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pH-Dependence of phase transition of the lipid bilayer of liposomes of stratum corneum lipids

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Abstract

Phase transition of the lipid bilayer of liposomes of stratum corneum lipids, consisting of ceramide, cholesterol, cholesteryl sulfate and palmitic acid, was examined at different pH values by monitoring the fluorescence anisotropy of diphenylhexatriene (DPH). The phase transition temperature decreased about 15°C with decrease of the pH from 6.2 to 4.0. The decrease depended on the content of palmitic acid in liposomes and corresponded with the protonation of the anionic form of fatty acid judging from the fluorescence intensity and fluorescence anisotropy of the cationic trimethylammonium derivative of DPH (TMA-DPH). These results and pH-dependent change in fluorescence anisotropy of 2-(9-anthroyloxy)palmitic acid (2-AP) indicate that perturbation of lamellar lipids is induced by the undissociated form of fatty acids. Stimulation of transdermal penetration of phenol at acidic pH was observed which was consistent with the results on liposomes. A pH-dependent decrease of the phase transition temperature of liposomes was also observed on replacement of palmitic acid by other saturated fatty acids, the decreases being in the order lauric, myristic > palmitic > stearic acid. In the presence of lauric acid at pH 4.0, the fluorescence anisotropy of DPH also decreased at physiological temperatures, this effect being similar to those of long-chain cis-unsaturated fatty acids.

Keywords: Stratum corneum; Liposome; Fluorescence anisotropy; Diphenylhexatriene; Fatty acid

1. Introduction

The stratum corneum lipid lamella, which is known to be a barrier to the transdermal penetration of hydrophilic solutes, mainly consists of ceramides, cholesterol, free fatty acids and cholesteryl sulfate (Wertz and Downing, 1989). This lipid composition is quite different from those of other biological membranes. As studies started only recently on the role of each lipid component in regulating the rigidity of the lamellar lipids of stratum corneum (Kitson et al., 1992; Thewalt et al., 1992), many points are still unknown. Liposomes are good models to clarify the factors governing the barrier function of the stratum corneum and the role of each lipid component (Abraham and Downing, 1991). They have also been used to determine the mechanisms of

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action of penetrating enhancers such as Azone, oleic acid and ethanol (Beastall et al., 1988; Ongpipattanakul et al., 1991; Krill et al., 1992a). They have also been revealed to represent the characteristics of drug skin permeability (Matsuzaki et al., 1993).

Using liposomes, we found that cholesterol is the main component causing solidity of the stratum corneum lipid bilayer (Kitagawa et al., 1993), whose behavior is quite different from that in lipid bilayers composed of phospholipids (Demel and De Kruyff, 1976). However, other lipid components may also have important roles in regulation of the rigidity of the lipid bilayer. For example, the effects of fatty acids in the stratum corneum on fluidity of lamellar lipids have not been studied extensively, although we showed that palmitic acid, which is a major fatty acid component in stratum corneum lipids (Friberg et al., 1991), had no effect on phase transition or fluidity of the stratum corneum lipid liposomal membrane at pH 7.5 (Kitagawa et al., 1993). Protonation of anionic forms of fatty acids possibly affects the fluidity of the lipid bilayer. Since the pH of skin is reported to be slightly acidic (Friberg et al., 1991), it is important to determine the effects of pH on the behavior of fatty acids in lamellar lipids. Moreover, as saturated fatty acids such as lauric acid have been found to enhance transdermal drug penetration (Aungust et al., 1986), we investigated the effects of various saturated fatty acids on phase transition and fluidity of lipid bilayers of stratum corneum lipid liposomes at different pH values by observing fluorescence anisotropy of diphenylhexatriene (DPH), which is a reporter of the fluidity in the hydrophobic core of the lipid bilayer (Kuhry et al., 1983).

2. Materials and methods

2.1. Materials

Ceramide (type IV), cholesterol, cholesteryl sulfate and DPH were purchased from Sigma Chemical Co. (St. Louis, MO). Fatty acids were from Wako Pure Chemical Industries, (Osaka, Japan). TMA-DPH and 2-(9-anthroyloxy)palmitic acid (2-AP) were obtained from Molecular Probes (Eugene, OR).

2.2. Preparation of liposomes

Ceramide, cholesterol, cholesteryl sulfate and palmitic acid were dissolved in chloroform at a weight ratio of 4.0:2.5:1.0:2.5 (Molar ratio, 3.2:3.2:1.0:4.4), as described previously (Kitagawa et al., 1993). The solvent was evaporated, the lipids were suspended in medium A (150 mM NaCl, 1 mM EDTA, 10 mM buffer described below) and multilammelar vesicles were prepared by vortex mixing. Tris-HCl buffer (pH 7.4), phosphate buffer (pH 3.0 and 6.2) and acetate buffer (pH 4.0 and 5.0) were used. The total lipid concentration was 1.2 mM. Then the vesicle suspension was sonicated with a probe-type sonicator at 70 80°C for 10 min at an output power of 80W under a stream of nitrogen. Liposomes containing the same molar ratio of other fatty acids instead of palmitic acid or no fatty acids were prepared by a similar method.

2.3. Measurement of fluorescence anisotropy

The fluorescence anisotropies of DPH, TMA-DPH and 2-AP, which are markers of fluidity of the lipid bilayer, were measured as described previously (Kitagawa et al., 1985, 1993). Liposome suspensions were diluted 10-fold just before measurement in medium A mentioned above. A solution of DPH, its cationic derivative, TMA-DPH, or 2-AP in dimethylformamide was added to the liposome suspension at a final concentration of 0.5 μM (DPH and TMA-DPH) or 1.0 μM (2-AP). The final concentration of organic solvent added was limited to 0.033% to avoid perturbation of the lipid bilayer. Liposomes were incubated with DPH for 5 min or with TMA-DPH or 2-AP for 2 min. Fluorescence in liposomes was measured in a 4010 spectrofluorometer (Hitachi Seisakusho, Tokyo, Japan). The excitation and emission wavelengths used for DPH, TMA-DPH and 2-AP were 363 and 428 nm, 365 and 428 nm, and 367 nm and 458 nm, respectively. Fluores-

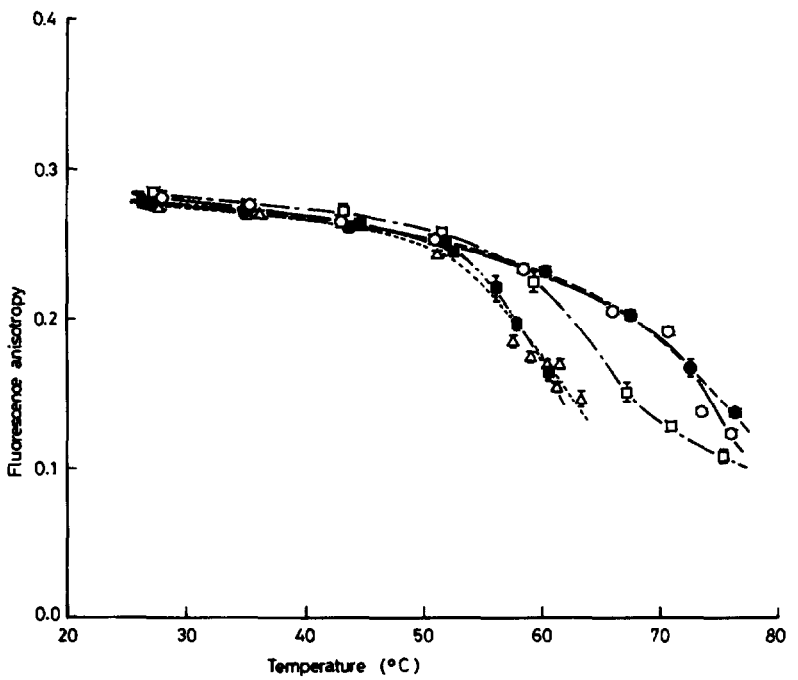

Fig. 1. Phase transition of lipid bilayer as monitored by the temperature dependency of fluorescence anisotropy of DPH in liposomes composed of four stratum corneum lipid components at pHs 7.4 (○), 6.2 (●), 5.0 (□), 4.0 (■) and 3.0 (△).

cence anisotropy was calculated as described previously (Kubina et al., 1987).

2.4. Measurement of in vitro skin penetration of phenol

Full thickness abdominal skin was excised from male guinea pig skin and mounted in the two-chamber diffusion cells with a water jacket (37°C) (available diffusion area was about 0.65 cm^2, each half-cell volume was about 5.4 ml). The donor and receiver cells, which were stirred by a magnetic stirrer were filled with either isotonic citrate buffer (pH 5.0) or isotonic phosphate buffer (pH 7.4). After 12-h pretreatment, 50 mM phenol dissolved in each buffer mentioned above was added to the donor cells and the penetration experiment was started. Every 1 h, 150 μl of samples were taken and analyzed spectrophotometrically and the permeation coefficient was calculated from the initial straight portion of the penetration curve (Itoh et al., 1992).

3. Results

3.1. pH-Dependency of phase transition temperature of lipid bilayer in liposomes of stratum corneum lipids

We first examined the pH-dependence of phase transition temperature in liposomes consisting of the four lipid components of the stratum corneum (ceramide, cholesterol, cholesteryl sulfate and palmitic acid) between pH 3.0 and 7.4. The results are shown in Fig. 1. As previously reported (Kitagawa et al., 1993), at pH 7.4, the fluorescence anisotropy of DPH decreased markedly at 70°C, indicating phase transition of the lipid bilayer from the gel to the liquid crystal state at this temperature. This phase transition temperature depended on pH. As shown in Fig. 1, the phase transition temperature decreased about 15°C with a decrease of pH from 6.2 to 4.0. These phase transition temperatures were similar to those reported in stratum corneum of various species

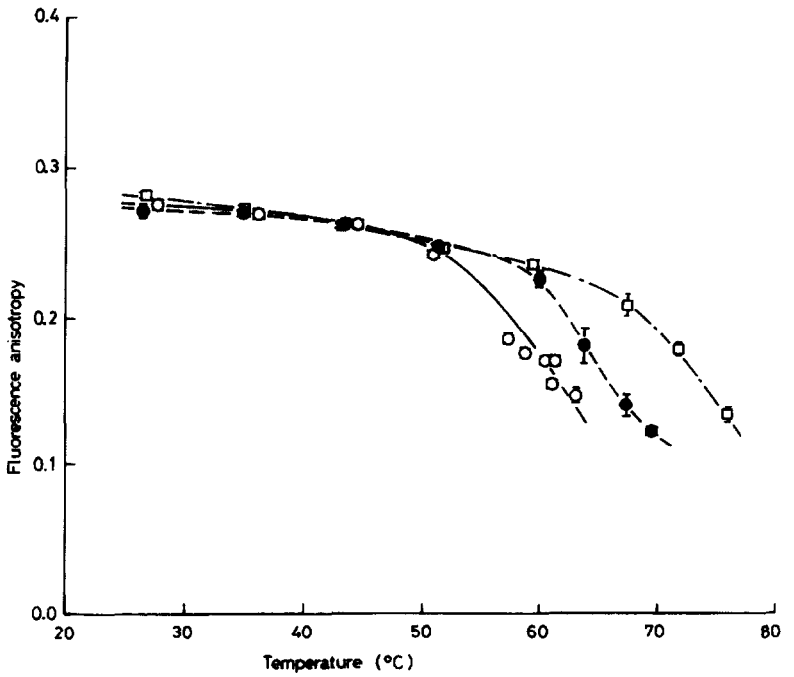

Fig. 2. Dependence of phase transition of lipid bilayer as monitored by temperature dependency of fluorescence anisotropy of DPH in stratum corneum lipid liposomes at pH 3.0 on the content of palmitic acid at a weight ratio in total lipids of 25% (control liposomes) (○), 12.5% (●) and 0% (□).

(Golden et al., 1987; Potts et al., 1991; Krill et al., 1992b). No significant difference was observed in the fluorescence anisotropies at physiological temperatures at the pHs observed.

This decrease of phase transition temperature at acidic pH clearly depended on the content of palmitic acid in the liposomes. As shown in Fig. 2, in liposomes without palmitic acid, phase transition at pH 3.0 was similar to that in liposomes with four lipid components, including palmitic acid at pH 7.4 as shown in Fig. 1. With the increase in the content of palmitic acid, the phase transition temperature decreased. These results indicated that lowering of the phase transition temperature of stratum corneum lipid liposomes at acidic pH was due to the presence of palmitic acid.

The results also suggested that the pH-dependent change in phase-transition temperature was probably due to change of dissociation of palmitic acid in the lipid bilayer. To examine this possibility, we next measured the fluorescence intensity and fluorescence anisotropy of the cationic trimethylammonium derivative of DPH, TMA-DPH. This quaternary ammonium probe has been shown to bind preferentially to negatively charged regions of the lipid bilayer due to electrostatic interaction and report the fluidity in the interfacial region of the lipid bilayer (Kuhry et al., 1983; Kitagawa and Hirata, 1992). As shown in Table 1, the fluorescence intensity of this probe decreased significantly with increase of pH between pH 4.0 and 6.2. The results are consistent with those on change of phase transition temperature shown in Fig. 1. These results indicate that dissociation of palmitic acid occurs between pH 4.0 and 6.2 in the liposomal membrane. The range is lower than the apparent pKa of fatty acids (7.2–7.4) in phosphatidylcholine bilayers (Ptak et al., 1980). Fluorescence anisotropy of TMA-DPH also increased slightly with increase of pH, also possibly due to the dissociation of palmitic acid.

Table 1
Effects of pH on fluorescence intensity and fluorescence anisotropy of TMA-DPH in liposomes composed of four stratum corneum lipid component at 37°C

pH	Fluorescence intensity[a]	Fluorescence anisotropy
3.0	414 ± 13	0.277 ± 0.001
4.0	411 ± 12	0.277 ± 0.002
4.4	442 ± 11	0.279 ± 0.001
5.0	476 ± 15	0.280 ± 0.002
6.2	571 ± 6	0.284 ± 0.003
7.4	600 ± 17	0.285 ± 0.002

Data are means ± S.D. for four experiments.
[a] Fluorescence intensities are listed as arbitrary units.

These results suggest that the unionized form of palmitic acid tend to fluidize the lipid bilayer. Therefore, we further examined the pH-dependence of fluorescence anisotropy of fluorescent palmitic acid derivative, 2-AP. As shown in Table 2, fluorescence anisotropy of 2-AP slightly decreased with decrease of pH between pH 3.0 and 7.4. This result indicates that unionized form of palmitic acid has more freedom in its molecular motion when the lipid bilayer is in gel state.

3.2. Effect of pH on in vitro skin penetration of phenol

In order to observe the relation between the pH-dependent effects on skin penetration of solutes and the pH-dependent effects on the dynamics of stratum corneum lipid bilayer mentioned above, we next examined the effect of pH on in vitro penetration of phenol through guinea pig skin. As shown in Fig. 3, penetration of phenol, almost all of which is present as unionized form at both neutral and acidic pH according to its pKa (10.0), proceeds more markedly at pH 5.0 than at pH 7.4. Permeability coefficients of phenol at pH 7.4 and 5.0 were 0.062 ± 0.008 (cm/h) and 0.092 0.020 (cm/h), respectively (n = 3). This result agrees with the estimation in the liposomes mentioned above that fluidization of lipid bilayer occurs at acidic pH in the stratum corneum of skin.

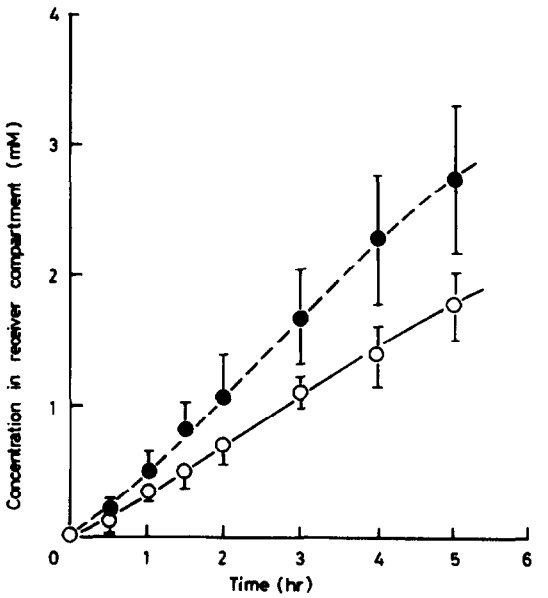

Fig. 3. Percutaneous absorption of 50 mM phenol through excised guinea pig skin at 37°C at pH 7.4 (○) and 5.0 (●).

Table 2
Effects of pH on fluorescence anisotropy of 2-(9-anthroyloxy)palmitic acid in liposomes composed of four stratum corneum lipid component at 37°C

pH	Fluorescence anisotropy
3.0	0.148 ± 0.003
4.0	0.150 ± 0.003
5.0	0.156 ± 0.003
6.2	0.160 ± 0.002
7.4	0.162 ± 0.002

Data are means ± S.D. for eight experiments.

3.3. Effects of other saturated fatty acids on fluidity and phase transition temperature

Saturated fatty acids such as lauric acid have been reported to enhance transdermal drug penetration (Aungst et al., 1986; Ogiso and Shintani, 1990; Takeuchi et al., 1992) as well as long-chain cis-unsaturated fatty acids such as oleic acid. As with cis-unsaturated fatty acids, perturbation of lamellar lipids in the stratum corneum has been suggested to be related with their mechanism of enhancement (Takeuchi et al., 1992). However, as shown in Fig. 4, at pH 7.4 lauric acid caused little change in the fluorescence anisotropy of DPH at any temperature, compared the same molar ratio

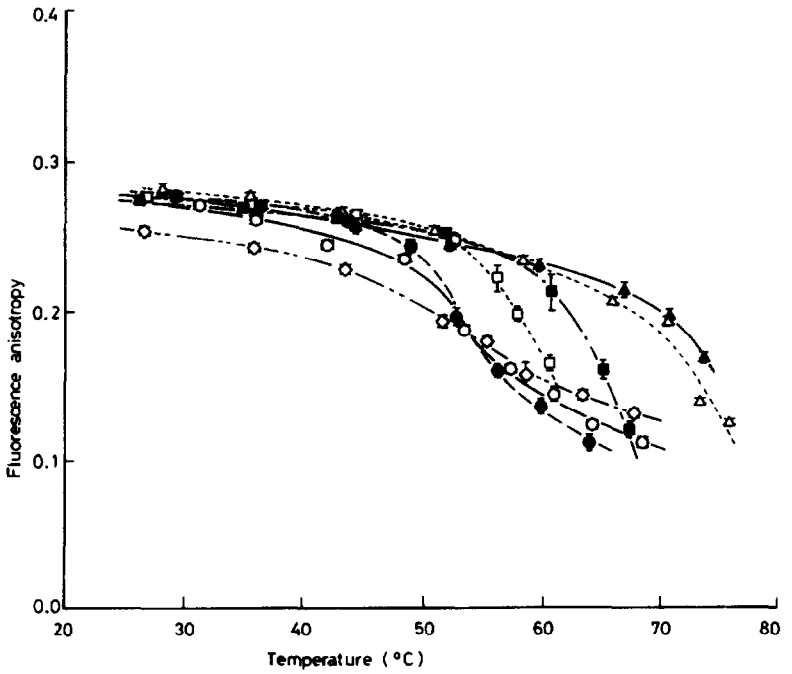

Fig. 4. Effects of substitution of palmitic acid for other saturated fatty acids or oleic acid on phase transition of the lipid bilayer of liposomes at pH 7.4 and 4.0 as monitored by temperature dependency of fluorescence anisotropy of DPH: palmitic acid (control liposomes) at pH 7.4 (△), lauric acid at pH 7.4 (▲), oleic aicd at pH 7.4 (◇), lauric acid at pH 4.0 (○), myristic acid at pH 4.0 (●), palmitic acid (control liposomes) at pH 4.0 (□), and stearic acid at pH 4.0 (■).

of palmitic acid in control liposomes at the same pH. As palmitic acid had no effect on fluorescence anisotropy at any temperature at pH 7.4 (Kitagawa et al., 1993), the results indicated that at this pH lauric acid also did not have any significant effect. Therefore, we next examined the effects of saturated fatty acids including lauric acid (from C_{12} to C_{18}) at pH 4.0 on the fluidity and phase transition temperature of stratum corneum lipid liposomes using liposomes containing various saturated fatty acids instead of palmitic acid.

As shown in Fig. 4, all the fatty acids decreased the phase transition temperature at pH 4.0, compared with that of control liposomes at pH 7.4. The decreases in the phase transition temperature by fatty acids were in the order lauric, myristic > palmitic > stearic. That is, at this pH, substitution of palmitic acid by stearic acid increased the phase transition temperature about 5°C, whereas its substitution by myristic acid or lauric acid decreased the phase transition temperature about 5°C. Moreover, phase transition occurred more gradually in the presence of lauric acid than in the presence of other fatty acids or in their absence. Thus, in addition to decreasing the phase transition temperature, lauric acid decreased the fluorescence anisotropy of DPH at physiological temperatures, as shown in Fig. 4 and Table 3. Considering the mechanisms of the enhancing effects of these fatty acids on transdermal drug penetration, their effects on the fluidity at physiological temperature seem to be more important than their effects in lowering the phase transition temperature described above. These characteristics of lauric acid at acidic pH values resemble those of long-chain cis-unsaturated fatty acids such as oleic acid shown in Fig. 4 for comparison, which we reported previously (Kitagawa et al., 1993). Myristic acid also decreased the value very slightly. These results are consistent with reports of the enhancement of these saturated fatty acids of transdermal drug penetration (Aungust et al.,

1986; Ogiso and Shintani, 1990; Takeuchi et al., 1992).

4. Discussion

The present study using a fluorescent probe indicated that the unionized forms of fatty acids differ from ionized forms in their interaction with other lipid components and result in lower phase transition temperature of the bilayer of stratum corneum lipid liposomes. This is probably due to the delocalization of the undissociated form of fatty acids in the lipid bilayer because of lack of an electric charge, as suggested by the study using 2-AP. The change of dissociation of palmitic acid, which is a major fatty acid component in stratum corneum (Friberg et al., 1991), occurred between pH 4.2 and 6.0. Since the pH of the skin is in the range of 4.5–6.0 (Friberg et al., 1991), significant parts of the fatty acids in the stratum corneum lipid lamella must be present in a nonionized form. Therefore, fatty acids whose major component is palmitic acid seem to lower the phase transition temperature of stratum corneum lipids. Stimulation of transdermal penetration of phenol at acidic pH suggests the possibility that fluidization of lipid bilayer actually occurs at acidic pH and enhances transdermal penetration of solutes.

Among the fatty acids examined, fatty acids with shorter alkyl chains lowered the phase transition temperature more and lauric acid (C_{12}) and myristic acid (C_{14}) had similar effects on the transition temperature. This was probably due to the balance of the membrane perturbing effects of the fatty acids and their affinities to the membrane. Lauric acid, which has a shorter alkyl chain, may perturb the lipid bilayer more than myristic acid, whereas its affinity to the lipid bilayer may be less than the latter and so it may be released in part from the lipid bilayer and be present as free fatty acids (Kittayanond et al., 1992). Lauric acid also increased fluidity of lipid bilayers significantly at physiological temperatures where the lipid bilayer was in the gel state, like long-chain cis-unsaturated fatty acids such as oleic acid.

It is still not clear that the mechanism of the effects of the fatty acids added as penetration enhancers is similar with that of the enhancement of drug penetration at acidic pH by fluidization of lipid bilayer due to protonation of anionic forms of fatty acids in stratum corneum lipid lamella as described above. However, the results described above corresponded with the enhancement effects of saturated fatty acids, especially that of lauric acid on transdermal drug penetration. That is, Aungust et al. (1986) examined the effects of a series of saturated fatty acids on in vitro naloxone penetration through human skin and found maximum enhancement effect for lauric acid. Ogiso et al. also reported significant enhancement effects of lauric acid and myristic acid on the in vitro and in vivo percutaneous absorption of propranolol (Ogiso and Shintani, 1990). Furthermore, Takeuchi et al. (1992) reported similar enhancement effects of these saturated fatty acids, especially of lauric acid, on the in vitro penetration of indomethacin. Therefore, saturated fatty acids such as lauric acid, whose significant parts seem to be present in a nonionized form in slightly acidic stratum corneum, may cause this perturbation of stratum corneum lipid lamella and induce enhancement of transdermal drug penetration, although further works are necessary to clarify the precise mechanism of the enhancement effects of fatty acids on transdermal drug penetration.

Table 3
Effect of omission of palmitic acid or its substitution by other fatty acids on fluorescence anisotropies of DPH in stratum corneum lipid liposomes at pH 4.0 at 37°C

Fatty acid	Fluorescence anisotropy
None	0.272 ± 0.003 (4)
Lauric	0.262 ± 0.002 (8)[a]
Myristic	0.268 ± 0.003 (8)
Palmitic (control)	0.272 ± 0.002 (4)
Stearic	0.270 ± 0.003 (4)

Data are means ± S.D. Numbers in parentheses indicate numbers of replicate experiments. Statistical significances of differences of values from that for control liposomes containing palmitic acid were determined by Student's t-test: [a]$P < 0.001$.

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