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Isolation and Identification of Previtamin D_3 from the Skin of Rats Exposed to Ultraviolet Irradiation†

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ABSTRACT: The process of the photolytic activation of vitamin D precursor(s) in the skin has been elucidated by a detailed analysis of the products formed after ultraviolet light exposure. The photolytic product isolated from the skin of rats exposed to ultraviolet irradiation was identified as previtamin D_3 by several criteria including its (a) characteristic ultraviolet absorption spectrum, (b) mass spectrum, and (c) thermal isomerization to vitamin D_3, which itself was identified also by mass spectroscopy. Vitamin D_3 per se was not formed by ultraviolet irradiation—vitamin D_3 arises exclusively from the thermal conversion of previtamin D_3. Detectable amounts of lumisterol$_3$ or tachysterol$_3$ were not seen.

As early as 1890, Palm and his colleagues (Palm, 1890) made the observation that sunlight played an important role in the maintenance of healthy bone. Subsequently, a number of investigators demonstrated that both animals and children with rickets could be cured by exposure to either mercury-vapor quartz lamps or sunlight (Huldshinsky, 1919; Goldblatt & Soames, 1923a,b; Steenbock & Black, 1924, Hess et al., 1925). These observations provided the impetus for the isolation and chemical characterization of the $\Delta^{5,7}$-diene sterols, which, after exposure to ultraviolet light, are photochemically converted to antirachitic substances characterized as 9,10-secosteroids with a 5,6-cis triene system (Askew et al., 1931; Windaus and Boch, 1936). Ergosterol (a plant $\Delta^{5,7}$-diene sterol) and 7-dehydrocholesterol (an animal $\Delta^{5,7}$-diene sterol) were isolated, purified, and exposed to ultraviolet irradiation. From the irradiation reactions were isolated ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3), respectively, from among the other photoisomeric products (Windaus et al., 1932, 1936; Askew et al., 1931).

Because 7-dehydrocholesterol is found in appreciable quantities in rat, pig, and human skin, it was assumed that this $\Delta^{5,7}$-diene sterol is photochemically converted to vitamin D_3 when skin is exposed to sunlight. In fact, several investigators provided chromatographic evidence (Okano et al., 1977) as well as structural evidence that includes an ultraviolet-absorption spectrum and mass spectrum (Esvelt et al., 1978) for the presence of vitamin D_3 in rat and human skin (Rauschkolb et al., 1969) exposed to ultraviolet irradiation.

However, when $\Delta^{5,7}$-diene sterols are irradiated with ultraviolet light in a quartz vessel, vitamin D is not one of the photolytic products. Instead, lumisterol, tachysterol, and

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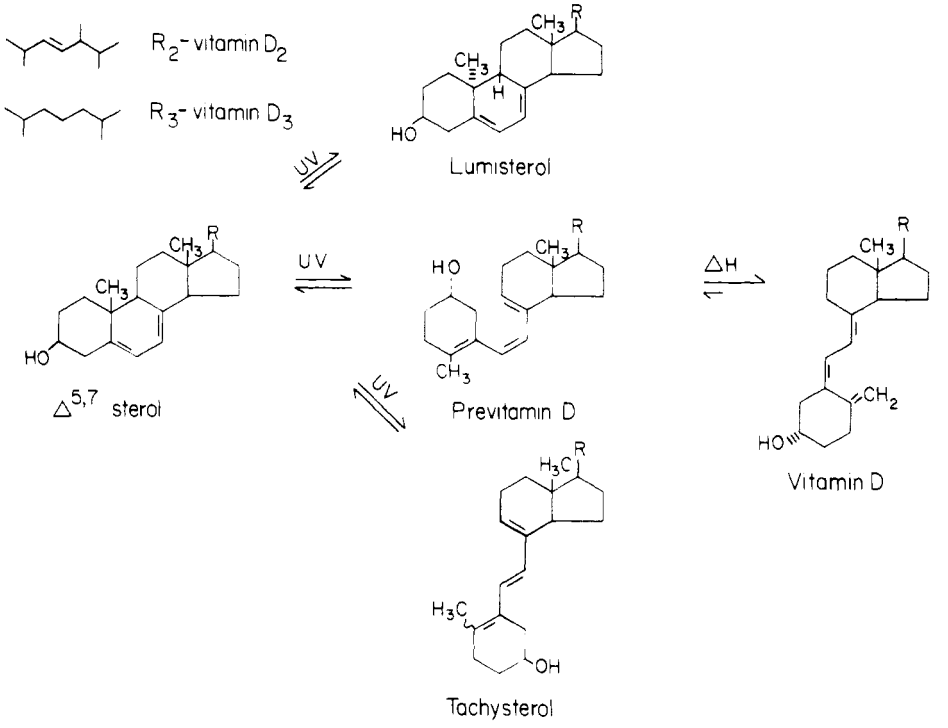

FIGURE 1: Structures of (i) ergosterol (R_2), 7-dehydrocholesterol (R_3), (ii) their photolytic products: lumisterol, tachysterol, and previtamin D, and (iii) the thermal products of previtamin D: vitamin D_2 or vitamin D_3.

previtamin D are the major products (Velluz et al., 1949; Havinga, 1973). It is previtamin D, a thermally unstable 6,7-cis isomer, that thermally converts to a thermally stable 5,6-cis isomer, vitamin D (Figure 1).

It is the purpose of this report to present data establishing that, when skin is exposed to ultraviolet irradiation in vivo, the major photolytic product is previtamin D_3 and that no tachysterol$_3$, lumisterol$_3$, or vitamin D_3 are detectable. The initial aspects of these studies were reported in preliminary form (Holick et al., 1977).

Materials and Methods

Radioactive determinations were carried out with a Packard Tri-Carb Model 3375 liquid scintillation counter equipped with an automatic external standard system. Samples were dried in plastic vial inserts (15 × 45 mm) by a stream of air and dissolved in 4 mL of Insta-Gel (Packard Instrument Co., Downers Grove, IL).

Ultraviolet absorption spectra were recorded with a Beckman DB-G recording spectrophotometer, and mass spectrometric determinations were carried out with an AEI MS-9 mass spectrometer using a direct-probe inlet at temperatures of 90–120 °C above ambient. Spectral irradiance was measured with an International Light No. 118 radiometer (International Light Inc., Newburyport, MA) at 297 nm. High-pressure liquid chromatography (LC) was carried out on a Waters high-pressure liquid chromatography system equipped with a stainless-steel column (30 cm × 4 mm) packed with μ-Porasil. The flow rate was from 1–4 mL/min (generating 600–1200 per in.^{2}), and the ultraviolet absorbance at 254 nm was measured and plotted automatically. All solvents were triply distilled; the hexane and isopropyl alcohol were LC grade.

Isotopically Labeled Compounds. [3α-^{3}H]-7-Dehydrocholesterol (sp act., 4.8 Ci/mmol) was synthesized by a method previously described (Holick et al., 1977). One hundred micrograms of [3α-^{3}H]-7-dehydrocholesterol was dissolved in 10 mL of diethyl ether in a quartz vessel and exposed to an

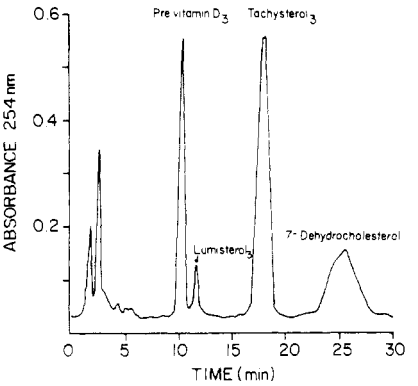

FIGURE 2: High-pressure liquid chromatographic profile on a μ-Porasil column (30 cm × 4 mm, developed with 0.25% isopropyl alcohol in hexane at 3.0 mL/min) of the products formed after 7-dehydro-[3α-^{3}H]cholesterol was exposed to ultraviolet irradiation.

Hanovia high-pressure mercury lamp, Model 654 A, for 1 min to generate radiolabeled photolytic isomers. The products were applied to LC and eluted with 0.25% isopropyl alcohol in hexane (Figure 2). [3α-^{3}H]Lumisterol$_3$, [3α-^{3}H]tachysterol$_3$, and [3α-^{3}H]previtamin D_3 were identified on the basis of characteristic ultraviolet absorption spectra (Havinga et al., 1955). A portion of [3α-^{3}H]previtamin D_3 was thermally converted to [3α-^{3}H]vitamin D_3 at various temperatures as described previously (Blunt & DeLuca, 1969).

Isolation of Previtamin D_3 from Rat Skin. Twenty-six rats were maintained for 4 weeks on a vitamin D deficient diet that was adequate in calcium and phosphorus. The hair was shaved from their backs, and 24 h later they were exposed to ultraviolet irradiation under a F40-T12 Westinghouse sunlamp (spectral range 253–400 nm) for a total irradiation dose of 0.2 J/cm^2. Immediately after irradiation the animals were killed, and the exposed epidermis and dermis were quickly separated from the subcutaneous fat and frozen at −60 °C. To minimize the thermal conversion of previtamin D_3 to vitamin D_3, the following extractions and chromatography were

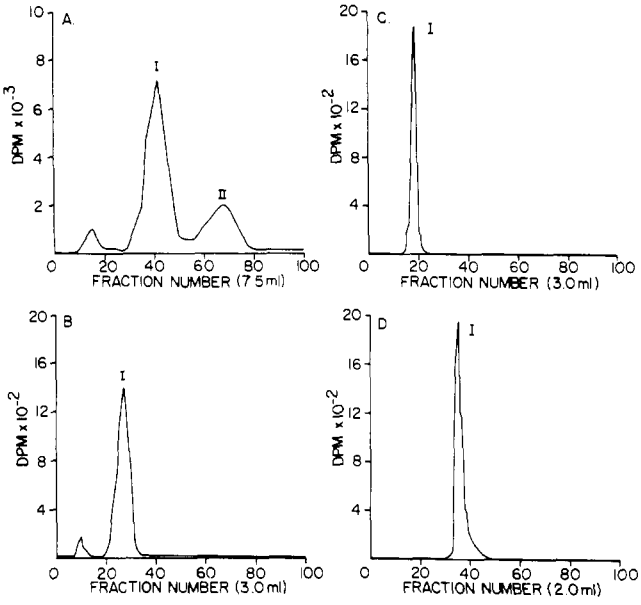

FIGURE 3: (A) Chromatographic profile on a 60-g Nedox 15-18-Sephadex LH-20 column (3 × 30 cm, packed and developed with 19:1 hexane/$CHCl_3$) of a lipid extract from rat skin exposed to ultraviolet light. Peak I (fractions 30–50) contains [3α-^{3}H]previtamin D_3 and [3α-^{3}H]lumisterol$_3$, and peak II (fractions 55–80) contains [3α-^{3}H]vitamin D_3 and [3α-^{3}H]tachysterol$_3$ (see text). (B) Chromatographic profile on an 18-g Nedox 15-18-Sephadex LH-20 column (1 × 60 cm, packed and developed with 19:1 hexane/$CHCl_3$) of peak I from A. (C) Chromatographic profile on a 40-g Biobeads SX-8 column (1 × 150 cm, packed and developed with 65:35 $CHCl_3$/hexane) of peak I from B. (D) Chromatographic profile on a 30-g Sephadex LH-20 column (1 × 100 cm, packed and developed with MeOH) of peak I from C.

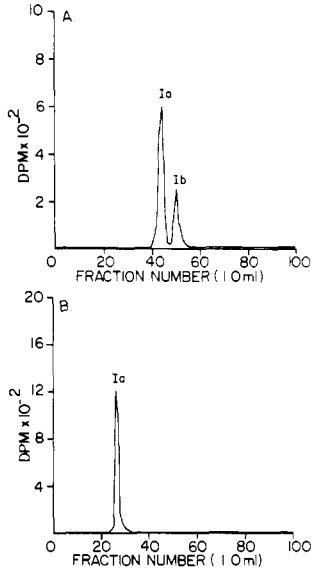

FIGURE 4: (A) High-pressure liquid chromatographic profile on a μ-Porasil column (30 cm × 4 mm, developed with 0.20% isopropyl alcohol in hexane) of tube no. 36 recovered from the Sephadex LH-20 column (1 × 100 cm) (Figure 3D). (B) High-pressure liquid chromatographic profile on a μ-Porasil column (30 cm × 4 mm, developed with 0.25% isopropyl alcohol in hexane) of peak Ia recovered from 4A.

carried out in a cold room at 5 °C. The frozen skins (80 g) were chopped into small pieces on dry ice and homogenized in 400 mL of 0.9% saline. The homogenate was extracted with 1:1 v/v of $CHCl_3$/MeOH (Bligh & Dyer, 1959), and the $CHCl_3$ phase was collected. The aqueous phase was reextracted with 100 mL of $CHCl_3$, and the $CHCl_3$ phases were combined and dried under N_2 to yield 15 g of an oily yellow residue. Three hundred nanograms of [3α-^{3}H]previtamin D_3, 100 ng of [3α-^{3}H]vitamin D_3, 100 ng of [3α-^{3}H]lumisterol$_3$, and 100 ng of [3α-^{3}H]tachysterol$_3$ were added to the residue, and it was dissolved in 30 mL of 19:1 hexane/$CHCl_3$ and applied in two batches to a glass column (3 × 30 cm) containing 60 g of Nedox 15–18-Sephadex LH-20, which is a C_{15}–C_{18} alkoxy derivative of Sephadex LH-20 synthesized according to the procedure of Ellingboe et al. (1970). This column packing was slurried and developed with the same solvent. Fractions of 7.5 mL were collected, and tubes no. 30–50 (Figure 3A), which contained [^{3}H]previtamin D_3 and [^{3}H]lumisterol$_3$, were combined and dried under N_2 to yield 2 g of a colorless oily residue. (Fractions 55–80, which contained [^{3}H]tachysterol$_3$ and [^{3}H]vitamin D_3, were combined, flushed with N_2, and stored at −60 °C for further evaluation.) The 2 g of oily residue was dissolved in 2 mL of 19:1 hexane/$CHCl_3$ and applied to a glass column (1 × 60 cm) containing 18 g of Nedox 15–18-Sephadex LH-20 that was slurried and developed with the same solvent. One hundred 3.0-mL fractions were collected, and the peak tubes 21–32 (Figure 3B) were combined and dried under N_2 to yield ~100 mg of an oily residue. The residue was dissolved in 0.5 mL of 65:35 $CHCl_3$/hexane and applied to a glass column (1 × 150 cm) packed with 40 g of Biobeads SX-8 (Bio-Rad Corp., Richmond, CA) in the same solvent, and 2.0-mL fractions were collected as previously described (Holick et al., 1971). Tubes 13–20 (Figure 3C) were combined and dried under N_2, and the 10-mg residue was dissolved in 0.5 mL of MeOH and applied to a glass column (1 × 100 cm) packed with 30 g of Sephadex LH-20 (Pharmacia Corp., Piscataway, NJ) in the same solvent, as described previously (Holick et al., 1971). The peak eluting between tubes 32–40 (Figure 3D) was dried under N_2 to yield less than 1 mg of a colorless oil. This sample was dissolved in 0.1 mL of MeOH and reapplied to the same Sephadex LH-20 column. The peak tube no. 36 (Figure 3D) was dried under N_2 and applied to LC in, and developed with, 0.20% isopropyl alcohol in hexane as described above in order to separate [3α-^{3}H]previtamin D_3 (peak Ia) from [3α-^{3}H]lumisterol$_3$ (peak Ib, Figure 4A). Peak Ia was collected and reapplied to LC in, and developed with, 0.25% isopropyl alcohol in hexane. The peak tube no. 26 (Figure 4B) was dried under N_2 and redissolved in 1.0 mL of MeOH, and its ultraviolet-absorption spectrum was recorded (Figure 5A). Half of this fraction was used for mass spectral analysis, and the other half was dissolved in 5 mL of MeOH and warmed at 60 °C for 6 h to convert it to vitamin D_3. The MeOH was evaporated under N_2, and the sample was applied to LC in, and developed with, 0.25% isopropyl alcohol in hexane. The peak tube no. 32 (Figure 6) was used for further mass spectral analysis.

Analysis for Lumisterol$_3$, Tachysterol$_3$, and *Vitamin D_3 in Rat Skin Exposed to Ultraviolet Light.* Peak Ib, which contained [3α-^{3}H]lumisterol (see Figure 4A), was rechromatographed on LC in, and developed with, 0.25% isopropyl alcohol in hexane. The peak eluting between 57–62 mL and containing 75% of the total [^{3}H]lumisterol$_3$ initially added was dried and redissolved in 0.5 mL of MeOH for ultraviolet-absorption spectral analysis. From the initial chromatography (see Figure 3A), tubes 55–80, which contained [3α-^{3}H]-tachysterol$_3$ and [3α-^{3}H]vitamin D_3, were combined and dried under N_2, and the residue was subjected to the same chromatographic separations employed for the isolation and purification of previtamin D_3 and lumisterol$_3$. The final product, which contained 80% of the total radioactivity added as [^{3}H]tachysterol$_3$ and [^{3}H]vitamin D_3, was taken to dryness

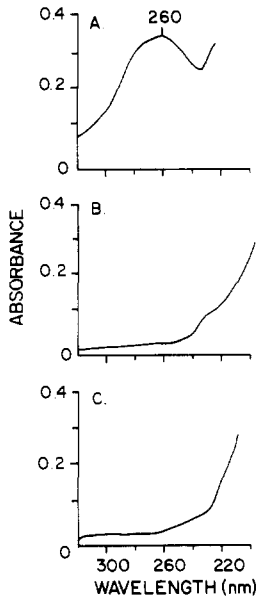

FIGURE 5: Ultraviolet absorption spectrum of (A) the lipid fraction in peak Ia (Figure 4A), which contained [^{3}H]previtamin D_3; (B) the lipid fraction in peak Ib (Figure 4A), which contained [^{3}H]lumisterol; and (C) the lipid fraction containing [^{3}H]vitamin D_3 and [^{3}H]-tachysterol$_3$ (Figure 3A).

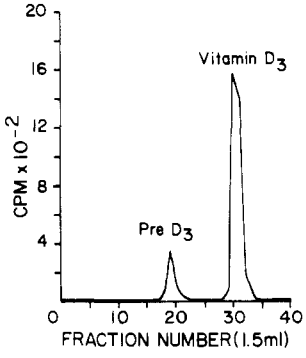

FIGURE 6: High-pressure liquid chromatographic profile on a μ-Porasil column (30 cm × 4 mm, developed with 0.25% isopropyl alcohol in hexane) of peak Ia (Figure 4B) after warming it at 60 °C for 6 h.

and redissolved in 1.0 mL of MeOH for ultraviolet-absorption spectral analysis.

Identification of the Photolytic Products in Rat Skin. After numerous chromatographic steps, peak Ia (Figure 4A), which contained only tracer amounts of [^{3}H]previtamin D_3 and the lipid fraction that comigrated with it, demonstrated an ultraviolet absorption spectrum with a λ_{max} 260 nm and λ_{min} 230 nm (Figure 5A), which is characteristic for the 6,7-cis triene chromophore of the pre-D vitamins (Velluz et al., 1949). The mass spectrum for the isolated product demonstrated a molecular ion *m/e* 384 and fragments *m/e* 271, 253, 136, and 118, as well as other fragments, of which many were presumed to be contaminants. For further purification, the product was thermally converted to vitamin D_3, which migrated separately from previtamin D_3 on LC, thus separating it from lipid contaminants that comigrated with previtamin D_3 (Figure 6). This fraction demonstrated a molecular ion at *m/e* 384 and fragments *m/e* 271 (M^+ – side chain, a C_{17}–C_{20} cleavage), 253 (271 – H_2O), 136 (A ring plus HC_7, HC_6, and $2HC_{19}$), and 118 (136 – H_2O), all of which are identical to vitamin D_3 (Figure 7) (Holick and DeLuca, 1974; Okamura et al., 1976).

Ultraviolet-absorption spectral analysis of peak Ib (see Figure 4A), which contained [3α-^{3}H]lumisterol$_3$, and of the lipid fraction that comigrated with it did not demonstrate any ultraviolet absorption characteristic for lumisterol (Figure 5B). Because tachysterol$_3$ and vitamin D_3 essentially comigrate with one another in each of the chromatographic systems described (Holick, unpublished results), the final purified product contained both [^{3}H]tachysterol$_3$ and [^{3}H]vitamin D_3, as well as the lipid fraction that comigrated with these tracer compounds. The final purified product had no ultraviolet absorption at either 281 nm or 265 nm, which are the characteristic λ_{max} for tachysterol$_3$ and vitamin D_3, respectively (Figure 5C).

Analysis of the Photolytic Products after Topical Application of 7-Dehydro[3α-^{3}H]cholesterol in Vivo. We employed an additional sensitive assay using 7-dehydro[^{3}H]cholesterol to carefully analyze which photolytic product(s) were produced in vivo after ultraviolet-light exposure. Vitamin D deficient rats were shaved, and, 24 h later, 0.5 μg of 7-dehydro[3α-^{3}H]cholesterol in 10 μL of Wesson oil was applied topically to their backs over a 1-cm^2 area. Five minutes after application of the isotope, the animals were placed under a Westinghouse sunlamp and exposed for a total irradiation dose of 0.2 J/cm^2. Immediately after exposure, the animals were killed, and the skins were removed, extracted, and stored at −60 °C. Twenty-thousand disintegrations per min from the extract was applied to LC in, and developed with, 0.25% isopropyl alcohol in hexane. Figure 8 shows that the major photolytic product was previtamin D_3 and, based on the recovery of radioactivity in the lumisterol$_3$, vitamin D_3, and tachysterol$_3$ regions (see Figure 2 for comparison), there was less than 2% of any of these compounds present.

Discussion

During the past decade, major research advances have been made in the areas of chemistry, physiology, and metabolism of vitamin D (Kodicek, 1974; Norman and Henry, 1974; Schnoes and DeLuca, 1976; Haussler and McCain, 1977; Holick and Clark, 1978). However, there has been very little new information regarding the details of the photolytic process by which vitamin D is produced in the skin after exposure to sunlight. A number of investigators have demonstrated the presence of vitamin D_3 in skin after ultraviolet light exposure and have correlated an increase in the amount of vitamin D_3 produced with an increase in the exposure time (Okano et al., 1977; Esvelt et al., 1978).

However, because vitamin D_3 is not one of the direct photolytic products of 7-dehydrocholesterol in vitro, we investigated the mechanism by which ultraviolet irradiation converts 7-dehydrocholesterol to vitamin D_3 in skin in vivo. At least two possibilities appeared likely: (a) that, as in vitro, ultraviolet-light exposure converts 7-dehydrocholesterol in the skin initially to previtamin D_3 and that this isomer thermally converts to vitamin D_3 or (b) that, as distinct from the in vitro process, ultraviolet-light exposure converts 7-dehydrocholesterol in the skin directly to vitamin D_3 by some active or passive process, thus bypassing the step of previtamin D_3 formation.

Our initial studies demonstrated that when 7-dehydro-[3α-^{3}H]cholesterol was applied topically to the surface of rat skin in vivo and exposed to ultraviolet light, the major radioactive photolytic product was previtamin D_3. Furthermore, we isolated and purified ~40 μg of endogenously formed previtamin D_3, unlabeled, from the skin of rats exposed to ultraviolet light. The purified product had an ultraviolet absorption spectrum that was similar to but not identical with the ultraviolet absorption spectrum for previtamin D_3. Therefore, the isolated previtamin D_3 was thermally converted to vitamin D_3 and repurified. The purified thermal product

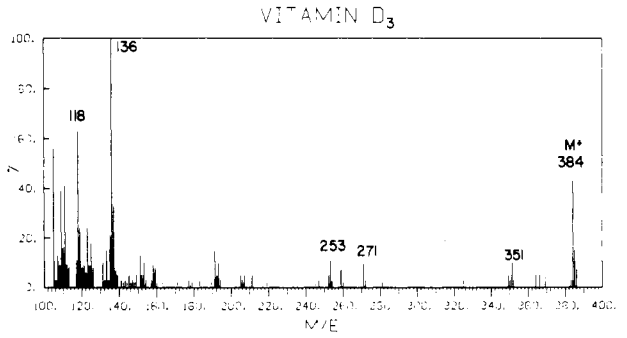

FIGURE 7: Mass spectrum of previtamin D_3 isolated from rat skin after it was thermally converted to vitamin D_3 (Figure 6).

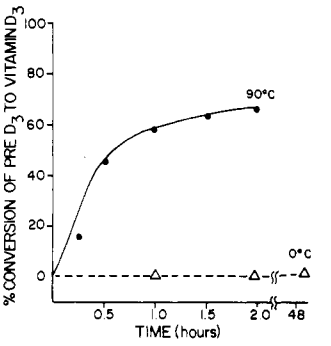

FIGURE 8: Thermal conversion of previtamin D_3 to vitamin D_3 as a function of time at 90 °C (-●-) and at 0 °C (--△--).

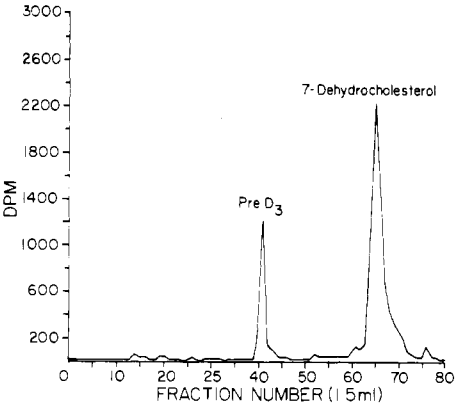

FIGURE 9: High-pressure liquid chromatographic profile on a μ-Porasil column (30 cm × 4 mm, developed with 0.25% isopropyl alcohol in hexane) of a lipid extract from the skin of a rat that received a topical application of 7-dehydro[3α-^{3}H]cholesterol and was then exposed to ultraviolet light.

had an ultraviolet absorption spectrum and a mass spectrum that were identical with those of vitamin D_3 (Holick et al., 1977). However, no evidence of initial production of [^{3}H]-vitamin D_3 in the skin by ultraviolet-light exposure was seen in these experiments, suggesting that all vitamin D_3 ultimately produced in the skin arises exclusively from the thermal conversion of previtamin D_3.

The experiments included in this report involved a more detailed analysis of the photolytic products resulting from ultraviolet irradiation of rat skin and resolve several issues: (1) unequivocal structural identification of previtamin D_3 photolytically produced in vivo; (2) elimination of the possibility that vitamin D_3, as well as previtamin D_3, is produced during ultraviolet irradiation; and (3) determinations of the extent of formation, if any, of tachysterol$_3$ and lumisterol$_3$ during photolysis of 7-dehydrocholesterol in vivo. These issues were examined by exposing 26 vitamin D deficient rats to ultraviolet light and making a lipid extract of the exposed skins. To this lipid extract was added tracer amounts of [^{3}H]-tachysterol$_3$, [^{3}H]lumisterol$_3$, [^{3}H]vitamin D_3, and [^{3}H]previtamin D_3. The lipid fractions containing the tracer compounds were separated, exhaustively purified, and analyzed for the presence or absence of endogenous product.

Previtamin D_3 (13.4 μg) was isolated from 80 g of rat skin and its structure was firmly established based upon (1) its ultraviolet absorption spectrum, λ_{max} 260 nm and λ_{min} 230 nm (Figure 5A), characteristic of the 6,7-cis triene chromophore of previtamin D_3; (2) its mass spectrum, which demonstrated the characteristic molecular ion and fragments for previtamin D_3; and (3) its thermal lability (Figure 6) and conversion to a product that has a mass spectrum identical to that of vitamin D_3 (Figure 7).

Because other investigators (Rauschkolb et al., 1969; Okano et al., 1977; Esvelt et al., 1978) have reported the presence of vitamin D_3 in skin, we added [^{3}H]vitamin D_3 to the lipid extract and exhaustively chromatographed the lipid fraction containing a tracer quantity of [^{3}H]vitamin D_3 and [^{3}H]-tachysterol$_3$. The final product, which contained ~80% of the added radioactivity, demonstrated no ultraviolet absorption for either vitamin D_3 or tachysterol$_3$ (Figure 5C), which meant that there was less than 1 μg of vitamin D_3 and/or tachysterol$_3$ present. The fact that no vitamin D_3 could be isolated from the lipid extract of 80 g of rat skin is in marked contrast to these reports. However, this can be easily explained by the fact that, in each case, in order to remove the large excess of contaminating lipids, the lipid extracts were saponified at refluxing conditions that would extensively convert previtamin D_3 to vitamin D_3. For example, when we incubated [^{3}H]-previtamin D_3 at 90 °C and analyzed the percent conversion of previtamin D_3 to vitamin D_3 as a function of time, at 2 h, 67% of previtamin D_3 had already converted to vitamin D_3, whereas, at 0 °C, less than 2% of previtamin D_3 had converted to vitamin D_3 at 48 h (Figure 8). During our isolation procedures, care was taken never to expose our extracts to temperatures above 5 °C, thus preventing any significant thermal isomerization of our isolated product.

Thus we have firmly established that the mechanism by which 7-dehydrocholesterol is converted to vitamin D_3 by ultraviolet irradiation of skin in vivo is through the initial formation of previtamin D_3, a thermally unstable intermediate that converts to vitamin D_3 at body temperature.

An additional finding of interest is that, unlike the products of ultraviolet irradiation in quartz vessels in vitro (Figure 2), little, if any, of either tachysterol$_3$ or lumisterol$_3$ was formed during photolysis of 7-dehydrocholesterol under the conditions of irradiation used in these in vivo experiments in which abundant previtamin D_3 is formed (Figure 9). While this phenomenon may simply be due to the decreased quanta of ultraviolet light that is able to be transmitted through the skin to 7-dehydrocholesterol in vivo, further investigations are required to rule out a possible selective photolysis reaction.

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Activation of Bovine Chymotrypsinogen A. Isolation and Characterization of μ- and ω-Chymotrypsin†

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ABSTRACT: Threonine-neochymotrypsinogen and alanine-neochymotrypsinogen were prepared by limited hydrolysis of bovine chymotrypsinogen A with α-chymotrypsin. The two neochymotrypsinogens were then activated under conditions designed to trap the immediate protein species arising from the cleavage of the Arg^{15}-Ile^{16} bond. Two trapping procedures were used. In one procedure the neochymotrypsinogen was activated with trypsin under classical rapid activation conditions but in the presence of a competitive inhibitor of chymotryptic action, β-phenylpropionate. In the other procedure, the neochymotrypsinogen was activated with an acid proteinase isolated from *Aspergillus oryzae* using pH conditions which inhibit autolytic activity. Both were successful in preventing autolytic attack of the initial protein and both yielded identical results. The immediate protein species obtained from activated threonine-neochymotrypsinogen, called μ-chymotrypsin, had Thr, Ile, and half-cystine as amino-terminal amino acids. The specific esterase activity of μ-chymotrypsin toward *N*-acetyl-L-tyrosine ethyl ester was two times greater than its stable autolytic product, α_1-chymotrypsin. Similarly, the immediate active species of alanine-neochymotrypsinogen, called ω-chymotrypsin, had Ala, Ile, and half-cystine as amino-terminal amino acids and also displayed an esterase activity which was two times greater than its stable autolytic product, α-chymotrypsin. The first-order rate constants of denaturation in 8 M urea for these two new enzyme species were 0.8 min^{-1} for μ-chymotrypsin and 1.4 min^{-1} for ω-chymotrypsin, respectively. These rate constants differ from all previously known species of chymotrypsin. The genesis of μ- and ω-chymotrypsin and their relationship to current schemes for the activation of bovine chymotrypsinogen A are discussed.

The classical rapid activation of bovine chymotrypsinogen A with trypsin occurs in two sequential steps: the mandatory tryptic hydrolysis of the Arg^{15}-Ile^{16} bond (open arrow in Figure 1) yielding an active but unstable intermediate chymotrypsin called π-chymotrypsin, followed by the rapid autolytic cleavage of the Leu^{13}-Ser^{14} bond (solid arrow in Figure 1) to produce δ-chymotrypsin (Jacobsen, 1947; Bettelheim & Neurath, 1955). Two other bonds in chymotrypsinogen are also subject to chymotryptic hydrolysis and are involved in the slow activation processes of bovine chymotrypsinogen to α-chymotrypsin (Rovery et al., 1957; Desnuelle, 1960), the conversion of δ-chymotrypsin to κ-chymotrypsin and to α-chymotrypsin (Miller et al., 1971; Avery & Hopkins, 1973; Sharma & Hopkins, 1978a), and the production of threonine-neochymotrypsinogen and alanine-neochymotrypsinogen from chy-

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