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QUANTITATIVE MICROSCOPY IN THE COSMETIC INDUSTRY

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ABSTRACT

A number of modern microscopical techniques and their possible adaptation to the specific problems encountered in cosmetic research are described. The question of quantification of the effects of a cosmetic treatment is discussed, and the principal approach is demonstrated with practical examples. Finally, the sensitivities of microspectrophotometry, interference microscopical methods, polarized light analysis and fluorescence microscopy are outlined to indicate their range of applicability to specific cosmetic problems.

Introduction

The cosmetic industry serves a highly competitive market. One answer to the demands of such a market is an intensely active research and development program. It may sound trivial to say that in research it is essential to express results in the form of quantitative data. But exactly this necessity constitutes one of the difficulties encountered in the field of cosmetics. It is often rather difficult to express the effects of a cosmetic treatment in terms of numerical values. In some cases, quantitative microscopy can give such answers. It is the purpose of this article to discuss a number of modern microscopic techniques, to point out their possible application in the field of cosmetic research and to describe their capabilities. Some of these techniques have originally been developed in totally unrelated fields of research. But it is typical for microscopic techniques that they can readily be adapted for other applications. The objects which are of interest here are of a widely differing nature. There are those portions of the human body which are to be subjected to a cosmetic treatment, such as hair, fingernails, skin and teeth. Then there are the

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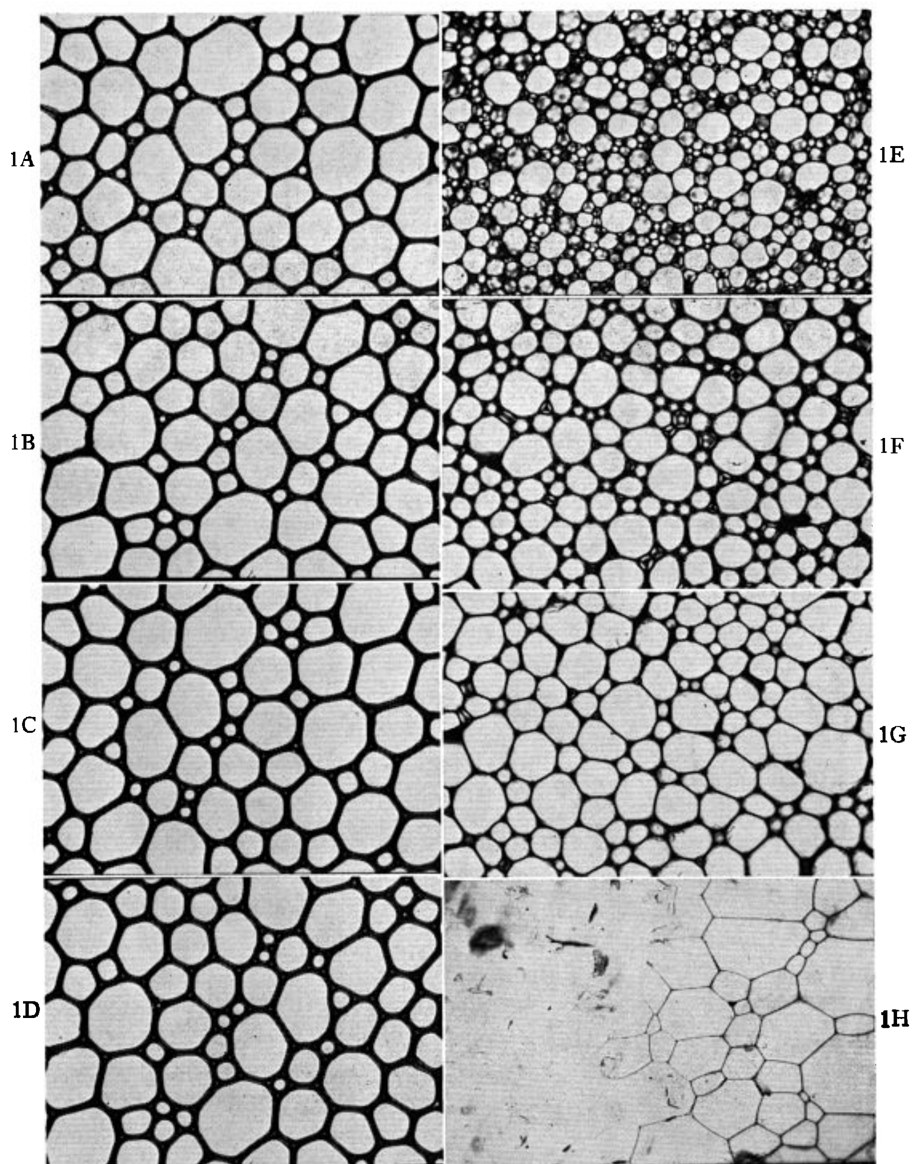

Figure 1.—Stabilized (1A, 1B, 1C, 1D) and unstabilized (1E, 1F, 1G, 1H) lather (bright field 125×). 1A and 1E represent fresh foam; 1B and 1F show foam after 4 min.; 1C and 1G show foam after 15 min.; 1D and 1H show foam after 25 min.

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products of the industry, such as creams, sprays, lotions, polishes, powders and emulsions. The purpose of the microscopic examination is not only to study the effect of one upon the other. It also is applicable to basic research and to research directed toward product improvement and toward ways of achieving the same purpose in a more economical way.

Microscopic techniques are by their very nature optical techniques, and the optical properties of the above mentioned specimens are used to describe their state and appearance or the dynamics of any change or interaction. A number of optical properties lend themselves to quantitative determination. First, there are the directly measurable material characteristics: spectral absorption and spectral reflectivity, refraction and dispersion, birefringence, dichroism, and all the related polarization-optical material constants. Finally, there are the spectral emission characteristics of fluorescing and phosphorescing materials.

Determinations of one or more of these characteristics can be used to obtain quantitative information on derived parameters, which directly describe product properties or the dynamics of certain processes. A typical example is the measurement of optical path differences in an interference microscope, which can be used to measure skin smoothness directly, as will be described later on. Sometimes it may become necessary to create and define new parameters in order to obtain a measurable quantity which shows a direct correlation to the process under examination. This technique will be illustrated with the aid of a practical example.

It is undesirable for a lather used for shaving purposes to collapse rapidly. It is well known that collapsing can be prevented by adding a stabilizing agent. The photomicrographs (Fig. 1) show two samples of shaving lather, one with and the other without stabilizer. The lather was prepared in a thin layer, and the pictures were taken over a period of approximately 30 minutes. Hardly any change occurs in the stabilized lather. The unstabilized preparation collapses completely. During this process a great many changes can be observed. The wall strength of the bubbles decreases, the bubble diameter increases, and the shape of the bubbles changes. Also, the number of bubbles per unit area decreases remarkably. Any one of these parameters may, of course, be used to describe the collapsing behavior of the lather. However, these various parameters may have different differential sensitivities to the effect under investigation, in this case to a change in the amount of stabilizing agent added. In other words, one should try to find that parameter which shows the highest coefficient of correlation to the variable under study.

In the specific example here it was the relative number of bubbles per unit area which showed better than the other parameters the high rate of collapse of the unstabilized lather, as shown in Fig. 2. Once such a parameter has been found, one can easily derive other quantities, such as a

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rate of collapse coefficient or time constants; and one can study the effects of temperature, water hardness, and similar external influences upon these derived parameters. By plotting one of these values against the amount of added stabilizer, one obtains numerical values for the minimum amount of stabilizer which will satisfy the requirements for product quality, e.g., by guaranteeing foam stability over a period of time not longer than is

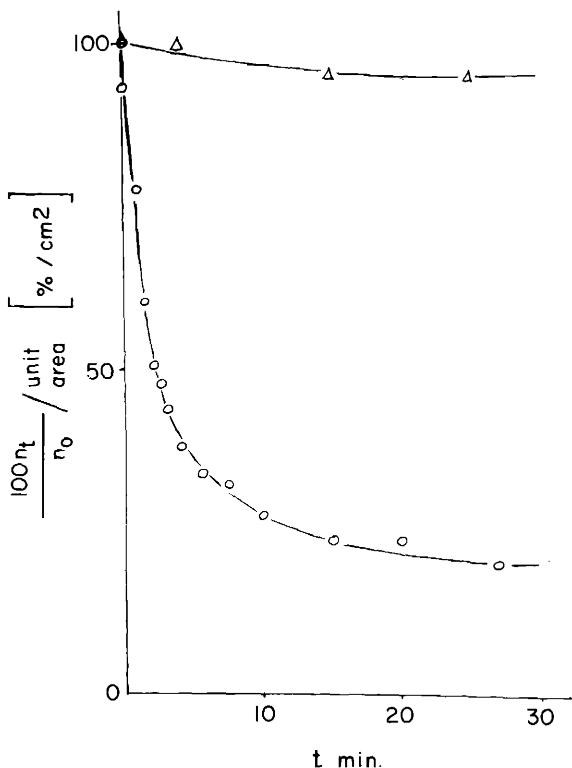

Figure 2.—Collapsing behavior of two samples of lather.

absolutely necessary. The stabilizer may be an expensive additive, and such studies can contribute to keep its use at a cost-saving minimum.

The previous example has purposely been exaggerated to demonstrate the usefulness of newly created derived quantities. In other cases, th determination of some of the optical properties leads directly to numerical values. Below, the sensitivities of a number of methods will be discussed. The numerical values given here are intended to indicate the possibilities and the limitations of the procedures. They represent a rough estimate based on reasonable values from experimental experience. In many cases, higher accuracies and higher sensitivities could be reached with more elab-

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orate techniques employed by those who in the patent literature are so aptly described as "skilled in the arts."

MICROSPECTROPHOTOMETRY

Spectrophotometry plays an important role in almost every aspect of chemical research. When the specimen has microscopic dimensions, the sample compartment of a standard spectrophotometer may be replaced by a microscope and the sample cell by the microscope slide. Such instruments are called microspectrophotometers. They have found widespread application in histochemistry, where minute amounts of substances are traced by their absorption, or where microchemical tests can be carried out even within individual cell components by means of color reactions. Microspectrophotometers permit identification of substances by means of their spectral absorption characteristics. Determinations of concentrations of the relative and sometimes even of the absolute amount of chemical compounds are possible. Sensitive microspectrophotometers have measuring areas as small as $1 \times 1\ \mu$. There are also microspectrophotometers for measurements in the ultraviolet spectral range (1, 2).

An estimate of the sensitivity of these methods leads to the following values. It can be assumed that a minimum absorption of 5% is sufficiently high above the noise level to exclude statistical errors. It shall furthermore be assumed that the specimen has a geometric thickness of 10μ and that the molecular coefficient of extinction at the maximum of the extinction curve has a value of 15,000 cm.^{2}/mMole. This is an average value. Dyes very often have molecular coefficients of extinction as high as 35,000 cm.^{2}/mMole, whereas substances of a simpler structure such as tryptophane have coefficients of around 8000 cm.^{2}/mMole. The concentration can be calculated from the formula,

$$I = I_0 10^{-\epsilon c d}$$

in which I is the transmitted intensity, I_0 the incident intensity, d stands for the geometric thickness of the specimen expressed in centimeters, c is the concentration expressed in Mole/10^3cm.^{3}, and ϵ is the molecular coefficient of extinction, which has the dimension cm.^{2}/mMole. Rearranging leads to

$$\frac{1}{\epsilon d} \log \frac{I_0}{I} = c.$$

The minimum detectable concentration, using the values given above, would then be

$$\frac{1}{15{,}000 \times 10 \times 10^{-4}} \log \frac{100}{95} = 1.2 \times 10^{-5} \times \frac{\text{Mole}}{10^3 \text{ cm.}^3}.$$

Assuming a measuring area of $4 \times 4\mu$ and a section thickness of 10μ, the

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measured volume would be $160\mu^3$. The minimum measurable amount of absorbing substance in this volume then is approximately 2×10^{-16} Mole. With a molecular weight of around 250, which again is an average value for a typical dye molecule, this sets the minimum detectable amount of such a dye at 5×10^{-14} g.

Certain precautions have to be taken. For instance, it is advisable to use an unstained but otherwise identical specimen to measure I_0, in order to compensate for light losses due to light scattering. If the absorbing material is not distributed homogeneously but in the form of granules, one has to use the two-wavelength method (3).

Microspectrophotometry has a number of applications in the cosmetic industry, especially so in histochemical work or in penetration studies. Some materials reveal more information in reflected light than through their absorption spectrum, as shown in Figs. 3 and 4. Figure 3 shows the absorption spectrum of human hair, covering the range from bleached, almost white hair to dark black. The absorption spectra in the visible range are not very informative. Figure 4, however, shows the spectral reflectivity. The technique is the same, in principle, as that employed in transmitted light, but the condenser of the microscope is replaced with a vertical illuminator. Since hair is highly birefringent, the same precautions have to be employed that are also taken in measurements of the reflectivity of ores and minerals (4, 5). Similarly, not only the relative spectral reflectivity can be measured, but also, in suitable cases, changes in reflectivity, e.g., after treatment of hair with cosmetic preparations. Again, once a measurable parameter is found, valid comparisons become possible.

Phase Contrast Microscopy

Many specimens, however, do not contain light absorbing structures. Their detail consists of structures with a refractive index different from that of their surroundings. In a normal brightfield microscope, such structures do not appear with any appreciable contrast. Such phase detail can be made visible by phase contrast (6, 7).

Differences in optical path are by this process converted into differences of light intensity. Inherent in the process, however, is the occurrence of light or dark seams wherever steep gradients of refractive index occur. These artifacts are called halos, and due to their presence the intensity distribution of a phase contrast image cannot be used to determine quantitatively the optical path differences in the specimen.

For qualitative observation, however, phase contrast is ideal in the study of pure phase objects, in the examination of washing agents, the

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interaction of detergents with fat films or deposits, the formation of myelin figures, the structure of creams, foams, lathers, and protective colloids, to name a few examples. Figure 5 shows the interaction between a hair-washing agent and a fat film.

The examination of oil, water or water/oil emulsions involves study of pure phase objects. Phase contrast permits a definite distinction between lipid and aqueous phases and their structural distribution (Fig. 6). A quantitative determination of the respective partial volumes of the two phases may employ the same principles as those used for the determination of partial volumes in mineralogy and ore microscopy (8, 9). It can be shown that, with certain assumptions, the relative areas are directly pro-

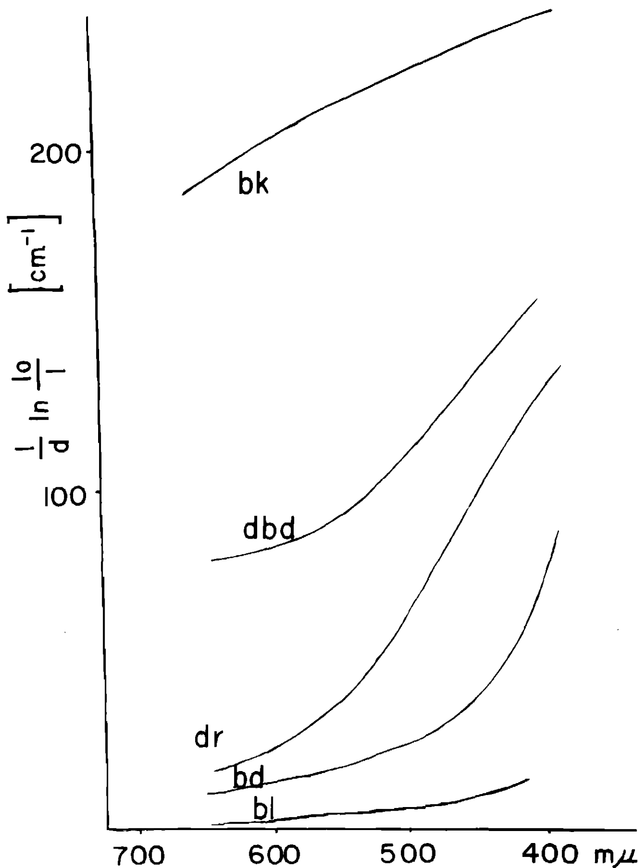

Figure 3.—Absorption spectra of human hair (coefficient of absorption = 1/d ln Io/I. bk = black; dbd = dark blond; dr = titian red; bd = blond; bl = bleached.

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portional to the partial volumes. Such an analysis is carried out either with an integrating stage, a point-counting device, or a photomicrograph.

Phase contrast techniques can also be used in reflected light. It is then necessary that the specimen surface be relatively smooth and not give too diffuse a reflection. The sensitivity for detection of relief, or optic path difference in general, is higher in reflected light than in transmitted light.

The sensitivity for detection of surface relief is approximately four times higher in incident than in transmitted light phase contrast. In incident or reflected light the path difference enters twice. This causes a gain of a factor of 2 in sensitivity. In transmitted light, one would have to examine a replica of the same relief. With a replica refractive index of 1.5, the effective path difference against air would only be $1.5 - 1.0 = 0.5$ of the

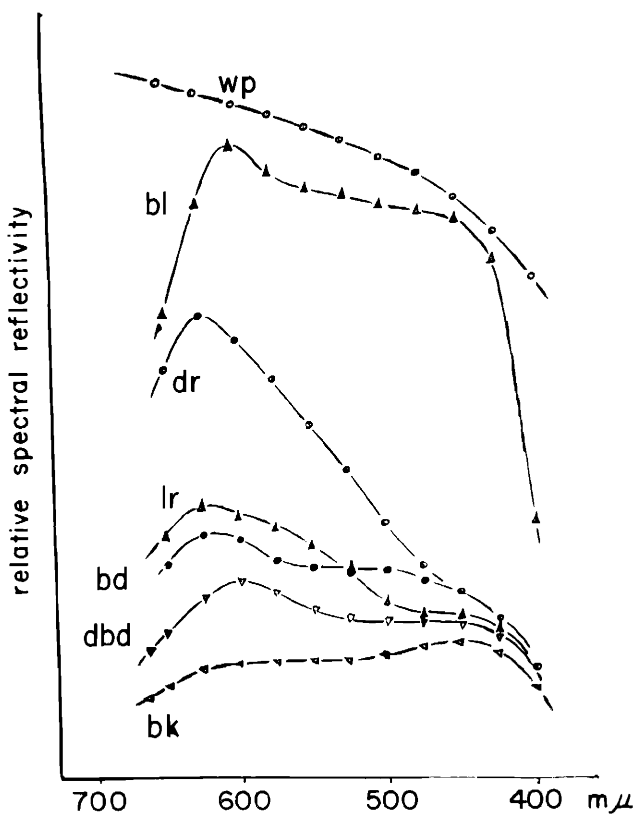

Figure 4.—Reflection spectra of human hair. bl = bleached hair; dr = titian red; lr = light red; bd = blond; dbd = dark blond; bk = black; wp = white paper surface.

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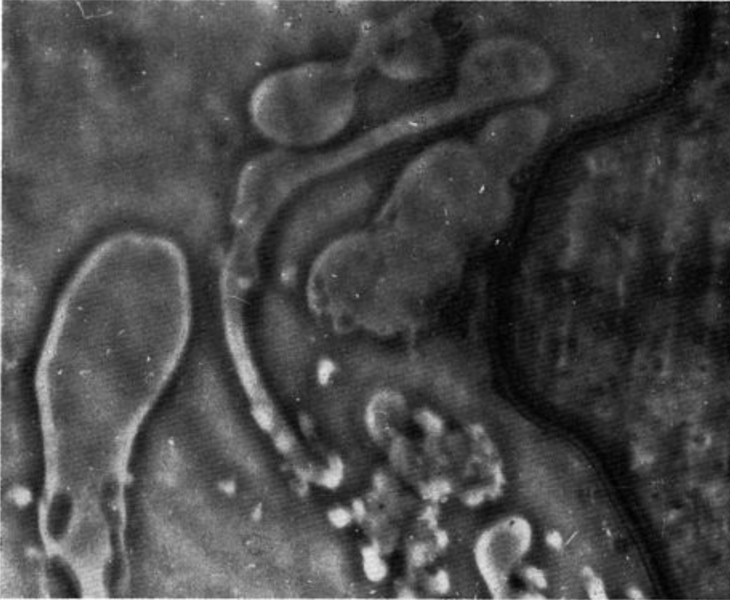

Figure 5.—Interaction between hair washing agent and fat film (phase contrast: 500×).

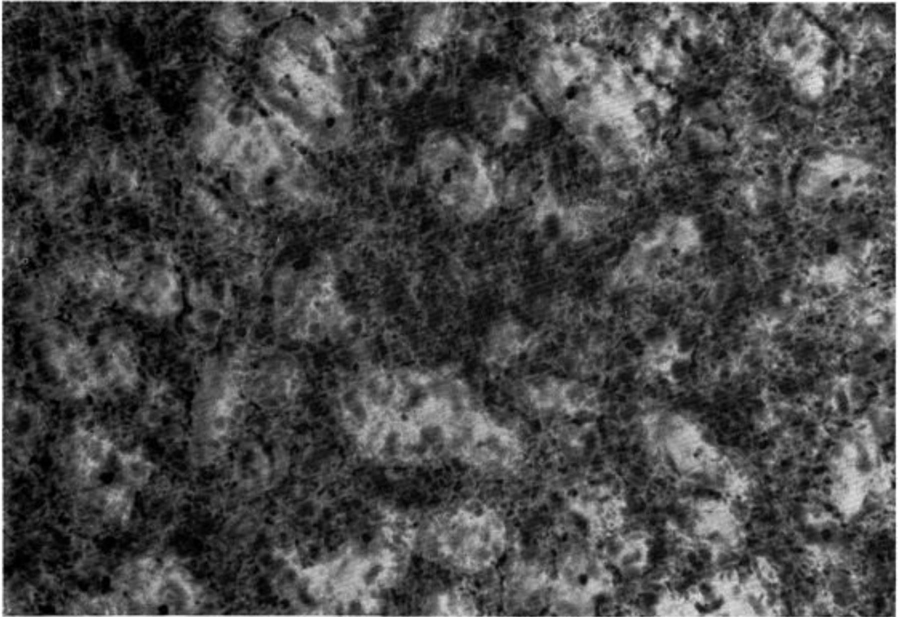

Figure 6.—Structural features of an emulsion; the oil is distributed in the form of lamellae (phase contrast: 500×).

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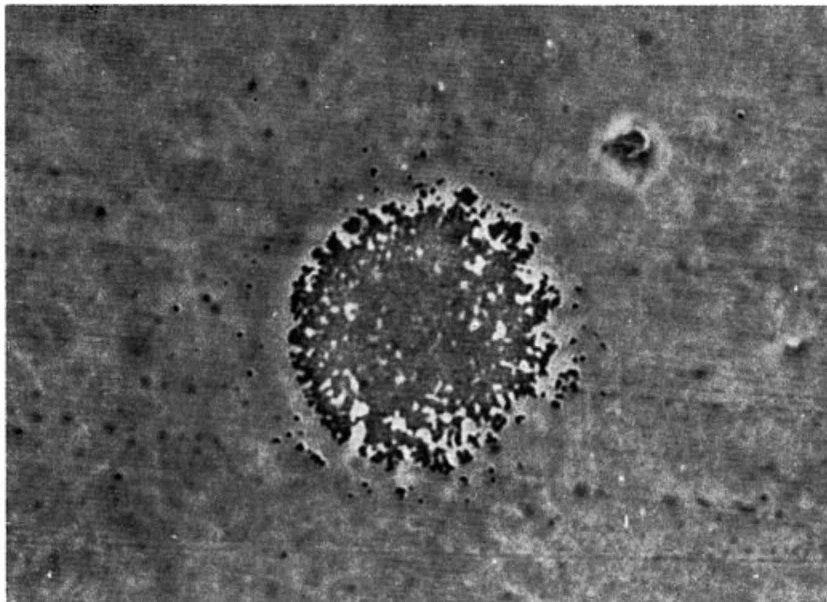

Figure 7.—Beginning corrosion (reflected light phase contrast: 500×).

actual relief depth. This makes reflected light phase contrast more sensitive by another factor of 2. The human eye can detect intensity difference of around 5%. With this approximate figure the contrast function leads

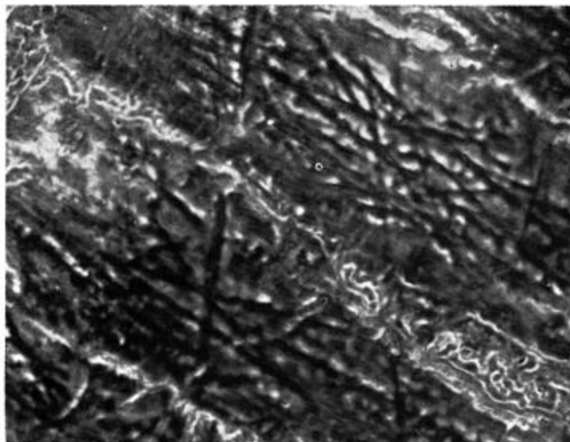

Figure 8.—Surface of human incisor treated with coarse abrasive (reflected light phase contrast: 500×).

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to a sensitivity of detection of optic path differences of around 15 A. for reflected light phase contrast. However, for practical purposes, a figure of approximately 25 A. should be considered the limit.

Incident phase contrast thus offers a possibility to observe the very beginning of corrosion (Fig. 7) in containers or on container material, the uniformity of sprayed-on films and protective coatings, or the effect of coarse abrasives on tooth surfaces (Fig. 8).

Phase contrast techniques are primarily methods of detection. Minute differences of optical paths caused by relief or change in refractive index in the specimen are made visible. Sudden changes of optic path are particularly accentuated. However, halo formation prevents quantitative evaluation of the intensity distribution in a phase contrast image. Exceptions to this rule are techniques which use the disappearance of the halo to detect a match of refractive indices, e.g., between specimen and medium (10–12).

Interference Microscopy

Interference methods are especially valuable because they yield quantitative results. The image in an interference microscope shows the specimen and a superimposed interference fringe system. In any interference microscope the light is separated into at least two coherent beams. One of these is called the measuring beam, the other the reference beam. How this separation is achieved depends on the specific design of the instrument. The light in the measuring beam is affected by the specimen; the reference beam is given an optical path that will leave it either completely unaffected by the specimen or which keeps the specimen influence at a minimum. Behind the object plane the two coherent beams are reunited and brought to interference. Even without a specimen in the microscope, one observes a system of interference fringes: in monochromatic light, a dark fringe where conditions prevail for destructive interference between measuring and reference beam, and a bright fringe where conditions exist for constructive interference between these two beams. Since these conditions occur alternately and periodically for optic path differences between the two beams of an uneven number of half-wavelengths (destructive interference, 180° phase shift), and of an even number of half-wavelengths (constructive interference, 0° or 360° phase shift), the distance from one dark fringe to the next represents a path difference of exactly one wavelength.

A specimen which introduces additional path differences for these rays in the measuring beam which have passed through it, will, in effect, change the location where conditions for destructive and constructive interference

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occur in the fringe system. An axially existing optic path difference, therefore, leads to a lateral fringe displacement or fringe shift. The amount of this lateral displacement is directly proportional to the optic path difference introduced by the specimen and can be compared directly to the one-wavelength units of optic path difference given by the distances from fringe to fringe in the undisturbed part of the field of view (13–15). Suitable instrument controls permit variation of the distance between the interference fringes. This does not, of course, change the existing path difference between them, i.e., one wavelength. The adjustment rather corresponds to a scale expansion, and the sensitivity of detection is directly proportional to the fringe width. In the most sensitive setting only one fringe covers the whole field of view. This condition is called homogeneous field or interference contrast. Optic path differences introduced by a specimen then appear as changes of image intensity, which again are a quantitative measure for the phase shift introduced by the specimen.

Measurements are taken with compensators, which are calibrated directly in mμ optic path and which permit an exact measurement of the lateral fringe displacement, or a photomicrograph is taken and the density recorded in a microdensitometer.

At this point it may be well to ask what kind of information the cosmetic chemist can obtain from micro-interferometric methods. Such information falls into two categories. First, there are the directly measurable data:

Measurements of optic path.
Measurements of refractive index.
Measurements of dispersion.
Measurements of geometric thickness.
Simultaneous measurement of thickness and refractive index.
Determination of total dry mass of an object.
Determination of total wet mass of an object.
Measurement of mass per unit area in the specimen.
Concentration of substances in the specimen.
Volume of specimen structures.

Second, there are the indirect data which follow from the interpretation of the directly measured values and for which only a few examples can be given here. Measurements of geometric thickness may be used to determine the thickness and uniformity of sprayed-on films, swelling effects and behavior of materials, or to measure the depths of scratches caused by polishing agents. A practical example is profile measurements, here applied to a determination of skin smoothness (16, 17). This can be carried out as a nondestructive test by taking a replica of the surface using transparent replication materials.

Figures 9 and 10 show photomicrographs of skin surfaces, one untreated

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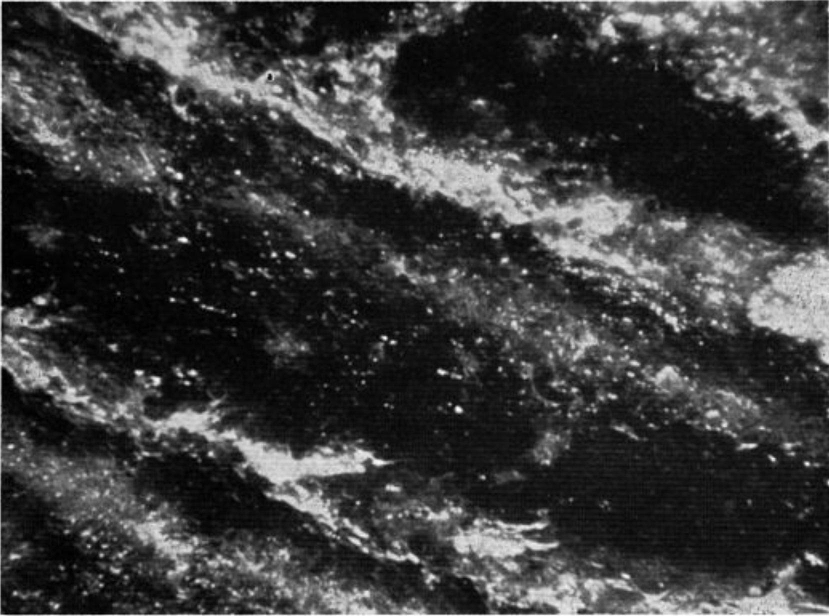

Figure 9.—Untreated skin (45×).

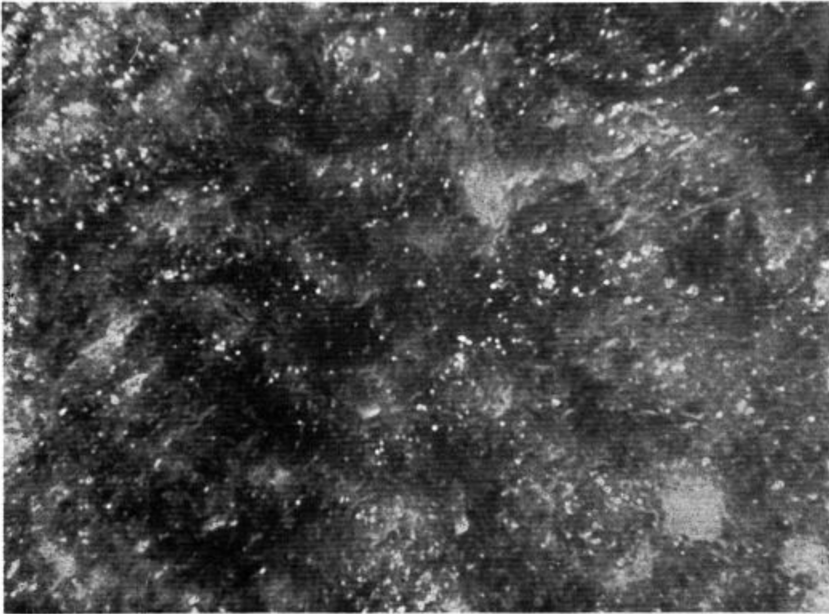

Figure 10.—Skin treated with proteolytic enzyme (45×).

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and one treated with a proteolytic enzyme preparation. Whereas the untreated skin very clearly shows high ridges (Fig. 9), these are missing in the treated skin, as can be seen in Fig. 10. However, the change in skin profile can be seen more clearly in the interferograms (Figs. 11 and 12) than in the photomicrographs in reflected light. Whereas the high profile in untreated skin leads to considerable fringe displacements in the interferogram (Fig. 11), the interferogram of treated skin shows fringe displacements to a much smaller extent (Fig. 12).

The lateral fringe displacement is a direct measure for the surface profile, and Fig. 13 shows the tracing of one such fringe. Such a trace is obtained simply by following the course of one certain fringe across the field. One may then compare the fringe displacements, which are indicative of and proportional to the profile depth, to a straight base line, for instance a line connecting the beginning of the fringe on the left and its end on the right.

As a measure of the smoothness of skin the same parameters can be used, which have long been employed in the machine and tooling industry. Here, the so-called h_{rms}-values are used to describe surface roughness, and these values are the averaged quadratic roughness (18). Again, once a measurable parameter has been established, the effect of cosmetic treatments can be expressed in numerical values. The most effective concentration of certain additives, application time, frequency of application, the effects of pH, temperature and the effect of storage upon the effectivity, all these free parameters become accessible to quantitative determination.

For applications of this sort not the lower but the upper limit of the measuring range is of interest. This upper limit has been widely extended recently by the invention of the Zehender cell (19) and of interference microscopes, which have an extremely wide measuring range. Macro-interferometers use almost parallel light and are, therefore, not so limited with respect to the largest measurable path difference as interference microscopes. Horn (20) describes an instrument which gives high contrast in monochromatic light for well over 1000 orders of monochromatic light. One order represents a path difference of 550 mμ. In a replica made of material with a refractive index of 1.5, this would represent a profile depth of 1100μ, or 1.1 mm. when measured against air.*

Two experimental difficulties may occur: First, the replica back surface may not be smooth; this can be overcome by mounting the replica on a slide and filling the space between the replica and the slide with an embedding liquid of the same refractive index as the replica material. Second, the replica may contain profiles, which are so steep and so narrow that a positive fringe identification, even in polychromatic light, becomes

* In the formula (24) $\Gamma = t(n_2 - n_1)$, Γ represents the path difference (here: 1000×550 mμ = 550μ); n_2 is the refractive index of the replica material ($n_2 = 1.5$), and n_1 that of the embedding material (here air, $n = 1.00$), so $n_2 - n_1 = 0.5$. This leads to $550/0.5 = 1100\mu$ profile depth t.

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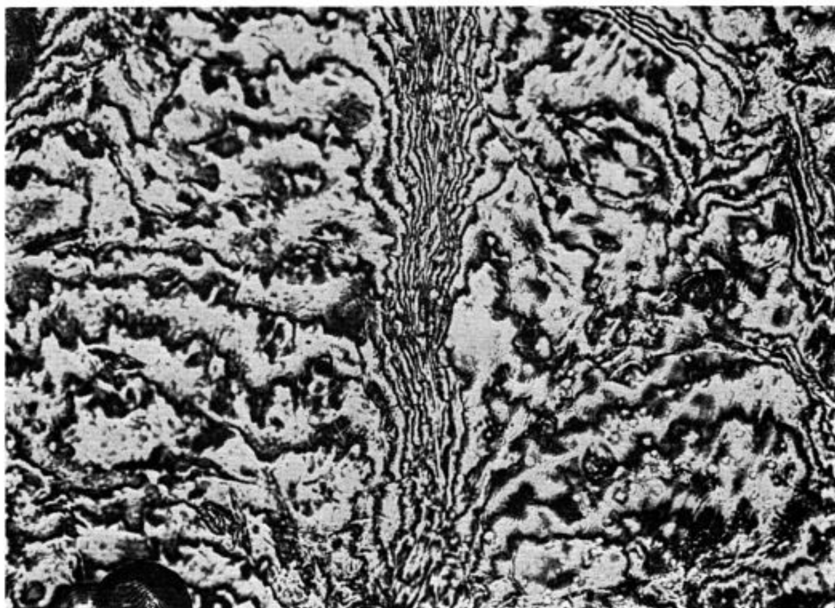

Figure 11.—Interferogram of untreated skin (200×).

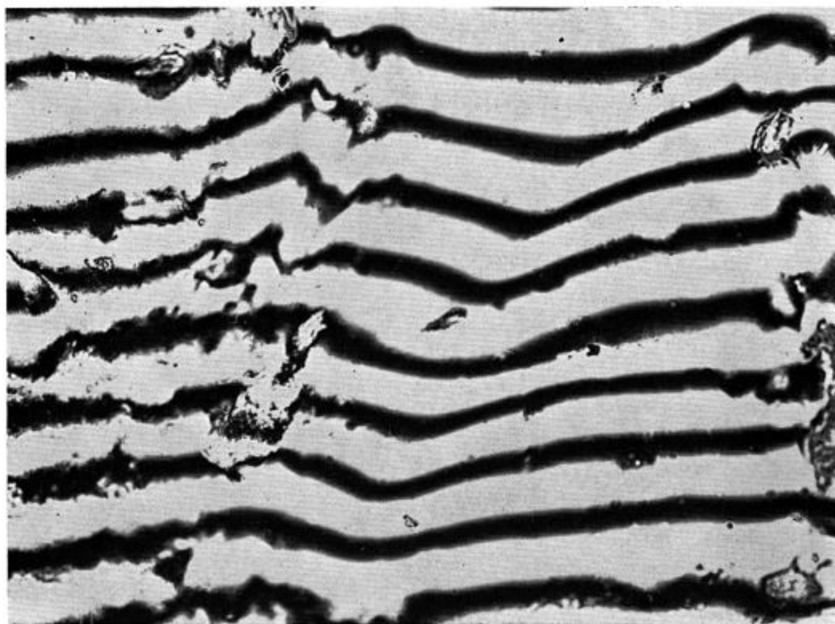

Figure 12.—Interferogram of skin treated with proteolytic enzyme (200×).

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impossible. It is then advisable to use the continuous scale method (21). The replica is mounted in a cell with two plane parallel plates, which is filled with a liquid of a known refractive index. The closer the refractive index comes to the index of the replica material, the more the scale can be expanded. This point can be illustrated by the following numerical example: If the path difference is 100 orders, i.e., 100 $\times$ 550 mμ, such a fringe displacement should be seen in the interferogram; if the replica has a refractive index of 1.5 and if one measures against air (n = 1.0), then t must be 110,000 mμ. The same profile depth of 110,000 mμ in a replica of refractive index 1.5, when measured against oil of 1.49 refractive index, would produce a path difference of only 110,000 $\times$ 0.01 or 1100 mμ.

Since a fringe shift of 100 orders in air is too high for positive fringe identification, the Zehender cell can be filled with a liquid of a refractive index of 1.49, thus lowering the fringe shift from 100 to 2 orders. Using the previous numerical example it can be seen that 55,000 mμ = 100 orders and that 1100 mμ = 2 orders.

Another application of interference methods is the determination of the depth of indentations made by a micro hardness tester (Fig. 14). Such tests are of interest, e.g., to establish the hardness of nail polish. Even very low loads on the Vickers diamond are occasionally too high for such relatively soft materials, and scratch tests seem to be more promising.

The depth of a regular Vickers indentation can easily be determined by a measurement of the length of its two diagonals (22). In scratch hardness tests, the scratch depth is proportional to the width of the scratch, which can be measured directly. A more accurate and direct measurement of the scratch depth is possible by means of determination of the fringe displacement in an interference microscope.

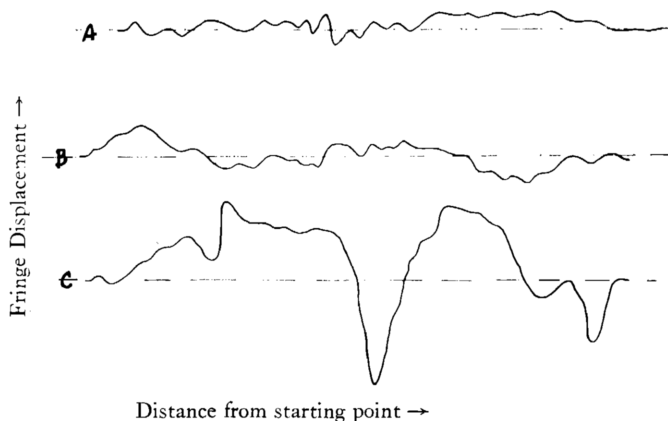

Figure 13.—Tracing of interference fringe (A and B = profiles of enzyme-treated skin; C = profile of untreated skin).

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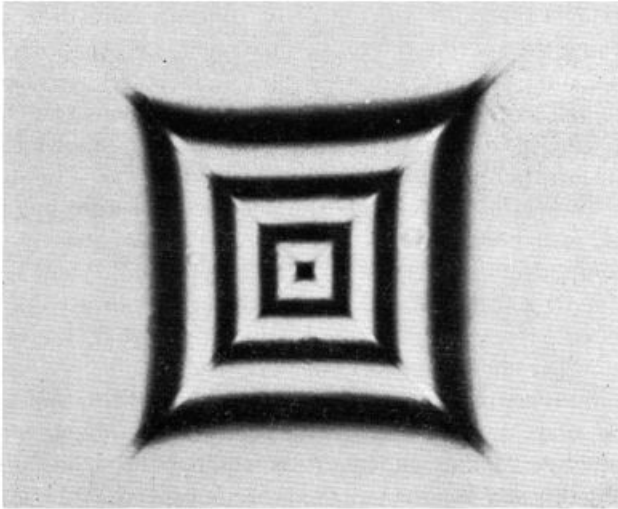

Figure 14.—Hardness test on nail polish (interference microscope: 200×).

The lower limit of the measuring range of interference microscopes becomes important in such applications of the cosmetics industry which deal with the pharmaceutical and cytological testing of materials, where determinations of dry and wet mass are made and concentration measurements are of interest. Most of these will be carried out in histological sections or in smear preparations. It may be interesting to calculate the limits of the method and the sensitivity of detection to evaluate the method for work in penetration studies.

To reach the lower limit of interference methods one must employ microdensitometric methods and use the interference contrast adjustment of the instrument. Only one interference fringe is spread out over the field of view. Its intensity distribution is recorded. The optic path introduced by the specimen causes it to appear darker than its surroundings. Locations of equal intensity—or equal density on the photographic plate—correspond to equal optical path, so that the path differences introduced by the specimen can be determined directly by a microdensitometric measurement. As a rule, a phase shift of approximately $^1/_{200}$ of a wavelength (about 20 A.) is the limit of detectable specimen influence.

A better idea of the sensitivity of the method is obtained when one expresses this measurable optic path difference in terms of detectable changes in dry mass or concentration. A 20 A. path difference in a histological section of 5μ thickness corresponds to a change in refractive index of

$$\Gamma/t = n_2 - n_1 = 2\ m\mu/5000\ m\mu = 4 \times 10^{-4}$$

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To convert this value into differences of concentration of a substance, one has to know the specific refractive increment, i.e., the increase of the refractive index of a solution for every 1% increase in concentration. For most biological materials, such as proteins, carbohydrates or lipids, this value is 1.85×10^{-3} (13). Using this value, it follows that an increase in concentration of about 0.2% could be detected. In order to obtain practical numerical values for the specific refractive increment the concentration is expressed in g./100 cm.^{3}. The volumes of biological cells or cell components are very small. A volume of $15\mu^3$ shall be assumed. Since a change of 200 mg. in 100 cm.^{3} is detectable, in a volume of $15\mu^3$ this amounts to only 3×10^{-11} g. In this application, the interference microscope thus becomes a highly sensitive optical balance.

Such a highly sensitive method is not only applicable to biological problems but also offers opportunities in the study of dissolving rates, of the effect of protective colloids added to soaps and lathers, and interactions between detergents and films of a fatty nature.

Interference microscopical methods thus offer a possibility to measure quantitatively those effects of a cosmetic treatment or preparation and of colloid chemical processes which produce a change in optical path. For practical purposes, measurements can detect changes of optical path from around 1 mm. to 20 A., a range of approximately six orders of magnitude.

Analysis in Polarized Light

One of the most revealing microscopic methods is the examination of microscopic structures in polarized light. Numerous materials are either crystalline, as, for example, many organic substances, or they have submicroscopic crystalline regions, for instance, gels (23, 24). Such structural anisometry leads to optical anisotropy. The anisotropy characteristics of such specimens can very accurately be measured in a polarizing microscope by analyzing the state of polarization of light which has passed through them. Even the molecular arrangements in the structures of these materials can be derived from such studies. Analysis in polarized light can lead to highly interesting information on the orientation of submicroscopic elements, micelles and gel components in anisotropic materials.

On the other hand, polarized light can also serve as a highly sensitive method of detection. In fact, in some specialized polarizing microscopes with rectified optics, optic path differences of as little as 0.1 A. have been detected (25). But even though the sensitivity of standard polarizing microscopes will not go quite as high, it is more than adequate for most purposes. The full measuring range of a 1/30 wavelength mica compensator covers 170 A., and its lower sensitivity limit is of the order of magnitude

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of a few Angstrom units. What then does an assumed sensitivity of 5 A. mean for a practical application in cosmetics research?

One practical example is the study of hair. Hair as such is rather highly birefringent, which makes it difficult to measure small changes against the fairly nonuniform background. Nevertheless, it may be interesting to calculate, as a numerical example, how thin a coating of birefringent material on the surface of hair can be measured in a polarizing microscope, or how much birefringent material would have to penetrate into the interior of a hair to change its anisotropy. The total birefringence of hair would, by the way, already be affected by any penetration (even of isotropic materials) due to a change of form birefringence. For the purpose of this calculation, a polymeric hair conditioning agent shall be assumed to coat the hair. Assuming a value for the birefringence of oriented polymer material of 0.02, we can then calculate the minimum thickness necessary for such a coating to be measurable. This follows from the equation $\Gamma = t\,(n_2 - n_1)$, in which Γ represents the retardation or optic path difference in mμ, t the geometric thickness, also expressed in mμ, and $n_2 - n_1$ the birefringence. Γ_{min} was assumed as 5 A., or 0.5 mμ, $n_2 - n_1$ as 0.02. The minimum value for t then becomes 25 mμ, or 250 A.

The total birefringence of a structure like a human hair is the result of the contributions of the different histological components, such as the medullary and the cortical cells, and the total birefringence of each of these components again is the sum of their textural and their intrinsic birefringence. The intrinsic birefringence is a material constant; the textural birefringence, also called form birefringence, is a function of the orientation of the intrinsically birefringent elements and of their partial volumes, compared to the partial volume of the unoriented matrix. Any mechanical influence, such as mechanical stress which leads to elastic or plastic deformation, will affect form birefringence (23), the changes of which can be used as sensitive indicators. Swelling and thermal treatment will not only affect form birefringence but also intrinsic birefringence.

Fluorescence Microscopy

Fluorescence microscopy in its various applications combines highest sensitivity of detection with extreme specificity. It utilizes the fact that many substances become self-luminous when irradiated with light of short wavelengths, i.e., with light from the energy-rich blue and ultraviolet range of the spectrum. This irradiating or "exciting light" stimulates fluorescence in such substances. The fluorescent light always has a longer wavelength than the exciting light.

Almost any microscope can be adapted for fluorescence microscopy. Some applications require hardly any, others demand more elaborate

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accessories. First, a light source is needed which yields a powerful flux of short-wavelength light. Maximum pressure mercury arcs are necessary for all work involving immuno-fluorescence (26, 27) and for work carried out under oil immersion with other staining techniques. For medium and low power work, where fluorochromes like Acridine Orange are employed, adequate excitation can often be obtained from a high intensity incandescent lamp. All such light sources do, of course, not only emit the desirable short wavelengths, but also light of longer wavelengths, which would completely mask any fluorescence. For this reason, all exciting light is filtered through a set of exciter filters, which transmit only the short wavelength range of the spectrum and completely absorb light of longer wavelengths. The exciting light is then concentrated on the specimen by a microscope condenser. In immuno-fluorescence this is normally a dark field condenser to give images of very high contrast, but in most other work a regular microscope condenser with all diaphragms wide open is sufficient. Fluorescence is stimulated in the specimen and both the remaining exciting light and the stimulated fluorescence enter the objective. To remove any of the remaining short wavelength exciting light, a barrier filter is mounted above the objective. It has a transmission curve complementary to that of the exciting filters and absorbs all light of shorter wavelengths than that of the fluorescence. The specimen, therefore, appears in brilliant luminosity against a dark background.

Many organic substances have the intrinsic property to fluoresce (28) and can be traced directly in a microscopic preparation. Others can be stained selectively with fluorescing dyes, so-called fluorochromes; this fluorescence is called secondary fluorescence. Finally, a fluorescing molecule can be used to tag an otherwise nonfluorescing complex, such as an antigen or antibody. It is in this last procedure that the high sensitivity of detection inherent in fluorescence techniques can be combined with the extreme specificity of serological methods. This technique is known as the fluorescent antibody technique, or immuno-fluorescence. It may be that the remarkable success and the exciting potential of this latter technique has diverted the interest in the field of fluorescence microscopy from those simpler staining techniques which can be used so successfully in routine work.

Among the many fluorochromes which are available for fluorescence staining, Acridine Orange has found some interesting applications. For example, under certain conditions, it permits differentiation between living and dead protoplasm (29). Acridine Orange, or tetramethyl-diamino acridine, is a basic dye. In the alkaline pH-range it is present as an uncharged base molecule, which selectively stains lipid phases with a dark, saturated green fluorescence. In the weakly alkaline, neutral or acid range, however, Acridine Orange is present in solution in the form of a univalent

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cation. In dilute solutions (10^{-6} M) the cation exists as a monomer and fluoresces bright green. With increasing concentration dimerization sets in. The dimer has an emission spectrum with its maximum in the red spectral region, so that the fluorescence color indicates the dye concentration. This so-called concentration effect has successfully (29) been employed to distinguish between living and dead protoplasm. This application permits rapid testing of the effectiveness of antibacterial agents in skin preparations, deodorants or toothpastes by bacterial counts and replaces, when applicable, the time-consuming growing of colonies. To obtain reproducible results it is necessary to control the pH carefully with buffers, to use excess dye solution, and to stay within a certain pH-range. Also, it is necessary to check whether the tested preparations contain fluorescence quenching agents and whether the bacteria under examination show the typical green-red transition (Fig. 15).

The uncharged molecules of several basic dyes accumulate readily in lipid phases. Addition of a fluorochrome to fatty substances allows determination of the uniformity of very thin films of these substances, or their presence, or their penetration.

One of the great advantages of fluorescence techniques is, as has been mentioned before, the very high sensitivity of detection. One can clearly recognize a particle as stained when the concentration of dye in it is as low as 1:10,000. For particles smaller than 1μ, which show up very clearly

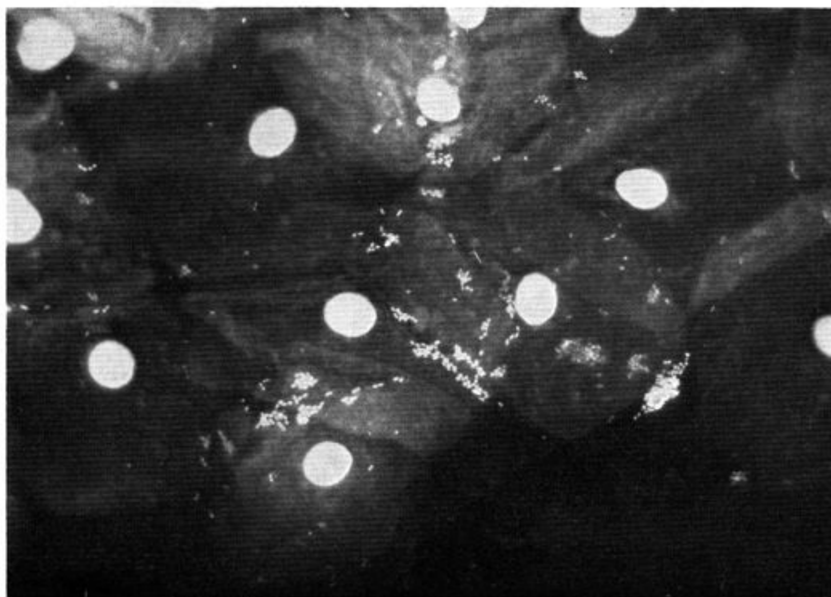

Figure 15.—Mucosa cells with bacteria stained with Acridine Orange (320×).

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in a fluorescence microscope, this means that as little as 10^{-18} g. of fluorescing material can be detected.

Summary

Problems of cosmetic research and development are highly complex and extremely diverse in nature. It is not the intent of this article to suggest certain microscopic techniques for the solution of specific problems nor to discuss in detail the intricacies and accuracies of any one of the described techniques. On the contrary, the article tries to show, with the aid of a few selected specific problems, a general concept of a systematic approach which could usefully be applied to a multitude of different problems. In this approach, the first step will be only qualitative, i.e., an attempt to make the process accessible to direct microscopic observation. This will very often be possible, if not directly, then in the form of models or simulated surroundings. The result of this observation will normally be that the method is found unsatisfactory. Modification in general preparative techniques and selection of a more suitable optical method are the next steps. This is followed by specific adaptations of the preparative technique to the selected and appropriate optical method.

Nothing is more revealing than a direct visual observation of "what happens," and one picture is worth not only a thousand words but, at least in the beginning, quite a few automatic sensing devices. This is particularly true in the study of dynamics, for example, by means of movies taken through the microscope.

The first attempt at quantitative analysis can now be made, encompassing a survey of all available parameters and investigation of each one of them. No hesitation should be shown to create concepts or parameters which describe directly observable facts or phases of the process under study, even if they have to be expressed in terms which are not normally found in the scientific literature. It could well be that the process cannot or has not yet been described quantitatively in physico-chemical terms such as cm./sec. or mol./sec. Numerical values for these terms can normally be obtained only after the process has been understood. From these developed parameters, one indicating parameter should be selected which shows the highest coefficient of correlation to the examined effect, and this correlation should be confirmed. Actually, the indicating parameter may not always be the most obvious one.

In all of these proceedings, it is advisable to use relative values only so that each experiment carries its own reference standards. This approach is much safer in a field where side effects may not have to be discovered and where the influence of systematic errors is not established. In many cases, relative values are all that may be of interest for the moment, and these may

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be obtained by comparing the above secured values with those obtained in the same manner from preparations of different compositions or made by other manufacturers. A set of well-secured relative values can easily be connected to an absolute scale by one single calibration which, as a routine method, may be too difficult and time consuming to carry out.

In the search for improved methods of observation and for indicating parameters, a surprising wealth o. information and techniques can be found in the literature, and often in the specialized literature published in totally unrelated fields. More often than not, such special techniques lend themselves to easy adaptations.

Very often, indicating parameters used in these fields of research can be applied to the cosmetic problem under study; then it is especially advisable to use these known parameters. Their behavior, sensitivities to errors or changes, and range of validity have often been studied and described. Frequently, one will find all of the mathematics, which also apply to one's own problems, already developed.

Cosmetic research with its highly diversified problems and demands for specialized knowledge seems to require even more imagination than research in any one particular field. Anybody actively engaged in research in this field will have to take advantage of every analytical method that might possibly be useful to him. Among these, quantitative microscopy plays an important role.

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