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Influence of Hydration on Dihydroxyacetone-Induced Pigmentation of Stratum Corneum

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Dihydroxyacetone, the browning ingredient in sunless tanning formulations, reacts with amino acids in the outer stratum corneum to form a mixture of high molecular weight pigments. Our initial observations indicated that high hydration of dihydroxyacetone-treated skin completely inhibited development of pigmentation. To investigate the mechanism underlying this effect, studies were carried out in isolated murine epidermis, polyvinyl alcohol/lysine films, and lysine in glycerol/water solvent. Murine epidermis treated with dihydroxyacetone showed a biphasic dependence on relative humidity: maximum pigmentation developed at 84% relative humidity and minimum pigmentation at 0% and 100% relative humidity. Filaggrin proteolysis, which shows a similar dependence on relative humidity and provides free amino acids in the outer stratum corneum, did not account for the relative humidity dependence of dihydroxyacetone pigmentation. A similar biphasic pigmentation response was obtained when polyvinyl alcohol film containing lysine was treated with dihydroxyacetone and incubated at various relative humidities, indicating that the structure of the stratum corneum was not a major factor. To remove the influence of the matrix, the reaction of dihydroxyacetone with lysine was followed at varying concentrations of water in mixed glycerol/buffer solvent. Again, greater pigment formation was found at an intermediate level of water (6% vol/vol) and little pigmentation at 0% and 100% water content. These results are consistent with a requirement for water at low relative humidity, which facilitates formation of free amine groups needed for the initial reaction with dihydroxyacetone, and with inhibition of the dehydration reactions by water through the law of mass action at high relative humidity. ***Key words: dihydroxyacetone/hydration/Maillard reaction/photoprotection/pigmentation/stratum corneum. J Invest Dermatol 120:655–661, 2003***

Dihydroxyacetone (DHA), a three-carbon sugar, is the browning ingredient in commercial sunless tanning formulations. DHA preparations have been used for more than 50 y and are currently highly popular for producing temporary pigmentation resembling an ultraviolet (UV) induced tan (Levy, 1992; 2000). Pigmentation develops over a period of hours after application of DHA and remains for several days. Similar to melanin, DHA-induced pigmentation absorbs light throughout the visible spectrum. DHA-induced pigment is much less photoprotective than melanin against UV radiation, however, because it has relatively lower absorption in the UV wavelength range. DHA pigment is only moderately protective against UVA radiation showing protection factors of approximately 2–5 (Fusaro and Johnson, 1975; Johnson and Fusaro, 1987). Recently, multiple applications of DHA have been shown to induce pigmentation that is sufficient to protect uninvolved skin during psoralen plus UVA (PUVA) therapy for psoriasis (Taylor *et al*, 1999). This approach allowed higher UVA doses to be used resulting in fewer treatments than standard PUVA regimens. A photoprotective effect of DHA pigment was also reported in patients with variegate porphyria (Asawanonda *et al*, 1999). In addition, pigmentation of vitiligenous skin using DHA has been reported to produce a cosmetically acceptable result (Fesq *et al*, 2001).

DHA-induced pigmentation forms in the stratum corneum, rather than in deeper epidermal layers, as demonstrated by removal of the pigmented layer by tape stripping (Maibach and Kligman, 1960). The chemistry leading to DHA pigments is believed to be similar to that established for reactions of other sugars with compounds containing amino groups and has been termed the Maillard reaction. This series of reactions has been extensively studied because it is largely responsible for the nonenzymatic browning of sugar-containing foods (reviewed in Labuza *et al*, 1977). The pigments formed, often called melanoidins, are a complex collection of high molecular weight, visible-absorbing chromophores that are produced through a series of nonenzymatic chemical steps. This process is initiated by condensation of the sugar molecule with an amino group, typically on an amino acid, as shown for DHA in **Fig 1**. Dehydration leads to formation of a Schiff base. This reaction and others in the series are reversible reactions. A rearrangement reaction follows leading to a compound called a Heyns product. This colorless rearrangement product subsequently undergoes a series of further condensations with amine-containing compounds, dehydrations, rearrangements, and other reactions to produce complex, visible-absorbing chromophores. The exact steps and products formed have not

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Abbreviations: DHA, dihydroxyacetone; RH, relative humidity; UV, Ultraviolet.

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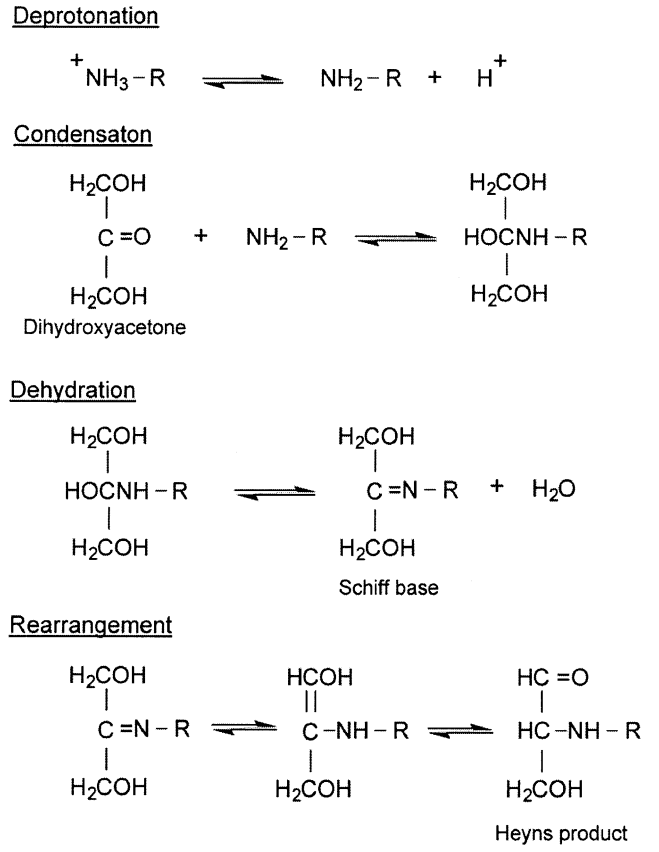

Figure 1. Reactions of DHA with amino groups leading to pigment formation.

been fully identified and vary with the sugar, the amino acid, and the reaction conditions. Free radicals have recently been reported to be involved, at least partially, in the production of melanoidins from the reactions of DHA with murine skin (Lloyd *et al*, 2001). Colored products from the reactions of DHA with amino acids have been isolated and partially characterized although their chemical compositions were not established (Meybeck, 1977). Similar products form between sugars and tissue protein amino groups under physiologic conditions (Njoroge and Monnier, 1989). These reactions are also believed to contribute to the formation of advanced glycation endproduct pigments (Monnier *et al*, 1992; Thorpe and Baynes, 1996). Accumulation of these pigments contributes to the gradual crosslinking, and subsequent reduction of elasticity, during aging of connective tissue collagen as well as to the pathophysiology of age-related diseases such as diabetes and atherosclerosis (Monnier *et al*, 1992; Thorpe and Baynes, 1996).

Different sources of the amino groups are potentially available in the stratum corneum to react with DHA and produce melanoidins. One source is the amino acids that are produced by the proteolysis of filaggrin and the breakdown products of these amino acids that accumulate within the corneocytes. The initial condensation reaction with DHA can occur with the α-amino group or with side-chain amino groups of free amino acids. The side-chain amino groups of the proteins of the cornified envelope (e.g., involucrin, loricrin), could react with DHA although the α-amino groups of the polypeptide chain are not available for the Maillard reaction.

We became interested in the mechanism for formation of DHA-induced pigmentation when preliminary studies showed that increasing the hydration of DHA-treated human skin entirely repressed the development of pigment formation (unpublished observations). This result indicated that simple manipulations of the cutaneous environment might lead to altered pigment formation. From an understanding of the mechanism for this phenomenon we might be able to control DHA pigment formation and potentially decrease the unevenness of color formation over skin areas. We also wondered whether the relative efficiency of DHA pigment formation might be used as an indicator of skin hydration.

MATERIALS AND METHODS

Preparation of murine epidermis Epidermal sheets were prepared from dorsal skin of adult Skh-1 albino hairless mice (Charles River Laboratories, Wilmington, MA). After euthanasia, the dorsal skin (approximately 25 cm^2) was excised and immersed in water at 60°C for 30 s, and the epidermis was gently peeled away from the dermis. The epidermal sheet was then floated on water to smooth it out before cutting into 2.5 cm × 2.5 cm strips. Each strip was lifted from the water onto a microscope slide and dried under vacuum over Drierite® in a desiccator overnight.

Controlled relative humidity (RH) The influence of moisture content on the development of DHA-induced color was investigated using RH chambers. Specific RH values were established in closed 500 ml jars, which contained 100 ml of various saturated salt solutions. RH of 0%, 55%, 75%, 84%, 90%, 97%, and 100% were obtained with Drierite® (anhydrous $CaSO_4$), $Mg(NO_3)_2$, $NaNO_3$, KBr, $BaCl_2$, K_2SO_4, and pure water, respectively. Epidermal samples on glass slides were placed in the vapor above the saturated salt solutions.

Fluorescence spectroscopy Fluorescence emission spectra were obtained with a Spex Flurolog 2 Model 212 spectrofluorometer (Spex Industries, Edison, NJ) with double excitation and emission monochromators and a spectral dispersion of 1.8 nm per mm. The spectrofluorometer was fitted with an optical fiber attachment containing a bifurcated quartz fiber optic bundle (Kollias *et al*, 1986). The excitation light is passed through a monochromator and into one arm of the fiber bundle to irradiate the sample. Fluorescence from the sample is collected by the same fiber and passed into the emission monochromator. The jointed end of the fiber bundle consisted of randomly mixed illuminating and receiving individual fibers. This end of the optical probe (≈6 mm diameter) was brought in close contact with the surface during measurements to eliminate specular reflectance. Fluorescence emission spectra were measured at fixed excitation wavelength (either 340 or 490 nm); whereas the emission monochromator was scanned.

Preparation of polyvinyl alcohol (PVA) films A solution (15 ml) of 10 wt% PVA (MW = 70,000–100,000, Sigma Chemical, St. Louis, MO) was prepared in distilled water with heating at 80°C until complete dissolution. The appropriate amount of L-Lysine–HCl (Sigma Chemical) corresponding to 0.01 M was added to the clear PVA solution and the mixture was allowed to cool under mild stirring. The solution was then poured into a 15 cm diameter Petri dish and allowed to dry at room temperature for 48 h. The dry clear film detached easily from the Petri dish and samples (2.5 cm × 2.5 cm) were cut out. The samples were ≈100 μm thick.

Water/ethylene glycol solutions Appropriate amounts of L-lysine and DHA to make 0.01 M solutions were dissolved in 5 ml of ethylene glycol or in 5 ml of ethylene glycol–aqueous 0.1 M phosphate buffer (pH 7) containing various proportions of phosphate buffer (2%, 4%, 6%, 8%, 10%, 20%, 30%, 40%, 50%, 80%, and 100% vol/vol).

RESULTS

Hydration inhibits DHA-induced pigment formation in *ex vivo* murine epidermis In pilot studies of the response of human skin to DHA *in vivo* and *ex vivo*, hydration was identified as a major factor inhibiting the development of pigmentation (unpublished observations). In order to examine the influence of hydration on the development of color induced by DHA in skin, a model system was developed that could be equilibrated at different RH. Dry epidermal sheets of Skh-1 hairless mouse skin were inverted and placed in contact with a solution of 5% DHA

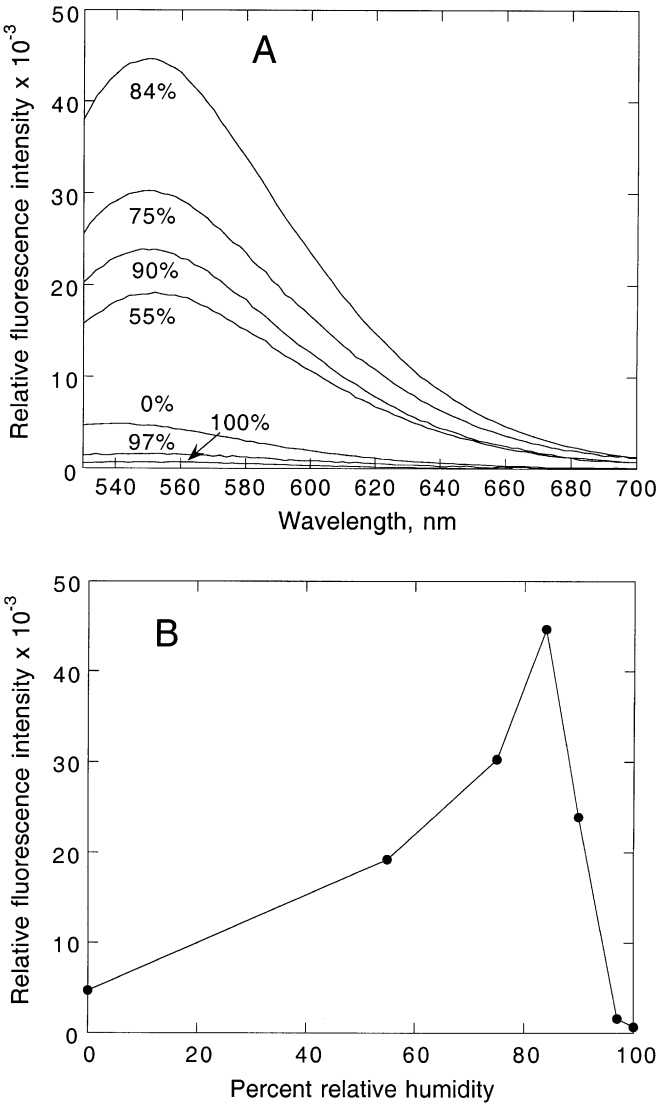

Figure 2. Influence of RH on the fluorescence of products formed between DHA and murine epidermis. (*A*) Relative fluorescence spectra obtained upon excitation at 490 nm of murine epidermal samples treated with DHA at various RH for 24 h. (*B*) Relative fluorescence at 550 nm of DHA-initiated pigment formed in murine epidermis *ex vivo* at different RH. Data shown are from one experiment of three that all gave a similar pattern.

in absolute alcohol for 30 s. Excess solution was blotted and the samples were allowed to dry in air for 15 min. Control samples were treated with absolute alcohol but otherwise handled identically. DHA-treated and control samples on glass slides were placed in atmospheres with different RH and incubated for 48 h.

The extent of pigment formation was assessed by fluorescence emission spectroscopy using excitation at 490 nm, the maximum wavelength in the visible excitation spectrum. Fluorescence developed over 48 h with the intensity reaching a relatively stable level between 24 and 48 h. Spectra of control samples have been subtracted from spectra of treated samples at the same RH to produce the spectra shown in **Fig 2**(***A***). Maximum fluorescence intensity was observed at about 550 nm for samples incubated at 55%, 90%, 75%, and 84% RH. The spectra observed at the various RH had the same general shape. Epidermis incubated at 0% RH showed a weak fluorescence whereas the spectra obtained from samples incubated at 97% and 100% RH were nearly flat. Significantly, an increase in RH did not necessarily correlate with an increase in fluorescence intensity.

Because the shape of the emission spectra was independent of the RH, the fluorescence intensities at 550 nm were plotted against RH as shown in **Fig 2**(***B***). The fluorescence intensity increased from 0% to 84% RH where it reached a maximum and then decreased at higher RH. A similar relationship between fluorescence and RH was obtained with isolated human epidermis although the maximum fluorescence was observed at 75% RH (unpublished results).

Hydration-induced proteolysis of filaggrin does not account for the relationship between RH and DHA-induced pigmentation in murine epidermis Two alternative hypotheses for the effect of RH on DHA-induced pigmentation were evaluated. One is based on the known effect of RH on the proteolysis of filaggrin to amino acids (Scott and Harding, 1986). The pool of free amino acids, and their derivatives, present in corneocytes derive from proteolysis of filaggrin shortly after it enters the lowest layers of the stratum corneum. The range of RH that allows proteolysis of filaggrin *ex vivo* is between 80% and 95% in isolated rat epidermis. As our experiments indicated that 84% RH led to maximum pigment formation, it was possible that the limiting factor in pigment formation was the availability of amino acids from breakdown of filaggrin. The second hypothesis is that the RH influences the amount of water or other reactants available for steps in the reaction between DHA and amino groups and thus controls the efficiency or the extent of pigment formation.

Two experiments were carried out to differentiate between the two hypotheses. In one experiment, epidermal samples were first incubated for 48 h at 84% RH, our optimal RH for pigment formation in murine epidermis, which is also in the range for filaggrin breakdown in *ex vivo* rat epidermis (Scott and Harding, 1986). The samples were then dried overnight over Drierite® *in vacuo*. Next, each sample was treated with DHA and incubated at 0%, 84%, or 100% RH for varying lengths of time. Formation of DHA-induced pigment was assessed as a function of incubation time by the fluorescence emission at 550 nm. If RH influences filaggrin breakdown to provide amino acids to react with DHA, all samples are predicted to have the same pigmentation, i.e., equal fluorescence intensities, as after the first incubation filaggrin would be proteolyzed into free amino acids to the same extent in all samples. In contrast, if the efficiency or extent of chemical reactions between amino acids and DHA is influenced by epidermal water content, the sample incubated at 84% RH during the second period is predicted to show much greater pigmentation (fluorescence) than those at 0% and 100% RH. **Figure 3**(***A***) shows that the sample held at 84% RH during the second incubation is markedly more fluorescent than the others. Samples held at 0% or 100% RH during the second incubation did not develop appreciable fluorescence. These results agreed with visual observation where the 84–84 sample was the only one that was pigmented. The results suggest that the chemical reaction of DHA with amino groups in the epidermis, rather than the availability of amino acids from humidity-dependent filaggrin proteolysis, is affected by the RH.

In a complementary experiment, samples were first equilibrated at 0%, 84%, and 100% RH for 48 h and then treated with DHA as above and re-incubated at 84% RH. If the first hypothesis is correct, the samples incubated during the first period at 84% RH should be much more fluorescent than samples incubated at 0% and 100% RH because 84% RH is near optimal for filaggrin proteolysis. If the second hypothesis is correct, all samples should show approximately the same fluorescence. All of the samples developed substantial fluorescence over the second incubation period (**Fig 3*B***) and all became pigmented, supporting the conclusion of the first experiment. The 84–84 sample showed the same magnitude of fluorescence as in the first experiment (**Fig 3*A***), as expected. The reason for greater pigmentation in the 100–84 samples is unclear. Possibly, the prior incubation at 100% RH allowed more rapid equilibration when the samples were subsequently

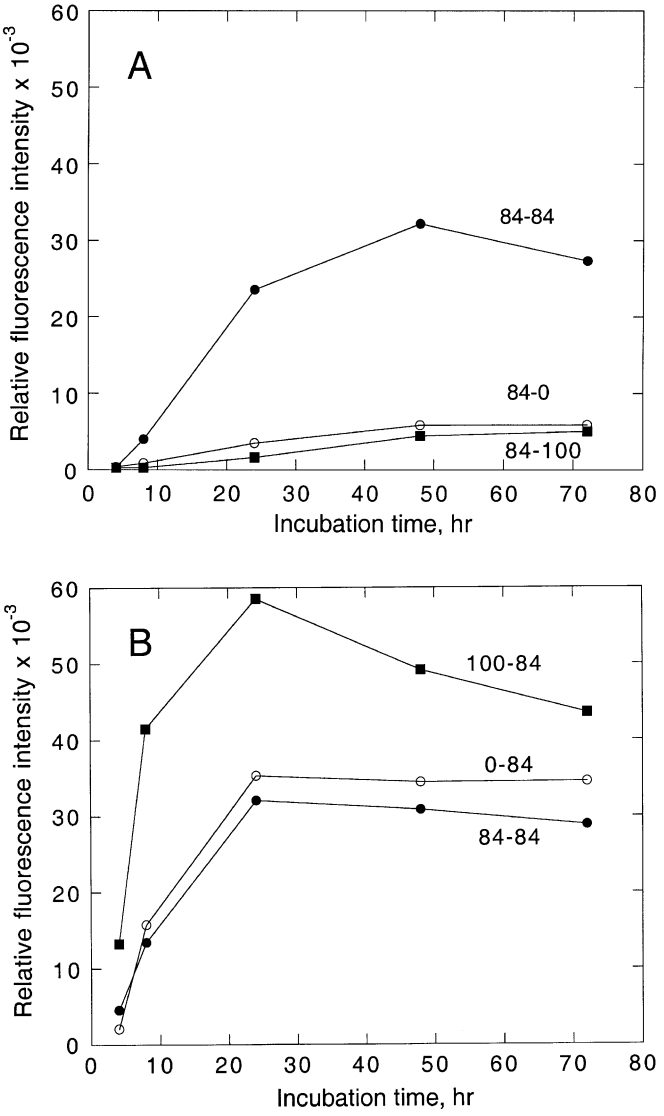

Figure 3. Effect of preincubation of murine epidermis at various RH on development of DHA-initiated pigmentation. Relative fluorescence intensities were measured at 550 nm upon excitation at 490 nm. (*A*) Epidermal samples were equilibrated at 84% RH for 24 h, and then DHA was applied and samples were re-incubated at 0% (84–0), 84% (84–84), and 100% (84–100) RH and fluorescence was measured at various times. (*B*) Epidermal samples were equilibrated at either 0% (0–100), 84% (84–100), or 100% (100–84) RH for 24 h, and then DHA was applied and samples were re-incubated at 84% RH and fluorescence was measured at various times. Data shown are from one experiment of three that gave similar patterns.

incubated at 84% RH as the hydration history has been shown to influence the rates of water absorption and desorption by human stratum corneum (Anderson *et al*, 1973). Thus, the 100–84 sample would have been at ≈84% RH for a longer period of time. These results indicate that the development of DHA-induced pigmentation is not limited by availability of basic amino acids at 0% and 100% RH. The water content of the stratum corneum, as influenced by the RH (Spencer *et al*, 1975), appears to control the DHA reactions leading to pigment formation.

Hydration influences the formation of fluorescent products from the reaction of DHA with lysine in PVA hydrogels We hypothesized that the influence of hydration on development of DHA-induced pigmentation might be attributable to changes in the highly organized structure of the stratum corneum as a function of water content that altered either the availability of the reactants or their rates of diffusion. The reactants are considered to include water, as well as DHA and basic amine-containing compounds (see equations in **Fig 1**). To evaluate this hypothesis, we used another solid matrix that is readily hydrated but does not have the specific complex lipid and protein structure of the stratum corneum. PVA films containing a high lysine concentration were chosen for this purpose. Similar to dry stratum corneum, dry PVA films readily regain moisture when placed in a high humidity atmosphere to form hydrogels. PVA films were formed from 10% wt/vol PVA solutions containing 0.01 M lysine so that the final concentration of lysine in the dry films was approximately 0.1 M. Two approaches were used. In one set of experiments, dry 2.5 × 2.5 cm PVA/lysine films were treated with 20 μl of an ethanolic solution of 0.1 M DHA, allowed to dry for 15 min, and then incubated at 0%, 75%, 90%, and 100% RH at room temperature. Control samples were treated with 20 μl of absolute ethanol but otherwise treated identically. In a second set of experiments, dry PVA/lysine films were first allowed to equilibrate for 2 d at 0%, 75%, 90%, and 100% RH and then treated with DHA or ethanol as above. The fluorescence spectra of all samples were measured at 0, 6, 20, and 46 h, with excitation at 340 (absorption maximum in UV range) and 490 nm and emission at 424 and 550 nm, respectively. The results presented in **Fig 4** were corrected for any emission in the control samples not treated with DHA but subjected to the same conditions.

Figure 4(***A***) shows the evolution of the reaction as a function of time at 75% RH. The results from both of the experiments were nearly identical. The shape of the spectra remained the same throughout the incubation period with a peak at 424 nm and a shoulder at about 475 nm. A similar set of spectra was generated upon excitation at 490 nm and emission at 550 nm (data not shown). The magnitude of the fluorescence that developed over 46 h with excitation at 340 nm was approximately a factor of 5 greater than the fluorescence excitation at 490 nm (**Fig 4*B***). The fluorescence of products formed by reactions between DHA and lysine in PVA films was influenced by the RH. As shown in **Fig 4**(***C***), after 46 h of incubation at 100% RH only very slight fluorescence was observed. In samples incubated at 0% RH a small peak developed at about 424 nm possibly due to residual water retained by the PVA film during the drying period. In samples incubated at 75% RH the fluorescence intensity increased considerably and was accompanied by the appearance of a shoulder at about 475 nm. The results from both sets of experiments were nearly identical. The maximum fluorescence intensities recorded at 424 nm at varying times are plotted as a function of RH in **Fig 4**(***D***). The relationship between fluorescence intensities and RH showed a pattern similar to that previously observed with murine epidermal samples (**Fig 2*B***). Thus, our hypothesis was not upheld, i.e., the effect of RH on the reaction between DHA and basic amino acids was not attributable to the specific structure of the stratum corneum.

Reactions between DHA and lysine in solution are influenced by the percentage water content of the solvent The basis for the influence of matrix structure on the reaction of DHA with basic amino acids is unclear, especially as the effect is nonlinear with respect to RH and therefore water content (Spencer *et al*, 1975). The availability of the reactants (DHA, lysine, and water) for reaction may vary in a complex manner due to differences in their binding to the polymer, protein, or lipid as a function of water content. Alternatively, interactions with the matrix may not be a major factor. Therefore, we hypothesized that the efficiency of the reaction between lysine and DHA is directly influenced by the availability of water as water is a reactant and a product in certain steps of the reaction mechanism (**Fig 1**). To test this hypothesis, we used a simple system, namely, DHA and lysine dissolved in ethylene glycol–aqueous phosphate buffer (0.1 M, pH 7) mixed solvent that varied in ethylene glycol content (0%, 2%, 4%, 6%, 8%, 10%, 20%, 30%, 40%, 50%,

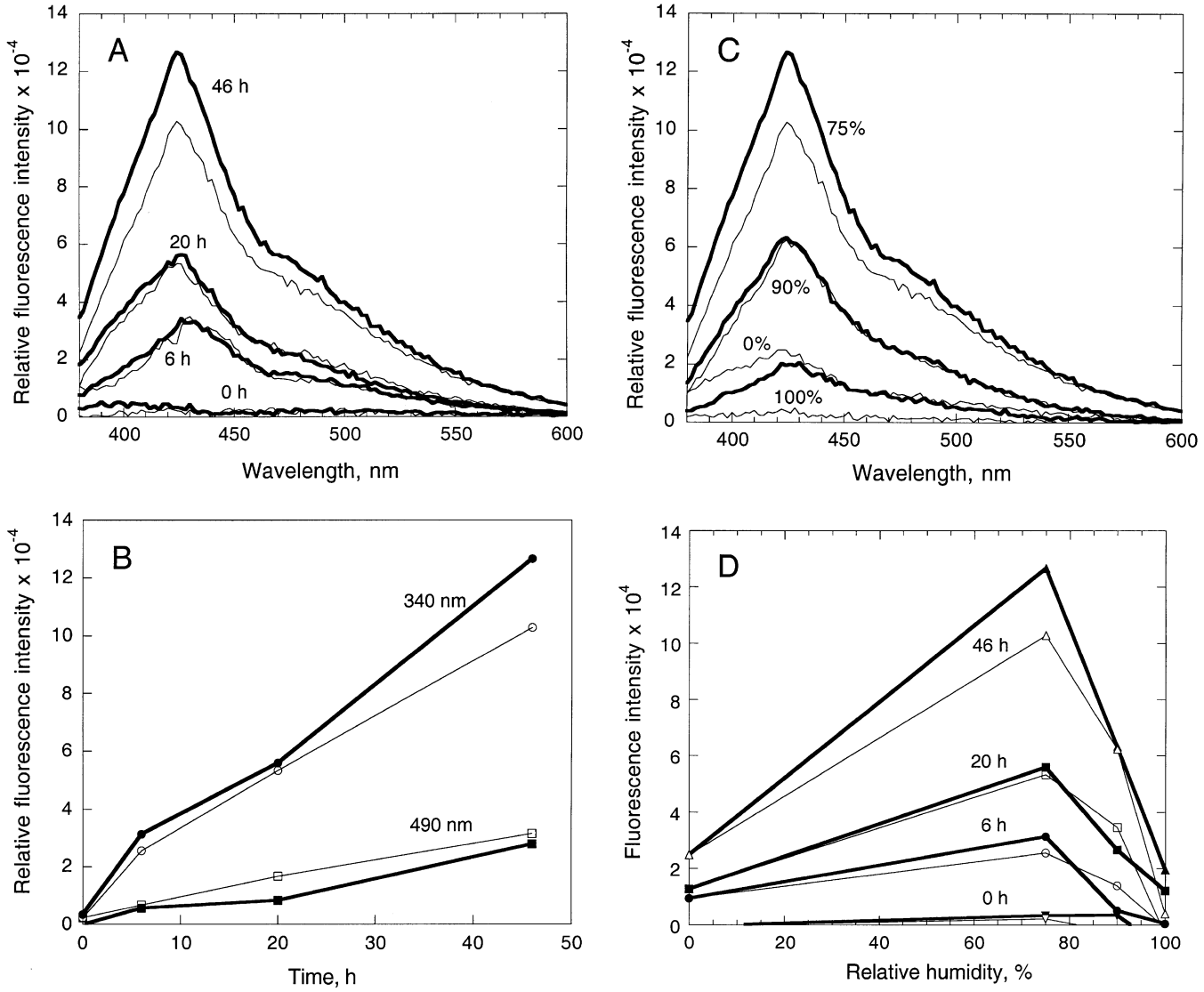

Figure 4. Influence of RH on formation of fluorescent products between DHA and lysine in PVA films. Fluorescence was excited at 340 nm. The heavy line indicates samples first incubated at 0%, 75%, 90%, or 100% RH that were treated with DHA and then re-incubated at the same RH. The thin line indicates samples treated with DHA and then incubated at 0%, 75%, 90%, or 100% RH. (*A*) Time course for development of fluorescence in DHA-treated PVA films containing lysine and incubated at 75% RH. (*B*) Comparison of development of fluorescence intensity upon excitation at either 340 nm or 490 nm in lysine–PVA films coated with DHA and held at 75% RH. (*C*) Relative fluorescence spectra of products formed after 46 h of incubation of DHA-treated PVA films containing lysine at varying RH. (*D*) Relative maximal fluorescence intensity at 424 nm at varying times (0, 6, 20, and 46 h) plotted as a function of RH. Data shown are from one experiment of three with similar results.

80%, and 100% vol/vol). The concentrations of DHA and lysine were constant at 0.1 M each.

The extent of color formation was followed by measuring the absorbance at 340 nm, near a peak in the absorption spectrum after 3, 4, and 5 h of incubation. Absorption in the UV at 340 nm appeared earlier and was greater than absorption in the visible range (460–520 nm) (unpublished results). As found for the reaction of DHA with amino groups in the hydrated solids, the amount of product formation was a nonlinear function of the water content. Very little product absorbing at 340 nm was formed in 100% ethylene glycol. Even a low concentration of water dramatically increased the amount of product, however. The 340 nm absorbance increased greatly between 0% and 6% water content at all three time points measured. At water contents greater than 6% the yield of product dropped almost as steeply as in the lower water content range. At 3 and 4 h of reaction time, virtually no 340 nm absorbing product was formed at 30% water content. The time-dependent appearance of absorption at 340 nm showed that, at 6% water content, the absorption increased by a factor of about 3 between 3 and 5 h. These results support our hypothesis that the availability of water directly influences the efficiency of the reaction between lysine and DHA and subsequent steps that lead to chromophore development.

DISCUSSION

DHA-induced pigmentation is not a simple function of hydration of the stratum corneum Our preliminary results in human skin *in vivo* suggested that hydration entirely inhibited DHA-induced pigment formation. Now we have shown, under controlled hydration conditions, that neither very low nor very high hydration allowed formation of DHA-induced chromophores. Instead pigment formation was optimal at an intermediate level of hydration achieved when the atmosphere was 84% RH, corresponding to 20% water content [based on measurements made on human stratum corneum (Spencer *et al*, 1975)]. These results suggest that at least two steps in the process leading to chromophore formation are subject to the influence of

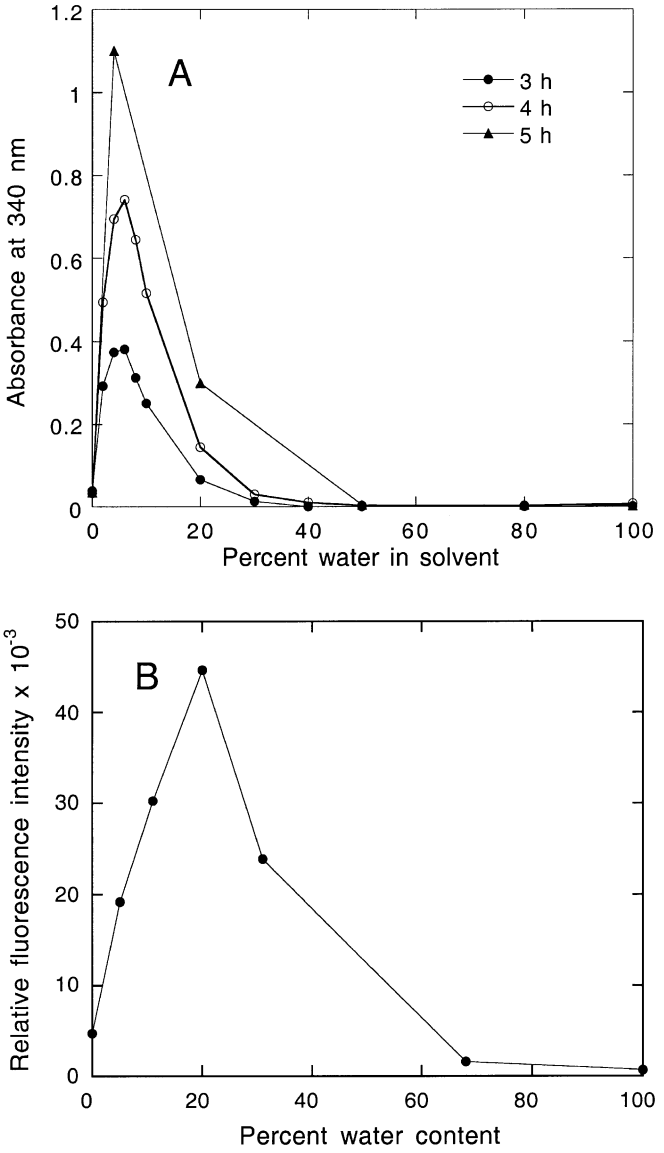

Figure 5. Relationship of percentage water content with efficiency of DHA reaction. (*A*) Effect of percentage water content in mixed ethylene glycol–aqueous buffer solvent on formation of products between DHA and lysine. The absorption at 340 nm is shown as a function of water content after 3, 4, and 5 h of incubation at room temperature. Data are from one experiment of three that all gave similar results. (*B*) Relationship between fluorescent product formation from reaction of DHA with murine epidermis as a function of the water content of the epidermis. Fluorescence was measured at 550 nm with excitation at 490 nm. These are the same data as shown in **Fig 2**(***B***).

water and that the water content has opposing effects on these steps. At low RH, increasing hydration promotes a process that enhances pigment formation, whereas at higher levels of hydration, water drives processes that inhibit chromophore development.

This pattern of hydration dependence contrasts with other effects of water content on stratum corneum. For example, increasing hydration of the stratum corneum enhances desquamation and chymotrypsin activity in stratum corneum (Watkinson *et al*, 2001) and decreases physical properties such as Young's modulus and fracture stress (Papir *et al*, 1975). On the other hand, the proteolysis of filaggrin in rat skin *ex vivo* has a maximum at 80%–95% RH and is inhibited at both low and high RH (Scott and Harding, 1986). As the amino acids formed from filaggrin provide a major source of basic amino groups to react with DHA, it appeared possible that hydration-controlled hydrolysis of filaggrin might be the determining factor in formation of DHA-induced pigmentation. Our results (**Fig 3**) did not support this hypothesis, however, as preincubation of epidermis at 84% RH, which promotes filaggrin proteolysis, prior to DHA treatment did not lead to greater DHA pigment formation compared to preincubation of epidermis at 0% and 100% RH.

The influence of hydration on DHA-induced pigmentation is not unique to stratum corneum The general pattern of DHA pigment formation as a function of RH was very similar in murine stratum corneum and PVA films (compare **Figs 2*B*, 4*D***) suggesting that similar factors may be controlling the reactions leading to pigmentation in both matrices. This was somewhat surprising as the matrices differ drastically in their composition and structure. The highly organized structure of the stratum corneum confines water and the hygroscopic free amino acids (and their derivatives) within the lumen of corneocytes. These low molecular weight species, produced by proteolysis of filaggrin in the inner layers of the stratum corneum, are termed the natural humetic factor because they account for the uptake of water by the stratum corneum. Hydration clearly would influence the availability of water to participate in the reactions leading to pigment formation but it could also influence the availability of the amino acids. Water molecules in stratum corneum are not all present in the same physical form. At low water content (10%), water molecules tightly associate with the natural humetic factor and corneocyte proteins by hydrogen bonds and do not readily participate in chemical reactions. At higher water content, the water molecules are mainly loosely bound ($\approx$10%–50% water content) or are present as bulk water (>50% water content). Water content of stratum corneum increases, but not linearly, with the RH (Spencer *et al*, 1975). The RH used in these experiments of 0%, 55%, 75%, 84%, 90%, 97%, and 100% correspond to water contents of 0, 5, 11, 20, 31, 68, and 100, based on studies in human stratum corneum. An increase in the water content of stratum corneum increases the intracellular volume of the corneocytes, which might both dilute the reactants, thereby decreasing the rate of bimolecular reactions, and increase the diffusion constants for the mobile reactants. As increasing water content up to 20% (84% RH) increases the amount of pigmentation, the latter effect of increasing water content may contribute more than the former at RH up to 84%.

Hydrated PVA film, a hydrogel, consists of one phase and does not limit the location of the reactants to defined volumes. Water molecules in PVA films also exist in different forms as they hydrogen bond to the hydroxyl groups of the PVA chain. At very low water content, water occupies these tight binding sites but at high water content it behaves as bulk water. In addition, water will also be hydrogen bonded to the polar amino and carboxyl groups of the lysine present at high concentration in the hydrated films. The high molecular weight PVA used in these experiments (70–100 kDa) restricts the diffusion of small molecules (Kojima *et al*, 1984). The diffusion coefficients increase as the film becomes more hydrated, which enhances reaction rates although, as for the stratum corneum, this effect is accompanied by a potential dilution effect. As the same relationship between pigmentation and hydration was observed in both stratum corneum and PVA hydrogel, it suggests that the increased mobility of reactants with increasing water content enhances pigmentation (up to 84% RH) in both matrices.

The similar correlation of pigment development with RH in the two matrices suggests that the water activity, a_w, is an important parameter. Water activity in a material is a measure of the availability of water for reactions and is directly related to the equilibrium RH. Thus, stratum corneum and PVA film equilibrated at the same RH have the same a_w, which validates using RH, rather than another parameter such as water content, for the comparison of DHA pigmentation in the two matrices (**Figs 2*B*, 4*D***).

Water influences the chemical reactions leading to DHA-induced pigment formation To remove the influences of matrix structure and composition, and to focus on the influence of a_w on the reaction mechanism, the reaction of DHA with lysine was carried out in mixed solvents containing a varying fraction of water. Our results indicate that, even in the absence of barriers to mobility and independent of viscosity and potential dilution effects, an optimal percentage water exists for development of pigmentation (**Fig 5*A***). The mechanism for initiation of DHA pigment formation shown in **Fig 1** indicates that at least two steps are subject to the influence of water. Water is necessary for the equilibrium between protonated and unprotonated amine groups because it provides a polar, hydrogen-bonding solvent. As the free, unprotonated amine is required for the subsequent nucleophilic attack on the carbonyl group of DHA, sufficient water molecules are needed to support this equilibrium. At 0% RH, this equilibrium cannot occur and development of DHA pigmentation is inhibited. Thus, water molecules enhance the DHA pigmentation reactions at low water content of the stratum corneum, which contributes to the increase in pigmentation with increasing RH up to 84% RH.

The second step in the initiation of DHA pigmentation influenced by water availability is the condensation/dehydration reactions of DHA with an amino group to form a Schiff base (**Fig 1**). These reactions are equilibria; the product of the condensation reaction is unstable and readily reverts to DHA and amino acid. Dehydration produces a Schiff base and a molecule of water. This reaction is also reversible so that, by the law of mass action, as the water content increases, the reaction is driven toward the condensation product, which can revert to DHA and the amino acid. Thus, in this step water plays an inhibitory role and can account for the decrease in DHA-induced pigmentation at RH > 84% in murine stratum corneum. In addition to the influence of water on these initial steps, the moisture content of skin could modulate additional later reactions as well as influence the relative reaction of DHA with free amino acids *versus* with protein-bound amino acids.

The influence of water on the Maillard reaction with glucose and other food sugars has been extensively studied in solution because of its importance in the food industry. For example, in studies of the Maillard reaction between xylose and glycine, maximum browning was observed when the water content was about 30% but no browning was observed when the water content was either 0% or above 90% (Wolfrom and Rooney, 1953). Our results follow the same pattern with a different optimal percentage water, which may be partly due to differences in the equilibrium constants for the condensation and dehydration reactions. When the extent of DHA pigmentation in murine stratum corneum is plotted against water content [using values that were measured for human stratum corneum (Spencer *et al*, 1975)], maximum pigmentation occurred at 20% water content (**Fig 5*B***). The difference between this value and the 6% found in water/glycerol mixed solvent has several sources including differences in the reactivity of the amino group with DHA. Subsequent condensation and dehydration reactions of intermediates lead to further loss of water molecules so that high water content also reverses these required for pigmentation (Eichner and Karel, 1972). The inhibition of browning at high water content has been shown to be more influenced by the reversal of the dehydration reaction than by dilution of the reactants (Eichner and Karel, 1972).

In summary, our results suggest that the biphasic relationship between RH and development of DHA pigmentation in stratum corneum has several sources. The inhibition of pigmentation at 0% RH is attributed to the lack of water molecules to support deprotonation of the free amine group. As the RH increases, water becomes more available to support this process and the water in the corneocytes may also increase the diffusion of small reactants. The decrease in pigmentation at RH > 84% is attributed to the reversal of the dehydration reaction by the high water content ($\approx$20%).

The results of this study have at least two potential applications. One implication of these results is that the moisture content of the stratum corneum is a factor in the development of an even tan, i.e., uniform intensity of pigmentation over the area treated with DHA. Thus, sunless tanning formulations containing DHA should be developed to control the moisture content of the stratum corneum over several hours while the pigmentation develops. Furthermore, the moisture content should be at or near the level that leads to maximum pigmentation. A second potential application of our results is that DHA-induced pigmentation might be used to monitor stratum corneum hydration over a period of several hours. As development of measurable pigmentation (or DHA-induced fluorescence) occurs over about a 4 h period (under our current conditions), an "instantaneous" or short-time measurement of stratum corneum hydration is not feasible. In certain situations, however, longer term measurements are useful, e.g., to measure the long-term effectiveness of a moisturizer. Development of these applications requires clinical studies.

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