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1,25-Dihydroxyvitamin D_3 Stimulated Increase of 7,8-Didehydrocholesterol Levels in Rat Skin†

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ABSTRACT: A convenient, accurate assay was developed for determining skin cholesta-5,7-dien-3β-ol (7,8-didehydrocholesterol) concentrations. Ultraviolet spectrophotometry provided quantitation of the sterol from rat skins following saponification and chromatography on Lipidex and high-performance liquid chromatography. Correction for recoveries was accomplished by using 7,8-didehydro[3α-^{3}H]cholesterol as an internal standard. Chronic dosing of vitamin D-deficient rats with 1,25-dihydroxyvitamin D_3 caused a 4-fold increase in skin 7-dehydrocholesterol content. This rise was not the result of changes in food consumption, body weight, or plasma calcium. Cholesterol concentrations were not significantly elevated although some of the other nonsaponifiable lipid components found in the high-performance liquid chromatogram appeared to be increased by the treatment. These results suggest that the vitamin D hormone 1,25-$(OH)_2D_3$ may exert a positive feedback regulation on the production of vitamin D_3 in skin.

Windaus and his co-workers (Windaus et al., 1935) synthesized cholesta-5,7-dien-3β-ol (7,8-didehydrocholesterol; Δ5,7) under the premise that it was the natural provitamin for vitamin D in animal tissues. Vitamin D_3 was identified in ultraviolet (UV) irradiation mixtures of synthetic Δ5,7 (Windaus et al., 1936). This group then spectrophotometrically detected Δ5,7 in several animal tissues, finding the highest concentrations in skin samples. They then isolated and identified Δ5,7 from pig skin (Windaus & Bock, 1937). This work led to the long held assumption that vitamin D_3 is the natural antirachitic substance produced in the skin of animals by irradiation with UV light. This idea was supported recently by the isolation and identification of vitamin D_3 (Holick et al., 1977; Esvelt et al., 1978) and its obligatory precursor, previtamin D_3 (Holick et al., 1979) from UV irradiated rat skins.

The skin is a highly active sterol biosynthesizing tissue which, unlike most tissues, accumulates significant quantities of sterol intermediates, including Δ5,7 (Kandutsch, 1964; DeLuca, 1971). In the skin, where the unsaturation at C-24 is reduced early in sterol biosynthesis (Clayton et al., 1963), Δ5,7 serves as a direct precursor of cholesterol. Δ5,7 is formed in an irreversible oxygen-dependent reaction from Δ7-cholestenol (Dempsey et al., 1964), and Δ5,7 can be converted to cholesterol in an irreversible nicotinamide adenine dinucleotide phosphate (NADP) dependent reaction (Frantz et al., 1964). The low level of Δ5,7 in other tissues (Dempsey et al., 1964;

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Kandutsch, 1964) and the irreversibility of the Δ7-reductase indicate that skin Δ5,7 content is entirely derived from biosynthesis in the skin. Nearly all of the Δ5,7 found in skin is unesterified. The highest skin concentrations of Δ5,7 are found in the epidermis (Reinerston & Wheatley, 1959), providing a convenient location for absorption of available UV light and isomerization to vitamin D_3. Skin Δ5,7 levels were recently reported to be 85 μg/g of dry rat skin (Okano et al., 1977).

The known functions of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] are all involved in calcium and phosphate homeostasis (DeLuca & Schnoes, 1976). Recently, however, Stumpf and co-workers (Stumpf et al., 1979) found specific localization of the hormone in the nuclei of cells in tissues, including epidermis, previously not considered to be target tissues in the vitamin D system. This discovery led to the present examination of the effect of 1,25-$(OH)_2D_3$ on the levels of Δ5,7 in skin. We have found that the skin may be a target tissue for 1,25-$(OH)_2D_3$, implying a positive feedback regulatory mechanism for vitamin D_3 production.

Materials and Methods

General. Radioactivity was determined with a toluene counting solution containing 0.2% diphenyloxazole and 0.01% 1,4-bis[2-(4-methyl-5-phenoxazolyl)]benzene in a Packard Model 3255 scintillation counter equipped with automatic external standardization for determination of counting efficiencies. Ultraviolet spectra were recorded in ethanol with a Beckman Model 24 recording spectrophotometer. High-performance liquid chromatography (high-performance LC) was performed on a Waters Model ALP/GPC 204 liquid chromatograph equipped with an ultraviolet absorbance detector monitoring at 254 nm, using columns and solvents as noted elsewhere.

Animals. Male weanling albino rats were purchased from the Holtzman Co. (Madison, WI) and were individually housed in hanging wire cages with free access to food and water. The animals were fed a vitamin D deficient diet containing 0.47% calcium and 0.3% phosphorus (Table I) or a rachitogenic diet containing 1.2% calcium and 0.1% phosphorus (Table III) (Suda et al., 1970).

Chemicals. [3α-^{3}H]Cholesta-5,7-dien-3β-ol ([3α-^{3}H]-Δ5,7) (14 Ci/mmol) was synthesized by sodium boro[^{3}H]hydride (48 Ci/mmol) (New England Nuclear) reduction of cholesta-3,5,7-trienyl 3-acetate (Heilbron et al., 1938) in ethanol for 1 h at 55 °C (Jones et al., 1975). The reaction mixture was extracted into ether and washed with water and brine. The [3α-^{3}H]-Δ5,7 product was purified on a 1 × 60 cm column of Lipidex 5000 (Packard Instruments) equilibrated in and eluted with 5% chloroform in hexane. Elution of Δ5,7 was at 165 mL. The product (14 Ci/mmol) showed the characteristic Δ5,7 UV absorbance spectrum (λ_{max} 294, 282, and 271 nm) and comigrated with crystalline Δ5,7 on a high-performance LC system using a 6.2 mm × 25 cm Zorbax SIL (Du Pont) column eluted with 1% 2-propanol in hexane.

Experimental. For experiment 1 (Table I) fourteen rats were maintained on the vitamin D deficient diet for 4 weeks. Then, one group of seven rats received two daily subcutaneous doses containing 120 ng of 1,25-$(OH)_2D_3$ in 50 μL of propylene glycol for 7 days. The control group of rats received two daily subcutaneous doses of 50 μL of propylene glycol for 7 days. Six hours following the last dose, the animals were weighed and killed by decapitation. For experiment 2 (Table III) 20 rats were fed the rachitogenic diet for 4 weeks. The rats were divided into two groups of 10 rats each and treated as in experiment 1 except food consumption and body weight gain were recorded. The skin was removed from the back and sides of each rat. The hair and dead cell keratin layer was scraped off the epidermal side, and the muscle and adipose tissue was scraped from the dermal side with a dull scalpel. The skins were individually frozen at −20 °C until further processing.

Table I: Concentrations of 7,8-Didehydrocholesterol and Cholesterol per Gram of Dry Skin in Vitamin D Deficient Rats and Rats Given 1,25-$(OH)_2D_3$[a]

group	7,8-didehydrocholesterol (μg/g dry weight)	cholesterol (mg/g dry weight)
control (n = 7)	42 ± 19	8.1 ± 0.8
1,25-$(OH)_2D_3$ (n = 7)	166 ± 92[b]	9.1 ± 0.6[c]

[a] Expressed as the mean ± SD. [b] Significantly different from controls at $P < 0.005$ by Student's t test. [c] Not significantly different from control level.

Each skin was cut into small pieces, homogenized in 2 volumes of water with a Brinkmann polytron homogenizer, and freeze-dried for weighing. [3α-^{3}H]-Δ5,7 (1.9×10^6 dpm) was added to each dried skin sample, and the samples were saponified in 50 mL of 3% KOH in 95% methanol by refluxing at 70 °C for 2 h with a continuous flow of N_2 gas through the mixture. The saponified mixtures were filtered through cheesecloth, adjusted to 10% water, and extracted 3 times with 60 mL of hexane. The hexane phases were evaporated, and the residues were dissolved in 5% $CHCl_3$ in hexane, filtered through glass wool, and sampled for recovery calculation. Each sample was chromatographed on a 1 × 20 cm Lipidex 5000 column eluted with 5% $CHCl_3$–hexane. The first 30 mL of column effluent was discarded, and the effluent volumes 30–90 mL (containing most sterols) were collected and sampled for recovery determination. Peak elution of [^{14}C]cholesterol was at 49 mL, and peak elution of [3α-^{3}H]-Δ5,7 was at 55 mL on this system.

One-tenth of each sample from the Lipidex column was chromatographed on a high-performance LC system consisting of a 6.2 mm × 25 cm Zorbax SIL column eluted with 1.2% 2-propanol in hexane at 3 mL/min and 900 psi. A narrow cut was taken out of the Δ5,7 peak eluting at 36 mL. The solvent was evaporated under N_2 and the residue dissolved in 1 mL of ethanol. Aliquots were taken for radioactivity and UV absorption spectral determination. Δ5,7 content was calculated from the absorbance at 282 nm (ϵ = 12000 M^{-1} cm^{-1}) with correction for recovery of [3α-^{3}H]-Δ5,7.

One-tenth of each sample from the Lipidex column was chromatographed on a high-performance LC system consisting of a 4.6 mm × 25 cm Zorbax SIL column eluted with 1.5% 2-propanol in hexane monitored at 220 nm. A broad fraction was collected, which included the cholesterol peak (elution volume 16 mL) and the Δ5,7 peak (elution volume 17.5 mL). The fractions were sampled for determination of [3α-^{3}H]-Δ5,7. Cholesterol was determined on $^1/_5$ of the fraction by the colorimetric method of Watson (1960). The Δ5,7 does not interfere in this analysis.

Results

The 1,25-$(OH)_2D_3$-dosed animals weighed 180 ± 17 g at the time of sacrifice while the controls weighed 167 ± 30 g (Table I). The average recovery of [3α-^{3}H]-Δ5,7 through saponification and extraction was 88%. Each sample was partially purified by batch elution on Lipidex columns. A broad elution was collected to include cholesterol, Δ5,7, and most 3-hydroxy sterols. An average of 86% of the [3α-^{3}H]-Δ5,7 was recovered. One-tenth of each of the samples from Lipidex was chromatographed on high-performance LC

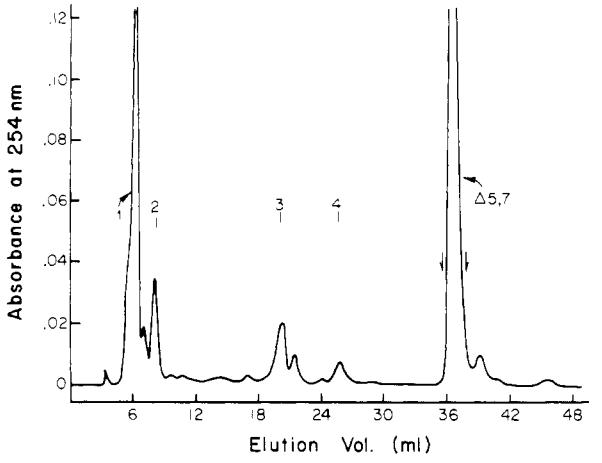

FIGURE 1: High-pressure liquid chromatographic trace of UV absorbance at 254 nm showing the peak used for quantitating skin 7,8-didehydrocholesterol and the other UV absorbing peaks detected in the chromatogram. The arrows show the fractions collected for 7,8-didehydrocholesterol analysis. This sample was from a 1,25-$(OH)_2D_3$-dosed rat, and the collected region contained 18 μg of 7,8-didehydrocholesterol; the original skin (Δ5,7) content was calculated at 210 μg/g dry weight.

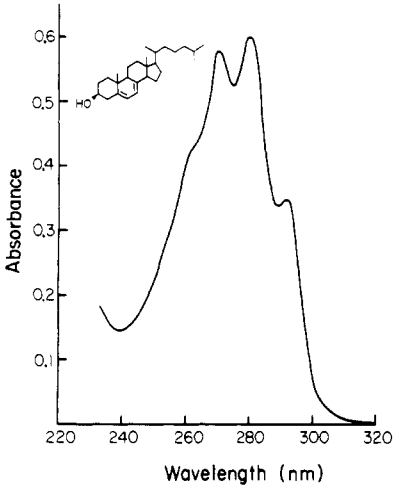

FIGURE 2: Ultraviolet absorption spectrum for the 7,8-didehydrocholesterol (shown in inset) isolated from rat skin. The high-performance LC trace of the sample which gave this spectrum is shown in Figure 1.

and a narrow cut of the Δ5,7 containing fractions (Figure 1) was taken in an attempt to minimize contamination with other UV absorbing compounds. Δ5,7 was quantitated by the absorbance at 282 nm. The purity of the Δ5,7 samples from high-performance LC was demonstrated by the typical Δ5,7 spectrum with sharp maxima at 293, 282, and 271 nm and the minimum between 235 and 245 nm (Figure 2). The samples were corrected for recoveries of added [3α-^{3}H]-Δ5,7 to reveal the quantity of Δ5,7 present in the original skin samples. The results are shown in Table I. Clearly, 1,25-$(OH)_2D_3$ dosing caused a 4-fold elevation in skin Δ5,7 content. By Student's t test this was highly significant ($P < 0.005$).

For analysis of the skin samples for cholesterol concentration, one-tenth of each sample from the Lipidex column was chromatographed on high-performance LC. A large fraction was collected to include Δ5,7 and cholesterol. It is not known if cholest-7-en-3β-ol was separated on this system. Cholesterol was analyzed colorimetrically and corrected for recovery of [3α-^{3}H]-Δ5,7 with the assumption that the two sterols were recovered equivalently. Δ5,7 at the concentrations present in these samples gave no absorbance in this assay. Despite an apparent slight increase in skin cholesterol levels with chronic 1,25-$(OH)_2D_3$ administration, this trend did not achieve significance (Table I).

Table II: Peak Heights[a] (Mean ± SD) of Some Prominent Peaks of UV Absorbance at 254 nm in High-Performance LC Chromatograms of Skin Samples from Lipidex Chromatography[b]

	peak no.			
group (n = 7)	1	2	3	4
controls	15.2 ± 1.5	3.1 ± 0.65	1.5 ± 0.68	0.60 ± 0.17
1,25-$(OH)_2D_3$	21.4 ± 2.4[c]	4.3 ± 1.20[d]	2.9 ± 0.82[e]	0.73 ± 0.15[f]

[a] In centimeters. [b] See text and Figure 1. [c-f] Significance of differences from controls: $P < 0.001$, $P < 0.05$, $P < 0.01$, and not significant, respectively.

Table III: Concentration of 7,8-Didehydrocholesterol in Skin of Rats Fed a Rachitogenic Diet with and without 1,25-$(OH)_2D_3$[a]

	−1,25-$(OH)_2D_3$	+1,25-$(OH)_2D_3$
gain in body weight (g)	27.5 ± 8.6	23.0 ± 11.4
food consumption	19.4 ± 4.8	20.7 ± 8.4
7,8-didehydrocholesterol (μg/g dry weight of skin)	79.7 ± 39.4	190.5 ± 94.6[b]

[a] Twenty rats were fed the 2% Ca and 0.1% P rachitogenic diet for 4 weeks and then divided equally into 2 groups. One group received 2 × 120 ng/day of 1,25-$(OH)_2D_3$ in 50 μL of propylene glycol for 7 days by subcutaneous injection while the other group received only the propylene glycol. The values are the mean ± SD for 10 rats. [b] Different from control, $P < 0.001$.

Other components absorbing at 254 nm in the chromatogram appeared to be increased in the skins from 1,25-$(OH)_2D_3$-dosed animals (Figure 1). The heights of UV absorbance peaks in an high-performance LC chromatogram are linear with the concentration of the absorbing species in the sample. Although there is no way to correct for recoveries in these samples and the peaks may not be homogeneous, a significant difference in peak heights was found between the vitamin D deficient and 1,25-$(OH)_2D_3$ samples for some of the peaks (Table II). It is reasonable to assume that the average recoveries of these compounds were the same in both groups since the samples all received identical treatments. The identity of these peaks is unknown.

Because serum calcium, food consumption, and body weight increase in rats on this diet given 1,25-$(OH)_2D_3$, it seemed possible that the changes observed in the skin could be related to these factors. A second experiment was therefore carried out in which body weight, food consumption, and serum calcium were not factors (Table III). With the 1.2% Ca and 0.1% P rachitogenic diet, body weight and food consumption are not changed by 1,25-$(OH)_2D_3$. Furthermore, serum calcium is high in the deficient state (10.1 mg/100 mL) and is only slightly increased by 1,25-$(OH)_2D_3$ (11.1 mg/100 mL) (Suda et al., 1970). With this diet a highly significant increase of 7,8-didehydrocholesterol is brought about by 1,25-$(OH)_2D_3$, strongly suggesting a direct action of 1,25-$(OH)_2D_3$ on the metabolism of sterols in skin resulting in an increase in 7,8-didehydrocholesterol content.

Discussion

An accurate assay for determining skin Δ5,7 concentrations was developed and used to demonstrate a significant ($P <$ 0.001–0.005) increase in skin Δ5,7 content with 1,25-$(OH)_2D_3$ administration to vitamin D deficient rats. Skin cholesterol levels were not significantly affected. Several considerations indicate that the hormone is acting directly. The strongest evidence for a direct action of 1,25-$(OH)_2D_3$ on skin is the

recent finding (Stumpf et al., 1979) that 1,25-$(OH)_2D_3$ specifically localizes in the nuclei of cells in the malphigian layer of rat epidermis. Specific nuclear localization is generally found only in target tissues for the hormone. Furthermore, a specific receptor for 1,25-$(OH)_2D_3$ has been demonstrated in rat skins (Simpson & DeLuca, 1980). It is also evident that the increase in Δ5,7 is not the result of such factors as changes in body weight, food consumption, or serum calcium levels since the 1,25-$(OH)_2D_3$-induced increases in Δ5,7 are clearly evident under circumstances where these are not factors (see Table III).

The skin is an active tissue in sterol biosynthesis which, unlike most tissues, accumulates sterol intermediates in significant levels (Kandutsch, 1964). Δ5,7 serves as an obligatory precursor to cholesterol in sterol biosynthesis in the normal animal (Clayton et al., 1963; Dempsey et al., 1964), and the skin is one of the few tissues which accumulates high concentrations of the sterol (Windaus & Bock, 1937; Kandutsch, 1964). These considerations would suggest that skin Δ5,7 content, and therefore the increase in skin Δ5,7 caused by 1,25-$(OH)_2D_3$, results from biosynthesis in the skin tissue. The effect of 1,25-$(OH)_2D_3$ on skin sterol biosynthesizing enzymes is under investigation.

The skin response to 1,25-$(OH)_2D_3$ seems directed toward Δ5,7 in particular, since cholesterol levels are not significantly elevated by 1,25-$(OH)_2D_3$. Certain other UV-absorbing fractions are increased to a lessor degree by 1,25-$(OH)_2D_3$. It is reasonable to assume that at least some of these components detected on high-performance LC are sterols, since the samples were saponified and the Lipidex chromatography selected for compounds with polarities similar to 3-hydroxy sterols. These peaks could, therefore, be precursors to Δ5,7 in skin sterol biosynthesis.

The increase in skin Δ5,7 content suggests a positive feedback control mechanism for vitamin D_3 formation. The view that vitamin D_3 is the antirachitic substance produced upon UV irradiation of skin was recently confirmed (Holick et al., 1977; Esvelt et al., 1978), which firmly establishes the role of Δ5,7 as the natural provitamin in skin. 1,25-$(OH)_2D_3$ is rapidly metabolized to polar products in rats (Esvelt et al., 1979), with 40–60% of dosed 1,25-$(OH)_2D_3$ found in the bile within 24 h (Onisko et al., 1980). These findings would indicate that 1,25-$(OH)_2D_3$ has a short half-life in vivo, and thus animals with an induced 25-hydroxyvitamin D_3 1α-hydroxylase would be expected to rapidly deplete their stores of 25-OH-D_3, the major circulating form of the vitamin. Provided that environmental UV light is available, the 1,25-$(OH)_2D_3$-stimulated increase in skin Δ5,7 content would be expected to result in increased vitamin D_3 production. Bearing in mind that the production of 1,25-$(OH)_2D_3$ is negatively feedback regulated by serum calcium and phosphorus directly or indirectly, the vitamin D endocrine system could have both negative and positive feedback features.

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