A STUDY OF THE COMPONENTS OF THE CORNIFIED EPITHELIUM OF HUMAN SKIN*

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INTRODUCTION

One of the most important functions of the human epidermis is the formation of a cornified layer about 40 μ thick over the entire 20,000 sq. cm. surface of the adult organism. This layer of the skin has a highly differentiated and organized structure (5, 10, 20) and its constituents have specific physical and chemical properties which are responsible for the protection this layer offers.

The cornified epithelium consists of tightly packed, inactive, horny cells, the main component of which is epidermal keratin, a "soft" type of keratin which contains only 2.3 to 3.8 per cent cystine (2, 7, 33) in comparison to 10 to 15 per cent found in the "hard" forms, such as hair, wool, horn, etc. (3 *a*). This epidermal keratin has been found to be insoluble in water, organic solvents, and aqueous solutions of neutral salts; and to be less stable toward alkalies and reducing substances, and more assailable by heat and proteolytic enzymes than the hard keratins (16). According to Spier and Pascher, 58.2 per cent of the dry cornified epithelium of human skin is epidermal keratin (30). Water-soluble substances, such as free amino acids and metabolic by-products of keratinization, have also been identified in the cornified epithelium (25, 26, 30). The lipids have been only partially identified (6, 18, 30).

Further investigation of the components of human cornified epithelium is of primary importance for better understanding of the mechanism of keratinization in epidermal tissues and of the protective function of the cornified layer. In this study, isolated human cornified epithelium has been broken down by a special grinding process into its cellular constituents, and these in turn have been separated and individually studied. Their properties are here described.

Methods

For ash determinations, 50 mg. samples of pulverized cornified epithelium were first dried at 110°C. to constant weight, then incinerated in silica crucibles. Total nitrogen was de-

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termined by Pregl's modification of the Kjeldahl method, and phosphorus by the procedure outlined by Fiske and SubbaRow (9). Carbohydrates were determined according to the method of Sørensen and Haugaard (29). For cystine determination, Block and Bolling's modification of the Winterstein-Folin reaction was used (3 *b*). (Since the cystine values obtained in this study appear too low, probably because of the presence of carbohydrate in the samples (3 *c*), they cannot be considered as final values. Cystine determinations by column chromatography are now in progress and will be reported in a subsequent paper.) Desoxyribose and ribose nucleic acids were determined according to the methods of Davidson and Waymouth (4), Euler and Hahn (8), and Schmidt and Thannhauser (27). Lipid were extracted with ethanol for 48 hours in the Soxhlet apparatus, and their weights determined after evaporation of the ethanol and drying in a desiccator at 60°C. for 48 hours. The absorption spectra of samples were measured in a Beckman spectrophotometer at room temperature.

Sørensen's phosphate, citrate, glycine[1]-HCl, and glycine[1]-NaOH standard mixtures (12 *a*), and Michaelis' veronal standard buffer (12 *b*) were freshly prepared for each experiment. The actual pH of each buffer was measured by the Beckman glass electrode pH-meter, model H2.

Three different methods were used for electrophoresis. (*a*) For zone electrophoresis, Grassmann and Hannig's apparatus[2] and technique (14) were used. Electrophoresis was carried out at room temperature for 2, 4, 8, and 12 hours with an E. M. F. of 110 volts. The paper strips (Whatman No. 1) were dried at 37°C. and then stained in a mixture of methanol–glacial acetic acid saturated with amido-black 10 B.[2] Density measurements along the paper strips were made in the Elphor densitometer.[2] (*b*) For continuous-zone electrophoresis, the Elphor V apparatus[2] was used. Electrophoresis was carried out at room temperature for 3 hours with an E. M. F. of 150 volts and a current of 14 ma. (13). After drying at 100°C., the filter paper sheets (Whatman No. 4) were sprayed with ninhydrin solution or stained with amido-black 10 B. The ninhydrin solution was always freshly prepared according to the technique of Moore and Stein (22). (*c*) For moving-boundary electrophoresis, a Perkin-Elmer model No. 38 Tiselius electrophoresis apparatus was used. Electrophoresis was carried out in the conventional manner at 0°C. with an E. M. F. of 110 volts and a current of 10 ma. Photographs were taken at the beginning of each experiment and after 15, 30, 45, 60, and 90 minutes. Mobility was calculated from the equation

$$\mu = \frac{dAK}{tIRm}$$

in which d = distance traveled by photographic image of the boundary; A = cross-section of electrophoresis cell; K = conductivity cell constant; t = time in seconds; I = current in amperes; R = resistance of buffer in ohms; m = magnification factor of optical system. The resistance of the solutions used for electrophoresis was measured by the use of a Leeds and Northrup electrolytic conductivity bridge No. 4960. 3 ml. of about 1 per cent protein solution was dialyzed for 24 hours in the cold against 1 liter of solution identical with that used in the chamber of the electrophoresis apparatus.

For paper chromatography, Whatman No. 1 filter paper sheets were used. The chromatograms were resolved at room temperature in a mixture of *n*-butyl alcohol, acetic acid, and water, 4:1:5, for 20 hours. The filter papers were dried at room temperature and sprayed with 0.1 per cent ninhydrin in *n*-butyl alcohol containing 5 per cent collidine.

Dowex 50–X8 (200 to 400 mesh) cation exchange resin[3] was purified before use according to the method of Moore and Stein (23).

The polarization optical studies were made with a Leitz research type polarizing microscope

[1] 7.505 gm. of glycine and 5.85 gm. of NaCl in 1 liter of solution.

[2] Purchased from Bender and Hobein, Munich, Germany.

[3] Purchased from The Dow Chemical Company, Midland, Michigan.

equipped with a retardation plate red I and Berek compensator. The double refraction ($n_{\parallel} - n_{\perp}$) of single cornified cells was determined according to Ambronn's immersion technique (1, 28), using non-swelling immersion media (ethanol, chloroform, *o*-xylene, and aniline). The refractive indices of these media were determined with the Abbé refractometer. The cornified cells were immersed on slides for 48 hours in each of the above media, and retardations (r) were measured with the Berek compensator in white light (optical center 550 mμ). The thickness (d) of the cells was determined by focusing first on the top then on the bottom of the cells, and reading the difference on the micrometer scale of the microscope. Double refraction was calculated from the equation $(n_{\parallel} - n_{\perp}) = r/d$.

Experimental Material

For the present study, the thick cornified epithelium of human plantar skin (callus) was used (Fig. 1 A). Isolated, large pieces of cornified epithelium were first washed in running distilled water for about a half hour, then cut into small pieces with scissors, and dried at room temperature in a desiccator under vacuum. Subsequently, the material was ground in an Abbé pebble type ball mill, using porcelain balls, at a constant speed of 115 R.P.M. for 17 hours. The resulting powder was passed through a 25 mesh sieve to remove large particles. Microscopic examination of the powder showed that the cornified epithelium was only partially destroyed and that the powder consisted of single cornified cells, a few clumps, and cell fragments. A photomicrograph of pulverized cornified epithelium is shown in Fig. 1 B.

For the purpose of establishing a reference standard for dry, pulverized cornified epithelium, analyses were made which gave the following data: ash content, 2.5 per cent; total carbohydrates, 1.7 per cent; total ethanol-extractable lipids, 7 to 9 per cent. The lipid-free, dry powder contained 15.6 to 16.6 per cent nitrogen, 0.08 per cent phosphorus, and 0.6 to 0.8 per cent cystine.

Identification of the Main Components of the Cornified Epithelium and Establishment of the Fractionation Method

To establish a fractionation method for cornified epithelium, the extractability of different components was investigated by using a series of solutions of different pH.

100 mg. samples of pulverized cornified epithelium, containing 26.2 per cent moisture, were put into a series of 10 ml. centrifuge tubes. These were then completely filled with Sørensen's glycine-HCl, glycine-NaOH standard mixtures, varying in pH between 1.2 and 13.0, tightly covered, and shaken continuously for 24 hours at room temperature. Subsequently, the tubes were centrifuged at 3,500 R.P.M. for 30 minutes, the supernatants were decanted, and the sediments washed three times with distilled water by repeated suspension and centrifugation. Finally, the dry weights of the sediments were determined after drying at 110°C. to constant weight. Table I shows the percentage of undissolved material recovered from the pulverized cornified epithelium after extraction with solutions of different pH and subsequently with distilled water.

The results show (Table I) that a fairly uniform amount of material, corresponding to 10 to 15 per cent of the original sample, may be extracted from pulverized cornified epithelium in the pH range 1.2–10.0. The fraction *extractable* by solutions within this range of pH has been called the "soluble fraction." In alkaline solutions between pH 10.3 and 13.0, the cornified epithelium showed increasing dissolution, and in a solution of pH 12.2, 92.5 per cent became

soluble. The fraction *solubilized* between pH 10.3 and 13.0 has been classed as "epidermal keratin." About 5 per cent of the pulverized cornified epithelium showed resistance toward strong alkali (pH 13.0). This fraction contained mainly cell membranes of the cornified cells and has been called the "resistant component" of the cornified epithelium.

From these observations, it was concluded that an optimal condition for extraction of the soluble fraction might best be achieved by using a neutral solution which would leave the epidermal keratin least affected. Furthermore, it was assumed that after extraction of the soluble fraction, the residue would contain epidermal keratin and the small resistant component. Epidermal

TABLE I

Percentages of Pulverized Cornified Epithelium Remaining after Extraction with Sørensen's Glycine-HCl and Glycine-NaOH Standard Mixtures of Different pH

pH of solution	Per cent of undissolved material
1.2	83.7
1.6	86.5
2.2	90.1
3.1	88.5
4.0	90.1
4.9	86.2
5.9	88.0
6.7	87.1
8.2	85.5
9.6	84.4
10.0	82.3
10.3	80.1
11.4	54.6
11.9	23.5
12.2	7.5
13.0	5.0

keratin could be separated from the resistant component and obtained in an altered soluble form by solubilizing the residue in solutions with a pH between 10 and 13. The resistant component can be isolated by treating the residue with a solution of pH about 13 and collecting the residual material.

On the basis of these observations and considerations, a procedure for fractionating pulverized cornified epithelium was worked out. This is shown in Text-fig. 1.

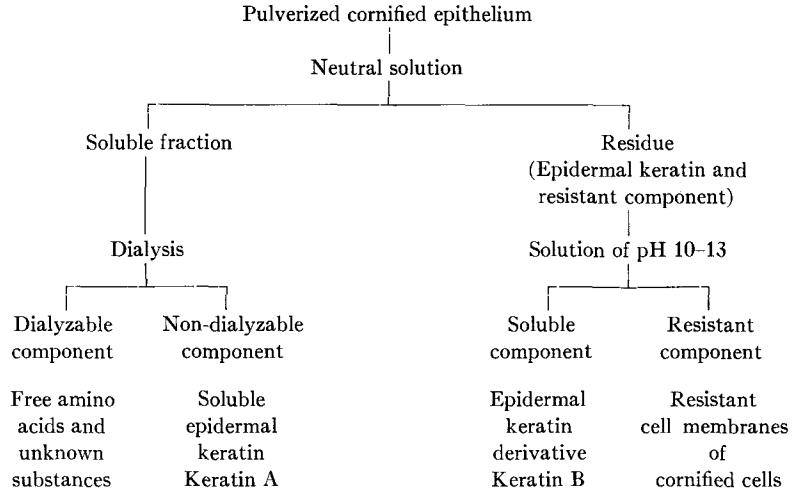
I

SOLUBLE FRACTION OF THE CORNIFIED EPITHELIUM

Preparation.—The soluble fraction of the cornified epithelium was prepared from about 1 gm. of pulverized cornified epithelium which was suspended in 500 ml. of Sørensen's standard

phosphate buffer at pH 7.1 and continuously agitated with a magnetic stirrer for 48 hours at 0°C. The suspension was then twice filtered through double filter paper and the filtrate centrifuged for 1 hour at 3,500 R.P.M.. The supernatant appeared as a slightly yellow, water-clear solution and was called the "soluble fraction." Dry weight determinations showed that by this method about 21 per cent of the original material can be extracted as compared to 12.9 per cent (pH 6.7) and 14.5 per cent (pH 8.2) extracted in the procedure described above (Table I).

Properties.—Information about the main components of the soluble fraction was obtained by continuous-zone electrophoresis. In trial experiments, Sørensen's standard phosphate buffers of pH 3.7, 5.2, and 7.1, as well as Michaelis' veronal buffer of pH 8.5, were used. The most satisfactory separation was ob-



TEXT-FIG. 1. Scheme of fractionation of pulverized cornified epithelium.

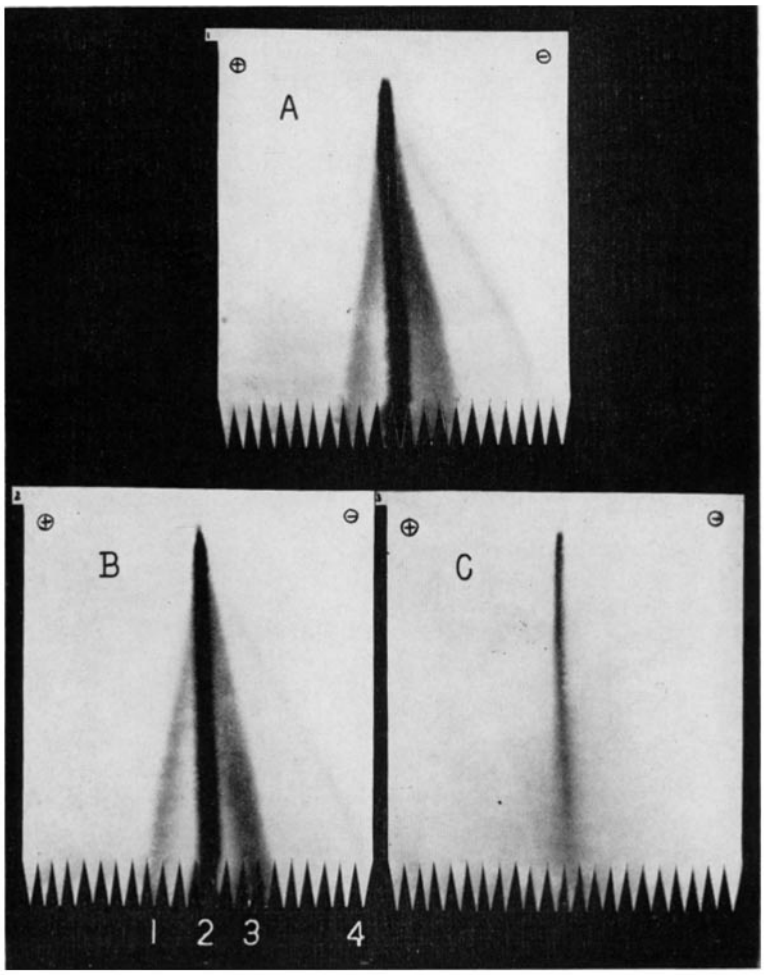
tained with phosphate buffer of pH 5.2. After the paper was sprayed with ninhydrin, four components were seen to have separated (Text-fig. 2 A).

Dialysis experiments on the soluble fraction showed that only a portion of it passes readily through the collodion bag, while approximately 60 per cent is non-dialyzable. Continuous-zone electrophoresis of the dialyzable component in phosphate buffer of pH 5.2 showed a pattern similar to that of the original soluble fraction (Text-fig. 2 B). The non-dialyzable component, however, formed only one central line (Text-fig. 2 C). This non-dialyzable component of the soluble fraction, which was found to be a protein, will be referred to as "soluble epidermal keratin," or keratin A.

Dialyzable Component of the Cornified Epithelium

The foregoing continuous-zone electrophoresis studies suggest that protein decomposition products, such as small peptides and free amino acids, might be present in considerable quantities in the dialyzable component of the cornified

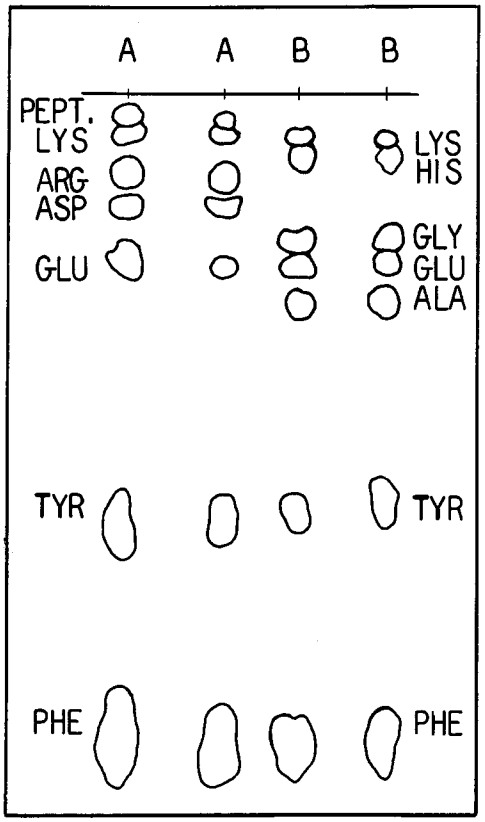
epithelium. If Text-fig. 2 B is interpreted according to Grassmann and Hannig's studies on the separation of amino acids by continuous-zone electrophoresis



TEXT-FIG. 2. Continuous-zone electrophoresis pattern of soluble fraction of cornified epithelium (A), dialyzable component of soluble fraction (B), and non-dialyzable component of soluble fraction (C). The patterns were obtained after 3 hours of electrophoresis in Sørensen's phosphate standard buffer of pH 5.2. The filter papers were developed by spraying with ninhydrin solution.

(13, 15), *line 1* can be assumed to indicate the presence of acidic amino acids, *line 2* might contain neutral, and *lines 3* and *4* basic amino acids. In order to get more definite information on the constituents of the dialyzable component, one dimensional paper chromatograms were made.

The dialyzable component was prepared by dialyzing the soluble fraction against several changes of distilled water. The combined dialysates were run through a Dowex 50 column (H^+ form) to adsorb the protein decomposition products. Subsequently, the adsorbed material was eluted with 1 N ammonium



TEXT-FIG. 3. Tracings of a chromatogram of the dialyzable component of cornified epithelium (A) and of a known amino acid mixture (B). Chromatogram was resolved in *n*-butanol, acetic acid, water, 4:1:5, and developed with 0.1 per cent ninhydrin in *n*-butanol containing 5 per cent collidine.

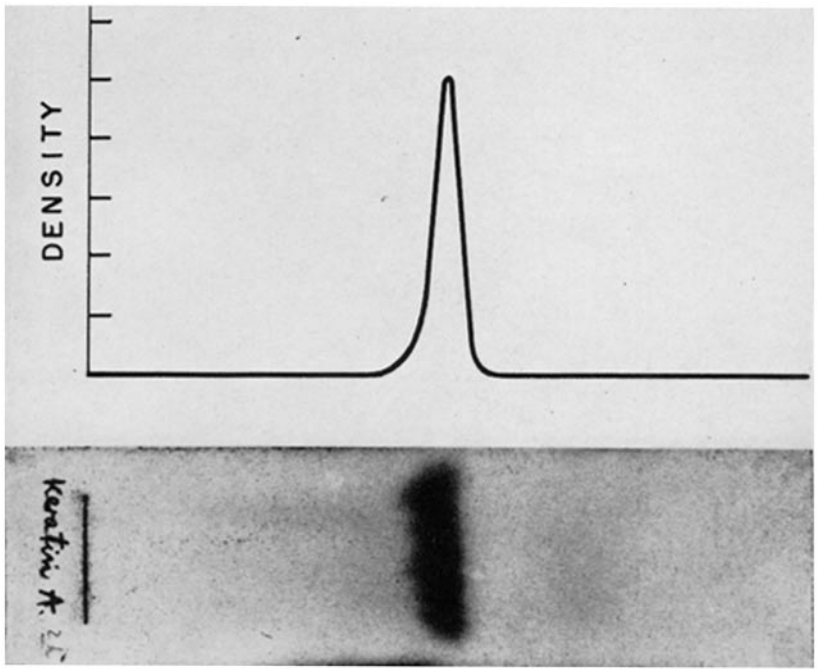
hydroxide, evaporated in a stream of air, and redissolved in distilled water. In the chromatogram of the eluted fraction, peptides, lysine, glutamic acid, tyrosine, and phenylalanine were identified (Text-fig. 3). Two spots, lying between lysine and glutamic acid, were suspected to be aspartic acid and arginine.

Tests for desoxyribose and ribose nucleic acids were negative, indicating the absence of these substances from the dialyzable component. Other constituents of the dialyzable component were not investigated.

Soluble Epidermal Keratin

Keratin A

Keratin A was prepared by two methods. The first consisted of prolonged dialysis of the soluble fraction against running distilled water. In the second, keratin A was precipitated at its isoelectric point by adding 0.1 N hydrochloric acid drop by drop to the soluble fraction. At pH 4.1, keratin A precipitated in the form of an extremely fine, particulate precipitate. This



TEXT-FIG. 4. Zone-electrophoresis pattern of keratin A obtained after 2 hours in Sørensen's citrate standard buffer of pH 12. Upper curve shows relative density values along the paper strip.

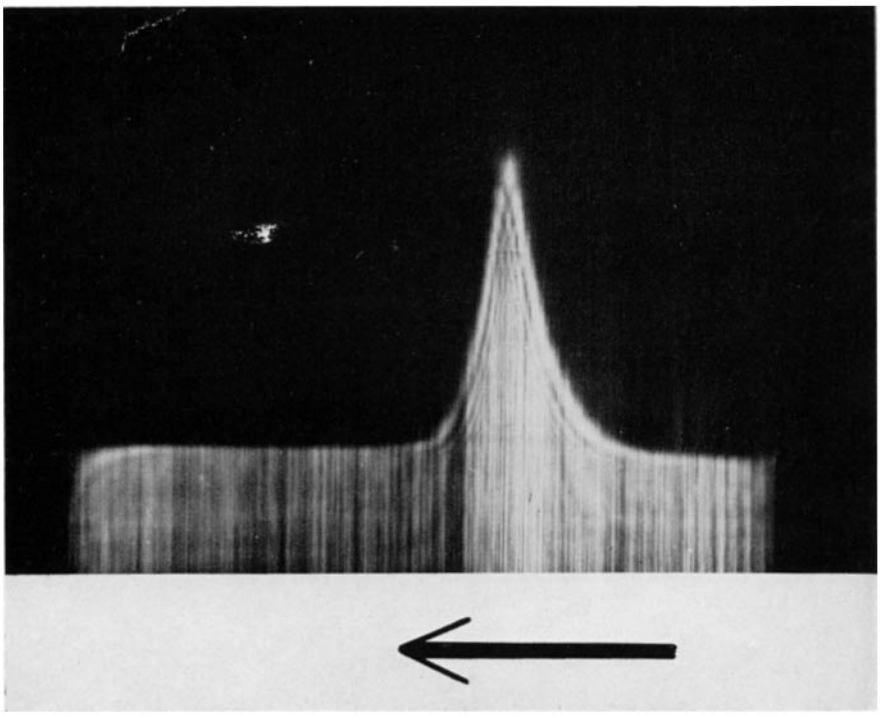
precipitate readily dissolved in Sørensen's standard phosphate buffer of pH 7.1 and could be reprecipitated and redissolved several times by repeating the processes already described

Keratin A preparations obtained by both of the above procedures were found to be electrophoretically homogeneous, as observed by zone electrophoresis. For electrophoresis, Sørensen's standard phosphate buffers of pH 5.5 and 7.1, citrate buffer of pH 12, and Michaelis' veronal buffer of pH 8.5 were used. In each of these buffers, a single band was seen on the filter paper strips, its rate of movement being determined by the pH of the buffer (Text-fig. 4).

For moving-boundary electrophoresis, once precipitated keratin A and Sørensen's glycine-HCl and glycine-NaOH standard mixtures, with the pH varied between 2 and 8, were used. In each solution, a single peak was seen on the photographic plates taken at different time intervals, also indicating that

this preparation is electrophoretically homogeneous. Text-fig. 5 shows an electrophoresis pattern of keratin A.

The results of mobility studies obtained from moving-boundary electrophoresis are shown in Text-fig. 11 (see page 356). It can be seen that the net electrical charge of keratin A is zero at pH 4.1. Consequently, pH 4.1 was considered to be the isoelectric point of keratin A.



TEXT-FIG. 5. Tiselius moving-boundary electrophoresis pattern of keratin A obtained after 30 minutes in Sørensen's glycine-NaOH standard mixture of pH 7.2.

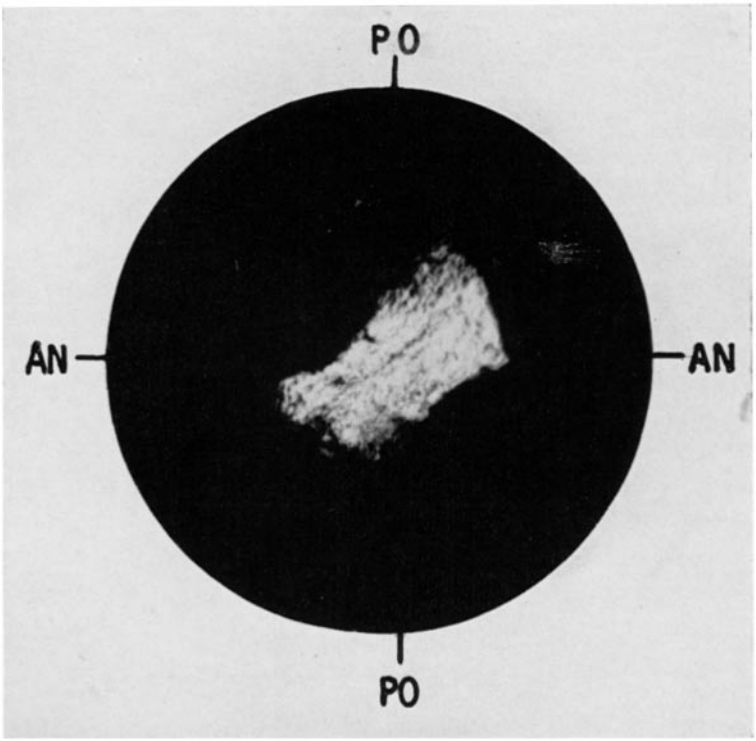
Chemical analyses of twice precipitated dry keratin A revealed 15.71 per cent total nitrogen, 1.7 per cent total carbohydrates, and 0.3 per cent cystine. Tests for phosphorus, ribose, and desoxyribose nucleic acids were negative. Furthermore, the maximum of the absorption spectrum (in phosphate buffer of pH 7.1) appeared at 277 mμ, indicating the presence of aromatic amino acids and the absence of purine or pyrimidine bases.

II

EPIDERMAL KERATIN

The residue of pulverized cornified epithelium, remaining after prolonged extraction with distilled water or Sørensen's standard phosphate buffer of pH

7.1, was used as experimental material for studying the properties of epidermal keratin. The single cornified cells of the extracted powder revealed intense double refraction under the polarizing microscope (Text-fig. 6), comparable to that of non-extracted cornified cells. This demonstrated that the original fibrous structure of epidermal keratin was not affected by the extraction procedures.



TEXT-FIG. 6. A single extracted cornified cell in polarized light.

By using extracted cornified cells, the following information was obtained about the physical and chemical properties of epidermal keratin.

The Structure of Epidermal Keratin

The submicroscopic structure of extracted cornified cells was investigated by polarization optical methods (1, 28). For this study, ten elongated, intensely birefringent cornified cells were selected, all of which showed positive double refraction with respect to the longitudinal axis. One of these cells is shown in Text-fig. 6. The average values of double refraction of the ten cells in various immersion media are shown in Table II. Since the values for double refraction in various media showed insignificant variations, it can be concluded that

double refraction is independent of the refractive power of the immersion medium and corresponds to crystalline double refraction (17, 32). The average value of crystalline double refraction of cornified cells, as calculated from the above results, is $+21.8 \times 10^{-4}$.

TABLE II

Average Values of Double Refraction of Ten Extracted Cornified Cells in Media of Different Refractive Index

Medium	Air	Ethanol	Chloroform	*o*-Xylene	Aniline
Refractive index (n_2)	1.00	1.36	1.44	1.50	1.58
Double refraction $\times 10^{-4}$	22.47 ±2.64	21.16 ±2.22	22.25 ±2.40	21.75 ±2.89	21.39 ±3.27

Since the birefringent component of the extracted cornified cells might be considered epidermal keratin, the observed crystalline double refraction may be related to axially oriented polypeptide chains of epidermal keratin. The lack of form double refraction in the cornified cells indicates that the immersion fluids do not penetrate appreciably between any keratin-containing oriented structural units which are small in relation to the wave length of light. This may betoken a dense submicroscopic structure in which the keratin particles are not separated by definite phase boundaries.

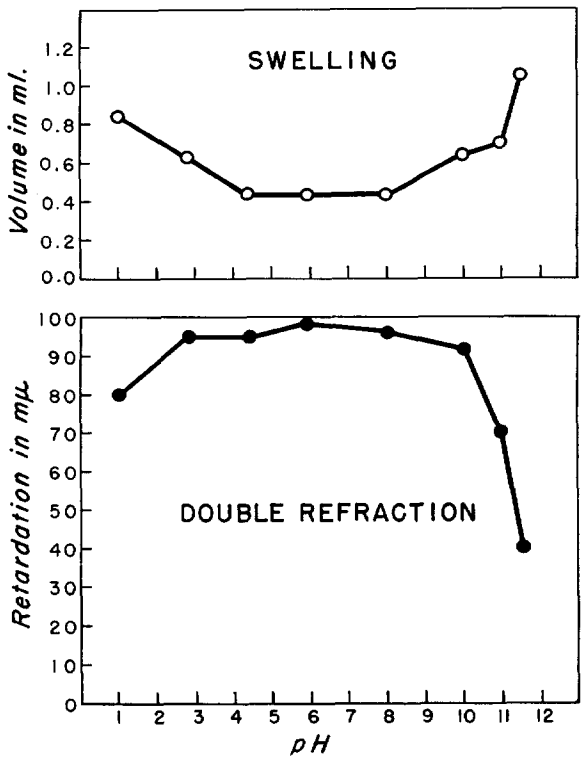
Swelling and Dissociation of Epidermal Keratin

To acquire information about the role of the main structural cohesive forces of epidermal keratin, such as salt linkages, hydrogen bonds, and disulfide cross-linkages, the reaction of extracted cornified cells toward solutions of different pH, concentrated urea, and reducing substances was investigated. It was assumed that as long as the double refraction and volume of extracted cornified cells are constant or reversibly altered, the structure of epidermal keratin remains intact or is only reversibly changed. Irreversible alteration or obliteration of double refraction, as well as decomposition of cells, indicates dissociation of the structure of epidermal keratin.

Effect of pH.—Extracted, single cornified cells were observed under the polarizing microscope while perfused for 20 minutes with Sørensen's glycine-HCl and glycine-NaOH standard mixtures of different pH. Retardation appeared fairly constant while the cells were immersed in solutions of pH 3–10 (Text-fig. 7). In solutions of pH 1 or 11, retardation was reversibly decreased, indicating some alteration of the structure of epidermal keratin. In solutions more alkaline than pH 11, retardation was irreversibly decreased and abolished, implying dissociation of epidermal keratin.

A comparative study of the volume changes of extracted cornified cells at

different pH was carried out by weighing 100 mg. samples of extracted cornified epithelium in a series of 10 ml. graduated centrifuge tubes (Corning No. 8360) and adding 10 ml. of glycine mixtures identical to those used in the above polarizing microscope study. The tubes were shaken for 24 hours at room temperature, centrifuged at 3,500 R.P.M. for 30 minutes, and immediately afterwards



TEXT-FIG. 7. Upper curve shows volume of 100 mg. pulverized cornified epithelium in Sørensen's glycine-HCl and glycine-NaOH standard mixtures of different pH. Lower curve shows values of double refraction (retardation) of a single cornified cell immersed in the same solutions.

the volume of the sediment in each tube was read. The volume of the sediment in all solutions between pH 4.0 and 8.0 was the same (Text-fig. 7), indicating a "stability zone" for epidermal keratin in this range of pH. "Acid swelling" occurred between pH 1.0 and pH 4.0, and "alkaline swelling" between pH 8.0 and pH 10.0. In alkaline solutions higher than pH 10.0, the single cornified cells were intensely swollen and gradually decomposed. In a solution of pH 11.9, the remaining material showed volume increases up to 500 or 600 per cent, indicating "unlimited swelling," dissociation, and solution of epidermal keratin.

Effect of Urea.—10 mg. samples of extracted cornified epithelium were suspended for 24 hours at room temperature in 10 ml. of urea solutions of different concentrations, or mixtures of urea and sodium hydroxide. Subsequently, smear preparations were made from the samples and compared under the polarizing microscope with samples which were suspended in distilled water. Unaltered double refraction of cornified cells indicated that the structure of epidermal keratin was intact (+); reduced double refraction that the structure was affected (±); and obliteration of birefringence was considered to mean dissociation of structure (−). The results of this study are shown in Table III. They indicate that epidermal keratin is not visibly affected by neutral solutions of urea, whereas it readily dissociates in alkaline urea, such as 50 per cent urea in 0.02 N sodium hydroxide.

TABLE III

Effect of Urea on Extracted Cornified Cells

Urea		50 per cent urea in NaOH solutions		Control	
Per cent	DR	NaOH Normality	DR	NaOH Normality	DR
10	+	0.005	+	0.005	+
25	+	0.01	+	0.01	+
50	+	0.02	−	0.02	+
		0.03	−	0.03	−
		0.04	−	0.04	−
		0.05	−	0.05	−

DR, double refraction.
+, unaltered DR.
−, lack of DR.

Effect of Reducing Substances.—The effect of reducing substances (sodium sulfide, potassium cyanide, thioglycolic acid, and their mixtures with sodium hydroxide) on extracted cornified epithelium was studied. The experiments were performed and evaluated in the same way as those described for the study of urea. The results show (Table IV) that for dissociation of epidermal keratin, mixtures of 10 mM potassium cyanide in 0.02 N sodium hydroxide and 2.5 mM thioglycolic acid in 0.02 N sodium hydroxide are most effective. Sodium sulfide is most effective in 0.05 M solution and potassium cyanide in extremely high concentration, such as 0.4 M solution.

Solubilization of Epidermal Keratin

The preceding investigations indicated that epidermal keratin dissociates in alkaline solutions between pH 10 and 13. It was also observed that this process is accelerated when urea or a reducing substance is added to the alkaline

solutions. In further studies, the point to be determined was whether in alkaline solutions epidermal keratin hydrolyzes into amino acids or non-dialyzable derivatives of high molecular weight. To study this problem, the cornified epithelium was extracted with Sørensen's standard phosphate buffer of pH 7.1, dialyzed until salt-free, and then dried in vacuum. After suspension of the extracted cornified epithelium in several concentrations of alkali, reducing

TABLE IV

Effect of Reducing Substances on Extracted Cornified Cells

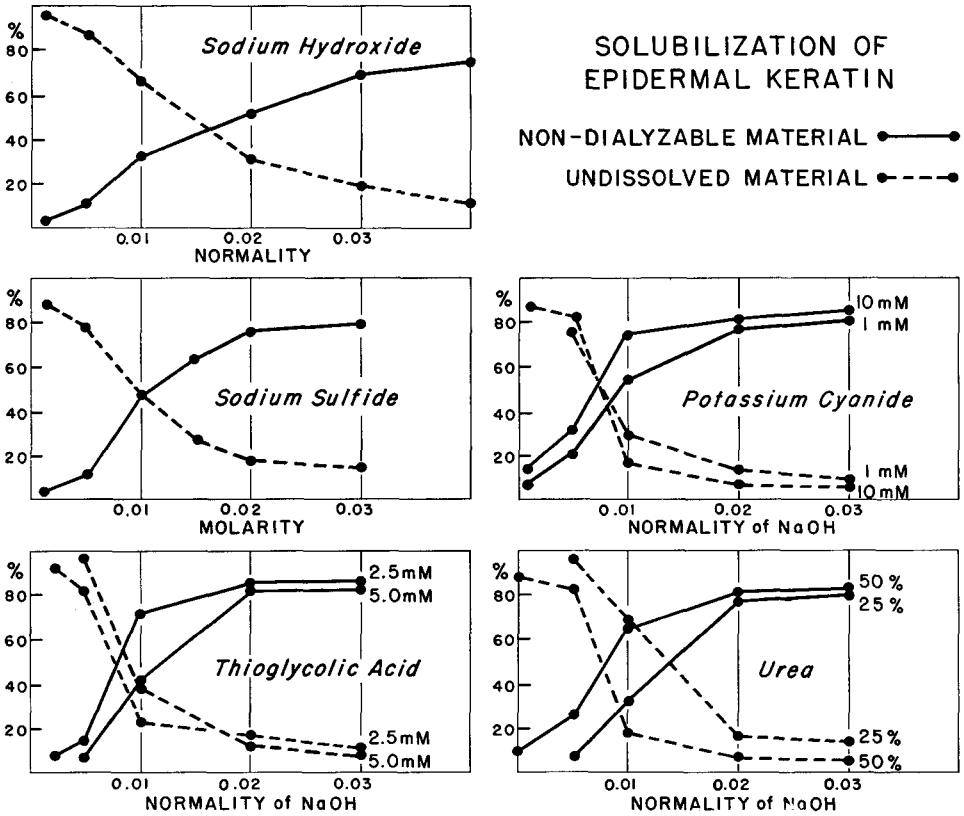
Na_2S		KCN		2.5 mM $CH_2SHCOOH$ in NaOH solution		10 mM KCN in NaOH solution		Control	
Molarity	DR	Molarity	DR	N NaOH	DR	N NaOH	DR	N NaOH	DR
0.005	+	0.005	+	0.005	+	0.005	+	0.005	+
0.01	+	0.01	+	0.01	+	0.01	±	0.01	+
0.02	+	0.02	+	0.02	−	0.02	−	0.02	+
0.03	±	0.03	+	0.03	−	0.03	−	0.03	−
0.04	±	0.04	+	0.04	−	0.04	−	0.04	−
0.05	−	0.05	+	0.05	−	0.05	−	0.05	−
0.06	−	0.06	+						
0.07	−	0.07	+						
0.08	−	0.08	+						
0.09	−	0.09	+						
0.10	−	0.10	+						
		0.20	±						
		0.30	±						
		0.40	−						
		0.50	−						

DR = double refraction.
+ = unaltered DR.
± = decreased DR.
− = lack of DR.

substance, mixtures of reducing substances with alkali, and alkaline urea, the remaining amounts of undissolved and non-dialyzable material were measured.

100 mg. samples of extracted and pulverized cornified epithelium of known moisture content were put into 10 ml. centrifuge tubes. Each tube was completely filled with solution, tightly covered, and shaken continuously at room temperature for 24 hours. It was then centrifuged for 30 minutes at 3,500 R.P.M. and the supernatant decanted. Both supernatant and sediment were dialyzed for 24 hours against running distilled water, lyophilized, and weighed when dry. Text-fig. 8 shows the percentages of undissolved and non-dialyzable materials recovered from pulverized cornified epithelium after it had been suspended in sodium hydroxide, ranging from 0.001 N to 0.04 N solutions; sodium

sulfide, ranging from 0.001 M to 0.03 M solutions; and mixtures of 1 mM and 10 mM potassium cyanide, or 2.5 mM and 5.0 mM thioglycolic acid, or 25 per cent and 50 per cent urea with sodium hydroxide ranging from 0.001 N to 0.03 N solutions.



TEXT-FIG. 8. Percentage of extracted cornified epithelium found as non-dialyzable and undissolved fractions after treatment of the cornified epithelium for 24 hours with alkali, reducing substance, mixtures of reducing substances with alkali, and alkaline urea.

The results show (Text-fig. 8) that, after treatment with these various solvents, most of the solubilized epidermal keratin can be recovered as a non-dialyzable material and that not more than about 20 per cent will be lost as a dialyzable fraction. Accordingly, it was concluded that the main process which occurs during solubilization is the production of a high molecular weight, soluble keratin derivative (keratin B). Hydrolytic decomposition appears to be of only secondary importance in this process.

Epidermal Keratin Derivative

Keratin B

The results shown in Text-fig. 8 indicate that efficient solubilization of epidermal keratin might be achieved by using any of the following alkaline solvents (pH between 10 and 13):

0.02 N NaOH
0.02 M Na_2S
10.0 mM KCN in 0.02 N NaOH
2.5 mM $CH_2SHCOOH$ in 0.02 N NaOH
50 per cent $(NH_2)_2CO$ in 0.02 N NaOH

Keratin B preparations were prepared in the following way: The pulverized cornified epithelium was first extracted with Sørensen's standard phosphate buffer of pH 7.1 for 48 hours. The residue was then dialyzed in running distilled water until salt-free and dried in a desiccator at room temperature under reduced pressure. 50 mg. samples were weighed in centrifuge tubes and suspended in 50 ml. of the above solutions. A few glass beads were added and the tubes were shaken at room temperature for 24 hours, centrifuged for 1 hour at 3,500 R.P.M., and decanted.

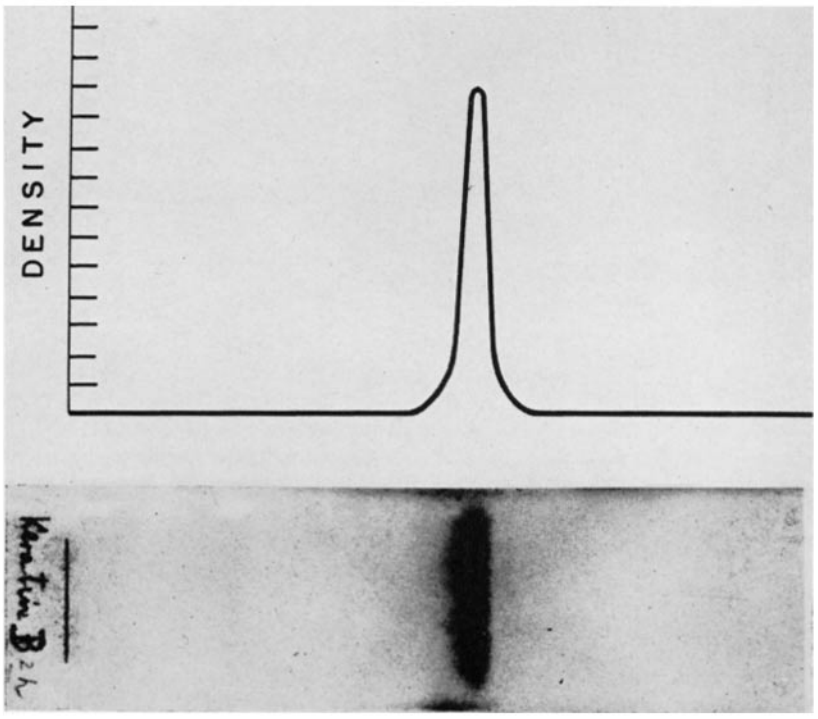
Keratin B preparations all appeared as slightly turbid solutions. After a few hours of dialysis against running distilled water, all formed large, flocculent precipitates on acidification with 0.1 N hydrochloric acid to pH 4.1. Precipitated keratin B did not dissolve in neutral or slightly alkaline solutions, but could be redissolved in 0.01 N sodium hydroxide by moderate stirring of the solution. The dissolved material could then be dialyzed against a weaker alkaline solution, such as 0.005 N sodium hydroxide or buffers of different pH and reprecipitated by repeating the processes already described.

In order to study the homogeneity of keratin B, the different preparations were dialyzed against Sørensen's glycine-NaOH standard mixture of pH 12.8 and their electrophoretic properties were studied in the Tiselius electrophoresis apparatus. Each photograph, taken 15 to 90 minutes after the beginning of electrophoresis, revealed a single broad peak. The mobility values calculated from these experiments are shown in Table V.

The electrophoretic properties of keratin B, obtained with 0.02 N sodium hydroxide, were studied in more detail. For zone electrophoresis, Sørensen's standard phosphate buffers of pH 5.5 and 7.1, citrate buffer of pH 12, and Michaelis' veronal buffer of pH 8.5 were used. In each of these buffers a single band, migrating with different speed, was seen on the paper strips (Text-fig. 9). For moving-boundary electrophoresis, once precipitated keratin B was dialyzed against Sørensen's glycine-HCl and glycine-NaOH standard mixtures varying in pH between 1.2 and 10. In each of these solutions a single electrophoretic component was seen, indicating that the preparation is electrophoretically homogeneous. Text-fig. 10 shows the electrophoretic pattern of keratin B in a solution of pH 10.

The results of mobility studies, obtained from moving-boundary electrophoresis, are shown in Text-fig. 11. Since zero mobility is reached at pH 4.1, this

value was considered as the isoelectric point of keratin B. If the mobility values of keratin B, as well as its isoelectric point, are compared to corresponding values for keratin A (Text-fig. 11) it can be seen that they are similar. This fact seems significant and indicates that by solubilization of epidermal keratin in alkali, a molecular species of keratin is obtained (keratin B) which is similar to that which occurs naturally in the cornified epithelium (keratin A).



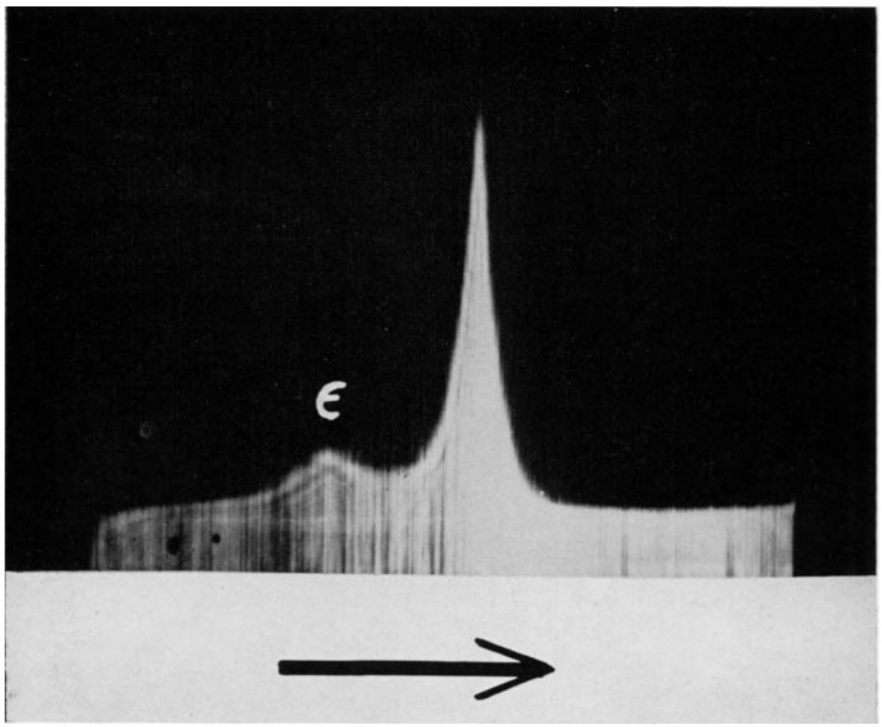
TEXT-FIG. 9. Zone-electrophoresis pattern of keratin B obtained after 2 hours in Sørensen's citrate standard buffer of pH 12. Upper curve shows relative density values along the paper strip.

The results of chemical analyses, obtained for twice precipitated dry keratin B preparations, are shown in Table V. For spectroscopic studies, the preparations were dissolved in Sørensen's glycine-NaOH standard mixture of pH 7.8. Each preparation showed an absorption maximum between 276 and 278 mμ, indicating the presence of aromatic amino acids.

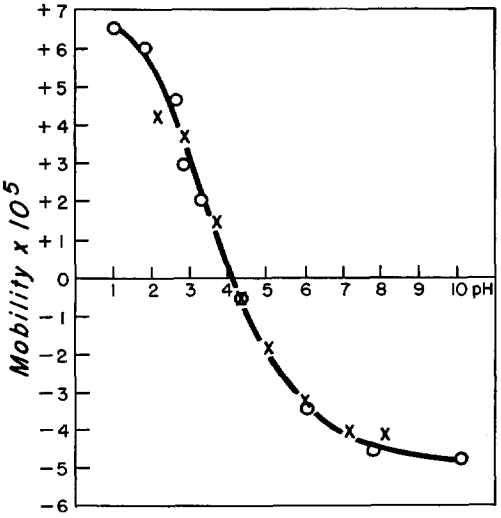
III

RESISTANT COMPONENT OF THE CORNIFIED EPITHELIUM

The resistant component was prepared by suspending 500 mg. samples of extracted cornified epithelium in 100 ml. of 0.1 N sodium hydroxide and shaking the suspension for 24 to 48 hours



TEXT-FIG. 10. Tiselius moving-boundary electrophoresis pattern of keratin B obtained after 60 minutes in Sørensen's glycine-NaOH standard mixture of pH 10. ϵ = buffer concentration boundary.



TEXT-FIG. 11. Electrophoretic mobility of keratin A and keratin B plotted as a function of pH. Keratin A, x, keratin B, O.

at room temperature. After this, it was centrifuged for 20 minutes at 3,500 R.P.M. and the small amount of sediment washed thrice with 100 ml. of distilled water by centrifugation and suspension. Hanging-drop preparations were then made from the resuspended sediment.

Microscopic examination of the preparations showed that the resistant component contains mainly cell membranes of cornified cells and granules in lesser amount. The cell membranes appeared as empty cell envelopes and often resembled the original form of cornified cells, suggesting a fairly rigid structure. Many were fragmented and wrinkled (Fig. 2). Under the polarizing microscope, the cell membranes appeared isotropic at all angles of rotation.

When suspended in a mixture of strong alkali and reducing substance, such as 0.5 M thioglycolic acid in 1.5 N sodium hydroxide, the cell membranes did not dissolve even after several days, denoting their extreme resistance.

TABLE V

Chemical and Physicochemical Data of Keratin B

Preparation obtained by	Per cent			Mobility $\times 10^5$
	N	Carbohydrates	Cystine	
0.02 N NaOH	15.88	0.91	0.23	7.85
0.02 M Na_2S	15.57	0.24	0.23	8.29
10 mM KCN in 0.02 N NaOH	15.72	0.68	—	9.84
2.5 mM $CH_2SHCOOH$ in 0.02 N NaOH	15.68	0.57	—	8.41
50 per cent $(NH_2)_2CO$ in 0.02 N NaOH	15.08	0.96	0.17	7.10

DISCUSSION

The cornified epithelium represents an end-product of keratinization. The results of this study suggest that the dry cornified epithelium consists of approximately 10 per cent soluble epidermal keratin, 10 per cent dialyzable substances, 65 per cent epidermal keratin, 7 to 9 per cent lipids, and 5 per cent resistant cell membranes. These data indicate that keratinization includes not only the formation of keratin, but also disintegration, resorption, and perhaps complete metabolic utilization or alteration of certain nuclear and cytoplasmic constituents of epidermal cells.

Although the structure of epidermal keratin has been satisfactorily explored by x-ray diffraction studies (5, 10), not much is known about the subunits of which it is composed or from which it is synthesized. Since epidermal keratin is an insoluble protein, its soluble derivatives, from amino acids to the higher peptides and their combinations, are of interest in understanding its structure.

Keratin B appears as a high molecular weight derivative of epidermal keratin. It seems to differ in the various solubilizing agents used in this study. Since keratin B preparations in many respects resemble soluble proteins, they can be

looked upon as a satisfactory experimental material for investigation of subunits of epidermal keratin.

With respect to keratin B it appears of interest to recall and compare some observations which were made on wool and feather keratin derivatives. Goddard and Michaelis (11) found that native sheep wool can be completely solubilized within a few hours at 30°C. in 0.5 M sodium sulfide or 0.5 M thioglycolate adjusted with alkali to pH 12 or in a mixture of 0.5 M sodium cyanide and 0.1 N sodium hydroxide (each of these solutions is of much higher concentration than those used for the solubilization of keratin B in this study (Text-fig. 8). The soluble wool preparations contained practically the same amounts of sulfur, nitrogen, and cystine as did the original material. The isoelectric point of soluble wool preparations was estimated to be between pH 4.1 and 4.7, which is comparable to pH 4.1 of keratin B (Text-fig. 11). Sedimentation and diffusion studies of Olofsson and Gralén (24) showed that solubilized wool keratin, prepared at room temperature with 3.5 N sodium sulfide, is polydisperse. The average molecular weight was calculated as 9,000, the average length of molecules was estimated to be 170 A, and the width 11 A. Accordingly, it appears that in a strong reducing substance wool keratin dissociates into single peptide chains. Mercer and Olofsson (21) found that wool, solubilized at 50°C. in saturated urea containing 5 per cent sodium bisulfite and adjusted with bicarbonate and carbonate to pH 8, also appears polydisperse. The average molecular weight of this preparation was calculated as 84,000. The approximate length of molecules was estimated to be 1,150 A, and the width 12.8 A.

Woodin solubilized white feathers of Leghorn hens in a mixture of 10 M urea, 0.1 M bisulfite, 0.05 M phosphate (pH 6.0–8.5) at 40°C. Solubilized feather keratin migrated with a single boundary on electrophoresis, and measurements of osmotic pressure, turbidity, sedimentation rate, and viscosity indicated a molecular weight of 10,000 (34, 35).

A soluble keratin, such as keratin A, extractable with a neutral buffer from the cornified epithelium, has not been previously described. The fact that the electrophoretic mobility of keratin A was found to be similar to that of keratin B (see Text-fig. 11) suggests that keratin A may represent a naturally occurring subunit of epidermal keratin. At the present time, it is not known whether keratin A corresponds to a precursor molecular species of epidermal keratin from which the final stabilized keratin molecules will be formed, or whether it represents a natural degradation product of decomposed epidermal keratin. For the solution of these problems, further characterization of both keratin A and keratin B appears necessary.

The presence of highly resistant cell membranes in the cornified epithelium seems to throw considerable light on the mechanism by which this layer protects the skin. The cell membrane can be looked upon as the specific protective element of each cornified cell, since it is much more resistant than epidermal

keratin. These resistant cell membranes, together with the tightly packed structure of epidermal keratin, appear to constitute a most efficient and resistant means of protection against physical and chemical agents which may impinge upon the surface of the human organism.

Highly resistant membranes in human epidermal tissue were previously described by Lagermalm, Philip, and Lindberg (19). These investigators treated pieces of skin, loosened from sunburned backs, with 1 per cent sodium sulfide at 50°C. for 6 days and at room temperature for 14 days. Examination of the residue under the electron microscope revealed wrinkled membranes 20 to 30μ in diameter, and 100 A in thickness.

With respect to the site of formation of resistant cell membranes in the epidermis, it appears interesting to recall Szodoray's observations (31). He treated skin sections with trypsin and observed that while the lower Malpighian cells were digested, the contours of upper Malpighian cells were still recognizable, indicating that the cell periphery becomes increasingly resistant as the epidermal cells move toward the surface. Accordingly, it appears that cell membranes acquire resistance in the upper Malpighian layer of the epidermis.

SUMMARY

Pulverized cornified epithelium of human skin was divided into a "soluble fraction" and a "residue." About half of the "soluble fraction" proved to be soluble epidermal keratin (keratin A); the remainder, dialyzable substances of low molecular weight. The "residue" contained epidermal keratin and resistant cell membranes of cornified cells. Epidermal keratin was found to form an oriented and dense submicroscopic structure in the cornified cells. It showed high resistance toward strong acid and moderately strong alkali solutions as well as concentrated urea. In strong alkali, reducing substances, alkaline urea, and mixtures of reducing substance with alkali, epidermal keratin dissociated and yielded a non-dialyzable derivative of high molecular weight (keratin B) which resembled true proteins. The cell membranes of cornified cells showed higher resistance toward strong alkali and reducing substance than did epidermal keratin.

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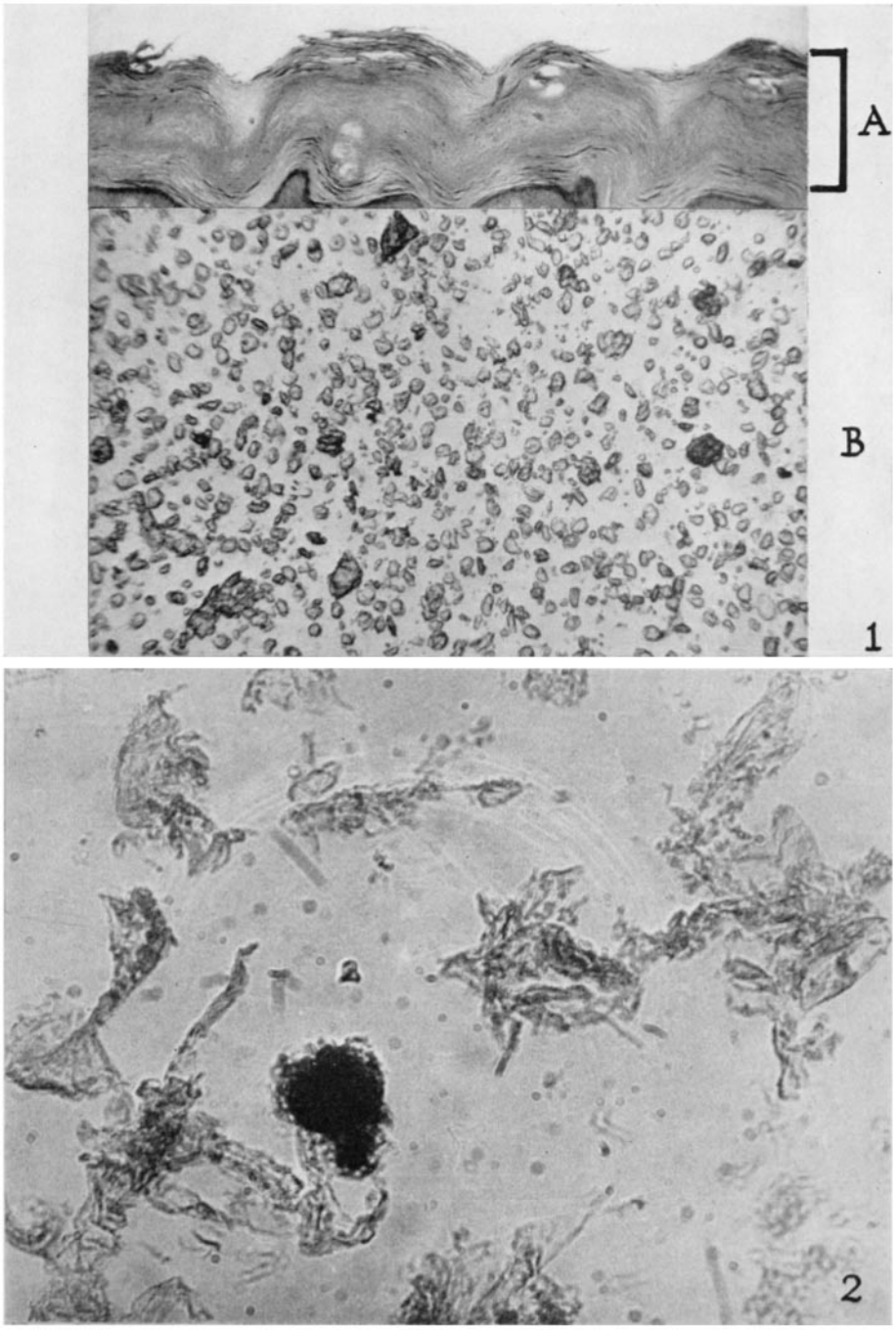
EXPLANATION OF PLATE 91

FIG. 1. A, cross-section of human plantar skin showing thick cornified epithelium. B, photomicrograph of pulverized cornified epithelium (callus) showing single cornified cells, clumps, and cell fragments.

FIG. 2. Isolated cell membranes of cornified cells.



(Matoltsy and Balsamo: Components of cornified epithelium)