Isolation and Identification of 24-Dehydroprovitamin D₃ and Its Photolysis to 24-Dehydroprovitamin D₃ in Mammalian Skin*

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Until now it had been assumed that mammalian skin contains only one provitamin D, 7-dehydrocholesterol, that is eventually converted to vitamin D₃ after the skin is exposed to sunlight. Examination by reverse phase high performance liquid chromatography of lipid extracts from young rat skin, however, led to the observation that 7-dehydrocholesterol is not the only provitamin D in rat skin. Another provitamin D, accounting for 22 ± 3% of the total provitamin content of the skin, was resolved from 7-dehydrocholesterol, and, on the basis of ultraviolet spectrophotometry, mass spectrometry, and nuclear magnetic resonance spectrometry, was identified as 24-dehydroprovitamin D₃ (cholesta-5,7,24-trien-3-ol). This new cutaneous provitamin D3 is not unique to the rat because it was also detected in the skin of reptiles, amphibians, birds, aquatic mammals, and humans. To be certain that the cutaneous 24-dehydroprovitamin D₃ was as susceptible as 7-dehydrocholesterol to ultraviolet photolysis, rat skin was exposed to ultraviolet radiation. A reverse phase high performance liquid chromatographic analysis of a lipid extract of rat skin previously exposed to ultraviolet radiation demonstrated the presence of both previtamin D₃ and 24-dehydroprovitamin D₃. Therefore, these observations demonstrate for the first time that mammalian skin has the capacity to produce not one but at least two different vitamin Ds.

When mammalian skin is exposed to ultraviolet radiation, cholesta-5,7-dien-3-ol, or 7-dehydrocholesterol (provitamin D₃), is photoisomerized to previtamin D₃ (1). Once formed in the skin, previtamin D₃ thermally converts to vitamin D₃, which is preferentially translocated into the circulation by the vitamin D binding protein (1). In 1957, Johnston and Bloch (2) were the first to suggest the possibility that cholesta-5,7,24-trien-3-ol (24-dehydroprovitamin D₃) might be an intermediate in cholesterol biosynthesis. Since then, this sterol has been detected in the intestine of guinea pigs treated with triparanol (3) and isolated and chemically characterized from the lung and liver of pigs treated with two inhibitors of cholesterol biosynthesis, AY-9944 and 20,25-diazacholesterol (4). Further evidence that this sterol was an intermediate in cholesterol biosynthesis was established when Scallen (5) demonstrated that [3a-3H]cholesta-5,7,24-trien-3-ol was metabolized to cholesterol in the rat in vivo. However, 24-dehydroprovitamin D₃ is present in only trace quantities in liver, lung, and intestine unless potent inhibitors of cholesterol biosynthesis are employed (3, 4). Until now, it was unknown whether mammalian skin contained 24-dehydroprovitamin D₃. An analysis of young rat skin has revealed that it contains two provitamin D₃. Approximately 75–80% of the total provitamin D content in the skin is 7-dehydrocholesterol, whereas 20–25% is another provitamin D that has been identified as 24-dehydroprovitamin D₃. These results suggest that young rats have the capacity to produce two vitamin Ds (vitamin D₃ and 24-dehydroprovitamin D₃) in their skin.

EXPERIMENTAL PROCEDURES

Materials and Methods—Five 14-day-old Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) that were housed in an ultraviolet β radiation-free environment were killed, and an area of 9 cm² was removed from each back skin, which had previously been shaved. The skins (total weight, 11.56 g) were minced with a razor blade at 5 °C under reduced light and extracted in 250 ml of diethyl ether at 5 °C, under argon and in the dark. After 1 h, 100 ml of distilled water was added. The flask was shaken and then stored under argon at −20 °C until the aqueous layer was frozen. The ether layer was decanted off, filtered through glass wool, and evaporated with absolute ethanol (25 ml) under nitrogen to yield a light-yellow oil.

High Performance Liquid Chromatography (HPLC)—HPLC was performed with a Waters M-45 pump (Waters Associates, Inc., Milford, MA) equipped with a 254-nm UV absorption detector monitor. A Waters μ-Porasil column (24 cm × 14 mm) eluted with 15% (v/v) ethyl acetate in n-hexane was used for preparative straight phase HPLC. A radial compression module with a Waters μ-Porasil cartridge (5-μm particle size, 10 cm × 15 mm) was used for analytical straight phase HPLC. Reverse phase HPLC was accomplished with a Rainin C₁₈-Microsorb column (5-μm particle size, 22 cm × 8 mm) (Rainin Instrument Co., Inc., Woburn, MA). HPLC solvents were purchased from Burdick & Jackson Laboratories, Inc. (Muskegon, MI).

Spectroscopy—A Perkin-Elmer (Oakbrook, IL) 552A UV-visible Spectrometer with 561 recorder was used for obtaining UV spectra. Samples for mass spectral analysis were applied to the glass tip of the direct insertion probe of a Nuclide (State College, PA) 12-90-G single-focus magnetic mass spectrometer and subjected to electron-impact conditions (70 eV) at a temperature of between 110 and 175°C. 1H NMR spectrometry was performed on samples dissolved in (99.96% D) CDC₃ (Stohler Isotopes, Waltham, MA), and spectra were recorded on a Bruker WM 270-MHz instrument (Billerca, MA) using CHCl₃ for internal standardization.
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Photolysis—The provitamin D samples were dissolved in hexane and exposed to $295 \pm 5$ nm radiation from a 2.5-kilowatt xenon-arc lamp as previously described (6).

RESULTS

The yellow oil obtained from the rat skin lipid extract was first subjected to preparative straight phase HPLC (Fig. 1). The peak containing provitamin D$_3$ (peak I in Fig. 1), with a retention time of approximately 15 min, was collected and concentrated under nitrogen. This peak was then dissolved in methanol (1.0 ml) and was chromatographed on reverse phase HPLC using methanol (Fig. 2). Peak I was resolved into two major peaks: peak Ia with a retention time of 9.5 min, and peak Ib (identified as provitamin D$_3$) with a retention time of 11.5 min. The peak Ia fractions were combined, dried under nitrogen, and redissolved in methanol. The UV absorption spectrum (Fig. 3) of peak Ia in methanol exhibited maxima at 295, 282, and 271 nm, typical of the 5,7-diene chromophore (7), and indicated an amount of 60 pg.

Likewise, the provitamin D$_3$ fractions (peak Ib) were combined, dried under nitrogen, and redissolved in methanol. The UV absorption spectrum indicated an amount of 231 pg.

Peak Ia was subjected to final purification on analytical straight phase HPLC (developed with 8% (v/v) ethyl acetate in hexane at 2 ml/min), where its retention time was 20 min (Fig. 4A). Examination of peak Ia by mass spectrometry indicated a molecular ion at $m/z$ 382, which was also the base peak. Therefore, the molecular mass of peak Ia was 2 mass units less than that of provitamin D$_3$ ($M$, 384), suggesting that it contains one more unsaturation than provitamin D$_3$ does.

For further proof that peak Ia was a 5,7-diene sterol, it was exposed to UV radiation to determine whether it could be photolyzed to a previtamin D structure in the way that other 5,7-diene sterols are (8). HPLC analysis of the photolysis reaction revealed that peak Ia was photolyzed into one major product, which is labeled pre-Ia (Fig. 4B). UV spectroscopic

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**FIG. 1.** Straight phase HPLC profile of the lipid extract of the skin from 14-day-old rats. The sample was applied to a preparative $\mu$-Porasil column and developed with 15% (v/v) ethyl acetate in hexane at a flow rate of 3 ml/min. Peak I represents the provitamin D region.

**FIG. 2.** Reverse phase HPLC profile of peak I. Peak I was applied to a C$_18$-Microsorb column and developed with methanol at a flow rate of 1.5 ml/min. The ratio of the amount of peak Ia to peak Ib (provitamin D$_3$) is 1:4.

**FIG. 3.** UV absorption spectrum of peak Ia in methanol. Maxima occur at 295, 282, and 271 nm.

**FIG. 4.** UV absorption spectra of peaks Ia and Ib (provitamin D$_3$) (top) and photolysis product pre-Ia (bottom).
before Ia was exposed to
with 8% for the previtamin Ds (Fig. 5). A mass spectral analysis of
at 230 nm, which is typical for the 6,7-cis-triene chromophore
analysis of peak pre-Ia revealed a $\lambda_{\text{max}}$ at 260 nm and a $\lambda_{\text{min}}$ at 230 nm, which is typical for the 6,7-cis-triene chromophore for the previtamin Ds (Fig. 5). A mass spectral analysis of peak pre-Ia (Fig. 6) showed a molecular ion $m/z$ at 382 (21.5%) and the following major peaks: $m/z$ 349 (22.5%), 335 (9%), 271 (9%), 253 (12%), 176 (14%), 158 (42%), 136 (40%), 118 (46%), 81 (58.5%), and 69 (100%). The relatively high intensity of the peak at $m/z$ 158 compared to 118 was indicative of the 6,7-cis-endocyclic-triene for previtamin D (18). The peaks at $m/z$ 271, arising from the loss of the side chain, and $m/z$ 253, arising from the loss of the side chain minus water, indicated that the additional unsaturation was in the side chain. Furthermore, the base peak at $m/z$ 69 was characteristic of a $\Delta^{24}$ unsaturation. It arises from the allylic cleavage of the 22–23 bond to produce an isoprene unit (9, 10).

Confirmation of the $\Delta^{24}$ unsaturation was provided by the 270-MHz $^1$H NMR spectrum of peak Ia. It showed the usual $\Delta^{25}$-3$\beta$-ol signals with three multiplets centered around 3.65, 5.38, and 5.57 ppm. An additional signal in the olefin region was a pseudo-triplet at 5.09 ppm, integrating for one proton. In the methyl region, two singlets at 0.62 and 0.94 ppm were in agreement with the expected value for the C-18 and C-19 methyl groups, respectively. However, the partly obscured doublet at 0.97 ppm for the C-21 methyl group represented a slight downfield shift. Finally, and most important, the C-26 and C-27 methyl groups were displayed at 1.68 and 1.60 ppm, typical values for allylic methyl protons (11). These values confirm that the unsaturation in the side chain is indeed at the 24-position and that peak Ia is in fact cholesta-5,7,24-trien-3$\beta$-ol (structure shown in Fig. 3), exhibiting $^1$H NMR signals comparable to those already reported for chemically synthesized cholesta-5,7,24-trien-3$\beta$-ol (12, 13).

To be certain that the cutaneous 24-dehydroprovitamin D$_3$ was as susceptible as 7-dehydrocholesterol to ultraviolet photolysis in the skin as it was in the test tube (Fig. 4), skins from three 14-day-old rats were exposed to simulated solar radiation as previously described (6). Lipid extracts from rat skin either exposed to ultraviolet radiation or shielded from light were chromatographed on straight phase HPLC (6). The previtamin D region was collected and chromatographed on reverse phase HPLC using methanol (Fig. 7). As can be seen in Fig. 7B, two major peaks, pre-Ia (retention time = 10.02 min) and pre-Ib (retention time = 11.64 min), were observed. Neither peak was present in the unexposed skin (Fig. 7A). Both peaks pre-Ia and pre-Ib had ultraviolet absorption spectra with $\lambda_{\text{max}}$ at 260 nm and $\lambda_{\text{min}}$ at 230 similar to that in Fig. 5, demonstrating that they were previtamin Ds. Pre-Ia and pre-Ib were further identified as 24-dehydroprovitamin D$_3$ and previtamin D$_3$, respectively, based on their chromatographic migration with authentic standards.

**DISCUSSION**

Cholesta-5,7,24-trien-3$\beta$-ol has an intermediary role in cholesterol biosynthesis (14). It is a precursor of 7-dehydrocholesterol and of 24-dehydrocholesterol (15) and has been isolated not only from mammalian intestine (3), liver (15), and lung (4), but also from fungi (16).

To date, it had been assumed that mammalian skin produces only one provitamin D, 7-dehydrocholesterol, that acts as the substrate for the sun-mediated formation of vitamin D$_3$. We now present unequivocal evidence that in rat skin there is an additional provitamin D whose structure has now been firmly established as cholesta-5,7,24-trien-3$\beta$-ol (24-dehydroprovitamin D$_3$) based on UV absorption, $^1$H NMR, and mass spectral analysis.

Previous reports suggested that cholesta-5,7,24-trien-3$\beta$-ol exists in trace quantities in liver, lung, and intestine and could be isolated from these tissues in sufficient quantities only
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FIG. 6. Mass spectrum of peak pre-Ia obtained from the UV radiation of peak I isolated from rat skin. For purposes of illustration, the intensities of all peaks in this spectrum are calculated relative to m/z 118 as the base peak. The relatively high intensity of the peak at m/z 158 compared to 118 is indicative of the 6,7-cis-endocyclic-triene for previtamin D (18). The peaks at 118 and 136 also may originate from the thermal rearrangement of 24-dehydroprevitamin D$_3$ to 24-dehydrovitamin D$_3$.

FIG. 7. Reverse phase HPLC profile of the previtamin D region collected from straight phase HPLC of lipid extracts from rat skin either shielded from (A) or exposed to ultraviolet radiation (B).

when animals were fed potent inhibitors of cholesterol biosynthesis (3, 4). We have found that, in the skin of 14-day-old rats, that 22 ± 3% (n = 10 ± S.E.) of the provitamin D content is represented by 24-dehydroprevitamin D$_3$. Inasmuch as 24-dehydroprevitamin D$_3$ and provitamin D$_3$ are equally susceptible to photolysis to form previtamin D$_3$ in the test tube and in rat skin (Figs. 4 and 7), our results suggest that, during exposure to sunlight, young rats are able to photosynthesize previtamin D$_3$ and 24-dehydroprevitamin D$_3$ that, in turn, can thermally isomerize to vitamin D$_3$ and 24-dehydrovitamin D$_3$, respectively. The physiologic function, if any, of 24-dehydrovitamin D$_3$ in the rat is unknown at present. It has been suggested that 24-dehydrovitamin D$_3$ is biologically active (17), but to a lesser degree than is vitamin D$_3$, suggesting that it indeed may be metabolized to a biologically active form similar to vitamin D$_3$. It is known, for example, that 3α,7α,12α-trihydroxy-5β-cholestanoic acid is hydroxylated at C-24 via the formation of a $\Delta^{24}$-unsaturated intermediate (19). Finally, the presence of 24-dehydrovitamin D$_3$ in the skin of the rat is not unique to this animal. Preliminary results from our laboratory suggest that 24-dehydrovitamin D$_3$ is also present in the skins of reptiles, amphibians, birds, aquatic mammals, and humans.

REFERENCES