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Investigations into biomechanisms of the moisturizing function of lanolin

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Synopsis

A study of the lanolin/human stratum corneum system has been made, both *in vitro* and *in vivo*, to investigate where lanolin absorbed by skin lies within the tissues, and to determine possible mechanisms of the moisturizing action. Skin treated with lanolin rendered electron-opaque by lipophilic doping with either lead lanolate or lead oleate was examined by freeze-fracture and transmission electron microscopy of tissue specimens in order to identify the location and state of lanolin within the stratum corneum. Evidence was found that most of the electron-opaque material, presumed to be doped lanolin, occupied intercellular spaces down as far as the stratum granulosum, replacing natural lipid structures lost during sample preparation. In some cases applied material had penetrated into corneocytes and also appeared to have been taken up into the lipid strata of some trilaminar cell envelopes, some of which remained intact. Lanolin appeared to have a special affinity for regions of cell junction. Although this could possibly have been lead marker which had migrated, there appears to be no clear reason why the marker should have greater affinity for natural skin lipids than for lanolin. There was further evidence that lanolin within intercellular spaces had spontaneously emulsified epidermal water as a w/o emulsion with a droplet size of approximately 40 nm, thus providing a possible moisture reservoir.

INTRODUCTION

The emollient or moisturizing action of lanolin on human skin is well established (1). Nothing is known of the biomechanisms involved, but considerable indirect evidence exists:

1. Microprofilometry and intracorneal cohesography of skin treated with lanolin or some lanolin derivatives demonstrated pronounced smoothing (2).
2. Reduction in TEWL of 30% to 32% after lanolin application has been reported (3,4).
3. It has been shown (2) that films of lanolin statically immersed in water become hydrated by spontaneous absorption of water to form a fine w/o emulsion and, conversely, that hydrous lanolin emulsions exposed to air lose water by migration through the substrate and evaporation from the surface; lanolin thus permits a two-way transport of water. The droplet size in spontaneously formed emulsions was measured at 50 nm to 6 μm, this dimension being significant in relation to observations described herein.

4. The penetration of topically applied lanolin down as far as the stratum lucidum has been demonstrated by tape stripping and chemical analysis (5).
5. Lanolin has some components in common with the natural intercellular lipids, such as free and esterified cholesterol and free and esterified fatty acids; some lanolic acids are hydroxy acids, and others are esterified with diols to form diesters with two long acyl chains, as in the case of ceramides.

This earlier work led to the hypothesis that the moisturizing effect of lanolin was a result of absorption into the stratum corneum, where it reduced TEWL by partial occlusion of the internal water pathways or other means, and possibly also of lanolin within the stratum corneum absorbing natural epidermal moisture and thus acting as a reservoir, releasing water if the moisture gradient across the stratum corneum should favor such loss. The present work explores this hypothesis in three separate stages.

EXPERIMENTAL

STAGE 1

This was an initial *in vitro* exploratory stage involving the application of lanolin to excised skin, which, after suitable fixing and sectioning, was examined by transmission electron microscopy. The lanolin was insufficiently electron-opaque, however, to be differentiated from other structures within the stratum corneum specimens. Therefore, this approach was discontinued and details are not reported here.

STAGE 2: OUTLINE

This stage of the study was subdivided into two sections involving different methodologies. Section 1 consisted of treating the surface of human skin *in vivo* with anhydrous lanolin, and after a time removing the surface lanolin and examining it by freeze-fracture and scanning electron microscopy in order to study the microstructural characteristics.

Section 2 involved the doping of lanolin with lead to increase its electron opacity before application to human skin *in vitro* at a specific loading. Prepared specimens of treated skin sections were then examined by transmission electron microscopy.

The principle of lead doping to increase electron opacity had been previously used by Ghadially and co-workers (6) in studies of skin penetration by petrolatum. In those studies, doping was carried out by adding lead nitrate in the form of a physical suspension. Lead nitrate is soluble in water but insoluble in petrolatum and other lipids, and thus there is the possibility of migration of some of the lead salt out of the petrolatum into hydrous areas.

In this part of our work, such a potential problem was avoided by doping lanolin with lead lanolate, which is the lead salt of the naturally occurring fatty acids of lanolin, at the level of 1.3% of lead as Pb. Lead lanolate has good solubility in lanolin but negligible solubility in water, and the possibility of migration into hydrous areas may be assumed to be negligible. There is at least the possibility of selective migration of lead salt into other lipids, but there seems to be no reason why these should have a greater affinity for lead salt than in the case of lanolin. Moreover, in a later section of this work, a different lead salt was used (lead oleate). Despite the fundamental differences between oleate and lanolate salts, results appeared to be similar.

In all stages of our work, the biological treatment and electron microscopy of specimens was carried out by Dr. Ashley J. Wilson at the Centre for Cell and Tissue Research, University of York, England, in cooperation with and under the sponsorship of Westbrook Lanolin Company. Lanolin used in the work was anhydrous lanolin Eur. Pharm.; the lead lanolate was prepared in house by base exchange between aqueous solutions of sodium lanolate and lead nitrate.

STAGE 1, SECTION 1

Methodology. A 50-mm square of inner forearm of a male volunteer subject was lightly delineated and 50 mg of undoped lanolin was gently rubbed into the test area for one minute. After five minutes, small portions of lanolin were carefully scraped from the surface of the skin, avoiding any abrasion, and prepared for freeze-fracture studies. As controls, specimens of the original lanolin and of lanolin containing 25% w:w of water (as a mechanically prepared w/o emulsion) were similarly examined. Samples were put into a pair of hollow rivets held together by special forceps, the whole assembly being rapidly frozen by quenching in subcooled nitrogen at 69 K. Samples were fractured and replicated in a Leybold Heraeus Biotech 2005 freeze-fracture apparatus. Platinum carbon was evaporated at an angle of 45°, with carbon from above to strengthen the replicas. These were then cleaned of lanolin in trichloroethylene, followed by 5% sodium hypochlorite solution, and examined in a JEOL 1200 EX transmission electron microscope.

Results. Specimens of original anhydrous lanolin showed fracture surfaces composed of a mix of relatively amorphous solid lanolin and areas of crystalline lamellae thought to be fractions of lanolin that had been liquid at room temperature.

Freeze-fractured 25% hydrous lanolin showed fracture surfaces of similar general appearance but superimposed on it were spherical water droplets varying in diameter from 0.5 to 3 μm. Lanolin removed from skin after five minutes likewise showed spherical water droplets of 50 to 300 nm in diameter (Figure 1), presumably epidermal water which had passed from the skin surface into the lanolin layer where it formed a w/o emulsion. These droplets formed *in vivo* are of the same order of magnitude as the water spontaneously emulsified in lanolin *in vitro*, as previously reported (2).

STAGE 1, SECTION 2

Methodology. Full-thickness skin resulting from breast reduction operations was used, after removing subcutaneous fat down to the dermis. Prior to actual use, the skin samples were kept moist by saline-soaked swabs. From each piece of skin, three circular specimens of 20-mm diameter were punched, and two of these were clamped in holders with the dermis side of the skin held in contact with a wad of three discs of filter paper soaked in saline solution. The top plate of the holder ensured that a defined area of 1 sq cm was exposed to the atmosphere. About 4 mg of lanolin doped with lead lanolate was applied to each exposed area and spread evenly by means of a piece of surplus skin. The third disc of skin was also mounted in a holder but left untreated as a control. All three holders were placed in a 25-cm diameter desiccator fitted with a hair hygrometer. The

Figure 1. Freeze-fractured lanolin showing emulsified transepidermal water.

base of the desiccator held a saturated solution of calcium chloride dihydrate to provide a relative humidity of 38% within the chamber. The desiccator was kept in a constant-temperature room at 37°C. Treated skin was sampled after 16 hours. At the end of the treatment period, skin was removed from the holders, and small, roughly cubical pieces about 1.5 mm per side were cut for fixation by the standard operating procedure as follows:

Primary fixation:	3% Glutaraldehyde solution in phosphate buffer
Buffer wash:	0.1 M Mixed phosphate buffer at pH 7.2
Secondary fixation:	1% Solution of osmium tetroxide in buffer
Wash:	Distilled water
Dehydration:	Ethanol series 50%, 70%, 90%, 100% (twice)
Infiltration:	Propylene oxide 50:50 Propylene oxide: resin 100% Resin at 37°C 100% Resin
Embedding:	100% Resin in flat embedding molds Polymerized at 65°C
Sectioning:	By Reichert OMU2 ultramicrotome
Staining:	Some sections unstained, others stained in uranyl acetate and lead citrate solutions
Observations and microscopy:	JEOL 1200 EX transmission electron microscope
Freeze-substitution medium:	Methyl alcohol (100%) 100 ml

Uranyl acetate 0.5 g
Osmium tetroxide 1.0 g
Glutaraldehyde stock solution (50%) 6.0 ml

Results. Figure 2 is a section of the outer layers of stratum corneum showing an excess of lanolin (grey) remaining on the surface (bottom of picture) after 16 hours, and also some lanolin (similarly grey) that has penetrated into intercellular spaces down to the third or fourth corneocyte. A dark line following the edge of some corneocytes suggests a particularly high concentration of lanolin (or at least lead marker) at those points, as though lanolin had become incorporated in the trilaminar outer membranes of corneocytes that in places had become detached from the cell. Most other intercellular lipids appear to have been lost during specimen processing. The surface lanolin is seen to contain some very electron-opaque particles, which are believed to be, in part, a residual impurity of unreacted lead nitrate in the doped lanolin. An examination of a thin film of this lanolin by electron microscopy showed similar small, very dense clumps. Figure 3 is of another skin section at greater magnification and shows even more clearly an accumulation of lanolin (and/or lead marker) in laminar structures.

The largest such feature is particularly interesting since is shows a distinct triple layer consisting of a central band about 200 Å thick sandwiched between two narrower (80 to 100 Å) bands that are more electron-opaque. This structure is in accordance with the findings of Swanbeck (9), who proposed the existence of fibrous protein layers surrounded by lipid bilayers, which, in our own case, have apparently been infiltrated by

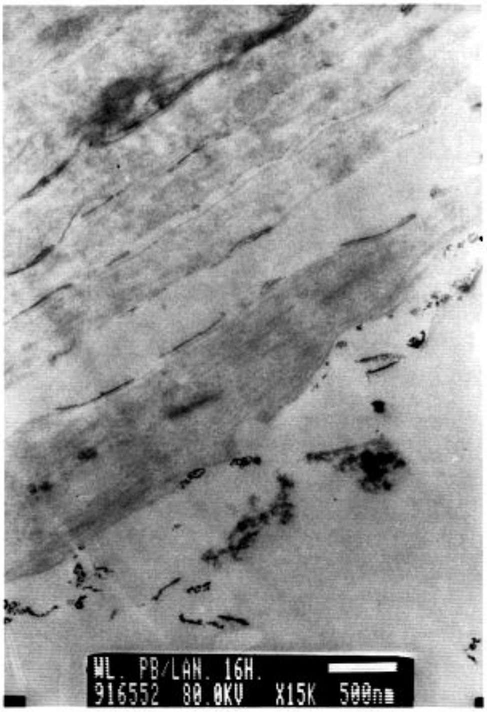

Figure 2. Lanolin on skin surface (bottom of picture) showing penetration into intercellular spaces and concentration at cell surfaces.

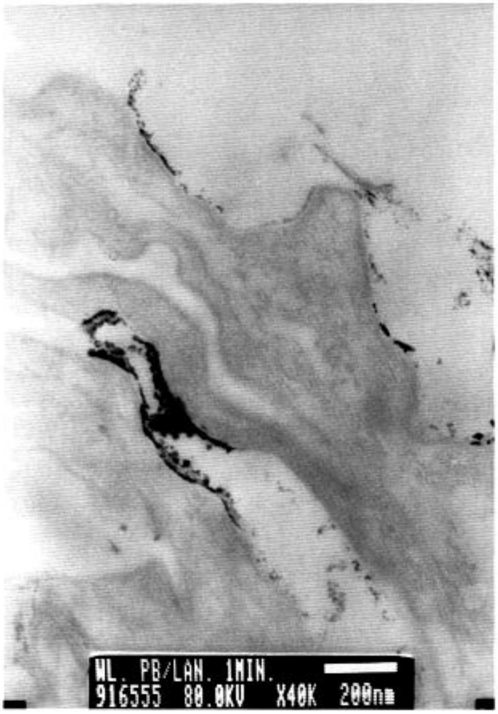

Figure 3. Concentration of lanolin/marker in trilaminar membrane of cell envelope.

lanolin and/or marker. The dimensions we see certainly agree well with Swanbeck's measurements. This trilaminar structure with a central protein band has been disputed, but lipid layers with a spacing of 50 to 80 Å were reported by Friberg *et al.* (10), who also showed that interaction of other lipids with the bilayers could affect the spacing. Other work reported by Ward and du Reau (11) also showed an alteration in spacing by the solubilization of oleyl alcohol into the bilayers and consequent increase in water capacity. It is possible in our case, therefore, that incorporation of applied substance into the lamellae has altered the spacing of lipid layers from that reported by Friberg *et al.* Trilaminar structures can also be detected in Figure 4, and here too, as in Figure 3, some of them have become detached from the cell surface. Trilaminar membranes have been previously reported by Odland and Holbrook (12) and Elias (13), and are now an accepted structural feature. In our case it appears that although most of the natural intercellular lipids have been lost during preparation of the specimens (except perhaps in Figure 10), the lamellae covalently bound to the corneocyte surfaces appear to have survived in places, although some have been detached from the cell surface. In an attempt to overcome this loss of lipids, further samples were prepared using a freeze-substitution technique that has been reported to fix and retain better, at low temperature, the natural lipids. This does not appear to have been so in our case, however, with most intercellular spaces still showing absence of natural lipids. Nevertheless, some interesting features are visible.

Figure 5 shows that lanolin (grey) has penetrated here into the intercellular spaces down as far as the stratum granulosum. Such stained material was not visible in the untreated

Figure 4. Lanolin/marker incorporated in membranes attached to, and detached from, the skin surface.

control (Figure 6), where the intercellular spaces appear white. There also appears to be a heavy concentration of electron-opaque material at a junction between the corneocytes (arrowed) in Figure 5 (perhaps a desmosome remnant), and more evidence of this is reported in Stage 3.

STAGE 3

Outline and methodology. Although this was basically a repeat of Stage 2, Section 2, freeze substitution was used throughout in the preparation of specimens, and there was a change in the lead-doping technique. Instead of using lead lanolate, doping was carried out with lead oleate, which has better lipid solubility and is easier to wash free from residual inorganic lead salts. Examination of thin films of the lead-oleate–doped lanolin by electron microscopy showed it to be quite homogeneous, with no dense particles. Tissue specimens were not stained, so that electron-opaque areas indicate the presence of lead oleate and presumably, therefore, of lanolin, unless selective migration of lead salt into other lipids has occurred, against expectation.

Results. Figure 7 confirms the penetration of lanolin deep into the stratum corneum. It also confirms findings reported in Stage 1, Section 2, of the existence of a structure with high lead and presumably lanolin content forming a layer on, or near, the surface of some corneocytes bounding intercellular spaces, more clearly seen in an enlargement of the area marked 'L' in Figure 8. Similar layers on or close to, and parallel with, corneocyte surfaces are very clear in Figure 9 and 10, where signs of a lamellar structure

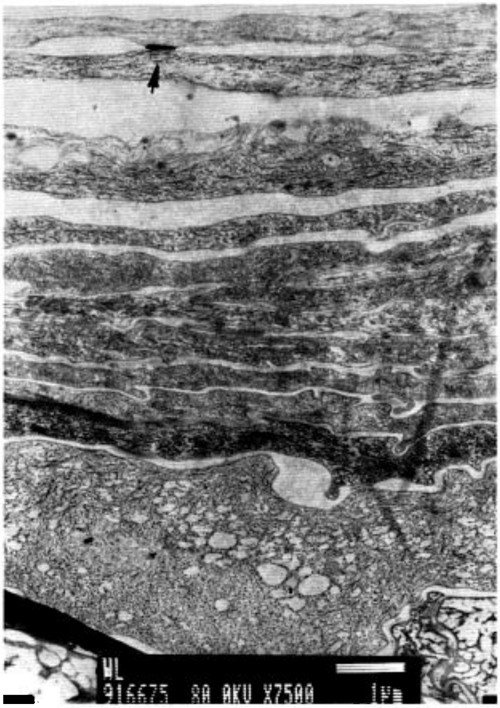

Figure 5. Concentration of lanolin/marker (arrowed) at cell junction, and lanolin visible in intercellular spaces down to the stratum granulosum.

can be seen. Also visible in Figure 10 is apparent evidence of water droplets emulsified in the lanolin.

The corneocytes themselves appear to be permeable to lanolin. Figures 11 and 12 show what seem to be concentrated accretions of lanolin (arrowed) within some corneocytes. The penetration of corneocytes by cholesterol was postulated by Garson *et al.* (14). Figure 12 also shows (arrows) concentrations of electron-opaque material at cell surfaces.

One of the objects of our work was to investigate the possibility of spontaneous emulsification of water in lanolin absorbed in stratum corneum, as is known to occur *in vitro*. Such an effect appears to be manifested not only in Figure 10 but also in Figure 13 (arrows), showing a mass of lanolin within an intercellular space, which is honeycombed with holes up to about 40 nm in diameter. These could be where emulsified water droplets had existed before the fixation of the specimen. Lead oleate without lanolin would not be expected to show this effect. The size of droplet observed is a close approximation to that of many of those shown in Figure 1, where epidermal water had migrated into a lanolin film on the skin surface, and strengthens the evidence pointing to spontaneous emulsion formation within the stratum corneum. If this does in fact occur, then lanolin would seem to provide a second mechanism by which essential moisture can be stored within the stratum corneum, in addition to the water held in the lipid bilayer structures.

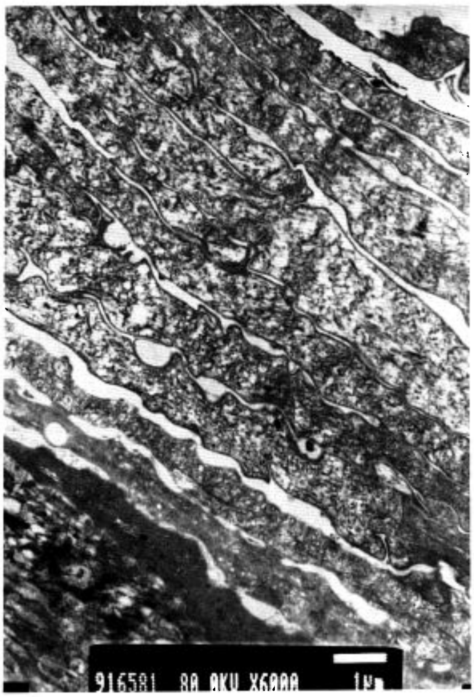

Figure 6. Section of control tissue showing no staining or electron-opaque material in intercellular spaces.

Figure 7. Concentration of lanolin/marker on cell surfaces bounding intercellular spaces L, M, and N.

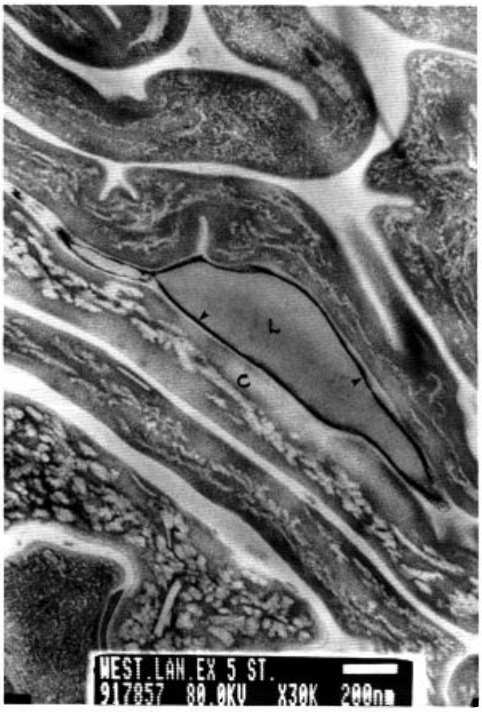

Figure 8. Enlargement of area L from Figure 11. (C indicates corneocyte about 11 layers from the surface.)

Figure 9. Lanolin/marker apparently concentrated in detached cell envelope membrane.

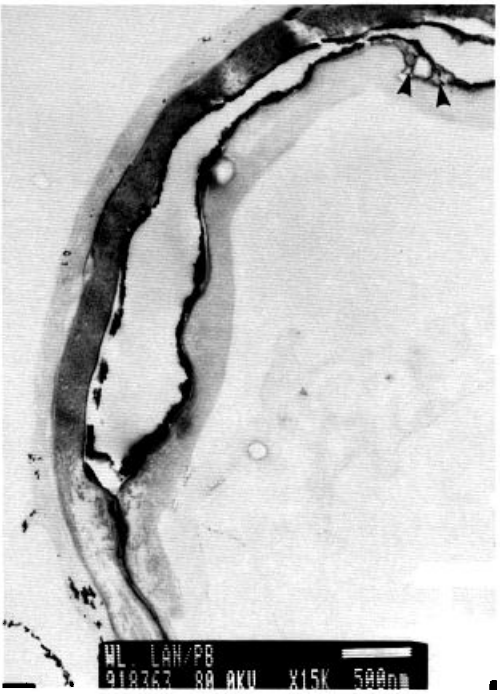

Figure 10. Lanolin/marker concentrated in trilaminar membrane, and possible emulsified water droplets in lanolin (arrowed).

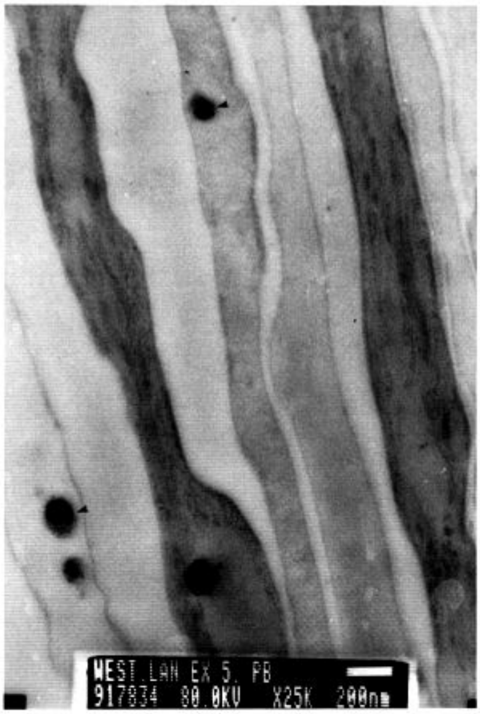

Figure 11. Accretions (arrowed) of lanolin/marker within corneocytes.

Figure 12. Lanolin penetration into intercellular spaces and apparent concentration at cell surfaces.

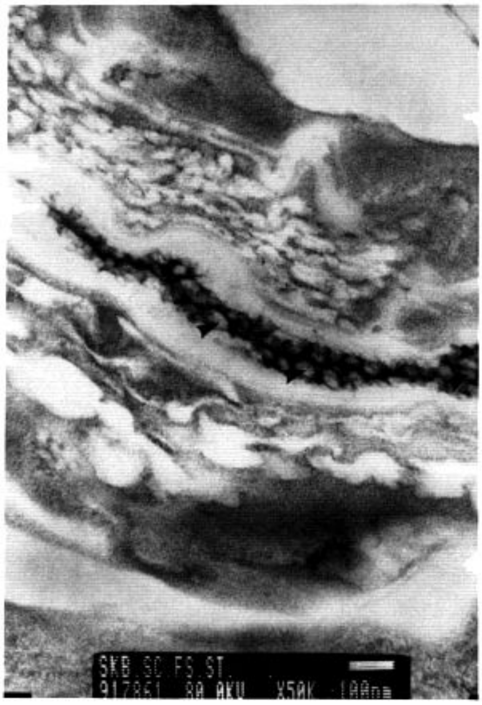

Figure 13. Evidence of emulsified water droplets in lanolin in intercellular spaces (arrowed).

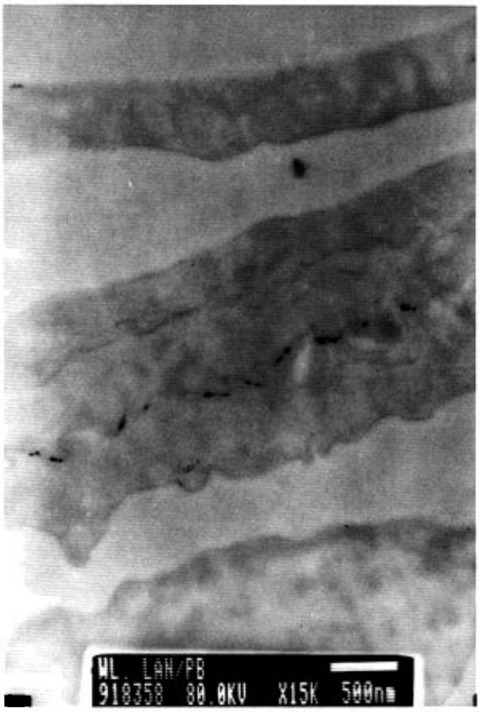

Figure 14. Lanolin/marker concentrated at long area of cell contact.

A feature of Figure 14 is a long line of contact between two corneocytes where lanolin or marker (arrows) has concentrated, presumably in the lipid layer on the surface of corneocytes. Evidence of this was seen earlier in Figures 2 and 12.

Figure 15 is also interesting, showing distinctly a number of short trilaminar structures, with a spacing of 25 to 50 nm, which have taken up a high concentration of lanolin or lead salt. These apparently lie within corneocytes about three layers down from the surface of the skin. This spacing seems too great for intercellular lipid/water bilayers, but it is closer to that of cell envelope material, although how fragments of envelope could penetrate to the interior of the corneocytes is not clear. It may perhaps be evidence of a lipid layer surrounding a short protein fibril.

CONCLUSIONS

Evidence has been found that is in agreement with the postulated mechanisms by which lanolin could act as an emollient and moisturizer:

1. Lanolin applied to the stratum corneum penetrates throughout its full depth, down to the stratum granulosum.
2. Lanolin located in intercellular spaces appears to form spontaneously a w/o emulsion with some of the epidermal water.
3. Lanolin (or lead marker) can penetrate to the interior of corneocytes.
4. Lanolin and/or lead marker appears to have the power to become incorporated in the

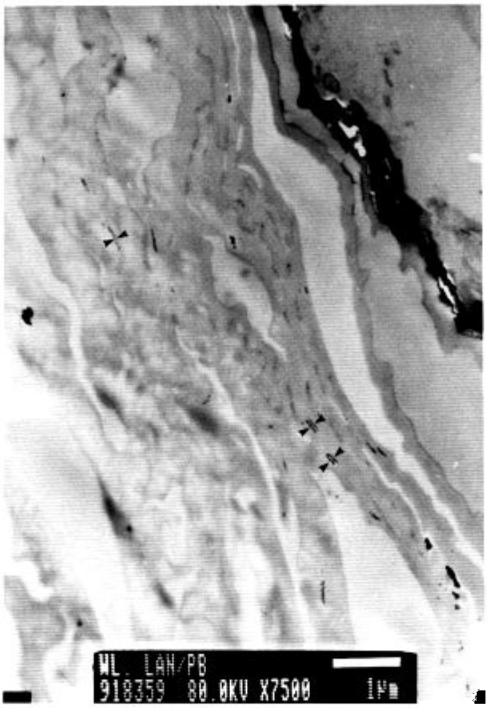

Figure 15. Small, apparently intracellular, laminar structures that contain lanolin/marker in their outer layers.

intercellular lipid bilayers, at least (apparently) in trilaminar structures forming the outer envelope of corneocytes.

This work suggests that lanolin achieves its moisturizing action by absorption deep into the stratum corneum. Once there, whether its action is partial occlusion of natural water pathways, or due to being taken up into natural lipid bilayers, or due to providing an additional water reservoir, is not yet entirely clear. There is, however, some evidence suggesting that lanolin may penetrate corneocytes and become incorporated into laminar structures such as cell envelope material. Evidence suggesting the spontaneous emulsification of epidermal water by intercellular lanolin has been seen.

ACKNOWLEDGMENT

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Near-infrared spectroscopy: A new approach to the characterization of dry skin

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Synopsis

A few years ago, Professor A. M. Kligman posed the question "Is dry skin dry?" Since that time, our knowledge of dry skin has improved considerably, but it is still not clear whether or not the water content of dry skin is lower than that of normal skin. This is, however, a fundamental question that involves one of the most important problems in cosmetics.

In the near infrared, water molecules show two clear absorption bands at 1450 and 1936 nm. The amplitudes are sufficiently high for them to be easily identifiable in the spectrum of the human skin *in vivo*. We have modified a commercial apparatus (Infra-Alyser 500; Bran and Luebbe) in order to obtain a system suited to spectroscopic analyses of all skin sites *in vivo*. Special optic fibers with a very low coefficient of attenuation and an integration sphere have been added to the original apparatus without modifying its capacities of function.

In a first set of *in vitro* experiments, we examined the penetration of IR waves into the various skin layers. The results showed the role of the stratum corneum (SC) in the amplitude of the two water peaks and that the amplitude is proportional to the water content.

The dry skin type known as winter xerosis is characterized by a marked alteration of the appearance and surface state of the SC. This led us to describe the clinical condition in terms of the following criteria: inflammation, roughness (smooth/rough), and presence of flakes and scales. We attributed a score of 0–4, and an overall score was then calculated. The water content of the skin was measured in the same area (external surface of the leg) in a number of subjects, using two biophysical methods—near-infrared spectroscopy and an electrical conductance method that provides only a very indirect measurement of water content. Correlations between the biophysical measurements and the clinical scores showed clearly that the skin judged as being the dryest gave the lowest peak amplitudes in the near infrared. This correlation was much better than that obtained using the clinical scores and the conductance method, particularly for very dry skin. A more thorough analysis of the correlations between the clinical scores and the NIR parameters provides further information on this phenomenon.

From the previous population, three groups of 19 subjects with very dry skin were further studied. The subjects in each group applied a moisturizing preparation to one leg, the other leg serving as a control. The state of the skin was tested after four weeks of daily application by means of the near-infrared and conductance methods as well as by using the clinical scoring system. The results indicate that the water content of the skin and its conductance changed in the direction opposite to the macroscopic aspect. Near infrared spectroscopy emerged as the most sensitive method for evaluating the efficacy of the preparations. We present the overall results in terms of the clinical description of dry skin, together with the actual efficacy of the moisturizing preparation relative to the expert evaluation.

INTRODUCTION

Despite thorough descriptions of dry skin, its causes are still poorly understood. Several biophysical methods are now available to characterize the state of the skin *in vivo* (1), but although progress has been made in our understanding of it functional properties (2), little is known of its precise nature, particularly with regard to lipid content, the reported variations of which are only minor (3,4). The fundamental question is whether or not dry skin is dehydrated. The lack of informative data is due to the fact that the numerous noninvasive methods used to described skin condition and to determine the efficacy of cosmetic preparations are based on measurements of physical parameters that are themselves dependent on the presence of water, lipids, and other components. In addition, the variation of the physical parameters studied is rarely directly related to differences in water content. In other words, these methods can provide adequate answers to the question "How much?" but not to the question "Why?"

Although water is the main determinant of the overall physical properties of the stratum corneum, other components can play an important role. This is particularly the case of lipids, which modify the relationship between light diffusion, reflection, and transmission, and which also influence the surface properties of the skin; in addition, they can give a smooth or rough feeling according to their nature. Cosmetics are complex compositions of numerous ingredients, but all are composed of water and oil. Most cosmetic products improve the smoothness of the skin and its overall condition, but we are not yet in a position to describe how they do so.

Infrared spectroscopy (5) can, in principle, provide valuable information on the water content of the skin. However, the infrared spectrum of water is wide and poorly defined. The amide I and II bands, the amplitudes of which are modified by the presence of water, are only indirect markers. In practice, measurements are made via a prism within which the rays undergo total reflection and show only poor penetration (less than one micron) into the tissue studied. Furthermore, the measurements take a considerable time, and the occlusive effect of the prism on the skin influences the water content.

The near-infrared spectrum (1100–2500 nm) appears to be more practical, since the water bands are precise. Two types of apparatus—an infrared sensor and a data analyser—are required; in addition, the radiation re-emitted by the skin cannot be determined precisely because of the nature of the applicator (6).

To overcome these difficulties, we have totally modified the optical elements of an integrating sphere of a near-infrared spectrophotometer (Infra-Alyser 500) in such a way as to record spectra at any skin site in a routine manner. We have conducted a study of the true nature of winter xerosis and the efficacy of various treatments, comparing the results with those provided by a simple and widely used electrical conductance method. In addition, the skin was characterized by trained experts in terms of several clinical criteria, both before and after four weeks of treatment. On the basis of the results obtained, we attempted to answer the following questions:

- Is dry skin insufficiently hydrated, or is it simply rough?
- How good is the agreement between clinical evaluation, and results provided by the physical measurement?
- Are the results of the two physical methods redundant or complementary?

MATERIALS AND METHODS

MATERIALS

Near-infrared spectrophotometer. Diffuse-reflection infrared spectroscopy has been considerably developed since the initial work by Norris in the 1970s (7). This rapid, noninvasive method can be used to obtain spectra of solid, opaque samples. In the agro-alimentary field, it is mainly used for *in vitro* analysis of water, lipids, and proteins (7). Apart from applications aimed at identifying raw materials and analyzing finished products, most uses in cosmetology (e.g., the assessment of hydration and the efficacy of moisturizing agents) involve *in vivo* measurements. The apparatus we have adapted to spectral measurements *in vivo* is an integrating sphere spectrophotometer (Infra-Alyser 500) coupled to a PC AT microcomputer. An external integrating sphere has been added (Figure 1) so as to acquire a full energy spectrum and to optimize the signal-to-noise ratio, without modifying the basic functions.

The external integrating sphere is identical to the internal sphere and is connected to the optical elements of the Infra-Alyser 500 via two special optical fibers with a very low attenuation coefficient (<0.5 db/m) throughout the study spectrum. The reference beam and the analytical beam are collected by the optical elements through a group of

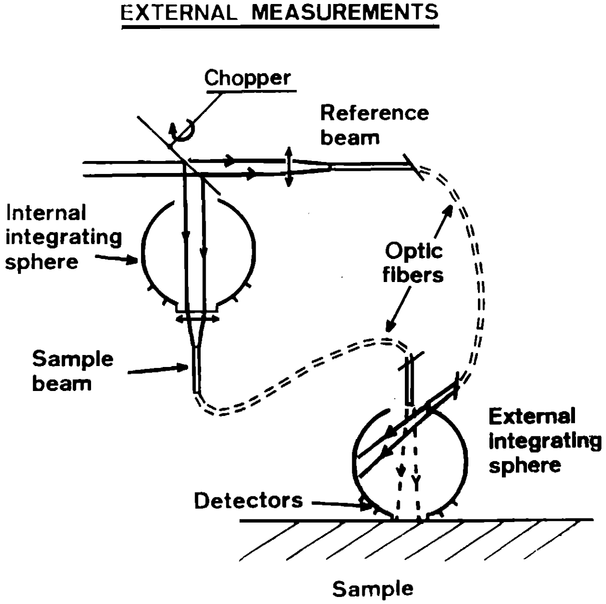

Figure 1. Modification scheme of the Infra-Alyser 500 to obtain *in vivo* measurement on all body sites.

adapted lenses, as well as under the internal sphere by a special optical system. The beam geometry (solid angles and incidence) in the external sphere is identical to that in the internal sphere. In preliminary studies, these modifications did not lead to a wavelength shift or change the absorption spectra of test materials. The fibers permit measurements to be made over the range 900–2500 nm with sufficient sensitivity, something that has not always been the case (6).

The spectra were acquired from 1100 to 2500 nm in 4-nm steps and expressed as absorbance (log 1/R), where R is the ratio between the energy reflected on the walls of the sphere and that retrodiffused by the sample. For homogeneous samples with little absorbance and no diffusion, the concentration of an analyte can be considered directly proportional to the absorbance (Ai) at a given wavelength (λi) according to an equation based on Beer-Lambert's law:

$$C = KAi = K \log (1/Ri)$$

In practice, particularly for measurements of the skin, the phenomena are far more complex. This law is no longer applicable because of interference due to analytes other than that under study and, above all, to diffusion due to the granulometry or surface state of the skin. The effects of this diffusion can be partly eliminated by calculation based on the difference in the absorbance at two wavelengths. The resulting equation is as follows:

$$C = K (A2 - A1) = K \log (R1/R2) \text{ (equation 1)}$$

Conductance measurements. Skin conductance was determined using a DermoDiag® apparatus (8), which operates at 10 MHz; the result is related to the degree of hydration, the surface state (contact impedance between the skin and the electrode), and the thickness of the stratum corneum.

EXPERIMENTAL STUDIES

In vitro. Samples of stratum corneum isolated by heat-trypsin treatment were equilibrated at various degrees of relative humidity and analyzed by means of near-infrared spectroscopy. The difference in absorbance at 1936 and 1100 nm was recorded, and the water content was deduced from the sorption isotherms determined by differential calorimetry and weighing (9). Spectroscopic analysis of the skin *in vitro* was performed before and after removal of the epidermis by heat treatment, in order to study the influence of the most superficial layers of the skin.

In vivo.

Study population. Dry skin was characterized by studying the external surface of both legs (just below the middle of the calf) in a panel of 159 women with a mean age of 40 years (range, 18–67), who had given their informed consent.

The efficacy of five cosmetic preparations was determined by studying five groups of about 20 women per group matched for age and the dryness score (>2). The treatment was applied twice daily for four weeks to one leg, the other serving as a control. Products were different O/W (products B, C, D) and W/O (products A, E) formulations containing various proportions of moisturizing agents except product D. The measurements and clinical evaluations were made at the beginning (T0) and end of the treatment period (T4).

Clinical scores. The aspect of the dry skin was evaluated by a trained expert on the basis of the following five criteria:

- "papyracé" state of the skin ("cigarette paper" aspect)
- roughness (tactile evaluation)
- presence of squames
- presence of scales ("snakeskin" aspect)
- irritation (subclinical inflammation: redness)

Each criterion was scored from 0 to 4 according to the degree of severity; half points were permitted. The average of the five criteria has been taken as the overall score of each individual. The score for each item was also calculated.

Statistical analysis. Correlations between the experimental values were sought by using Spearman's test for nonparametric variables and Pearson's test for parametric variables. Regression lines were constructed from the mean scores (half-point steps), together with the corresponding standard error of the mean (SEM). Three-factors analysis of variance, including a hierarchical analysis [time, product; subject (product)], was used to determine the statistical significance of the treatment effects, followed by the Newman-Keul test. The results are presented as means ± SEM.

RESULTS

IN VITRO STUDIES

The absorbance of the dermis was greater than that of total skin (Figure 2), the difference, i.e., the spectrum of the epidermis, showing the two characteristic water peaks at 1450 and 1936 nm. As expected, the absorbance of the skin increased after removal of the epidermis, partly because of the removal of the least-hydrated layer, the stratum corneum, and partly because of an increase in the volume analyzed due to the greater infrared penetration.

Figure 3 shows the correlation ($r = 0.98$, $p < 0.001$) between the difference in absorbance at 1936 and 1100 according to the water content of the stratum corneum. The difference in absorbance at the wavelengths used clearly increased with water content, as did the water peak (1936 nm). This difference in absorbance is computed according to the modified Beer-Lambert's law (equation 1) and is better than any other one computed using the water peak wavelengths and references.

CHARACTERIZATION OF DRY SKIN *IN VIVO*

Figure 4 shows the mean spectra obtained for the study population according to the overall clinical score. Absorbance fell gradually with the increase in the clinical score, and this was particularly marked for the two water peaks (1450 and 1936 nm) and less marked in the region between 2000 and 2500 nm.

Finally, a multiple correlation study has shown that the absorbance (1936–1100 nm) was better correlated to the dry skin score than the 1450–1100 nm one ($r = 0.789$

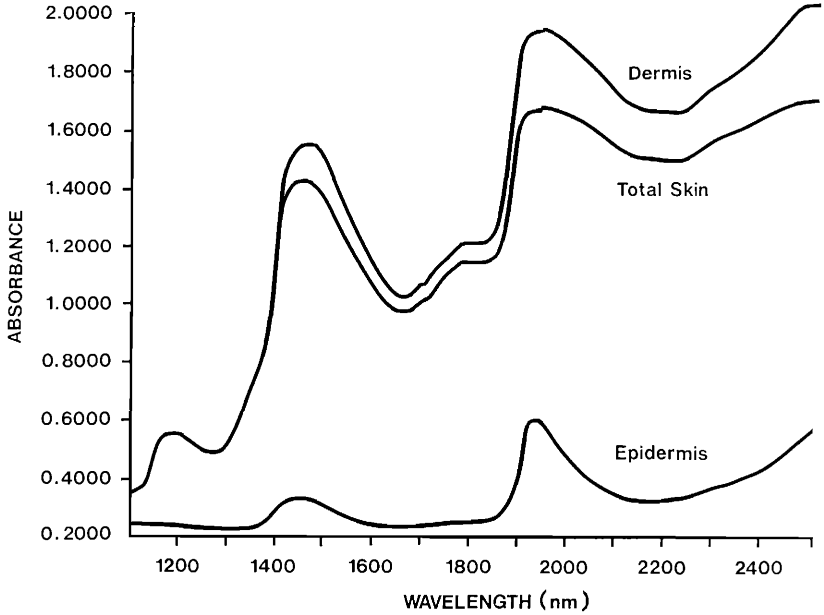

Figure 2. NIR absorption spectra of total skin, dermis, and epidermis.

versus r = −0.720 for n = 106). The band at 1100 nm is the wavelength at which the absorbance of skin is minimal. We therefore used the difference in absorbance at 1100 and 1936 nm in the rest of the study, which is, as in the *in vitro* experiment, better than other ones.

The regression line in Figure 5, constructed using the overall data obtained at T0 (before beginning treatment), is a mathematical representation of the decrease in the spectra illustrated in Figure 4. Despite the large number of values (n = 310), the correlation coefficient (r = −0.536, $p < 0.001$) remained very high.

For the overall data at T0, the changes in the overall clinical score (Figure 6) as a function of the individual "scaling" and "roughness" scores correlated strongly with the presence of scales (r = 0.76), but weakly with roughness (r = 0.43). The roughness score no longer changed above an overall score of 3, a phenomenon reflected by the correlations between absorbance and these two individual scores: the "scaling" score was still strongly correlated (r = −0.409) with IR absorbance (Figure 7a), while the "roughness" score was only weakly correlated (Figure 7b) (r = −0.344). It is worth noting that the three other descriptive scores ("papyracé," squames, irritation) are not correlated either to the difference in absorbance (r = −0.15, −0.19, −0.13) or to the overall score (r = 0.25, 0.20, 0.28).

The correlation between electrical conductance and the clinical score was significant but linear only for scores below 2.5 (Figure 8). Beyond the conductance is independent of the score. The correlations between descriptive scores values and conductance concern the "papyracé" scores (r = −0.331, $p < 0.01$) and roughness (r = −0.370, $p < 0.01$).

Figure 9 compares the overall results obtained with the five cosmetic preparations, comparing the treated and untreated legs, for the three following parameters: change in

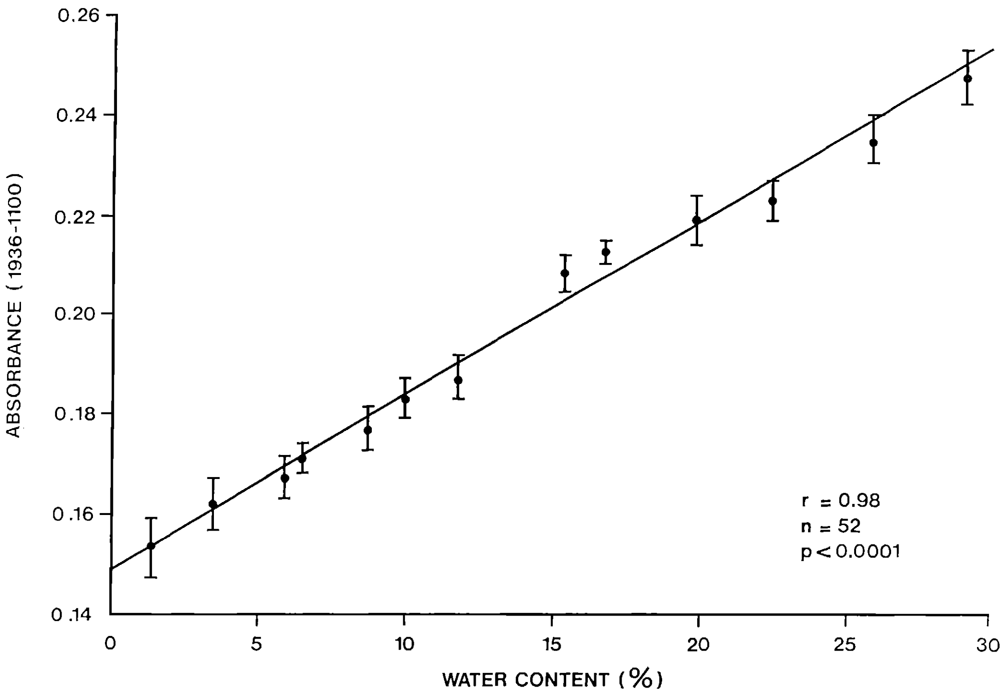

Figure 3. *In vitro* experiment: Influence of the water content of stratum corneum samples on the NIR absorption measured at 1936 nm and 1100 nm.

near-infrared absorbance, change in conductance, and change in clinical dryness score. The change in the near-infrared parameter (Figure 9a) showed that all the treatments except preparation D had statistically significant activity. Products A, C, and E were significantly more effective than product B, which, in turn, was more effective than product D. In terms of conductance, product D was again statistically ineffective, but products A, C, and E were significantly more effective than products B and D, which had comparable effects (Figure 9b). Finally, the reduction in the dryness score (Figure 9c) was significant for products A, B, C, and E, which showed similar activity, but not for product D. On the basis of near-infrared absorbance, which correlated with the clinical score, product B was distinguished from products A, C, D, and E.

DISCUSSION

With the equipment described, the near-infrared radiation penetrated deeply into the skin, although the superficial layers had a clear influence, as shown in Figures 2 and 4. The study of isolated stratum corneum samples with varying degrees of relative humidity showed that the difference in absorbance at 1936 and 1100 nm indeed reflected water content, since, for values between 0 and 30% (at which water is bound), there was a linear relationship between absorbance and water content. It was also this difference in absorbance that was best correlated with the overall skin dryness score, which, as mentioned above, was the sum of several clinical parameters (roughness, scaling, "cigarette paper" aspect, etc.).

The correlation was far better than that between conductance values and the degree of

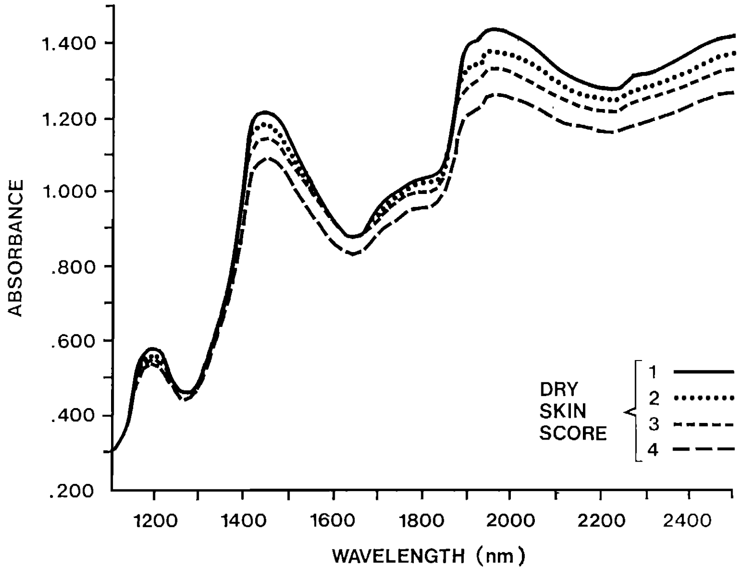

Figure 4. NIR absorption spectra of skin showing different scores of dryness (intermediate scores not presented).

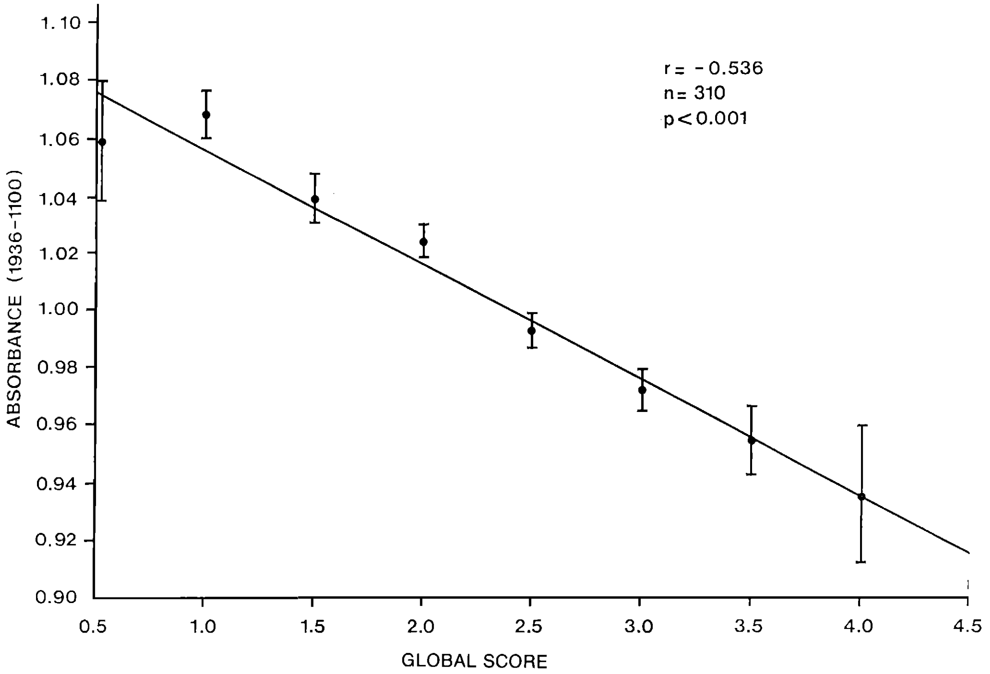

Figure 5. Linear correlation between the NIR absorbance (measured at 1936–1100 nm) and the global score.

skin dryness: above an overall score of 3, no further changes in conductance were observed. A closer examination of the clinical data shows that electrical conductance was related to the roughness of the skin: skin roughness no longer varied above an overall

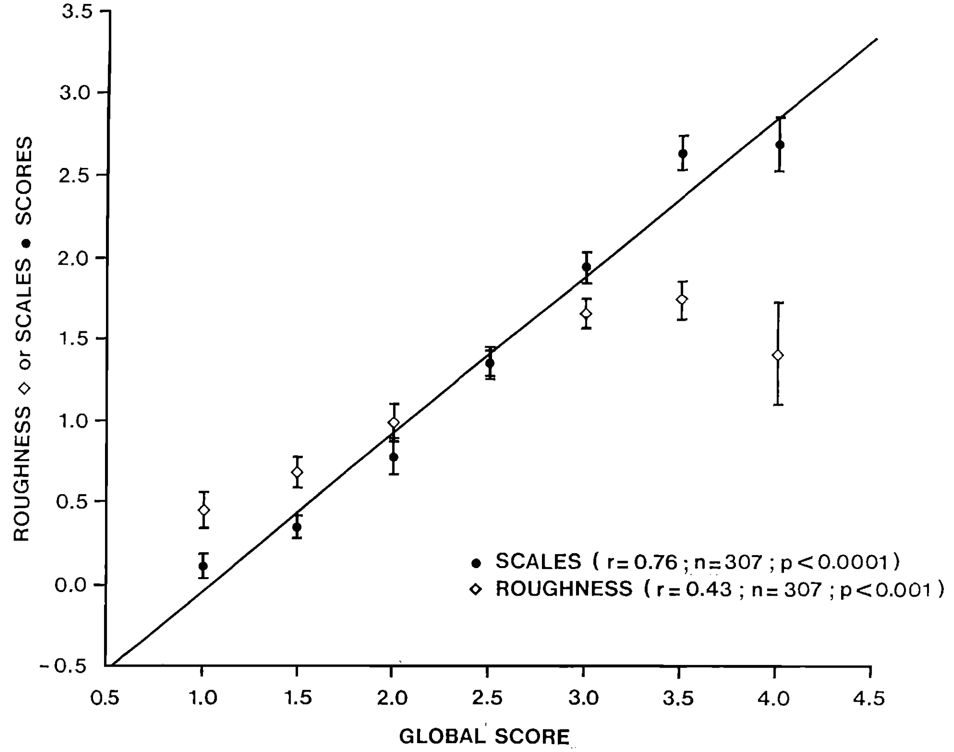

Figure 6. Correlation between roughness or scales scores and the global score (note that beyond a global score of 3, the roughness score no longer increases).

score of 3; higher scores were mainly due to marked scaling. The lack of correlation between conductance and roughness was clearly due mainly to the poor quality of the electrical contact between the skin surface and the measurement electrode. Needless to say, this poses a problem for the precise interpretation of the results of electrical conductance measurements. Such difficulties have previously been pointed out (8), but our data provide a concrete illustration of the problem.

The use of an integration sphere that collects all the radiation re-emitted probably explains why the NIR absorbance method was so sensitive. Above a clinical score of 3, scaling probably corresponds to a reorganization of the stratum corneum or the epidermis in response to relatively deep dehydration, which the Infra-Alyser recorded linearly up to scores of about 4.5.

With regard to the efficacy of the cosmetic preparations, product D, which does not contain moisturizing agents, was totally ineffective, regardless of the parameter considered. The results for the other products varied according to the measurement techniques used. The clinicians considered them to be equally effective, whereas product B was ranked between the group A/C/E and product D in the NIR method and on a level with product D in the electrical conductance method. This latter result was probably erroneous since, despite a degree of subjectivity and a lack of accuracy, the clinical scores could not have been so far from the real situation. It is more likely that the unexpected classification of product D by the impedance method was due to its lack of sensitivity above clinical scores of 3.

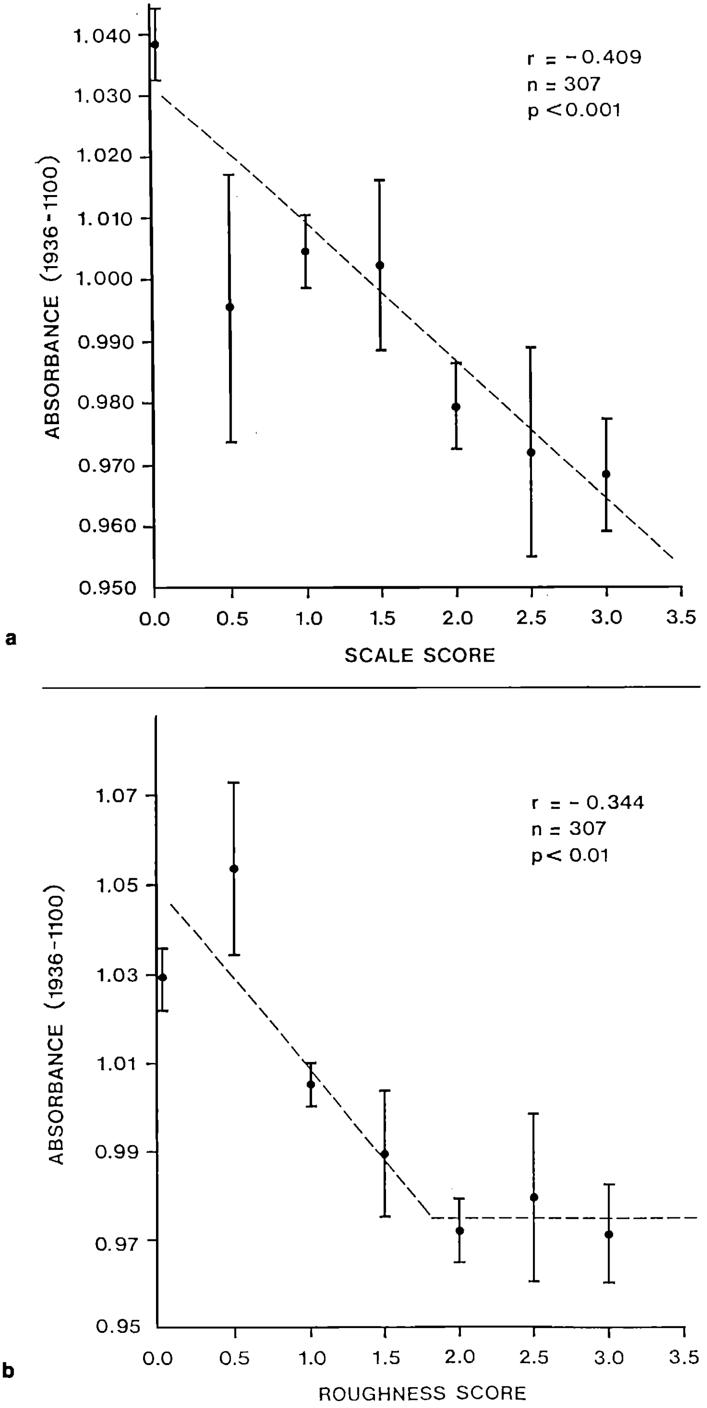

Figure 7. a: Correlation between the NIR absorbance and the "scale" score. b: Correlation between the NIR absorbance and the "roughness" score.

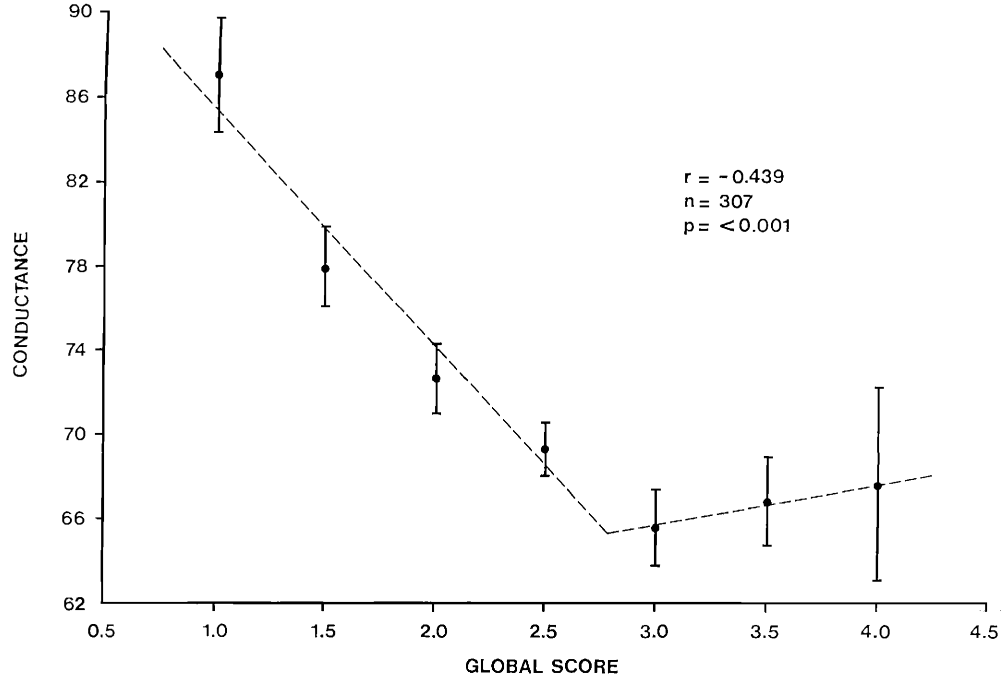

Figure 8. Correlation between the electrical conductance in voltage and the global score. The statistical parameters correspond to a linear correlation obtained with the whole data.

The correct classification of products in terms of their efficacy on dry skin was therefore given by the NIR absorption method and the clinical scoring technique. As the statistical analysis showed a significant difference between NIR results for product B and products A, C, and E, this is probably the most sensitive technique for distinguishing between different cosmetic preparations.

The conclusions of our study concern both the nature of dry skin and its assessment (and consequently the efficacy of topical agents). The results clearly show that dry skin is indeed dehydrated, and confirm hypotheses based on indirect methods showing that the stratum corneum of extremely dry skin is almost two times less elastic than that of normal skin (2). This fundamental observation should now form the basis for studies of the physiological phenomena that give rise to abnormal keratinization, and should also lead to the development of new and more effective moisturizers.

The results of the NIR absorption method, which can be applied *in vivo* to all skin sites, were strongly correlated with the clinical scores; the intrinsic property it measures can be directly interpreted in terms of water content, something that was not previously possible. Indeed, although the electrical conductance method is simple and informative, the present study clearly shows its limitations.

We have often insisted on the complementary nature of the various methods used to study the nature of dry skin and the efficacy of cosmetic products (10); the results of this work confirm our view but illustrate the exceptional value of the NIR absorption method for measuring a parameter fundamental to cosmetology, i.e., skin water content.

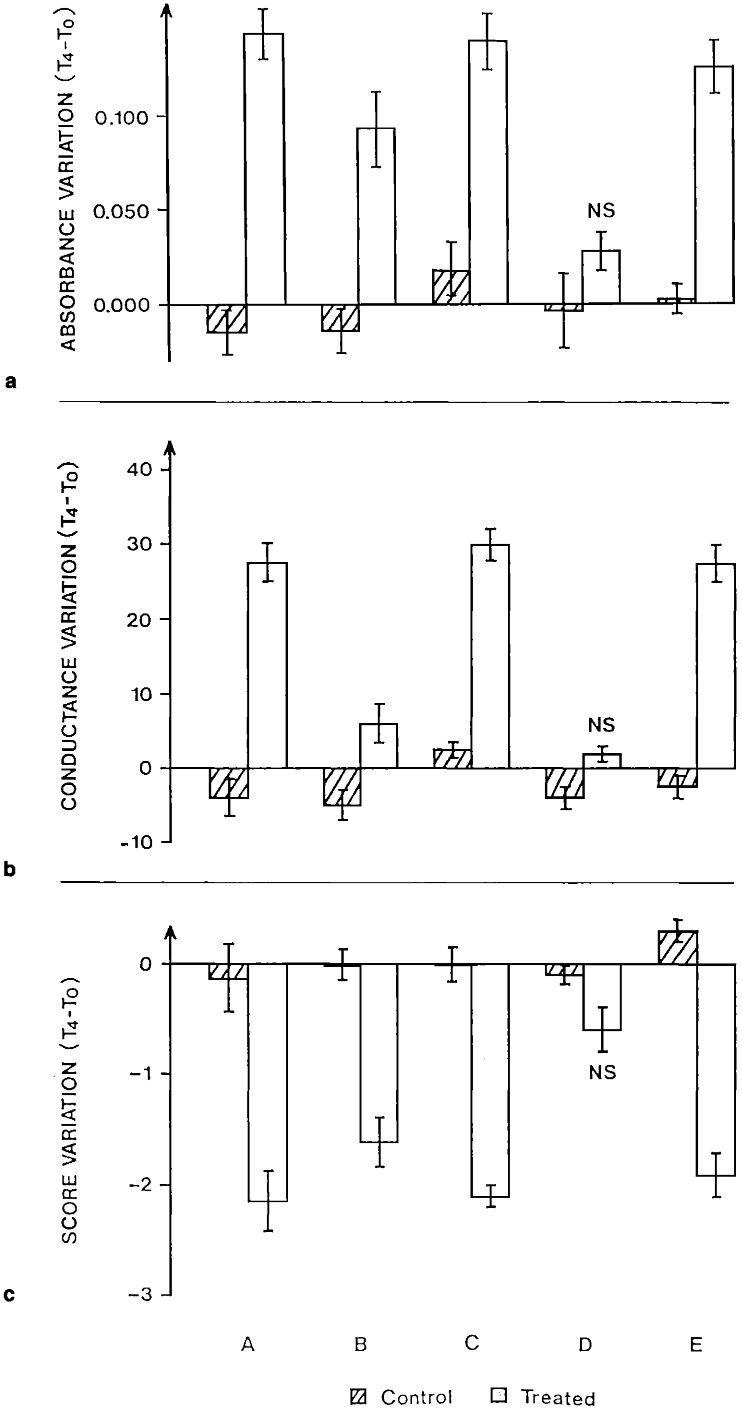

Figure 9. a: NIR absorbance variation for the control and treated leg corresponding to the treatment for 4 weeks by the five products A, B, C, D, and E. b: Conductance variation in the same conditions. c: Score variation in the same conditions.

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Growth inhibition of coryneform bacteria by a mixture of three natural products—Farnesol, glyceryl monolaurate, and phenoxyethanol: HGQ

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Synopsis

Axillary malodor is produced by secretions of the apocrine sweat glands that are contaminated by coryneform bacteria. One of the mechanisms of deodorant action is to inhibit bacterial growth. This investigation was designed to study the bactericidal effect of the mixture of three natural products, HGQ,* on wild strains of axillary coryneform bacteria. Thirty corynebacteria species could be isolated and identified biochemically from 530 underarm swabs. Their sensitivity to the HGQ mixture was determined by means of the minimum bactericidal concentration. It ranged from 0.025% to 1% HGQ. 47% of the strains were inhibited by less than 0.1% HGQ, 30% by less than 0.3%, and 23% by less than 1.0% HGQ. The sensitivity to HGQ did not correlate with specific corynebacteria strains but with the survival time of the strains, i.e., the most sensitive strains had the shortest survival times at 4°C, both on blood agar and in the stab culture.

Our studies show that HGQ, a mixture composed of three products occurring in plant or animal species, farnesol, glyceryl monolaurate, and phenoxyethanol, kills corynebacteria and that it can be recommended for use as an effective deodorant, as has already been confirmed by its successful use in practice.

HGQ and its components are biologically degradable to more than 60% within 28 days. Only CO_2 and H_2O are formed, since there is no nitrogen contained in HGQ.

Furthermore, this finding indicates that although the synergistically acting HGQ mixture has a selective bactericidal effect, it is degraded naturally after use, which is not true of the first generation of deodorants.

INTRODUCTION

The underarms play an important role in the generation of body odor. Odor is generated here from secretions of the apocrine sweat glands, which are primarily contaminated by coryneform bacteria (20). C_3-fatty acids, (iso)butyric acid, isovaleric acid, and androgen steroids such as 16,5-Ó-androsten-3β-ol and 16,5-Ó-androsten-3-1 have been identified as the odorous substances. The latter are excreted in odorless axillary sweat as water-

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* Farnesol Plus®, Dragoco Gerberding & Co. GmbH, D-3450 Holzminden, Germany.

soluble sulfates or glucuronides and then liberated by hydrolytic enzymes of bacteria and/or the skin as volatile steroids.

A gas chromatographic analysis of axillary sweat, performed parallel to a sniff test with perfumers, gave 20 different odorous substances. A few by these substances characterized only by retention times occurred in all persons studied (2). Moreover, amino acids with a characteristic odor have been identified in eccrine sweat (15).

Sweat secretion, the bacteria population, and a moist environment are the three major components contributing to odor production by the skin (4,6,14,21,22). The bacteria flora of the skin vary within broad limits both qualitatively and quantitatively (5,14). In a review, the relationships between the bacteria population, the host, and the environment have already been described (7).

From the foregoing it becomes understandable why it is possible to inhibit sweat odor by different mechanisms. These include:

- inhibition of sweat secretion by systemic administration of sedatives, ataractics, parasympatholytics, and saluretics
- application of topical antiperspirants, such as formaldehyde, glutaraldehyde (danger of sensitization), and formulations containing aluminum hydroxycholoride and tannins
- binding of odorous substances by mixtures of zinc ricinoleate and other zinc compounds that act synergistically (17)
- environmental control, e.g., by body hygiene (soap or surfactants), and by absorbent, loose underwear
- deodorizing by means of an antibacterial therapy with strong disinfectants such as halogenated phenol compounds or quarternary ammonium compounds that influence virtually all of skin flora in the same way (12)

Inhibition of esterases is an alternative mechanism of deodorant action. Glyceryl triacetate, triethyl citrate, and other rapidly saponifying esters represent substances acting according to this mechanism. However, most other esters are bacteriologically inert, i.e., have no measurable antibacterial effect, when tested according to conventional methods.

The aryl sulfatases and β-glucuronidases can also be inhibited by Cu^{++}- and Zn^{++}-compounds. For example, even concentrations of 10–100 μ Cu or zinc glycinate have an effect (3). If proliferation of bacteria is prevented by antimicrobial substances, the production of skin odor caused by bacterial decomposition of sweat is also largely reduced. However, it is still possible that the deodorant effect could be a result of a regulatory role of the substance in the biochemical processes on the skin surface.

For years the deodorant HGQ, which is recommended as a natural synergistic complex of active substances (9), has proved successful in everyday use. It is offered as a concentrate as well as a 50% solution in dipropylene glycol or ethanol, and consists of three individual components:

- 34% *farnesol,* which has been identified in cotton bud oil, cabreuva oil, musk seed oil, neroli oil, tuberose blossom oil and other volatile oils
- 11% *glyceryl monolaurate,* found in the feather fat or marabous (Leptoptilos crumeniferus)
- 53% *phenoxyethanol,* which occurs in tropical fruits, in Cichorium endivia and in Camellia sinensis (green tea)

In a concentration of 0.3%, HGQ completely inhibits the growth of *Staphylococcus aureus, Staphylococcus epidermidis,* and *Propionibacterium acnes* (10). The present study was designed to test the bactericidal properties of HGQ against wild strains of corynebacteria isolated from human axillary swabs. Based on biochemical characterization, 30 species of coryneform bacteria were identified from 530 human axillary swabs. These were tested for their sensitivity to HGQ.

MATERIALS AND METHODS

ISOLATION AND IDENTIFICATION OF CORYNEBACTERIA STRAINS

Smears freshly taken with cotton swabs from axillary skin were plated on blood and endoagar. After incubation for 24 and 48 hours at 37°C, potential corynebacteria strains were isolated as individual colonies. They were identified by their typical macromorphological appearance, being usually gray, opaque colonies, and by gram staining as gram-positive rods. Different strains were identified by examination of the following properties: catalase and oxidase production capacity, β-hemolysis on sheep blood agar, nitrate reduction, pigment formation, ureases, gelatin hydrolysis, mobility, esculin hydrolysis, serum response, and glucose, lactose, maltose, rhamnose, arabinose, trehalose, saccharose, xylose, and manitol fermentation. Tests were performed and interpreted according to methods described by Coylectal and Lipsky (1), Lenette *et al.* (13), and Lipstick *et al.* (16). Reference strains in the tests were a *C. diphtheriae* Park William-8-strain and a *C. pseudotuberculosis strain.* The purity of the strains to be identified was checked on blood agar plates before and after application of the inoculum to the test series.*

TESTING THE SENSITIVITY OF IDENTIFIED SPECIES OF CORYNEBACTERIA STRAINS TO HGQ

The only feasible method proved to be determination of the minimum bactericidal concentration (MBC). 10, 3, 1, 0.5 and 0.25% HGQ stock solutions were prepared in 70% ethanol. Nine milliliters of glucose nutrient broth (Berlin-Weißensee Institute of Immunopreparations and Nutrient Media) were added to 1 ml of each of the HGQ stock solutions, giving a final HGQ concentration of 1 to 0.025% and an ethanol content of 7%. An inoculating loop was used to inoculate the 6-h preculture, which had been adjusted to 1 million viable microorganisms/ml. An inoculated glucose nutrient broth tube with 7% ethanol served as the positive control, and uninoculated tubes of each of the HGQ concentrations were the negative controls. To ensure a maximum homogeneous distribution of HGQ in the nutrient medium, the tubes were shaken at a moderate rate during the entire 18-h incubation period at 37°C. Aliquots from the shaken culture tubes were spread on blood agar (5% human blood) by means of an inoculating loop. After incubating for 48 h at 37°C, those smears in which less than 0.1% of the original inoculum could be counted as individual colonies were taken as the MBC.

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* We are grateful to Dr. Med. Lehmann of the Institute of Medical Microbiology and Epidemiology of the University of Leipzig for his assistance with species identification.

RESULTS

The study of 530 axillary swabs and their bacteria isolates reveals a total of 30 coryneform bacteria (Figure 1). These include *C. jeikeium,* pathogenic bacteria resistant to antibiotics. All of the C. species listed in Table I are virtually destroyed in the suspension test in the range of 0.025% to 1% HGQ (99.99% reduction of the initial microbial count).

Table I shows that six of the strains studied were already inactivated by a HGQ concentration below 0.025%, another 8 below 0.1%, 9 below 0.3%, and 7 strains below a HGQ concentration of 1%. Although no correlation could be determined between the HGQ sensitivity and specific corynebacteria, there was definitely one between the HGQ sensitivity and the survival time of individual strains. Strains 1–6, which were especially sensitive to HGQ, had the shortest survival times, both on blood agar plates and in the stab culture when stored in the refrigerator.

Assessment of the inhibitory (deodorizing) effect of a formulation on skin odor should not be based on the antimicrobial range of the preparation alone. It should also include other specifically use-related tests to enable decisions appropriate to the composition of the preparation.

Deodorants are usually assessed by means of the sniff test (8), a sensory method that is based on the evaluation of the underarm odor of a consumer group consisting of at least 30 subjects by a panel of experts with a reliable judgment of odors. The active complex, HGQ, whose effect is to be evaluated in this case, is composed of three natural products that possess some bacteriostatic properties. When the whole group is evaluated after regular use of deodorants and the formulations containing the active ingredient are compared with those containing no active ingredient, the following results are obtained:

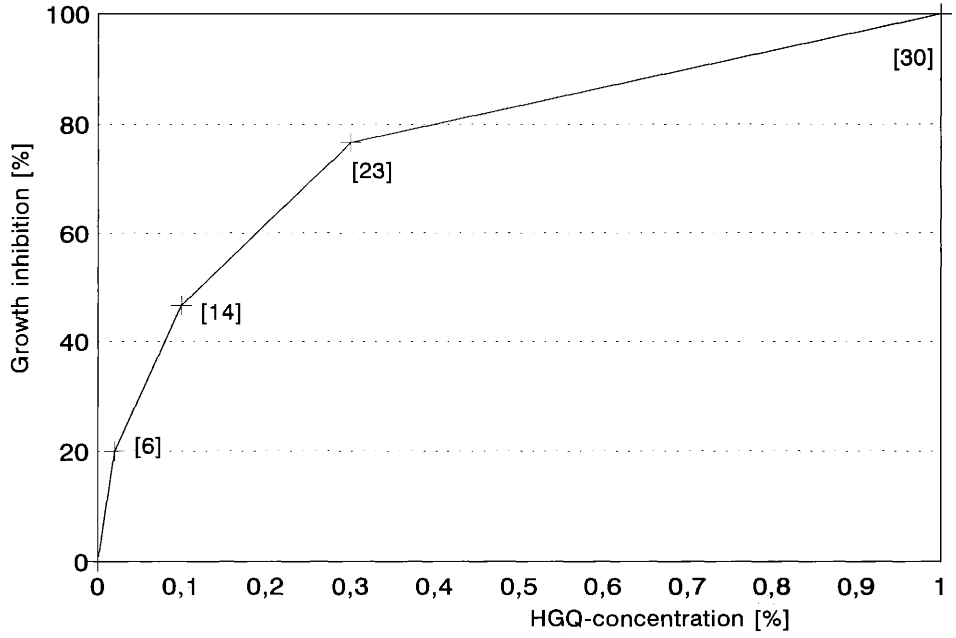

Figure 1. Minimal bactericidal concentration of HGQ against 30 strains of corynebacteria.

Table I
Minimum Bactericidal Concentration (MBC) of HGQ for 30 Species of Coryneform Bacteria Isolated From Human Underarms

Strain no.	Species	MBC (% HGQ)
1	*C. jeikeium*	<0.025
2	*C. pilosum*	<0.025
3	*C. pilosum*	<0.025
4	*C. pyogenes*	<0.025
5	Group I	<0.025
6	B-1	<0.025
7	*C. jeikeium*	0.1
8	*C. kutscheri*	0.1
9	*C. pilosum*	0.1
10	Group I	0.1
11	Group I	0.1
12	Group I	0.1
13	Group I	0.1
14	B-3	0.1
15	*C. cystidis*	0.3
16	*C. jeikeium*	0.3
17	*C. minutissimum*	0.3
18	*C. xerosis*	0.3
19	*C. xerosis*	0.3
20	*C. xerosis*	0.3
21	Group A-4	0.3
22	Group F-2	0.3
23	Group I	0.3
24	*C. jeikeium*	1.0
25	*C. xerosis*	1.0
26	Group I	1.0
27	Group I	1.0
28	Group I	1.0
29	Group I	1.0
30	B-1	1.0

the group with strong body odor, i.e., those really in need of a deodorant, give better results with soap containing the active ingredient than with a placebo (Table II, Figure 2). The differences are highly significant. The deodorant effect is comparable to that obtained with soaps containing trichlorocarbanilide (24).

It can thus be concluded (Table III) that the deodorant effect of HGQ is also comparable to that of 2,4,4'-trichloro-2'-hydroxydiphenyl ether in the practical test. The alleged natural deodorant principle does not necessarily depend on an antimicrobial effect in the sense of killing bacteria (bactericidal effect). Metabolic deactivation and growth inhibition alone (bacteriostatic effect) should also give satisfactory results.

ECOCOMPATIBILITY

HGQ and its individual components and 2,4,4'-trichloro-2'-hydroxydiphenyl ether and 2,2'-methylenebis(6-bromo-4-chlorophenol) were tested for their biodegradability. The

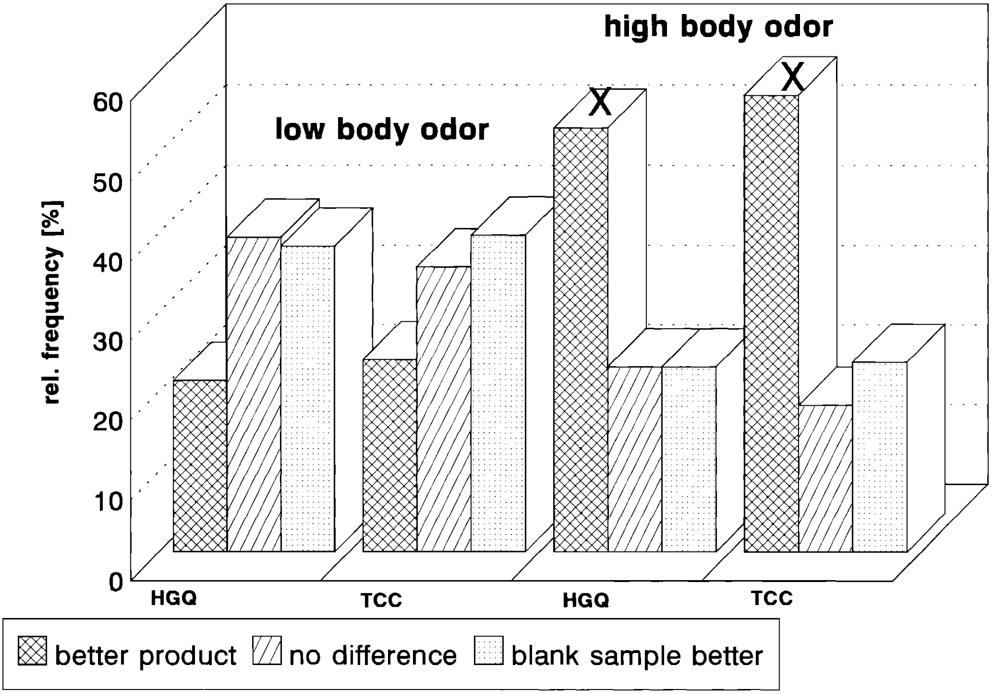

Figure 2. Comparison of the deodorant effect of soaps containing either a natural active substance mixture (HGQ) or TCC (0.26%), solubilized with nonylphenolethoxylate 9 (24) (x = statistically significant difference).

Table II
Sniff Test: Sniff Potential Assessment Scale

0	Fresh, pleasant odor (faint scent of perfume, conditioner)
1	Fresh, pleasant odor
2	Faint musty odor
3	Unpleasant, distinctly musty odor
4	Unpleasant, faint odor of sweat
5	Unpleasant, strong odor of sweat
P	Strong perfume masking everything else

The sniff potential is assessed by three experts.

latter two substances were widespread, especially in the deodorant sector, and served as the standards (4).

Biological degradability is tested by measuring the amount of oxygen required for oxidation. A distinction is made between the chemical oxygen demand (COD) and the biological oxygen demand (BOD).

Chemical oxygen demand is a constant and corresponds to the amount of chromium(VI) compounds—expressed as oxygen relative to a 1-liter sample of water—consumed under defined conditions by the reducing components of contaminated water.

Biological oxygen demand is defined as the amount of oxygen required by microorganisms from activated sludge basins for oxidative degradation of the organic substances

Table III

Results of a Comparison of Three Spray Preparations

Product A: contains 2,4,4′-trichloro-2′-hydroxydiphenyl ether (0.26%, solubilized)
Product B: contains HGQ (0.3%)
Product C: placebo

Sniff test

A><B	6 h: no difference in sniff potential
A><B	12 h: no difference in sniff potential
A><B	24 h: no difference in sniff potential

Self-assessment

Evaluation of the questionnaire revealed that the subjects reported no preference for either of the two products.

Sniff test

B><C	3 h: B significantly better than C in sniff potential
B><C	12 h: B significantly better than C in sniff potential
B><C	24 h: B significantly better than C in sniff potential

Self-assessment

Evaluation of the questionnaire revealed: After 3 h and 12 h the subjects clearly preferred B. After 24 h the subjects reported that B seemed more effective.

contained in a 1-liter water sample under given test conditions (time, temperature, etc.) (23).

The BOD subscript indicates the test period in days. The BOD_5/COD ratio is often formed, which represents the relative biological degradability.

The results of these types of test are naturally subject to variations. The information obtained on the degradation behavior, i.e., return of the component substance to the natural cycle, does, however, allow an estimation of the impact on the environment.

Two grams of each product were mechanically shaken for 24 hours, the undissolved fraction separated, and the chemical and biochemical oxygen demand of the dissolved components determined. For this reason, the results are not related to the product itself but to the aqueous solution prepared as described. The data permit estimation of the degradability on the basis of the BOD_5/COD ratio (4).

Table IV shows that the soluble fraction of 2,2′-methylenebis(6-bromo-4-chlorophenol) has a very poor biological degradability. For 2,4,4′trichloro-2′-hydroxydiphenyl ether there is apparently a total inhibition of degradation by the microbiocidal effect.

The natural product active substances named above have an acceptable degree of degradability. The degradation products are only CO_2 and H_2O, as HGQ does not contain any nitrogen. (Also see Figure 3.) Furthermore, this finding indicates that although the synergistically acting HGQ mixture has a selective bactericidal effect, it is degraded naturally after use, which is not true of the first deodorant generation.

DISCUSSION

A major component of odor production is eliminated by growth inhibition of coryneform bacteria. It seems probable that the coryneform bacteria do not themselves produce

Table IV
Test Results

Sample name	Chemical oxygen demand COD (mg/l)	Biochemical oxygen demand BOD_5 (mg/l)	BOD_5/COD	%
Glyceryl monolaurate (H)	1755	1069	0.61	61
Farnesol (G)	483	217	0.45	45
Phenoxyethanol (Q)	3205	975	0.30	30
HGQ	2880	728	0.25	25
2,4,4′-Trichloro-2′-hydroxydiphenyl ether	453	<5	—	0
2,2′-Methylenebis-(6-bromo-4-chlorophenol)	778	15	0.02	2

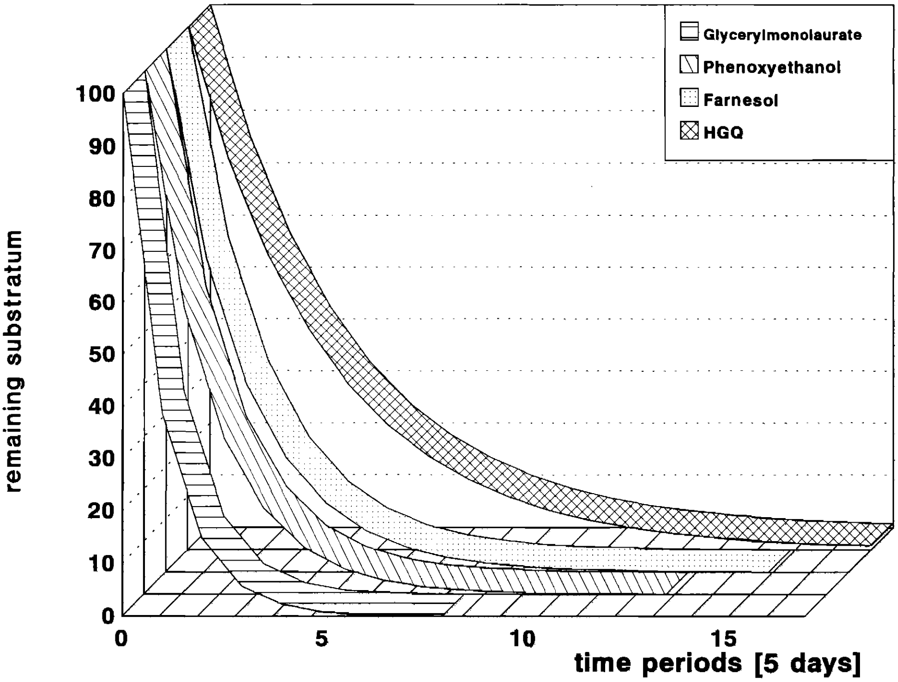

Figure 3. Biological degradability of HGQ and its ingredients (computed from BOD_5/COD).

short-chain fatty acids (18) but that their enzymes release these compounds from apocrine sweat or physiological skin surface lipids.

Apparently only coryneform bacteria are capable of releasing odorous substances such as short-chain fatty acids (19) or steroids. It is remarkable that the bacteria population of the axillae is distinguished by two types of flora. There is a coccal dominance and a corynebacteria dominance (at least 75% of all aerobic bacteria are of the coryneform type). The population density of the coryneform flora is six times that of the coccal dominance (11).

In an application concentration of 0.3% to 0.6%, HGQ acts as an effective deodorant by reliably inhibiting the growth of coryneform bacteria, the main producers of odorous substances from underarm sweat and skin surface lipids. In addition, it is composed of natural products and has shown no signs of sensitization, i.e., of allergic contact eczema (3).

HGQ and its individual natural active substances are biologically degradable to an acceptable degree. Only CO_2 and H_2O are formed, since there is no nitrogen contained in HGQ.

Furthermore, this finding indicates that although the synergistically acting HGQ mixture has a selective bactericidal action, it is degraded naturally after use, which is not true of the first generation of deodorants.

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Light scattering and shine measurements of human hair: A sensitive probe of the hair surface

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Synopsis

In this paper, an instrumental method for measuring hair shine is presented and shown to exhibit excellent correlation with a large series of subjective evaluations of shine. In addition to providing shine values, the light-scattering methods developed are shown, in many cases, to provide a sensitive means of following changes to the hair surface, including deposition (soiling), particle removal (cleaning), and even interactions on the fiber surface. Employing the developed methods, the effect of washing hair tresses with a series of commercial shampoos is investigated. The effects examined are shown to fall into three classes: Shampoos without highly substantive ingredients left hair in its cleanest and shiniest state. Shampoos containing ingredients substantive to hair (polycationics and soap) left deposits on the fiber surface and dulled the hair. The worst dulling was observed when particles deposited on the hair from one shampoo formed a complex with particles contained in a second product.

INTRODUCTION

Shiny hair is consistently cited by consumers in panels and surveys as one of the most desirable of cosmetic attributes. As a result, much effort has been expended in recent years to understand the physical phenomena that give rise to shine and also to quantitate this desirable hair attribute.

The most effective means described in the literature to study hair shine has been the use of a goniophotometer to measure light scattering by hair fibers (1–4). Goniophotometric techniques have been used by the authors of references 1–4 to relate light-scattering patterns to hair morphology and also to develop formulas relating goniophotometric data to hair shine.

Unfortunately, the bulk of the effort in the above studies has been concerned with explaining the light-scattering patterns; only cursory efforts were made to relate calculated shine values to panelists' subjective evaluations. In addition, little effort was made to systematically apply the methods developed in these papers to problems related to hair care.

In this paper, a large series of subjective evaluations of hair shine is reported and shown to exhibit excellent correlation with a formula developed to calculate hair shine using goniophotometric measurements. In addition to providing a quantitative measure of shine, the light-scattering methods developed in this work are shown to provide a means of following changes to the hair surface, including deposition (soiling and buildup), removal of particles (cleaning), and even interactions at the fiber surface.

Single-fiber techniques are presented and shown to provide a rapid, qualitative means of determining the effects on hair fibers of various treatments. The results of shine measurements of tresses exposed to various treatments of interest are then shown to confirm and make quantitative the foregoing single-fiber results.

Finally, the shine of hair tresses treated with various shampoos is measured and shown to be a function of the nature of the substantive ingredients found in particular shampoos.

The results in this paper show that light scattering can be used not only to obtain quantitative information about shine but that it can also serve as a sensitive probe of the hair surface, providing information difficult or impossible to obtain using other surface techniques.

EXPERIMENTAL

SHAMPOO EXPERIMENTS

All tresses for shampoo experiments were prepared using three grams of human hair purchased from DeMeo Brothers, New York. In order to minimize scattering from the rear cuticle, only dark brown Oriental hair was used (1).

For each comparison series, a minimum of three tresses was prepared for each shampoo treatment. In addition, three control tresses, treated only with 20% sodium lauryl sulfate (SLS) and representing "clean hair," were prepared for each series. In comparison experiments, succeeding tresses in a series received different treatments so that any particular treatment was staggered throughout the entire series.

All tresses were subjected to five pretreatment washes with SLS, followed by five treatments with a test shampoo. In those cases where shampoo combinations were employed, five treatments with the first shampoo were followed by three treatments with a second.

Pretreatment cycles were performed by immersing tresses for five minutes in 250 ml of 5% SLS in a graduated cylinder. The tresses were then removed, rubbed by hand for two minutes, and then rinsed twice for one minute each in graduated cylinders containing 500 ml of deionized water.

Treatment cycles were performed in the same manner as the above, except that a 2:1 tap water, shampoo solution was substituted for SLS, while tap water rather than deionized water was employed in rinses. Note that the tap water used in these experiments had a water hardness level of 80 ppm.

SUBJECTIVE SHINE EVALUATIONS

Subjective evaluation of tress shine was performed on groups of six tresses each, with three tresses mounted for each treatment. Figure 1 shows a photograph of the frame used for mounting tresses for subjective comparisons. Each tress is clamped at the root end, stretched over the cylinder, and secured at the tip. This setup insures that all tresses are oriented in the same manner toward the viewing light and also minimizes orientation differences among the individual hairs comprising the tresses.

Tresses were viewed under two lamps, each of which contained two cylindrical, 60-watt tungsten bulbs. The lamps were placed side by side so that all four bulbs were in a single row approximately ten inches above the tresses. This configuration maximizes the chances that each tress receives the same amount of light.

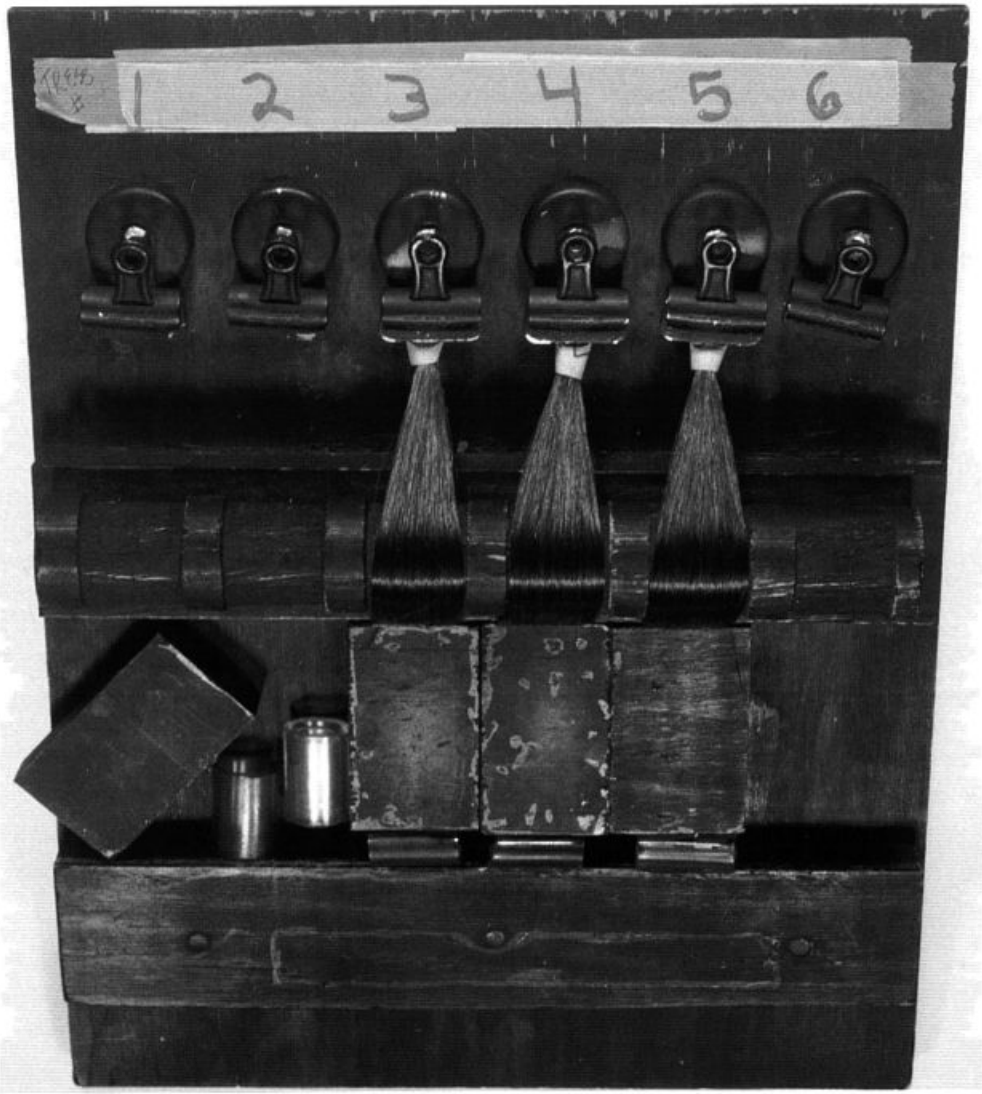

Figure 1. Photograph of frame used for subjective shine assessments. Tresses are clamped at the root end and stretched over the cylinder. A wooden block is then placed over the tip end and held secure by wedging a small piece of flexible tubing between the frame and the block.

Following mounting, tresses were evaluated by sixteen to twenty panelists, each of whom was asked to rank the tresses in order of relative shine. After evaluation, tress positions were interchanged and a new set of evaluations performed. This was done in order to minimize any positional biases.

The data from the above rankings were evaluated statistically by the Friedman test (5). This is a non-parametric test and has several advantages over the corresponding parametric methods since it does not require a normal distribution and makes no assumptions concerning variance of the data.

GONIOPHOTOMETRIC MEASUREMENTS

The bulk of the goniophotometric measurements were performed using a Brice-Phoenix light-scattering photometer (Virtis Co., Gardiner, NY), modified by attaching a recorder motor to the graduated disc in order to automate scanning of receiving angles.

Subsequent to the research described in this paper, a Murakami GP-1R automatic goniophotomer was purchased (Hunter Associates Laboratory, Inc., Reston, VA). This instrument is not subject to the scanning limitations experienced with the Brice-Phoenix photometer. The sample light-scattering curve presented in Figure 2 was produced with the GP-1R photometer.

In order to maximize the accuracy of the diffuse scattering measurement (D in Figure 2), polarizers having directions of polarization perpendicular to the plane of incidence were

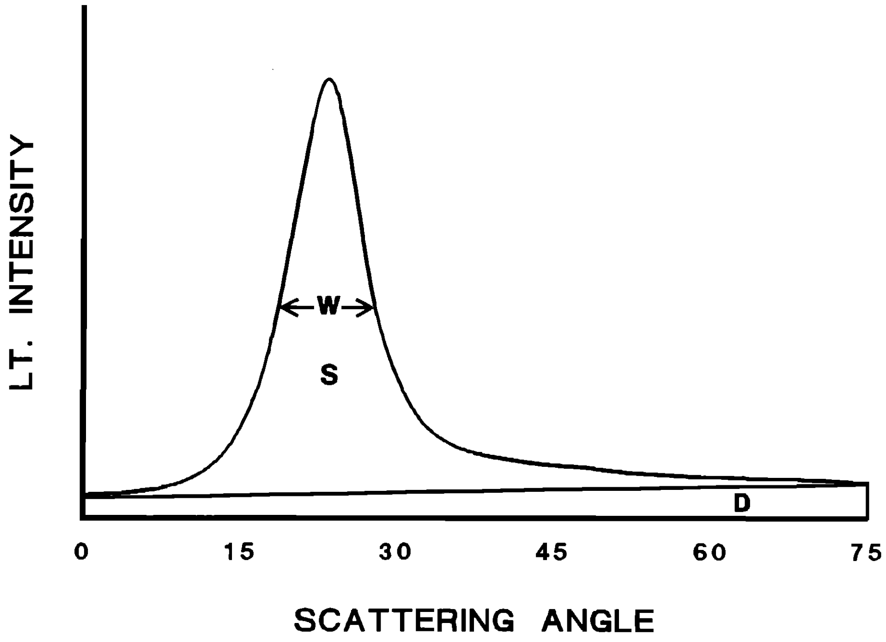

Figure 2. A typical light-scattering curve for a virgin Oriental hair. D is the diffuse reflectance and is calculated by measuring the area under the straight line connecting the light intensities at 0 and 75 degrees. S is the specular reflectance, and is the total area under the curve minus the diffuse region. W is the width of the curve at half height.

placed before the samples and photomultiplier tubes of the goniophotometer in all light-scattering experiments (2).

The sample holder for the Brice-Phoenix photometer consisted of two supports, indented at the top and 4 cm apart, across which a single hair could be laid. A sample hair fiber was secured at one end and kept under constant tension by attaching a 2-gram alligator clip to the other end. Hair fibers had to be at least four inches long to conveniently fit on the sample holder.

Because of the short distance between sample and photomultiplier tube in the Brice Phoenix photometer, samples run at an incident angle of 30° with respect to the perpendicular to the hair could only be scanned between 15° and 75°. This proved adequate for qualitative work, but for quantitative measurements, the incident angle was changed to 37.5° so that scans could be run between 7.5° and 75°. The scanning time for the latter interval was 72 seconds.

For all experiments, hair fibers were oriented so that the direction of the incident light was toward the tip end of the hair (RER orientation in reference 1).

The output from the goniophotometric measurements was sent to a Bascom-Turner 3120T electronic recorder (Bascom-Turner Instruments, Norwood, MA) that digitized each scan into 500 points. These points were then sent to an IBM PC computer for processing.

SINGLE-FIBER SCREENING TESTS

For single-fiber screening tests, single hair fibers were mounted on the goniophotometer sample holder and held taut with a 2-gram alligator clip. Treatments were then applied by dropping one or two drops of a test solution onto a glass microscope slide supported under the hair by a platform high enough so that the slide just missed touching the hair.

After application of test solutions, the slides were moved back and forth under the hair for 60 seconds. Fibers were then rinsed with tap water in the same fashion. Following this, light-scattering curves were run, and the fiber was then either treated again or discarded.

Note that some fibers are sufficiently irregular in cross section that movement of the sample holder from the instrument to the lab bench could cause the fiber to twist and change the light-scattering curve. A change in a measured curve was thus only considered to be real if it could be reproduced after movement of the fiber holder.

DYE-STAINING EXPERIMENTS

Dye-staining experiments were performed using Sirius Red F3BA New, C. I. No. 35780. This is a high-molecular-weight (1372) anionic dye and was obtained from Mobay Chemical Co., Rock Hill, SC.

Experiments were performed by wetting a 3-inch by 4.5-inch wool swatch (wool challis, Test Fabrics Inc., Middlesex, NJ) with water, applying 3 ml of a test shampoo, rubbing for one minute, and then rinsing under 100°F running tap water for one minute. Following this, swatches were either treated with dye solution or treated with 3 ml of a second shampoo, rinsed, and then treated with dye.

Dye treatments consisted of placement of a swatch in 40 ml of a 0.5% solution of dye for 30 seconds. The swatches were then rinsed under 100°F running tap water for 30 seconds and hung up to dry.

MEASUREMENT OF SHINE

Shine, or luster, is perceived when an object scatters much more light at a particular angle or in a particular direction than in other directions. Under these conditions, surface highlights or brightness contrasts appear and the object is seen to be shiny (see, for example, references 6–8).

In order to measure shine, therefore, one needs to be able to measure scattered light intensity as a function of angle. This is accomplished with a goniophotometer. Figure 3 shows, in rudimentary fashion, the operation of such an instrument with a hair fiber sample.

In this case, the fiber is assumed to be held taut and is irradiated with light at an incident angle, i, that is prechosen and is measured with respect to the perpendicular to the fiber.

The intensity of that portion of the incident light that is scattered by the hair is measured by rotating a photomultiplier tube, or light detector, from 0° to 75°. The illustration shows two of the positions of the photomultiplier tube.

Typical results from a goniophotometer measurement are shown in Figure 2 for an undamaged Oriental hair. The light-scattering scan is presented as a plot of scattered light intensity as a function of angle.

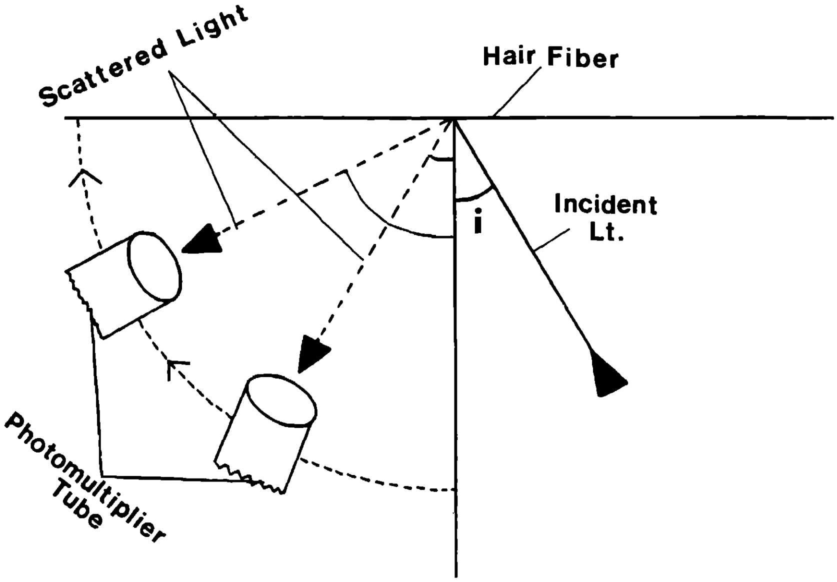

Figure 3. Rudimentary diagram of a goniophotometer experiment. A hair fiber is held taut and struck by incident light at angle i. Scattered light is then detected by rotating a photomultiplier tube from 0 to 90 degrees with respect to the perpendicular to the fiber. Two of the photomultiplier positions are shown.

The incident light in this experiment was set at 30°, and one can see a maximum in the measured curve near this angle. This is termed *specular reflection.* Light is also scattered at angles other than the specular; this is termed *diffuse reflection.* This latter type of scattering is caused by light hitting the scale edges of the hair. It can also be caused by small imperfections on the hair surface and, in addition, by deposited particles. An excellent discussion of the morphological features of hair that give rise to observed light-scattering patterns can be found in reference 1.

The question now arises as to how one can use the goniophotometric results to measure shine or luster. From the definition of shine, it appears obvious that luster increases with increasing specular reflection and decreases with increased diffuse scattering. Any functions used to estimate shine must therefore take these two relationships into account.

Several workers have developed shine functions, employing these relationships for applications such as textile fibers, polymer surfaces, etc. [see, for example, (2,6–10)].

In this work, several functions were tested both from the literature and also devised by ourselves. The best agreement with subjective evaluations was found using the relationship

$$L = S/DW(\tfrac{1}{2}) \qquad \text{(Eq. 1)}$$

where L equals luster or shine. D in this expression is the integrated diffuse reflectance and is obtained, as in reference 2, by connecting the scattered light intensities at 0° and 75° and measuring the area under the resulting line. S in equation 1 is the integrated specular reflectance and is obtained by measuring the area of the specular peak, while $W(\tfrac{1}{2})$ is the width of the specular peak at half-height. All three of these quantities are illustrated in Figure 2.

It has been pointed out (2) that use of expressions such as equation 1 with D in the denominator are valueless for cases where diffuse reflectance goes to zero. For most cases involving hair, however, scattering off the scale edges insures a minimum value for D, so that equation 1 is broadly applicable.

In the current experiments, hair fibers from treated tresses were scanned one at a time in the goniophotometer. There is tremendous variation from hair to hair, even from a single head of hair, so that in order to obtain meaningful shine values for a particular treatment, an average of many hairs must be taken. In the current case, 21 hairs were taken from each tress, while three tresses were employed for each treatment. Each shine value, therefore, represents an average taken from 63 hairs.

RESULTS AND DISCUSSION

SINGLE-FIBER SCREENING TESTS

Figures 4–6 show typical light-scattering scans taken after a series of shampoo treatments of single hair fibers. These types of single-fiber experiments are useful as a means of rapidly screening the effects of various treatments on hair.

The results from these experiments can only be treated qualitatively, however, since they represent treatments on single hairs and there is too much variation among hairs for

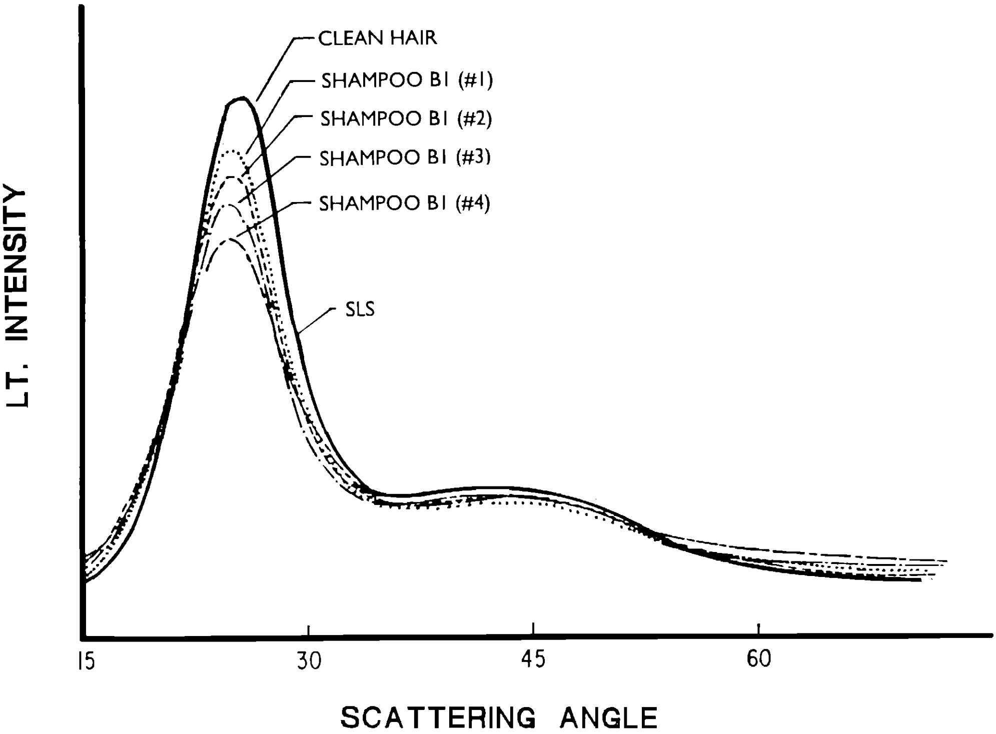

Figure 4. A single-fiber experiment testing the effect on hair of shampoo B1. The hair was washed with SLS at the beginning of the experiment (clean hair) and at the end. The same curve was obtained in both cases.

quantitative results from a single hair to be meaningful. A particular result must therefore be repeated several times on different hairs in order to be considered real.

Bearing this in mind, and also the fact that single-fiber treatment conditions are very different from tress and in-shower treatments, one can use single-fiber screening tests as a convenient means of determining the possible effects of many different products on hair. This method is especially useful when hairs are undergoing a series of treatments, since after each particular step in the series, a light-scattering scan can be run in order to determine the effect of that particular step. This can be very helpful in elucidating the mechanism of a particular effect.

Figure 4 shows the results of a series of treatments of an Oriental hair with shampoo B1. This is a commercial product that, at the time these experiments were performed, contained Polyquaternium-10, a polycationic well known to be substantive to hair, in a trideceth-7 carboxylic acid detergent system.

The hair was first washed with 20% sodium lauryl sulfate (SLS). The resultant light-scattering curve is considered that of a clean hair. Following this, successive treatments with shampoo B1 (followed by water rinses) caused dulling, indicated by decreases in the peak height along with increases in diffuse scattering. This loss of shine was caused by deposition of shampoo residue, probably Polyquaternium-10, on the hair surface.

After four treatments with B1, the hair was again treated with 20% SLS. The resulting light-scattering curve was congruent with the original, clean hair curve, implying that

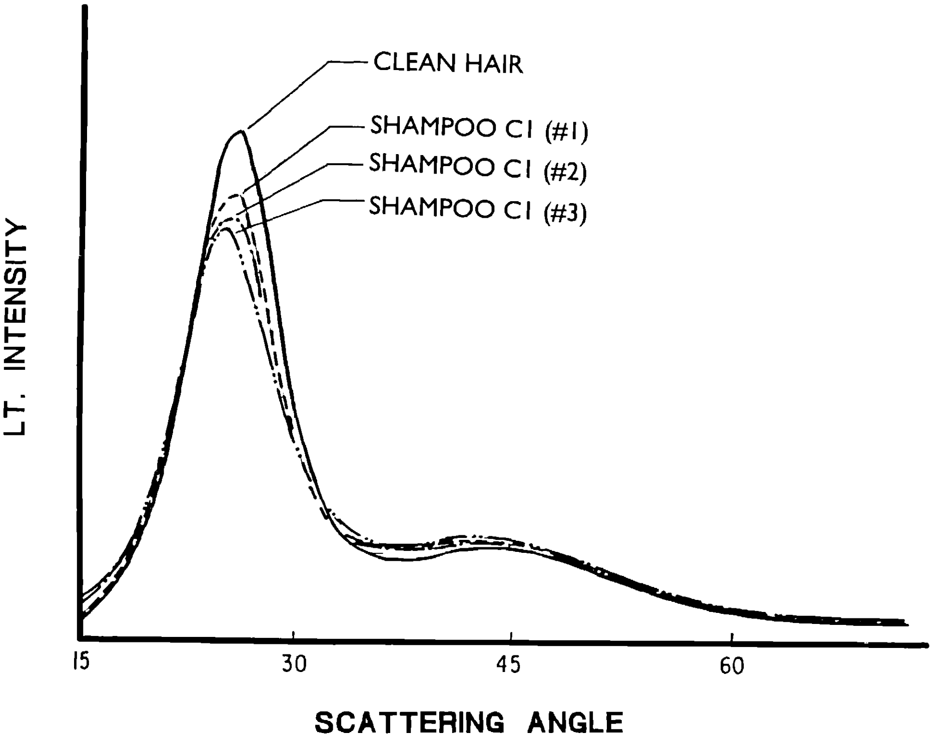

Figure 5. A single-fiber shampoo C1 experiment.

the dulling B1 residue was removed from the hair. These experiments demonstrate that light scattering can be used to follow both deposition (soiling) and removal of particles (cleaning) from the hair surface.

Figure 5 shows another single-fiber experiment using shampoo C1, a commercial product that at the time of sale contained sodium myristate, a component of soap that is well known to deposit on and dull hair (11). As with the B1 experiments, successive treatments with shampoo C1 caused dulling, in this case a result, probably, of deposition of sodium myristate particles on the hair. Again, as with the preceding experiment, after treatment of the dulled hair with 20% SLS, the original, clean hair curve was recovered.

Figure 6 shows the results of a single-fiber experiment in which a clean hair was treated twice with shampoo B1, resulting in dulling from shampoo residue. An attempt was made to clean this residue with shampoo C1, with the expectation that initially the C1 would remove accumulated B1 residue and that further applications would lead to accumulation of new C1 deposits.

Surprisingly, application of shampoo C1 did not lead to an initial increase in shine but, unexpectedly, to a great increase in dullness, much larger than the sum of the individual shampoo effects. This implies that the deposits from the two shampoos are interacting to form a new residue that is more dulling to the hair than the former deposits. That a new type of residue has formed is also supported by the observation that attempting to reverse the increased dullness by washing with SLS did not lead to a change in the

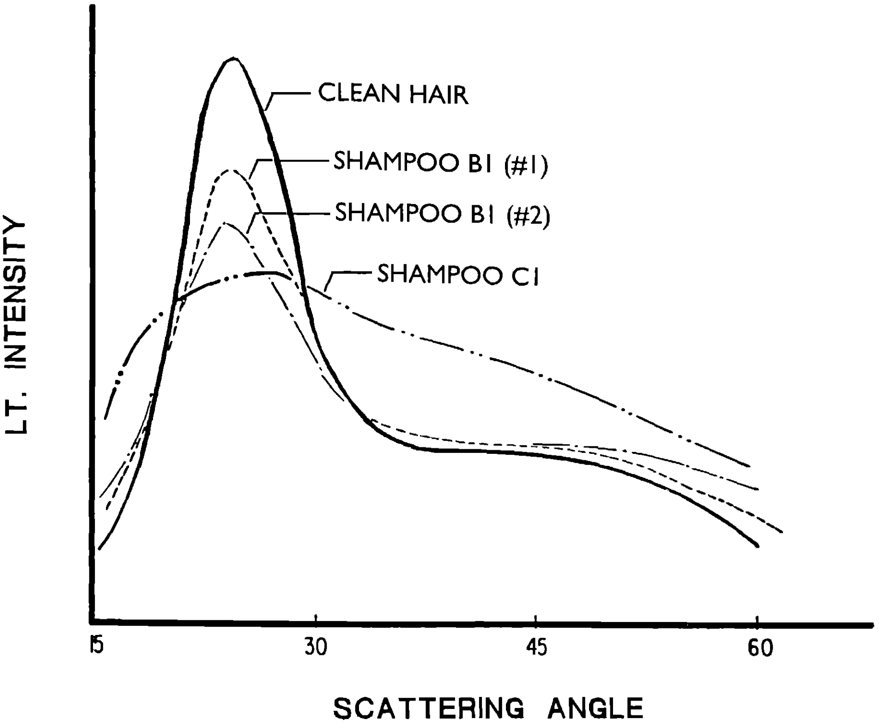

Figure 6. A single-fiber experiment measuring the effect of washing with two different shampoos. Note the huge increase in diffuse scattering and the great decrease in shine after treating the hair with shampoo C1.

light-scattering curve, indicating that, unlike the individual residues, the combination residue is resistant to removal by SLS.

Formation of a B1:C1 complex, presumably between the Polyquaternium-10 cation and the myristate anion, was further tested by performing a dye-staining test, the results of which are shown in Figure 7. The wool swatch on the left in this figure was washed with shampoo B1 and exhibits a pink color as a result of subsequent treatment with Sirius Red. This dye binds to wool only in the presence of bound cationic (12); the color, therefore, indicates that some Polyquaternium-10 was deposited on the wool surface from shampoo B1.

The swatch on the right in Figure 7 was washed with shampoo B1, then shampoo C1, and then treated with Sirius Red. In this case, the swatch retained almost no red dye, indicating that after treatment with C1, very few cationic binding sites were left on the swatch. Since, from the single-fiber experiments, it is known that Polyquaternium-10 still remains on the surface, one must conclude that it is no longer available for binding to Sirius Red as a result of complexation with myristate anion. The conclusion from light scattering that a complex was formed on the hair surface is thus confirmed.

TRESS TREATMENT EXPERIMENTS

As stated previously, single-fiber results, such as those in the preceding section, cannot be treated quantitatively because of the tremendous variation among single hairs. In

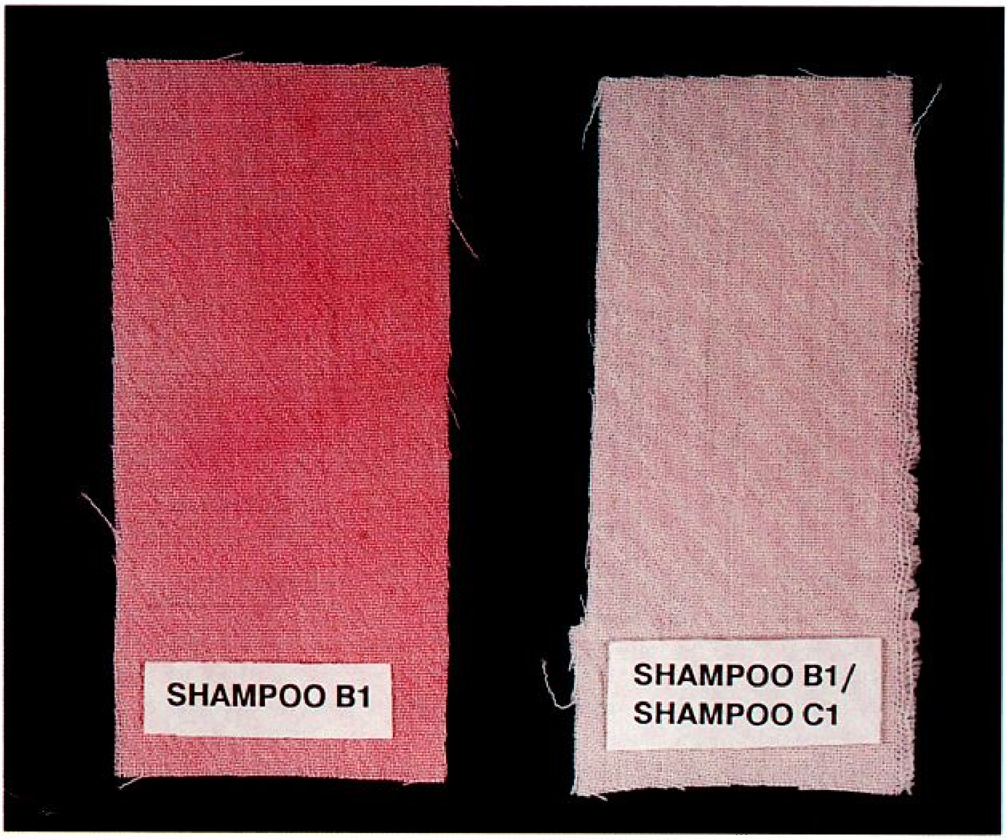

Figure 7. A dye-staining experiment testing the effects of washing with shampoos B1 and C1. The pink color of the swatch on the left indicates the presence of Polyquaternium-10 after washing with shampoo B1. The absence of a pink color for the swatch on the right indicates the lack of cationic binding sites as a result of complexation of Polyquaternium-10 from B1 and myristate anion from C1.

order to obtain meaningful instrumental shine values, therefore, it is necessary to treat tresses, rather than single hairs, and to then measure the shine values for a number of hairs from each tress and average the results.

Aside from the resultant quantitative data, there are two other advantages to treating tresses rather than single fibers. First, unlike the case with individual hairs, the shine of treated tresses can be subjectively assessed and, if desired, compared to instrumental data.

In addition, the conditions employed in treating tresses are much closer to actual in-shower conditions than is true for single-fiber tests. In the latter case, even though a hair is exposed to only one to three drops of treatment solution, this is still more exposure than it would receive buried in a head of hair.

Thus, although results from single-fiber tests are valid for the conditions employed, they would not necessarily be observed in normal usage. To confirm whether an observed effect on single hairs would actually occur under in-shower conditions, it is necessary to treat tresses (containing more than a thousand hairs) rather than single fibers.

In order to test the validity of equation 1, and also to confirm the preceding single-fiber results, a series of tresses was treated with shampoos B1 and C1 along with four other commercial shampoos and SLS. Table I lists the detergents contained in the shampoos employed, along with any ingredients substantive to hair that they contain.

Each of the tresses employed in the current experiments was washed at least five times with a particular shampoo in order to simulate buildup. Following this treatment, hair samples were taken from each tress for light-scattering measurements. Tresses were then mounted on the evaluation frame for assessment by panelists.

A large series of shine evaluation panels was run to determine treatment differences among all seven of the shampoos employed. The results are tabulated in Table II, where the rankings are listed in order of decreasing shine. Those treatments connected with a vertical line are not significantly different from each other.

Table II also lists the instrumental shine values measured for each treatment, along with the associated standard deviations. The numbers were obtained by calculating an average shine for each tress and then averaging the shine numbers for all tresses treated with the same shampoo.

The agreement between the subjective rankings and the instrumental values in Table II was tested using the Spearman rank correlation coefficient method (13). The value calculated for the Spearman rank correlation coefficient was 1.00, which is significant at a level greater than 99%. The agreement between panelists' assessments and instrumental measurements is thus excellent, and one can conclude, therefore, that the numbers calculated from equation 1 are a reliable measure of hair shine.

The results in Table II also indicate that the single-fiber effects reported in the previous section can be expected to be observed under actual usage conditions. Tresses washed with shampoo B1 were found to be duller than clean tresses, while tresses treated with shampoo B1 followed by C1 were found to be duller than those treated with B1 alone.

In general, the shampoo treatments in Table II were found to fall into three main groupings. In the first group, indicated by the first vertical line in Table II, none of the shampoos had ingredients highly substantive to hair. Treatments with these shampoos resulted in the shiniest hair observed, and we consider this hair to be essentially clean.

The second group of shampoos in Table II all contained Polyquaternium-10 (a cationic polymer) or fatty acid salts (soap). These ingredients are substantive to hair and can

Table I
Shampoos Employed in Shine Experiments[1]

Shampoo	Primary detergent	Substantive ingredients
A1	Ammonium lauryl sulfate	—
A2	Sodium laureth sulfate	—
SLS	Sodium lauryl sulfate	—
B1	Trideceth-7 carboxylic acid	Polyquaternium-10
B2	Sodium laureth sulfate	Polyquaternium-10
C1	Sodium laureth sulfate	Sodium myristate
C2	Sodium lauryl sulfate	Coconut acid (sodium salts)

[1] All shampoos except SLS and A2 were commercial products on sale in the United States. Shampoo A2 was a product available in England.

Table II

Subjective and Instrumental Assessments of Shampoo Treatments[1]

Subjective rank[2,3]	Instrumental shine[3]
Shampoo A1	0.762 (0.10)
Shampoo A2	0.714 (0.09)
SLS	0.696 (0.03)
Shampoo C2	0.602 (0.04)
Shampoo B2/shampoo C2	0.582 (0.02)
Shampoo B2	0.551 (0.08)
Shampoo B1	0.486 (0.03)
Shampoo B1/shampoo C1	0.427 (0.03)

[1] Assessments were made with a minimum of three tresses per treatment.

[2] Treatments are listed in order of decreasing shine.

[3] Vertical lines connect treatments that are not statistically different.

build up with repeated use. As a result of this particle deposition, the shampoos in group 2 all caused dulling.

The third type of treatment effect occurred when the negatively charged fatty acid salts in one shampoo formed a complex with the positively charged Polyquaternium-10 previously deposited on the hair by another shampoo. These types of particles caused the greatest degree of dulling on hair. Although only one example of Polyquaternium-10/myristic acid dulling is shown in Table II (B1/C1), a second example of this type of interaction and shine loss was observed in single-fiber tests between shampoos B2 and C1. The former product contains Polyquaternium-10 in a sodium laureth sulfate detergent system.

CONCLUSIONS

In this paper, goniophotometric measurements of shine were presented and shown to exhibit excellent correlation with a large series of subjective assessments. In addition to providing a quantitative measure of hair shine, the light-scattering methods presented were also shown to serve as a sensitive probe of the hair surface, permitting one to monitor deposition, removal, and even interaction of particles on hair. Employing the light-scattering methods developed, a group of shampoos containing highly substantive ingredients was shown to dull hair as a result of deposition on the fiber surface, while even worse dulling was observed as a result of interaction on the fiber surface of incompatible particles from different shampoos.

It should be noted that the incidence and magnitude of dulling effects from deposition depends on the size of the particles deposited on the hair surface (14). The greatest degree of diffuse scattering and, consequently, the greatest degree of dulling, occurs from particles of the order of 0.4 to 0.7 microns. Particles of the order of 4 microns or larger, on the other hand, will reflect light specularly.

Thus, although the substantive ingredients in the shampoos tested in this work caused dulling, it is conceivable that substantive ingredients from other products might be deposited in the form of a film. Depending upon the orientation of its various parts, such a film might cause little or no dulling and, in fact, might even increase hair shine.

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Chlorophyllin copper complex: Quality control

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Synopsis

The currently employed spectrophotometric method in its present version is not reliable to control the quality of commercially available chlorophyllin copper complex (CCC). This quantitative assay may significantly overestimate the purity of CCC compared to that obtained on the basis of elemental analysis (copper and nitrogen content). The presence of uncoppered chlorophyll derivatives and carotenoids could account for the erroneous data. These results are discussed in the context of approaches for the improvement of CCC quality control.

Chlorophyllin copper complex (CCC) is a modified plant product known for decades to be used as a natural colorant (1) and as an internal deodorant and wound healing agent (2). It is obtained from chlorophyll by saponification and replacement of chelated magnesium with copper (3). Commercially available CCC is not a chemically pure compound but a mixture of several water-soluble chlorophyll derivatives, the composition of which varies depending upon the manufacturing conditions employed (4). A spectrophotometric assay was suggested for the quantitative analysis of CCC (5). This method, however, has been shown to be unreliable. Voigtlander and Henning (6), following the established procedure, found in some samples a purity reaching up to 160%. As discussed later, even in studies where the calculated amount of chlorophyll derivatives in CCC did not exceed 100%, the observations made by spectrophotometry contradict the data obtained from elemental analysis.

The following explanation can be given for the erroneous data resulting from the spectrophotometric assay of CCC. This method is based on the optical properties of CCC in the blue region of the spectrum. The absorptivity of CCC in this area increases significantly if other coloring compounds are present with the coppered chlorophyll derivatives. Here we will analyze results obtained elsewhere to demonstrate that metal-free chlorophyll analogs and carotenoids may account for the errors in the spectrophotometric quantitative assay of CCC.

Trisodium copper chlorin e_6 ($C_{31}H_{31}N_4Cu\ (COONa)_3$) and disodium cooper isochlorin e_4 ($C_{31}H_{32}N_4Cu\ (COONa)_2$) are the main chlorophyll derivatives reported in CCC (7–9). Based on these observations, we calculated that the theoretical content of chelated copper and nitrogen in a 100% pure CCC is 9.2% and 8.1%, respectively. The data have been compared with the values of these elements (3.9–4.3%) found in CCC characterized by the spectrophotometric assay to be a good quality product (7). While the purity of CCC, as calculated from spectrophotometric analysis, was in a range of 78.0–94.4%, the amount of the CCC estimated by the content of copper and nitrogen was only between 42.4–46.7% and 48.2–53.1%, respectively. The poor quality of the CCC is confirmed by fractionation analysis, which reveals that the main bulk of the chlorophyll-derived constituents represents only 50% of the total product (9).

No substantial analytical data on CCC have been reported in recent years. The most intense studies on the quality of CCC have been carried out by Voigtlander and Henning (6). These authors investigated commercially available CCC, samples prepared in their laboratory, and purified individual chlorophyll derivatives. The following discussion is based on these data, some of which have been modified and recalculated.

It is implicit that the copper content in CCC reflects the level of chlorophyll derivatives complexed with metal. We attempted to correlate the amount of copper determined in CCC with the purity of the samples established by spectrophotometric assay (Table I). The comparison did not reveal any relationship between these two parameters. An alteration in the copper content of the samples was not accompanied by an appropriate change in their purity. While for some samples the increase in the amount of copper led to a higher quality of CCC, others were characterized by significant reduction of the purity values.

The ratio between copper and nitrogen content (Cu/N index) can also be a reliable indication of the quality of CCC. Our calculations indicate that the Cu/N index for 100% coppered chlorophyll derivatives is 1.1. We estimated the Cu/N index for different commercial samples of CCC (Table II). The data presented show that for the majority of these samples, the Cu/N index is far from the standard value of 1.1. Only four of the nine samples could be considered good quality products, since their Cu/N indices were found to be between 0.9 and 1.1. These values correspond to purities of CCC not less than 81.8%.

From the lack of correlation between copper content and purity determined by spectrophotometric assay, and from the inconsistent results relative to the Cu/N index, it can be concluded that in most samples of CCC studied, copper-containing chlorophyll derivatives are accompanied by their metal-free analogs. Spectrophotometric evaluation

Table I
Comparative Data on the Copper Content and Spectrophotometrically Determined Purity of Commercial CCC*

Sample number	Cu (%)	Purity (%)
1	0.80	42
2	1.27	23
3	2.90	30
4	3.05	48
5	3.10	43
6	3.33	102
7	3.68	160
8	3.86	89
9	4.70	82

* From reference 6, Table 1, section a.

Table II

Copper/Nitrogen Index and Content of Metal-Free Chlorophyll Derivatives in Commercial CCC*

Sample number	Cu/N	Metal-free derivatives as % of the total
1	0.3	72.7
2–4	0.6	45.5
5	0.7	36.4
6–7	0.9	18.2
8	1.0	9.1
9	1.1	0

* Based on data from reference 6, Table 1, section a.

of purified trisodium copper chlorin e_6 (one of the main components of CCC) and its metal-free analog, following the procedure currently used for CCC (5), revealed the purity as 106% for the former compound and 380% for the latter (6). By comparison of these data, it may be estimated that 100% copper-free trisodium chlorin e_6 exhibits a value of specific absorbance 3.6 times greater than that of its coppered counterpart. Investigations on the spectral properties of various metal-containing chlorophyll derivatives corroborate the conclusion of higher absorptivity if the metal is eliminated from these compounds (10). Hence, specific extinction coefficients determined at the absorption maxima between 400–450 nm for pheophytin a and its coppered analog were 126 and 67.8, respectively. Similarly, values of 182.2 and 93.9 were obtained for pheophorbide a and its coppered derivative. Therefore, the presence of even small quantities of metal-free chlorophyll-derived compounds in CCC can result in increased values of apparent purity, determined by spectrophotometric assay.

Valuable conclusions can be made from the study of chlorophyll-derived compounds intended to mimic commercially available CCC (Table III). Although the Cu/N indices of such samples are close to 1.1 (as required for completely coppered chlorophyll derivatives), the purity determined spectrophotometrically and on the basis of copper content is different. The values of purity exceeding 100% may be due to the presence of carotenoids, which have an intense absorptivity at 350–500 nm (11). On the other hand, those values below this level may come about because of samples containing colorless impurities. These two groups of compounds have been already found in CCC (7,9).

Table III

Percent Purity of Laboratory Preparations of CCC*

Sample number	Determined by: Copper content	Determined by: Spectrophotometry
1	52	62
2	62	89
3	78	107
4	88	115
5	92	147
6	96	163

* Based on data from reference 6, Table 1, section b.

The present data clearly indicate that the currently used spectrophotometric assay erroneously estimates the purity of CCC as a result of metal-free chlorophyll derivatives having even higher extinction coefficients than their coppered counterparts and/or carotenoids. In order to control the quality of CCC, the Cu/N index should be employed in conjunction with the absolute percentage of copper and nitrogen content. These values have to be in range of those calculated for a 100% pure CCC. Interference by carotenoids can be avoided by obtaining the total absorptivity of the red peak for the preparation, rather than spectrophotometric measurements in the blue region of the spectrum, as presently employed.

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