assignment as 25,26-$(OH)_2D_3$. The metabolite showed a molecular ion of m/e 416 [relative intensity, 42] with fragment ions at m/e 271 [16], 253 [15], 136 [100] and 118 [87]; a pattern essentially identical with that of synthetic 25,26-$(OH)_2D_3$ which gives m/e 416 [relative intensity, 48] 271 [13], 253 [12], 136 [100] and 118 [85] (2). These results, therefore, conclusively demonstrate that kidney tissue obtained from vitamin D repleted chickens possesses a 25-OH-D_3 26-hydroxylase. The results also demonstrate that vitamin D deficient chicken homogenates on the other hand do not possess this activity.

That the 26-hydroxylase is a regulated system, is demonstrated not only by its dependence on vitamin D administration but also by its suppression by parathyroid hormone administration. Parathyroid hormone injected into the vitamin D repleted chickens suppresses the activity of the 25-OH-D_3 26-hydroxylase (Figure 3).

The importance of 25,26-$(OH)_2D_3$ in terms of biological function of vitamin D_3 is at the present time unknown. From the present results, it is clear that renal tissue is at least one of the sites of its synthesis and furthermore, its biogenesis is regulated by substances that regulate calcium metabolism. It seems likely that, because of the regulation, the 25,26-$(OH)_2D_3$ might possess important biological activity or on the other hand it may represent an important inactivation mechanism for vitamin D. In either case, the 26-hydroxylase system could assume a position of significant importance in the regulation of calcium and phosphorus metabolism.

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