Photoconversion of 7-Dehydrocholesterol to Vitamin D_3 in Synthetic Phospholipid Bilayers

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ABSTRACT: The incorporation of 7-dehydrocholesterol into synthetic phospholipid bilayers altered the distribution of products after photolysis. In liposomes, the relative amounts of 7-dehydrocholesterol and lumisterol were elevated, and tachysterol was reduced from the levels observed in hexane solution. *Z* to *E* isomerization of the previtamin to tachysterol is favored in organic solvents. The inhibition of this process is evidence that an ordered lipid matrix places a new constraint on the conformation of the ring B fission product—one in which the configuration is favorable for a return to a cyclized diene. Further, rate enhancements of up to 15-fold were observed for the thermal isomerization of the previtamin to vitamin D_3 in liposomes. The free energies of activation for the reaction at 25 °C were reduced by 1.3–1.5 kcal/mol in the bilayer environment compared to that of hexane. As this reaction involves the concerted transfer of a hydrogen via a cyclic intermediate, it provides additional evidence for membrane stabilization of an all-cis conformation of the previtamin. Photoproduct ratios were also studied for 7-dehydrocholesterol adsorbed to a variety of solid supports. That nonspecific interactions of 7-dehydrocholesterol with lipid can influence product formation may have important implications with respect to the mechanism of vitamin D_3 biosynthesis.

The photochemical transformation of 7-dehydrocholesterol (DHC)[1] to vitamin D_3 (cholecalciferol$_3$) in solution has been studied in detail by Havinga and co-workers (Havinga, 1973; Havinga & Schlatmann, 1961; Havinga et al., 1960). Irradiation between 250 and 310 nm results initially in ring opening to yield the 6*Z* hexatriene derivative, previtamin D_3 (Figure 1). This compound can undergo either of the conrotatory ring closures to DHC or to lumisterol, the 9β,10α isomer. In addition, $Z \rightleftarrows E$ photoisomerization to the 6*E* isomer tachysterol occurs readily. Vitamin D_3 itself is formed upon reversible thermal (20–80 °C) rearrangement of the previtamin via antarafacial [1,7]-sigmatropic hydrogen migration (Woodward & Hoffmann, 1965). Upon continued irradiation or at higher temperatures, a number of other products are formed irreversibly and with low quantum yields.

As yet, the in vivo processes are poorly understood. Early reports suggested that the biological reaction was more selective; previtamin D_3 was the only significant photoproduct (Holick et al., 1977, 1979). The photochemistry can occur throughout the epidermal and dermal cell layers of skin which can filter and attenuate the incident radiation. As previtamin undergoes its slow thermal isomerization, the product vitamin D_3 is available for binding to specific proteins and translocation into the circulation. Thus, the importance of the competing photoreactions is unknown, particularly since the system may never reach an equilibrium.

Nonspecific interactions with the epidermal environment could conceivably influence product yield. The key intermediate in vitamin D_3 biosynthesis is a ring fission product with increased rotational degrees of freedom. Restriction of single bond rotation by carrying out the reaction in a relatively ordered milieu should inhibit or reduce side-product formation and decrease the activation energy required to form the cyclic transition state for the subsequent thermal rearrangement. The photolysis of DHC incorporated into synthetic phospholipid bilayers was studied as a potential model for the biological reaction.

Materials and Methods

DHC, cholesterol (Chol), vitamin D_3, and phospholipids (PL) were obtained from commercial sources. Lumisterol was prepared by irradiation of DHC at 300 nm in anhydrous diethyl ether and purified by high-performance liquid chromatography (HPLC) (see below). Phospholipid purity was monitored by thin-layer chromatography (TLC) in two solvent systems: chloroform/methanol/7 M ammonia, 230:90:15 (by volume) (Papahadjopoulos & Miller, 1967); chloroform/methanol/acetic acid/water, 50:15:4:2. The extent of lipid oxidation was detected by using the oxidation index of Klein (1970). Precoated TLC plates were from commercial sources. Gel-Bond is a product of Bio-Rad Laboratories. Cross-linked polystyrene film was a gift from Stephen Willging (University of Minnesota). The polyethylene film was manufactured by Union Carbide Corp.

Preparation of Lipid Bilayers and Multibilayers. Large unilamellar vesicles were prepared by the reverse-phase evaporation technique of Szoka & Papahadjopoulos (1978). A total lipid mixture of 33 μmol was dissolved in 5 mL of diethyl ether. When fully saturated phospholipids were used, they were dissolved in 10 mL of 1:1 chloroform/methanol. The aqueous phase was composed of 1–1.5 mL of 10 mM HEPES–10 mM NaCl, pH 7.4, buffer. After the annealing

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[1] Abbreviations: DHC, 7-dehydrocholesterol; Chol, cholesterol; PL, phospholipids; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; REVs, reverse-phase evaporation vesicles; BHT, butylated hydroxytoluene; DPPC, dipalmitoylphosphatidylcholine; EPC, egg phosphatidylcholine; DSPC, distearoylphosphatidylcholine; PE, polyethylene; PS, polystyrene; GB, Gel-Bond; SG, silica gel; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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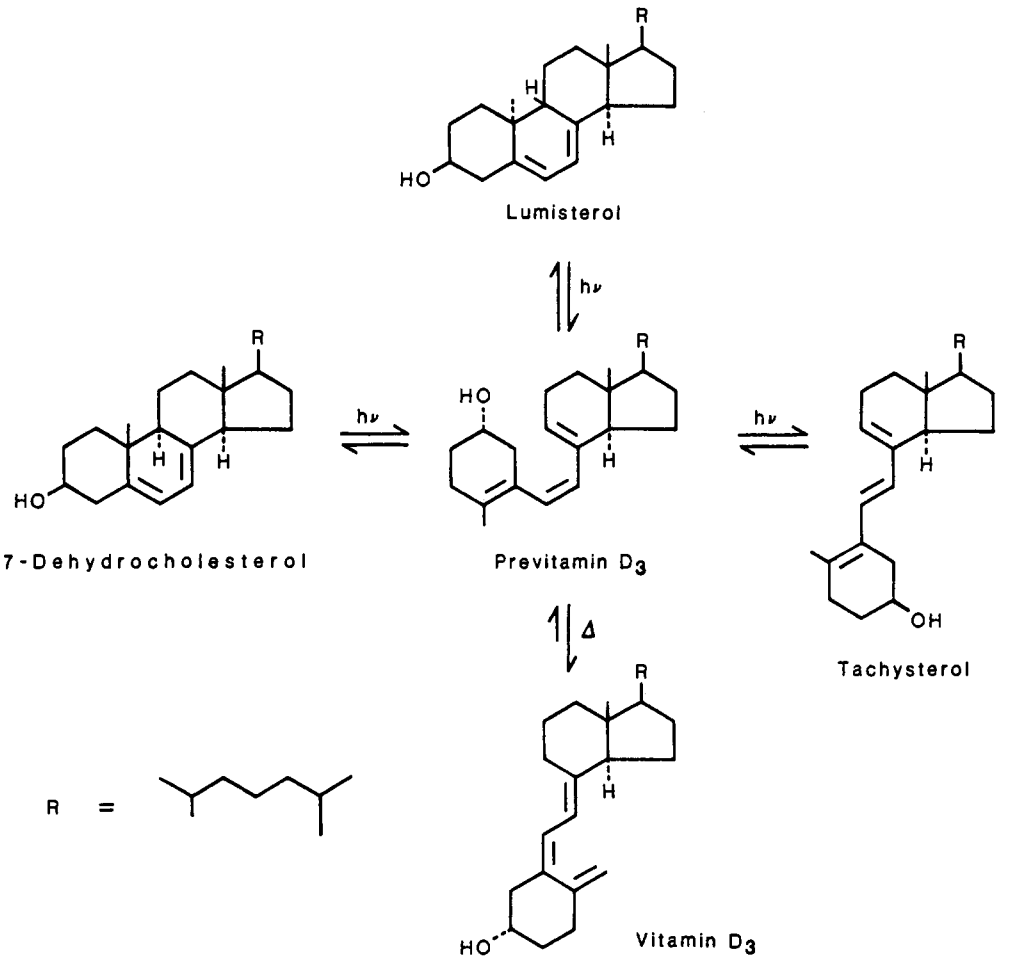

FIGURE 1: Major products of 7-dehydrocholesterol photolysis.

step, the liposome dispersions were allowed to equilibrate at room temperature for at least 10 h, after which they were centrifuged at 1000*g* for 15 min and then at 5400*g* for 45 min at 5 °C. Failure to equilibrate the liposomes for a suitable interval resulted in pelleting most of the lipid in the centrifugation step. The liposomes presumably undergo fusion to form larger, more stable structures (Prestegard & Fellmeth, 1974). The resulting dispersions eluted as single bands over Bio-Gel A-50m (Bio-Rad Laboratories). Oriented multibilayers were prepared essentially as described by Moriarty et al. (1980). Phospholipid concentrations were determined by measuring inorganic phosphorus using the Bartlett method (1959).

For estimating encapsulation volume, glycine or glucose was added to the aqueous phase to a final concentration of 0.1 M. The liposomes were chromatographed over Sephadex G-50 (Pharmacia Fine Chemicals) or Bio-Gel A-15m (Bio-Rad Laboratories) to remove unentrapped solute. Glycine was analyzed by the ninhydrin assay (Harding & MacLean, 1916), and glucose was determined by the phenol–sulfuric acid assay (Dubois et al., 1956).

Photochemical Reactions. All photolyses were carried out under nitrogen atmosphere and in quartz tubes, with irradiation at 300 nm in a Rayonet photochemical reactor (RPR 100). The bandwidth at half-height for the energy intensity of the ultraviolet sources was approximately 30 nm. In control experiments, DHC was dissolved in either anhydrous diethyl ether or *n*-hexane to a concentration of 5×10^{-5} M.

Reverse-phase evaporation vesicles (REVs) were prepared with phospholipid/sterol (DHC or DHC + Chol) ratios between 10/1 and 1/1. Where feasible, the concentrations of parallel suspensions were adjusted with buffer to an arbitrary value of 0.38–0.40 absorbance unit at 340 nm in order to minimize differences in light scattering during the photolysis reaction. Aliquots were removed at intervals and immediately frozen in a dry ice/acetone bath (−78 °C). The aqueous solutions were extracted once with chloroform/methanol, 1:1, and twice with chloroform alone. The pooled extracts were concentrated and passed through a silicic acid column with chloroform as eluent. This procedure removed ~99% of the phospholipid from the preparations.

Oriented multibilayers with 8/2 lipid/sterol ratios were formed on the wall of a standard 1-cm quartz cuvette. Lipid opacities were recorded at 400 nm. Each sample was irradiated for 1 min. The lipid was dissolved in chloroform and filtered through silicic acid to remove phospholipid.

DHC was also irradiated while adsorbed to several solid supports, including silica gel (SG), neutral alumina, reverse-phase (C8), and cellulose TLC plates. Precoated silica gel TLC plates (250 μm) were saturated with ammonia vapor prior to application of 10^{-3} M DHC in *n*-hexane. The DHC was misted onto the surface by using a TLC reagent sprayer. In some analyses, a final concentration of 0.01% butylated hydroxytoluene (BHT) and/or 1.0% squalene was added to the solution to prevent oxidative degradation (Hanewald et al., 1968). All plates were dried under nitrogen. Most of them were irradiated in a stream of nitrogen at ambient temperature, but in certain cases, the nitrogen was cooled by passage through a copper coil immersed in a dry ice/acetone bath. After photolysis, the plates were scraped and the adsorbent was extracted with diethyl ether and filtered. DHC loading was estimated by extraction of unirradiated plates followed

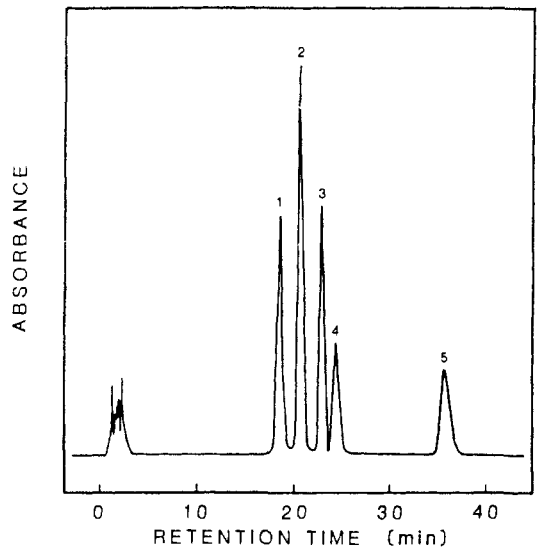

FIGURE 2: Reverse-phase HPLC separation of the major vitamin D_3 isomers. Isocratic elution in 95:5 methanol/water; flow rate of 2.0 mL/min; detection at 254 nm. Peak 1, previtamin D_3; peak 2, vitamin D_3; peak 3, tachysterol; peak 4, lumisterol; peak 5, 7-dehydrocholesterol.

by analysis of the extract by HPLC and comparison with standard curves.

In a similar manner, DHC in hexane solution was applied to glass microscope slides, Gel-Bond (GB), and polyethylene (PE) films for photolysis studies. GB, designed as an agarose gel support medium, has a highly hydrophilic surface. A cross-linked polystyrene (PS) film was swollen in dichloromethane and then rinsed successively in solutions with increasing concentrations of hexane, all 10^{-3} M in DHC. In hexane, the film was restored to a nonswollen state and was removed and dried at room temperature for 2 h followed by vacuum desiccation for 30 min. After irradiation, the photoproducts were extracted with dichloromethane/hexane solutions in the reverse order of above. The film was extremely fragile in the swollen state.

Thermal Isomerization Reaction. REVs were prepared from dipalmitoylphosphatidylcholine (DPPC) or egg phosphatidylcholine (EPC) with an 80/15/5 (mol %) ratio of (DPPC or EPC)/Chol/DHC. The lecithin and DPPC dispersions were adjusted to the same turbidity at 340 nm. Solutions under a nitrogen atmosphere were equilibrated to 5 °C in quartz tubes and irradiated for 90 s to produce previtamin D_3. Under these conditions, minimal vitamin D_3 formation occurred. The kinetics of the previtamin to vitamin D_3 conversion were studied as a function of incubation temperature. Aliquots sampled at each time interval were immediately cooled to −78 °C in a dry ice/acetone bath and subsequently worked up in the usual manner. The oxidation index for egg lecithin in the liposomes remained unchanged during the course of the experiment. The rate constants and thermodynamic activation parameters were calculated by using standard methods for reversible first-order reactions (Jencks, 1969).

HPLC Analysis. Photoproduct ratios were obtained from reverse-phase HPLC using a Dupont Zorbax ODS column (25 × 0.46 cm). Base-line separation of the five major products of DHC irradiation was obtained with isocratic elution of 95:5 methanol/water at 2.0 mL/min and with detection at 254 nm (Figure 2). Peak identities were assigned on the basis of their characteristic UV absorption spectra and by enhancement with standards. Authentic vitamin D_3 was incubated at 80 °C, and the product formed was shown to have the same retention time as previtamin D_3. The identity of tachysterol was confirmed by its faster rate of addition to maleic anhydride (Windaus et al., 1932; Verloop et al., 1957; Hanewald et al., 1968). Integrated peak areas were corrected for differences in extinction at 254 nm.

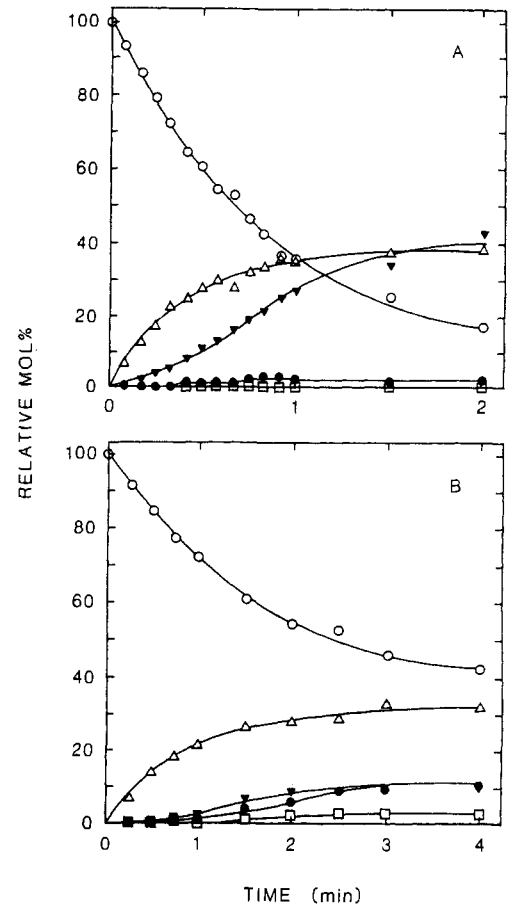

FIGURE 3: Relative molar percentages of the photoproducts of 7-dehydrocholesterol as a function of irradiation time: (○) 7-dehydrocholesterol, (△) previtamin D_3, (▼) tachysterol, (●) lumisterol, (□) vitamin D_3. (A) Irradiation of 5.4×10^{-5} M 7-dehydrocholesterol in *n*-hexane at 0 °C. (B) DPPC/DHC, 75:25 (mol %), liposomes irradiated at ambient temperature; $A_{340nm} = 0.111$; 2.9×10^{-4} M dispersed phospholipid.

RESULTS

Liposomes. The reverse-phase evaporation technique was chosen for the incorporation of DHC into a membrane bilayer because the method provided reproducible liposome suspensions with a high degree of stability, independent of lipid composition. Captured volumes in our studies ranged from 6 to 11 μL/μmol of PL compared to 11–22 μL/μmol of lipid reported in the literature (Szoka & Papahadjopoulos, 1978). Photoproduct ratios from irradiation of DHC in organic solution vs. phospholipid bilayers were studied as a function of time (e.g., see Figure 3). In general, the overall conversion of DHC to photoisomers is significantly greater in hexane than in the vesicle preparations. However, although tachysterol is a major product in Figure 3A, its contribution is reduced while that of lumisterol is increased, in the bilayers. The product ratios showed no dependence on either solute concentration or solution turbidity over a 4-fold range.

A true photostationary state cannot be attained in this system due to the inevitable formation of overirradiation products (Sanders et al., 1969; Verloop et al., 1955). However, the relative photoproduct ratios appear to attain limiting values (Table I). DPPC and, in some cases, DSPC were used to form the REVs because, at temperatures low enough to inhibit

Table I: Relative Product Ratios at the Steady-State Level of Previtamin

system	T (°C)	relative product (mol %)[a] previtamin	vitamin	tachysterol	lumisterol	DHC	previtamin/tachysterol ratio
DHC in hexane (N = 4)	0 or ambient	39 ± 2	0	45 ± 7	1.7 ± 0.6	13 ± 8	0.9
lumisterol in hexane (N = 1)	0	48 ± 3	0	30 ± 2	20 ± 1	0	1.6
5–15 mol % sterol in DPPC liposomes[b] (N = 7)	5 or ambient	30 ± 2	0.9 ± 0.7	16 ± 4	6 ± 2	47 ± 5	2.0
25 mol % sterol in DPPC liposomes[c] (N = 4)	ambient	32 ± 2	1.8 ± 0.5	13 ± 5	9 ± 2	44 ± 8	2.5
25–50 mol % sterol in DPPC or DSPC liposomes[d] (N = 3)	3–4	24 ± 1	0	10 ± 1	5.7 ± 0.6	61 ± 2	2.6
25 mol % lumisterol in DPPC liposomes (N = 1)	3	37 ± 2	0	19 ± 1	35 ± 2	10 ± 1	1.9

[a] Standard deviations of the mean are shown for $N \geq 3$. [b] Sterol: 5–10 mol % DHC plus 0–5 mol % Chol. [c] Sterol: 5–25 mol % DHC plus 20–0% Chol. [d] Sterol: DHC only.

significant isomerization of previtamin D_3 to vitamin D_3 (5–25 °C), these lipids are below their characteristic gel to liquid-crystalline transition temperatures. The previtamin/tachysterol (P/T) ratio for DHC photolysis was significantly higher in liposomes. The combined percentages of DHC plus lumisterol were much greater in the liposome experiments (53–67 mol %) as opposed to hexane solution (~15 mol %). Lumisterol in liposomes also gave reduced tachysterol and increased levels of closed-ring diastereomers. This enhancement of cyclized products is likely induced by the ordered environment of the lipid membrane.

The inclusion of up to 50 mol % cholesterol increases the degree of ordering within a membrane and decreases the permeability to small molecules (Oldfield & Chapman, 1972; Papahadjopoulos, 1976; Papahadjopoulos & Kimelberg, 1974; Phillips, 1972). Ergosterol, the plant analogue of DHC, also increases the degree of order of phospholipid bilayers at concentrations of up to 20 mol %, but this trend decreases between 20 and 40 mol %. In contrast, vitamin D_3 disorders lipid bilayers and increases membrane permeability toward sodium ions (Butler & Smith, 1978). Therefore, as DHC is depleted during photolysis, the bilayer should become more fluid. Cholesterol was added to some of the liposome preparations summarized in Table I to ensure the continuous presence of a minimum amount of sterol during the irradiation period. However, 5–50 mol % starting sterol yields identical product ratios regardless of whether the sterol was comprised of DHC alone or a mixture of DHC and cholesterol, presumably because the conversion levels remained relatively low in liposomes (<50%). At the higher sterol concentrations, overall conversion of DHC to photoisomers was reduced at 3–4 °C compared to ambient temperature irradiation. Distearoylphosphatidylcholine (DSPC)/DHC, 75/25 (mole ratio), liposomes at 4 °C gave the same results as the corresponding DPPC liposomes. The effect of increased sterol content and decreased temperature was also reflected in the decrease in first-order rate constants for the disappearance of DHC upon irradiation (Table II). In contrast, liposomes containing low levels of sterol produced rate constants which agreed with those in hexane.

Solid Supports. In the photolysis of DHC on glass, PE, PS, and GB, the total conversion to photoproducts remained below 20 mol % at the apparent steady-state levels. The major product was previtamin D_3, with the additional formation of 1–2 mol % tachysterol. Irradiation for periods greater than 1 min for PE and 2.5 min for glass and GB resulted in significant degradation of the products. However, for the photolysis of DHC on SG TLC plates, at least 50 mol % conversion to photoproducts with minimal degradation was consistently observed. Solute load had no effect between 0.4 and 2.8 μg of DHC/cm^2. On SG, the relative steady-state product levels were in good agreement with the values obtained for vesicles containing 25 mol % sterol (Table I). The rate constants for the disappearance of DHC on glass, PE, and GB were 1.3×10^{-3}, 2.5×10^{-3}, and 1.7×10^{-3} s^{-1}, respectively. On SG, the rate constant was $\sim 6.6 \times 10^{-3}$ s^{-1}. Surprisingly, the rate constant for lumisterol disappearance on SG (1.6×10^{-2} s^{-1}) was as fast as for DHC in hexane, and faster than lumisterol in both hexane and liposomes. Lumisterol on SG was converted to almost 50 mol % previtamin D_3, 30% tachysterol, and 0% DHC. The axial hydroxyl group on the β-face and the boat conformation of ring C in lumisterol may contribute to its relatively fast rate of ring opening on SG. Several modifications in the experimental procedure were used including photolysis in a cold nitrogen atmosphere, the addition of antioxidants (BHT and/or squalene), saturation of the plates with ammonia vapor, and other particulate supports (reverse phase, neutral alumina, and cellulose-coated TLC plates) in an unsuccessful attempt to increase the stability of the photoproducts. As DHC itself is stable on SG plates for several hours if oxygen is excluded, the decomposition was probably due to interactions of the photoproducts with the support medium and/or conversion to overirradiation products.

Table II: Rate Constants for the Disappearance of 7-Dehydrocholesterol (or Lumisterol) upon Irradiation

system	T (°C)	rate constant, $k \times 10^3$ (s^{-1})[a]	$t_{1/2}$ (s)	$k/k_{DHC(hexane)}$
DHC in hexane (N = 4)	0 or ambient	13 ± 3	53	1
lumisterol in hexane (N = 1)	0	3.6	192	0.28
5–15 mol % sterol, in DPPC liposomes[b] (N = 6)	5 or ambient	11 ± 2	63	0.85
25 mol % sterol in DPPC liposomes[c] (N = 4)	ambient	4.9 ± 0.2	115	0.38
25–50 mol % sterol in DPPC or DSPC liposomes[d] (N = 3)	3–4	2.2 ± 0.2	315	0.17
25 mol % lumisterol in DPPC liposomes (N = 3)	3	2.2	315	0.17

[a] Standard deviations of the mean are shown for $N \geq 3$. [b] Sterol: 5–10 mol % DHC plus 0–5 % Chol. [c] Sterol: 5–25 mol % DHC plus 25–0 % Chol. [d] Sterol: DHC only.

Oriented Multibilayers. Moriarty et al. (1980) studied DHC photochemistry in synthetic lipid multibilayers oriented on a surface vs. DHC on glass and in hexane solution. The results of irradiations carried out at 280 and 310 nm gave uniformly low conversions from DHC for the multibilayers (12–18%) with P/T ratios between 2 and 12. No conversion on glass was reported. In our experiments, a wide variation of P/T was typical at low conversion levels since small changes in tachysterol percentages produce large changes in the ratio.

Table III: Relative Product Ratios from Oriented Multibilayers[a]

photoproducts	rel % products from multibilayers consisting of DPPC/DHC (80:20)	DSPC/DHC (80:20)	DPPC/Chol/DHC (80:10:10)	DSPC/Chol/DHC (80:10:10)	liposomes DPPC/DHC (75:25) (3–5 °C)	DHC in hexane (0 °C)
previtamin	23	20	28	27	23	25
vitamin	2	1	0	<1	0	0
tachysterol	9	7	8	10	7	13
lumisterol	4	3	2	7	4	0
DHC	62	69	62	55	65	65
P/T ratio	2.6	2.9	3.5	2.7	3.3	1.9

[a] Irradiation was for 60 s at 300 nm (ambient temperature except where indicated).

Table IV: Summary of Kinetic Data for the Previtamin to Vitamin D_3 Conversion

thermodynamic parameters	previtamin $\rightleftarrows^{k_1}_{k_2}$ vitamin hexane solution	egg lecithin liposomes	DPPC liposomes
k^a (s^{-1}) at			
5.5 ± 0.2 °C	3.17×10^{-7} (25 days)[b]	3.40×10^{-6} (57 h)	4.52×10^{-6} (43 h)
25.2 ± 0.2 °C	2.93×10^{-6} (66 h)	2.95×10^{-5} (65 h)	4.28×10^{-5} (4.5 h)
40.0 ± 0.2 °C	1.25×10^{-5} (15 h)	1.10×10^{-4} (1.7 h)	1.55×10^{-4} (1.2 h)
60.0 ± 0.5 °C	1.19×10^{-4} (1.6 h)	ND	7.52×10^{-4} (0.26 h)
k/k_{hexane} at			
5.5 °C		11	14
25.2 °C		10	15
40.0 °C		9	12
60.0 °C			6
Ea_1 (kcal/mol)	19.5	17.2	16.8
Ea_2 (kcal/mol)	23.7	21.6	21.1
ΔH_1^* (kcal/mol)[c]	18.9	16.6	16.6
ΔH_2^* (kcal/mol)	23.1	21.0	20.5
ΔS_1^* (cal deg^{-1} mol^{-1})[c]	−20.6	−23.9	−24.6
ΔS_2^* (cal deg^{-1} mol^{-1})	−11.2	−13.9	−15.0
ΔG_1^* (kcal/mol)[c]	25.0	23.7	23.5
ΔG_2^* (kcal/mol)	26.4	25.1	25.0

[a] $k = k_1 + k_2$. [b] Values in parentheses indicate $t_{1/2}$. [c] At 298 K.

Our oriented multibilayers (Table III) gave results consistent with those obtained from liposomes at similar conversion levels (not at the steady state). Tachysterol levels were slightly lower, and lumisterol levels were slightly higher in multibilayers than in comparable hexane solutions. Due to the difficulties of this technique, no attempt was made to obtain steady-state product ratios.

Thermal Isomerization of Vitamin D_3. Vitamin D_3 is formed from the previtamin via a thermally allowed cyclic transition state. A change in the conformational equilibrium of previtamin D_3 might be reflected in a change in the activation energy for the hydrogen migration. Therefore, the rate dependence of the previtamin to vitamin conversion was studied at several temperatures for DHC in hexane solution and EPC/Chol/DHC (80:15:5) and DPPC/Chol/DHC (80:15:5) liposomes.

The results (Table IV) show modest rate enhancements in the liposome preparations. In the DPPC liposomes, the largest rate increase of 14–15 times that in hexane was observed at the lower temperatures. This same enhancement was seen in DPPC/DHC (90:10) liposomes at 5 °C (data not shown). Cholesterol levels near 30 mol % eliminate the gel to liquid-crystalline phase transition of phospholipids (Papahadjopoulos, 1976; Papahadjopoulos & Kimelberg, 1974). The transition temperature (T_c) of DPPC is 41 °C (Poste et al., 1976), but at 40 °C, the rate enhancement was 12-fold for DPPC/Chol/DHC (80:15:5) liposomes, suggesting that a high degree of order was maintained in the environment of the previtamin molecules. In contrast, DPPC/DHC (90:10) liposomes with no cholesterol showed only a 6-fold rate increase at 40 °C. At 60 °C, the rate was increased only 6 times over hexane; this temperature may be sufficiently high to increase the fluidity of the bilayer. EPC has a transition temperature between −7 and −15 °C (Poste et al., 1976), and the rate enhancements in the corresponding liposomes were all approximately 10-fold increases between 5 and 40 °C. The thermodynamic activation parameters calculated from the kinetic data showed a decrease in the free energies of activation for the forward and reverse reactions of between 1.3 and 1.5 kcal/mol at 25 °C in the bilayer systems compared to that of hexane.

Discussion

Several conformations derived from single bond rotations are possible for previtamin D_3, but the cis form cannot be fully coplanar due to steric restrictions. It has long been assumed that the molecule exists primarily as two diastereomeric conformers achieved by rotation around the 5,6 bond, with interconversion proceeding via the quasi-planar form α (Figure 4). The conformer arising via a right-handed rotation about the 5,6 bond is designated as the β(R) conformer, and that arising via a left-handed rotation is designated β(L). Thus ring opening of dehydrocholesterol and lumisterol would first generate either the β(L) or the β(R) conformer, respectively. Inspection of molecular models suggests that the β(L) should be less stable than the β(R) conformer because of the unfavorable C_{18}–C_{19} methyl–methyl interaction in the former. Furthermore, β(L) $\rightleftarrows$ β(R) interconversion should proceed preferentially through the α conformer. Although there is significant crowding in the α conformer due to the mutual repulsion of hydrogen atoms at C_4 and C_9, direct interconversion would require an even worse steric interaction between the C_{19} methyl and C_9 hydrogen or extensive deconjugation of the triene.

It is accepted that DHC and ergosterol (precursor of vitamin D_2 in plants) can be included in lipid monolayers, bilayers, and

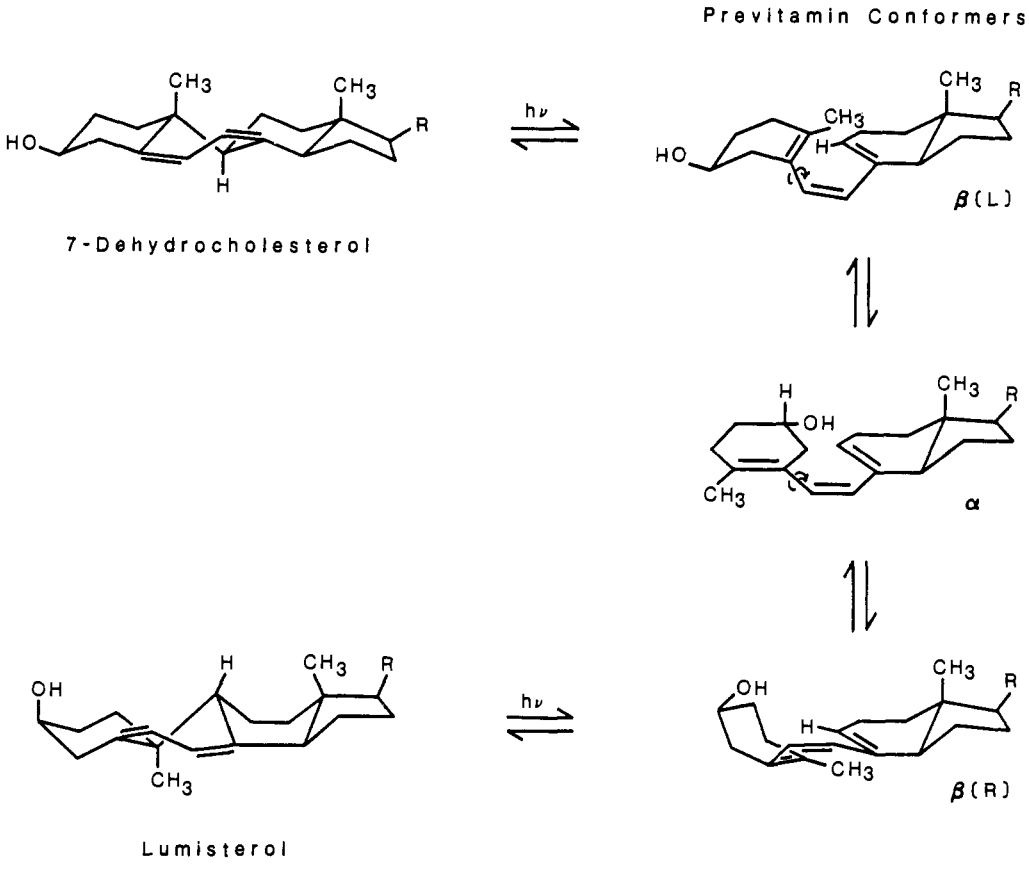

FIGURE 4: Interconversion of 7-dehydrocholesterol and lumisterol via previtamin D_3.

aqueous dispersions, in a manner similar to cholesterol (Rogers et al., 1979; Kasumov et al., 1975), and that ergosterol increases the ordering of lipid molecules and decreases sodium ion permeability (Butler & Smith, 1978). We chose to noncovalently incorporate the cyclic diene into a phospholipid matrix to restrict the mobility of the ring-opened product and prevent further isomerization to the less desirable photoproducts. With the hydroxyl group of DHC, and thus ring *A*, anchored near the lipid–water interface of a membrane bilayer, if rotation about the 5,6 bond is not completely inhibited in the *B* ring fission product, the activation energy for such a process should be greater than in solution. In a highly ordered environment, tachysterol formation should be inhibited.

Solution quantum yields (Havinga, 1973) indicate that irradiation of DHC should give tachysterol as the major product at the quasi-stationary state. This was observed in hexane, but the decreased proportions of tachysterol in the liposome preparations suggest that $Z \rightarrow E$ isomerization is inhibited by the ordered bilayer structures (Table I). The combined percentages of DHC plus lumisterol were significantly greater in the liposome experiments as opposed to hexane solution. Therefore, the β conformers of the previtamin, particularly the initially formed β(L), are stabilized relative to $Z \rightarrow E$ isomerization, thus facilitating the reverse ring closure reaction to regenerate starting material. According to Havinga, the quantum efficiency for the photoconversion of lumisterol to previtamin is quite high (0.42) compared to that of the reverse reaction (0.030). In contrast, once tachysterol is formed, with a quantum yield of 0.48, it reverts poorly to previtamin with a yield of 0.10. Therefore, while tachysterol formation is essentially nonproductive, lumisterol is a good precursor of previtamin. In studies on isolated human skin, Holick et al. (1981) observed very high levels of lumisterol after prolonged exposure to simulated solar irradiation. Differences in actual product ratios reported in the literature may be due in part to the wavelength of light used (MacLaughlin et al., 1982; Malatesta et al., 1981; Moriarty et al., 1980).

The study of conformational considerations can be extended to the generation of vitamin D_3 itself. The influence of substituent groups on the previtamin D_3–vitamin D_3 interconversion has been the subject of recent interest (Moriarty & Paaren, 1980; Sialom & Mazur, 1980; Sheves et al., 1979; Sheves & Mazur, 1977). The thermal isomerization proceeds via concerted, intramolecular H transfer from C_{19} to C_9 (i.e., a [1,7]-sigmatropic shift) (Woodward & Hoffmann, 1965; Legrand & Mathieu, 1957; Verloop et al., 1957; Velluz et al., 1957; Schlatmann et al., 1964). If the β conformers of the previtamin are stabilized within the phospholipid bilayers, with C_9 and C_{19} in close proximity, the activation energy for the reaction should be lower than in hexane solution. Rate enhancements and lowered activation energies were observed in the vesicle systems (Table IV). As expected, the less fluid membranes were more efficient in promoting the formation of the vitamin. The entropies of activation were negative due to the loss of rotational degrees of freedom with the introduction of a high degree of order in the cyclic activated complex.

At 40 °C the $t_{1/2}$ for approaching equilibrium in the previtamin–vitamin interconversion in hexane was 15 h as opposed to 1.2–1.7 h in liposomes. Although the rate enhancement is small in catalytic terms, it may be of practical benefit to an organism. In vivo, the isomerization of DHC could be influenced by protein–lipid or lipid–lipid type interactions. The latter is more likely since no protein has been isolated that has significant binding affinity for DHC, compared to the circulating forms of the vitamin (Hadad & Walgate, 1976; Imawari et al., 1976; Bouillon et al., 1976). Photolysis of the sterol within an ordered matrix results in decreased side-product

formation. Although the generation of previtamin is less efficient, an increased rate of isomerization to vitamin D_3 may promote its removal from skin by carrier proteins which in turn would allow further photoisomerization of DHC and lumisterol to previtamin. The net result might be more efficient use of precursor material.

The interaction of DHC with nonspecific adsorbents was expected to produce results similar to that of the bilayers. Noncovalent interaction of the C_3-hydroxyl group with a hydrophilic surface was postulated to anchor ring *A* of the sterol. After photolytic opening of ring *B*, isomerization to tachysterol and lumisterol would require a rotation of the fused *C–D* rings around the 5,6 bond. The activation energy for such a rotation may be high if the reaction is carried out on a support in the absence of solvent. Alternatively, lipophilic binding of the hydrocarbon portion of the sterol on a nonpolar surface may produce similar effects by anchoring the opposite end of the molecule. The low conversion of DHC to photoproducts observed on the two-dimensional films demonstrates that the immediate reclosure of previtamin back to DHC is overwhelmingly favored as opposed to further isomerization requiring rotation of the fused ring system around a bond. If vitamin D_3 can form from previtamin on a support, a repeated cycle of irradiation and heating has the potential of forming vitamin with minimal side-product formation. The use of nonspecific adsorbents to direct vitamin D_3 photochemistry merits further investigation.

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Registry No. DHC, 434-16-2; Chol, 57-88-5; DPPC, 2644-64-6; DSPC, 4539-70-2; PE, 9002-88-4; PS, 9003-53-6; GB, 83589-62-2; hexane, 110-54-3; previtamin D_3, 1173-13-3; vitamin D_3, 67-97-0; tachysterol, 115-61-7; lumisterol, 474-69-1.

REFERENCES

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.
Bouillon, R., VanBaelen, H., Rombauts, W., & DeMoor, P. (1976) *Eur. J. Biochem. 66*, 285–291.
Butler, K. W., & Smith, I. C. P. (1978) *Can. J. Biochem. 56*, 117–122.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.
Hadad, J. G., & Walgate, J. (1976) *J. Biol. Chem. 251*, 4803–4809.
Hanewald, K. H., Mulder, F. J., & Keuning, K. J. (1968) *J. Pharm. Sci. 57*, 1308–1312.
Harding, V. J., & MacLean, R. M. (1916) *J. Biol. Chem. 24*, 503–517.
Havinga, E. (1973) *Experientia 29*, 1181–1192.
Havinga, E., & Schlatmann, J. L. M. A. (1961) *Tetrahedron 16*, 146–152.
Havinga, E., deKock, R. J., & Rappoldt, M. P. (1960) *Tetrahedron 11*, 276–284.
Holick, M. F., Frommer, J. E., McNeill, S. C., Richtand, N. M., Henley, J. W., & Potts, J. T., Jr. (1977) *Biochem. Biophys. Res. Commun. 76*, 107–114.
Holick, M. F., Richtand, N. M., McNeill, S. C., Holick, S. A., Frommer, J. E., Henley, J. W., & Potts, J. T., Jr. (1979) *Biochemistry 18*, 1003–1008.
Holick, M. F., MacLaughlin, J. A., & Doppelt, S. H. (1981) *Science (Washington, D.C.) 211*, 590–593.
Imawari, M., Kida, K., & Goodman, D. S. (1976) *J. Clin. Invest. 58*, 514–523.
Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp 555–614, McGraw-Hill, New York.
Kasumov, K. M., Liberman, E. A., & Kholodova, Y. D. (1975) *Biofizika 6*, 831–833.
Klein, R. A. (1970) *Biochim. Biophys. Acta 210*, 486–489.
Legrand, M., & Mathieu, J. (1957) *C. R. Hebd. Seances Acad. Sci. 245*, 2502–2504.
MacLaughlin, J. A., Anderson, R. R., & Holick, M. F. (1982) Science (*Washington, D.C.*) *216*, 1001–1003.
Malatesta, V., Willis, C., & Hackett, P. A. (1981) *J. Am. Chem. Soc. 103*, 6781–6783.
Moriarty, R. M., & Paaren, H. E. (1980) *Tetrahedron Lett. 21*, 2389–2392.
Moriarty, R. M., Schwartz, R. N., Lee, C., & Curtis, V. (1980) *J. Am. Chem. Soc. 102*, 4257–4259.
Oldfield, E., & Chapman, D. (1972) *FEBS Lett. 23*, 285–297.
Papahadjopoulos, D. (1976) *Lipids 1*, 187–195.
Papahadjopoulos, D., & Miller, N. (1967) *Biochim. Biophys. Acta 135*, 624–638.
Papahadjopoulos, D., & Kimelberg, H. K. (1974) in *Progress in Surface Science* (Davision, S. G., Ed.) pp 141–232, Pergamon Press, Oxford.
Phillips, M. C. (1972) in *Progress in Surface Science* (Danielli, J. F., Rosenberg, M. D., & Cadenhead, D. A., Eds.) pp 139–221, Academic Press, New York.
Poste, G. D., Papahadjopoulos, D., & Vail, W. J. (1976) *Methods Cell Biol. 14*, 33–71.
Prestegard, J. H., & Fellmeth, B. (1974) *Biochemistry 13*, 1122–1126.
Rogers, J., Lee, A. G., & Wilton, D. C. (1979) *Biochim. Biophys. Acta 552*, 23–27.
Sanders, G. M., Pot, J., & Havinga, E. (1969) *Fortschr. Chem. Org. Naturst. 27*, 131–157.
Schlatmann, J. L. M. A., Pot, J., & Havinga, E. (1964) *Recl. Trav. Chim. Pays-Bas 83*, 1173–1184.
Sheves, M., & Mazur, Y. (1977) *J. Chem. Soc., Chem. Commun.*, 21–22.
Sheves, M., Berman, E., Mazur, Y., & Zaretskii, Z. V. I. (1979) *J. Am. Chem. Soc. 101*, 1882–1883.
Sialom, B., & Mazur, Y. (1980) *J. Org. Chem. 45*, 2201–2204.
Szoka, F., Jr., & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4194–4198.
Velluz, L., Amiard, G., & Goffinet, B. (1957) *Bull. Soc. Chim. Fr.*, 882–886.
Verloop, A., Koevoet, A. L., & Havinga, E. (1955) *Recl. Trav. Chim. Pays-Bas 74*, 1125–1130.
Verloop, A., Koevoet, A. L., & Havinga, E. (1957) *Recl. Trav. Chim. Pays-Bas 76*, 689–702.
Windaus, A., Werder, F. V., & Lüttringhaus, A. (1932) *Liebigs Ann. Chem. 499*, 188–200.
Woodward, R. B., & Hoffmann, R. (1965) *J. Am. Chem. Soc. 87*, 2511–2513.