sensitivity reactions in patients with breast carcinoma but not in healthy controls (*2*). The findings of previous studies in conjunction with the immunomorphological detection of a putative carcinoma-associated antigen (T antigen) described in our report suggest a possible link between neoplastic transformation and the development of tumor immunity.

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Photosynthesis of Previtamin D_3 in Human Skin and the Physiologic Consequences

Abstract. *Photosynthesis of previtamin D_3 can occur throughout the epidermis and in the dermis when hypopigmented Caucasian skin is exposed to solar ultraviolet radiation. Once previtamin D_3 is formed in the skin, it undergoes a temperature-dependent thermal isomerization that takes at least 3 days to complete. The vitamin D-binding protein preferentially translocates the thermal product, vitamin D_3, into the circulation. These processes suggest a unique mechanism for the synthesis, storage, and slow, steady release of vitamin D_3 from the skin into the circulation.*

About 60 years ago, exposure of children to radiation from a mercury-vapor quartz lamp (*1*) or to sunlight (*1*) was reported to prevent or to cure rickets. Subsequent investigations (*2*) culminated in the structural identification of vitamin D_3 (D_3) and its photochemical precursor 7-dehydrocholesterol (7-DHC) (*2*). It had been assumed that when skin is exposed to sunlight, part of the stores of 7-DHC in the epidermis undergoes a photochemical reaction resulting in the eventual formation of D_3. This concept was supported by the isolation of D_3 from human and rat skin (*3*). Until recently, however, the exact sequence of steps leading to the cutaneous photoproduction of D_3 was unknown, including the sites in the skin where D_3 formation occurs and the mechanism for entry of the vitamin into the circulation. Recently, we (*4*) established, in rat skin in vivo, that previtamin D_3 (preD_3) is the principal photoproduct that results from exposure of 7-DHC to a physiological dose of ultraviolet radiation. Because preD_3 is thermally labile and readily isomerizes in vitro by a temperature-dependent process to D_3 (*2*), we suspected that preD_3, once formed in the skin in vivo, would isomerize to form D_3.

We report on (i) the cutaneous sites where preD_3 formation occurs, (ii) the temperature dependence of the thermal equilibration of preD_3 to D_3 in vitro and in vivo, and (iii) the role of an α_1-globulin (the vitamin D–binding protein for translocating D_3 from the skin into the circulation.

We synthesized [3α-^{3}H]7-DHC, [3α-^{3}H]preD_3, and [3α-^{3}H]D_3, each with a specific activity of 4.8 Ci/mmole (*4*). Ten milligrams of 7-DHC was exposed to ultraviolet B radiation to generate sufficient quantities of preD_3 for the assay of binding protein. Chromatography of the photolytic products, skin lipid extracts, and thermal equilibration reactions was performed in 8 percent ethyl acetate in *n*-hexane on a high-performance liquid chromatography system (HPLC) equipped with a radial compression module containing a Radial-Pak-B (10 by 0.8 cm) coupled with an ultraviolet absorption detector at 254 nm (model 440, Waters Associates) and a printer-plotter data module integrator (Waters Associates). This chromatographic system permits complete resolution to baseline of 7-DHC, preD_3, and D_3. Quantitation of the concentrations of 7-DHC and preD_3 in skin lipid extracts was based on measurement of the integrated area under the appropriate peak and comparison with standard curves that were generated by plotting the integrated area under the peak as a function of a known concentration of pure compound. Identification of the skin lipid peak designated as 7-DHC was based on its ultraviolet absorption spectrum (maximum wavelength at 295, 282, and 271 nm), which is characteristic for $\Delta^{5,7}$-diene sterols, and its mass spectrum with M^+ at 384. Identification of the skin lipid peak designated as preD_3 was based on (i) its appearance only after skin containing 7-DHC was subjected to ultraviolet B radiation (spectral range, 290 to 320 nm), (ii) comparison chromatography studies with a preD_3 standard, and (iii) its thermal lability (*4*).

The location in human skin where preD_3 is initially formed after exposure to ultraviolet B radiation was determined by separating layers of skin without altering the integrity of the cellular structure. Surgically obtained hypopigmented Caucasian human leg skin with the subcutaneous fat removed was cut into samples (6.25 cm^2) and separated by incubation with staphylococcal exfoliatin [a substance that specifically cleaves the epidermis at the stratum granulosum–stratum spinosum interface (*5*)] (20 mg/ml) or by immersion in a 60°C water bath for 30 seconds (*6*) (this technique cleaves the epidermis at the stratum spinosum–stratum basale interface). After either treatment, but before physical separation of the skin layers, the stratum corneum side of the skin samples was exposed to ultraviolet B radiation (0.5 J/cm^2) in a narrow wave band centered at 295 nm with a 10-nm half-bandwidth. This radiation was obtained from a 6.5-kW xenon-mercury arc lamp coupled to a holographic grating monochromator (HL-300, Jobin-Yvon). Control skin samples were kept in an ultraviolet-free environment. Immediately after irradiation, the toxin-treated skin was separated into a top layer (stratum corneum and stratum granulosum) and a bottom layer (stratum spinosum, stratum basale, and dermis), whereas the heat-treated skin was differently separated into a top layer (stratum corneum, stratum granulosum, and stratum spinosum) and a bottom layer (stratum basale and dermis). The basal cells of the stratum basale were collected by mechanically scraping them off the dermis. Contents of all skin layers were confirmed by histological examination. The separated layers of skin were extracted with 8 percent ethyl acetate in *n*-hexane for 24 hours at −20°C. The extracts were centrifuged, the supernatant was dried under nitrogen and weighed, and a portion of each sample was chromatographed by HPLC in duplicate to determine 7-DHC and preD_3 concentrations.

To examine the thermal isomerization of preD_3 to D_3 in vitro, 1 μCi of [3α-^{3}H]preD_3 was dissolved in methanol, flushed with argon, and incubated in triplicate at 0° ± 1°, 25° ± 1°, or 37° ± 1°C. At various times, portions were removed

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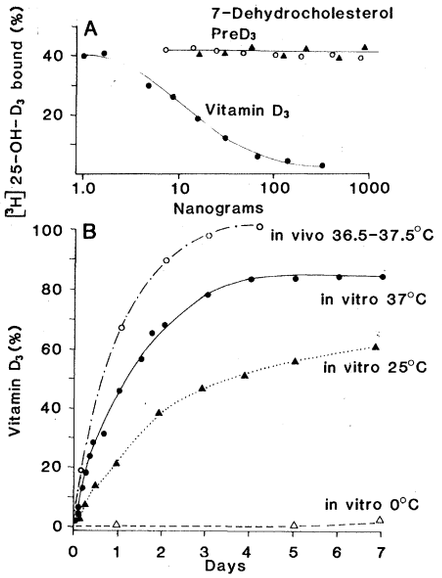

Fig. 1. (A) Displacement of tritiated 25-hydroxyvitamin D_3 ([^{3}H]25-OH-D_3) from rat vitamin D-binding protein by vitamin D_3, previtamin D_3 (*PreD*$_3$), and 7-dehydrocholesterol. Each data point represents an average of two separate determinations. (B) Thermal conversion of previtamin D_3 to vitamin D_3 as a function of time. Each time point represents two experiments, each determined in triplicate. There was excellent agreement for each of the data points, with less than 2 percent variation.

and chromatographed by HPLC in triplicate to determine the amount of [3α-^{3}H]preD$_3$ that had converted to [3α-^{3}H]D$_3$. The time course of the thermal isomerization reaction in vivo was determined by two methods: (i) [3α-^{3}H]preD$_3$ (1 μCi) in 10 μl of 95 percent ethanol was topically applied to the backs of shaved vitamin D-deficient rats, or (ii) [3α-^{3}H]7-DHC in 10 μl of 95 percent ethanol was topically applied to similar rats, and these rats were then exposed to broad-band ultraviolet radiation (0.2 J/cm^2) (*4*) to partly convert, in vivo, [3α-^{3}H]7-DHC to [3α-^{3}H]preD$_3$. At various intervals, two rats from each group were killed, and whole skin that received the topical radiolabel was excised entirely and immediately extracted with 8 percent ethyl acetate in *n*-hexane at −20°C. The extracts were dried under nitrogen at 4°C and chromatographed by HPLC; 1-ml fractions were collected for determination of radioactivity (*4*).

The affinity of vitamin D-binding protein for 7-DHC, preD$_3$, and D$_3$ was determined (*7*) after each of the compounds had been purified to homogeneity by HPLC. There was no detectable displacement of tracer 25-hydroxyvitamin D$_3$ (25-OH-D$_3$) when either 7-DHC or preD$_3$ (up to 1000 ng per tube) was added (Fig. 1A). Fifty percent of tracer 25-OH-D$_3$ was displaced with D$_3$ at a concentration of 30 ng per tube (Fig. 1A).

Table 1 illustrates the average concentration of 7-DHC in the various strata of the Caucasian skin sample examined. Although all of the epidermal strata and the dermis contained 7-DHC, the highest concentration of 7-DHC per milligram of lipid was located in the stratum basale. The largest amount of this sterol per unit surface of skin, however, was present in the stratum spinosum. Thus, the stratum spinosum and stratum basale potentially have the highest capacity for preD$_3$ formation. When human hypopigmented Caucasian skin was exposed to ultraviolet B radiation, photosynthesis of preD$_3$ occurred throughout the entire epidermis and, to a small extent, in the dermis (Table 1); the stratum spinosum and stratum basale had the highest concentrations of preD$_3$. Similar results were obtained when we exposed Caucasian skin to simulated solar ultraviolet radiation, which was obtained from a 2.5-kW xenon arc lamp with a dichroic mirror and a 3-mm filter (WG 305-C, Schott Optical) which simulates noon sunlight at the equator at sea level.

An analysis of the influence of temperature on the equilibration of preD$_3$ to D$_3$ in vitro is illustrated in Fig. 1B. At 0°C there was no detectable (< 2 percent) formation of D$_3$ after 1 week, whereas at 25°C approximately 50 percent of preD$_3$ equilibrated to D$_3$ in 48 hours. When preD$_3$ was incubated at 37° ± 1°C, approximately 50 percent of the preD$_3$ converted to D$_3$ by 28 hours, and equilibrium was reached after 4 days with 83 percent of preD$_3$ converted to D$_3$. In vivo, the surface temperature of rat skin (as determined with a thermocouple) was 36.5° ± 0.5°C and that of the dermis was 37.5° ± 0.5°C. Fifty percent of the radiolabeled preD$_3$ that was topically applied to the shaven backs of rats converted to D$_3$ in 18 hours, and by 3 days there was a 95:5 ratio of D$_3$ to preD$_3$ remaining in the skin (Fig. 1B). A similar time course for the conversion of preD$_3$ to D$_3$ was seen when preD$_3$ was generated from topically applied [3α-^{3}H]7-DHC exposed to ultraviolet B radiation.

Table 1. Concentration of 7-DHC and preD$_3$ in the various strata of a hypopigmented Caucasian whole-skin sample after exposure to ultraviolet radiation at 0.5 J/cm^2. The concentrations represent the average of two determinations from the same thigh-skin sample. Analysis of the 7-DHC concentration in the various strata of skin obtained from different areas of the body or from different individuals indicates that although the absolute amount of 7-DHC may vary, the stratum basale contains the highest concentration of 7-DHC per milligram of lipid and the stratum spinosum has the highest content of 7-DHC per unit surface area.

Skin strata	7-DHC (nanogram per milligram of lipid)	7-DHC (nanogram per 6.25 cm^2)	PreD$_3$ (nanogram per 6.25 cm^2)
Stratum corneum + stratum granulosum	180	360	20
Stratum spinosum	630	2459	114
Stratum basale	1720	1892	113
Dermis	88	1670	3

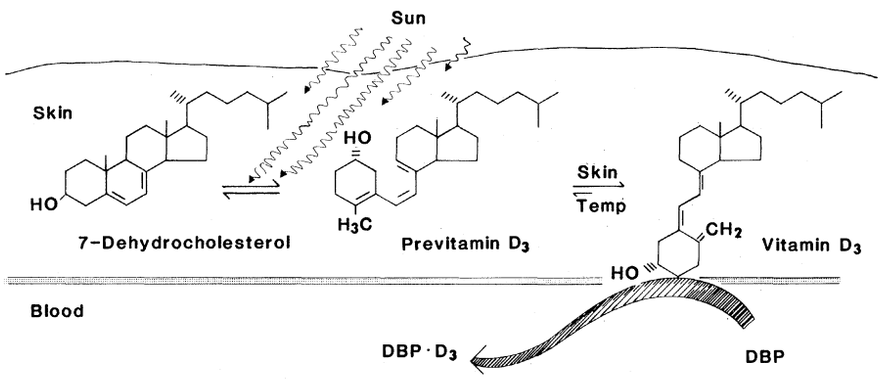

Fig. 2. Diagrammatic representation of the formation of previtamin D$_3$ in the skin during exposure to the sun and its subsequent thermal conversion to vitamin D$_3$, which, in turn, is bound to the vitamin D-binding protein (*DBP*) in plasma for transport into the circulation.

Collectively, these data provide important insights concerning basic details of the photochemical production of D$_3$ in vivo. When hypopigmented Caucasian skin is exposed to sunlight, ultraviolet B radiation (spectral range, 290 to 320 nm) penetrates into the skin and causes the photoconversion of 7-DHC to preD$_3$ throughout the epidermis and in the dermis. The regions of skin that potentially have the highest capacity for preD$_3$ formation per unit area are the stratum spinosum and stratum basale. However, the

amount of $preD_3$ that is formed in the various layers will be dependent on the amount of ultraviolet radiation reaching each layer. Once formed in the skin, $preD_3$ is isomerized to D_3 by a nonphotochemical rearrangement of the $preD_3$ triene system at a rate dictated by the temperature of the skin. At 37° ± 1°C, this thermal isomerization reaction appears to be more rapid in vivo than in vitro (Fig. 1B). This difference seems most likely to be due to the specific translocation, by the vitamin D-binding protein, of the thermal product, D_3, from the skin into the circulation (in the capillary bed) beneath the dermoepidermal junction. The net effect of this highly specific translocation would be to remove the product D_3 (as it is being formed) from the reaction, thereby promoting the conversion of $preD_3$ to D_3. Therefore a true equilibrium is never established (as occurs in vitro) and essentially 100 percent of $preD_3$ converts to D_3. It is unlikely that the differences between the thermal isomerization of $preD_3$ to D_3 in vitro and in vivo are due to a cutaneous catalytic process (such as an enzymatic, lipid-lipid, or lipid-protein interaction), since thermal equilibration time courses for incubations of plasma and skin homogenates with $preD_3$ at 37° ± 1°C were identical to those with $preD_3$ in methanol at 37° ± 1°C. Furthermore, there was no evidence that $preD_3$ was metabolized more rapidly than D_3 in the skin, nor was there any difference in the disappearance of radiolabeled $preD_3$ and D_3 given intravenously, a finding which suggests that there is no significant difference in the metabolic clearance rates of these two secosterols.

The physiological advantage of photosynthesizing $preD_3$ in the skin during exposure to the sun is illustrated in Fig. 2. During exposure to sunlight, the ultraviolet B portion of the solar spectrum produces the photochemical conversion of cutaneous 7-DHC to $preD_3$. Immediately upon its formation, $preD_3$ begins to isomerize by a temperature-dependent process to D_3. This process permits the skin to continually synthesize (from its previtamin) and release D_3 into the circulation for up to 3 days after a single exposure to sunlight. In warm-blooded animals, this equilibrium reaction remains relatively unchanged inasmuch as the body temperature is closely regulated; in cold-blooded species, the body temperature fluctuates greatly and therefore this equilibrium reaction would be highly variable. Once D_3 is formed, the vitamin D-binding protein translocates it preferentially into the circulation and ensures the efficient conversion of the small quantities of $preD_3$ to D_3 by shifting the $preD_3 \rightleftharpoons D_3$ reaction to the right at the time when equilibrium is being approached. Thus, the skin serves as (i) the site for the synthesis of 7-DHC, (ii) a reservoir for the storage of the primary photoproduct, $preD_3$, and (iii) the organ where the slow thermal conversion of $preD_3$ to D_3 occurs.

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Asymmetry of the Acetylcholine Channel Revealed by Quaternary Anesthetics

Abstract. *Tissue-cultured rat myoballs were examined electrophysiologically with a suction pipette, which was used for voltage clamping and internal perfusion. The lidocaine derivative QX-314 caused a time- and membrane potential–dependent block of acetylcholine-induced current only when applied from the extracellular membrane surface. The same compound caused a use-dependent block of the sodium channel only from the intracellular membrane surface. These experiments demonstrate a fundamental asymmetry of the acetylcholine receptor-channel complex.*

Pharmacological agents are frequently used to examine the inside-outside symmetry of membrane-bound proteins. The strategy is to examine the effects of a drug when applied to either the intracellular or extracellular membrane surface. Recent electrophysiological data suggest that, in the acetylcholine (ACh) channel, ion permeation occurs through a symmetrically shaped pore (*1*). We tested this postulate by studying the action of ACh channel blockers on both membrane surfaces of tissue-cultured rat muscle cells. We report an asymmetrical action of the lidocaine derivatives QX-314 (quaternary *N*-ethyl) and QX-222 (quaternary *N*-methyl) on the ACh-induced current of these cells.

Myoballs were grown from tissue-cultured muscle of neonatal rat thighs (*2*).

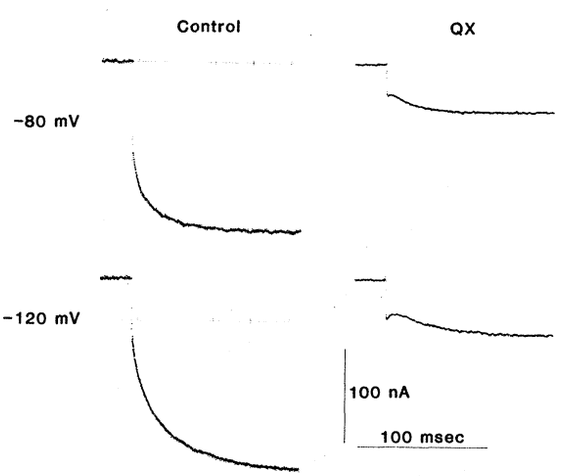

Fig. 1. Effect of external QX-314 (0.1 m*M*). The control traces (left) show the ACh current relaxations for voltage jumps to − 80 mV and − 120 mV from the holding potential of − 40 mV. In each case the current was fitted by a single exponential [$\tau(-80) = 15.3$ msec; $\tau(-120) = 22.9$ msec]. Five minutes after 0.1 m*M* QX-314 was added to the bath, the traces on the right were recorded. The iontophoretic current was approximately the same as in the control case. Derivative QX-314 caused a time- and voltage-dependent reduction of the ACh current (temperature, 18°C).

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