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Fourier transform Raman spectroscopy of interactions between the penetration enhancer dimethyl sulfoxide and human stratum corneum ☆

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Abstract

The stratum corneum, the outermost layer of human skin, is the major barrier to transdermal delivery of most drugs. Dimethyl sulfoxide (DMSO) is an established penetration enhancer. To assess its mechanism of flux enhancement, Fourier transform (FT) Raman spectroscopy was used to study the effects of a series of aqueous solutions of DMSO on hydrated human stratum corneum following treatment for 1 h. The results showed changes in the stratum corneum keratin from an α-helical to a β-sheet conformation. In addition, at concentrations $\geq 60\%$ v/v, at which DMSO enhances drug flux, there was evidence of interactions with stratum corneum lipids. These observations suggest that the skin penetration enhancement produced by DMSO not only involves changes in protein structure but may also be related to alterations in stratum corneum lipid organization, besides any increased drug partitioning effects.

Keywords: Penetration enhancer; Human stratum corneum; Dimethyl sulfoxide; Water; Fourier transform Raman spectroscopy

1. Introduction

Human skin functions as an excellent barrier in two directions, controlling the loss of water and other body constituents while preventing the entry of noxious substances from the external environment. The percutaneous route for drug administration holds several advantages over the oral or systemic routes such as the avoidance of first pass gut and hepatic metabolism, the ability to deliver drugs continuously, potentially fewer side effects, better patient compliance and ease of rapid cessation of therapy (Barry, 1983; Weissinger, 1993). Widespread use of the skin for drug delivery is, however, limited because of the aforementioned barrier properties.

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Human skin consists essentially of three tissue layers, the multi-layered epidermis, the underlying dermis containing a matrix of connective tissue woven from fibrous protein and the deep subcutaneous fatty layer. The outermost stratum of the epidermis, the stratum corneum or horny layer, is recognized as contributing the rate-limiting step in the barrier function of human skin to most drugs (Blank, 1953; Barry, 1983). This tissue typically consists of 10–15 layers of flattened, keratinized dead cells embedded in a lipid-rich matrix and may be about 10 μm when dry but usually swells to several times this thickness when hydrated. The barrier properties of the stratum corneum are controlled by its composition: 75–80% proteins, 5–15% lipids and 5–10% unidentified material on a dry weight basis (Wilkes et al., 1973).

There has been considerable research effort devoted to improving the percutaneous penetration of drugs either for local therapeutic effect or for systemic therapy. One approach has been the use of penetration enhancers which are chemicals that can decrease reversibly the barrier properties of the stratum corneum. Dimethyl sulfoxide (DMSO) is a dipolar aprotic solvent with a wide range of physical and chemical properties to which its diverse physiological and pharmacological activities are attributable. It is the earliest and the most widely studied skin penetration enhancer. It has been found to improve the permeation of a wide range of ionic and non-ionic compounds of molecular weight below 3000 at concentrations exceeding 60% (Ritschel, 1969) and a product containing a 5% solution of the anti-viral agent idoxuridine in DMSO is available for clinical use. Tetracycline hydrochloride in DMSO is also commercially available for the treatment of Acne vulgaris and various other patents for dermatological formulations containing DMSO have been filed.

Several theories have been advanced to explain the mechanisms of action of DMSO enhancement of skin permeability including: extraction of skin lipids (Allenby et al., 1969; Embery and Dugard, 1971); denaturation of stratum corneum proteins (Elfbaum and Laden, 1968); formation of hydrogen-bonded complexes with stratum corneum lipids (Al-Saidan et al., 1987); and the distortion and intercellular delamination of stratum corneum as a result of high osmotic stresses caused by transportation of both DMSO and water into the tissue from admixtures containing both solvents (Chandrasekaran et al., 1977). More recent studies suggest that DMSO exerts its rôle in enhancement of drug permeation by not only extracting soluble components of the horny structure but also by delaminating the horny layer and denaturing the proteins (Kurihara-Bergstrom et al., 1986, 1987). It has been proposed that part of the effects of DMSO arises from its solvent properties and thus, at high concentrations, it may promote partitioning of lipophilic drugs into the stratum corneum (Barry, 1987). Results of Fourier transform infrared (FTIR) spectroscopic investigations suggest that DMSO changes stratum corneum protein conformations (Oertel, 1977). Based on results of recent differential scanning calorimetric studies, it has been suggested that DMSO acts by displacing bound protein water, leaving a looser structure (Barry, 1987). Despite the wide ranging studies that have been performed with DMSO, its mechanisms of action as a penetration enhancer still remain unclear.

Fourier transform (FT) Raman spectroscopy has recently been used to characterize human skin (Williams et al., 1992). Wavenumber positions (υ, cm^{-1}) of bands in a Raman spectrum depend on both the atomic masses and the force constant. Vibrations involving light atoms occur at higher frequency positions than those of heavy atoms; for example, the stretching vibration of H_2O is seen at approx. 3400 cm^{-1} while that of D_2O occurs at approx. 2400 cm^{-1}. The force constant is a measure of molecular bond stiffness. Tightly bound groups have stronger force constants and vibrations involving them appear at higher frequencies than those involving looser bound groups, so that, for example, C = C stretching modes occur at around 1500 cm^{-1} whereas C-C modes are seen at around 1000 cm^{-1}.

A comparison of several Raman spectroscopic techniques demonstrated minimal inter-and intra-cadaver variations in molecular vibrations

arising from human stratum corneum constituents (Williams et al., 1993). Recently, we have shown the versatility of the technique in studies of terpene penetration enhancer action on human skin (Anigbogu et al., 1993). Since Raman scattering by water is weak, the interference from water seen in infrared studies of hydrated human stratum corneum is minimized. In the study presented here, FT Raman spectroscopy was used to probe molecular interactions between aqueous solutions of DMSO and human stratum corneum constituents. The technique was also applied to studies of interactions between DMSO and water.

2. Materials and methods

2.1. Chemicals

Spectroscopic grade dimethyl sulfoxide (DMSO) was obtained from Sigma Chemical Co., Poole, UK with a stated purity of 99 + %. Deuterium-exchanged dimethyl sulfoxide (DMSO-d_6) and deuterium oxide (D_2O) were supplied by Aldrich Chemical Company, Gillingham, UK, both with stated purities of 99.9%. Bovine keratin powder was purchased from ICN Biomedicals, Inc., Ohio, U.S.A. The chemicals were used without further purification. Mixtures of DMSO or DMSO-d_6 with deuterium oxide or distilled water were made ranging from 10 to 90% v/v in increments of 10% v/v.

2.2. Preparation of stratum corneum

Caucasian abdominal skin was obtained post mortem and stored in double-sealed evacuated polyethylene bags at −20°C prior to use (Harrison et al., 1984). The samples used in this study were from eight donors, 54% female, and had a mean age of 79 ± 9 (S.D) years. Epidermal membranes were prepared by heat separation (Kligman and Christophers, 1963); excess subcutaneous fat and connective tissue were removed from the skin which was then immersed in water at 60°C for 45 s. The epidermal membrane was gently teased off and floated stratum corneum side up overnight at 22 ± 1°C on an aqueous solution of trypsin (0.0001% w/v) and sodium hydrogen carbonate (0.5% w/v). The digested epidermal remnants were removed by swabbing. The stratum corneum membranes were washed with distilled water, rinsed in cold acetone for 10 s to remove surface contaminants and then stored over silica gel in an evacuated desiccator until required.

The stratum corneum samples were hydrated to a water content of ≥ 60% w/dry weight over a saturated aqueous solution of sodium sulfate which provides a relative humidity of 97% at 25°C. The hydrated samples of whole stratum corneum were treated by complete immersion in the desired concentration of aqueous DMSO or DMSO-d_6 for 1 h after which any excess enhancer was removed by blotting. All treatments were performed in triplicate.

2.3. Lipid extraction of stratum corneum and keratin powder

Sheets of human stratum corneum membranes prepared as described above were exhaustively delipidized by a modification of the protocol detailed by Roberts and Lillywhite (1983). The stratum corneum membranes were soaked at 32°C in (i) chloroform/methanol (2:1) for 24 h followed by (ii) soaking in acetone for 4h and then (iii) hexane for 24 h followed by (iv) ethanol/diethyl ether (8:92) for 24 h. At all stages during the lipid extraction, the flasks containing the samples and the solvents were gently shaken to aid the extraction. The samples were evacuated to remove excess solvent. Lipid-extracted stratum corneum did not take up appreciable water by the method used in hydrating whole stratum corneum. Samples were therefore floated in water to hydrate before analysis. Bovine keratin powder was subjected to the same rigorous extraction process to ensure the removal of any lipid contaminants.

2.4. Fourier transform Raman spectroscopy

FT Raman spectra of aqueous solutions of DMSO, DMSO-d_6, untreated whole human stratum corneum, lipid-extracted stratum corneum

and DMSO-d_6-treated whole stratum corneum were obtained using a Bruker FRA 106 FT Raman accessory mounted on an IFS 66-FTIR optical bench. Several Raman bands are common to molecules containing the same functional groups, termed 'characteristic frequencies', such as the C-H bands common to organic species. Therefore, to examine the C-H vibrational frequencies of stratum corneum constituents without interference from those of DMSO, fully deuterated DMSO was used. The atomic masses of the deuterated form are higher and thus the C-D bonds vibrate at lower frequencies than the C-H bonds and in a region where the skin is devoid of any vibrations, so allowing interference-free assessments of vibrational modes.

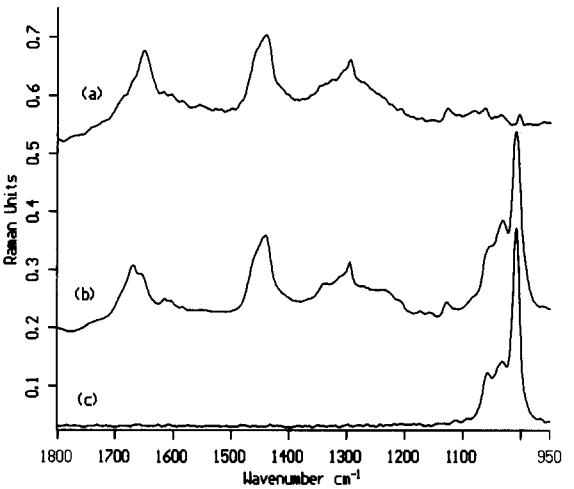

Fig. 2. FT Raman spectra of (a) untreated stratum corneum, (b) stratum corneum treated with DMSO-d_6 for 1 h and (c) pure DMSO-d_6. The spectra clearly show the presence of dimethyl sulfoxide in the skin.

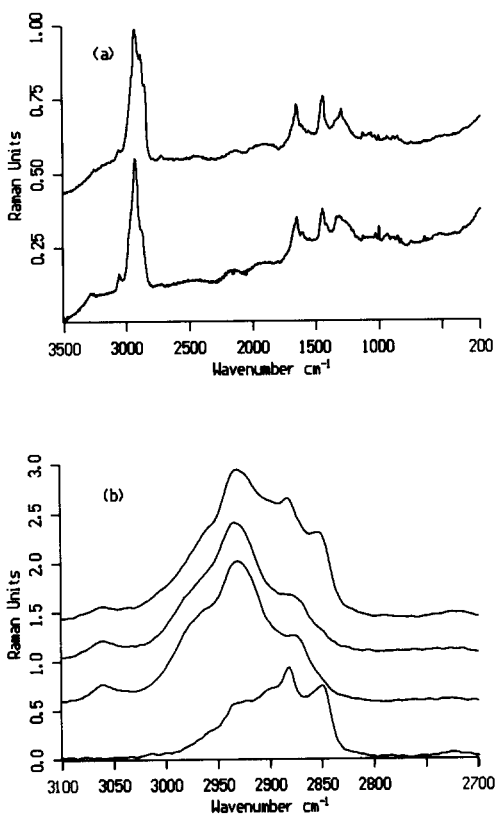

Fig. 1. FT Raman spectra of (a) hydrated whole stratum corneum (upper trace) and lipid-extracted human stratum corneum (bottom trace) over the wavenumber range 3500–200 cm^{-1} and (b) whole stratum corneum (top trace), lipid-extracted stratum corneum, lipid-extracted keratin powder and lipid fraction of stratum corneum (bottom trace) over the 3500–2700 cm^{-1} range.

The liquid samples were presented in a 2 cm^3 quartz cuvette with a mirrored rear surface while the stratum corneum samples were presented in a stainless-steel cup of diameter approx. 2 mm to a near-infrared Nd:YAG laser operating at a wavelength of 1.064 μm with an output power of 750 mW. The liquid samples were subjected to full laser power and typically 200 scans were collected at a resolution of 4 cm^{-1}. The stratum corneum samples were, however, exposed to a laser power of approx. 450 mW to avoid fluorescence and sample degradation and the spectra represent an average of 4000 scans at a resolution of 4 cm^{-1}. The FT Raman accessory is equipped with a liquid-nitrogen cooled germanium diode detector with an extended spectral bandwidth which covered the wavenumber range 3500–50 cm^{-1}. Spectral response was corrected for white light and the observed band wavenumbers, calibrated against the internal laser frequency, were correct to better than ± 1 cm^{-1}.

2.5. Curve fitting

Opus data files generated on the Bruker FRA 106 FT Raman accessory were translated into

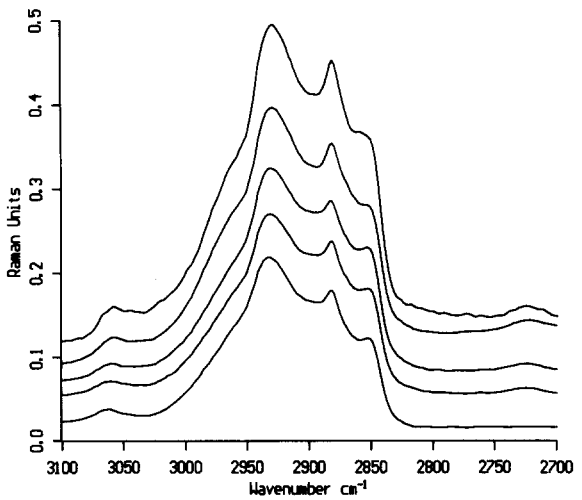

Fig. 3. FT Raman spectra of the CH stretching modes in untreated stratum corneum (bottom trace) and stratum corneum treated with 20, 40, 60% and pure DMSO-d_6 (top trace).

JCAMP files and curves were fitted using Lab Calcl software (Galactic Industries Corp.). The program is flexible and allows the user a choice of parameters to input for the curve fitting. At the start, initial approximations were made about the number of bands present, their positions, widths and types (i.e., Gaussian, Lorentzian, log-normal or mixture). For the present study, the band type specification was a Gaussian/Lorentzian mixture. The program fitted a linear baseline to each spectrum and used the initial approximations and iteration to find a combination of the heights, positions, widths and areas that best fitted the data.

3. Results

All samples of stratum corneum used in this study were prepared by trypsin digestion of heat-separated epidermal membranes and all spectra were corrected for instrument response. Because of the natural variability that occurs in human skin, there were some minor differences in the wavenumber positions of some bands from sample to sample but the trends observed for each treatment were the same. The results presented here are therefore representative and not the mean values from all replicates.

Fig. 1a shows typical FT Raman spectra of hydrated whole (untreated) and lipid-extracted human stratum corneum respectively over the wavenumber range 3500–200 cm^{-1} and Fig. 1b illustrates spectra over the range 3500–2700 cm^{-1} for whole (untreated), lipid extracted stratum corneum, lipid-extracted bovine keratin powder and the lipid fraction of stratum corneum obtained by subtracting the spectrum of the lipid extracted stratum corneum from that of whole (untreated) stratum corneum. There is a marked similarity between the spectrum of lipid-extracted stratum corneum and lipid-extracted bovine keratin powder.

Fig. 2 illustrates FT Raman spectra over the 1800–950 cm^{-1} range of untreated stratum corneum, stratum corneum treated with pure deuterated DMSO, and pure deuterated DMSO. This figure shows that the DMSO is devoid of vibrational modes which would interfere with the amide I band (C = O stretching around 1650 cm^{-1}) in the stratum corneum. Fig. 3 details the C-H stretching region (3100–2700 cm^{-1}) of untreated stratum corneum and stratum corneum treated with different aqueous concentrations of

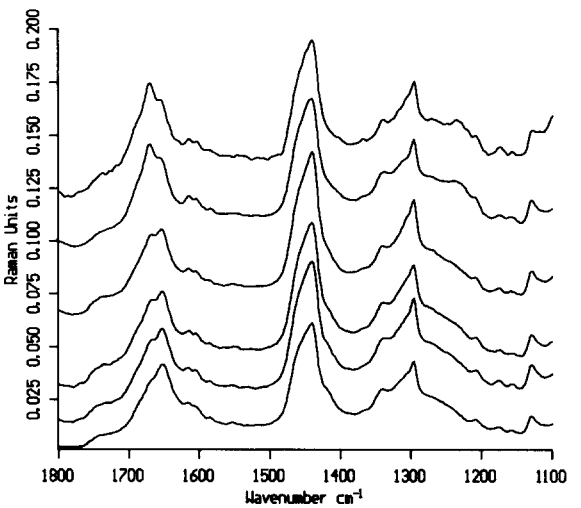

Fig. 4. FT Raman spectra over the wavenumber range 1800–1100 cm^{-1} of untreated stratum corneum (bottom trace), and stratum corneum treated with 20, 40, 60, 80% and pure DMSO-d_6 (top trace).

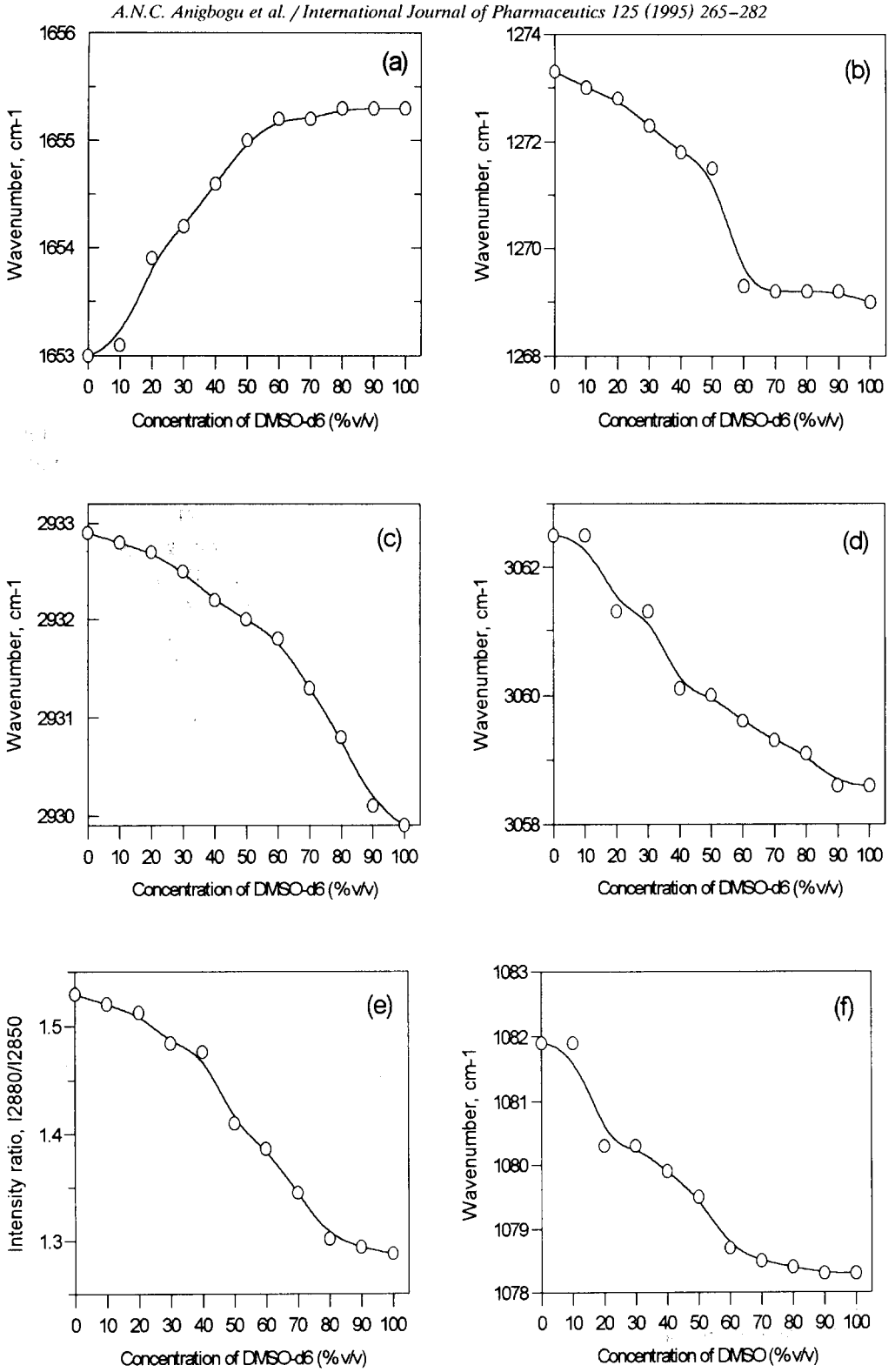

Fig. 5. Frequency and intensity ratio changes in stratum corneum as a function of aqueous DMSO-d_6 (a–e) and DMSO concentration (f): (a) amide I band of protein, (b) amide III band of protein, (c) CH_3 symmetric stretching vibration of stratum corneum, (d) C-H (olefinic) stretching mode of stratum corneum, (e) I_{2880}/I_{2850} ratio of stratum corneum lipids, and (f) C-C skeletal stretch (random conformation) of stratum corneum lipids.

deuterium-exchanged DMSO (DMSO-d_6). Fig. 4 provides FT Raman spectra in the 1800–1100 cm^{-1} range of untreated stratum corneum and stratum corneum treated with various concentrations of aqueous deuterium-exchanged DMSO ranging from 20% v/v to pure. Fig. 5 shows graphs derived from Fig. 1–4.

Fig. 6 represents profiles obtained from curve fitting of the amide I band in whole and lipid-extracted human stratum corneum. Fig. 7a is a graph showing the proportions of Raman signal arising from the various vibrational modes of keratin at different DMSO concentrations. The graph shows an initial steady increase in the signal due to the symmetrical parallel amide I mode of the anti-parallel β-pleated sheets being formed up to 60% v/v DMSO with a sudden increase to 70% v/v DMSO. The signal from the asymmetrical parallel amide I mode of anti-parallel β-sheets was fairly constant between 0 and 40% v/v DMSO with a slight increase thereafter up to 70% v/v DMSO. In stratum corneum

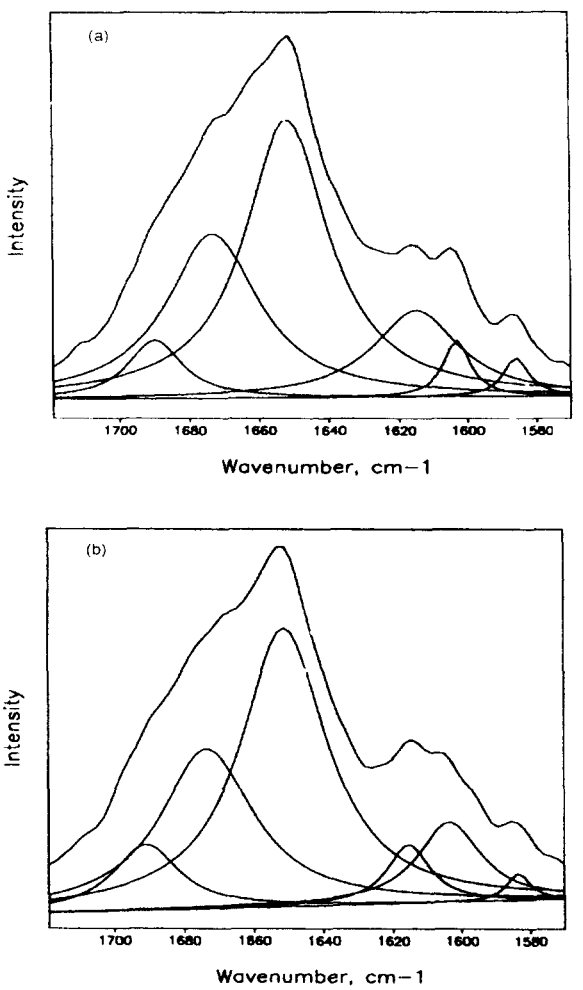

Fig. 6. Profiles obtained from curve-fitting the amide I band in (a) whole stratum corneum and (b) lipid-extracted stratum corneum.

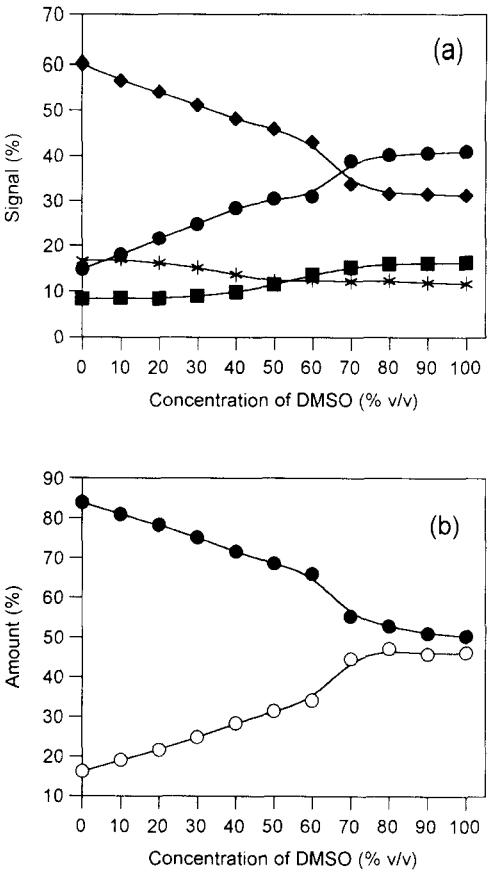

Fig. 7. (a)Changes in the Raman signal arising from the amide I mode of α-helical keratin (♦), the symmetrical parallel mode of anti-parallel β-pleated sheets (●), the asymmetrical parallel mode of anti-parallel β-pleated sheets (■) and unidentified protein residues (*), in human stratum corneum with different concentrations of aqueous DMSO. (b)Profile showing amount of α-helical keratin (●) and β-pleated sheets (○) in human stratum corneum at different concentrations of DMSO, expressed as the percentage of total amount, α-helical + β-pleated keratin.

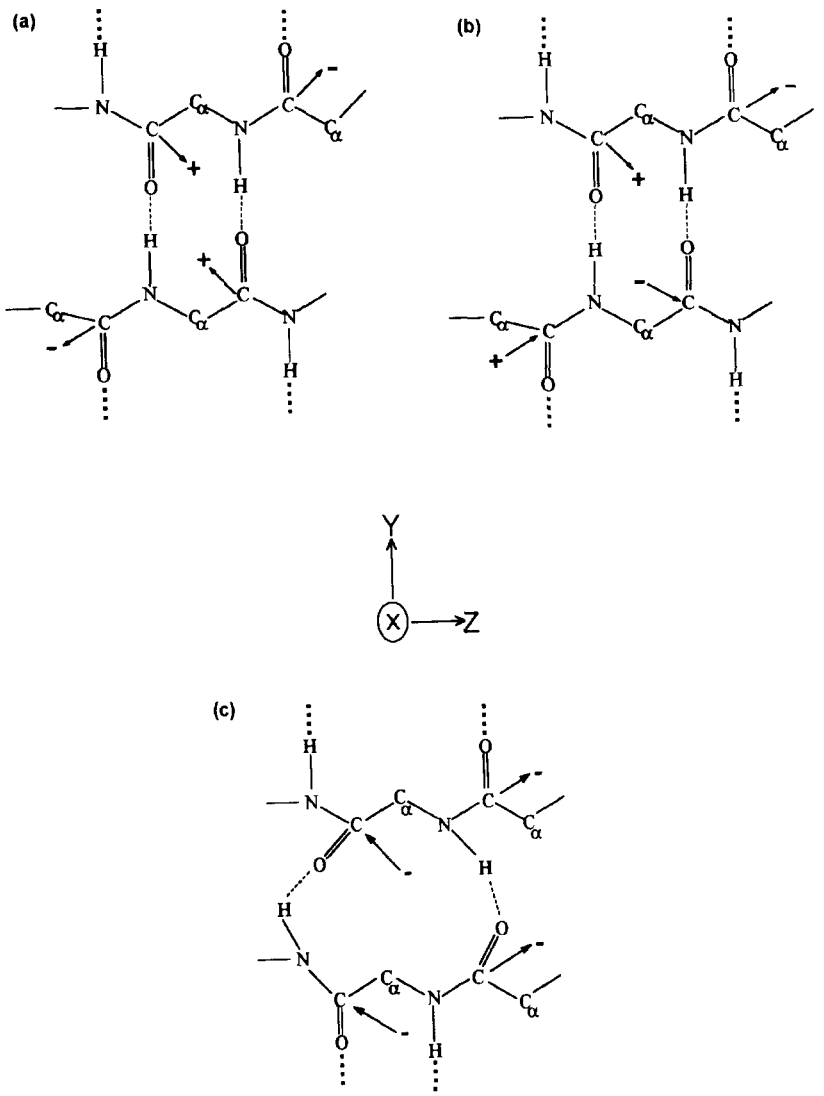

Fig. 8. A schematic illustration of the vibrational modes for (a) symmetrical parallel amide I mode of anti-parallel β-pleated sheets, (b) asymmetrical parallel amide I mode of anti-parallel β-pleated sheets and (c) perpendicular amide I mode of parallel β-pleated sheets observed in human stratum corneum (redrawn from Miyazawa, 1960).

treated with 80% v/v to pure DMSO, the signal was again fairly uniform. There was a slight but steady decrease in the signal arising from the unidentified protein residues between 0 and 60% v/v DMSO beyond which there was no appreciable further decrease in the signal. The amount (%) of α-helical keratin and β-pleated sheets relative to one another with different concentrations of aqueous DMSO are shown in Fig. 7b. With increasing concentrations of DMSO, the amount of α-helix in the stratum corneum decreased while the amount of β-sheet structures increased.

Fig. 8 shows the actual vibrational modes of parallel and anti-parallel β-sheets redrawn from Miyazawa (1960).

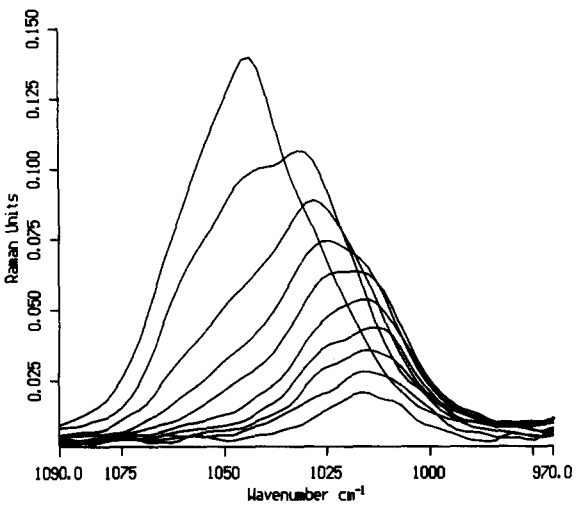

Fig. 9. FT Raman spectra of DMSO-water mixtures in the S = O stretching region of dimethyl sulfoxide in concentrations ranging from 10% v/v (bottom trace) to pure (top trace) in 10% v/v intervals.

Fig. 9 and 10 show spectra of DMSO-deuterium oxide mixtures in the S = O stretching and O-D stretching regions respectively.

4. Discussion

Assignments of the bands in the spectra for human stratum corneum (Fig. 1a) consistent with the observed Raman active vibrational modes have been made (Barry et al., 1992), and the main features are summarized in Table 1.

Of particular importance to this study are the CH stretching modes in the 3100–2700 cm^{-1} region; the C = O stretching mode at about 1650 cm^{-1} (amide I band) arising predominantly from the α-keratin in the stratum corneum corneocytes; the band at about 1274 cm^{-1} assigned as the CN stretching and NH deformation of protein (amide III band) and weaker bands at about 1030, 1062, 1082 and 1126 cm^{-1} assigned as C-C stretching modes which yield information about skeletal structures in a molecule. These bands (1030–1126 cm^{-1}) have been used to assess lipid bilayer packing in model membrane systems (Carey, 1982). The amide I and III bands have been shown to arise from 'in-plane' vibrations of the peptide bond -CONH- [24] and differences in position, dichroism and intensity of these bands have been used extensively in conformational analysis of proteins (Carey, 1982; Hudson and Mayne, 1987).

The bands in the C-H stretching region, 3100–2700 cm^{-1}, are clearly complex and contain overlapping vibrational modes. In previous literature reports detailing infrared spectroscopic investigations of human stratum corneum (Golden et al., 1986; Knutson et al., 1986; Krill et al., 1992), it had been assumed that the intercellular lipids were the major contributors to all the bands arising from the C-H groups. To investigate this assumption, we extensively extracted the lipids, adopting a protocol that would ensure the removal of most of the different classes of lipids found in the stratum corneum, rather than just applying chloroform-methanol (2:1) as usually used for lipid extraction. Even though some covalently bound lipids in the stratum corneum may not be removed, our method of extraction ensured that most of the lipids were removed and this process was usually complete within 72 h. Extension of the time period for lipid extraction to 168 h did not remove further lipids as shown by Raman spectroscopy.

Our results show that following extensive lipid extraction, the C-H olefinic stretching mode for stratum corneum at about 3060 cm^{-1} remained invariant suggesting that it mostly derives from the keratin, while the C-H aliphatic stretching mode at about 2725 cm^{-1} was entirely removed, indicating it arises essentially from the intercellular lipids. The band at about 2852 cm^{-1} assigned as a CH_2 symmetric stretching mode, is not present in the spectrum of the extracted stratum corneum, suggesting that this band arises mostly from intercellular lipids. The band at about 2883 cm^{-1}, assigned as a CH_2 asymmetric stretching mode is markedly reduced in intensity but a fraction of this band remains, indicating that it is mainly due to the intercellular lipids but may have a contribution from the keratin. The band at about 2931 cm^{-1} which has been assigned as a CH_3 symmetric stretching mode is however only slightly reduced in intensity suggesting that most of the vibrations responsible for this band arise

Table 1
FT Raman spectral assignments of the main vibrational modes for human stratum corneum

Wavenumber (cm^{-1}) [a]	Assignments and approximate description of vibrational modes [b]
3060w	υ(CH) olefinic
2958m, sh	$\upsilon(CH_3)$ Asym
2931s	$\upsilon(CH_3)$ Sym
2883m	$\upsilon(CH_2)$ Asym
2852m	$\upsilon(CH_2)$ Sym
1652s	υ(C = O) amide I (α-helix)
1585w	υ(C = C) olefinic
1552w	δ(NH) and υ(CN) amide II
1438s	$\delta(CH_2)$ scissoring
1296m	$\delta(CH_2)$
1274m	υ(CN) and δ(NH) amide III (α-helix)
1244w, sh	$\delta(CH_2)$ wagging; δ(CN) amide III, disorder
1172w	υ(CC)
1126mw	υ(CC) skeletal, *trans* conformation
1082mw	υ(CC) skeletal, random conformation
1062mw	υ(CC) skeletal, *trans* conformation
1031mw	υ(CC) skeletal, *cis* conformation
1002m	υ(CC) aromatic ring
644w	υ(CS)
623w	υ(CS)

Data are wavenumber positions of bands in a representative spectrum resulting from 4000 scans at a resolution of 4 cm^{-1}. Asym, asymmetric; Sym, symmetric. Abstracted from Barry et al. (1992).
[a] s, strong; m, medium; w, weak; sh, shoulder.
[b] υ, stretch; δ, deformation.

from the keratin component of the stratum corneum with a minor contribution from the lipids.

Similarly, of the four C-C skeletal stretching modes in the spectrum of stratum corneum, the one found at about 1031 cm^{-1} remained prominent after lipid extraction, suggesting it is derived from keratin, while the other three at about 1062, 1082 and 1126, cm^{-1} were markedly reduced, indicating they arise mainly from the intercellular lipid alkyl chains.

All the changes discussed above are summarized in Table 2. The spectrum of the resultant extracted stratum corneum we obtained is in fact markedly similar to the spectrum of commercially available keratin powder (Fig. 1b) which we also subjected to extensive solvent extraction to remove lipid contaminants.

Even though stratum corneum lipids contain very long chain (C_{24}–C_{35}) saturated ceramides as well as long chain acids, cholesterol esters, triglycerides and hydrocarbons, an examination of the amino acid composition of the stratum corneum keratin shows that at least between 65 and 75% of the amino acid residues found in the stratum corneum have aliphatic side chains (Crounse, 1963; Baden and Bonar, 1968; Wood and Blandon, 1985; Krill et al., 1992). If we consider that the stratum corneum, in addition to

Table 2
FT Raman spectral assignments for some vibrational modes in human stratum corneum deduced following extensive lipid-extraction

Wavenumber (cm^{-1}) [a]	Assignments and approximate description of vibrational modes [b]	Origin of vibration
3060w	υ(CH) olefinic	predominantly protein
2931s	$\upsilon(CH_3)$ Sym	mostly protein but with significant lipid contribution
2883m	$\upsilon(CH_2)$ Asym	mostly lipids with minor protein contribution
2852m	$\upsilon(CH_2)$ Sym	essentially lipids
2725w	υ(CH) aliphatic	essentially lipids
1126mw	υ(CC) skeletal, *trans* conformation	mainly lipid with minor protein contribution
1082mw	υ(CC) skeletal, random conformation	mainly lipid with minor protein contribution
1062mw	υ(CC) skeletal, *trans* conformation	mainly lipid with minor protein contribution
1031mw	υ(CC) skeletal, *cis* conformation	mostly protein with small lipid contribution

Samples were exhaustively delipidized as described in section 2. Data are wavenumber positions of C-H and C-C vibrational modes from representative spectra of 4000 scans obtained at resolution of 4 cm^{-1}.
[a] s, strong; m, medium; w, weak; sh, shoulder.
[b] υ, stretch; Asym, asymmetric; Sym, symmetric.

Table 3
FT Raman spectral assignments of the main vibrational modes for dimethyl sulfoxide (DMSO) and deuterium exchanged dimethyl sulfoxide (DMSO-d_6)

Wavenumber (cm^{-1}) [a]		Assignments and approximate description of vibrational modes [b]
DMSO	DMSO-d_6	
2997s		$\nu(CH_3)$ Asym.
2914vs		$\nu(CH)$ Sym.
2891w sh		$\nu(CH_3)$ Sym.
	2249s	$\nu(CD_3)$ Asym.
	2125vs	$\nu(CD)$ Sym.
	1996w	$\nu(CD_3)$ Sym.
1420m		$\delta(CH)$
1310w		$\delta(CH_3\text{-}S)$ Sym.
	1057m sh	$\delta(CH)$ degenerate
	1032m sh	
1044m		$\nu(S=O)$
	1008s	$\nu(S=O)$
955w		$\delta(CH_3)$ rock
	821vw	$\delta(CH_3)$ rock
	761m	$\delta(CH_3)$ rock
700s		ν(C-S-C) Asym.
670s		ν(C-S-C) Sym
	614vs	ν(CS) Asym.
384m		δ(C-S=O)
335s	341m	δ(C-S=O)
308m	308s	δ(C-S-C)
	264ms	δ(C-S-C)

Asym., asymmetric; Sym., symmetric. Samples of DMSO and DMSO-d_6 were presented in a 2 cm^3 quartz cuvette to a near-infrared Nd:YAG laser. Data represent wavenumber positions of bands in spectra generated from 200 scans at a resolution of 4 cm^{-1}.
[a] v, very; s, strong; m, medium; w, weak; sh, shoulder.
[b] ν, stretch; δ, deformation.

some unidentified material, consists mainly of proteins (75–80%) and lipids (5–15%), a large contribution from the proteins to the vibrations in the C-H regions is to be expected.

DMSO possesses several distinctive and characteristic bands and assignments for the observed Raman active vibrational modes of non-deuterated and deuterium-exchanged DMSO are in Table 3. Of particular interest are the bands in the 1050–1000 cm^{-1} region assigned as S = O stretching modes. The appearance of these bands in the spectrum of stratum corneum following a 1 h treatment indicates the presence of DMSO in the tissue. In the 1700–1250 cm^{-1} region where the amide I and III bands occur in the stratum corneum, DMSO is devoid of any vibrations and hence changes observed in the stratum corneum spectra after treatment with DMSO must arise from changes in the molecular environment of the amide bonds and are not due to interference from DMSO bands.

Contrary to the conclusions of a recent X-ray diffraction study by Garson et al. (1991) which indicated that human stratum corneum keratin was mainly in the β form, FT Raman vibrations recorded for human stratum corneum show that the proteins exist predominantly in the α-helix conformation as indicated by the positions of the amide I and III bands at about 1650 and 1274 cm^{-1}, respectively (Barry et al., 1992). The solvents used in our study for lipid extraction did not change protein conformation as the amide I band in the extracted stratum corneum was also at about 1650 cm^{-1}.

At low applied concentrations of DMSO (around 20% v/v), there were minor yet real changes in spectral features arising from C-H stretching modes of stratum corneum (Fig. 3). Deuterated DMSO was used for this investigation and the CD modes were clearly separated from the CH vibrational modes of stratum corneum. The bands at about 2881 and 2850 cm^{-1} in untreated stratum corneum are asymmetric and become more asymmetrical in stratum corneum treated with various concentrations of DMSO. Generally, all the bands in the 3100–2700 cm^{-1} region broaden in stratum corneum treated with DMSO.

Fig. 4 is a comparison of the FT Raman spectra over the 1800–1100 cm^{-1} wavenumber range of untreated stratum corneum and stratum corneum treated with various concentrations of DMSO. Compared with an untreated sample, following application of increasing concentrations of DMSO, the amide I band shifts significantly and reproducibly by about 2 cm^{-1} to higher wavenumbers (Fig. 5a). There was a small but steady shift in the position of the amide I band to higher wavenumbers from 10 to 60% v/v DMSO. From 70% v/v to pure DMSO there was no appreciable shift in the position of this band as

shown by the plateau in the graph. The intensity of the amide I band relative to the neighbouring CH_2 scissoring mode decreases markedly with the appearance of a new band at about 1673 cm^{-1}. The amide III band shifts to a lower wavenumber by about 4 cm^{-1} from approx. 1273 cm^{-1} to approx. 1269 cm^{-1} (Fig. 5b). This shift was gradual from 10 to 50% v/v DMSO with a sudden increase in the magnitude of shift at 60% v/v DMSO as seen from the change in the slope of the graph. Thereafter, there was no further appreciable shift in the position of the amide III band. These changes are consistent with conversion of the protein from an α-helical conformation to β-pleated sheets that has been observed in other natural proteins and synthetic polypeptides (Tu, 1982). These changes were observed with concentrations of DMSO as low as 20% v/v. Treatment with increasing concentrations of DMSO produced increasing amounts of β-pleated sheet form relative to α-helix, and with pure DMSO, the intensity of the β-pleated sheet form predominated.

In the absence of calibration data for proteins with known α-helical and β-sheet composition, we employed curve-fitting for the first time, to separate the bands in the amide I region of untreated stratum corneum and stratum corneum treated with various concentrations of aqueous DMSO in order to determine semi-quantitatively the proportions of α-helical and β-sheet proteins present in stratum corneum before and after treatment. Although curve fitting has limitations, with careful choice and use of parameters, overlapping bands found in heterogeneous systems such as human skin can be effectively and correctly separated (Maddams, 1980). In the work reported here, for all the bands curve-fitted, the statistical goodness of fit was better than 99.9% but visual inspection was still employed as suggested by Maddams (1980).

Using X-ray diffractometry to study changes in palmar cuttings caused by heat, Baden et al. (1973) reported that in addition to a predominantly α-helical content, epidermis and stratum corneum contains 5–10% anti-parallel β-sheet and that parallel β-sheets (initial concentration not stated), formed as a result of heat-induced conformational changes in the α-helices. The profiles in Fig. 6a and b show the experimentally generated Raman spectra from human stratum corneum (top traces) with the derived composites from curve fitting underlying each. These profiles resulting from curve fitting of our spectra show that the observed amide I band in human stratum corneum following mild heat separation is a composite of three bands at about 1650, 1670 and 1695 cm^{-1} attributed to α-helices, the symmetrical parallel Amide I mode of anti-parallel β-pleated sheets and the asymmetrical parallel amide I mode of anti-parallel β-pleated sheets respectively (Walton and Blackwell, 1973; Tu, 1986). Anti-parallel β-pleated sheets actually have four Raman-active vibrational modes of which the symmetrical parallel amide I mode of anti-parallel β-pleated sheets, at about 1670 cm^{-1}, is the strongest and most easily observable. The other three modes are usually much weaker and may in fact not be detectable. From the profiles in Fig. 6a we were also able to detect the asymmetrical parallel amide I mode of anti-parallel β-pleated sheets at about 1695 cm^{-1}. The remaining two, symmetrical and asymmetrical perpendicular amide I vibrational modes of the anti-parallel β-pleated sheets, were not detectable in the spectra of human stratum corneum before and after treatment with DMSO. The bands at about 1585, 1606 and 1618 cm^{-1} are presumed to arise from unidentified protein residues.

From the areas under the bands, we calculated the proportion of the Raman signal in untreated stratum corneum arising from α-helical keratin to be approx. $60 \pm 9\%$, from the symmetrical parallel amide I mode of anti-parallel β-pleated sheets to be approx. $15 \pm 3\%$, and from the asymmetrical parallel amide I mode of anti-parallel β-pleated sheets to be approx. $8 \pm 2\%$; approx. $17 \pm 4\%$ was from unidentified protein residues [(S.E.), $n = 4$]. These results are presented graphically in Fig. 7a. Anti-parallel β-pleated sheet in human stratum corneum gives rise to two vibrational amide I modes at approx. 1670 and 1695 cm^{-1} whereas there is a single amide I mode at about 1652 cm^{-1} arising from the α-helical keratin. To confirm that the two observed modes of

anti-parallel β-pleated sheets are indeed due to the same species, we calculated the ratio of the areas of symmetrical/asymmetrical parallel amide I modes of anti-parallel β-sheets in stratum corneum treated with different concentrations of DMSO. If the two modes arise from different molecular species, we would expect a trend in the ratios, either increasing or decreasing with increasing concentrations of DMSO. Regression analysis, however, showed no correlation between DMSO concentration and symmetrical/asymmetrical β-keratin ratio ($r = 0.065$); the mean ratio was 2.53 ± 0.21. This is strong evidence that the two modes (symmetrical and asymmetrical) arise from a single β-keratin molecule.

In order to estimate the relative proportion of β-keratin to α-helical keratin in the stratum corneum, we averaged the areas under the two modes arising from anti-parallel β-keratin. This average value was then added to the area of the α-helical band to obtain the total area of α-helical and anti-parallel β-sheet keratin produced following treatment with different concentrations of DMSO. To calculate the amounts of α-helical keratin and β-pleated sheets present in stratum corneum from the areas under the bands, we assumed a linear relationship between amount and the signal arising from various modes of the two conformations following treatment with DMSO. The amount of each type of conformation was then obtained as percent fraction of the total.

The amounts of α-helical and anti-parallel β-pleated sheet keratin relative to one another in the stratum corneum at different DMSO concentrations are shown in Fig. 7b, expressed as a percentage of α-helical plus β-pleated keratin. On first observation, the amount of β-pleated sheets so calculated in untreated stratum corneum (approx. 16%) as detected by Raman spectroscopy coupled with curve fitting appeared somewhat higher than the previously reported amounts of 5–10% (Baden et al., 1973). To assess if this high proportion arises as an artefact from our method of preparation of stratum corneum (trypsinizing epidermal membranes previously heat-separated at 60°C), we also collected Raman spectra from samples of stratum corneum prepared by scraping abdominal skin thus avoiding such treatment. Following curve fitting and calculation of the areas under the bands, similar levels of symmetrical parallel amide I mode of anti-parallel β-pleated sheets and asymmetrical parallel amide I mode of anti-parallel β-pleated sheets and α-helical keratin were found in stratum corneum scrapings as in heat-separated stratum corneum. This suggests that the temperature we used for our sample preparation together with the extremely mild trypsinization procedure did not change stratum corneum proteins from α-helices to β-sheets.

The amide I band arises predominantly (about 80%) from C = O stretching vibrations of the CONH group and to determine if C = O vibrations from the fatty acids were contributing significantly to this band we also curve-fitted the amide I band in lipid-extracted stratum corneum. Similar profiles (Fig. 6b) and values were obtained as for whole stratum corneum suggesting that indeed there are more β-pleated sheets in stratum corneum than have previously been reported (Baden et al., 1973).

Following treatment with increasing concentrations of DMSO solutions, the signals arising from symmetrical parallel amide I mode of anti-parallel β-pleated sheets and asymmetrical parallel amide I mode of anti-parallel β-pleated sheets increased while the signal from the α-helical keratin decreased (Fig. 7a). There was initially a small but steady increase in the amounts of anti-parallel β-pleated sheets formed up to 60% v/v DMSO. Then there was an abrupt increase in the amount of anti-parallel β-pleated sheets as shown by the change in the slope of the graph in Fig. 7b; beyond 70% v/v the amount formed was essentially constant. With pure DMSO, the amount of anti-parallel β-pleated sheets structures in the stratum corneum was approx. 46% (Fig. 7b).

The band attributed to the perpendicular amide I mode of the parallel β-pleated sheets at about 1632 cm^{-1} appeared for the first time in the Raman spectrum of stratum corneum treated with 90% v/v aqueous DMSO. The area of the parallel β-pleated sheets formed, was added to that of α-helical keratin and anti-parallel β-pleated sheets and the amount calculated as per-

cent fraction of the total. The amount of parallel β-pleated sheets represents about 3% of the total α-helical and β-sheet structures in human stratum corneum. Similar amounts were produced by pure DMSO. The parallel amide I mode of the parallel β-pleated sheets at about 1645 cm^{-1} was not observed. The band at about 1652 cm^{-1} arising from the α-helical keratin was however, still present at approx. 51% showing that the conversion to β-sheets was not complete even with pure DMSO.

The vibrational modes of the parallel and anti-parallel β-sheets discussed above are shown in Fig. 8. In anti-parallel β-sheets, each unit cell contains four peptide groups arising from two adjacent chains. Adjacent chains can move in phase or out of phase. Specifically, in the symmetrical parallel amide I mode of anti-parallel β-sheets at approx. 1670 cm^{-1}, adjacent peptide groups move in phase. Similarly, adjacent groups in the asymmetrical parallel amide I mode of anti-parallel β-sheets at approx. 1695 cm^{-1} also move in phase. Adjacent groups across interchain hydrogen bonds in the perpendicular amide I mode of parallel β-pleated sheets move out of phase. The plus and minus signs represent the x components of the transition moments of the peptide groups pointing upward and downward, respectively, in the plane of the paper.

The changes outlined above suggest that DMSO, in addition to altering the protein conformation, also affects the intercellular lipids in stratum corneum. The lipids in the stratum corneum are arranged in a multiply bilayered structure. At physiological temperatures and in their unperturbed state, stratum corneum lipids exist in various phases; crystalline, gel and liquid crystalline forms with the gel phase predominating (White et al., 1988). In the gel phase, lipid backbone C-C bonds are arranged in a zig-zag manner such that the alkyl chains are maximally extended, affording close packing. This is the all-*trans* structure which has the lowest energy and lateral motion is highly restricted (Lee, 1975). With thermal or chemical perturbation, *trans* conformers convert to *gauche* conformers along the alkyl chains. The energy associated with the structure is higher and the carbon atoms are less rigidly held together and thus C-C single bonds along the alkyl chains vibrate with a greater degree of motional freedom. This increasing mobility along the alkyl chain is associated with a decrease in the microviscosity of the hydrocarbon region of the lipid bilayer. The lipids are thus thought to exist in a more fluid-like state and this is termed the liquid crystalline phase.

The force constants of the C-C bonds in *trans* and *gauche* conformations are different and as such the stretching vibrations occur at different frequencies. It has been shown that C-H stretching vibrations are sensitive to changes in their environment and are thus significantly affected by changes in the conformation of the adjoining C-C bonds (Snyder et al., 1982; Casal and Mantsch, 1984). Changes observed in the frequencies of the C-H vibrations are therefore an indirect measure of structural changes in the lipids. Shifts to lower wavenumbers of up to 3 cm^{-1} in the frequency of the CH_3 symmetric stretching vibration of stratum corneum (from approx. 2933 cm^{-1} to approx. 2930 cm^{-1}) were observed after treatment with different concentrations of DMSO (Fig. 3). As mentioned earlier, there are contributions from both the lipids and proteins to this vibrational mode and these changes observed following treatment with DMSO may be indicative of interactions between DMSO and stratum corneum lipids and proteins (Fig. 5c shows a graphical representation of these shifts). The C-H olefinic stretching mode, mainly due to stratum corneum keratin, also shifted by 4 cm^{-1} to lower frequencies from approx. 3062 cm^{-1} to approx. 3058 cm^{-1} over the range 0–100% DMSO (Fig. 5d).

The intensity ratio (I_{2880}/I_{2850}) of the peak heights of the bands at about 2880 and 2850 cm^{-1}, corresponding to the C-H asymmetric and symmetric stretching frequencies of the methylene groups arising mainly from the lipids, has been used to study the structure and phase transitions of the hydrocarbons in model phospholipid membranes such as dipalmitoyl phosphatidylcholine (DPPC) and polymethylene chains (Gaber and Peticolas, 1977; Snyder et al., 1978). The I_{2880}/I_{2850} ratio measures both lateral packing in the extended chains of the lipid bilayer and conformational order/disorder seen in gel and

liquid crystalline phases. A broad asymmetric band underlies the CH_2 asymmetric stretching mode at approx. 2880 cm^{-1} (Snyder et al., 1978). This broad band is involved in Fermi resonance interaction with the first overtone of a methylene bending mode at approx. 1440 cm^{-1} and contributes to the intensity of the 2880 cm^{-1} band. When the population of *gauche* conformers in the lipid bilayers of the stratum corneum increases following treatment with DMSO-d_6, the chain symmetry disappears and with it the resonance condition ceases. The contribution from the first overtone of the 1440 cm^{-1} band disappears and therefore, the relative intensity of the 2880 cm^{-1} band decreases, whereas the 2850 cm^{-1} is not affected with its intensity remaining essentially constant. Consequently, the intensity ratio (I_{2880}/I_{2850}) decreases with an increase in the number of *gauche* conformers in the system. Fig. 5e shows a plot of the I_{2880}/I_{2850} ratio of stratum corneum as a function of DMSO concentration. Although the magnitude of this change is less than that obtained for thermally induced conformational changes in DPPC (Brown et al., 1973; Litman et al., 1991), it is reproducible and does suggest the production of intramolecular chain disorder arising from *trans-gauche* isomerization of the lipid alkyl chains within the bilayer. There was initially a small decrease in the intensity ratio from the untreated control to stratum corneum treated with 40% v/v DMSO. This was followed by a more rapid decrease between 40 and 80% v/v DMSO after which the rate of change fell.

Further evidence for this disorder is seen in the changes in the C-C skeletal stretching region, whereby the alkyl C-C backbone yields information about membrane structure and fluidity. The 1062 and 1126 cm^{-1} bands arise from the *trans* conformation of the C-C stretch while the 1082 cm^{-1} band is thought to be mainly due to the *gauche* conformation of the C-C stretch. Deuterated DMSO, however, also has bands in the 1000–1050 cm^{-1} region and to study changes in the C-C modes of the skin, aqueous solutions of non-deuterated DMSO were therefore used. With increasing concentrations of DMSO, the intensities of the 1126 and 1062 cm^{-1} bands greatly diminish, consistent with fewer *trans* conformers, whereas the 1082 cm^{-1} band broadens, increases in intensity and shifts to lower frequencies by up to 7 cm^{-1} (Fig. 5f) consistent with a greater number of *gauche* conformers. These results are in good agreement with those obtained for chloroform-induced disordering of DPPC chains (Gaber and Peticolas, 1977). It is observed, however, that the changes in the C-C skeletal band at 1082 cm^{-1} occurred between 0 and 60% v/v DMSO after which there was no further appreciable change. It may well be that since this band arises mainly from gauche conformers of the lipids, the maximum fluidity attained by stratum corneum lipids denoted by changes in this band were achieved at 60% v/v DMSO and hence no further shift in the frequency of this band at higher DMSO concentrations. In a previous FTIR study of the effect of DMSO on stratum corneum constituents, Oertel (1977) was unable to show these changes in the alkyl backbone region of the lipids because vibrational features below 1300 cm^{-1} in the infrared spectrum of stratum corneum are usually very weak and poorly resolved.

Overall, the results from this study show a concentration-dependence in the action of DMSO on stratum corneum lipids and proteins and correlates with data from earlier DSC studies (Barry, 1987).

DMSO is a powerful dipolar aprotic solvent which forms an association complex with water at a concentration of about 67% v/v DMSO through dipole-dipole interactions and through hydrogen bonding interactions which are stronger than those formed between water molecules (Cowie and Toporowski, 1961). Figs. 9 and 10 illustrate changes in the S = O stretching mode of DMSO and the O-D bands of D_2O, respectively. The S = O stretching Raman band in pure DMSO occurs at about 1045 cm^{-1}. With increasing amounts of water, the position of this band gradually shifts to lower frequencies until, in the 10% v/v aqueous solution, it occurs at approx. 1014 cm^{-1}. There were also changes in the O-D bands of water when present with different proportions

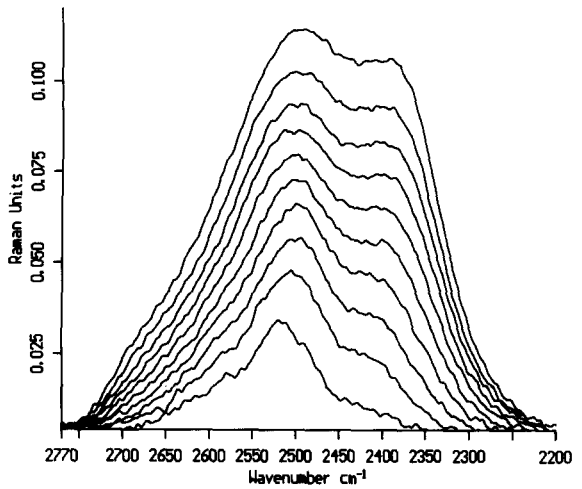

Fig. 10. FT Raman spectra of DMSO-water mixtures in the O-D stretching region of deuterium oxide in concentrations ranging from 10% v/v (bottom trace) to pure (top trace) at intervals of 10% v/v.

of DMSO. Both of these phenomena correlate with results from other studies (Scherer et al., 1973; Bertoluzza et al., 1979).

The nature of DMSO-water binary mixtures has been the subject of many investigations and it is generally agreed that at high concentrations, DMSO breaks up the structure of water. It is likely that the structural effects of DMSO on water present in biological systems can help explain its various biological properties. The obvious site for such interactions in the skin is at the polar head group regions of the lipids, and with keratin where water is present. It is worth noting that in the skin, similar shifts were observed in the S = O stretching mode of DMSO as those observed when it was mixed with water. Results from small angle X-ray scattering (SAXS) studies have shown that the repeat distance between lipid lamellae found in untreated human stratum corneum remained unchanged upon treatment with water, indicating that no swelling between the lipid bilayers occurred and therefore water did not intercalate between the bilayers (Bouwstra et al., 1991). This implies that water in extensively hydrated skin is mainly associated with the keratin component of the corneocytes. In the native form, the conformational integrity of a protein is dependent upon bound water which forms a hydration sheath. DMSO may substitute or displace this bound water and in so doing alter the protein conformation as observed in the present study.

However, these protein structural changes cannot fully explain the actions of DMSO as at concentrations similar to those at which it is known to enhance drug flux, DMSO was shown to affect stratum corneum lipids and the intercellular domain is presumed to be the main site of the resistance to solute transport for most drugs. In addition, DMSO may promote the partitioning of lipophilic drugs into the stratum corneum.

In conclusion, therefore, DMSO appears to absorb into the corneocytes whose keratin conformation tends to alter from an α-helix to β-sheets. In the lipid domains, DMSO appears to disturb the multilamellar lipid bilayers by causing conformational changes from an all *trans* gel phase to a *trans-gauche* liquid crystalline phase. Overall, the action of DMSO on the keratin and lipids thus results in looser or more permeable structures which are presumably responsible at least in part for the observed increases in the flux of very many drugs following DMSO treatment.

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