PHOTOMETABOLISM OF 7-DEHYDROCHOLESTEROL TO PREVITAMIN D_3 IN SKIN

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SUMMARY: The photometabolism of [3α-^{3}H]-7-dehydrocholesterol in skin was studied in groups of rats exposed to ultraviolet irradiation. The major photolytic product was identified as previtamin D_3 by its identical migration with authentic previtamin D_3 on high-pressure liquid chromatography. Furthermore, this photometabolite was isolated in pure form from endogenous precursors in skins of rats exposed to ultraviolet irradiation; identification as previtamin D_3 was based on its ultraviolet absorption spectrum, mass spectrum and its thermal conversion to vitamin D_3.

INTRODUCTION: Vitamin D, a fat-soluble factor that acts to heal rachitic lesions, was discovered in the early 1900's (1,2). Subsequently, Steenbock (3) and Hess et al. (4) independently demonstrated that this factor could be generated simply by irradiating either rachitic animals or their food. Further work led to the isolation and structural identification of vitamins D_2, D_3, and their precursors, ergosterol and 7-dehydrocholesterol, respectively (5—7).

Photolysis of ergosterol or 7-dehydrocholesterol in vitro yields, at equilibrium, at least three isomeric products: tachysterol, lumisterol, and a 9,10-seco-steroid with a 5,6-cis-triene conformation. This latter compound was shown to have antirachitic activity and, depending on the starting material, ergosterol or 7-dehydrocholesterol, was named vitamin D_2 or vitamin D_3, respectively. At first it was believed that 7-dehydrocholesterol and ergosterol were converted directly to vitamin D_3 and vitamin D_2 by ultraviolet photolysis (8). In 1949, however, Velluz et al. (9) demonstrated that a 6,7-cis isomer, which they named previtamin D, was the initial irradiation product and that, at varying rates as a function

Abbreviations: HPLC, High-Pressure Liquid Chromatography; TLC, Thin Layer Chromatography

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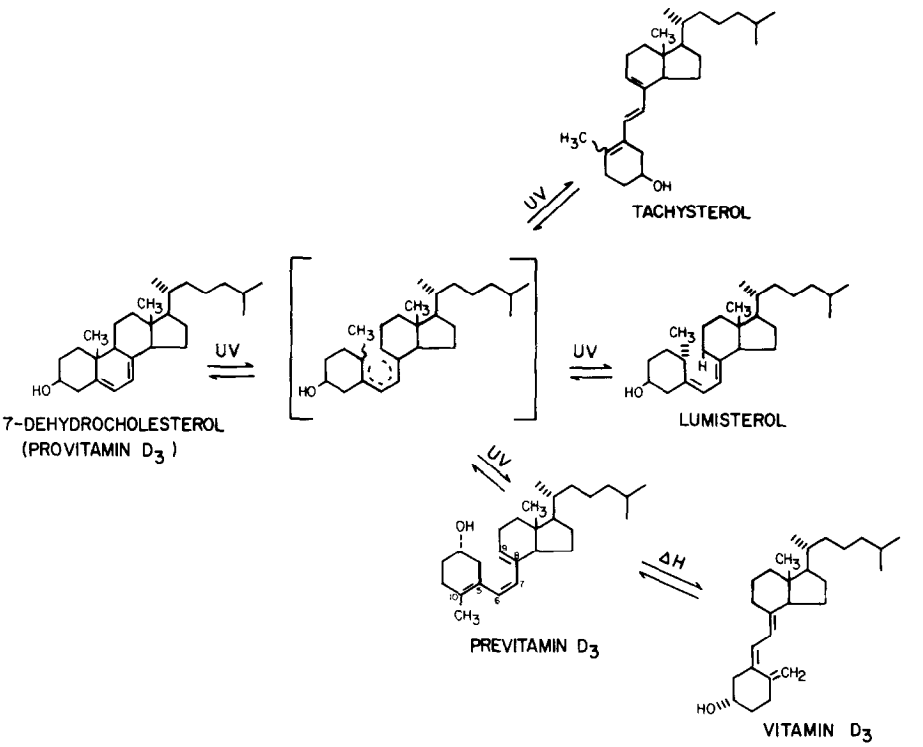

Fig. 1. Photometabolism of 7-dehydrocholesterol to lumisterol, tachysterol, and previtamin D_3, which is converted to vitamin D_3 by thermal energy.

of temperature, previtamin D converted to vitamin D with the equilibrium position in favor of vitamin D. Thus, under controlled conditions, ultraviolet irradiation of 7-dehydrocholesterol generates three major products: tachysterol, lumisterol, and previtamin D_3 (Fig. 1). Previtamin D_3 is then converted to the thermodynamically favored 5,6-cis isomer, vitamin D_3, by thermal equilibration.

Although it has been generally accepted that vitamin D_3 is generated *in vivo* by ultraviolet irradiation of the 7-dehydrocholesterol in the skin, the mechanism by which this occurs has not been investigated. We have examined this mechanism and clearly demonstrated that 7-dehydrocholesterol is initially converted to previtamin D_3 in the skin *in vivo*, as it is in the test-tube model, by the action of ultraviolet light.

METHODS:

Synthesis of [3α-^{3}H]-7-dehydrocholesterol. Synthesis of this isotope was carried out as described for [3α-^{3}H]-ergosterol (10). Crystalline 7-dehydrocholesterol was oxidized to 4,7-cholestadiene-3-one (λ_{max} 240 nm) by the method of Shepherd et al. (11). This α-β unsaturated ketone was acetylated with acetic anhydride and pyridine, and the isolated product exhibited λ_{max} 331, 316, and 303 nm, which is characteristic for the $\Delta^{5,7}$-enol acetate. The enol acetate (10 mg) was dissolved in 10 ml isopropanol containing 100 µl H_2O and to this solution was added 1 mg [^{3}H]-sodium borohydride (S.A. 8.0 Ci/mM) purchased from New England Nuclear, Boston, Massachusetts. The mixture was stirred at 25°C for 2 h and then extracted with ether:water. The product was dried under N_2 and applied to preparative thin-layer chromatographic plates (Uniplate, Analtech Inc., Delaware) and developed in n-hexane: ethylacetate (8:2 v/v). The compound with an R_f of 0.6 was eluted and exhibited an ultraviolet spectrum with λ_{max} 295, 282, and 271 nm, which is identical to the ultraviolet spectrum for 7-dehydrocholesterol (10). Furthermore, this product migrated identically with authentic 7-dehydrocholesterol on thin-layer chromatography (TLC) and on high-pressure liquid chromatography (HPLC) using a 0.4-mmx30-cm µ-Porasil column developed with 1% isopropanol in n-hexane. An additional product with an identical ultraviolet-absorption spectrum but with an R_f of 0.8 was also recovered; the latter did not migrate with 7-dehydrocholesterol on HPLC or TLC. This additional compound was presumed to be [3β-^{3}H]-3-epi-7-dehydrocholesterol, a predicted side-product of the reduction reaction.

Topical application and irradiation of [3α-^{3}H]-7-dehydrocholesterol. Areas (3 X 3 cm) of the backs of two groups of vitamin-D-deficient rats were shaved. Two µCi of [3α-^{3}H]-7-dehydrocholesterol dissolved in 0.1 ml of Wesson oil was applied to this exposed skin 24 h after shaving. The control group was kept in the dark; the treatment group, after an additional 24-h delay, was placed under an ultraviolet light of wave-length 250—350 nm for 3 h (0.23 joules/cm^2 at $\lambda_{280\ nm}$). Immediately after irradiation of the treatment group, all animals were killed by exsanguination, and the 3x3-cm area of skin was removed and frozen on Dry Ice.

Isolation and identification of radioactive photolysis products. Individual skins were homogenized in 10 ml 0.9% saline with a Polytron homogenizer for 30 sec and extracted with 40 ml cholorform:methanol (1:1). The cholorform phase was collected, and the aqueous phase was re-extracted twice with 10 ml chloroform. The chloroform phases were combined, dried under N_2 at 4°C, dissolved in n-hexane:chloroform (19:1), and applied to a 1x60-cm glass column packed with 15 grams of a Neodox-1518 alkoxyl derivative of Sephadex LH-20 prepared according to the procedure of Ellingboe et al. (12). The column was eluted with n-hexane: chloroform (19:1). Fractions (2 ml) were collected, dried under air, and dissolved in Instagel (Packard) prior to counting of their tritium content in the Packard Tricarb 3375 scintillation counter.

The various peaks of radioactivity were identified by taking individual peak fractions from the Neodox column and subjecting them to chromatography simultaneously with standard compounds on HPLC equipped with a 0.4-cmx30-cm µ-Porasil column using 1% isopropanol in n-hexane as solvent. The peak assigned as previtamin D_3 was mixed with 100 µg authentic previtamin D_3, chromatographed on HPLC as described above, and collected in 1-ml fractions. Half of each fraction was dried and counted for tritium. The remaining fractions containing the previtamin-D_3 peak were pooled and warmed at 60°C in methanol for 3 h before re-chromatography on the same column under identical conditions.

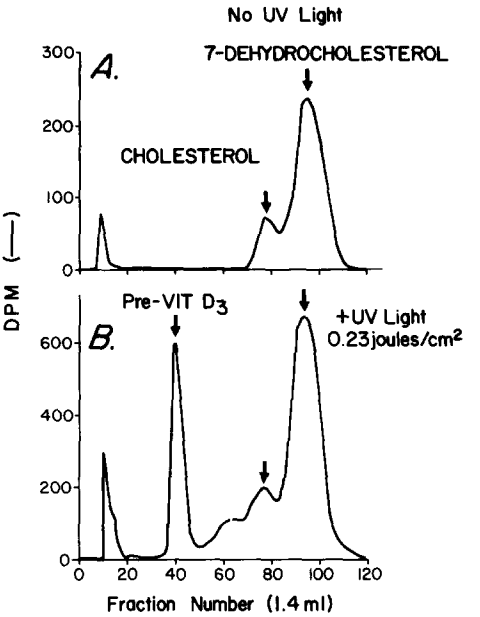

Fig. 2. Neodox 1518 Sephadex column (1 x 60 cm packed in 19:1 n-hexane: chloroform) profiles of skin lipid extracts from animals treated with topical application of 2 μCi [3α-^{3}H]-7-dehydrocholesterol and then killed 24 h later (A) or irradiated with 0.23 joules/cm^2 and then killed (B).

Isolation and identification of a sterol formed from photolysis of endogenous 7-dehydrocholesterol. 50 rats previously maintained on a vitamin-D-deficient diet for 3 weeks were shaven as before. 24-h later they were exposed to 0.23 joules/cm^2 at $\lambda_{280\ nm}$ of ultraviolet light over a period of 2 h and then immediately killed by exsanguination. Topical doses of [3α-^{3}H]-7-dehydrocholesterol were applied to 3 rats as described above, before irradiation, to act as a radio-active marker. The shaved skin from all animals was collected, homogenized and extracted as previously described. The extract was dried under N_2 in the cold room and the lipid residue was re-extracted in 800 ml water:n-hexane (1:1). The n-hexane phase was collected, and the aqueous phase was re-extracted twice with 100 ml n-hexane. The n-hexane phases were combined and dried under N_2 at 4°C, and the residue was dissolved in n-hexane:chloroform (19:1) and applied to a 3x40-cm glass column packed with 60 g Neodox-1518 derivative of Sephadex LH-20 in the same solvent. The previtamin-D_3 peak was collected, dried under N_2, redissolved in 10 ml methanol, and warmed at 60°C for 3 h. The product was applied to the HPLC, and 50 μl from the resulting 1-ml fractions was counted for tritium. Ultraviolet-absorption spectra were made on the previtamin-D_3 peak before and after heating, on a Beckman DB-G spectrophotometer.

RESULTS: The chromatographic profiles of the skin extracts from animals given topical applications of [3α-^{3}H]-7-dehydrocholesterol and then exposed to 0.23 joules/cm^2 ultraviolet radiation (A) or confined in the dark (B) are

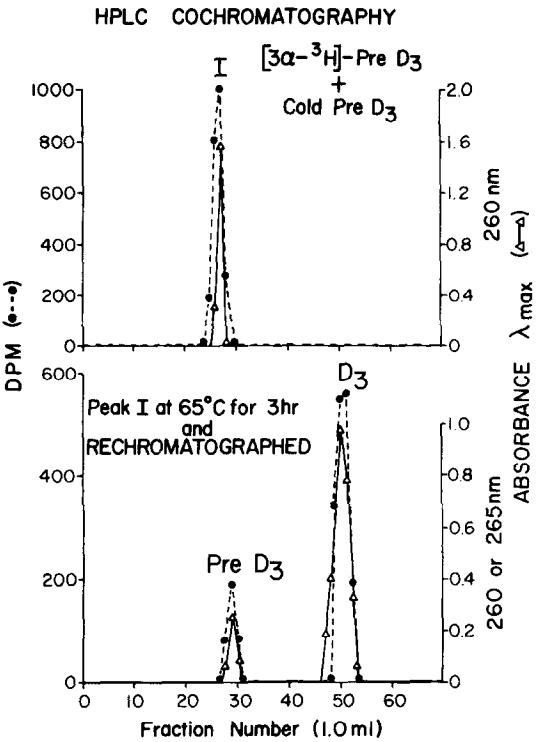

Fig. 3. Chromatography of fractions 35—45 from Fig. 2B with 100 µg of authentic previtamin D_3 on HPLC (A). Peak 1 was collected and warmed at 65°C for 3 h and rechromatographed on the same column (B), which demonstrated conversion to vitamin D_3.

shown in Fig. 2. The chromatogram from the confined animals shows three peaks. The earliest peak (fractions no. 10—20) is presumed to be an ester(s) of the starting material. The two additional peaks were tentatively identified as 7-dehydrocholesterol and its Δ^7 reduction product, cholesterol. This identification was confirmed by cochromatography with authentic compounds on HPLC. The chromatogram from animals that received ultraviolet irradiation demonstrated one additional major peak labeled as previtamin D_3 (Fig. 2B). The identification of this radioactive peak as previtamin D_3 was established by (i) identical mobility with authentic previtamin D_3 on the high-resolution HPLC system and (ii), after warming, identical mobility with vitamin D_3 formed by thermal conversion of authentic previtamin D_3 (Fig. 3).

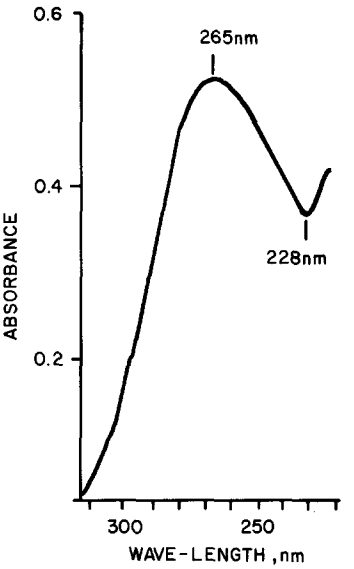

Fig. 4. Ultraviolet absorption spectrum of the isolated product from the skin that was warmed at 65° for 3 h and chromatographed on HPLC as shown in Fig. 3.

DISCUSSION: Although it has been generally accepted that vitamin D_3 is made in the skin by the action of solar ultraviolet irradiation on 7-dehydrocholesterol, the mechanism by which this occurs has never been investigated. In this paper we present evidence that 7-dehydrocholesterol in the skin is converted to previtamin D_3 and not vitamin D_3, a result similar to that seen *in vitro*. This was demonstrated by two methods. In the first, [3α-^{3}H]-7-dehydrocholesterol applied topically was converted to a compound that migrated identically with authentic previtamin D_3 on high-resolution separation systems. The concern that topically applied 7-dehydrocholesterol might not enter epithelial cells where the photolytic reaction naturally occurs was lessened when it was demonstrated that cholesterol was generated from its precursor 7-dehydrocholesterol. Therefore, at least some of the 7-dehydrocholesterol had to enter viable epithelial cells for the 7-dehydrocholesterol-reductase to work.

It could be argued, however, that the photolytic products recovered were similar to those that would be found if we applied 7-dehydrocholesterol

to a piece of glass and irradiated it. Therefore, the previtamin D_3 that we observed in our isotope experiments might in fact have been an artefact generated on the surface of the skin and may not have entered the cells or be a physiological product.

To eliminate this possibility an alternate approach was used. We irradiated 50 vitamin-D-deficient rats and isolated a product with an ultraviolet absorption spectrum λ_{max}257 nm and λ_{min}230 nm that was similar to previtamin D_3 (λ_{max}260 nm, λ_{min}230 nm). This product, formed from endogenous 7-dehydrocholesterol, migrated identically with authentic previtamin D_3 and, when warmed at 60°, chromatographed identically with vitamin D_3. Furthermore, the product after heating had an ultraviolet absorption spectrum λ_{max}265 nm, λ_{min}228 nm, which is identical to that of the 5,6-cis-triene system for vitamin D_3 (Fig. 4). To confirm that the isolated 5,6-cis-triene was vitamin D_3, a mass spectrum of the isolated product was analyzed. The compound was shown to have a molecular ion 384 (m/e) and fragments 369 (M^+-CH_3), 366 (M^+-H_2O), 271 (M^+-side chain), 253 (M^+-side chain-H_2O), 136 (A ring + C_6 and C_7), and 118 (136-H_2O), which are identical to authentic vitamin D_3 (13).

In this paper we report the isolation of a photolytic product from skin and its identification as previtamin D_3. Therefore we suggest that the mechanism for the photoconversion of 7-dehydrocholesterol to vitamin D_3 in the skin occurs through an intermediate product, previtamin D_3, which is further converted to vitamin D_3 presumably by the thermal action of body heat. Qualitative or quantitative differences, if any, in the overall process of photolytic activation of 7-dehydrocholesterol *in vitro* versus *in vivo* await further study, especially the issue of biological control of the *in vivo* process.

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REFERENCES

1. Mellanby, E. (1919) Lancet, 1, 407—412.
2. McCallum, E.V., Simmonds, N., Becker, J.E., and Shipley, P.G. (1922) Bull. John Hopkins Hosp., 33, 229—232.
3. Steenbock, H. (1924) Science, 60, 224—227.
4. Hess, A.F., Weinstock, M., and Helman, F.D. (1925) J. Biol. Chem., 63, 305—308.
5. Askew, F.A., Bourdillon, R.B., Bruce, H.M., Jenkins, R.G.C., and Webster, T.A. (1931) Proc. Roy. Soc. Med., B107, 76—78.
6. Windaus, A., Lettre, H., and Schenck, F. (1935) Justus Leibigs Ann. Chem., 520, 98.
7. Windaus, A., Schenck, F., and von Werder, F. (1936) Hoppe-Seyler's Physiol. Chem., 241, 100—104.
8. Rosenheim, O., and King, H. (1935) Chem. Ind. (London), 54, 699—671.
9. Velluz, L., Amiard, G., and Petit, A. (1949) Bull. Soc., 16, 501—503.
10. Jones, G., Schnoes, H.K., and DeLuca, H.F. (1975) Biochemistry, 14, 1250—1254.
11. Shepherd, D., Donia, R.A., Cambell, J.A., Johnson, B.A., Holysz, R.P., Slomp, G., Stafford, J.E., Pederson, R.L., and Ott, A.C. (1955) J. Am. Chem. Soc., 77, 1212—1216.
12. Ellingboe, J., Nystrom, E., and Sjovall, J. (1970) J. Lipid Res., 11, 266—270.
13. Holick, M.F., and DeLuca, H.F. (1974) Steroids Biochemistry and Pharmacology, Vol. 4, M.H. Briggs and G.A. Christie (eds), pp. 111—155, Academic Press, New York.