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Physical Techniques For Assessing Skin Moisturization

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Synopsis: An overview is presented of some PHYSICAL TECHNIQUES currently available for use in SKIN MOISTURIZATION studies. Water soaking of unmodified versus ether-extracted stratum corneum, for example, causes a marked alteration in the BIOMECHANICAL properties of these tissues (i.e., swelling capacity, elastic modulus, relaxation function, and work index). Differences in moisture binding properties as measured by GRAVIMETRIC and SCANNING CALORIMETRIC analyses of the tissue at various relative humidities are related. The correlation of changes in these traits with changes in the pliability and strength of corneum tissue and its capacity to retain moisture is discussed. Criteria for judging dry versus hydrated skin *IN VIVO* are also reviewed through the utilization of TRANSPIROMETRY, PHOTOGRAPHY, and SCANNING ELECTRON MICROSCOPY (SEM). Analysis via these techniques of the effect of humectants and occlusive oils on water retention within skin is presented.

Introduction

A wide variety of *in vitro* and *in vivo* physical procedures are available for investigating phenomena associated with moisturization of the stratum corneum. This presentation will touch on the usefulness of gravimetric, scanning calorimetric and, mechanical techniques in quantitating levels of moisture retention and pliability obtained after treating corneum tissue with various materials *in vitro*. In addition, *in vivo* evaluations by means of transpirometry low magnification photography, and scanning electron microscopy (SEM) of skin replicas before and after treatment of human skin with moisturizing formulations will be reviewed in detail.

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In vitro methodologies

A. *Gravimetric Measurement of Water Binding*

This widely used method of assessing the affinity of isolated stratum corneum for water consists of equilibrating corneum tissue at a fixed temperature in a constant relative humidity (RH) chamber until a nonvarying weight is attained. Temperatures in the range of 0° to 35°C and RH in the range of 10 to 90 per cent have been commonly used (1-5). A period of 5 to 7 days is generally required for attaining constant weights at 10 to 90 per cent RH. The samples are then desiccated over a dehydrating agent until a dry weight is reached. The data are expressed as the per cent moisture uptake (i.e., weight per cent gained) with respect to the dry weight.

The capacity of callus tissue to remain soft and flexible was shown by gravimetric assay of water uptake to be directly correlated with the presence of natural moisturizing factors (NMF) in the tissue (1, 2). Human callus, extracted with diethyl ether and water, and then allowed to equilibrate in chambers at 35 per cent RH, gained 5 per cent less absolute weight (i.e., moisture) than callus which was just water-soaked and equilibrated. At 80 per cent RH, this differential increased to as much as 20 per cent water uptake. Laden and Spitzer were able to identify the major humectant in NMF as being sodium 2-pyrrolidone-5-carboxylate (2). Since that study, Middleton has further substantiated the role of an NMF (e.g., lactic acid) in influencing the state of stratum corneum hydration (6). These investigations support the hypotheses that NMF within the cornified cells of the epidermis maintain the flexibility of this tissue (a) through enhancing the rate of water migration from lower living cell layers and (b) by hindering the release of moisture from the skin surface by reinforcing the water retaining capacity at very low RH.

B. *Differential Scanning Calorimetry*

A direct measure of the levels of "bound" (nonfreezing) and "unbound" (freezing) water in animal and human corneum strips was described by Walkey (7) in 1972 using a differential scanning calorimeter.* She was able to quantitate the level of hydration after equilibration of dried strips at various RH from latent heat of melting curves. Walkley showed that when dry human corneum attained a 45 per cent moisture regain above dry weight, approximately two-thirds of that water (0.35 mg/mg dry corneum) was nonfreezable (i.e., bound). Her results were confirmed by the findings of Anderson *et al.* (5), based on proton magnetic resonance and infrared spectroscopy, which demonstrated the presence of 0.35 to 0.50 mg of bound water per mg

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of dry corneum. Both Walkley (7) and Anderson *et al.* (5) hypothesized that the freezable fraction was held only by diffusional barriers, whereas the non-freezable (i.e., bound) fraction was strongly associated with the polar groups of corneum proteins and NMF. Walkley further found that the effect of extracting lipids with diethyl ether and NMF with water allowed for an increase in the portion of bound water from 0.29 to 0.41 mg/mg of dry animal foot pad corneum. Ether-water extraction caused a dramatic lowering of corneum diffusional barriers and allowed for a greater proportion of sorbed water to be bound by polar residues of the remaining proteins and lipids. In a study of swelling properties of unmodified and ether-water extracted stratum corneum via biomechanical analyses as is described below, Wolfram *et al.* (8) have confirmed Walkley's finding.

C. *Biomechanical Analyses*

As several investigators have pointed out (4, 6, 8, 9), the elastic modulus of stratum corneum is directly correlated with the amount of water retained in the tissue. Water retention, in turn, has been demonstrated to depend on the surrounding temperature and relative humidity and on the structural integrity of the cornified cells (10-14). It is widely observed that, in winter, the rate of moisture replacement from beneath the corneum becomes inadequate in comparison to the rate of transpiration from the surface. Moreover, exposure to organic solvents or aqueous detergents damages the skin and allows for dehydration of the outermost cell layers. As these cornified layers become progressively more dehydrated, they become inflexible and less extensible than the deeper layers causing the surface to stiffen, flake, and crack, while the person involved perceives the tight, drawn, and itching sensations of chapped and dry skin.

Changes in the reversible stretching properties of animal corneum may be evaluated by the method of Elfbaum and Wolfram (9) who used the extensometer.* Their results have been expressed as the work index (i.e., the ratio of the work required to reversibly stretch a strip of corneum to a 5 per cent displacement in a given solvent versus preliminary 5 per cent displacement of the same strip in water). In this way, aqueous dimethyl sulfoxide (DMSO) concentrations greater than 50 per cent cause a reversible stiffening (increase in the work index) of animal corneum together with extensive swelling in the cells of the cornified tissue. A concomitant increase in the tautness and hardness of the samples is observed at the macroscopic level. In a similar manner, ether-delipidized tissue has been water-swollen and reversibly stretched (8). Unlike the dimethyl sulfoxide treatment, exposure of stratum corneum to

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ether and then water causes about a 30 per cent dry weight loss and nearly a two-fold decrease in stiffness as measured by the work index.

A contacting probe balance, developed by E. M. Buras at our laboratory,* has been employed for measuring the cross-sectional swelling of specimens of stratum corneum (8). This instrument permits the rapid and accurate recording of the displacement of a probe placed in contact with the dry surface of dry cornified tissues. After measuring the dry state thickness, each sample is submerged in 0.1 per cent aqueous Triton X-100,† and the displacement due to swelling is continuously monitored. The final thickness is determined after equilibrium is reached, usually within 10 min. The percentage swelling is calculated by comparing the initial displacement with that following imbibition.

Changes in the remaining two dimensions (termed in-plane swelling) are measured directly for square samples (20 x 20 mm) before and after immersion in 0.1-per cent aqueous Triton X-100. After 16 h, all squares are removed, and their perimeters remeasured to the nearest 0.1 mm to determine the percentage change. To test the effects of delipidization, squares have been preextracted with diethyl ether for 1.5 h, air dried, incubated as above for 16 h, and then remeasured.

The pronounced weakening of the ether-pretreated specimens as reported in the Instron study correlates well with distinct increases in both cross-sectional and in-plane swelling. Ether-water extraction and concomitant loss of NMF causes about a 3-fold increase in thickness when subsequently reswollen, but only a 5 per cent enhancement in area (8), as compared with water-soaked stratum corneum which is not preextracted. Ether pretreatment, therefore, alters not just the lipid content and moisture retaining capacity through loss of NMF, but the physical dimensions, strength, elasticity, and membrane permeability as well. The high swelling and reduction in the rate of strain recovery (i.e., decrease in the viscous component of elasticity) of the ether-treated samples may be explained by marked alteration in the conformation of keratin molecules, which is brought about by the breakdown of hydrogen bonds and accompanying aqueous exposure of previously buried

*The probe balance consists of a freely moving aluminum arm suspended on aluminum-coated Mylar flexures. The balance has a 2 mm^2 probe at one end, which contacts the corneum specimen placed on a flat surface. At the other end of the balance arm, there is a position transducer which consists of a vane and a proximity probe. Displacement is measured by change in capacitance which varies with the length of a cylindrical probe inserted into the vane. The proximity probe of this dynamic balance is wired into a commercial driver unit, and then into the Y-axis of the recorder. An aluminum cylinder coil fastened to the balance arm above a magnet constitutes a damping system. The instrument has also been used to measure diametrical swelling of hairs.

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hydrophilic and hydrophobic groups within the keratin of the delipidized corneum cells.

Instead of the work index, Rieger and Deem (13, 14) have analyzed the elastic modulus (i.e., the ratio of stress imposed on stratum corneum to the strain applied at a constant strain rate) and the relaxation function (i.e., the decay in the strain rate of the tissue while a constant stress is imposed). Both the elastic modulus and the time constant of relaxation of unmodified stratum corneum decreases with increasing RH, providing an objective characterization of pliability (13). Upon application of known humectants such as 4 per cent sodium pyrrolidone carboxylate and 50 per cent glycerol in water, there is a decided increase in elasticity and a faster relaxation time in comparison with dried tissue (14). Light mineral and safflower oils have the opposite effect, suggesting an increase in the stiffness and a decrease in the pliability of the treated samples.

In Vivo Methodologies

A. *Transpirometry*

Some of the current techniques, which we utilize *in vivo*, have aided us greatly in directly evaluating cutaneous moisturizers. Several types of instrumentation are described in the literature for application in the direct determination of transpiration rates (15-21). We employ an apparatus designed by Slegers and Dobson for measuring the rate of moisture release from the skin into a stream of dry nitrogen (Fig. 1).* This stream, passing in a flow-through chamber on the skin, and a stream of identical pressure flowing independently of the skin are compared for their thermal conductivity in a gas chromatograph. Two of these systems, each equipped with integrators, allow for simultaneous measurement of the rate of moisture loss at two separate sites (i.e., a control and a test).

We have observed all three sources of water (i.e., surface moisture, transpired water, and eccrine sweating) which Berube *et al.* have mentioned (15). After an equilibration and "calm-down" period of 30 min, surface moisture is eliminated and most panelists become sufficiently conditioned to a room tem-

*The transpirometer consists of two thermal conductivity gas chromatographs. For each unit, streams of dry nitrogen at 200 ml/min/cm^2 are split into two equal components, one passing directly into the chromatography unit, while the other streams into a flow-through probe on the skin before entering this thermal conductivity analyzer. The difference in the conductance between the split streams is measured, and a signal from each chromatograph is sent to a dual pen recorder. The latter is equipped with two repeating potentiometers allowing for integration of each signal. Standard curves are obtained for each system before use each day by application of known quantities of water (0.1 to 1.0 μl, for the 0.05 mV sensitivity range) to filter paper sealed within each chamber.

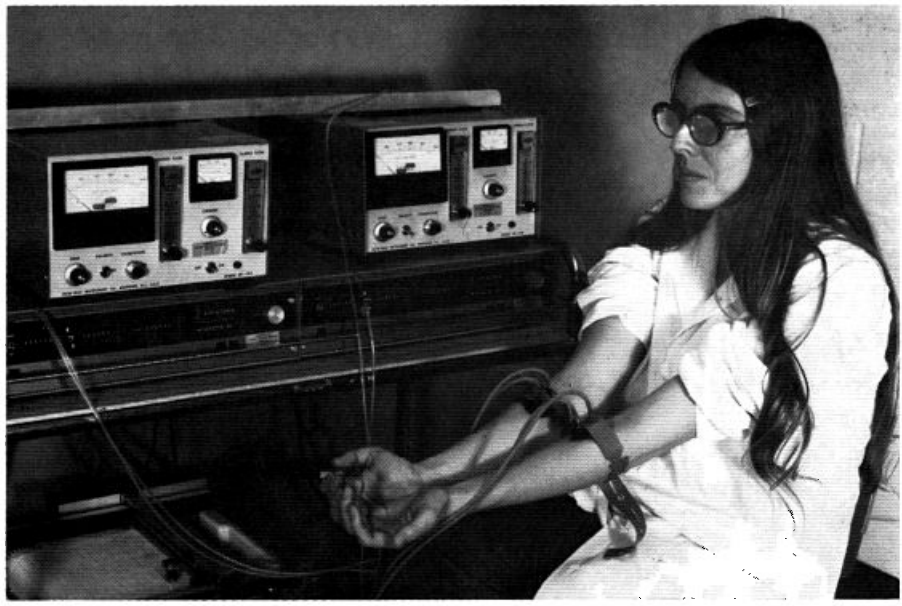

Figure 1. Dual-recording transpiration analyzer as designed by Slegers and Dobson (23)

perature of 20°C and RH of 50 per cent or less so as not to demonstrate significant eccrine sweating. Those panelists, who are not stabilized after this period, are eliminated from testing that day.

Upper forearms and the calves of the lower legs of 12 panelists of both sexes were examined, with one site serving as an untreated control and a contralateral site being used for the tests. Occlusion of the skin for 5 min with a water-saturated gauze patch followed by tissue blotting of the excess water produces an initial 20 to 40 per cent increase in water loss, which decreases steadily over a 30-60 min period to the level of transpiration on the opposite side. Similar application and blotting of a commercial emollient cream reverses this trend, giving a 20 per cent decrease in transpired water after 1 h and about a 10 per cent decrease after 4 to 6 h.

We interpret these results with an emollient cream as indicative of temporary retardation of water loss afforded by an oil barrier. Once the dry corneum imbibes moisture from lower living cell layers and attains a new equilibrium water content, the temporary effect of the diffusional barrier of the cream is slowly overcome, and the original transpiration rate is reestablished. The effect of the barrier cream is then to raise the moisture concentration within the dead cells without effecting the final equilibrium transpiration rate too much.

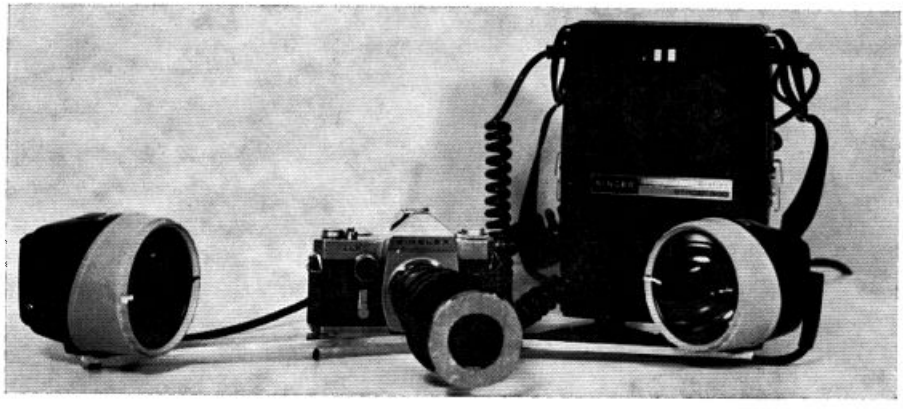

Figure 2. Photographic apparatus for producing low magnification prints

B. *Low Magnification Photography*

Low magnification photography of skin sites before and at specific intervals after treatment provides a rapid subjective means of evaluating the moisturizing potency of emollient creams. Kodacolor II® (ASA 80) film* and a Ricoh® 35 mm reflex camera† with a 55 mm lens and extension tube system give a final magnification of 3.5-fold. The front of the lens is equipped with a gridded disc fixed onto 95 mm spacing bars. This system allows the correct focus to be obtained for an area of 33 x 23 mm^2 with minimal readjustment (Fig. 2). A camera aperture of f16 gives optimal exposure and depth of field at 1/60 of a second. Two strobe heads (7100 ecps) on a Graflex 500® flash unit‡ are fixed to the camera so as to be set 220 mm from the photographic site at an angle of 25° above the plane of the skin. To minimize glare, polarizing filters are placed over the strobe heads and oriented perpendicular to a polarizing filter placed over the camera lens.

A panel of three independent judges evaluates the photographs based on the level of white lines and flakes discernible in these pictures (Fig. 3). A rating scale (from 6 equals no white margins nor scales to 0 equals only white margins and many lifted scales) is utilized by the assessors. This rating scheme is similar, but opposite in value to that described by Gibson (22) and Middleton (6). The major advantage of our technique is to provide an extremely helpful low magnification (3.5 X) of each site studied (Fig. 3).

*Eastman Kodak Co.,Rochester, NY 14650.
†Braun North America, Cambridge, MA 02141.
‡The Singer Co., Graphics Systems Division, San Leandro, CA 94577.

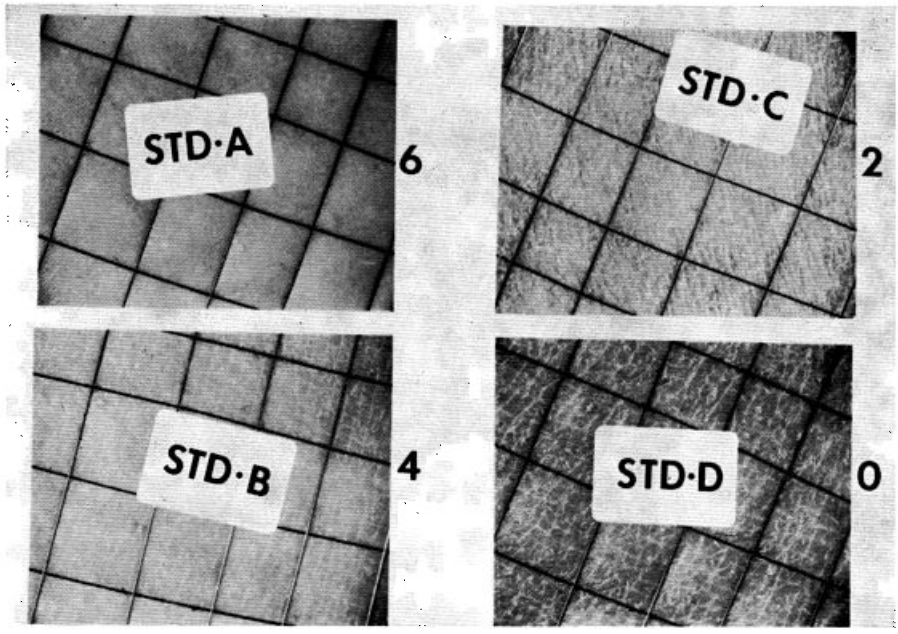

Figure 3. Photographic rating scale: Standard A: 6 equals no white margins nor uplifted dry flakes; Standard B: 4 equals a few white margins, but no uplifted dry flakes; Standard C: 2 equals many white margins and a few uplifted dry flakes; Standard D: 0 equals totally distinct white margins and many uplifted dry flakes

In an initial investigation, 30 panelists were treated at 1 skin area with an emollient cream, while a site on the opposite side remained untreated. Photographs were taken of both sites after 6 and 24 h, and evaluated by 3 judges. Their scores were averaged and analyzed by the t-test statistic (23). The treated sites were scored nearly 3 points higher at 6 h and 1 point higher at 24 h (Table I), indicating that the visual benefits provided by the emollient cream could be readily discerned from the photographs.

More recently, we have been examining the effect of various camera color filters on the quality of black-and-white photographs. Dent, in 1941, published an elegant study (24) on skin photography as a function of the wavelength of light reflected from the skin. He determined that detailed texture, definition and lines discerned under violet-blue lighting (300-450 nm) result from the fact that very little of this light penetrates below the stratum granulosum.

Table 1

Mean Photographic Scores

Hours After Start	Skin Sites: Treated	Skin Sites: Untreated	Null Probability[a]
6	5.06	2.43	< .001
24	3.72	2.55	< .001

[a]Based on the t-test statistical comparison.

Thus, light directed into the camera can only be reflected from within the corneum, the lucidium and to a lesser extent the granulosum layers. Light of the green through red wavelengths (480 to 800 nm), however, is able to penetrate further into the dermis. Photographs obtained under this light show veins and blood vessels, but no surface detail or texture. Gibson (25) has reported on a direct viewing system of goggles equipped with a monochromatic vision filter (MV812)* which converts colors to shades of gray. A gray photographic scale is then used for evaluating levels of erythema or changes in skin pigmentation.

We have confirmed Dent's observations, in particular, for Caucasian skin after elution with acetone for 30 sec. Photography using Panatomic-X® black-and-white film† and a Kodak CC50C-cyan filter† (passing mainly <550 nm and >740 nm) gives pictures with significantly more surface detail than photography using the same film but with a yellow filter equivalent to Kodak 81C (passing mainly >450 nm) (Fig. 4).

C. SEM

SEM investigations of panelists' skin have been used to correlate the influence of the chemical composition of various preparations with the moisturizing efficacy of these formulations *in vivo*. Bernstein and Jones (26, 27) have developed a replication method which would neither damage the skin nor become destroyed by the electron beam. Initially, a negative impression of the skin is formed by polymerization of 10:1 mixture of Silastic® 382 Elastomer‡ and RTV-Thinner‡ with stannous octoate. From this impression,

*Ilford Inc., Ciba-Geigy Co., Paramus, NJ 07652.
†Eastman Kodak, Rochester, NY 02142.
‡Dow Corning Corp., Midland, MI 48640.

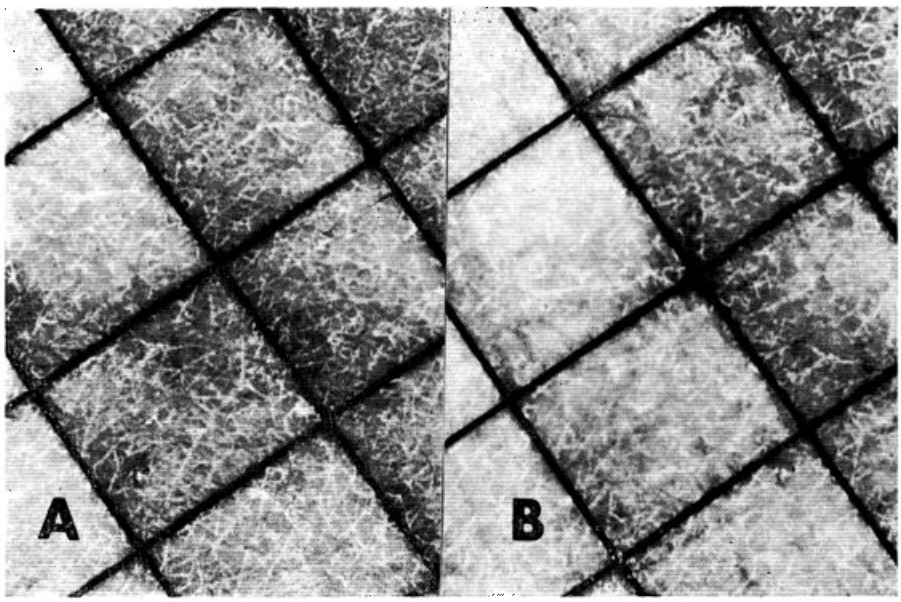

Figure 4. Repetitive photography of a site on Caucasian skin. Recorded with (A) a Kodak CC50C-cyan filter; (B) a yellow filter equivalent to Kodak 81C

a positive replica is made by melting polyethylene pellets* *in vacuo* at 180°C. After shadowing with Au-Pd to a thickness of 150 Å as was previously described (26), metal coated 9 mm punches of the polyethylene replicas are examined with a JEOL JSM-2† instrument at 30 to 3,000 X magnifications with accurate reproduction of details and greatly enhanced depth of field compared to conventional light microscopy.

Unmodified and solvent-extracted human skin *in vivo* have been examined in this manner (8). Impressions are obtained from the backs of panelists' hands before treatment in order to obtain control photographs. Half of each hand is then extracted with diethyl ether for 60 sec. The hands are dried and water-soaked with a damp flannel patch for 1 h. Following removal of the patches and blotting away the excess water with a paper tissue, Silastic‡ negative impressions are taken of the ether-water and water-only soaked areas. Together with the adjacent samples derived from unmodified skin, positive polyethylene casts are taken of each of the two extracted skin specimens.

*Union Carbide, Chemicals and Plastics Division, New York City, NY 10017.
†JEOL U.S.A. Inc., Medford, MA 02155.
‡Dow Corning Corp., Midland, MI 48640.

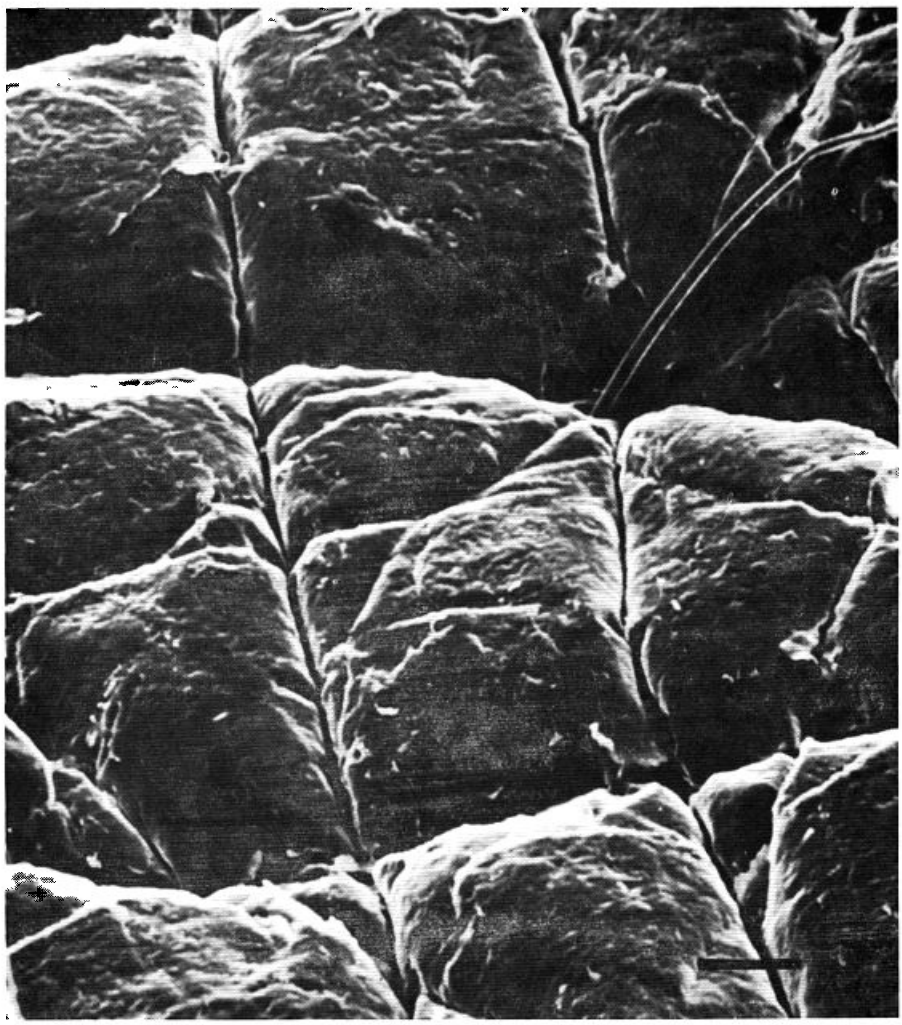

Figure 5. Scanning electron micrograph of untreated skin. Wolfram, Wolejsza and Laden (8). Bar represents 167μm

Photographs of these specimens under SEM examination reveal a surprising level of detail and contrast (8). Triangular, roughly rectangular, and polygonal cell clusters, having lengths of 600 to 1,200 μm, can be seen readily in the unmodified skin sample (Fig. 5). Earlier, Bernstein (28) demonstrated an enhanced rounding in the divisional contours with progressive increases in the degree of hydration. Water soaking the skin compared to the unmodified skin causes an increased plumping up of the subdivisional contours, while

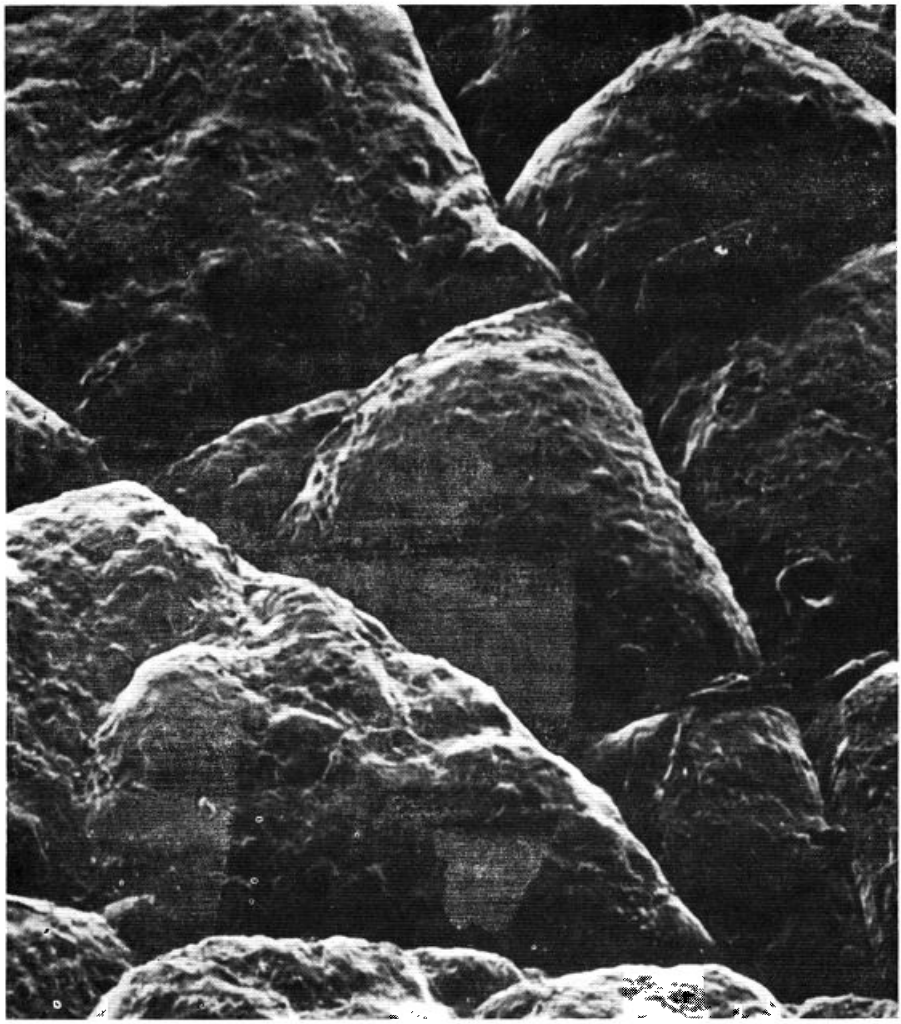

Figure 6. Scanning electron micrograh of water-soaked skin. Wolfram, Wolejsza and Laden (8). Bar represents 167 μm

simultaneously enhancing the roughness and bumpiness within each cluster (Fig. 6). Ether extraction followed by water elution causes a further elevation in the height and number of protuberances (Fig. 7). The grooves between the subdivisions spread and have become accentuated, while the surface has become stretched and made taut. These SEM observations (8) strongly substantiate the conclusion drawn from gravimetric (1, 2) and biomechanical (7-9) analyses that removal of lipids and NMF results in enhanced water

Figure 7. Scanning electron micrograph of ether-extracted and water-soaked skin. Wolfram, Wolejsza and Laden (8). Bar represents 167 μm

permeability, roughness, and swelling of the corneum cells concomitant with a weakening of the cellular membranes. This swelling, however, disappears within 1 h.

In order to avoid removal of desquamous material from winter-dried skin through adhesion to the Silastic polymer upon repetitive replication of a single area, we have routinely chosen to reproduce immediately adjacent

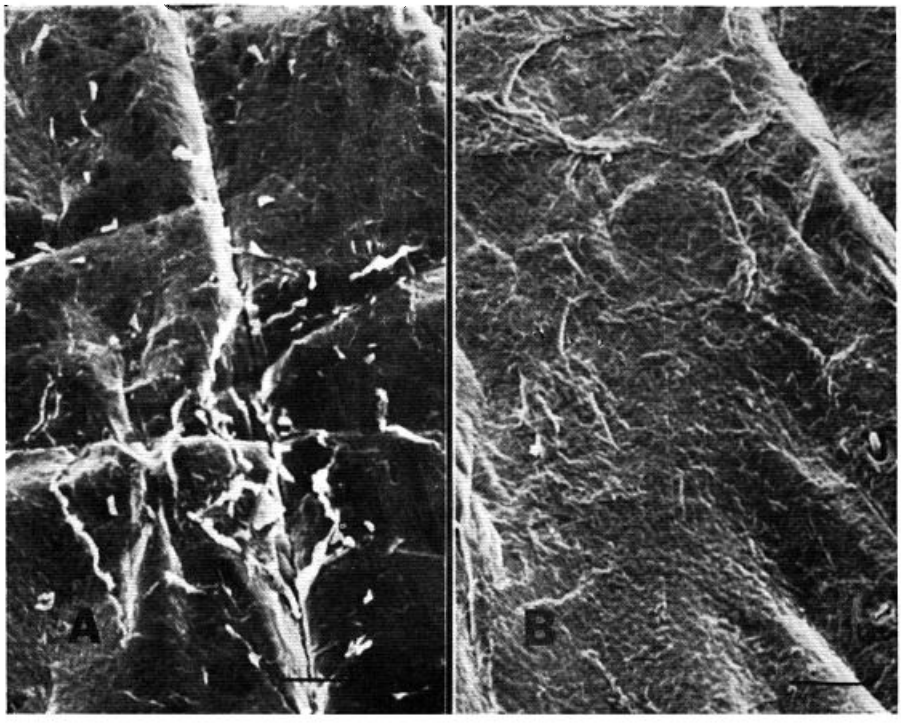

Figure 8. Scanning electron micrographs of immediately adjacent lateral sites on the calf of woman's leg. Each site was replicated simultaneously with (A) no treatment, and (B) 8 h after application of a commercial emollient cream. Each bar represents 167 μm

sites from grossly dry areas (e.g., the calf of a leg). A panel of women with chapped legs have recently been examined via this SEM technique before and up to 8 h after application of an emollient cream. Representative photographs of a single individual's dry untreated leg show a severely desquamous and fissured surface embossed with laminae of accumulated dried cells (Fig. 8(A)). At higher magnification, the shrunken, scaly and fractured vista is more pronounced (Fig. 9(A)). About 8 h after application of an emollient cream, the subdivisional interstices and white edges of uplifted corneum plaques had nearly disappeared, while the overall topography appeared to be partially coated and distinctly granular in texture (Fig. 8(B)). Enhanced magnification demonstrated the near absence of crevices, flaky edges and flatten scales, and accentuated a swollen, indented texture (Fig. 9(B)). An evaluation of the duration of relief and the efficacy of skin moisturization afforded by a product can thus be made by extending the periods of replication so as to provide several adjacent samples for an SEM time study.

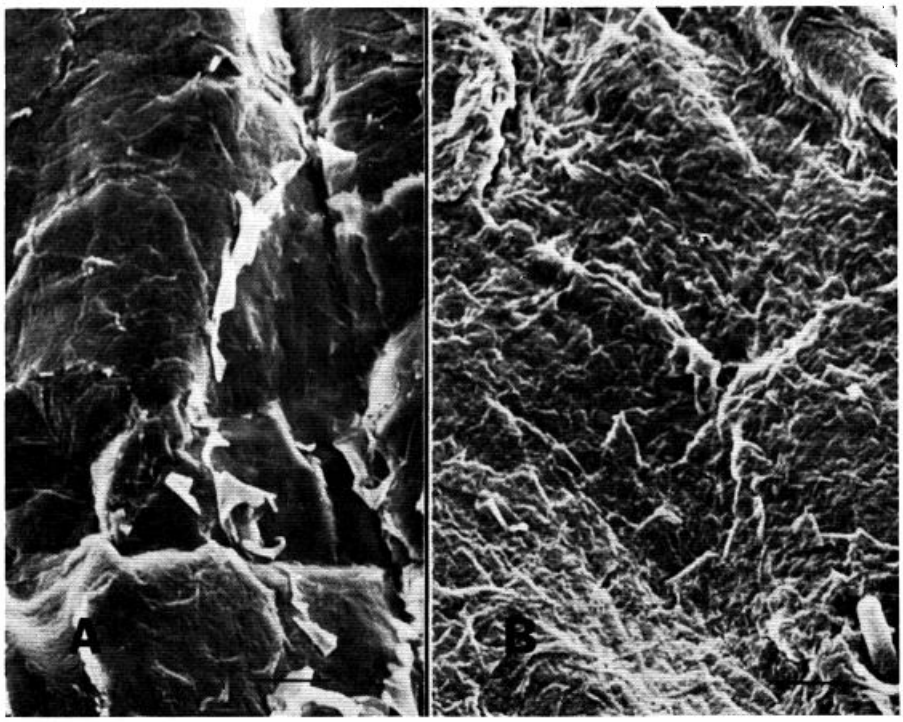

Figure 9. Higher magnification scanning electron micrographs of skin sites in Fig 8. Each bar represents 55.6 μm

Conclusions

In conclusion, the *in vitro* techniques of biomechanical, gravimetric and scanning calorimetric analyses provide valuable background information concerning the substances and dynamics involved in moisture uptake and retention by stratum corneum. Moreover, *in vivo* investigations by means of transpirometry and particularly low magnification photography and scanning electron microscopy are quite critical in the assessment of benefits derived from any cutaneous moisturizer or treatment. These latter methodologies afford a direct assessment of the physical condition of the living skin, and provide very nearly objective means for evaluating the efficacy of present and new formulations designed to moisturize or relieve dry and chapped skin.

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