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Percutaneous Transport in Relation to Stratum Corneum Structure and Lipid Composition

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Despite the acknowledged importance of the stratum corneum in limiting water loss and in controlling skin permeability, the basis for these functions remains unknown. To pinpoint those factor(s) of importance for cutaneous barrier function, we correlated the thickness, number of cell layers, and lipid composition of leg vs. abdominal stratum corneum samples with penetration of ^{3}H-water and ^{14}C-salicylic acid across the same tissue sample. Viable upper epidermal sheets were obtained by incubating fresh autopsy or amputation full-thickness skin with staphylococcal exfoliatin. Each sheet was divided into 3 portions. The first piece was mounted in a diffusion cell for penetration studies. The second stratum corneum sample was frozen sectioned, stained with the fluorochrome, ANS, and measured with a micrometer eyepiece. The 3rd piece was pooled with other leg (n = 6) and abdomen (n = 15) specimens for determination of lipid weight percent. In all cases, leg stratum corneum was ≅ 2 times more permeable than abdominal stratum corneum to water and slightly more permeable to salicylic acid, as well. Penetration of both substances correlated inversely with lipid weight % of leg (mean = 3.0%) vs. abdomen (mean = 6.8%), but neither the penetration of water nor of salicylic acid was influenced by the number of cell layers or the thickness of the stratum corneum. We conclude that: differences in the thickness and the number of cell layers in the stratum corneum are insufficient to account for differences in percutaneous transport across leg and abdomen, and that total lipid concentration may be the critical factor governing skin permeability.

Although the importance of the stratum corneum for skin barrier function is generally acknowledged (reviewed in references 1 and 2), it was the physical chemical measurements of Monash and Blank [3] and Scheuplein [4], demonstrating that the entire stratum corneum functions as an effective barrier, that first alerted epidermal cell biologists that this layer might not simply be a loosely desquamating tissue. Subsequently, the elegant geometry and cohesive structure of this layer was demonstrated [5–7]. More recently, the heterogenous structure of the stratum corneum has been emphasized by standard ultrastructural [8–10], freeze-fracture [9, 11], histochemical [12, 13], and biochemical [13, 14] methods. Yet, few studies have attempted to link these new structural and chemical insights with percutaneous transport phenomena. The extreme sensitivity of the permeability barrier to damage by lipid solvents [15–18], and the selective penetration of most nonpolar materials across the stratum corneum (reviewed in references 1, 2) have long suggested that lipids are important determinants of skin penetration. In addition to lipids, several other stratum corneum structural parameters, including thickness [22], number of cell layers [22, 23], and geometric organization [6], are currently under consideration as determinants of stratum corneum permeability. However, none of these earlier studies have linked permeability phenomena directly with either stratum corneum structural parameters or lipid content and composition. In this study, we have correlated the *in vitro* penetration of water and salicylic acid across 2 different regions of the skin surface with both the lipid composition and the structure of the stratum corneum of the same samples. The results suggest that percutaneous penetration of both of these substances correlates with the lipid content by weight of the sample [24]. Although Fick's Law predicts that thickness of the sample should influence penetration inversely, we were unable to discover any relationship between absorption and either the thickness or the number of cell layers of the sample.

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METHODS

Source of Human Tissues

All skin samples were obtained from males. Abdomen samples were obtained from fresh autopsy specimens (less than 3 hr postmortem), and leg skin samples comprised fresh tissue from shins of patients undergoing amputation. In both instances, only skin that was grossly normal was utilized, and charts were reviewed to exclude the presence of co-existent skin diseases, such as psoriasis or eczema.

Preparation of Upper Epidermal Sheets

Both leg and abdominal skin specimens were cleaned of subcutaneous fat by blunt dissection and scraping. The specimens were then floated dermis-side downward in Waymouth's MB752/1 Medium containing L-glutamine, amphotericin B 25 mg/ml, penicillin 100 Units/ml, streptomycin 100 mg/ml (all from Grand Island Biological Corp., Grand Island, N.Y.), containing a partially purified [25] fraction of staphylococcal exfoliatin (10 mg/ml). Incubations were carried out for 4 hr at 37°C, following which homogeneous sheets of stratum corneum and stratum granulosum could then be peeled off from the underlying epidermis and dermis [25].

Sheets obtained in this way were then divided into 3 portions: The largest, most intact portion was frozen in a hydrated state at −70° until used for the permeability studies detailed below. An almost equal portion was treated with trypsin to remove underlying stratum granulosum [13] and extracted for the lipid biochemical studies detailed below, while several small fragments were snap-frozen in liquid nitrogen for frozen sectioning, fluorescence microscopy and the quantitative studies described below.

Diffusion Experiments

Pieces of epidermal sheets were removed from the freezer, thawed, spread on water, and examined under a dissecting microscope for tears or holes. Pilot studies demonstrated that frozen-thawed and freshly obtained sheets exhibited identical penetration characteristics. The sheets were then mounted in a diffusion cell with a diffusion area of 0.122 cm^2 and covered with a glass plate to maintain a constant state of hydration. All samples were run simultaneously in triplicate after allowing at least 1 hr for equilibration, except samples #178, 179, and 184, which provided enough material for only a single measurement.

Test Solutions

At the beginning of each experiment, the radioactive solutions were gently placed in the chamber on top of the sample, and the chamber

was again covered with the glass plate to maintain hydration. The aqueous test solution comprised ^3H water, synthesized from pure tritium (Oak Ridge National Laboratories) by oxidation, at a final concentration of 80 μCi/ml. The more nonpolar test solution consisted of ^{14}C salicylic acid (SA, AmershamSearle), diluted with unlabeled salicylic acid (Fischer Biochem.) to a final concentration of 5% in 95% propylene glycol (USP). Salicylic acid was chosen as the lipophilic permeant because of current interest in its derivatives as a new class of topical anti-inflammatory agents. Since the kinetics of absorption of salicylic acid remained constant during the experiment, it is unlikely that salicylic acid degraded the sample. Propylene glycol was chosen as the vehicle because it is a common constituent in many topical preparations. Although propylene glycol undoubtedly introduces a certain amount of artifact, some artifact would be expected from any vehicle. The final concentration of ^{14}C SA was 0.4 μCi/mg SA.

Distilled water flowed unidirectionally under the stratum granulosum side at a rate of 3.5 ml/hr and was collected every 2 hr in scintillation vials mounted in a fraction collector. The flow rate of 3.5 ml/hr was chosen because the volume of blood normally bathing a 0.122 cm^2 sample is approximately 35 μl. Thus, this flow rate guarantees replacement of the volume under skin about 100×/hr. All test and collecting solutions were maintained at 22°C. The outflow samples were counted in a Packard liquid scintillation spectrometer. The scintillation counting solution (10 ml), added to each 7.0 ml sample, consisted of Triton X-100, 6 vol and 7 vol of cocktail stock solution consisting of 2,5-diphenyloxazole (PPO, 18Gm) and 1,4-bis-2 (4-methl-5-phenyloxazolyl)-benzene (POPOP, 0.16 Gm) in toluene.

The data expressed in Tables I and II for penetration of water and salicylic acid represent the total amounts absorbed during the 24 hr test period. However, comparable results pertain at all time periods, since absorption followed the same general pattern (Fig 1). Under the conditions of this study, the amount of tracer remaining in the sample was negligible.

Lipid Extraction

The pooled stratum corneum sheets were extracted overnight at 25°C with redistilled chloroform:methanol:0.1 M KCl in distilled H_2O (1:2:0.8 vols, all solvents analytical grade, Mallinckrodt Inc., Paris, KY) in the manner of Bligh and Dyer [26], using a ratio of approximately 1 ml solvent/10–15 mg tissue. After overnight soaking the tissue was transferred to a ground glass homogenizer and extracted further with fresh solvent. After thorough homogenization, the suspension was agitated in a modified Burrel wrist-action shaker for an additional 30 min at room temperature. The centrifuged pellet (2, 000 rpm, × 10 min) was discarded. The extraction media were pooled and converted to a 2-phase system with equal volumes of chloroform and water (extraction medium: chloroform:0.1 M KCl in H_2O, 7.6:2:2 vols), again according to Bligh and Dyer [26], and the 2 phases were separated by centrifugation (2,000 rpm, × 10 min), and the upper aqueous phase was saved. The lower phase was washed again with upper phase of wash solution (4:4:3.6:chloroform:methanol:0.1 M KCl in H_2O). Both phases were saved separately. The collected upper phases were washed twice with equal volumes of chloroform. The chloroform washes were combined with the lower phases of the original extraction media and taken to dryness under dry nitrogen at 37°C. The dried chloroform-soluble lipids were then resuspended in absolute benzene and stored at −20°C.

Fractionation and Biochemical Analysis

Cholesterol sulfate was subfractionated from total lipid extract on silica gel G in chloroform:methanol:water (90:10:1) followed by petroleum ether: diethylether:acetic acid (70:60:1). Phospholipids and neutral lipids were scraped from the first plate and separated on thin-layer chromatograms (silica gel G, home-made with silica gel H from E. Merck, Darmstodf, Germany), utilizing chloroform:methanol:water: acetic acid (60:35:4.5:0.5, v/v/v/v) as the solvent for phospholipids, and petroleum ether:diethyl ether:acetic acid (80:20:1, v/v/v) for neutral lipids. The quantities of individual lipid fractions were calculated per weight of total recovered lipid. Lipids were visualized under UV light after spraying them with a 0.25% aqueous solution of 8-anilino-1-naphthalene sulfonic acid (ANS). Individual compounds were identified by biochemical analysis and/or co-chromatography against authentic standards, as described previously [12].

Determination of Stratum Corneum Thickness and Cell Layers

Four-micron frozen sections of stratum corneum were stained with 8-anilino-1-naphthalene sulfonic acid (1 mg/ml in PBS), and examined with a Leitz Ortholux II equipped for epifluorescence [12, 13]. This fluorochrome intensely stains the cell membranes of the stratum corneum, but not the stratum granulosum. All sections were coded, ex-

TABLE I. *Penetration of water and salicylic acid across abdominal stratum corneum in relation to quantitative parameters*

Sample #	Penetration (±SEM)		Stratum corneum parameters (±SEM)	
	Water (mM/cm^2/24 hr)	Salicylic acid (μM/cm^2/24 hr)	Thickness (μM)[a]	# Cell layers average[b]
176	5.4 ± 1.7	4.9 ± 0.4	—	—
178	2.77 (1 exp.)	3.7 (1 exp.)	14.8 ± 1.0	25.6 ± 1.3
179	1.95 ± 0.63	0.7 (1 exp.)	20.0 ± 1.4	25.6 ± 0.9
184	5.47 ± 0.81	9.7 ± 7.8	—	—
191	1.77 ± 0.34	1.2 ± 0.6	19.2 ± 1.4	20.8 ± 1.0
193	2.03 ± 0.48	1.4 ± 0.8	20.8 ± 2.0	24.0 ± 0.7
211	—	—	20.4 ± 3.0	17.0 ± 0.9
227	—	—	11.6 ± 0.8	16.8 ± 1.5
Mean (±SEM)	3.2 ± 0.8	3.6 ± 0.6	21.8 ± 2.2	20.6 ± 1.5

[a] Thickness represents mean of 5 narrowest (most perpendicular) thicknesses of stratum corneum encountered in frozen section (see Methods).
[b] Number of cell layers represents average of 5 separate locations along frozen section (see Methods).

TABLE II. *Penetration of water and salicylic acid across leg stratum corneum in relation to quantitative parameters*

Sample #	Penetration (±SEM)		Stratum corneum parameters (±SEM)	
	Water (mM/cm^2/24 hr)	Salicylic acid (μM/cm^2/24 hr)	Thickness (μM)[a]	# Cell layers average[b]
169	10.7 ± 3.9	8.7 ± 1.9	21.2 ± 0.8	22.0 ± 2.1
170	12.7 ± 4.67	7.9 ± 1.8	32.4 ± 2.6	24.4 ± 1.6
172	4.5 ± 1.2	1.9 ± 0.4	19.6 ± 1.8	20.2 ± 1.2
173	5.5 ± 1.86	6.8 ± 4.1	32.0 ± 2.6	22.4 ± 1.4
174	7.6 ± 1.7	5.0 ± 0.8	28.4 ± 2.4	23.2 ± 1.0
180	7.6 ± 2.15	4.1 ± 1.9	—	—
Mean (±SEM)	8.1 ± 2.6	5.7 ± 1.8	26.8 ± 2.6	22.4 ± 0.7

[a] Thickness represents mean of the 5 narrowest (most perpendicular) thicknesses of stratum corneum encountered in frozen section (see Methods).
[b] Number of cell layers represents average of 5 separate locations along frozen section (see Methods).

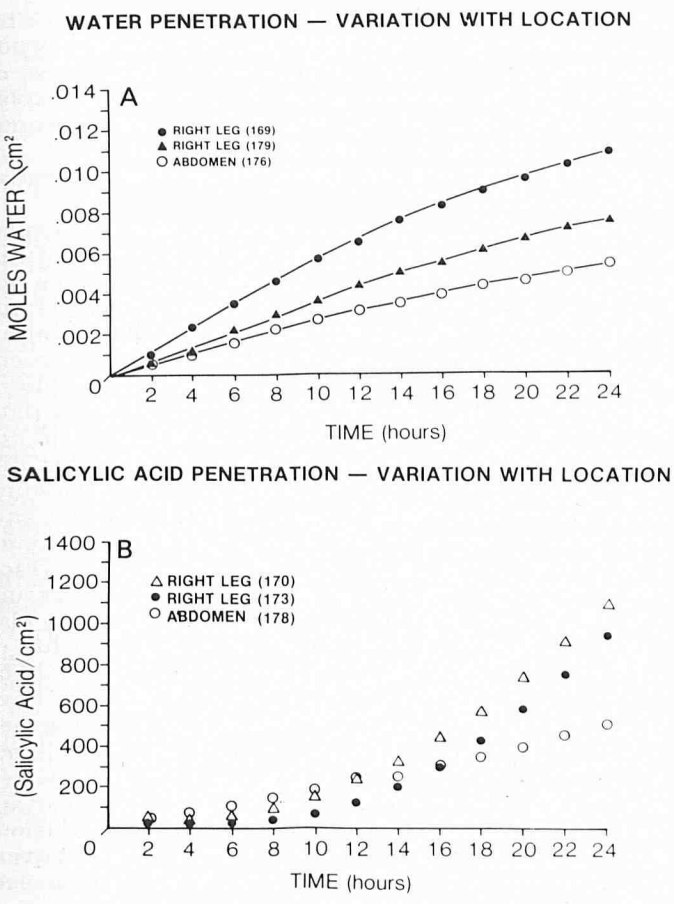

FIG 1. Transport kinetics of water (*A*) and salicylic acid (*B*) through representative samples of abdomen and leg stratum corneum. Note essentially linear kinetics for water (*A*) and lag period for salicylic acid (*B*). The differences in penetration rate of each substance across abdomen vs. leg stratum corneum also can be seen.

amined, and measured without the knowledge of the observer as to either the source or penetration kinetics of the tissue specimen.

The thickness of the stratum corneum was determined by measuring the thinnest sectioned regions utilizing an eyepiece reticle. Only regions of intact stratum corneum were measured; regions where a portion of the outer stratum corneum was disloged were easily recognized and excluded. The thinnest regions presumably represent the sections that were cut most normal to the stratum corneum, and therefore, that most closely approximate its true width. Four separate areas were measured from each specimen and the mean and standard error of the mean was determined. The number of cell layers in the same stratum corneum was counted simultaneously in the same samples, and the mean and standard error of the mean of 5 separate sites was determined. The site-to-site variation within a given sample never exceeded ± 10%.

Statistical Methods

A Monroe statistically programmable printing calculator (Monroe 1860) was used to compile the raw data yielding means and standard error of the means, and to apply the Student *t*-test to illustrate the significant differences between cell number, thickness, and penetration of salicylic acid and water across the stratum corneum samples.

RESULTS

Transport Kinetics in Relation to Topographic Site

Water transport observed essentially linear kinetics over the 24-hr test period in specimens derived from both leg and abdomen (Fig 1). In contrast, salicylic acid initially crossed both sites slowly, but the rate steadily increased between 10 and 20 hr, thereafter tending to level out (Fig 1). As with water, both abdominal and leg samples exhibited similar, parallel patterns of transport.

The penetration rates of water and salicylic acid across abdominal and leg stratum corneum are summarized graphically in Fig 2. Although water clearly permeated leg stratum corneum more readily than it did abdominal stratum corneum ($p < 0.025$), the disparity for salicyclic acid across these 2 sites was not so great, and was not statistically significant ($p < 0.1$, Table III).

Transport in Relation to Stratum Corneum Structural Parameters

Tables I and II contain the raw penetration data for both water and salicylic acid for each site. Although the number of cell layers of abdominal and leg stratum corneum are comparable (difference $-p < 0.35$), leg stratum corneum is distinctly thicker than abdominal stratum corneum (significance—$p < 0.01$).

When the penetration data for each experiment were plotted against either the stratum corneum thickness and cell layers, no clear correlation emerged (Fig 3).

Transport in Relation to Lipid Composition

Lipid concentration by weight: When the permeation data for each site are compared to lipid composition a distinct relationship emerged (Table III). Leg stratum corneum was

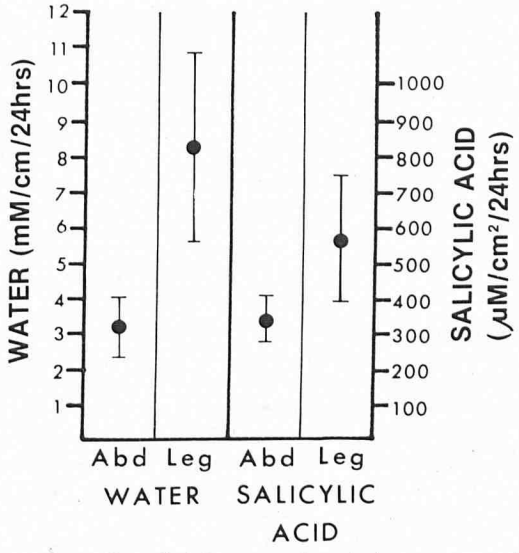

FIG 2. Permeability of abdomen vs. leg stratum corneum to water and salicylic acid is compared. *Brackets* indicate mean plus standard error of mean for all samples (Tables I, II).

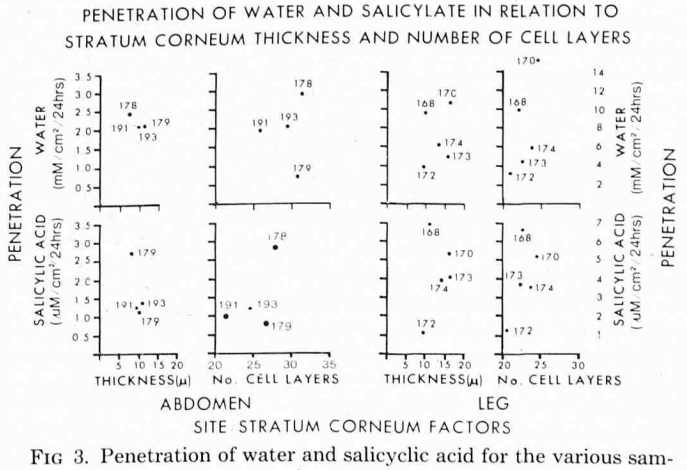

FIG 3. Penetration of water and salicyclic acid for the various samples in this study is plotted against thickness and number of cell layers. Instead of expected inverse relationship, there is either no correlation or an opposite, direct relationship.

TABLE III. *Mean penetration in relation to total lipids*[a]

Penetration	Leg[b]	Abdomen[b]	Significance
Water (mM/cm^2/24 hr)	8.1 ± 2.6	3.2 ± 0.8	$p < 0.0025$
Salicylic acid (μM/cm^2/24 hr)	5.7 ± 1.8	3.6 ± 0.6	$p < 0.1$
Lipid (% wet weight)	3.0%	6.8%	

[a] Lipid expressed as percent of lipid weight/tissue wet weight.
[b] Number of samples (abdomen = 15; leg = 6).

TABLE IV. *Lipid weight percents of randomly obtained abdomen vs. leg stratum corneum*

Sample	Abdomen	Sample	Leg
1	7.5	1	2.2
2	6.6	2	3.4
3	6.5	3	3.8
4	5.6	4	2.2
5	8.2		
Total (±SEM)	6.8 ± 0.4[a]	Total (±SEM)	2.9 ± 0.5[a]

[a] Difference is statistically significant: $p < 0.0025$.

TABLE V. *Comparison of major classes of lipids isolated from abdomen and leg stratum corneum*[a]

	Leg[b]	Abdomen[b]
Phospholipids	14.9	10.6
Neutral Lipids	75.6	76.8
Sphingolipids[c]	12.4	9.4
Total	102.9	96.8

[a] Mean of 2 separate chromatographic separations. Values determined by sequentially subtracting weight fractions on polar and neutral lipid plates from weight of total lipid applied.
[b] Number of pooled skin samples: leg = 6; abdomen = 15.
[c] Both sites contained less than 0.1% cholesterol sulfate in the sphingolipid fraction.

consistently lipid-depleted in comparison to abdomen. Although data on lipid composition were not available for each of the individual samples, the permeation of water, and to a lesser extent of salicylic acid, appeared to be correlated with the marked difference in lipid weight percent of these 2 regions (Table III, Fig 2). In a separate group of samples, identical in origin to the samples used in this study, we showed that the difference in mean lipid weight percent of abdomen vs. leg stratum corneum samples was statistically significant (Table IV). These difference are not affected when the numbers are expressed either in terms of dry weight or protein concentration.

Qualitative lipid composition: In order to determine whether the transport data might be correlated not only with quantitative differences, but also with qualitative differences in lipid composition at the 2 sites, the lipids from the 2 sites were separated into their major constituents by thin-layer chromatography. There were no major differences in the quantities of phospholipids, neutral lipids, and sphingolipids at these two sites (Table V).

DISCUSSION

In this study we have attempted to address several possible parameters deemed important for percutaneous transport. An important aspect of this study is the correlation of several different stratum corneum parameters with *in vitro* penetration data from the same hydrated tissue sample [27]. Water was chosen as the aqueous permeant, while salicylic acid was chosen as a model of a relatively lipid-soluble substance.

Our data indicated a consistent difference in the permeability of leg vs. abdominal skin to water, and suggest a comparable difference for salicyclic acid. Although direct comparisons of these 2 sites are not found in the literature, it is not surprising that such striking differences were encountered, since even greater topographic variation is known to occur [28–30]. The reason for these great regional differences in penetration is not clear. Since there are no great differences in the hairiness of abdomen and leg stratum corneum, this is an unlikely explanation. In any case, although penetration via pilosebaceous structures may occur in certain circumstances [1, 2, 29], the pilosebaceous orifices are not considered important for steady-state diffusion [1, 2, 31].

Since the pilosebaceous route can not be offered as an explanation, other factors, such as the thickness and number of cell layers, have been pondered as regulators of percutaneous absorption (reviewed in reference 22). The most unexpected finding in this study was the apparent noncorrelation of penetration of both model substances with either stratum corneum thickness or number of cell layers. While it would be desireable to have additional data points, it seems clear from available data that the inverse correlation, expected from Fick's Law, did not apply. Although there was a small, statistically significant difference in the thickness of abdomen and leg stratum corneum, the penetration was actually greater across the thicker skin site. That thickness may have little to do with stratum corneum permeability is also underscored by the relative ease with which certain substances penetrate regions with the thickest stratum corneum, namely the palms and soles [28–30]. There was also a small, statistically insignificant difference in the number of cell layers across these sites, again the reverse of what would be expected. Although attempts to relate there parameters have been made [22], physiological experiments were not performed on the same specimens. Nevertheless, Holbrook and Odland, on the basis of measurements of ultrathin sections, suggested that stratum corneum thickness and cell layers were important for barrier function [22]. The differences in the conclusions drawn may be methodological; e.g., in order to avoid distortion from fixation and embedding we employed unfixed frozen sections, rather than embedded tissues. Moreover, we measured the narrowest intact regions of stratum corneum to eliminate variation from oblique sectioning, rather than averaging multiple sites without consideration of sectioning angle.

We do not know how to reconcile the above data with accepted mathematical models of percutaneous absorption [1, 2]. However, one possible explanation would be that other factor(s), such as lipid content (see below), are proportionally much more significant than stratum corneum thickness and/or number of cell layers for percutaneous absorption.

Role of Lipids in the Barrier

Still prevalent physical-chemical dogma (reviewed in references 1 and 2) equates the stratum corneum barrier with the keratin-phospholipid complex inside the stratum corneum cell. In the past, penetration of polar molecules was presumed to occur near the outer surface of intracellular protein filaments, while nonpolar molecules were thought to diffuse through the lipid matrix between the filaments (reviewed in reference 2). The intercellular spaces were dismissed as a potentially significant pathway because of their small volume [1].

An overwhelming array of recent information is now aligned against this model: First, histochemical studies show that the stratum corneum cell is devoid of lipid [12, 13]. Second, since most of the lipid present is highly nonpolar [13, 14] there is no candidate to serve as a "sleeve" or matrix for intracellular keratin filaments. Third, the intercellular volume fraction is much larger than previously recognized [15], and it is probably expandable either by solvent/detergent extraction or by inspissation of nonpolar vehicles, carriers or permeators. Fourth, there is also experimental evidence from recombinant experiments that almost all of the lipid of the stratum corneum lies between the cells of the stratum corneum [16]. Fifth, there is now direct evidence that epicutaneously applied substances permeate via the intercellular spaces [21].

The importance of lipids in the control of percutaneous

TABLE VI. *Comparison of lipid weight percent from 4 skin sites*[a]

Site	Number of Specimens	Lipid weight %
Abdomen	5	6.8 ± 0.4
Leg	4	2.9 ± 0.5
Soles	2	1.3 ± 0.5
Face	4	7.2 ± 0.6

[a] Total equals percent of wet weight ± standard error of the mean.

transport has been appreciated for 3 decades. But these earlier studies [17–19], and more recent investigation on lipid-solvent treatment of stratum corneum (e.g., reference 20) employed drastic means to derange penetration. Our study suggests that relatively small inherent variations in lipid concentration may explain observed differences in permeation across different topographic regions. We would predict, therefore, that regions of high permeability such as palms and soles would have a low lipid weight percent, whereas those of low permeability such as facial or perineal stratum corneum, would possess a relatively high lipid weight percent. The preliminary data presented in Table VI fully support this conclusion.

On the other hand, we found no evidence that *qualitative* differences in lipids in these 2 locales could account for the observed differences in penetration. Further work on the characterization of lipids from different skin sites is underway in this laboratory. Even if no differences are found in the major species of phospholipids, neutral lipids, and glycosphingolipids, it is still possible that variations in fatty acid composition could contribute to variations in penetration. For example, in light of the notable importance of linoleic acid for normal barrier function [34], it is still possible that differences in the distribution or quantities of this important substance could play a role.

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