Differential Scanning Calorimetric Studies on the Melting Behavior of Water in Stratum Corneum

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The melting behavior of water in human stratum corneum (s. corneum) has been studied by using differential scanning calorimetry (DSC) in the temperature range from −40° to 20°C. The DSC thermogram was analyzed in terms of the amount of bound water and the melting temperature of water in s. corneum. Extraction of the s. corneum with the mixed solvent of chloroform:methanol (2:1, v/v) or 0.5% sodium dodecyl sulfate aqueous solution decreased the bound water content, whereas extraction with water did not change the bound water content. The melting temperature of water in the s. corneum was lowered as the water contents decreased. Extraction of the water-soluble components from the s. corneum increased the melting temperature of water when the water contents were constant. The results suggest that 20–30% of water in the s. corneum is bound water interacting strongly with the protein or lipids in the s. corneum, and the excess of water over the bound water content is unbound water solubilizing the water-soluble components such as amino acids and urea in the s. corneum. The thermodynamic theory for freezing-point depression is favorably applied to the melting temperature change of the unbound water, which implies that the water-soluble components are present as an aqueous solution in the s. corneum. Measurements of the melting-point depression of water in s. corneum provide us the quantitative information on the amount of water-soluble components in the s. corneum. This technique is a sensitive and useful tool to evaluate the hydration behavior of s. corneum. *J Invest Dermatol 86:689–693, 1986*

The important role of water for plasticity of stratum corneum (s. corneum) has been well known since Blank [1,2] reported it. After his work, several studies on the content and the state of water in s. corneum were made [3–6]. Hansen and Yellin showed from the nuclear magnetic resonance and infrared techniques that there are 2 kinds of water in s. corneum: one is bound water, the amount of which is 0.30–0.35 g/g of dry s. corneum, and the other is unbound water [6]. This result was supported by Walkley [7] from differential scanning calorimetry (DSC) determinations of the relative amounts of unbound and bound water in human limb s. corneum. He showed that extraction of lipids and water-soluble substances from guinea pig footpad s. corneum resulted in the increase of bound water content [7]. The DSC technique could provide us not only the enthalpy of fusion but also the melting temperature of water in the s. corneum, which would give useful information on the activity of the water solubilizing the low-molecular-weight water-soluble components such as natural moisturizing factor (NMF) [8] in the s. corneum.

The present work deals with the hydration behavior of untreated, water-extracted, detergent-extracted, and solvent-extracted s. corneum. The DSC technique has been utilized to measure the relative amounts of bound and unbound water and the melting-point depression of unbound water in s. corneum. Thermodynamic treatment of the melting-point depression phenomena has been made to understand the physicochemical state of water and also to estimate the amount of water-soluble components present in hydrated s. corneum.

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Abbreviations:
DSC: differential scanning calorimetry
NMF: natural moisturizing factor
s. corneum: stratum corneum

MATERIALS AND METHODS

Stratum Corneum Human s. corneum samples were obtained from the plantar skin of a healthy individual by excising thin sheets with a razor. The specimens were cut into pieces smaller than 1 mm^3, and stored in a desiccator over P_2O_5 in vacuo.

Extraction from and Incorporation into Tissues of Low-Molecular-Weight Moisturizing Compounds The prepared samples of plantar s. corneum were extracted with distilled water, or aqueous detergent solution (0.5% sodium dodecyl sulfate), or organic solvent (chloroform:methanol, 2:1, v/v). The extraction was done by stirring the sample at 4°C for 24 h, and the treated samples were collected by centrifugation at 3000 rpm for 30 min at 4°C. The sedimented tissues were reextracted 5 times in the same manner. The s. corneum was dried and stored in a desiccator over P_2O_5 in vacuo.

Some of the detergent-extracted samples of human plantar s. corneum were immersed in an aqueous solution of glycerol to examine the effect of water-soluble moisturizing substances incorporated into the extracted s. corneum on the DSC thermograms. The weighed detergent-extracted s. corneum was immersed in aqueous glycerol solution (10 wt%) for 4 h at 20°C, rinsed with distilled water for 10 s, put on a filter paper for 30 s, and dried in a desiccator over P_2O_5 in vacuo for 24 h at 20°C. The amount of glycerol sorbed in the s. corneum was calculated from the difference of dry matter weight before and after the glycerol treatment.

Hydration of Samples and DSC Measurements DSC was performed with a high-sensitive heat-flux type DSC apparatus (Seiko Instrument & Electronics Ltd., type SSC-544). The ap-

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paratus was calibrated using the melting temperature and the heat of fusion of distilled water (79.6 cal g^{-1}). The weighed dry s. corneum of 3–5 fragments (typically 1–2 mg) was put into the aluminum capsule and hydrated in the atmosphere of constant relative humidity (90.7% at 20°C) obtained by saturated $BaCl_2$ solution in a desiccator. The s. corneum sample was weighed repeatedly to calculate the water uptake. After the water uptake attained the desired amount, the capsule was hermetically sealed and reweighed to calculate the final water uptake. The total water content was defined as grams of water uptake per 100 g of dry s. corneum sample. The sealed capsule was kept overnight at 20°C and then transferred to the calorimeter and cooled to −40°C at the rate of 0.3–0.4°C min^{-1} and held for 10 min at that temperature prior to starting measurements. The samples were heated to 20°C at a rate of 0.6°C min^{-1}. In order to examine the effect of cooling rate of the sample on the thermogram, the following experiments were performed. Two untreated samples, both hydrated to 50% water content, were prepared. One sample was quickly frozen in liquid nitrogen before placing in a cold DSC instrument; the other was cooled in the DSC instrument as usual. Two thermograms obtained from the above samples were almost identical, which implies that the equilibration can be attained by even rapid cooling with liquid nitrogen. The melting behavior of the water in s. corneum was registered on a chart recorder. The amplitude of endothermic heat flow due to the melting was normalized by the weight of the dry sample, and the peak area between the trace and the baseline was calculated with a microcomputer (Nippon Electric Co., type PC-9801E). The endotherm characteristics are defined as the peak temperature, t_p, and the extrapolated conclusion temperature of the melting peak, melting point t_m, which is the intersection between the extrapolated decaying endothermic curve and the baseline. The melting generally occurs in a range of temperature for a mixing system of multicomponents. In an eutectic system to which the freezing-point depression theory can be applied, the crystal of the pure solvent (pure ice in our case) is precipitated from the solution when the temperature is decreased. As a result of the above, the remaining solution is concentrated, i.e., the activity of water in the solution becomes smaller. In the freezing process of the solution, therefore, the activity of water is changing continuously. This explains why the thermogram shows a range of melting temperature in the mixing system. In such cases, the upper limit of the temperature range is taken as the melting point and used for thermodynamic analysis. The reproducibility of the measured melting points was within ±0.5°C. Repeated measurements (at least 3) of the enthalpy of fusion for the same sample agreed to within 5% of the mean.

RESULTS

Effect of Extraction Treatments on DSC Thermograms Fig 1 shows the representative DSC thermograms of human plantar s. corneum containing 35% total water content (35 g water per 100 g dry s. corneum) treated in 4 different ways. Four thermograms are, interestingly, quite different one from another. The untreated s. corneum sample shows 2 endothermic peaks at −14°C and −9°C and the melting point of −6°C. This melting point shifts to higher temperature in the sample extracted with water (Fig 1*b*). In the solvent-extracted sample, on the other hand, the melting point is the same as, but the peak area is larger than, that of the untreated one (Fig 1*d*). The detergent-extracted sample gives both larger peak area and higher melting point than the untreated and the water-extracted ones (Fig 1*c*). These results indicate that this technique is a sensitive and useful tool to obtain data on the hydration behavior of s. corneum.

Fig 2*a* shows a series of thermograms for the untreated s. corneum having different total water contents. When the water content is less than about 30%, no peak is observed in the thermogram. Whereas the water content exceeds 30%, a small endothermic peak appears at about −10°C, which is much lower than the melting point of pure water. For the samples of higher water

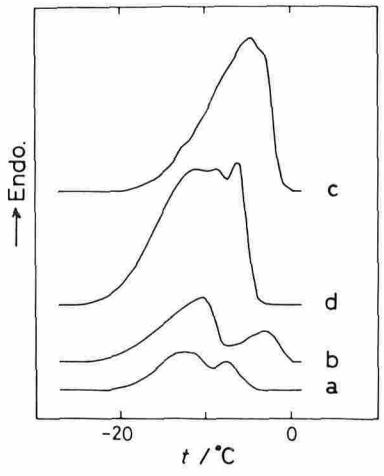

Figure 1. Effect of extraction treatments on DSC thermograms of human plantar s. corneum; the untreated (*a*), water-extracted (*b*), detergent-extracted (*c*), and solvent-extracted (*d*) human plantar s. corneum with 35 wt% total water content.

contents, the magnitude of both the peak area and the peak temperature increase with increasing water content. Similar phenomena are observed in the extracted samples (Fig 2*b–d*).

The transition enthalpy can be calculated from the peak area of each thermogram. Fig 3 shows a plot of the enthalpy change

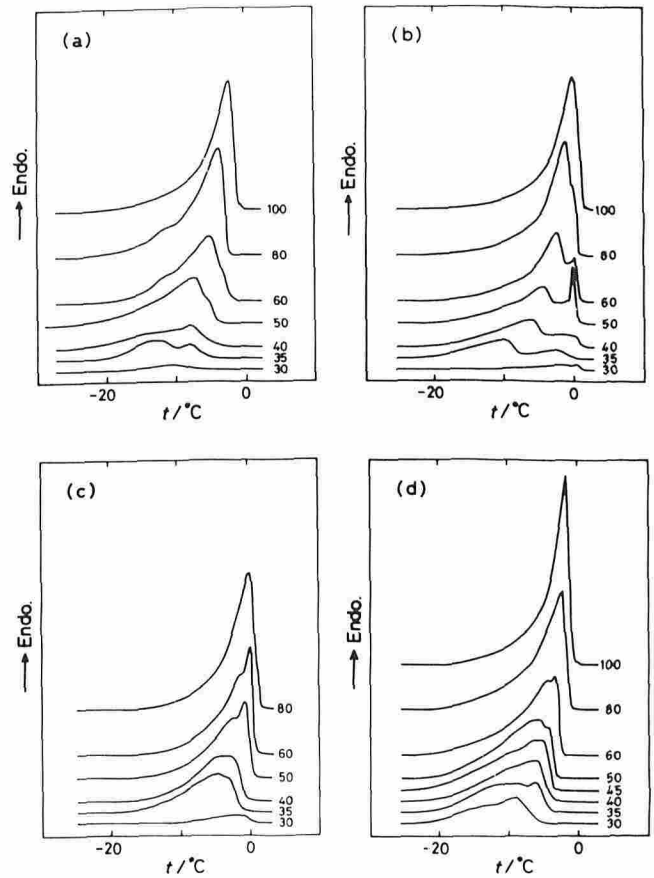

Figure 2. Effect of total water content on DSC thermograms; the untreated (*a*), water-extracted (*b*), detergent-extracted (*c*), and solvent-extracted (*d*) human plantar s. corneum. The number designated in each curve represents the total water content (wt%).

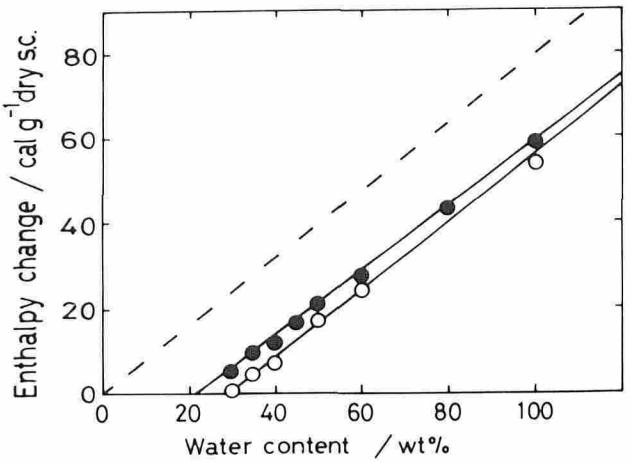

Figure 3. The melting enthalpy of ice plotted against the total water content in the s. corneum for untreated (○) and solvent-extracted (●) s. corneum. The *dashed line* indicates the theoretical value of the enthalpy change assuming that all the water in the s. corneum can be frozen.

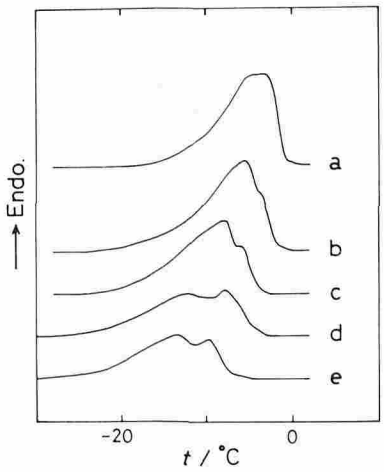

Figure 4. DSC thermograms of glycerol-incorporated s. corneum samples. The amount of glycerol sorbed in the detergent-extracted corneum were 0% (*a*), 1.8% (*b*), 4.9% (*c*), 10.2% (*d*), and 13.6% (*e*) by weight of dry s. corneum.

of the transition in the hydrated plantar s. corneum per gram of dry s. corneum against the total water content in the corneum. The dashed line is the theoretical curve for the transition enthalpy, when it is assumed that all the water in the sample to be present as unbound water. The transition enthalpy change for the untreated s. corneum appears from the water content of 29%, and increases linearly in parallel with the dashed line. This water content of 29% represents the maximum quantity of bound water that may interact strongly with dry s. corneum [7].

Linear plots similar to the case of the untreated s. corneum are also obtained for the extracted samples, one of which is shown in Fig 3 by solid circles. The values of the bound-water content are summarized in Table I, together with the temperatures at peak maximum in DSC thermograms, t_p, and the melting point, t_m. The bound-water content of the water-extracted sample is the same as that of the untreated one, but becomes smaller by the detergent- or solvent-extraction treatment.

The DSC thermograms of the glycerol-incorporated s. corneum show a lower melting point of water than that in the detergent-extracted s. corneum, as shown in Fig 4.

DISCUSSION

Bound Water and Lipid Component in Stratum Corneum As mentioned previously, there are 2 kinds of water coexisting in s. corneum, in agreement with previous studies [6,7]. One is the bound water present up to the water content of 20–30%, depending upon the conditions of sample preparations, and the other is unbound water which appears when the water content exceeds the above value. The bound water may interact strongly with fibrous proteins and/or lipids, whereas the unbound water is essentially the free water. The bound water may be important for moisturizing the s. corneum since the water content of living s. corneum is about 10–30%.

The maximum content of bound water depends highly upon the treatment of the s. corneum, as understood from Table I. The bound water content remains unchanged by the water-extraction, but decreases considerably by solvent-extraction treatment. These results indicate that the components soluble in the chloroform and methanol mixture contribute to enhance the bound-water content, whereas the water-soluble components do not. The main component of the solvent-soluble ones may be the lipids located in the intercellular spaces of keratinocytes in the form of lamellar structures [9–11], since the amphiphilic compounds can hold the bound water in the bimolecular lamellar structure of lyotropic liquid crystals [12]. Possible denaturation of the insoluble fibrous protein components by the solvent treatments could also decrease the bound water content. The decrease of the bound-water content (29–23%) by the solvent extraction is in opposition to result obtained by Walkley [7]. The above discrepancy could be due to the difference between the sample of s. corneum and the extraction solvent we used and those used by Walkley, although the reason is not clear.

Table I. Effect of Extraction Treatments on DSC Thermograms

Extraction[a]	Bound Water Content[b] (wt% vs dry s. corneum)	Peak Temperature[c] (t_p/°C)	Melting Point[d] (t_m/°C)
Untreated	29	−14, −9	−5.7
Water	29	−12, −4	−0.8
Detergent	26	−6, −4	−2.2
Solvent	23	−13, −8	−5.3

[a] See *Materials and Methods*.
[b] Calculated from enthalpy change of water in the treated corneums (Fig 1).
[c] Two main endothermic peaks (Fig 1).
[d] Extrapolated concluding temperature of the peak.

Melting Temperature of Unbound Water and the Water-Soluble Components In the previous DSC study by Walkley on the melting behavior of ice in guinea pig footpad s. corneum, only one endothermic peak was observed at about 0°C, and the melting-point depression phenomenon was not reported [7]. Contrary to his results, the melting temperature of unbound water is markedly affected by the water content and the amount of water-soluble components in the s. corneum (Figs 1, 2, and 4). The dependence of the melting temperature on the water content (Fig 2) and the concentration of water-soluble component (Fig 4) may indicate that the melting behavior of water observed in the s. corneum is the freezing-point depression phenomenon.

According to the thermodynamic theory for freezing-point depression [13]:

$$d \ln a_i/dT = \Delta H^\circ_i/RT_m^2 \quad (1)$$

where a_i and ΔH°_i are the activity in the liquid phase and the enthalpy of fusion of the i component (water in this case), respectively, and T_m is the melting point expressed in absolute

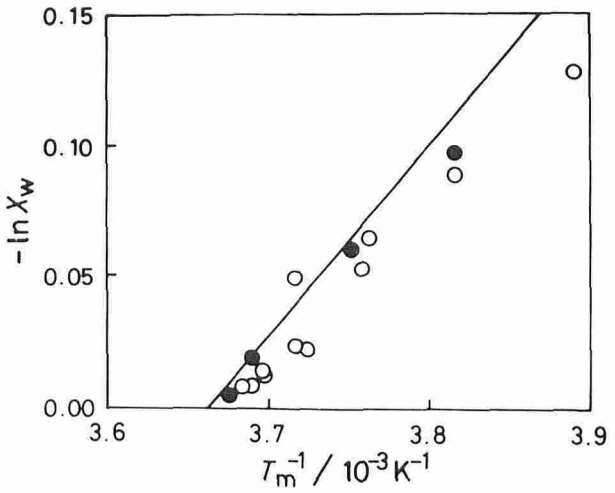

Figure 5. Plots of $-\ln X_w$ against T_m^{-1} for the aqueous solution of glycerol and the hydrated s. corneum incorporated with glycerol. The *line* indicates the theoretical curve of the melting-point depression of water.

temperature. If one assumes that ΔH°_i is independent of temperature, Eq. (1) is integrated to give Eq. (2):

$$-\ln a_i = (\Delta H^\circ_i/R)\,(1/T_m - 1/T^\circ_i) \quad (2)$$

where T°_i is the melting point of the pure component, i. Assuming ideal mixing of the water-soluble components and the water in the s. corneum, we obtain Eq. (3):

$$\ln a_i = \ln x_w \quad (3)$$

where x_w is the mole fraction of water in the solution. From Eqs. (2) and (3), we obtain Eq. (4):

$$-\ln x_w = (\Delta H^\circ_w/R)\,(1/T_m - 1/T^\circ_w) \quad (4)$$

where T°_w is the melting point of pure water; ΔH°_w, the enthalpy of fusion of pure water; R, the gas constant.

We show next that the freezing-point depression theory, Eq. (4), can be applied to our s. corneum systems, using the glycerol-incorporated s. corneum samples. The mole fraction of water, x_w, can be written as:

$$x_w = m_w/(m_w + m_s) \quad (5)$$

where m_w and m_s are the moles of water and of water-soluble components (glycerol in this case), respectively, present in unit weight of dry s. corneum. The mole fraction of water-soluble components, x_s, is, of course, written as:

$$x_s = 1 - x_w = m_s/(m_w + m_s) \quad (6)$$

The m_w and m_s values can be calculated from Eqs. (7) and (8):

$$m_w = W_w/M_w \quad (7)$$

$$m_s = W_s/M_s \quad (8)$$

Table II. Melting-Point Depression of Water in Stratum Corneum

Extraction	W_w[a] (g/g s. corneum)	t_m[b] (°C)	x_s[c]	m_s[d] (mmol/g s. corneum)	W_s[e] (mg/g s. corneum)
Untreated	0.30	−6.7	0.064	1.145	115
	0.35	−5.7	0.055	1.127	113
	0.40	−5.1	0.049	1.146	115
	0.50	−4.6	0.044	1.286	129
	0.60	−3.3	0.032	1.095	110
	0.80	−3.0	0.029	1.324	132
	1.00	−2.2	0.021	1.205	121
				[1.197 ± 0.092][f]	
Solvent	0.30	−6.2	0.060	1.055	106
	0.35	−5.3	0.051	1.044	104
	0.40	−4.9	0.047	1.099	110
	0.50	−4.3	0.041	1.199	120
	0.60	−3.7	0.036	1.232	123
	0.80	−2.5	0.024	1.098	110
	1.00	−2.0	0.019	1.094	109
				[1.128 ± 0.072][f]	
Water	0.30	0.0	0.000	0.000	0
	0.35	−0.8	0.008	0.152	15
	0.40	−0.3	0.003	0.065	7
	0.50	−0.6	0.006	0.162	16
	0.60	−0.1	0.001	0.032	3
	0.80	−0.2	0.002	0.086	9
				[0.083 ± 0.064][f]	
Detergent	0.30	−0.2	0.002	0.032	3
	0.35	−2.2	0.021	0.422	42
	0.40	−1.6	0.015	0.349	35
	0.50	−0.5	0.005	0.135	14
	0.60	−0.2	0.002	0.065	7
	0.80	0.4	−0.004	−0.171	−17
				[0.138 ± 0.218][f]	

[a]Grams of total water per gram of dry sample.
[b]Melting point of water evaluated in Fig 2.
[c]Mole fraction of the water-soluble components in the s. corneum calculated using Eq. (9).
[d]Mole of water-soluble components per unit weight of dry s. corneum calculated using Eq. (10).
[e]Amount of water-soluble components calculated assuming M_s = 100 using Eq. (8).
[f]Mean ± SD

where W_w and W_s are the weight (grams) of water and of water-soluble components (glycerol) in 1 g of dry s. corneum, M_w and M_s the molecular weight of water and of water-soluble components, respectively. The mole fraction x_w and the melting point T_m can then be readily obtained experimentally. Fig 5 shows the $-\ln X_w$ vs T_m^{-1} plots for the glycerol-incorporated s. corneum (Fig 4) and the glycerol/water mixture. The solid line is the theoretical curve calculated from Eq. (4). The experimental values are in fair agreement with the theoretical values as understood from Fig 5, which means that the activity of water in the s. corneum is decreased in the same fashion as that expected from the thermodynamic relation expressed in Eq. (4).

The mole fraction of water-soluble components present in the s. corneum, now, can be estimated from Eqs. (6) and (9):

$$-\ln(1-x_s) = (\Delta H^\circ_w/R)\,(1/T_m - 1/T^\circ_w) \qquad (9)$$

The mole, m_s, and the weight, W_s, of water-soluble components per unit weight of dry s. corneum can be estimated from Eqs. (8) and (10), taking an assumed molecular weight as $M_s = 100$ [8]:

$$m_s = (W_w/M_w)\,(x_s/x_w) \qquad (10)$$

Table II shows the mole fraction of water-soluble components, x_s, and the amount of water-soluble components, W_s, calculated from the observed melting point, T_m, in the thermograms for the treated s. corneum (Fig 2) with different water contents using Eqs. (5)–(10).

The s. corneum samples with the same treatment should, of course, contain the same amount of water-soluble components, which is substantiated for the samples of the untreated and the solvent-extracted s. corneum as shown in Table II. The water- and detergent-extracted samples, on the other hand, contain virtually no water-soluble component. The amount of water-soluble components in the s. corneum, W_s, can be estimated to be about 10% by weight, taking their average molecular weight as $M_s = 100$ [8].

Finally, we may safely say that the DSC technique is a sensitive and useful tool to obtain the quantitative information on the activity of water and the amount of water-soluble components in biologic systems without any process of extraction, isolation, or chemical analysis.

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