An Experimental Approach to Study the Binding Properties of Vitamin E (α-Tocopherol) during Hairless Mouse Skin Permeation

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An experimental approach to study the binding properties of vitamin E has been developed. Total vitamin E solubility in the skin was determined by a partition study, followed by *in vitro* skin permeation studies with whole skin and stripped skin. The amount of freely diffusable vitamin E in the diffusion process was determined from the permeation profiles of whole skin and stripped skin by employing a bi-layer model. The concentrations of vitamin E in the stratum corneum and viable dermis were determined separately. By subtracting this amount from the total concentration of vitamin E in the skin, as determined by the solubility study, the amount of bound vitamin E was determined. After skin permeation reached a steady state, the donor solution was removed and the permeation study continued (desorption study). During the entire period of the desorption experiment, the amount of vitamin E in the receptor solution hardly increased and remained constant. After the desorption experiment, vitamin E still remaining in the skin was determined by extracting with tissue solubilizer, SOLABLE, and is considered as the amount of vitamin E strongly bound in the skin. The concentrations of bound vitamin E determined by permeation and desorption studies coincided relatively well. To further investigate skin binding of vitamin E, a differential scanning calorimetry study was performed. Vitamin E-treated stratum corneum showed phase transitions at 76 and 85 °C, associated with lipid transitions. The thermal transitions associated with the lipid transition suggested interactions of vitamin E with lipid components of the skin. During skin permeation, vitamin E forms a very strong reservoir in the skin tissue and this amount of vitamin E, about 30%, exists as a bound-form with the lipid components of the stratum corneum.

Key words skin permeation; vitamin E (α-tocopherol); binding; skin reservoir; differential scanning calorimetry

Vitamin E acts as an antioxidant and protects molecules, especially polyunsaturated fats, against attack with free-radicals and the initiation of peroxidation.[1,2] It has been generally accepted that vitamin E plays an important role in the stabilization of biological membranes that contain high levels of polyunsaturated fatty acids.[3—5] Therefore without sufficient amounts of vitamin E, the arrangement of the hydrocarbon regions of biological membranes become disordered and oxidative destruction of the polyunsaturated fatty acids may be facilitated. The effect of vitamin E on the skin permeability is more significant when the phospholipid contains a higher content of arachidonic acid residues. Vitamin E forms a complex with the phospholipids having arachidonyl residues that are present in cellular membranes. Diplock and Lucy reported that α-tocopherol could reduce the permeability to glucose and chromate ions when the liposome consisted of phospholipids containing arachidonic acid residues.[6,7] In studies to understand the role of vitamin E in our body, many researches have stressed the structural link between vitamin E and phospholipids in cellular membranes.

For most drugs, after removing the donor solution after achieving a steady state of permeation rate, the profile shows an increase in skin permeation rate due to some reservoir capacity of the skin.[7—9] Miselinicky[10] reported that materials with good lipid solubility or a high affinity for protein would be retained within the skin structure to the greatest extent since increasing lipophilicity and protein binding promote reservoir formation. However, vitamin E which is a very lipophilic compound (partition coefficient, octanol/water: 437) did not show any increase after desorption study indicating no reservoir formation or very strong chemical binding which can not be explained by simple Langmuir-type binding.[9,11]

To determine the amount of vitamin E which attends in the diffusion process (the free diffusable drug concentration), skin permeation studies with whole skin and stripped skin were performed. From the lag times and the steady state skin permeation rates, the drug solubility, diffusivity and partition coefficient in each skin layer were determined by employing a bi-layer model.[12] From our previous experiments, it was shown that vitamin E metabolized into α-tocopherol quinone and α-tocopherol quinol in hairless mouse skin. To understand the binding property of vitamin E after topical application, tritium labeled vitamin E was employed. Tritium labeled vitamin E in the skin can be either vitamin E, α-tocopherolquinone or α-tocopherolquinol.[13] Since the molecular weights of these three compounds are similiar; they differ by only one hydroxyl functional group and two hydrogen atoms respectively, α-tocopherol ($C_{29}H_{50}O_2$=430.7), α-tocopherol quinone ($C_{29}H_{49}O_3$=445.7), α-tocopherolquinol ($C_{29}H_{51}O_3$= 447.7). We investigated the binding property of vitamin E during skin permeation by employing the bi-layer model.[12] Differential scanning calorimetry (DSC) was performed to study the chemical interactions of vitamin E with stratum corneum. In this report, an experimental approach to study the binding property of the drug and the results of the DSC study of vitamin E are discussed.

Experimental

Vitamin E (α-tocopherol), and radio-labelled vitamin E, ([^{3}H]^2-*dl*-α-tocopherol, 2.0 mCi/mM) were kindly provided by Hoffmann-La-Roche (Nutley, NJ, U.S.A.). The purity of radio-labelled compound was tested by TLC or HPLC in line with a Liquid Scintillation Counte (LSC) (Rack Beta 1214-001, LKB instruments Inc., Gaithersburg, MD, U.S.A.). Tween-80 (ICI Americas, Inc., DE, U.S.A.) and Silicone Fluid (Dow Corning 360, 20 cp)

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were used as obtained. All other chemicals were reagent grade. Water was purified by a Nanopure water purification system (Sybron/Barnstead, Boston, MA). Bioflour (E.I. Dupont NEN Research, MA, U.S.A.) was used as a liquid scintillation cocktail. SOLABLE (E.I. Dupont NEN Research) was used to digest the skin tissue for sorption and desorption study. A differential scanning calorimeter (Delta Series DSC 7; TAC 7/3 Instrument Controller; Graphic Controller, Perkin Elmer) was used to investigate the effects of vitamin E on the thermal transition behavior of the stratum corneum.

Radio-Activity Counting LSC was used to quantitate the concentration of radio-labelled vitamin E. For the assay, samples (30—80 μl) were taken and mixed well with Bioflour scintillation cocktail (5—10 ml). The amount of radioactivity (disintegration per minute, dpm) was determined and the correction for quenching was made automatically by comparison against a standard quench curve. The concentration of vitamin E in receptor solution at each sampling time was calculated based on the corrected values of dpm and the specific activity (dpm/mg) of radio-labelled vitamin E in the donor solution at time zero.

Permeation Study The procedure was followed as reported previously.[14] In brief, a freshly excised, full thickness, female hairless mouse skin (5—7 weeks old, Jacksonb Lab. HRS/J Strain) was mounted between the half cells of the *in vitro* skin permeation system. A specimen of the stripped skin was prepared by 16-time strippings using an adhesive tape (3M Scotch Tape). Vitamin E spiked with [^{3}H]^2-*dl*-α-tocopherol in silicone fluid was employed as a donor solution. An aqueous solution of Tween-80 (5 mM) was used as a receptor solution after considering the critical micelle concentration of Tween-80 in water (0.4 mM) and the possible skin damage by the surfactant at high concentration. The solubility of vitamin E in the donor solution , Silicone Fluid, was 39 mg/ml. At predetermined time intervals, 30 μl of receptor solution was withdrawn and assayed for drug concentration by HPLC or LSC. The total amount of drug that permeated through the skin was plotted as a function of time.

Tissue Solubilization Method To extract the total amount of drug (bound+free) from the skin tissue, a tissue solubilizing agent, SOLABLE were used. Skin tissues were digested into clear solution and the concentration of drug strongly bound to skin tissue, as well as the free drug concentration, were determined by analyzing the total radioactivity of solution by LSC. The solubilization procedure was reported previously.[14]

Desorption Study After reaching steady state in the skin permeation experiment, the donor solution was quickly removed and the surface of the stratum corneum was washed with water and methanol to remove any residual drug on the surface of the skin. Since Silicone Fluid was used as donor solution, an organic solvent to wash out the residual vitamin E on the surface of the skin was required and methanol was selected. We controlled the washing time to less than one minute in order to reduce the possibility that vitamin E was extracted from the skin. After washing, the skin surface was covered with aluminum foil and the desorption study was carried out. At the end of the desorption experiment, the skin was removed from the diffusion cell and drug still remaining in the skin were extracted with tissue solubilizer and the total tritium-labeled vitamin E concentration was determined.

Determination of Vitamin E Solubility in the Skin (Sorption Study) A piece of freshly excised whole skin or stripped skin (30—60 mg) was put in a test tube containing 10 ml of elution medium with silicone fluid containing one fifth of the saturation concentration (1/5Cs) to the saturation concentration (Cs: 7.9—38.48 mg/ml). The density of skin (1.04 g/ml) was employed for calculation. After equilibration for 24 h at 37 °C, the skin surface was washed with water and methanol and the amount present in the skin was determined by total extraction with tissue solubilizer. The amount extracted from the skin was then plotted against drug concentration in the elution medium to study the skin binding of vitamin E. The amount extracted from the skin was also compared with that calculated from the steady-state concentration profile assuming no binding.

DSC Study Equilibrated skin with drug solution (13 mg/ml of vitamin E) was mounted on filter paper, and wetted with 0.5% (pH 7.4 phosphate buffer) trypsin solution in a covered Petri dish overnight at room temperature. Stratum corneum layer was carefully separated from viable skin with forceps. A clear and thin layer of stratum corneum was then put into the sampling cell for DSC study. The temperature was increased from 30 to 170 °C at the rate of 5 °C per minute.

Each DSC analysis was repeated at least 5 times since DSC thermal profile can be affected by sample treatment and water content of the skin. The skin samples treated with vitamin E provided reproducible thermal profiles.

Data Treatment α-tocopherol is metabolized to α-tocopherolquinone by accepting one hydroxyl functional group, and can be reduced to α-tocopherol quinol by accepting two hydrogen atoms. Thus, we have assumed the same diffusion coefficient for these three compounds to determine the total concentration of tritium labeled vitamin E in the skin. The concentrations of vitamin E in the skin with different donor solution concentrations were determined by the extraction method. By subtracting free diffusable drug concentration from the total soluble vitamin E in the skin, we determined the amount of bound vitamin E in the skin (C-bound). C-bound was compared with the value determined by the extraction method with the skin specimen after the desorption study.

Results and Discussion

Figure 1 shows the permeation and desorption profiles of vitamin E (donor: 6.5 mg/ml). After the permeation experiment for 24 h, the donor solution was completely removed and thereafter the concentration of the drug in the receptor solution was continuously monitored until 72 h. For most drugs, an increase in cumulative amount is commonly observed for a short period of time after removing the donor solution due to some reservoir capacity of the skin.[8,9,15] Simple Langmuir type binding would release most of the drug out even after removing the donor solution as observed in progesterone and its derivatives.[9] However, this is not the case for vitamin E: there is no increase in permeation profile observed after removing the donor solution (t>24 h). During the entire period of the desorption experiment, the amount of vitamin E hardly increased and remained almost constant after removal of the donor solution. This finding suggests two possibilities; either no residual vitamin E is present in the skin or vitamin E is present as a very strong-bound form which can not diffuse out during the desorption experiment. The amount of vitamin E remaining in the skin after the 72 h desorption study was determined by extracting vitamin E using a tissue solubilizing agent (SOLABLE). Significant amounts of vitamin E (0.27 ± 0.005 mg/ml) were extracted from the skin. The extracted amount of vitamin E after the desorption study presumably represents the concentration of vitamin E bound strongly in the skin.

A skin permeation study with different concentrations of vitamin E in the donor solution were conducted to investigate how this strong binding property of vitamin E influenced the skin permeation profile. Figure 2 shows the skin permeation profiles when the concentration of vitamin E in the donor solution was 2-fold higher (donor: 13 mg/ml). The skin permeation rates through whole skin and stripped skin show an increase and the permeation rate proportionally increases. We

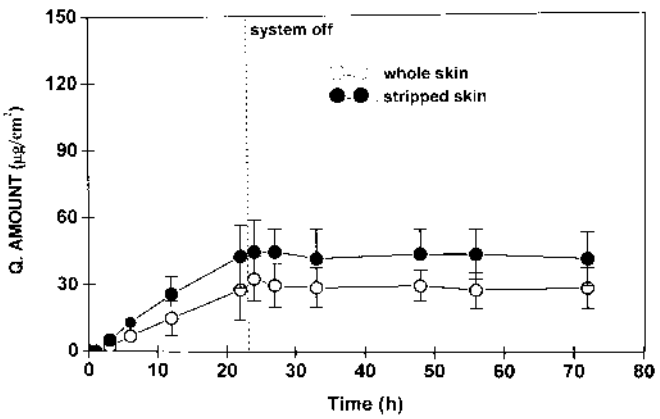

Fig. 1. Permeation of [^{3}H]^2-3,4-α-Tocopherol (6.5 mg/ml) through Whole Skin and Stripped Skin

After 24 h, donor solution was removed and the desorption study continued.

also observed a linear relationship between the donor solution concentration and the permeation rate: 39 mg/ml of vitamin E in the donor solution showed about a 3-fold increase in skin permeation profile as compared with that for 13 mg/ml (data not shown). Based on this proportional relationship between concentration and skin permeation rate, it is reasonable to analyze the skin permeation profiles of vitamin E by assuming skin is a homogeneous membrane for simplicity of analysis.

The concentration of vitamin E involved in the diffusion process was determined from the skin permeation profiles of vitamin E with whole skin and stripped skin. Physico-chemical properties such as diffusivity, partition coefficient and solubility in each skin layer were determined from the steady state skin permeation profiles with radio-labeled compound by using a bi-layer model.[12] Table 1 summarizes the steady-state rate and the time lag of skin permeation of tritium labeled vitamin E through whole skin and stripped skin. The time lag was found to be approximately 3 times longer than that through stripped skin. Diffusivity, solubility and partition coefficient in each skin layer are summarized in Table 2.

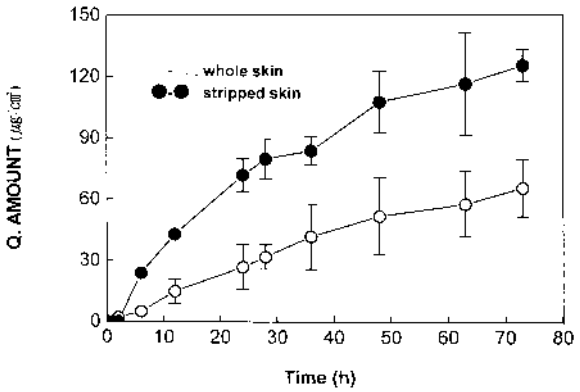

Fig. 2. Permeation of [^{3}H]^2-3,4-α-Tocopherol (13 mg/ml) through Whole Skin and Stripped Skin for 72 h

Table 1. Steady-State Rate and Time Lag of Permeation of Radiolabeled α-Tocopherol ([^{3}H]^2-*dl*-α-Tocopherol) across Hairless Mouse Skin

Vitamin E ($C_{29}H_{50}O$)		
Molecular weight		430.7
Partition coefficient (octanol/water)		476
Donor solution (13 mg/ml)		33% Cs
Permeation rate (g/cm^2/h)	Whole skin	1.58±0.58
	Stripped skin	3.34±1.68
Time lag (h)	Whole skin	2.95±0.72
	Stripped skin	0.9±0.1

The diffusivity through the stratum corneum is about 1000 times less than through the viable skin. Due to its high lipophilicity, vitamin E showed higher solubility (*ca.* 19 times) in the stratum corneum than in the viable skin. The stratum corneum functions as a major barrier, even for this very lipophilic vitamin E (partition coefficient of octanol/water=476). These parameters were used to determine the free drug concentration which attends in the diffusion process. The effect of the donor concentration on the skin uptake was studied. A piece of intact skin, weighing about 30—60 mg, was put into the elution medium containing various concentrations of vitamin E. After equilibrium, vitamin E was extracted from the skin with a tissue solubilizer. Figure 3 shows that the amount extracted from the skin, which is the total soluble drug concentration (bound+free), is approximately proportional to the donor solution concentration. With the 6.5 mg/ml donor solution, the total soluble drug concentration was found to be 1.04±0.04 mg/ml. We calculated the free drug concentration based on the steady state permeation rate, as explained above. When the concentration of donor solution was 6.5 mg/ml, the free drug concentration in the skin was calculated to be 0.724 mg/ml.

Figure 4 shows the thermal profile of the stratum corneum isolated from normal hairless mouse skin which was prepared by 0.5% trypsin treatment for 17 h. The DSC thermal profile of normal skin showed a broad endothermic change from 50 to 150 °C. After investigating the DSC thermal profile of extracted lipid and protein from the stratum corneum, Potts[16] reported that lipid transition in the stratum corneum of hairless mouse occurs primarily at temperature below about 70 °C, whereas protein transitions occur at higher tem-

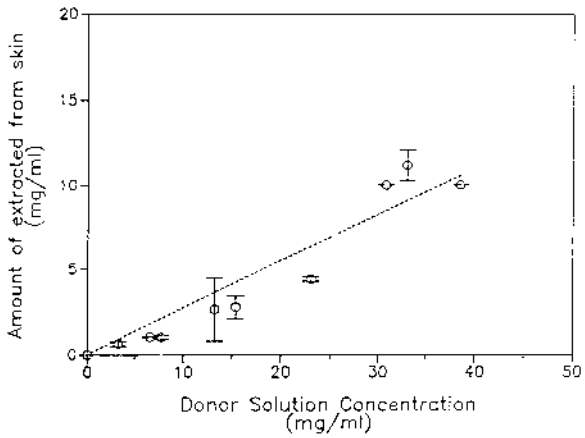

Fig. 3. Solubility of Vitamin E in Whole Skin

As the concentration of vitamin E in the donor solution increased, the solubility of vitamin E increased proportionally. The correlation coefficient is r^2=0.9245.

Table 2. Diffusivity, Solubility and Partition Coefficient of Vitamin E in Hairless Mouse Skin

Diffusivity (cm^2/s)	Stratum corneum	8.42±1.31 ($\times10^{-11}$)
	Viable skin	6.96±0.08 ($\times10^{-8}$)
Solubility (g/ml)	Viable skin	492.88±204.6
	Whole skin	724.15±221.78
Partition coefficient (Stratum corneum/donor solution)		0.73±0.50
Partition coefficient (Viable skin/donor solution)		0.04±0.002
Partition coefficient (Stratum corneum/viable skin)		18.25

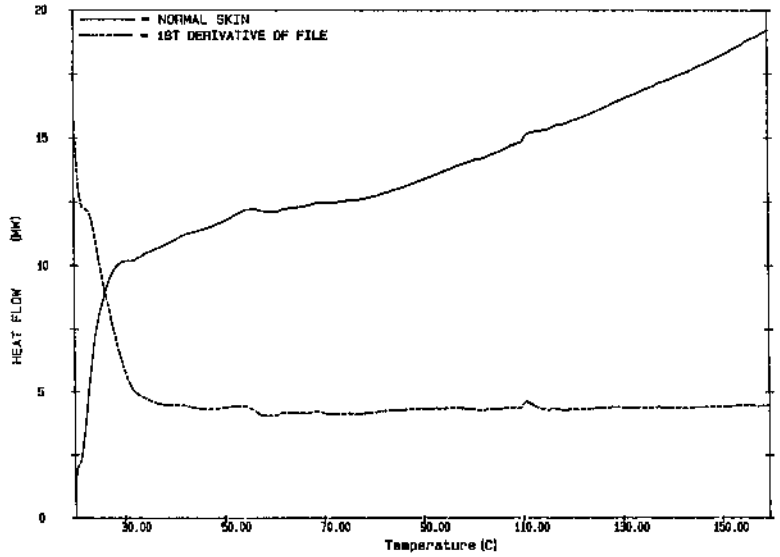

Fig. 4. DSC Thermal Profile of the Stratum Corneum of Normal Skin

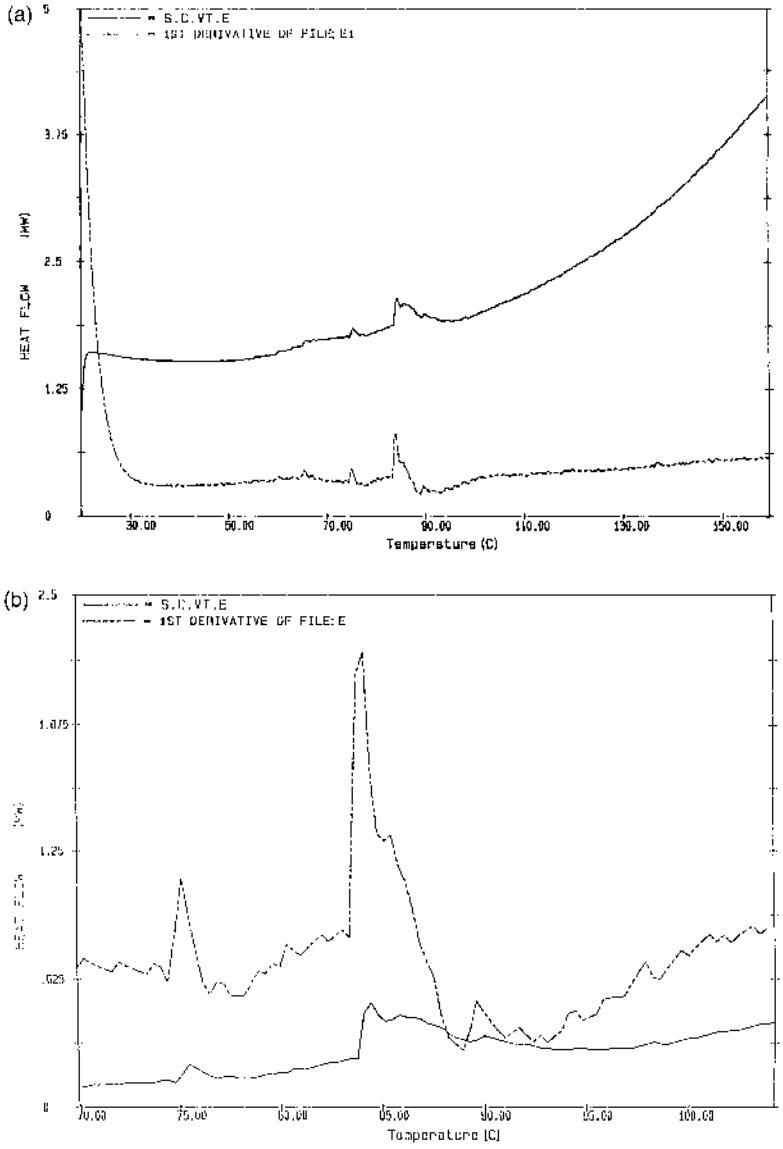

Fig. 5. (a) DSC Thermal Profile of Vitamin E Treated Stratum Corneum and (b) Close-up View of Lipid Phase Transitions of Vitamin E Treated Stratum Corneum

peratures. Thermal transitions were observed near 60 and 100 °C. It has been well recognized that the transitions near 60 °C are associated with lipids, whereas the peaks near 100 °C are associated with irreversible protein denaturation.[17—20] The amount of vitamin E also contains its metabolites.

Figure 5a shows the DSC thermal profile of stratum corneum equilibrated with vitamin E. Figure 5b is a close-up view of the lipid phase transitions (70—100 °C). There are clear phase transitions at 76 and 85 °C which are associated with lipid transitions, and vitamin E itself showed no transitions. DSC studies indicated that vitamin E changes the properties of the lipid domain of the stratum corneum. Vitamin E treated skin showed another broad peak at about 90 °C in addition to two peaks at 60 and 110 °C for normal skin.

Diplock and Lucy reported that vitamin E, a very important antioxidant in our body, has a chemical interaction with arachidonic acid in skin lipid components.[7] The thermal transitions associated with lipid transitions observed in the DSC study of vitamin E may be due to interactions of vitamin E or binding with lipid components of the skin. Barber *et al*., also reported that the inhibition of peroxidation by vitamin E correlated with its binding to complexes of unsaturated lipids.[21,22] Thiele and coworkers[23] observed significantly higher levels of the antioxidant α-tocopherol in the upper stratum corneum which has been recognized as the main cutaneous oxidation target of oxidative stressors such as ultra violet radiation, ozone and chemicals. They proposed that the high concentration of vitamin E[24] in the stratum corneum suggested the important role of this vitamin to protect skin surface lipids and the upper stratum corneum from harmful oxidation. Their explanation can be applied to understand our experimental results which show that about 30% of topically applied vitamin E is present as a very strong chemically bound form rather than simple partitioned into the skin layers.

We could not identify the specific binding sites of vitamin E, although it is possible that vitamin E can bind to arachidonic acid which is present in the skin as Lucy and Diplock proposed. This proposed localization of vitamin E needs to be confirmed. More rigorous analysis considering simultaneous diffusion and binding is under investigation.

Conclusion

An experimental approach to study the binding property of vitamin E has been proposed. This approach can determine free drug and bound drug concentrations based on skin permeation profiles. An appreciable amount (about 30%) of total soluble vitamin E in the skin was found to be bound strongly to the skin. The thermal transitions associated with lipid transitions in DSC thermograms of vitamin E treated skin implies that there are some interactions of vitamin E with the lipid domain of the stratum corneum.

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