Apparent pK_a of the Fatty Acids Within Ordered Mixtures of Model Human Stratum Corneum Lipids

Renate Lieckfeldt,[1] José Villalaín,[2] Juan-Carlos Gómez-Fernández,[2] and Geoffrey Lee[1,3]

Received March 7, 1995; accepted July 25, 1995

Purpose. The apparent pK_a of the fatty acids within hydrated (30 % w/w) model human stratum corneum (SC) lipid mixtures should be measured.

Methods. The degree of ionisation of the fatty acids was calculated as a function of pH using Fourier transform infra-red spectroscopy. The relative intensity of the stretching bands of the unionized and ionized carboxylic groups was determined and fitted to the relevant expression for ionic equilibrium of a monoprotic acid. The pK_a was then calculated for increasing proportion of unsaturated fatty acid in the lipid mixture.

Results. Values for pK_a in the range 6.2-7.3 were found, increasing with greater proportion of oleic acid. These are some 1.5-3 pH units higher than the pK_as of fatty acids in molecular solution.

Conclusions. As there exists a pH-gradient across the SC, the degree of ionisation will also vary. In the innermost SC layers, a pH of 7 will produce 90% ionization of the fatty acids and head-group repulsion will be great. At the SC surface, the pH of 5 will cause almost minimal head-group repulsion, tending to increase crystallinity and promote a bilayer structure.

KEY WORDS: pK_a; stratum corneum; fatty acid; ionization.

INTRODUCTION

The lipid present within the extracellular space of human stratum corneum (SC) is vital for maintaining the barrier property of this membrane. Although its composition is complex, the major components have been identified as free fatty acids, cholesterol and sphingolipids. The fatty acids represent with approx. 45 mol% (1) the major component and are equally divided between saturated (mainly stearic, palmitic and myristic) and unsaturated (mainly oleic, linoleic and palmitoleic) acids (2). Studies of the polymorphic behavior of this SC-lipid combination have shown how a bilayer structure is formed. The hydrated fatty acids produce by themselves a biphasic structure (3), comprising a crystalline phase of saturated fatty acid and an H_{II} phase of unsaturated fatty acid (4). A lamellar gel phase is first formed on the addition of cholesterol (5), which apparently promotes incorporation of the unsaturated fatty acids into a bilayer that would otherwise be geometrically unfavorable for them. This bilayer structure is maintained on addition of ceramides, but now shows an additional high-temperature L_α - H_{II} transition directly subsequent to the L_α - L_β transition observed without ceramide (5).

It is well known that the polymorphism of hydrated, charged lipids can be influenced by the degree of ionisation of their head groups. The question arises, therefore, if the degree of ionisation of the 45 mol% fatty acids present in the SC lipids is an important factor for bilayer structure and, hence, for maintaining barrier property. The pH within the SC gradually changes from pH 5 on the SC-surface to pH 7 in the upper viable epidermis (6). As a consequence of this pH-gradient, the degree of ionisation of the fatty acids within the SC-lipid bilayers may also vary across the SC. The pK_a for medium- and long-chain ($C \geq 10$) fatty acids molecularly dispersed in water is approx. 4.8 (7). Recent work has shown, however, that both pure fatty acid bilayers (8) and fatty acids contained within phospholipid bilayers (9, 10) have pK_as of ≥ 8.0. Does such a pKa shift occur with SC-lipid bilayers, and, if so, are the fatty acids still ionized? In this paper we determine the pK_a of two representative fatty acids within model SC lipid bilayers as a function of the ratio of unsaturated/saturated acid. The results help provide insight into the role of pH and fatty acid ionization in maintaining the barrier property of the SC's lipid fraction.

MATERIALS AND METHODS

Model Stratum Corneum Lipid Mixtures. The composition of the model SC lipid mixtures used in this study (Table 1) is based on that of abdominal human SC (2). Only a single representative saturated (palmitic) and a single representative unsaturated (oleic) fatty acid were selected. The mole proportion of unsaturated component to a total free fatty acid was 0.1, 0.5 or 0.9. Palmitic and oleic acids were both obtained from Sigma (Munich, Germany), cholesterol from Aldrich (Steinheim, Germany), and the ceramide from Avanti Polar Lipids (Birmingham, AL-USA). They all had a stated purity of ≥ 99 % and were stored under N_2. The lipids (approx. 400 mg in total) were dissolved together in 0.5 ml of chloroform: methanol (1 : 1), and the solution pipetted on to a microscope slide and allowed to dry at room temperature. The lipid film formed was then hydrated with 30 % w/w aqueous buffer and warmed to 80 °C with continued stirring using a fine glass rod. The buffer pH was in the range 4 - 11 and of 1.0 M ionic strength (pH 4 - 7.5: potassium biphosphate buffer; pH 8 - 11: tris buffer). Each hydrated sample was examined immediately after its preparation and equilibration at the temperature of measurement.

Spectroscopic Measurements. Infrared spectra were obtained at 25 °C ± 0.2 °C using a Phillips PU 9800 Fourier transform infrared (FT-IR) spectrometer equipped with a DTGS detector. Each lipid sample was placed in a thermostatted Specac 20710 cell having CaF_2 windows and a 25 μm teflon spacer. After temperature equilibration, the FT-IR spectrum of the sample was calculated from 50 interferograms with a nominal resolution of 2 cm^{-1} and a triangular apodization. A sample shuttle accessory was used to average

[1] Department of Pharmaceutical Technology, Erlangen University, Germany.

[2] Department of Biochemistry and Molecular Biology, University of Murcia, Murcia, Spain.

[3] To whom correspondence should be addressed at Lehrstuhl für Pharmazeutische Technologie, Universität Erlangen-Nürnberg, Cauerstr. 4, 91058 Erlangen, Germany.

0724-8741/95/1100-1614$07.50/0

Table 1. Model Stratum Corneum Lipid Mixtures[a]

ϕ_{un}	Palmitic acid	Oleic acid	Cholesterol (anhydrous)	Ceramide	$\Delta\log[A^-]/[HA]/\Delta pH$	pK_a^{app}	r^2
0.1	40.5	4.5	27.5	27.5	1.1	6.16	0.992
0.5	22.5	22.5	27.5	27.5	0.80	6.34	0.975
0.9	4.5	40.5	27.5	27.5	0.51	7.32	0.887

[a] Each mixture contained 30% w/w water and was buffered to a pH in the range 4–11. All values are mol %. ϕ_{un} represents the relative molar proportion of unsaturated free fatty acid (oleic) to total free fatty acid. The results for pK_a^{app} were calculated from Fig. 3 according to Eq. 2.

the background spectra over the time of measurement. The complete optics were continuously purged with dry, CO_2-free air.

The apparent pK_a of the fatty acids within each lipid mixture was determined from its infrared spectrum (10). The degree of ionization of the fatty acids was first calculated from the infrared stretching bands of the un-ionized carboxylic groups at 1725 and 1530 cm^{-1} for $\nu(c=o)_{OH}$ and $\nu(c=o)_{0^-}$, respectively. The area under each band was calculated using the 'Spectra-Calc' program and normalized relative to the band area of the symmetric CH_2 stretching vibration, $\nu_s(CH_2)$, at 2850 cm^{-1}. In this manner the effects of any varying sample thickness were compensated. The ratio of the areas $\nu(c=o)_{0^-}/\nu(c=o)_{OH}$ gives the value of $[A^-]/[HA]$ for the fatty acids, and was calculated as a function of both pH and the proportion of unsaturated fatty acid to total free fatty acid, ϕ_{un} (Table 1).

RESULTS AND DISCUSSION

Change in pH has a slight, but measurable, effect on acyl chain fluidity. The same behavior is seen for the CH_2 symmetric stretching frequency, $\nu_s(CH_2)$, at each value of ϕ_{un} examined (Fig. 1). With increasing pH there is at first no change in $\nu_s(CH_2)$, which lies at 2849.2 cm^{-1} for ϕ_{un} = 0.1 and 0.5, and is a little higher at 2850.1 cm^{-1} for ϕ_{un} = 0.9. These values are all characteristic of rigid acyl chains for the fatty acids and ceramide (11). The higher value for ϕ_{un} = 0.9 indicates a slightly greater fluidity. In the range pH 7 - 8, $\nu_s(CH_2)$ jumps by approx. 1 cm^{-1}, this occurring at slightly lower pH with increasing ϕ_{un}. The resulting $\nu_s(CH_2)$ remain too low to indicate a change to fully-fluidized acyl chains, which lie above 2853 cm^{-1} for an L_α phase (11). The jumps in $\nu_s(CH_2)$ coincide with those pH values determined for the pK_as of the hydrated lipid mixtures. At pHs below 6 the fatty acids will, therefore, be almost completely unionised. A sharp increase in degree of ionisation of the carboxylic acid groups is then to be expected between pH 6 and 8. The resulting increase in headgroup repulsion may, therefore, lead to the observed increased acyl chain fluidity. This occurs despite the high ionic strength used here, which may induce in itself some crystallinity, owing to decreased head group repulsion.

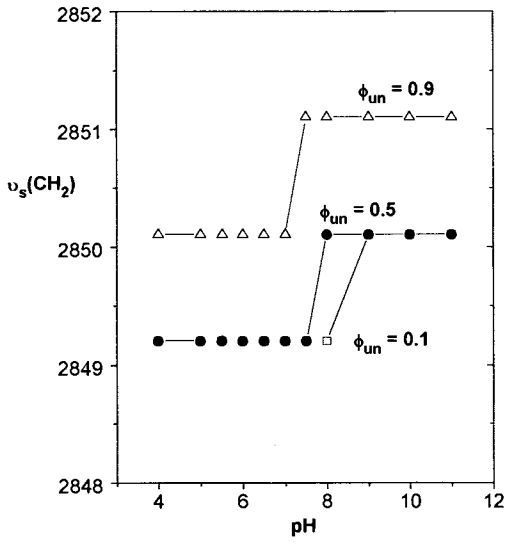

Fig. 1. pH-dependence of the CH_2 symmetric stretching band, $\nu_s(CH_2)$, of hydrated mixtures containing 27.5 mol% ceramide, 27.5 mol% cholesterol and 45 mol% free palmitic and oleic acids. Relative molar proportions of oleic to total acid ϕ_{un} = (□) 0.1, (●) 0.5 and (△) 0.9.

An hydrated mixture of palmitic acid, cholesterol and ceramide (i.e. free of unsaturated fatty acid) forms a lamellar gel phase (12). Fig. 1 shows that replacing up to 50 mol% of the palmitic acid with oleic acid (ϕ_{un} = 0.5) does not induce greater fluidity. Only after replacing 90 mol% with oleic acid (ϕ_{un} = 0.9) is a slight increase in fluidity observed, owing to partial formation of an H_{II} phase (13). In contrast, increasing ϕ_{un} within an hydrated mixture of the six major SC fatty acids (i.e. free of cholesterol and ceramide) already produced some H_{II} periodicity at ϕ_{un} = 0.1 (4). We conclude that cholesterol promotes incorporation of unsaturated fatty acids within the lamellar structure. Their phase-separation as an H_{II} phase is thereby hindered. The cholesterol induces sufficient fluidity of the saturated fatty acids acyl chains to allow incorporation of the bulky unsaturated fatty acids.

The bands for $\nu(c=o)_{OH}$ and $\nu(c=o)_{0^-}$ at 1725 cm^{-1} are clearly identifiable at all values of ϕ_{un} (see Figs. 2a-c). With increasing pH the intensity of the $\nu(c=o)_{OH}$ band gradually decreases, whilst that of $\nu(c=o)_{0^-}$ correspondingly increases. This type of spectral behavior has been observed with ^{13}C-palmitic acid incorporated within a dipalmitoylphosphatidyl choline bilayer (where $\nu(^{13}c=o)_{OH}$ = 1665 cm^{-1} and $\nu(^{13}c=o)_{0^-}$ = 1513 cm^{-1}) and attributed to an increase in degree of ionisation with increasing pH (10). At pH 5, for example, Figs. 2a-c show that the $\nu(c=o)_{OH}$ band is much more intense than the $\nu(c=o)_{0^-}$ band; the carboxyl groups of the palmitic and oleic acids are almost fully protonated. At pHs > 8, the $\nu(c=o)_{OH}$ band has almost completely disappeared, whereas the $\nu(c=o)_{0^-}$ band has increased greatly because the carboxyl groups are now almost fully ionised. We exploit this change in relative intensity of

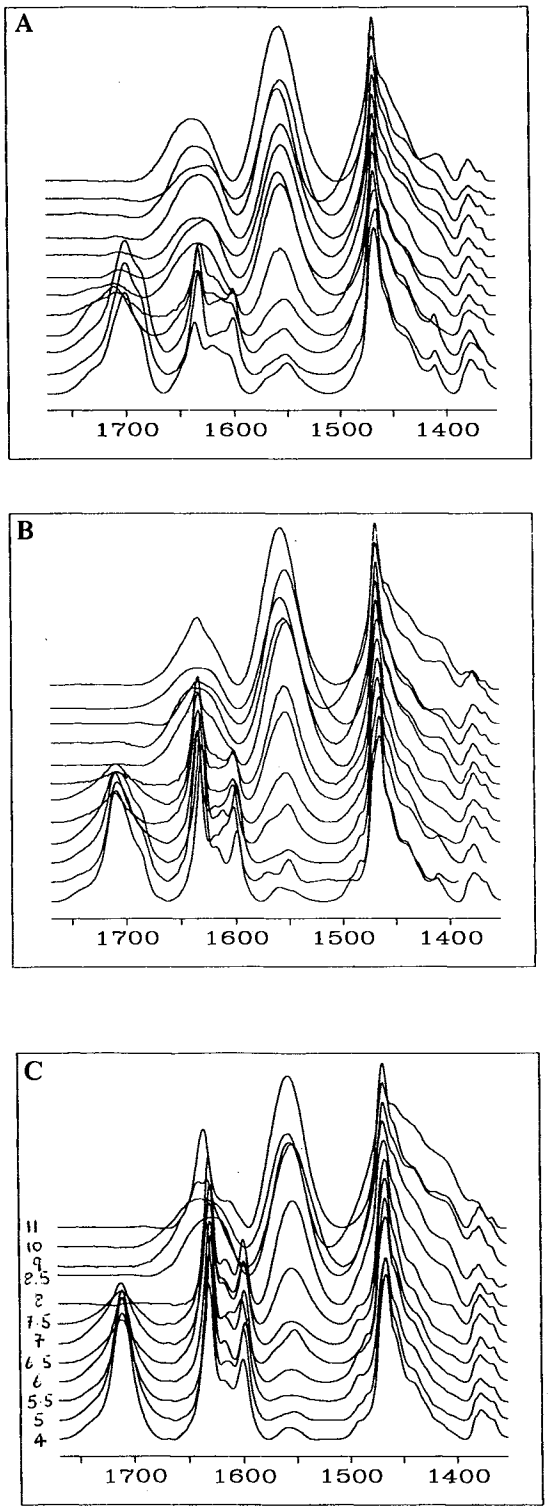

Fig. 2. Infrared spectra of the 1800 - 1350 cm^{-1} region of hydrated lipid mixtures containing different relative molar proportions of oleic to total acid. (A) ϕ_{un} = 0.1, (B) ϕ_{un} = 0.5 and (C) ϕ_{un} = 0.9. The bands corresponding to unionized carboxylic stretching, $\nu(c=o)_{OH}$, at approx. 1725 cm^{-1}, to ionized carboxylic stretching, $\nu(c=o)_{OH}$ at approx. 1530 cm^{-1}, and CH_2-scissoring, $\sigma(CH_2)$, at approx. 1470 cm^{-1}, can be observed. pH values are (from the bottom up): 4, 5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 10, 11. $\sigma(CH_2)$ showed the same pH-dependency as $\nu_s(CH_2)$ in Fig. 1.

the two bands to calculate the pK_a of the fatty acids within the mixture. The intensities of the $\nu(c=o)_{OH}$ and $\nu(c=o)_0$-bands cannot be resolved into the individual contributions from the palmitic and oleic acids. The ratio of band intensity, $\nu(c=o)_0$-/$\nu(c=o)_{OH}$, at any pH is, therefore, equal to the sum of the contributions from both acids: $([A^-_{palm}] + [A^-_{ol}])/([HA_{palm}] + [HA_{ol}])$. The equation for the relative concentrations of the ionized and unionized species of a mixture of two independent weak acids as a function of pH (14) cannot be solved for this factor. Consequently, we cannot determine a separate pK_a for each of the two fatty acids in the mixture. We assign to the lipid mixture an apparent pK_a equivalent to that for a single monoprotic acid:

$$K_a^{app} = \frac{[H_3O^+]([A^-_{palm}] + [A^-_{ol}])}{([HA_{palm}] + [HA_{ol}])} \quad (1)$$

This gives the following equation for a straight line:

$$\log \left\{ \frac{[A^-_{palm}] + [A^-_{ol}]}{[HA_{palm}] + [HA_{ol}]} \right\} = -pK_a^{app} + pH \quad (2)$$

Fig. 3 shows the plots of log $\{(\nu(c=o)_0$-/$\nu(c=o)_{OH})\}$ versus pH in accordance with Eq. 2 for the three mixtures with ϕ_{un} = 0.1, 0.5 and 0.9.

Increasing the proportion of unsaturated fatty acid has a marked effect on ionisation behavior (Fig. 3). For ϕ_{un} = 0.1, the co-ordinates fit closely to Eq. 2 and give a slope of best fit of unity (Table 1), as predicted by Eq. 2 for a monoprotic weak acid. Although Eq. 1 is only an approximation for these lipid mixtures, it does, therefore, accurately describe the ionisation behavior using a single apparent pK_a. With increasing ϕ_{un}, however, the scatter of the coordinates around the straight lines of best fit increases and the slopes fall below unity. The latter can only occur if ionisation is being hindered with increasing ϕ_{un}. For example, the slope of 0.5 at ϕ_{un} = 0.9 intimates that only every second carbox-

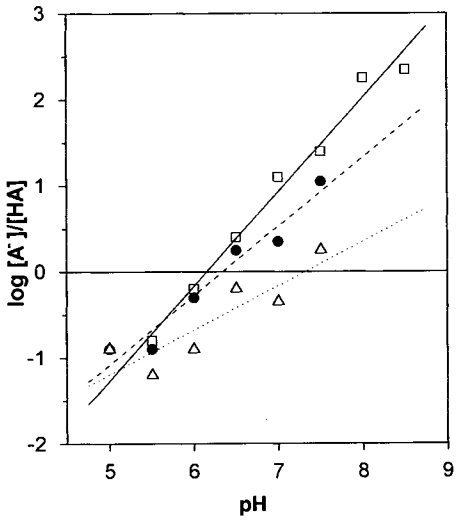

Fig. 3. Plot of log $\{(\nu(c=o)_0$-/$(\nu(c=o)_{OH}\}$ versus pH for the model stratum corneum mixtures containing 27.5 mol% ceramide, 27.5 mol% cholesterol and 45 mol% free palmitic and oleic acids. Relative molar proportions of oleic to total acid ϕ_{un} = (□) 0.1, (●) 0.5 and (△) 0.9.

ylic acid group can ionise with increasing pH. Thus, the palmitic and oleic acids behave differently, with the oleic being less effected by the titration than is the palmitic acid. We conclude that if the mixtures behave ideally (as appears to be the case from Table 1) then each palmitic acid molecule looses a single proton during ionisation, whereas each oleic acid molecules looses only a half proton. We find no evidence to suggest that this arises from the structural changes occurring with increasing ϕ_{un}, whereby the L_β phase of the hydrated cholesterol, ceramide and palmitic acid (12) alters to an H_Π phase of hydrated cholesterol, ceramide and oleic acid (4, 13). Whatever the cause, it is evident from the positions and slopes of the lines in Fig. 3 that the reduced ease of ionisation of the oleic acid compared with palmitic acid causes pK_a^{app} to increase with greater ϕ_{un}. The pK_a^{app} values (Table 1) all lie within the neutral pH-range and are, therefore, at least 1.5 pH units larger than those for these two fatty acids in molecular solution (7). This shift in pK_a^{app} of fatty acids incorporated within colloidal aggregates is caused by dielectric double-layer effects of the nonpolar lipid environment, as discussed in detail by Cistola et al. (8).

The finding that the pK_a^{app} of the fatty acids in the model SC lipid mixtures is higher than previously supposed is important for understanding their phase behaviour. Consider the lipid mixture corresponding most closely to that within intact human SC, i.e. that with $\phi_{un} = 0.5$ (cf Table 1). This mixture has a pK_a^{app} of 6.3 (Table 1). Tape-stripping studies have shown that a vertical pH gradient exists within human SC (6). A pH of 7 in the viable epidermis decreases across the SC to pH 5 on the SC surface. In the innermost SC layers, the fatty acids will, therefore, be approx. 90 % ionized, and head-group repulsion will be almost maximal. Moving outwards, across the SC, the drop in pH will reduce the degree of ionization to < 10 % at pH 5, and head-group repulsion will be almost minimal. This decrease in head-group repulsion of the charge lipids will tend to increase crystallinity and also maintain a lamellar geometry. A hydrated mixture of cholesterol, ceramide and the six major SC fatty acids (5) shows at pH 5 a lamellar periodicity (X-ray) and rigid acyl chains (FT-IR). On the basis of the results presented here, this must be considered to be at least partly a result of the pK_a shift of the fatty acids (from approx. 5 to 6.3) in the SC lipid bilayers.

REFERENCES

1. P. Elias, in Topical Drug Delivery Formulation (Eds., Osborne, D. and Ammon, A.), Marcel Dekker, New York (1990), pp. 13-28.
2. M. Lampe, A. Burlinghame, J. Whitney, M. Williams, B. Brown, E. Roitman, and P. Elias, Human stratum corneum lipids: characterisation and regional variation, *J. Lipid Res.*, 24: 120-130, 1983.
3. S. Friberg, I. Kayali, and L. Rhein, Direct role of linoleic acid in barrier function, *J. Disp. Sci. Technol.*, 11: 31-46, 1990.
4. R. Lieckfeldt, J. Villalaín, J. Gómez-Fernández, and G. Lee, Influence of oleic acid on the structure of a mixture of hydrated model stratum corneum fatty acids and their soaps. *Coll. and Surfaces*, 90: 225-234, 1994.
5. R. Lieckfeldt, J. Villalaín, J. Gómez-Fernández, and G. Lee, Diffusivity and structural polymorphism in mixtures of some model stratum corneum lipids, *Biochim. Biophys. Acta*, 1151: 182-193, 1993.
6. H. Öhman and A. Vahlquist, In vivo studies concerning the pH gradient in human stratum corneum and upper epidermis. *Acta Derm. Venerol.*, 74: 375-379, 1994.
7. A. Spector, *J. Lipid Res.*, 16: 165-179, 1975.
8. D. Cistola, J. Hamilton, D. Jackson, and D. Small, Ionisation and phase behaviour of fatty acids in water: Application of the Gibbs Phase Rule, *Biochemistry:* 27: 1881-1888, 1988.
9. J. Hamilton, and D. Cistola, Transfer of oleic acid between albumin and phospholipid vesicles, *Proc. Natl. Acad. Sci. USA*, 83: 82-86, 1986.
10. J. Villalaín, and J. Gómez-Fernández, Fourier transform infrared spectroscopic study of mixtures of palmitic acid with dipalmitoyl phosphatidylcholine using isotopic substitution. *Chem. Phys. Lipids*, 62: 19-29, 1992.
11. H. Casal, and H. Mantsch, Polymorphic phase behaviour of phospholipid membranes studied by infra-red spectroscopy, *Biochim. Biophys. Acta*, 779: 381-401, 1984.
12. W. Abraham, and D. Downing, Deuterium NMR investigation of polymorphism in stratum corneum lipid, *Biochim. Biophys. Acta*, 1068: 189-194, 1992.
13. P. Häusler, and C. Müller-Goymann, Mutual interactions between selected lipid fractions of stratum corneum and liquid crystalline microstructure of semisolid formulations, *Eur. J. Pharm. Biopharm.*, 405: 126, 1994.
14. A. Martin, J. Swarbrick, and A. Cammerata, *Physical Pharmacy*, 3rd. Ed., Lea and Febiger Philadelphia (1993), 205, Eq 108.