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Biological Activity of 1,25-Dihydroxyvitamin D_2 in the Chick†

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ABSTRACT: 1,25-Dihydroxyvitamin D_2 has been prepared from 25-hydroxyvitamin D_2 using rachitic chick kidney mitochondria. This metabolite was highly purified by Sephadex LH-20 chromatography and by preparative high-pressure liquid chromatography. Its purity was assessed by analytical high-pressure liquid chromatography which revealed no other 254-nm absorbing material and by mass spectrometry. The concentration of dilute solutions of 1,25-dihydroxyvitamin D_2 was determined by high-pressure liquid chromatography and deflection of the 254-nm column monitor. The 1,25-dihydroxyvitamin D_2 was then shown to be $\frac{1}{5}$ to $\frac{1}{10}$ as active as 1,25-dihydroxyvitamin D_3 in the chick while it had previously been shown to be equal in activity in the rat. Thus, discrimination against the vitamin D_2 side chain by the chick persists in the metabolically active 1,25-dihydroxyvitamin D compounds.

The discrimination between vitamin D_3 and vitamin D_2 that has been observed in chicks (Steenbock et al., 1932; Chen and Bosmann, 1964) is thought to be due to the rapid excretion of vitamin D_2 and its metabolites by the biliary route (Imrie et al., 1967). In support of this theory are the extremely low levels of 25-hydroxyvitamin D_2 (25-OH-D_2)[1] (Drescher et al., 1969) and 1,25-dihydroxyvitamin D_2 (1,25-$(OH)_2D_2$) (Jones et al., 1975) that are observed in the rachitic chick after the in vivo administration of physiological doses of radioactive vitamin D_2. Using in vitro techniques we were able to show that these reduced levels of hydroxylated vitamin D_2 metabolites are not due to a failure of the enzymic machinery to carry out the reactions (Jones et al., 1976). In this present paper we now show that 1,25-$(OH)_2D_2$ is only $\frac{1}{5}$ to $\frac{1}{10}$ as active as 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) in the chick, demonstrating that the mechanism of discrimination applies also to the metabolically active form.

Materials and Methods

Chemicals. 25-OH-D_2 was prepared by the method of Suda et al. (1969) as modified by Jones et al. (1975). 1,25-$(OH)_2D_3$ was the synthetic material prepared by Semmler et al. (1972).

General Procedures. High-pressure liquid chromatography (HPLC) was performed on a Dupont 830 LC fitted with a 254-nm ultraviolet (uv) detector (Dupont Instruments, Wilmington, Del.) and a U6K injector (Waters Associates, Milford, Mass.). When two analytical columns (25 cm × 2.1 mm) containing Zorbax-SIL (Dupont) were arranged in series, a pressure of 4400 psi gave a solvent (15% 2-propanol–Skellysolve B) flow rate of 0.8 ml/min. Using a semipreparative column of Zorbax-SIL (25 cm × 7.9 mm) a flow rate of 1.8 ml/min (15% 2-propanol in Skellysolve B) was achieved with a pressure of 500 psi.

Mass spectrometric determinations were made with an AEI MS-9 mass spectrometer using a direct probe inlet at temperatures of 118–130 °C above ambient. All solvents were reagent grade and those used in the HPLC and mass spectrometry were doubly distilled before use.

Radioactive determinations were carried out with a Nu-

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[1] Abbreviations used are: 25-OH-D_2, 25-hydroxyvitamin D_2; 1,25-$(OH)_2D_2$, 1,25-dihydroxyvitamin D_2; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; HPLC, high-pressure liquid chromatography.

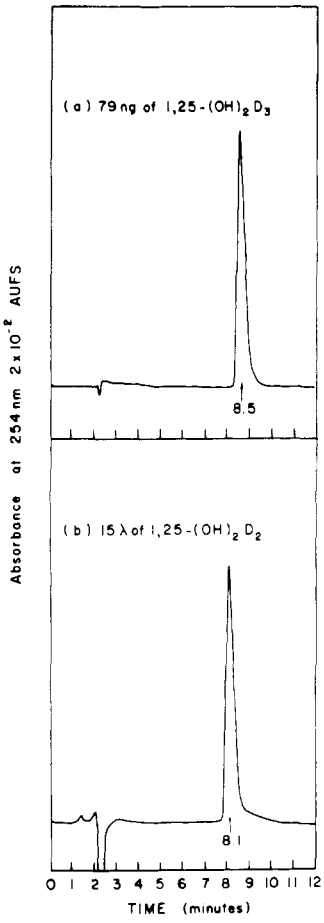

FIGURE 1: HPLC of: (a) 79 ng of crystalline 1,25-$(OH)_2D_3$; and (b) 15 μl of purified 1,25-$(OH)_2D_2$ run under identical conditions: pressure, 4400 psi; flow rate, 0.8 ml/min; solvent, 15% 2-propanol in Skellysolve B; two 25 × 2.1 mm Zorbax-SIL columns in series.

clear-Chicago Isocap 300 liquid scintillation counter. One-hundred microliter samples of aqueous ^{45}Ca solutions were mixed with 1 ml of water and 15 ml of Aquasol (New England Nuclear, Boston, Mass.) so that counting efficiencies (around 40%) could be checked on a standard quench curve.

In Vitro Preparation of 1,25-$(OH)_2D_2$. The method used was a simplified version of the procedure of Jones et al. (1975). The metabolite 1,25-$(OH)_2D_2$ was generated from 25-OH-D_2 using kidney mitochondria from rachitic chicks incubated in the media of Ghazarian and DeLuca (1974) under scaled up conditions. Erlenmeyer flasks (125 ml) each containing 3 ml of mitochondrial suspension and 1.5 ml of succinate–magnesium acetate in buffer were used. From 20 such flasks (each containing 1.5 μg of 25-OH-D_2) incubated for 30 min, a combined lipid extract was prepared (Jones et al., 1975).

Chromatography of the lipid extract on Sephadex LH-20 (Holick and DeLuca, 1971) was carried out using a 1 cm × 60 cm column packed in and eluted with 65:35 chloroform–Skellysolve B. Fractions 21–40 (5 ml), which contained the 1,25-$(OH)_2D_2$, were pooled and evaporated to dryness before redissolving in a volume of 60 μl of 15% 2-propanol in Skellysolve B. At this point the lipid had an intense yellow color.

The 1,25-$(OH)_2D_2$ preparation was divided into three aliquots (20 μl each) which were each subjected to HPLC on a semipreparative Zorbax-SIL column using 15% 2-propanol–Skellysolve B and collecting 0.5-min fractions using a Gilson microfractionator set on the time mode (Gilson Medical Electronics, Inc., Middleton, Wis.). The uv_{254} peak of 1,25-$(OH)_2D_2$ appeared in fractions 33–36 relatively free of the other uv_{254} absorbing lipids. Fractions 33–36 from HPLC of each of the three aliquots of the Sephadex LH-20 peak were pooled, evaporated to dryness, and redissolved in 1.2 ml of ethanol.

Table 1: Biological Activity of 312 pmol of 1,25-$(OH)_2D_2$ in the Chick Duodenal Assay System.

Time Elapsed after Administration of Dose (h)	% ^{45}Ca Transported in 30 min[a]		
	Ethanol Dosed	1,25-$(OH)_2D_2$ (312 pmol)	1,25-$(OH)_2D_3$ (312 pmol)
6	22.3 ± 3.9 (5)[b]	30.2 ± 11.2 (5)	49.9 ± 5.2[c] (5)
9	21.0 ± 5.4 (5)	31.2 ± 6.2[d] (5)	50.8 ± 6.1[c] (5)
24	20.8 ± 1.6 (4)	23.4 ± 1.4 (3)	45.9 ± 9.6[d] (3)

[a] ± standard deviation. [b] Numbers in parentheses represent number of animals in each group. [c] Significantly different from ethanol control; P less than 0.001. [d] Significantly different from ethanol control; P less than 0.05.

This material was pure by HPLC standards. It gave a single 254-nm absorbing peak when run on the two-column analytical system described under general procedures. Furthermore, the purity was further assessed by mass spectrometry (Figure 2).

Estimation of the Concentrations of 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ Dosing Solutions by HPLC. HPLC resolves 1,25-$(OH)_2D_2$ from 1,25-$(OH)_2D_3$ (Jones and DeLuca, 1975). Using the 15% 2-propanol–Skellysolve B system employed here it is possible to retain resolution of 1,25-$(OH)_2D_2$ (8.1 min) from 1,25-$(OH)_2D_3$ (8.5 min) and yet obtain sharper, more quantitative peaks.

Estimation involves running the 1,25-$(OH)_2D_2$ of unknown concentration under identical conditions that were used for a standard solution of 1,25-$(OH)_2D_3$ (Figure 1). Figure 1a represents the injection of 79 ng of 1,25-$(OH)_2D_3$ and Figure 1b the back-to-back run of 15 μl of the unknown solution of 1,25-$(OH)_2D_2$. Using peak areas (peak height × half peak base) the concentration of 1,25-$(OH)_2D_2$ can be calculated as 85 ng/15 μl.

The total preparation of 1,25-$(OH)_2D_2$ thus contained 17 μg of metabolite which amounted to a 20–25% conversion. Though the material was free of contaminants detectable on HPLC, it nevertheless was not totally devoid of impurities in the 220–230-nm range on spectrophotometric analysis. These made quantitation by conventional means difficult. Nevertheless, the peak from the analytical HPLC run was collected and subjected to mass spectrometric analysis. The spectrum depicted in Figure 2 shows all the peaks present in the original mass spectrum of 1,25-$(OH)_2D_2$ (Jones et al., 1975), i.e. 428 (M); 410 (M − H_2O); 352 (M − 18 − 58); 287 (M-side chain); 269 (M − side chain − H_2O); 251 (M − side chain − $2H_2O$); 152 (cis triene cleavage); and 134 (cis triene cleavage, then − H_2O. The mass spectrum suggests that the 220–230-nm absorbing contaminant is minor. Therefore, it was with confidence that this material was used in the bioassay using the chick duodenal loop method.

Bioassay of 1,25-$(OH)_2D_2$ in Chicks. Purified 1,25-

Table II: Biological Activity of 625 and 1250 pmol of 1,25-$(OH)_2D_2$ in the Chick Duodenal Assay System.

Time Elapsed after Administration of Dose (h)	% ^{45}Ca Transported in 30 min[a]				
	Ethanol Dosed	1,25-$(OH)_2D_2$ (625 pmol)	1,25-$(OH)_2D_2$ (1250 pmol)	1,25-$(OH)_2D_3$ (62.5 pmol)	1,25-$(OH)_2D_3$ (312 pmol)
9 h	22.7 ± 6.2 (8)[b]	54.1 ± 9.0[c] (8)	58.1 ± 4.5[c] (4)	40.1 ± 7.9[c] (7)	58.9 ± 6.8[c] (6)

[a] ± standard deviation. [b] Numbers in parentheses represent number of animals in each group. [c] Significantly different from ethanol control; P less than 0.001.

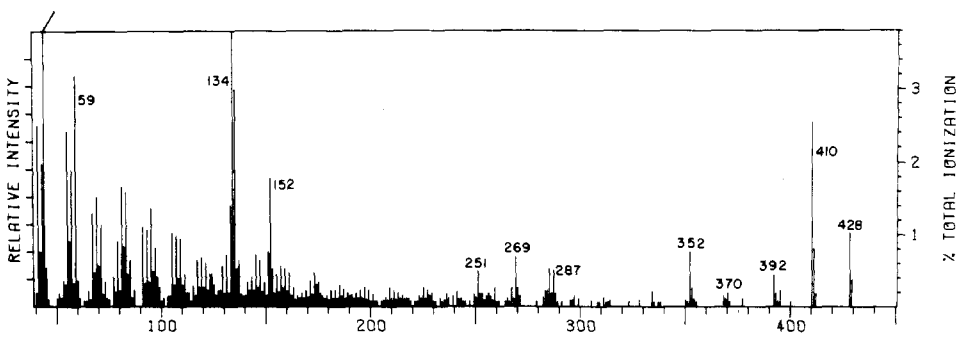

FIGURE 2: Mass spectrum of 1,25-$(OH)_2D_2$ eluted from HPLC as in Figure 1b.

$(OH)_2D_2$ and crystalline 1,25-$(OH)_2D_3$ were dissolved in ethanol at a concentration to give the desired dosage in 50 μl. One-day old white Leghorn cockerel chicks were obtained from Northern Hatcheries (Beaver Dam, Wis.). They were maintained on a vitamin D deficient purified soy protein diet for 4 weeks before use (Omdahl et al., 1971).

Twenty-four hours before ^{45}Ca transport was measured, the animals were weighed and arranged into representative weight groups of 6–10 animals. At 24, 12, 9, or 6 h prior to ^{45}Ca transport measurement, the test compound was administered in 50 μl of ethanol by wing vein injection.

^{45}Ca transport from a 10-cm duodenal loop in situ was carried out as described by Omdahl et al. (1971). The intestinal loops containing residual ^{45}Ca were then removed from the animals and ashed, the ash dissolved in 2 N hydrochloric acid, and this solution was neutralized with 2 M Tris. Duplicate 100-μl aliquots were counted as described above. Results are expressed as percent ^{45}Ca transported, this representing initial ^{45}Ca minus remaining ^{45}Ca divided by initial ^{45}Ca times 100.

Results

The duodenal loop method is a reliable, sensitive method for the assay of vitamin D compounds in the chick. As shown in Tables I and II, the baseline calcium absorption is around 20% in 30 min for a rachitic chick whereas it rises to almost 60% in a vitamin D treated animal.

In early experiments the biological activity of small amounts of 1,25-$(OH)_2D_2$ (such as 62.5 pmol) gave virtually no calcium transport, whereas 62.5 pmol of 1,25-$(OH)_2D_3$ gave a significant but less than maximal response (Table II). Larger dose levels (i.e., 312 pmol) of 1,25-$(OH)_2D_2$ were thus administered and it was possible to see a small but significant response at 9 h after administration. At this dose level 1,25-$(OH)_2D_3$ gave maximal ^{45}Ca transport at 9 h with significant values at 6 and 24 h (Table I). This is in agreement with the work of Omdahl et al. (1971).

In order to demonstrate the complete biological potency of 1,25-$(OH)_2D_2$ it was necessary to administer 625 or 1250 pmol to chicks 9 h before measurement of ^{45}Ca absorption (Table II). From the combined data of Tables I and II it becomes apparent that 1,25-$(OH)_2D_2$ is about 5–10 times less active than 1,25-$(OH)_2D_3$ (62.5 pmol of 1,25-$(OH)_2D_3$ is more active than 312 pmol of 1,25-$(OH)_2D_2$ but less active than 625 pmol of 1,25-$(OH)_2D_2$).

Discussion

The present report demonstrates that chicks discriminate against 1,25-$(OH)_2D_2$ as well as other forms of vitamin D_2. The biopotency of 1,25-$(OH)_2D_2$ was found to be $\frac{1}{5}$ to $\frac{1}{10}$ that of 1,25-$(OH)_2D_3$ while it is equal in activity in the rat (Jones et al., 1975). This observation is in line with the D_3/D_2 activity ratio of five- to eightfold (Chen and Bosmann, 1964) and the 25-OH-D_2/25-OH-D_3 ratio of tenfold (Drescher et al., 1969) as seen previously. From these results it seems clear that the discrimination against D_2 or its metabolites is not due to a single specific block in metabolism of vitamin D_2 or 25-OH-D_2 to its active forms since the discrimination should have been eliminated by administration of the final active form of vitamin D_2 or 1,25-$(OH)_2D_2$. Instead, discrimination is expressed as a lowered biopotency of all of the metabolites of vitamin D_2. It is, therefore, likely that the metabolic discrimination reaction occurs in large measure on vitamin D_2 and 25-OHD_2 resulting in rapid excretion and formation of only small amounts of 25-OH-D_2 and 1,25-$(OH)_2D_2$. These metabolites are then also susceptible to the same discriminatory mechanism.

The recognition portion of the D_2 molecule for the discrimination is obviously the C_{22-23} double bond or the C_{24} methyl group of the vitamin D_2 side chain. Holick et al. (1976) have suggested that the chick rapidly excretes 24-substituted vitamin D compounds possibly as a protective mechanism against vitamin D intoxication. In support of this concept, it is known that vitamin D_4 (22,24-dihydroxyvitamin D_2) which differs from vitamin D_3 only by the pres-

ence of the C_{24} methyl group is also only $1/10$ as active as vitamin D_3 in chicks (DeLuca et al., 1968; Windaus and Trautman, 1937).

The mechanism of discrimination by which the chick recognizes 24-substituted D compounds may involve the blood carrier proteins shown to be selective at least against vitamin D_2 compounds by Belsey et al. (1974). In the experiments described here, intravenously administered 1,25-$(OH)_2D_2$ might be only poorly picked up and transported to its target tissues enabling some degradative mechanism to rapidly inactivate and excrete it.

The present results could also be due to target tissue discrimination against the vitamin D_2 side chain. Experiments with ^{3}H-labeled 1,25-$(OH)_2D_2$ are now planned to test this possibility. Inasmuch as the D_2 compounds are rapidly excreted (Imrie et al., 1967) and only small amounts of 25-OH-D_2 (Drescher et al., 1969) or 1,25-$(OH)_2D_2$ (Jones et al., 1975) are found even though the enzymatic machinery can metabolize vitamin D_2 normally (Jones et al., 1976), it is likely that discrimination is at the metabolic level in which all vitamin D compounds with a C_{24} substitution are rapidly eliminated. However, the full elucidation of such a mechanism must await identification of the site of such discriminatory metabolism.

The advent of HPLC will be of great assistance to those who are generating vitamin D metabolites for biological assay. As shown in this paper it serves not only as a purification method, but also as an accurate method of estimating the concentration of dilute solutions. The 254-nm uv detector is particularly suited for vitamin D since the vitamins' uv absorption maximum is at 265 nm. Any ambiguity as to the identity of the peak of 1,25-$(OH)_2D_2$ was dispelled by the application of mass spectrometry. A combination of HPLC and mass spectrometry is a very powerful tool in purification and identification which must certainly gain more importance when a continuous interface apparatus becomes commercially available.

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CORRECTIONS

ATP ↔ P_i Exchange and Membrane Phosphorylation in Sarcoplasmic Reticulum Vesicles: Activation by Silver in the Absence of a Ca^{2+} Concentration Gradient, by Leopoldo de Meis* and Martha M. Sorenson, Volume 14, Number 12, 1975, pages 2739–2744.

Page 2741: a line was omitted. The last sentence in the first column should read: "The concentration of Ag^+ required for maximal activation of ATP ↔ P_i exchange increases with the SRV protein concentration (Figure 3, left)".

^{1}H Nuclear Magnetic Resonance of Modified Bases of Valine Transfer Ribonucleic Acid (*Escherichia coli*). A Direct Monitor of Sequential Thermal Unfolding, by Rodney V. Kastrup and Paul G. Schmidt, Volume 14, Number 16, August 12, 1975, pages 3612–3618.

Page 3613, second column, line 6 from bottom: the authors made incorrect reference to data of Dr. P. Sattsangi. The correct statement should be: "In dimethyl sulfoxide the methyl peak of N^6-methyladenosine is found at 3.0 ppm from internal Me_4Si".

Degradation of Fibrinogen by Plasmin. Isolation of an Early Cleavage Product, by Elizabeth J. Harfenist* and Robert E. Canfield, Volume 14, Number 18, 1975, pages 4110–4117.

Page 4114, in Table II, the residues in the major sequence have not been underlined. The major sequence is given directly under the numbers.

Page 4115, first column, first paragraph: line 7 should be omitted and the paragraph should read ". . . which was entirely consistent with this effluent curve and which was composed of at least . . .".