Biochimica et Biophysica Acta, 1153 (1993) 289–298
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BBAMEM 76122

Enhanced permeation and stratum corneum structural alterations in the presence of dithiothreitol

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(Received 2 April 1993)
(Revised manuscript received 22 July 1993)

Key words: Stratum corneum; Percutaneous absorption; Absorption; Disulfide crosslinking

Stratum corneum protein biochemical and biophysical structural contributions to the barrier properties of human epidermis were determined in the presence of the reducing agent dithiothreitol (DTT). Mannitol and sucrose permeation through human epidermis in the presence of 0 to 50 mM DTT in PBS (pH 7.4) was measured in symmetric, side-by-side diffusion cells (32°C). DTT enhancement ratios, $K_{P(DTT)}/K_{P(PBS)}$, ranging from 1.6 to 32, were dependent on skin donor and DTT concentrations. DTT did not alter stratum corneum uptake of mannitol or sucrose nor mannitol solubility in DTT/PBS solutions. Stratum corneum biophysical structure was ascertained by FTIR in solvent replacement experiments. DTT-induced protein conformational alterations were apparent in the emergence of an Amide I band near 1615 cm^{-1}, which is generally associated with β-sheet-like conformers. Therefore, DTT alters stratum corneum biophysical structure through interactions with proteins. After exposure of stratum corneum protein sheets to DTT/PBS solutions, the free thiol concentration increased from < 1 nmol SH/mg protein sheet to approx. 130 nmol/mg. The enhanced permeation which increased with increasing concentrations of DTT, was associated with diffusion mechanisms involving the cornified cells of the stratum corneum. These results indicate that corneocyte protein integrity does contribute to barrier function of the skin and influences the transport of polar solutes.

Introduction

Percutaneous absorption of polar compounds is limited by transport through the outermost layer of the epidermis, a highly impermeable, multicomponent membrane known as the stratum corneum. The complex histological morphology of normal stratum corneum can be perceived as protein-rich domains or regions within the corneocyte cells surrounded by lipid-rich regions [1]. The keratinization and extensive crosslinking of proteins during stratum corneum maturation is thought to contribute to the barrier function of the stratum corneum by making the corneocytes practically impermeable to most diffusing solutes, thus forcing diffusion of topically applied compounds along primarily a lipophilic pathway [2]. Experimental studies have shown that the permeation of most compounds through the stratum corneum is highly dependent on their lipophilicity and molecular weight, suggesting a significant dependence on the barrier properties of the stratum corneum lipid matrix [3]. Very hydrophilic compounds (log(diethyl ether/water) < -2.0), however, do not show a significant correlation between lipophilicity and ability to permeate the stratum corneum [4]. Percutaneous transport of these compounds may depend on the barrier properties of the keratinized cells as well as the lipid matrix, both of which contain hydrophilic regions favorable to the partitioning and diffusion of hydrophilic compounds.

To increase absorption rates through the skin, compounds may be applied which effectively decrease the barrier properties of the stratum corneum membrane, either through decreased membrane resistance or increased solute partitioning into the membrane. Most effective penetration enhancers interact with both stratum corneum lipids and proteins [5–7]. By selecting a penetration enhancer that interacts specifically with stratum corneum proteins through the reduction of protein crosslinks, the contributions of such crosslinks to polar compound transport may be determined.

Stratum corneum proteins are noted for their insolubility, which results from extensive crosslinking of both cell envelope and intracellular proteins [8–11]. The cell envelope, in particular, is stabilized by a

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considerable number of isopeptide crosslinks and disulfide bridges [9,10]. The intracellular stratum corneum proteins appear to contain only modest amounts of disulfide bridges. Cell envelope proteins are reported to contain approx. 4% cystine [10,12], whereas epidermal keratin proteins contain less than 2% cystine [13,14]. The high degree of crosslinking between stratum corneum proteins may be responsible for the apparent impermeability of the corneocytes to the majority of solutes.

The importance of crosslinking density in synthetic membranes to diffusion mechanisms has been studied extensively. Increased crosslinking density within amorphous polymer networks leads to a gradual reduction of solute diffusion through the network. With sufficiently high crosslinking density, the solute may be completely excluded [15]. If polar compound permeation through stratum corneum were capable of accessing intracellular pathways, then decreased crosslinking density within the cells may increase intracellular free volume regions available for diffusion. Thus, increased diffusion through the corneocytes (transcellular transport) may result from either (1) a decrease in crosslinking density at the cell envelope, thereby allowing access to the cells, or (2) a decrease in crosslinking density within the intracellular keratins and matrix proteins, thereby increasing diffusivity through the cellular regions. Hence, changes in protein crosslinking density could be expected to contribute to altered barrier function of the stratum corneum.

To determine the contributions of intracellular protein crosslinking density to polar compound diffusion mechanisms, the disulfide reducing agent, dithiothreitol (DTT), was selected. DTT efficiently reduces protein disulfides to the free thiol form [16] and is not expected to significantly alter the biophysical structure of the stratum corneum lipid matrix. The ability of DTT and a second reducing agent, ascorbic acid, to increase transport of an ionic compound, diclofenac, through rodent skin has been evaluated [17]. Although increases in permeation rates were found to correlate with increased epidermal free thiol concentrations, increases in free thiols within the rate-limiting stratum corneum membrane were not determined independently. Confounding factors, such as epidermal free thiols, reoxidation of free thiols, and residual DTT free thiols, were apparently not taken into consideration. Changes in stratum corneum biophysical structure, which may be brought about by the change in protein crosslinking density, were also not examined in that study.

The influence of DTT-induced reduction of protein disulfides on the permeation of two hydrophilic compounds, mannitol and sucrose, across human epidermis was studied. The thermodynamic parameters of membrane partitioning and permeant solubility in bulk solution were monitored to determine their possible contributions to DTT-enhanced diffusion. Identification of altered protein conformation as well as confirmation of the reduction of stratum corneum protein disulfide crosslinks were achieved through concurrent biophysical and biochemical analyses.

Materials and Methods

Skin preparation

Skin bank human skin, female abdomen and back, was donated by Ciba-Geigy, Corporation. The skin bank skin, stored frozen at −70°C, was thawed in 25°C saline 1 to 2 h prior to use. Human epidermis used in permeation studies was isolated by heat separation in 60°C saline for 2.0 min to remove the dermis, leaving the stratum corneum and epidermal layers intact. Stratum corneum sheets were isolated from heat-separated epidermis by incubation in a 0.5% solution of trypsin (from porcine pancreas, Type IX Sigma, St. Louis, MO) in PBS (pH 8.0) for 4 h at 37°C. After rinsing the stratum corneum sheets repeatedly with distilled water to remove trypsin and excess epidermal cells, they were vacuum dried (10^{-4} Torr) at room temperature and stored desiccated over Drierite (Aldrich, Milwaukee, WI).

Permeation experiments

Diffusion of the polar compounds mannitol and sucrose through human epidermis from five skin donors was determined in the presence of 0, 12.5, 25, or 50 mM dithiothreitol (DTT, Sigma, St. Louis, MO) in PBS (0.05 M, pH 7.4, 32°C). Using side-by-side diffusion cells, two cells per solution were assembled for each of the five skin donors ($n = 30$). Epidermis was mounted between the half cells with the stratum corneum facing the donor chamber.

An initial test of membrane integrity, the tilt test [18], involved adding 1 ml of DTT/PBS solution to the donor chamber. Accumulation of solution in the receiver chamber over 30 to 60 min was indicative of a damaged membrane. After testing membrane integrity, both the donor and receiver chambers were filled with 2.0 ml of DTT/PBS solution (with 0.01% gentamicin sulfate solution, Sigma, to inhibit bacterial growth). A preliminary spike (2–3 μCi) of either mannitol (L-[1(n)-^{3}H]mannitol, 30 Ci/mmol, New England Nuclear, Boston, MA) or sucrose ([U-^{14}C]sucrose, 0.363 Ci/mmol, ICN Biochemicals, Fullerton, CA) was added to the donor solution for a second check of membrane integrity. The minimal polar solute conctration used in testing for membrane integrity is insufficient to drive permeation across an intract stratum corneum membrane; therefore, detectable flux of the radiolabel across the epidermal membrane within 1–2 h was indicative of a damaged membrane. For an intact membrane, detectable levels of these radiolabeled per-

meants are attained only after 4 h with a donor spike of 30–50 μCi due to the inherently low permeation rates of polar compounds across skin. When detected, damaged membranes were replaced and again tested for integrity.

After confirming membrane integrity, each intact diffusion cell was spiked with both [^{3}H]mannitol and [^{14}C]sucrose (30–50 μCi each). Samples were withdrawn from the receiver chambers (200 μl) every 3 to 9 h over a 50 h period. The receiver volume was replenished with fresh solution after each sample was taken. Donor samples were taken periodically throughout the experiment without replacement. Mannitol and sucrose concentrations were determined by liquid scintillation counting (LS 1801, Beckman Instruments, Fullerton, CA).

Permeability coefficients, K_P, were calculated using the equation:

$$K_P = V_R(dC_R/dt)/A\Delta C$$

where V_R is the receiver chamber volume, dC_R/dt is the change in receiver concentration with time, A is the diffusional area of the cell (average = 0.8 cm^2) and ΔC is the donor-to-receiver concentration gradient. Linear regression of receiver sample data, collected after attainment of steady-state flux, was used to calculate dC_R/dt. The dilution effect of sample replacement in the receiver chamber was accounted for in the calculations. Permeation rates for mannitol and sucrose were also determined independently to assure the independence of permeant transport.

The final test for assessing membrane integrity involved determining whether the membrane in each diffusion cell was size-selective for mannitol (mol. wt. 182.17) and sucrose (mol. wt. 342.3). Mannitol should diffuse through the skin at a higher rate than sucrose based on molecular weight considerations [19,20]. Therefore, by simultaneously determining the flux of mannitol and sucrose the integrity of each epidermal membrane could be monitored. If both mannitol and sucrose penetrated the skin at nearly the same rate, the resulting permeability coefficients were high and the skin was then classified as damaged. That is, when $K_{P(mannitol)}/K_{(sucrose)} \leq 2.0$ the membrane was considered damaged and when $K_{P(mannitol)}/K_{(sucrose)} > 2.0$ the membrane was considered intact. Only a limited number of the membranes did not meet this third criterion for an intact membrane. A complete set of permeability coefficients, i.e., two cells for each DTT concentration, was not available from each skin donor due to membrane damage as detected by the above criteria.

Solubility experiments

Solubility of mannitol was determined from saturated solutions containing 0, 12.5, 25, or 50 mM DTT in PBS. An excess of unlabeled mannitol (Sigma) was added to 5.0 ml of freshly-prepared DTT solutions and stirred 24 h at room temperature (27–28°C) to saturate. After centrifugation, the supernatant was filtered and diluted with H_2O prior to high performance liquid chromatographic (HPLC) analysis. Mannitol concentrations were determined relative to a mannitol standard curve using anion exchange HPLC on a Dionex BioLC (Dionex, Sunnyvale, CA) fitted with a pulsed electrochemical detector. The retention time of mannitol was 3.35 min using a Dionex Carbo Pak PA1 column with a flow rate of 1.0 ml/min and a mobile phase of 15 mM NaOH.

Membrane uptake experiments

Stratum corneum disks punched from intact stratum corneum sheets with a cork bore (approx. 1.3 cm diameter) were hydrated 1 h in solutions of 0, 12.5, 25, or 50 mM DTT in PBS. The hydrated stratum corneum disk thickness, measured with a lightwave micrometer (1.0 μm accuracy, Van Keuren, Waterford, MA), and surface area, determined from the average disk diameter, measured to the nearest 0.1 cm, were used to calculate an estimated stratum corneum volume. The disks were then incubated at 32°C for 48 h in 3 ml of the corresponding DTT/PBS solution (containing 0.01% (w/v) gentamicin sulfate) spiked with [^{3}H]mannitol and [^{14}C]sucrose. After equilibration, stratum corneum disks were removed from solution, blotted dry, rinsed and blotted dry twice in fresh water, and vacuum dried 24–48 h. The samples were weighed, digested with Soluene 350 tissue solubilizer (100 μl/mg tissue) at 37°C, and submitted for liquid scintillation counting.

Stratum corneum uptake, K_m, of mannitol and sucrose was determined by calculation of apparent partition coefficients, K_m, according to the equation:

$$K_m = C_{\text{stratum corneum}} / C_{\text{donor solution}}$$

where concentrations are given in mmol/ml. Solute concentration in the stratum corneum was determined by dividing the amount of radiolabeled drug in the stratum corneum by the hydrated stratum corneum volume. Stratum corneum uptake of mannitol and sucrose was determined at least once for each skin donor per DTT/PBS solution.

Spectroscopy experiments

Infrared absorbance spectra were obtained under iso-thermal conditions in the presence of deuterated water (D_2O, 99.9%, Cambridge Isotope Laboratories, Woburn, MA) and 12.5, 25, and 50 mM DTT in D_2O solutions to mimic permeation experiment conditions. Stratum corneum sheets were hydrated overnight in D_2O vapor and then equilibrated in D_2O for 2–3 h. The D_2O-hydrated stratum corneum sheet was sealed

in an excess of D_2O between two ZnSe crystals and placed in a Harrick flow-through transmission sample holder (Harrick Scientific, Ossining, NY) equipped with two solvent ports. Spectra were obtained with an FTIR spectrometer (Digilab FTS 20/80) equipped with a liquid nitrogen cooled mercury-cadmium-telluride narrow band detector at 2 cm^{-1} resolution (1024 scans, triangular apodization, 0.1 noise level, and zero fill factor of 4) under nitrogen purge. After obtaining an initial absorbance spectrum of the stratum corneum sheet in D_2O, the D_2O in the sample cell was gradually replaced with 2.0 ml of fresh D_2O or DTT in D_2O over a 2.5 h period. After solvent replacement, FTIR absorbance spectra of the stratum corneum sheet were obtained hourly for up to 9 h to monitor solvent-induced changes in stratum corneum structure. DTT did not absorb in the infrared regions of interest.

Solvent-induced alterations in stratum corneum biophysical structure were monitored by following changes in band shape. Band shape changes were determined through absorbance difference spectra (SpectraCalc software). In solvent replacement experiments each sample is its own control; therefore, detection of minor structural alterations was possible due to elimination of sample-to-sample and intrasample variability.

Absorbance difference spectra were calculated for each solvent replacement experiment:

Difference spectrum = Sample spectrum(DTT)

$-$ Sample spectrum(D_2O)

Negative bands in a difference spectrum indicate a decrease in the conformational population of a given molecular group as a result of perturbation, while positive bands indicate an increase in population.

Free thiol assay

The actual reduction of intracellular disulfide crosslinks was confirmed by colorimetric assay of the free thiols [21] generated within stratum corneum proteins before and after reaction with DTT. The reactions associated with free thiol formation by DTT and free thiol assay by reaction with 2,2′-dithiodipyridine (DTP) are given in Fig. 1. A significant advantage of DTT disulfide reduction over other disulfide reducing agents, such as β-mercaptoethanol, is that the reaction proceeds through the mixed protein-DTT disulfide to the free thiol. DTP is then used to assay free thiols generated by DTT reduction. A quantitative release of the thione occurs in the presence of free thiols.

Within a stratum corneum sheet, the quantity of proteins is dependent on the amount of lipid present, which varies from donor to donor [22]. Therefore, to obtain a constant, known protein weight for the analysis of intracellular disulfide reduction, delipidized stratum corneum sheets were prepared as described previously [23] using a modification of the Bligh and Dyer [24] lipid extraction method. Desiccated stratum corneum sheets were extracted twice with a solution of chloroform/methanol/water (1:2:0.8, v/v) for 24 h each, at room temperature with rotation. The sheets

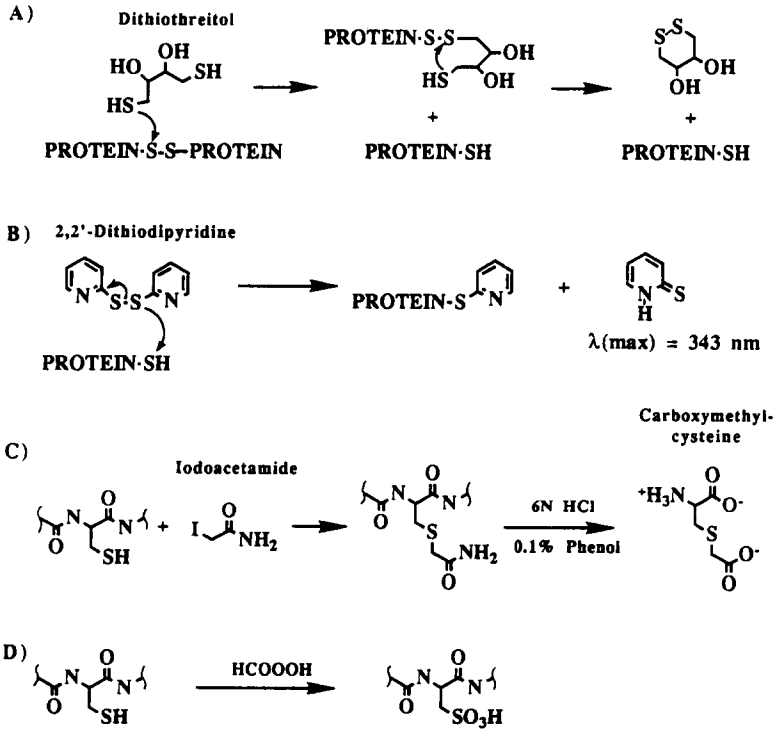

Fig. 1. Formation of protein free thiols through reduction of disulfide bonds by DTT (A), followed by reaction with DTP (B) or iodoacetamide (C). Oxidation of free thiols and disulfides (D) will prevent reactions A–C.

were then further extracted with methanol for a period of 14 days with rotation to remove all extractable stratum corneum lipids. The delipidized sheets were vacuum dried and stored desiccated over Drierite.

Stratum corneum protein sheets (weighed to the nearest 10 μg on a Mettler M3 Microbalance, Mettler, Hightown, NJ) were equilibrated in solutions of 0, 12.5, 25, or 50 mM DTT in PBS on a sample rotator (Cole Parmer Instrument, Chicago, IL). To approximate conditions of the diffusion experiments, 4 ml solvent were added for every 1.0 mg stratum corneum or 0.67 mg stratum corneum protein. After 68 h, the protein sheets were blotted dry and placed in a rinse solution (30% ethanol in 0.05 M sodium acetate buffer (pH 4)) on a sample rotator to inhibit further disulfide exchange and wash out excess DTT. The protein sheets were changed to a fresh rinse solution every 24 h for 7 days for maximal removal of unbound DTT.

To quantify the extent of DTT binding, stratum corneum protein sheets were first treated with performic acid to oxidize both free thiols and disulfides to the sulfonic acid (Fig. 1) [25]. The oxidized sheets were then treated with DTT and rinsed as described above. Free thiols detected within these oxidized stratum corneum samples were ascribed to bound DTT that was not removed during the rinse sequence.

For analysis of free thiol content, stratum corneum protein samples (controls, DTT-treated, and oxidized then DTT-treated) were reacted with DTP as described by Grassetti and Murray [21]. The stratum corneum protein sheets were added to a solution containing 2 ml 8 M urea (in 0.05 M sodium acetate buffer (pH 4) with 2 mM EDTA) and 0.4 ml DTP stock solution (saturated). The denaturing agent 8 M urea is recommended to unfold the stratum corneum proteins and allow DTP access to all free thiols within the sample. Addition of EDTA for complexation of ions that may auto-oxidize DTP is also recommended [26]. The DTP stock solution was prepared by dissolving >20 mg DTP in 50 ml deionized water. The stock solution was filtered prior to use. After 4 h, UV absorbance was measured at 343 nm on a PE Lambda 19 UV/VIS/NIR Spectrometer (Perkin-Elmer, Norwalk, CT) to determine the amount of thiopyridine released. Literature values for the molar absorption coefficient, $\epsilon(343\text{nm}) = 7060\ \text{M}^{-1}\text{cm}^{-1}$, were used to calculate the amount of free thiols per mg stratum corneum proteins. Glutathione was used as a free thiol standard to confirm the molar absorption coefficient of the thione and linearity of response to free thiols present as they react with DTP.

A second method for free thiol analysis involved the use of iodoacetamide to form the alkylated cysteine (Fig. 1C), which is detectable by amino acid analysis. To augment results of the DTP assay and determine whether an excess of DTT was used in these experiments, a second set of stratum corneum protein sheets was first treated with 25 mM DTT as described previously. After rinsing the samples at low pH for 72 h, the samples were reacted with a 10-fold excess of iodoacetamide (Sigma). Prior to amino acid analysis, the protein sheets were hydrolyzed 24 h in 6 M HCl with 0.1% phenol in vacuo at 110°C. Amino acid analysis was performed by HPLC on a 6300 Beckman analyzer with postcolumn ninhydrin derivatization according to the manufacturer's specifications. Free thiol content, measured as mole percent carboxymethylcysteine, was then determined relative to the stratum corneum protein sample weight.

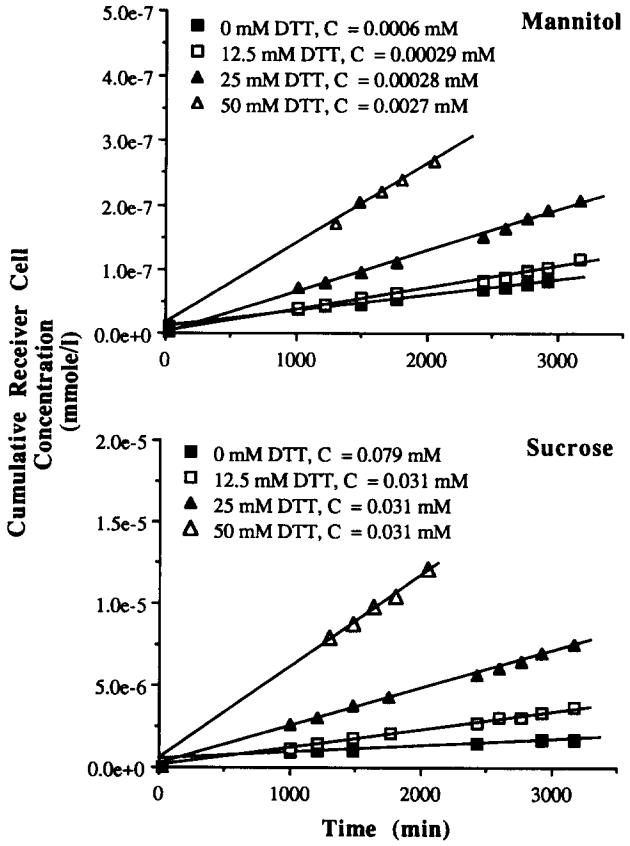

Fig. 2. Representative plots of receiver cell concentration versus time for mannitol and sucrose diffusion through human epidermis in the presence of 0 to 50 mM DTT. Donor concentrations were variable as indicated.

Results

Permeation experiments

The apparent steady-state flux of both mannitol and sucrose through human epidermis was generally achieved in less than 4 h and maintained for over 50 h (Fig. 2) during the diffusion experiments. For each of the five skin sources studied, the presence of DTT enhanced skin permeability to mannitol and sucrose. Overall, average permeability coefficients of both man-

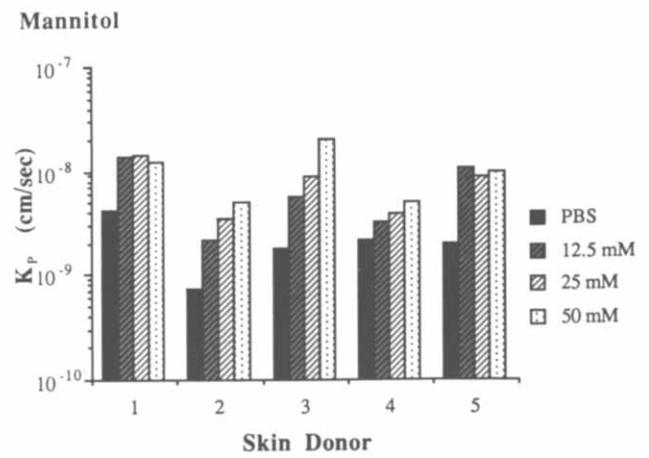

Fig. 3. Mannitol permeability coefficients through human epidermis in the presence of 0, 12.5, 25, and 50 mM DTT/PBS solutions ($n = 1$–2 for each K_P reported). Note the donor-dependence for increases in permeability coefficients.

nitol and sucrose increased with increased DTT concentrations (Figs. 3 and 4). However, in two of the skin sources, mannitol and sucrose permeation appeared to reach a limiting value at concentrations above 12.5 mM DTT. These results suggest that DTT-induced increases in both mannitol and sucrose permeation are dependent on skin donor. The cysteine content of stratum corneum proteins is inherently low [13,14,27, 28]; therefore, any variation in cysteine content from donor to donor may influence the ability of DTT to enhance permeation.

The degree of permeation enhancement for each donor was assessed through enhancement ratios, ER:

$$ER = K_{P(DTT)} / K_{P(PBS)}$$

where $K_{P(DTT)}$ is the average permeability coefficient in the presence of DTT (12.5, 25, or 50 mM) and

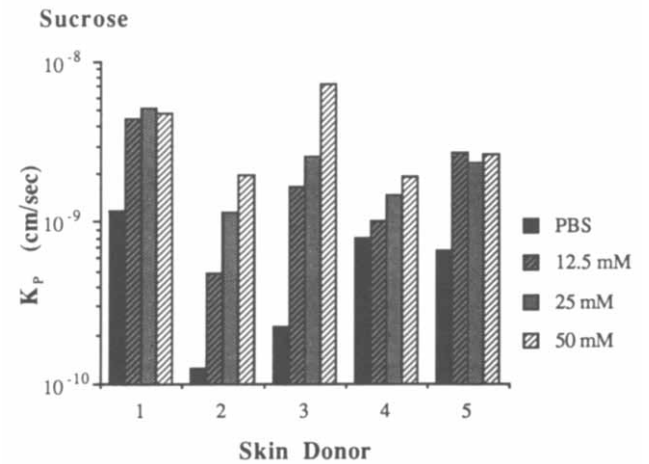

Fig. 4. Sucrose permeability coefficients through human epidermis in the presence of 0, 12.5, 25, and 50 mM DTT/PBS solutions ($n = 1$–2 for each K_P reported). Note the donor-dependence for increases in permeability coefficients.

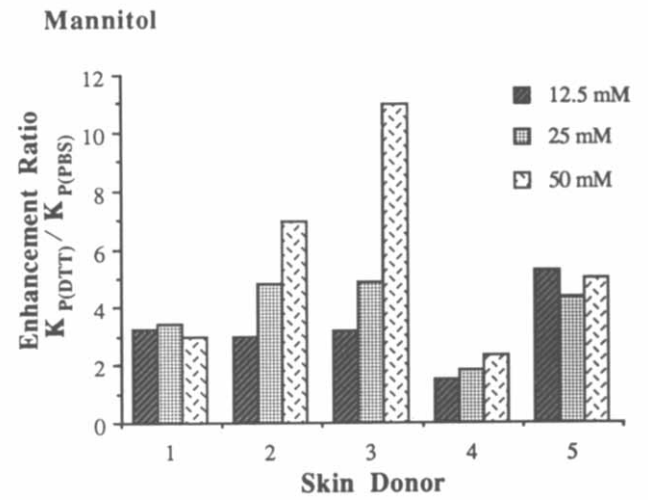

Fig. 5. Mannitol enhancement ratios for permeation through human epidermis in the presence of 12.5, 25, and 50 mM DTT in PBS.

$K_{P(PBS)}$ is the average permeability coefficient in PBS. Again, the degree of enhancement was dependent on the skin source and the concentration of DTT employed (Figs. 5 and 6). In general, mannitol permeation rates were increased 3- to 6-fold in the presence of DTT, while sucrose permeation rates were increased 3- to 10-fold. The size-selectivity of the epidermal membranes was maintained despite the decreased cross-linking density, suggesting size selectivity for polar solutes to be mediated by other stratum corneum components.

Solubility experiments

Mannitol solubility was not altered in the presence of DTT concentrations up to 50 mM. Mannitol concentrations at saturation were 224 ± 14, 222 ± 19, 235 ± 20, and 230 ± 12 mg/ml (mean ± S.D., $n = 4$) in solutions of PBS and 12.5, 25, or 50 mM DTT in PBS, respectively. Therefore, DTT-enhanced permeation is not

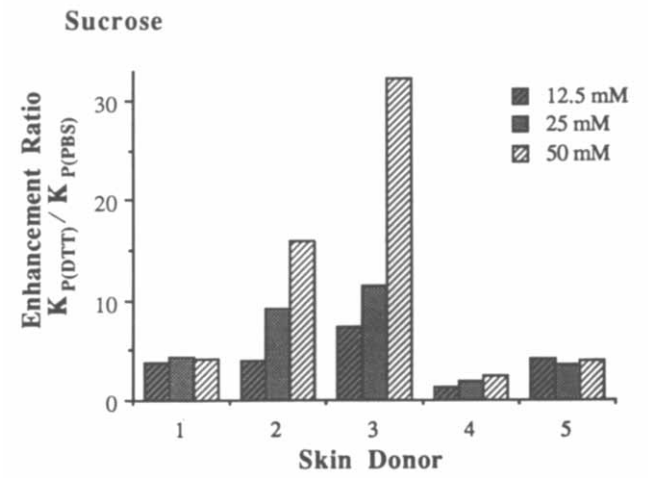

Fig. 6. Sucrose enhancement ratios for permeation through human epidermis in the presence of 12.5, 25, and 50 mM DTT in PBS.

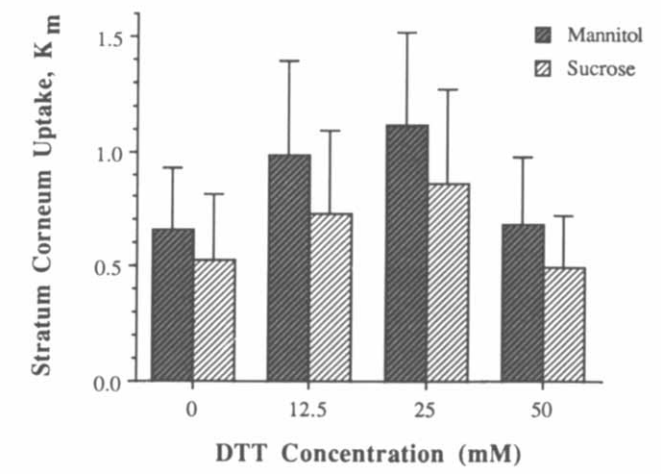

Fig. 7. Stratum corneum uptake of mannitol and sucrose in the presence of 0 to 50 mM DTT/PBS. The uptake is reported as $K_m = C_{\text{stratum corneum}} / C_{\text{donor solution}}$ (C = mmol/l), mean ± S.D.

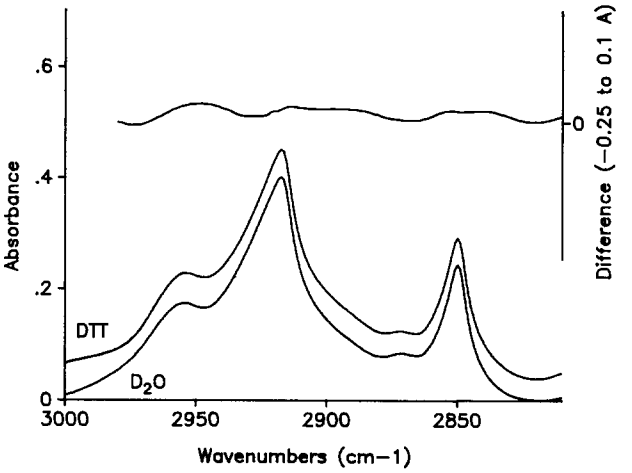

Fig. 8. Absorbance changes in stratum corneum C-H stretching bands during DTT solvent replacement. Representative spectra in the presence of 25 mM DTT and D_2O and the resultant difference spectrum are presented.

associated with changes in thermodynamic solution activity of mannitol in DTT/PBS solutions.

Membrane uptake experiments

Mannitol and sucrose uptake into human stratum corneum is reported as volume-based apparent partition coefficients in Fig. 7. For all of the skin sources tested, mannitol and sucrose uptake reached a maximum near 25 mM DTT and then decreased for 50 mM DTT solutions. The reported values for apparent partition coefficients, K_m approx. 0.5 to 1.0, suggest that mannitol and sucrose partition into the same stratum corneum regions as water, thus providing further support for diffusion through the corneocytes as well as the lipid matrix. Differences in uptake with DTT concentration were not always significant. The lack of significant change in membrane uptake of mannitol or sucrose in the presence of DTT does not reflect the mechanism for the noted 3- to 6-fold increased permeation of mannitol and sucrose in the presence of DTT.

Spectroscopy experiments

The order, mobility and conformation of the stratum corneum lipid alkyl chains was not significantly affected by D_2O solvent replacement with DTT. No significant change in band position or bandwidth was noted which would suggest a change in alkyl chain conformation or mobility [29,30]. Difference spectra resulted in essentially flat baselines in the C-H stretching region, confirming the absence of alterations within the stratum corneum lipid alkyl chains (Fig. 8).

Dithiothreitol-induced conformational changes within stratum corneum proteins were often apparent in the infrared spectra at concentrations of 25 and 50 mM, though not at 12.5 mM. Although there were no detectable shifts in band position or changes in bandwidth, emergence of a weak Amide I band near 1615 cm^{-1} was apparent in the difference spectra (Fig. 9). Amide I absorbances in the 1621 to 1640 cm^{-1} region are generally associated with β-sheet-like conformations [31].

Biochemical analysis of free thiol formation

The reduction of intracellular disulfide crosslinks within the rate-limiting stratum corneum membrane may be determined through biochemical analysis of free thiol formation. It is essential in such analyses that the increase in free thiols be attributed solely to disulfide reduction within the rate-limiting membrane. In the permeation studies, both stratum corneum and epidermal cells were exposed to and capable of reacting with the DTT solution. Thus, the concentration of DTT available for reduction of stratum corneum protein disulfide crosslinks during the diffusion experi-

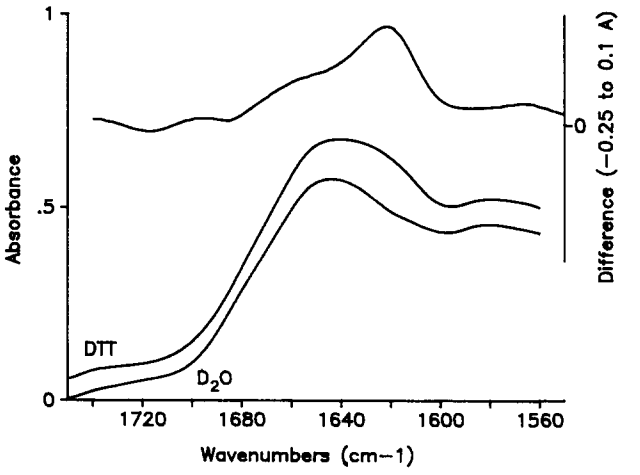

Fig. 9. Absorbance changes in the Amide I band during DTT solvent replacement. Spectra of stratum corneum in 25 mM DTT and D_2O and the resultant difference spectrum are presented.

TABLE I

Free thiol content of stratum corneum proteins after treatment with DTT as determined by DTP assay

Binding of DTT is given by free thiol content after oxidation.

DTT concn. (mM)	nmol SH/mg protein
0	0.23 (0.23)
12.5	133 (33)
25	141 (14)
50	143 (11)
25	(oxidized) 11.7 (2.2)

Free thiol content reported as mean (S.D.).

ment is diminished by the presence of epidermal protein disulfide crosslinks. In order to attribute disulfide crosslink reduction to corneocyte proteins, the stratum corneum sheets were first isolated from the epidermis and then the intercellular lipid matrix was removed through solvent extraction. By first extracting stratum corneum lipids, the free thiol formation was determined relative to a known corneocyte protein weight. However, such extraction may increase the partitioning of DTT into the stratum corneum proteins, resulting in somewhat higher values for free thiol formation than would occur within untreated stratum corneum. By treating every 1.0 mg of stratum corneum (or 0.67 mg of protein sheet) with 4 ml DTT solution, the effective exposure of stratum corneum proteins to DTT solution was matched to that of the diffusion experiments in which 1 mg of whole epidermis (approx. 1/2 of which is stratum corneum weight) is exposed to 4 ml DTT solution.

For protein sheets treated with PBS, the inherent free thiol content was found to be < 1 nmol/mg (Table I). After treatment with DTT (12.5, 25, or 50 mM), the free thiol content increased to approx. 140 ± 10 nmol/mg. Protein sheets treated first with performic acid for oxidation of thiols and then with DTT gave an average free thiol content of 12 nmol/mg. Therefore, of the free thiol content measured, approx. 12 nmol/mg could be ascribed to bound DTT. The remaining free thiols (approx. 130 nmol/mg) are due to protein free thiols. The increase in free thiols was not DTT concentration dependent above 12.5 mM ($P > 0.05$), suggesting that DTT concentrations were excessive of the disulfide bond concentration in the stratum corneum samples. Amino acid analysis of carboxymethylcysteine gave free thiol contents ranging from 160 to 400 nmol/mg stratum corneum protein. Therefore, DTT exposure is > 400-fold in excess of the free thiol content.

Discussion

Dithiothreitol provided modest increases in permeability relative to other chemical permeation enhancers. For example, DTT-enhanced permeation of mannitol averaged less than 10-fold, whereas application of propylene glycol with 2% Azone or 15% decylmethylsulphoxide, 2-propanol, or *N*-methylformamide results in 600- to 1200-fold increases in mannitol flux through human skin [5]. Therefore, DTT-induced perturbation of stratum corneum biochemical and biophysical structure and/or polar compound uptake within diffusion-accessible regions is limited. The degree of permeation enhancement generally increased with increasing concentrations of DTT and was found to be highly dependent on the skin source (Figs. 5 and 6). Changes in stratum corneum uptake of the permeants did not account for the increased permeation. Furthermore, mannitol solution activity was not altered by DTT.

Similar results were obtained by Nishihata et al. who have examined the permeation of the ionic compound diclofenac through rat dorsal skin [17]. Diclofenac permeation rates gradually increased for DTT concentrations up to 12.5 mM and remained constant at 25 mM DTT to give a maximum 20-fold enhancement. It was also shown that the stratum corneum/epidermal free thiol content, relative to soluble proteins within the sample, increased [17]. Disulfide crosslinking density and localization may vary between species, which may be reflected in the greater enhancement noted for rodent skin compared to human epidermis. The physicochemical properties of ionic permeants may also influence the ability of DTT to enhance their permeation above that of neutral compounds such as mannitol.

Stratum corneum biophysical structure is suggested to play a key role in barrier function of the skin [32–34]. DTT treatment (> 12.5 mM) often resulted in conformational changes within the stratum corneum proteins yielding an increase in β-sheet-like structures, i.e., emergence of an Amide I component near 1615 cm^{-1}. The lack of detectable alterations in absorbance bands arising from alkyl chains suggests stratum corneum lipids were not significantly affected by DTT solutions. In D_2O spectra of concanavalin A and β-lactoglobulin B, an Amide I component absorbing near 1624 cm^{-1} has been associated with β-type structures located in more 'exposed' regions of the β-sheets [35,36]. Therefore, formation of an Amide I component near 1615 cm^{-1} may also be associated with formation of β-sheet-like conformations within the more exposed regions of stratum corneum proteins. Before enhancement mechanisms can be evaluated on the basis of FTIR analysis alone, additional investigations correlating stratum corneum biophysical structure to barrier function of skin must be completed. Therefore, additional experiments were performed to assess DTT-induced biochemical changes in the stratum corneum membrane.

DTT effected a significant reduction of disulfide crosslinks within stratum corneum protein sheets. Disulfide crosslinks are present in both cell envelope and intracellular proteins [10–14,37]. In addition to disulfide crosslinks, the cell envelopes are further stabilized by ϵ-(γ-glutamyl)lysine crosslinks [9]. These crosslinks involve approx. 18% of the lysine residues found in samples of human plantar callus stratum corneum [9]. Physical entanglement of stratum corneum proteins may also effectively function as crosslinks and contribute to decreased corneocyte permeability [38]. A decrease in the number of protein disulfide crosslinks would only affect permeation if such a reduction altered permeant-accessible regions of the stratum corneum membrane. Even greater permeation enhancement may be achieved through enzymatic reduction of the iso-peptide bonds or disentanglement of protein strands.

Summary

By specifically reducing disulfide bonds within the epidermis, the contributions of reduced protein disulfide crosslinks and the concurrent biophysical structural alterations to enhanced permeation have been evaluated. Permeation of mannitol and sucrose was enhanced 3- to 30-fold depending on the concentration of DTT employed and the skin source. This was accompanied by alterations within the stratum corneum of secondary protein structure and no apparent alteration of lipid alkyl chain conformation or mobility. Stratum corneum protein disulfide crosslinks were reduced in the presence of DTT, providing over a 100-fold increase in free thiol content. Overall, the dramatic decrease in disulfide crosslinking density was able to significantly affect permeation through human skin. These investigations provide the first definitive data to demonstrate that, in the presence of DTT, polar solutes permeate the stratum corneum via pathway(s) that include the stratum corneum proteins. The mechanisms of DTT-enhanced permeation may involve reduction of disulfide crosslinks within existing diffusion pathways. Although unlikely, DTT may also open new pathways for diffusion through the reduction of disulfide crosslinks in regions previously inaccessible. The majority of stratum corneum proteins are found primarily within the corneocytes, with a high concentration of sulfur-rich proteins in the cell envelope. Thus, corneocyte protein integrity does contribute to barrier function of the skin and transport of polar solutes.

Acknowledgements

The authors are grateful to T. Kurihara-Bergstrom, B. Berner and L.K. Pershing for helpful discussions. Human skin samples were donated by Ciba-Geigy. This research was funded by Ciba-Geigy and NIH Grant HD R01-23000. C.Y.G. appreciates the support of the PMA Foundation through an Advanced Predoctoral Fellowship.

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