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METABOLISM OF 25-HYDROXYVITAMIN D_3 IN KIDNEY HOMOGENATES OF CHICKS SUPPLEMENTED WITH VITAMIN D_3

YOSHIKO TAKASAKI, NOBORU HORIUCHI, and TATSUO SUDA*

Department of Biochemistry, Showa University Dental School, 1-5-8, Hatanodai, Shinagawa-ku, Tokyo 142, Japan

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Summary: Kidney homogenates from chicks fed a vitamin D-deficient diet for 10 days and supplemented with 6.5 nmol of vitamin D_3 48 hr prior to sacrifice metabolized in vitro [^{3}H]-25-hydroxyvitamin D_3 (25-OH-D_3) to 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2$-D_3] and 3 other metabolites (peaks A, C and E). When the homogenates were incubated with purified [^{3}H]-24,25-$(OH)_2$-D_3, 3 similar metabolites (peaks A', C' and E') were produced. On high pressure liquid chromatography, peaks A, C and E migrated to exactly the same respective positions as peaks A', C' and E'. Kidney homogenates from D-deficient chicks failed to produce these metabolites from [^{3}H]-25-OH-D_3 or [^{3}H]-24,25-$(OH)_2$-D_3. These results strongly suggest that the new metabolites reported here are synthesized via 24,25-$(OH)_2$-D_3 in the kidney of chicks supplemented with vitamin D_3.

INTRODUCTION

It is now well established that vitamin D_3 is first metabolized to 25-hydroxyvitamin D_3 (25-OH-D_3) in the liver, and subsequently in the kidney to 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2$-D_3] or 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2$-D_3](1). 1α,25-$(OH)_2$-D_3 has been thought to be an active form, since this metabolite induces a remarkable change in the levels of plasma calcium and phosphate (1). The biological significance of 24,25-$(OH)_2$-D_3, however, has not been established. The physiological circumstances associated with the selective formation of these metabolites has been investigated extensively. More 1α,25-$(OH)_2$-D_3 was produced when animals were in need of calcium or phosphate, or during vitamin D depletion, and more 24,25-$(OH)_2$-D_3 was produced in the opposite situation (2-6).

In the course of investigating renal 24-hydroxylase activity, we found that kidney homogenates from chicks supplemented with vitamin D_3 metabolize in vitro [^{3}H]-25-OH-D_3 to 3 other metabolites besides 24,25-$(OH)_2$-D_3. The

* To whom all correspondence should be addressed.
Abbreviations used: 25-OH-D_3, 25-hydroxyvitamin D_3; 1α,25-$(OH)_2$-D_3, 1α,25-dihydroxyvitamin D_3; 24,25-$(OH)_2$-D_3, 24,25-dihydroxyvitamin D_3.

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present report describes that these new metabolites are synthesized from 25-OH-D_3 via 24,25-$(OH)_2$-D_3 in the kidney of chicks supplemented with vitamin D_3.

MATERIALS AND METHODS

Animals: One-day-old White Leghorn cockerel chicks were maintained for 10 days on a vitamin D-deficient purified soy protein diet (7) containing 3% calcium and 0.45% phosphorus. Most of the animals were orally predosed with 6.5 nmol of vitamin D_3 48 hr prior to sacrifice. The kidneys were quickly removed, rinsed, minced with a garlic press, and homogenized in 4 volumes of 0.2 M sucrose containing 15 mM Tris-Cl (pH 7.4), 2 mM $MgCl_2$, 0.4 mM NADP, and 5 mM sodium succinate.

Incubation: The homogenates (1.5 ml) were incubated in 20 ml-Erlenmeyer flasks with 10 pmol (0.1 μCi) of either [26,27-^{3}H]-25-OH-D_3 (Radiochemical Centre, Amersham) or [26,27-^{3}H]-24,25-$(OH)_2$-D_3. The iucubation was carried out at 37°C for 5-60 min under 100% oxygen. The reactions were terminated by the addition of 9 ml of methanol-chloroform (2:1 v/v). Extraction was performed as reported by Gray et al. (8).

Chromatography: Chromatography of the extracts was carried out on a 1 x 30 cm column of Sephadex LH-20 (10 g) using a solvent of 65% chloroform - 35% hexane according to Holick and DeLuca (9). High pressure liquid chromatography(HPLC) was performed on a Waters HPLC system (Model 440) equipped with a Zorbax-Sil column (2.1 mm x 25 cm, Du Pont). The solvent system was either 0.67% or 2% methanol in dichloromethane according to Ikekawa and Koizumi (13). Thirty-four 0.25 ml fractions were collected at a flow rate of 0.5 ml/min. Determination of radioactivity was carried out with a Packard liquid scintillation counter (Model 3255) as previously reported (10).

Preparation of [26,27-^{3}H]-24,25-$(OH)_2$-D_3: Kidney homogenates of chicks predosed intravenously with 312.5 pmol of 1α-hydroxyvitamin D_3 24 hr prior to sacrifice were incubated with [^{3}H]-25-OH-D_3 at 37°C for 20 min. [^{3}H]-24,25-$(OH)_2$-D_3 was purified on the Sephadex LH-20 and HPLC column described above. Crystalline vitamin D_3 was purchased from Tokyo Chemical Industry, and 25-OH-D_3 from Phillips-Dupher Co., Amsterdam. Crystalline 1α,25-$(OH)_2$-D_3 and 24R,25-$(OH)_2$-D_3 were gifts from Dr. M. R. Uskokoviĉ, Hoffman-La Roche Inc., New Jersey. 1α-hydroxyvitamin D_3 was a gift from Dr. I. Matsunaga, Chugai Pharmaceutical Co., Tokyo.

RESULTS AND DISCUSSION

Administration of 6.5 nmol of vitamin D_3 to D-deficient chicks completely suppressed renal 25-OH-D_3-1α-hydroxylase and enhanced 24-hydroxylase activity (Table I). In the course of investigating renal 24-hydroxylase activity, we found that the 25-OH-D_3 and the 24,25-$(OH)_2$-D_3 fractions isolated from Sephadex LH-20 columns were both heterogeneous when purified by HPLC. The heterogeneity of the 24,25-$(OH)_2$-D_3 fraction on Sephadex LH-20 column has been reported by Garabedian et al. (11) and Gray et al. (12). When the 25-OH-D_3 and the 24,25-$(OH)_2$-D_3 fractions from Sephadex LH-20 columns (Fig 1. (a)) were individually applied to HPLC column, the former was separated into

Table I. Failure of synthesis of the new metabolites by kidney homogenates from D-deficient chicks.

Predose of D_3 nmol	Incubation* Substrate	Peak A %	25-OH-D_3 %	Peak C %	24,25-$(OH)_2$-D_3 %	Peak E %	1α,25-$(OH)_2$-D_3 %	Others %
0	Control							
	25-OH-D_3	0	17.6	0	0	0	73.6	8.8
		0	24.8	0	0	0	63.2	12.0
	24,25-$(OH)_2$-D_3	Trace	0	0	87.2	Trace	0	5.4
		Trace	0	0	89.6	Trace	0	3.2
6.5	Control							
	25-OH-D_3	15.8	39.6	23.3	9.5	5.1	0	6.7
		15.9	22.2	25.8	23.1	8.0	0	5.0
	24,25-$(OH)_2$-D_3	15.4	0	45.2	25.9	8.4	0	5.1
		13.9	0	47.6	27.3	8.8	0	2.4
	Boiled**							
	24,25-$(OH)_2$-D_3	0	0	0	97.7	0	0	2.3
		0	0	0	98.5	0	0	1.5
	100% N_2***							
	24,25-$(OH)_2$-D_3	0	0	0	92.0	0	0	8.0
		0	0	0	91.7	0	0	8.3

The 25-OH-D_3 and the 24,25-$(OH)_2$-D_3 fractions from Sephadex LH-20 columns were separately applied to HPLC columns. Figures in Table I are expressed as percentage of the sum of the radioactivity recovered in each 1-34 fractions on HPLC.

* All incubations were performed for 60 min.

** boiled homogenates for 5 min at 100°C prior to incubation.

*** incubated for 60 min under 100% N_2.

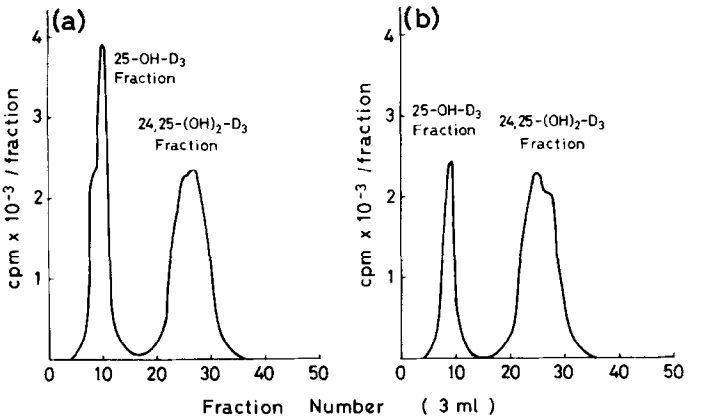

Fig 1. Sephadex LH-20 chromatographic profiles of extracts of kidney homogenates from chicks orally dosed with 6.5 nmol of vitamin D_3. The reaction conditions are described under MATERIALS AND METHODS. The columns containing 10 g of Sephadex LH-20 were eluted with chloroform : n-hexane (65:35, v/v) and 3 ml fractions were collected. Panel (a) represents a chromatographic profile of the extracts when the homogenates were incubated for 60 min with 10 pmol (0.1 μCi) of [^{3}H]-25-OH-D_3 and panel (b) with 10 pmol (0.1 μCi) of [^{3}H]-24,25-$(OH)_2$-D_3.

2 peaks: an unknown peak referred to as peak A, and peak B which coincided with authentic 25-OH-D_3 (Fig 2. (a)). On HPLC the latter was separated into 3 peaks: 2 unidentified peaks C and E, and peak D which coincided with authentic 24R,25-$(OH)_2$-D_3 (Fig 2. (b)). Some of these new metabolites resemble chromatographically those recently reported by Gray et al. (12). However, they demonstrated that prior administration of 1α,25-$(OH)_2$-D_3 produced similar metabolites from [^{3}H]-25-OH-D_3 to those reported here, whereas administration of vitamin D_3 or 25-OH-D_3 failed to produce them (12). Thus, new metabolites in this study might be different from those by Gray et al. (12).

When the homogenates were incubated with purified [^{3}H]-24,25-$(OH)_2$-D_3, a radioactive peak appeared in the 25-OH-D_3 fraction on Sephadex LH-20 columns (Fig 1. (b)). HPLC showed that the radioactive peak was homogeneous, which was referred to as peak A'. The homogenates did not produce 25-OH-D_3 from [^{3}H]-24,25-$(OH)_2$-D_3 (Fig 2. (c)). Peak A' migrated to exactly the same position as peak A on HPLC. The 24,25-$(OH)_2$-D_3 fraction was also separated into 3 peaks: 2 unidentified peaks C' and E', and peak D' which coincided with

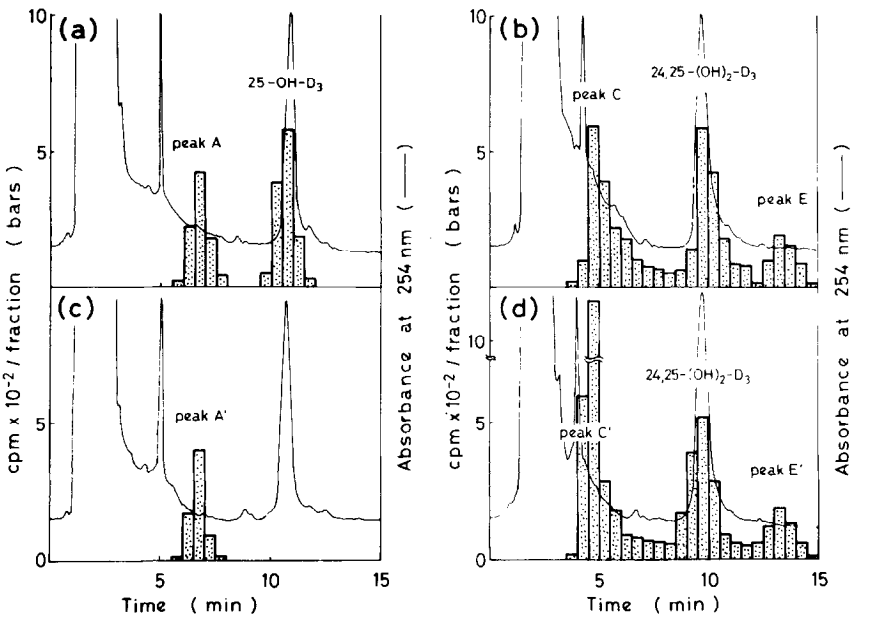

Fig 2. HPLC profiles of the 25-OH-D_3 and the 24,25-$(OH)_2$-D_3 fractions collected from the Sephadex LH-20 columns. Panels (a) and (c) represent HPLC profiles of the 25-OH-D_3 fraction (tubes 6-13) shown on Fig 1. (a) and (b), respectively. Panels (b) and (d) indicate those of the 24,25-$(OH)_2$-D_3 fraction (tubes 16-35) on Fig 1. (a) and (b), respectively. Before applying to HPLC columns, 500 pmol of authentic 25-OH-D_3 (in panels (a) and (c)) or 24R, 25-$(OH)_2$-D_3 (in panels (b) and (d)) were added to each extract. The columns were eluted with a solvent of either 0.67% methanol (in panels (a) and (c)) or 2% methanol (in panels (b) and (d)) in dichloromethane.

authentic 24R,25-$(OH)_2$-D_3 (Fig 2. (d)). On HPLC peaks C' and E' migrated to exactly the same respective positions as peaks C and E. When the homogenates were preheated at 100°C for 5 min, neither peaks A, C and E nor peaks A', C' and E' were produced, indicating that these metabolites are synthesized enzymatically. Production of these metabolites was also suppressed completely under 100% nitrogen (Table I).

Figure 3 indicates time course of change in the amounts of 24,25-$(OH)_2$-D_3 and peaks A, C and E when the homogenates were incubated with [^{3}H]-25-OH-D_3 for 5-60 min. Amounts of 24,25-$(OH)_2$-D_3 produced attained a maximal level in 5-10 min and decreased thereafter. On the other hand, amounts of peaks A, C and E gradually increased during the incubation period of 60 min. When the

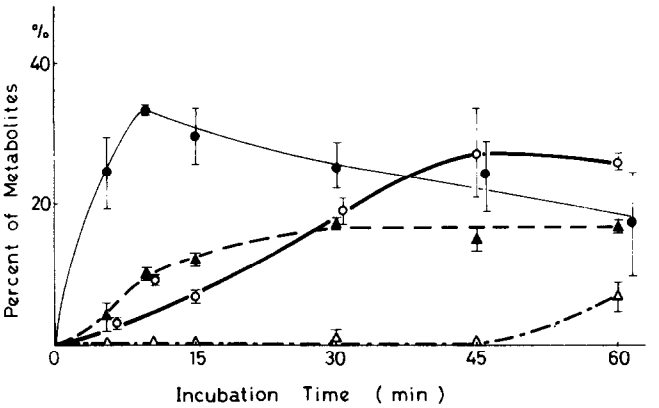

Fig 3. Time course of change in the amounts of the 25-OH-D_3 metabolites produced by kidney homogenates from chicks dosed with 6.5 nmol of vitamin D_3. Incubations were carried out with 10 pmol (0.1 μCi) of [^{3}H]-25-OH-D_3 as described under MATERIALS AND METHODS. Results are expressed as percentage of the radioactivity recovered as peak A (— —▲— —), peak C (——○——), 24,25-$(OH)_2$-D_3 (——●——), and peak E (— -△- —). The vertical bar indicates standard error of the mean of 3-4 experiments.

homogenates were incubated with [^{3}H]-25-OH-D_3 or [^{3}H]-24,25-$(OH)_2$-D_3, the major metabolite was peak C (or C'), and peaks A (or A') and E (or E') were relatively minor ones (Table I). Kidney homogenates from D-deficient chicks failed to produce these metabolites from 25-OH-D_3 or 24,25-$(OH)_2$-D_3 (Table I).

The results reported here indicate that the metabolites of 25-OH-D_3 (peaks A, C and E) are synthesized via 24,25-$(OH)_2$-D_3 in the kidney of chicks supplemented with vitamin D_3. First, peaks A', C' and E' with chromatographic behaviors similar to peaks A, C and E, respectively, are produced from purified [^{3}H]-24,25-$(OH)_2$-D_3. Second, on HPLC peaks A, C and E migrate to exactly the same respective positions as peaks A', C' and E'. Third, the homogenates do not produce 25-OH-D_3 from 24,25-$(OH)_2$-D_3. Fourth, kidney homogenates from D-deficient chicks fail to produce these metabolites from 25-OH-D_3. Finally, the time course of change in the amounts produced of 24,25-$(OH)_2$-D_3 and peaks A, C and E fits with the above hypothesis.

Biological significance of these metabolites is of considerable interest in view of the control mechanism of 25-OH-D_3 metabolism, which is currently under investigation in our laboratory.

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