
CELL BIOLOGY AND METABOLISM:

# A Liposomal Model That Mimics the Cutaneous Production of Vitamin $D_3$: STUDIES OF THE MECHANISM OF THE MEMBRANE-ENHANCED THERMAL ISOMERIZATION OF PREVITAMIN D3 TO VITAMIN D3

Xiao Quan Tian and Michael F. Holick

*J. Biol. Chem. 1999, 274:4174-4179.*
*doi: 10.1074/jbc.274.7.4174*

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 7, Issue of February 12, pp. 4174–4179, 1999

*Printed in U.S.A.*

# A Liposomal Model That Mimics the Cutaneous Production of Vitamin $D_3$

## STUDIES OF THE MECHANISM OF THE MEMBRANE-ENHANCED THERMAL ISOMERIZATION OF PREVITAMIN $D_3$ TO VITAMIN $D_3$*

(Received for publication, July 13, 1998, and in revised form, November 25, 1998)

**Xiao Quan Tian‡ and Michael F. Holick§¶**

*From the Vitamin D, Skin, and Bone Research Laboratory, Endocrinology Nutrition and Diabetes Section, §Departments of Medicine and ‡Physiology, Boston University Medical Center, Boston, Massachusetts 02118*

**We reported previously that the rate of previtamin $D_3$ (preD$_3$) ⇌ vitamin $D_3$ isomerization was enhanced by about 10 times in the skin compared with that in organic solvents. To elucidate the mechanism by which the rate of this reaction is enhanced in the skin, we developed a liposomal model that mimicked the enhanced isomerization of preD$_3$ to vitamin $D_3$ that was described in human skin. Using this model we studied the effect of changing the polarity of preD$_3$ as well as changing the chain length and the degree of saturation of liposomal phospholipids on the kinetics of preD$_3$ ⇌ vitamin $D_3$ isomerization. We found that a decrease in the hydrophilic interaction of the preD$_3$ with liposomal phospholipids by an esterification of the 3β-hydroxy of preD$_3$ (previtamin D$_3$-3β-acetate) reduced the rate of the isomerization by 67%. The addition of a hydroxyl on C-25 of the hydrophobic side chain (25-hydroxyprevitamin $D_3$), which decreased the hydrophobic interaction of preD$_3$ with the phospholipids, reduced the rate by 87%. In contrast, in an isotropic *n*-hexane solution, there was little difference among the rates of the conversion of preD$_3$, its 3β-acetate, and 25-hydroxy derivatives to their corresponding vitamin $D_3$ compounds. We also determined rate constants (*k*) of preD$_3$ ⇌ vitamin $D_3$ isomerization in liposomes containing phosphatidylcholines with different carbon chain lengths. The rates of the reaction were found to be enhanced as the number of carbons ($C_n$) in the hydrocarbon chain of the phospholipids increased from 10 to 18. In conclusion, these results support our hypothesis that amphipathic interactions between preD$_3$ and membrane phospholipids stabilize preD$_3$ in its "cholesterol like" cZc-conformer, the only conformer of preD$_3$ that can convert to vitamin $D_3$. The stronger these interactions were, the more preD$_3$ was likely in its cZc conformation at any moment and the faster was the rate of its conversion to vitamin $D_3$.**

During evolution, many poikilothermic and homeothermic terrestrial vertebrates including humans acquired the ability to photosynthesize vitamin $D_3$ in their skin (1, 2) and to use vitamin $D_3$ to enhance the efficiency of dietary calcium absorption to maintain a healthy and mineralized skeleton (3, 4). Cutaneous synthesis of vitamin $D_3$ consists of both photo- and thermal reactions (5, 6) (Fig. 1). When exposed to sunlight, ultraviolet-B (UV-B) (290–315 nm) radiation photolyzes provitamin $D_3$ (7-dehydrocholesterol (7-DHC)[1]) (Fig. 1*A*), a $\Delta^{5,7}$-sterol synthesized in the skin, into a 9,10-*seco* B sterol, cZc-previtamin $D_3$ (cZc-preD$_3$) (Fig. 1*B*). This novel structure enables the *seco*-sterol to change its configuration between the preD form and the vitamin D form via a 1,7-sigmatropic hydrogen shift (Fig. 1, *B* and *C*) (7–11), one of the pericyclic processes defined by Woodward and Hoffmann (8). This thermally dependent reaction is the final step to produce vitamin $D_3$ in the skin and represents one of the best known examples of a concerted reaction that occurs *in vivo*. Although it has long been noted that, like other concerted reactions, the interconversion between preD$_3$ ⇌ vitamin $D_3$ is not influenced by a solvent effect when carried out in isotropic solutions (12), our recent data have revealed that both kinetics and thermodynamics of this reaction may change significantly in many anisotropic microenvironments (13, 14). Therefore, this simple yet physiologically important reaction provides us an ideal model to study the mechanism by which the kinetics of a concerted reaction is modified by an anisotropic medium. The *seco*-B ring of preD$_3$ consists of a conjugated triene system, which confers preD$_3$ with high conformational mobility (Fig. 1). Although the middle double bond in the triene system of preD$_3$ is in the *cis* (*Z*) configuration, two conformations arise from rotation around the single bonds $C_5$-$C_6$ and $C_7$-$C_8$ within the triene system *i.e.* cZc or s-*cis*,s-*cis* conformations (Fig. 1*B*) and tZc or s-*trans*,s-*cis* conformations (Fig. 1*D*) (9–11). It is known that to chemically isomerize to vitamin $D_3$, preD$_3$ is required to be in the cZc conformation (Fig. 1*B*) (9–11). From a structure-reactivity point of view, it is important to know whether there is any effect of the conformational restraints imposed by anisotropic media on the chemical transformation of preD$_3$ into vitamin $D_3$. We have previously shown that the rate of the formation of vitamin $D_3$ from preD$_3$ was enhanced by about 10-fold in the skin of terrestrial vertebrates compared with the rate of the same isomerization in an isotropic solution (1, 2, 13). Based on the finding that the major fraction of cellular 7-DHC and preD$_3$ are present in the cell membrane (13), we proposed a mechanism for the membrane-enhanced isomerization of preD$_3$ to vitamin $D_3$. We hypothesized that within the anisotropic membrane bilayers, amphipathic interactions between preD$_3$ and phospholipids stabilized the "cholesterol-like" cZc

* This work was supported in part by National Institutes of Health Grant RO1-AR-36963 and NASA Grant NAGW4936. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Tel.: 617-638-4545; Fax: 617-638-8882; E-mail: mfholick@bu.edu.

[1] The abbreviations used are: 7-DHC, 7-dehydrocholesterol; preD$_3$, previtamin $D_3$; 25(OH)7-DHC, 25-hydroxy-7-dehydrocholesterol; 25(OH)L$_3$, 25-hydroxylumisterol$_3$; 25(OH)T$_3$, 25-hydroxytachysterol$_3$; HPLC, high performance liquid chromatography; DDPC, didecanoylphosphatidylcholine; DLPC, dilauroylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; DAPC, diarachidoylphosphatidylcholine.

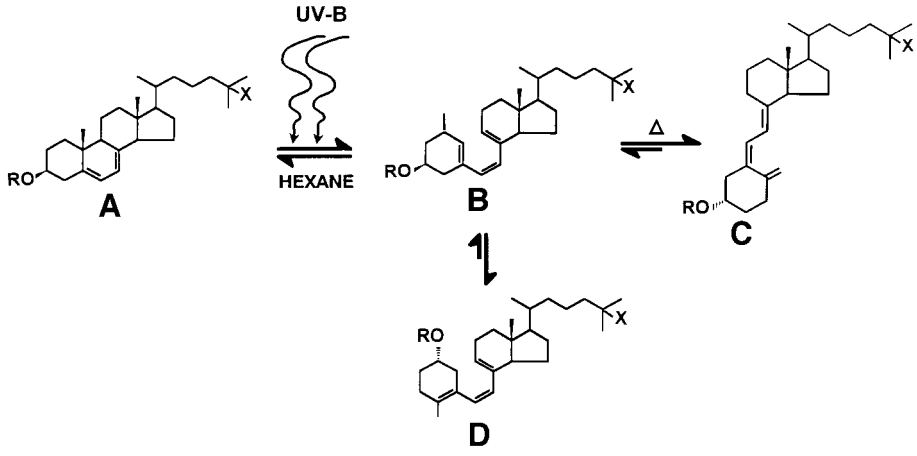


FIG. 1. **Photolysis of 7-dehydrocholesterol and its analogs to previtamin $D_3$ and its analogs and their subsequent thermal isomerization to the corresponding vitamin $D_3$ forms.** *A*, $R$ = H; $X$ = H: 7-dehydrocholesterol; $R$ = H, $X$ = O: 25-hydroxy-7-dehydrocholesterol; $R$ = $CH_3CO$-, $X$ = H: 7-dehydrocholesterol-3$\beta$-acetate. *B*, $R$ = H, $X$ = H: cZc-previtamin $D_3$; $R$ = H, $X$ = OH: cZc-25-hydroxyprevitamin $D_3$; $R$ = $CH_3CO$-, $X$ = H: cZc-previtamin $D_3$-3$\beta$-acetate. *C*, $R$ = H, $X$ = H: vitamin $D_3$; $R$ = H, $X$ = OH: 25-hydroxyvitamin $D_3$; $R$ = $CH_3CO$-; $X$ = H: vitamin $D_3$-3$\beta$-acetate; *D*, $R$ = H; $X$ = H: tZc-previtamin $D_3$; R = H, $X$ = OH: tZc-25-hydroxyprevitamin $D_3$; $R$ = $CH_3CO^-$, $X$ = H: tZc-previtamin $D_3$-3$\beta$-acetate.

conformation of $preD_3$ (Fig. 2) and shifted the conformational equilibrium of $preD_3$ toward cZc-$preD_3$, the only conformation of $preD_3$ that isomerizes to vitamin $D_3$. Therefore, the rate of $preD_3 \rightleftharpoons$ vitamin $D_3$ reaction is enhanced. To test this hypothesis, the effect of changing the polarity of 7-DHC as well as changing the chain length and the degree of saturation of the liposomal phospholipids on the kinetics of $preD_3 \rightleftharpoons$ vitamin $D_3$ reaction were investigated. We have demonstrated for the first time that there is a positive correlation between the strength of the amphipathic interactions of $preD_3$ with liposomal phospholipids and the rate of $preD_3 \rightleftharpoons$ vitamin $D_3$ isomerization.

## EXPERIMENTAL PROCEDURES

### *Materials*

7-DHC and vitamin $D_3$ were purchased from Sigma. 7-DHC-3$\beta$-acetate was purchased from Steroids Inc. (Wilton, NH). 25-Hydroxy-7-dehydrocholesterol (25(OH)7-DHC) was a kind gift from Dr. Rick Gray (Amoco Bioproducts, Naperville, IL). All the above chemicals were purified by high performance liquid chromatography (HPLC) and stored at −80 °C before use. Saturated lipids including didecanoylphosphatidylcholine (DDPC, C10:0) (99%), dilauroylphosphatidylcholine (DLPC, C12:0) (99%), dipalmitoylphosphatidylcholine (DPPC, C16:0) (99%), distearoylphosphatidylcholine (DSPC, C18:0) (99%), diarachidoylphosphatidylcholine (DAPC, C20:0) (99%), and unsaturated lipids dipalmitoleoylphosphatidylcholine (C16:1, [*cis*]-9) (99%) were obtained from Sigma.

HPLC was performed with a P1000 pump equipped with a UV2000 UV-visible absorption detector (Thermo Separation Products, San Jose, CA). An Econosphere silica column (250 × 4.6 mm, 5 $\mu$m; Alltech Associate, Inc., Deerfield, IL) was used to separate the various vitamin D metabolites except their 3$\beta$-acetate derivatives, which were separated by a Cyclobond I 2000 column (100 × 4.6 mm, 5 $\mu$m; Advanced Separation Technologies Inc., Whippany, NJ).

UV spectra of vitamin $D_3$, $preD_3$ and their photoisomers as well as their 25-hydroxylated derivatives were recorded either by a UV spectrometer (U-2000, Hitachi Instruments, Inc., Stoughton, MA) or by an online HPLC UV detector (UV2000).

### *Methods*

*Preparation of Liposomes and Human Skin Samples*—Liposomes were prepared by a modified procedure reported by Wiseman *et al.* (15, 16), whereby 7-DHC and vitamin $D_3$ were incorporated into liposomes. The different lipids were dissolved in pure chloroform to give a final concentration of 20 mg/ml. An aliquot of the stock solution was taken and mixed with an equal volume of one of the following standard solutions of 7-DHC, 25(OH)7-DHC, and 7-DHC-3$\beta$-acetate. The organic solvent was evaporated by a stream of nitrogen. When necessary, the vials containing lipids were left under high vacuum overnight to remove trace solvent. The thin lipid film was resuspended in a 10 mM phosphate buffer (pH = 7.4) followed by vortexing and sonication. The resulting liposomes (lipid concentration, 6.75 mM; molar ratio of lipid to incorporated sterol, 100:2) were sealed in ampules that had previously been flushed with argon. The preparation of liposomes (hydration and vortexing) was performed above the transition temperature ($T_m$) of the corresponding lipid mixture (DMPC, 23 °C; DPPC, 41.5 °C; and DSPC, 58 °C) (17).

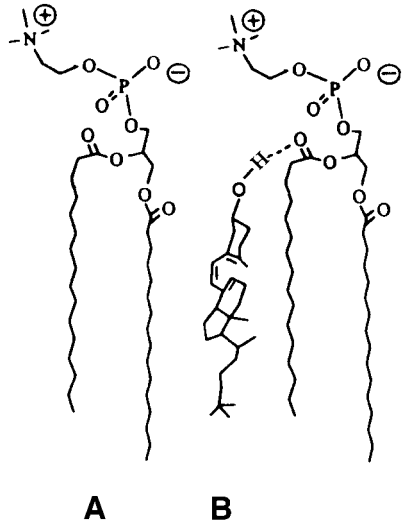


FIG. 2. **Proposed theoretical structural model for the localization of the cZc-previtamin $D_3$ in the phospholipids of a membrane.** Based on the amphipathic nature and conformational mobility of previtamin $D_3$, we proposed the following model to show the spatial relationship between previtamin $D_3$ and phospholipids. We postulated that in the membrane, the cholesterol like cZc-previtamin $D_3$ is aligned parallel to its neighboring phospholipids, with its polar 3$\beta$-hydroxy interacting with the polar head groups of the phospholipids through hydrogen bonding, and the hydrophobic rings and side chain interacting with the nonpolar acyl chains of the lipids through hydrophobic and van der Waals interactions. *A*, phosphatidylcholine; *B*, cZc-previtamin $D_3$.

Human skin samples were prepared by a previously described method (5, 13). Briefly, neonatal foreskin was cleaned from subcutaneous tissues and cut into small pieces (about 0.3 $cm^2$). Before UV irradiation, skin samples were immersed in a water bath at 60 °C for 30 s (this technique allows the separation of the dermis from epidermis at the stratum basale).

*Photoreaction and Thermal Isomerization*—Heat-treated skin samples, ampules containing liposome preparations, ampules containing a solution of 7-DHC or its analogues in *n*-hexane (0.135 mM), and ampules containing a solution of 7-DHC-DPPC in *n*-hexane (7-DHC, 0.135 mM; DPPC, 6.75 mM) were placed on ice and irradiated by UV Medical Sunlamps (National Biological Corp., Cleveland, OH) for 3 min (13). Spectral output for these lamps peaked at about 314 nm, and about 56% of the total output was within UV-B range (13). UV-irradiated samples were incubated at 37 °C, and aliquots were taken at different time intervals. The formation of vitamin $D_3$ from $preD_3$ was analyzed by the HPLC method described below.

*HPLC Analysis*—Immediately after incubation, the heat-treated skin was separated into epidermis and dermis according to a previously described method (5). Epidermis and liposomes were extracted three times with 8% ethyl acetate in *n*-hexane (5, 13). The organic phase

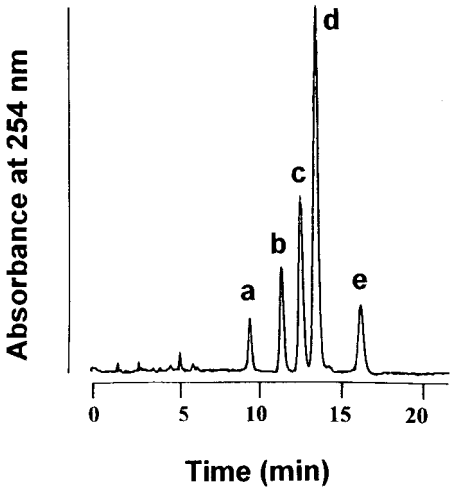


FIG. 3. **HPLC separation of 25-hydroxy-7-dehydrocholesterol, its photoisomers, and 25-hydroxyvitamin $D_3$.** Peak *a*, 25(OH)$L_3$; peak *b*, 25(OH)$D_3$; peak *c*, 25(OH)pre$D_3$; peak *d*, 25(OH)7-DHC, and peak *e*, 25(OH)$T_3$.

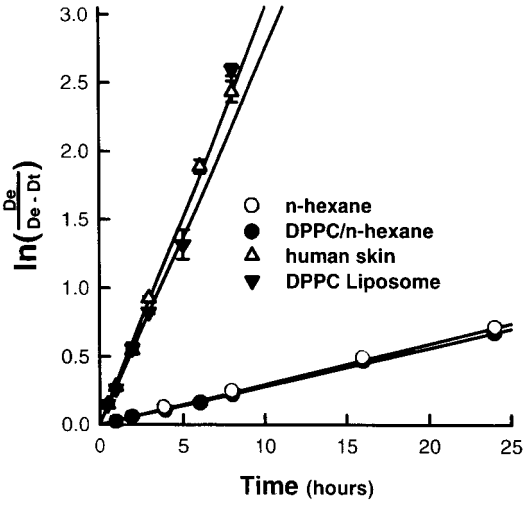


FIG. 4. **Kinetic plot of pre$D_3$ ⇌ vitamin $D_3$ isomerization in isotropic (*n*-hexane and DPPC/*n*-hexane solution) *versus* anisotropic microenvironments (human skin and DPPC liposomes).**

(upper layer) was transferred into a test tube and dried under a stream of nitrogen. The residue was reconstituted with mobile phase and centrifuged briefly to remove insoluble particles before HPLC analysis.

Methods of HPLC separation and quantification of unmodified pre$D_3$ and vitamin $D_3$ have been described previously (2, 13, 14). To the best of our knowledge, there have been no reports on HPLC separation of 25(OH)pre$D_3$ as well as pre$D_3$ acetate from their photo- and thermal isomers in the literature. We therefore developed the following HPLC systems for the separations. Complete separation of 25(OH)pre$D_3$ from its photo- and thermal isomers was obtained with isocratic elution of 2% 2-propanol in *n*-hexane on an Alltech Econosphere silica column (250 × 4.6 mm, 5 $\mu$m). Separation of the various pre$D_3$ acetate isomers was achieved by using an Astec Cyclobond I 2000 column (100 × 4.6 mm, 5 $\mu$m) with 0.03% 2-propanol in *n*-hexane as a mobile phase.

*Kinetic Studies of the Thermal Isomerization*—The thermal conversion between pre$D_3$ and vitamin $D_3$ is a first order reversible process. The integrated rate equation is expressed as

$$k = 1/t \ln[(D_e - D_o)/(D_e - D_t)] \quad \text{(Eq. 1)}$$

where $D_e$, $D_o$, and $D_t$ are concentrations of vitamin $D_3$ at time $t$ equals equilibrium, zero and $t$, respectively. The total rate constant ($k$) is calculated from the slope of the plot of $\ln[(D_e - D_o)/(D_e - D_t)]$ *versus* time $t$. The equilibrium constant ($K$) of the reaction is expressed as:

$$K = [\text{vitamin } D]_{eq}/[\text{preD}]_{eq} \quad \text{(Eq. 2)}$$

where $[\text{vitaminD}]_{eq}$ and $[\text{preD}]_{eq}$ are equilibrium concentrations of vitamin $D_3$ and pre$D_3$, respectively.

## RESULTS

*Separation of Vitamin $D_3$, Previtamin $D_3$, and Its Photoisomers as Well as Their 25-Hydroxylated Derivatives*—Pre$D_3$ was completely separated from its photoisomers and vitamin $D_3$, as described previously (13). 25-Hydroxyprevitamin $D_3$ (25(OH)pre$D_3$), 25-hydroxyvitamin $D_3$ (25(OH)$D_3$), 25(OH)7-DHC, 25-hydroxylumisterol$_3$ (25(OH)$L_3$), and 25-hydroxytachysterol$_3$ (25(OH)$T_3$) were completely separated from each other by HPLC (Fig. 3). Peak identities of 25(OH)7-DHC and 25(OH)$D_3$ were assigned on the coelution of both compounds with standards on both normal and reversed phase HPLC. 25(OH)$L_3$ and 25(OH)$T_3$ were identified by their characteristic UV absorption spectra (3, 5, 6). 25(OH)pre$D_3$ was identified by both its UV absorption spectrum ($\lambda_{max}$ = 260 nm) and its ability to thermally isomerize to 25(OH)$D_3$. Base-line separation of vitamin $D_3$-3$\beta$-acetate, pre$D_3$-3$\beta$-acetate, and 7-DHC-3$\beta$-acetate was accomplished with the normal phase HPLC method described under "Experimental Procedures."

*Kinetics of PreD$_3$ ⇌ Vitamin $D_3$ Reaction in Isotropic (n-Hexane and DPPC/n-Hexane) Versus in Anisotropic Microenvironments (Human Skin and DPPC Liposomes)*—Solutions of 7-DHC in *n*-hexane (0.135 mM), 7-DHC in DPPC/*n*-hexane (7-DHC, 0.135 mM; DPPC, 6.75 mM), DPPC liposomes containing 7-DHC (7-DHC, 0.135 mM; DPPC, 6.75 mM, *i.e.* 2 mol % of 7-DHC) as well as samples of human skin were exposed to UV radiation on ice to generate pre$D_3$. The isomerization of pre$D_3$ into vitamin $D_3$ was followed by means of HPLC analysis. The results showed that the reaction was of first order whether it was carried out in *n*-hexane, DPPC/*n*-hexane, skin, or liposomes (Fig. 4). The rate constants of the isomerization were calculated from the integrated rate equation (Eq. 1) using the least squares method. It was found that in anisotropic microenvironments (human skin and DPPC liposomes), the rates of the isomerization were about 10 times larger than those in isotropic ones (*n*-hexane and DPPC/*n*-hexane). At 37 °C, they were 8.62 ± 0.24 × $10^{-5}$ $s^{-1}$ (regression coefficient, $r$ = 0.999) and 8.72 ± 1.11× $10^{-5}$ $s^{-1}$ ($r$ = 0.990) in human skin and DPPC liposomes, respectively, *versus* 8.08 ± 0.07 × $10^{-6}$ $s^{-1}$ ($r$ = 0.999) and 8.06 ± 0.13 × $10^{-6}$ $s^{-1}$ ($r$ = 0.995) in *n*-hexane and DPPC/*n*-hexane, respectively. The kinetic plot of the isomerization is shown in Fig. 4. The equilibrium constants (defined by the ratio $k_1/k_2$) were calculated from Eq. 2. They were 11 and 10 in human skin and DPPC liposomes, respectively, *versus* 6 and 7 in *n*-hexane and DPPC/*n*-hexane, respectively.

*Temperature Dependence on Rate Constants of the Isomerization in DPPC Liposomes*—The rate constants of pre$D_3$ ⇌ vitamin $D_3$ reaction in DPPC liposomes was determined at 0, 15, 30, 37, 50, 60, and 70 °C, and they were 2.40 × $10^{-6}$ $s^{-1}$, 1.25 × $10^{-5}$ $s^{-1}$, 4.26 × $10^{-5}$ $s^{-1}$, 8.72 × $10^{-5}$ $s^{-1}$, 2.12 × $10^{-4}$ $s^{-1}$, 4.37 × $10^{-4}$ $s^{-1}$, 1.00 × $10^{-3}$ $s^{-1}$, respectively. The activation energy for the isomerization was determined according to the Arrhenius equation,

$$\ln k = \ln A - E_a/RT \quad \text{(Eq. 3)}$$

where $k$ is the rate constant for the isomerization, ln A is a constant, $E_a$ is activation energy, R is the molar gas constant, and $T$ is the temperature in degrees Kelvin. The Arrhenius plot (Fig. 5) for the isomerization in DPPC liposomes showed a straight line ($r$ = −0.999) for the entire temperature range examined, which included temperatures well above or below the phase transition temperature ($T_m$) of DPPC liposomes ($T_m$ = 41.5 °C). This result indicated that the mechanism for the isomerization remained the same whether the DPPC liposomes were in the liquid-crystalline phase or in the gel phase. The

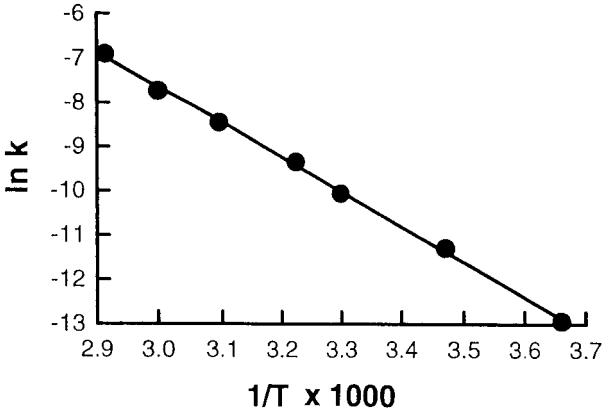


FIG. 5. **Arrhenius plot of preD$_3$ ⇌ vitamin D$_3$ isomerization in DPPC liposomes.**

calculated $E_a$ for the isomerization in DPPC liposomes is 66 kJmol$^{-1}$, which is similar to the value determined in human skin (73 kJmol$^{-1}$), and both of them are significantly lower than that determined in *n*-hexane (87 kJmol$^{-1}$) (13).

*Kinetics of the Isomerization of PreD$_3$ and Its Analogues in n-Hexane and DPPC Liposomes*—Ampules containing 7-DHC, 25(OH)7-DHC, and 7-DHC-3$\beta$-acetate in *n*-hexane and in DPPC liposomes were placed on ice and irradiated with UV radiation to generate preD$_3$ and preD$_3$ analogues. Immediately after irradiation, the exposed ampules were incubated at 37 °C. The formation of vitamin D$_3$ from preD$_3$ was monitored by HPLC. It was found that in an isotropic *n*-hexane solution, a decrease in the polarity of preD$_3$ at the C$_{3\beta}$ position by esterification (preD$_3$-3$\beta$-acetate) or an increase in polarity with the addition of a hydroxyl at C$_{25}$ (25(OH)preD$_3$) had little effect on the rate of the thermal isomerization. The rates for the isomerization of preD$_3$, preD$_3$-3$\beta$-acetate, and 25(OH)preD$_3$ in an isotropic solution (*n*-hexane) were 8.08 ± 0.07 × $10^{-6}$ $s^{-1}$, 7.05 ± 0.42 × $10^{-6}$ $s^{-1}$, and 8.36 ± 0.07 × $10^{-6}$ $s^{-1}$, respectively (Fig. 6*A*). The rates of the thermal isomerization of these preD$_3$ derivatives were drastically different from each other when incorporated into liposomes (Fig. 6*B*). In liposomes, the unmodified preD$_3$ had the highest rate of the isomerization, $k$ = 8.72 ± 1.11 × $10^{-5}$ $s^{-1}$. In contrast, the rates for the isomerization of preD$_3$-3$\beta$-acetate and 25(OH)preD$_3$ in liposomes were greatly reduced by 67% ($k$ = 2.86 ± 0.40 × $10^{-5}$ $s^{-1}$) and 87% (1.16 ± 0.30 × $10^{-5}$ $s^{-1}$), respectively.

*Effects of Lipid Composition of Liposomes on the Rate of the Isomerization*—To examine the effects of lipid composition of liposomes on the rate of preD$_3$ ⇌ vitamin D$_3$ conversion, 7-DHC was incorporated into liposomes containing phospholipids varying in hydrocarbon chain length *i.e.* DDPC (C10:0), DLPC (C12:0), DPPC (C16:0), DSPC (C18:0), and DAPC (C20:0). 7-DHC was also incorporated into the liposomes made of *cis*-unsaturated phospholipids, *i.e.* dipalmitoleoylphosphatidylcholine (C16:1, [*cis*]-9). Ampules containing each of the above liposomal preparations were placed on ice and irradiated with UV radiation to produce preD$_3$. Exposed samples were incubated at 37 °C for different time intervals. The formation of vitamin D$_3$ from preD$_3$ showed that the rate of the isomerization correlated positively with the length of the hydrocarbon chain of lipids from C10 to C18 and followed the order DDPC (C10:0) ($k$ = 3.93 ± 0.05 × $10^{-5}$ $s^{-1}$) < DLPC (C12:0) ($k$ = 5.13 ± 0.16 × $10^{-5}$ $s^{-1}$) < DPPC (C16:0) ($k$ = 8.72 ± 1.11 × $10^{-5}$ $s^{-1}$) <DSPC (C18:0) ($k$ = 1.04 ± 0.02 × $10^{-4}$ $s^{-1}$). The linear regression equation for the rate constants of the isomerization *versus* carbon chain length of lipids (Fig. 7) was deduced from the above data and was written as

$$\ln k = -14.02 + 1.68 \ln C_n \qquad \text{(Eq. 4)}$$

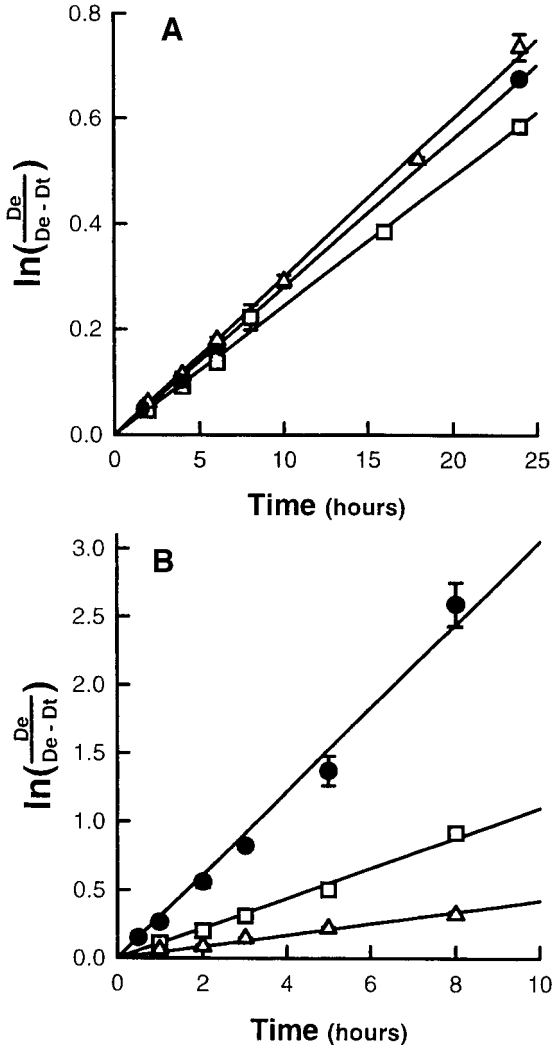


FIG. 6. **Kinetic plot of the isomerization of preD$_3$ (●), 25(OH)preD$_3$ (△), and preD$_3$-3$\beta$-acetate (□) to their corresponding vitamin D$_3$ analogs in *n*-hexane (*A*) and in DPPC liposomes (*B*).**

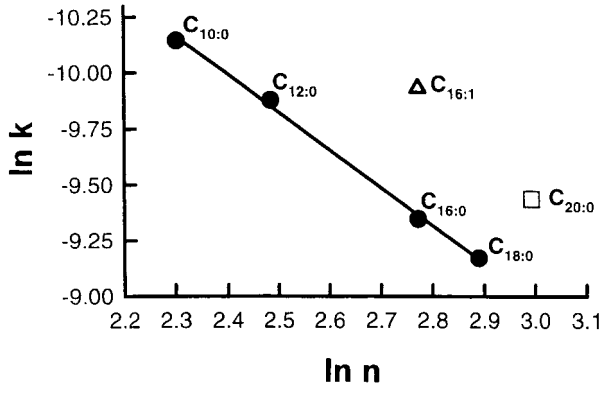


FIG. 7. **Effects of phospholipid carbon chain length and saturation on the rate of preD$_3$ ⇌ vitamin D$_3$ isomerization in liposomes.** $k$, rate constant; $n$, carbon number of phospholipid chain.

where $k$ ($s^{-1}$) was the rate constant of the isomerization, and $C_n$ was the number of carbons in the hydrocarbon chains of phosphatidylcholines. The correlation coefficient ($r$) of the determined regression equation was 0.999. Eq. 4 allows the calculation of rate constants of the isomerization in various liposomes and is valid for liposomal lipids containing 10 to 18 carbon atoms in hydrocarbon chain. Increasing the length of

hydrocarbon chain further from C18 to C20 did not enhance but decreased the rate constant ($k_{C18} = 1.04 \pm 0.02 \times 10^{-4}$ $s^{-1}$ *versus* $k_{C20} = 8.01 \pm 0.08 \times 10^{-5}$ $s^{-1}$). The effect of incorporating *cis*-unsaturated phospholipids into the liposomes on the rate of the isomerization was also examined. It was found that by introducing a *cis* double bond at carbon 9 of hydrocarbon tails of DPPC liposomes, the rate of the reaction was significantly reduced by more than 40%, *i.e.* $k = 4.86 \pm 0.63 \times 10^{-5}$ $s^{-1}$ for liposomes prepared by unsaturated phospholipids (C16:1, *cis*-9) *versus* $k = 8.72 \pm 1.11 \times 10^{-5}$ $s^{-1}$ for liposomes constructed by saturated phospholipids of the same carbon chain length (DPPC, C16:0).

## DISCUSSION

The first objective of this study was to develop a simple model by which the mechanism of the synthesis of vitamin $D_3$ in the skin could be studied. Based on our previous finding that 7-DHC and its photolyzed product $preD_3$ were present in the skin cell membrane, we incorporated 7-DHC into liposomes to mimic the membrane phospholipid bilayers. We found that the rate for the $preD_3 \rightleftharpoons$ vitamin $D_3$ isomerization in DPPC liposomes and human skin was essentially the same. The rate of the reaction was about 10-fold faster in liposomes ($8.72 \pm 1.11 \times 10^{-5}$ $s^{-1}$) compared with that in *n*-hexane ($8.08 \pm 0.07 \times 10^{-6}$ $s^{-1}$) and similar to human skin ($8.62 \pm 0.24 \times 10^{-5}$ $s^{-1}$). The equilibrium constant was also significantly higher in liposomes than the values obtained from the isotropic *n*-hexane solution and similar to human skin. Thus our liposome model closely mimicked the kinetic properties of the $preD_3 \rightleftharpoons$ vitamin $D_3$ isomerization in the skin (1, 2, 13). This is in contrast to the cholesteric liquid-crystalline model used by Cassis and Weiss (18) in which the determined equilibrium constant was smaller instead of larger than that determined in organic solvents.

Next, we examined the effects of isotropic *versus* anisotropic interactions of DPPC with $preD_3$ on the rate of $preD_3 \rightleftharpoons$ vitamin $D_3$ isomerization. We found that in an isotropic solution of DPPC/*n*-hexane, the rate of $preD_3 \rightleftharpoons$ vitamin $D_3$ isomerization is essentially the same as in a solution of pure *n*-hexane ($8.06 \pm 0.13 \times 10^{-6}$ $s^{-1}$ *versus* $8.08 \pm 0.07 \times 10^{-6}$ $s^{-1}$) (Fig. 4). In contrast, the rate of $preD_3 \rightleftharpoons$ vitamin $D_3$ isomerization in a solution of DPPC liposomes is enhanced by about 10 times compared with that in *n*-hexane (Fig. 4). Our results indicate that isotropic interactions of phospholipids with $preD_3$ do not affect the rate of $preD_3 \rightleftharpoons$ vitamin $D_3$ conversion, but in an anisotropic microenvironment such as liposomes and skin, the rate of the isomerization is greatly enhanced (Fig. 4).

We used the liposomes as a simple model for the membrane bilayers to study the mechanism by which the rate of $preD_3 \rightleftharpoons$ vitamin $D_3$ conversion was greatly enhanced in the skin. It is known that phospholipids and $preD_3$ are amphipathic molecules (ones with both hydrophilic and hydrophobic parts). Based on structural similarity, it was proposed (1, 19, 20) that, within the ordered lipid bilayer, $preD_3$ molecules oriented themselves in a way similar to cholesterol molecules, *i.e.* with their hydrophilic $C_{3\beta}$ hydroxyl group close to the polar head groups of lipids, whereas their hydrophobic rings and the side chain aligned along the hydrocarbon chains of membrane lipids (Fig. 2). However, unlike the rigid cholesterol, the *seco*-B ring sterol, $preD_3$, is very conformationally mobile. Rotation about the single $C_5$-$C_6$ bond of $preD_3$ generates a wide array of conformations extending from cZc-$preD_3$ to tZc-$preD_3$ (Fig. 1). It was well established that the thermal isomerization between $preD_3 \rightleftharpoons$ vitamin $D_3$ was a conformation-controlled process and required a cyclic transition state, possible only for cZc conformers but not possible for tZc and other conformers (Fig. 1) (10, 11).

We hypothesized that the conformational restraints imposed by amphipathic interactions between $preD_3$ and phospholipids stabilized the "cholesterol like" cZc-$preD_3$ conformer (Fig. 2). Therefore, the conformational equilibrium of cZc-$preD_3 \rightleftharpoons$ tZc-$preD_3$ was shifted to the cZc conformation. According to the hypothesis, the population of $preD_3$ molecules existing in the cZc form at any instant was higher in a lipid bilayer compared with an isotropic solution, and consequently, the rate of the thermal isomerization of $preD_3$ was enhanced (1). If this hypothesis was correct, it was expected that there would be a positive correlation between the strength of the amphipathic interactions and the rate of this thermal isomerization. To evaluate the hypothesis, we examined the effect of decreasing the polarity of the $preD_3$ by acetylation of $C_{3\beta}$-OH on the kinetics of the thermal conversion of $preD_3$ in liposomes. It was found that by reducing the strength of the amphipathic interactions between the amphiphiles, the rate of the isomerization of $preD_3$ was markedly reduced. The rate of the isomerization of $preD_3$-3$\beta$-acetate was reduced to less than one-third the rate of its unesterfied counterpart, $preD_3$ (Fig. 6). The reduced rate of the isomerization probably reflected that the less polar $C_{3\beta}$ ester group was less effective than free $C_{3\beta}$-OH of $preD_3$ in hydrophilic interaction with polar head groups of liposomal phospholipids.

We speculated that the free 3$\beta$-OH of $preD_3$ would interact with adjacent polar phospholipid head group via hydrogen bonding as cholesterol likely does (21, 22). Hydrogen bonding can lead to tight packing of phospholipid bilayers and, thus, enhance the strength of the amphipathic interaction of liposomal lipids with $preD_3$. Because of the absence of a free 3$\beta$-OH, $preD_3$-3$\beta$-acetate was unable to form a hydrogen bond with the carbonyl groups of phospholipids. This weakened the hydrophilic interactions between $preD_3$-3$\beta$-acetate, and membrane lipids probably reduced the efficiency of stabilizing the $preD_3$-3$\beta$-acetate in its cZc conformer and led to a slower rate of its isomerization to vitamin $D_3$-3$\beta$-acetate. This observation emphasizes the important role of $C_3$ hydroxyl group for the correct orientation of the $preD_3$ molecule within phospholipid bilayers and its optimal hydrophilic interactions with polar head groups of membrane phospholipids.

We also investigated whether a disruption in the hydrophobic (van der Waals) interactions between $preD_3$ and hydrophobic tails of phospholipids had an effect on the rate of $preD_3 \rightleftharpoons$ vitamin $D_3$ conversion by the addition of a hydroxyl group on the hydrophobic side chain of $preD_3$ at $C_{25}$. It is known that the liposomal phospholipid bilayers are held together mainly by hydrophobic interactions. The presence of hydrophilic $C_{25}$ hydroxyl group in the hydrophobic core disorganized phospholipid bilayers (22, 23), and thus, the strength of van der Waals interactions between the hydrophobic tails of phospholipids and 25(OH)$preD_3$ could be significantly weakened. It is also possible that 25(OH)$preD_3$ can be folded so that both 3$\beta$- and 25-hydroxyl groups align close to polar head groups of liposomal phospholipids (23) and further disorganize phospholipid bilayers. Accordingly, the stabilizing effect of phospholipid bilayers on the cZc-25(OH)$preD_3$ would be much less effective. Therefore, the rate of the 25(OH)$preD_3 \rightleftharpoons$ 25(OH)$D_3$ isomerization was anticipated to be reduced more markedly than the rate of $preD_3$-3$\beta$-acetate $\rightleftharpoons$ vitamin $D_3$-3$\beta$-acetate isomerization. Our results demonstrated that the rate of the isomerization of 25(OH)$preD_3$ in liposomes was reduced by about 7-fold compared with the rate of the isomerization of $preD_3$ in liposomes, which approached the rate determined in an isotropic solution (Fig. 6).

The strength of the amphipathic interactions between the incorporated $preD_3$ and liposomal phospholipids depends not

only on the polarity of $preD_3$ but also on the hydrophobicity of phospholipids. To evaluate the effect of changing structures of phospholipids by altering either the carbon chain length or chain saturation on the rate of $preD_3 \rightleftharpoons$ vitamin $D_3$, isomerization was examined.

We first carried out kinetic studies of $preD_3 \rightleftharpoons$ vitamin $D_3$ isomerization in liposomes prepared by phospholipids with different hydrocarbon chain lengths. We observed a chain length-dependent rate enhancement of the isomerization. Fig. 7 showed that the rate of the thermal isomerization in the different liposomes followed the order DSPC (C18:0) > DPPC (C16:0) > DLPC (C12:0) > DDPC (C10:0). Least squares analysis of the data revealed a positive linear relationship ($r$ = 0.999) between ln k ($k$, rate constant) and ln $C_n$ ($C_n$, number of carbon atoms in the hydrocarbon chains of phospholipids) (Fig. 7). Because the strength of the amphipathic interactions is directly related to the chain length of phospholipids, our observation further supports the hypothesis that there is a positive correlation between the strength of the anisotropic interactions between the amphiphiles and the rate of the isomerization.

We found that DSPC (C18:0) liposomes had the maximum effect on the rate enhancement of $preD_3 \rightleftharpoons$ vitamin $D_3$ isomerization (Fig. 7). It indicated that the optimal amphipathic interactions between $preD_3$ and phospholipids were achieved in DSPC (18:0) liposomes. Based on this information, we estimated that the "effective hydrophobic length of $preD_3$" corresponded to the length of a 18-carbon chain, which was similar to the reported value of cholesterol in phospholipid bilayers (the length of a 17-carbon chain or about 17.5 angstroms) (24–27).

To gain further insight into the mechanism of membrane-enhanced $preD_3 \rightleftharpoons$ vitamin $D_3$ isomerization, we carried out kinetic studies of the reaction in liposomes prepared with unsaturated phospholipids. We found that the rate of the isomerization was reduced by more than 40% when the reaction was carried out in C16:1 (*cis*-9) liposomes compared with the reaction in saturated C16:0 liposomes. It is known that the *cis* double bonds cause rigid kinks of 30° in the hydrocarbon chains. These kinks cause disorder in the packing of the hydrophobic chains and increase the distance between hydrocarbon chains of phospholipids and the incorporated $preD_3$, which are expected to reduce the strength of van der Waals interaction between these amphiphiles (28). This reduced van der Waals interaction would be less effective in stabilizing the cholesterol-like cZc-$preD_3$ conformer. Accordingly, the rate of the isomerization was more than 40% slower in *cis*-unsaturated liposomes than that in saturated ones of comparable carbon chain length (Fig. 7).

We concluded that in an ordered lipid bilayer, conformational restraints imposed by amphipathic interactions stabilized the cholesterol-like cZc conformer of $preD_3$. The stronger the amphipathic interactions were, the more $preD_3$ was in cZc conformation, and the faster was the rate of its conversion to vitamin $D_3$.

*Acknowledgments*—We thank Dr. James A. Hamilton for his critiques and recommendations on experimental design of liposomal studies and Dr. David M. Jackson for his help for preparing the figures.

REFERENCES

1. Holick, M. F., Tian, X. Q., and Allen, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 3124–3126
2. Tian, X. Q., Chen, T. C., Lu, Z., Shao, Q., and Holick, M. F. (1994) *Endocrinology* **135,** 655–661
3. Holick, M. F. (1989) in *Vertebrate Endocrinology: Fundamentals and Implications* (Pang, P. K. T., and Schreibman, M. P., eds) Vol. 3, pp. 7–34, Academic Press, Inc., Orlando, FL
4. Holick, M. F. (1994) *Am. J. Clin. Nutr.* **60,** 619–630
5. Holick, M. F., MacLaughlin, J. A., Clark, M. B., Holick, S. A., Potts, J. T., Jr., Anderson, R. R., Blank, I. H., Parrish, J. A., and Elias, P. (1980) *Science* **210,** 203–205
6. MacLaughlin, J. A., Anderson, R. R., and Holick, M. F. (1982) *Science* **216,** 1001–1004
7. Havinga, E. (1973) *Experientia* (*Basel*) **29,** 1181–1192
8. Woodward R. B., and Hoffmann, R. (1965) *J. Am. Chem. Soc.* **87,** 2511–2513
9. Okamura, W. H., Midland, M. M., Hammond, M. W., Rahman, N. A., Dormanen, M. C., Nemere, I., and Norman, A. W. (1995) *J. Steroid Biochem. Mol. Biol.* **53,** 603–613
10. Dauben, W. G., and Funhoff, D. J. H. (1987) *J. Org. Chem.* **53,** 5070–5075
11. Dauben, W. G., and Funhoff, D. J. H. (1988) *J. Org. Chem.* **53,** 5376–5379
12. Schlatmann, J. L. M. A., Pot, J., and Havinga, E. (1964) *Rec. Trav. Chim. Pays-Bas* **80,** 1173–1185
13. Tian, X. Q., Chen, T. C., Matsuoka, L. Y., Wortsman, J., and Holick, M. F. (1993) *J. Biol. Chem.* **268,** 14888–14892
14. Tian, X. Q., and Holick, M. F. (1995) *J. Biol. Chem.* **270,** 8706–8711
15. Wiseman, H., Cannon, M., Arnstein, H. R. V., and Halliwell, B. (1990) *FEBS Lett.* **274,** 107–110
16. Wiseman, H. (1993) *FEBS Lett.* **326,** 285–288
17. Woodle, M. C., and Papahadjopoulos, D. (1989) *Methods Enzymol.* **171,** 193–217
18. Cassis, E. G., and Weiss, R. G. (1982) *Photochem. Photobiol.* **35,** 439–444
19. Moriarty, R., Schwartz, R. N., Lee, C., and Curtis, V. (1980) *J. Am. Chem. Soc.* **102,** 4257–4259
20. Tian, X. Q., Holick, M. F., and Allen, M. (1995) in *Biologic Effects of Light 1995* (Holick, M. F., and Jung, E. G. eds) pp. 39–48, Walter de Gruyter & Co., Berlin
21. Villalain, J. (1996) *Eur. J. Biochem.* **241,** 586–593
22. Theunissen, J. J. H., Jackson, R. L., Kempen, H. J. M., and Demel, R. A. (1986) *Biochim. Biophys. Acta* **860,** 66–74
23. Verma, S., Philippot, J. R., and Wallach, D. F. H. (1983) *Biochemistry* **22,** 4587–4591
24. Wu, W.-G., and Chi, L.-M. (1991) *J. Am. Chem. Soc.* **113,** 4683–4685
25. McMullen, T. P. W., Lewis, R. N. A. H., and McElhaney, R. N. (1993) *Biochemistry* **32,** 516–522
26. McMullen, T. P. W., Lewis, R. N. A. H., and McElhaney, R. N. (1994) *Biophys. J.* **66,** 741–752
27. Bittman, R. (1997) *Subcell. Biochem.* **28,** 145–171
28. Lancée-Hermkens, A. M. W., and Kruijff, B. (1977) *Biochim. Biophys. Acta* **470,** 141–151