

THE CHEMICAL NATURE OF KERATOHYALIN GRANULES OF THE EPIDERMIS

A. GEDEON MATOLTSY and MARGIT N. MATOLTSY

From the Department of Dermatology, Boston University School of Medicine, Boston,
Massachusetts 02118

ABSTRACT

Keratohyalin granules were isolated in the native form from the epidermis of newborn rats by the use of citric acid and a detergent. The isolated granules revealed a fine granular substructure in the electron microscope similar to that seen *in situ*. Analyses of amino acids by automated column-chromatography showed that proline and cystine are present in large proportions whereas histidine is present in a small amount. Accordingly, it was concluded that keratohyalin represents a sulfur-rich amorphous precursor of the horny cell content, rather than a sulfur-poor side product of the keratinization process, or a unique histidine-rich protein as proposed by *in situ* histochemical and radioautographic studies.

INTRODUCTION

Cytoplasmic granules were noted in differentiating epidermal cells by Auffhammer (1) as early as 1869, and a granular layer was described in the epidermis by Langerhans in 1873 (20). Since the granules appeared between the germinative and cornified layers of the epidermis, they were generally regarded as precursors of "keratin." The granules did not stain specifically; thus it was debated as to whether they were composed of lipid or protein. Ranvier (31) assumed lipid nature and proposed to call them "eleidin"; others postulated that the granules were composed of protein such as "hyalin." The popular name "keratohyalin" was introduced by Waldeyer (38) in 1882.

Histochemistry contributed little to the better understanding of the chemical composition of keratohyalin. After Giroud and Bulliard (16) emphasized the importance of —SH and —S—S— bonds in keratinization, attempts were made to demonstrate the presence of sulfur-containing amino acids in keratohyalin granules. While

Chévremont and Frédéric (10) observed a faint reaction by the ferricyanide method, Barnett and Sognnaes (3) were unable to demonstrate —SH or —S—S— bonds with the dichlorodiphenyldichloroethane (DDD) reagent. Since then, some investigators have thought that keratohyalin represented a cytoplasmic debris, a side product of keratinization (3), whereas others believed that it was either a sulfur-poor (7, 24, 27, 35) or a sulfur-rich precursor (8, 10) of the horny cell content. Recently, it was proposed that these granules contained histidine in large proportions. Reaven and Cox (32, 33) and Nagy-Vezekényi (29) demonstrated a strong reaction with the Pauly reagent in support of this view.

On the basis of radioautographic studies, the view was advanced that keratohyalin granules were formed by a unique protein synthesized by granular cells of the epidermis (4, 5, 15). This was based on the observation that tritiated glycine, histidine, serine, arginine, and cystine were taken

up preferentially by granular cells whereas other tritiated amino acids were incorporated preferentially by basal and spinous cells (11-15). Fukuyama and Epstein (13, 14) characterized the unique protein as being rich in histidine and poor in cystine since silver grains appeared concentrated over keratohyalin granules after the use of histidine-³H, whereas after the use of cystine-³H they appeared only at the "edge" of the granules. Fukuyama and Epstein's view on keratohyalin received support by biochemical studies of Bernstein et al. (4, 5, 17, 18, 37). These investigators extracted proteins from the horny and granular layers of the epidermis, and found one fraction that showed a relatively high histidine content; sulfur-containing amino acids were only demonstrable in traces.

Little effort has been made to study keratohyalin granules in vitro. Thus far, only two isolation methods have been established, but neither has proved adequate for collecting keratohyalin in a sufficient amount for amino acid analysis. Recently, Ugel (36) used 1.0 M phosphate buffer at pH 7 for the isolation of these granules from the hoof epidermis of the cow. In his view, the keratohyalin granules were solubilized by this reagent. After clearing the extracted material by ultracentrifugation and dialysis against distilled water, Ugel noted aggregates which resembled keratohyalin granules in density and shape seen in thin sections of the hoof epidermis. Earlier, we (24) found that keratohyalin granules of the newborn rat epidermis were insoluble in the usual solvents of proteins, including buffers in the range of pH 2.9-8.6, 1-3 M urea solutions, and 0.1-1.0% solutions of trypsin. Consequently, we (22) used a mixture of 2 M urea and 1% trypsin to free the keratohyalin granules from epidermal strips. We noted that this mixture did not attack the horny layer, but dispersed the entire noncornified part of the epidermis. Keratohyalin granules obtained after repeated suspension and centrifugation of the dispersed epidermis revealed "sticky" surfaces; most were seen in the electron microscope joined together in clumps. The yield was very small because most of the released granules adhered to tissue fragments and were lost during the preparative procedure.

During the past years our main effort has been to establish a new method whereby keratohyalin granules can be obtained in large quantities for analysis of amino acids. The epidermis of the new-

born rat was selected as experimental material because electron microscopy indicated that keratohyalin granules did not incorporate filaments in this tissue (6, 34) as in humans and other mammals (9, 26, 30). According to Bonneville (6) the keratohyalin granules of the rat epidermis after birth consisted of homogeneous dense material, and tonofilaments seemed to blend into this material only at the surface of the granule. Rhodin and Reith (34) noted that keratohyalin granules of the rat epidermis were frequently surrounded by ribosomes, and filaments abutted their surface. In an effort to find a new reagent for extraction of keratohyalin granules, we studied the solubilizing effect of a wide variety of reagents on the epidermis in the light microscope, and examined structural integrity of the released keratohyalin granules in the electron microscope. As a result, citric acid proved favorable because it disrupted the granular cells efficiently, and set free keratohyalin granules in the native form from their environment. It was also found that clumping of isolated granules could be reduced by the use of a detergent such as Brij 35. These observations formed the basis of a new method whereby keratohyalin granules can be isolated in large quantities for in vitro studies. The new method is described in this paper and data are presented on the amino acid composition of the protein contained by keratohyalin granules as determined by automated column chromatography.

MATERIALS AND METHODS

Experimental Tissue

Newborn white rats, not older than 24 hr, were used. At this age, hair follicles have not yet fully developed and the rat skin is virtually "hairless." After decapitation, the entire skin was dissected from the trunk area, placed with dermal surface on a filter paper, and stored at -20°C in the freezer.

Method of Isolation of Keratohyalin Granules

Preliminary studies showed that keratohyalin granules, about 10 mg in wet weight, can be obtained from the skin of 15 newborn rats. Since this amount proved satisfactory for a single run in the amino acid analyzer, the isolation procedure was worked out for epidermal samples obtained from 15 rats, and was prepared as follows: To separate the epidermis from the dermis, the frozen rat skin with the filter paper was immersed in veronal acetate buffer pH 7.2 of Michaelis containing 0.5% trypsin, 0.45% sucrose,

and 0.3% Brij 35 (polyoxyethylene-lauryl-ether) preheated to 37°C. After about 1 hr, the epidermis was gently lifted in the peripheral region of the skin sample under the dissecting microscope with fine forceps and carefully pulled off in one sheet. Epidermal sheets from 15 rats were rinsed three times in saline and then chopped into small fragments with scissors. After the addition of 15 ml 2% solution of citric acid containing 0.3% Brij 35, the epidermal fragments were homogenized by 20 strokes in a glass tissue grinder. The homogenate was diluted to 150 ml with the same reagent and stirred with a magnetic bar for 10 min. Tissue fragments were allowed to settle for about 5 min, and the supernatant was filtered through double paper S + S No. 588. The remaining tissue fragments were resuspended in citric acid, stirred, and the supernatant was filtered as described. The filtrates were pooled and centrifuged for 30 min at 3000 rpm. The supernatant was discarded, and the sediment was dispersed with a magnetic stirrer in 100 ml of veronal acetate buffer pH 7.2 of Michaelis containing 0.45% sucrose and 0.3% Brij 35. Stirring was continued until test samples revealed keratohyalin granules singly or in small clumps under the phase-contrast microscope. Following this, the isolated material was resuspended and sedimented by the same procedure and the sediment was examined under the phase-contrast and electron microscopes for impurities.

In our estimate, about 90% of the material isolated by the above procedure consisted of keratohyalin granules; the rest was made up of cell debris (see Fig. 7). Impurities markedly increased when keratohyalin was isolated from the epidermis of rats older than 1 day. From rats over 1 day old, the samples were heavily contaminated by small hair fragments which could not be separated from keratohyalin granules as they settled with the same velocity. Furthermore, it was also observed that carelessly prepared epidermal sheets yielded samples with abundant dermal contaminants such as collagen fibres, etc.

Amino Acid Analysis

The material obtained from 15 newborn rats was prepared for amino acid analysis as follows: It was washed twice in 100 ml of distilled water by repeated sedimentation and suspension, and transferred to a thick-walled test tube containing 6 N HCl. After replacing air with nitrogen, the tube was sealed and placed in an oven at 110°C. Prior to hydrolysis, some of the samples were treated with performic acid according to Moore's technique (28) to convert cystine into cysteic acid. Hydrolyzed materials were twice concentrated to dryness with a rotary evaporator, and the residue was dissolved in 0.1 N HCl and

placed on top of the column of the Technicon automated amino acid analyzer.

The analyses presented in this paper were obtained from a pooled sample collected from 90 newborn rats. The sample was divided into three equal parts: one part was hydrolyzed for 24 hr, the second part for 48 hr, and the third part for 72 hr. The hydrolyzate of each part yielded sufficient material for duplicate runs in the amino acid analyzer. The analytical data were in good agreement with those obtained from individual samples prepared from the epidermis of 15 newborn rats. Cysteic acid values of 48- and 72-hr hydrolyzates were close to being comparable to those prepared from performic acid samples, whereas the 24-hr hydrolyzates gave different values. The data, shown in Table I, were not corrected.

Light and Electron Microscopy

For light microscopic studies, samples of the back skin of newborn rats were fixed in 4% neutral formaldehyde solution and processed routinely for paraffin embedding. Sections were stained with hematoxylin and eosin.

For electron microscopic studies, small fragments of the back skin of the newborn rats, and pellets of isolated keratohyalin granules embedded in agar, were fixed for 1 hr in cold, 1% osmium tetroxide buffered to 7.2 with veronal acetate. The samples were embedded in Epon 812 after washing in distilled water, and dehydration in increasing ethanol series. Sections were cut with a diamond knife in the Porter-Blum microtome. Thick sections were stained with toluidine blue, and thin sections with uranyl acetate and lead hydroxide. An RCA EMU 3F electron microscope was used to study structures seen in thin sections.

RESULTS

Light Microscopy

The epidermis of the newborn rat skin consists of a single layer of basal cells, two layers of spinous cells, three to five layers of granular cells, and 10-12 layers of horny cells. Thus, about half or more of the nucleated cells are represented by granular cells in this epidermis (Fig. 1). The lowermost granular cells are relatively small and flat, and contain many small and round keratohyalin granules. The cells are somewhat larger in the mid portion of the granular layer and comprise both small and large granules. The upper granular cells are filled with large granules and irregularly shaped masses of keratohyalin.

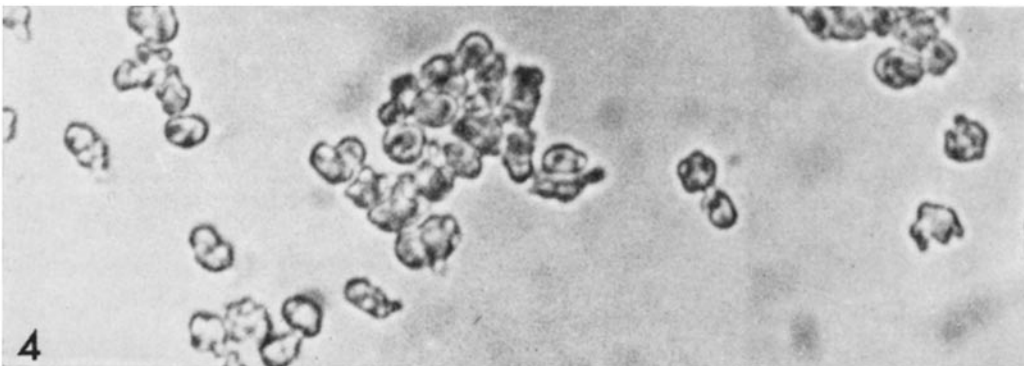
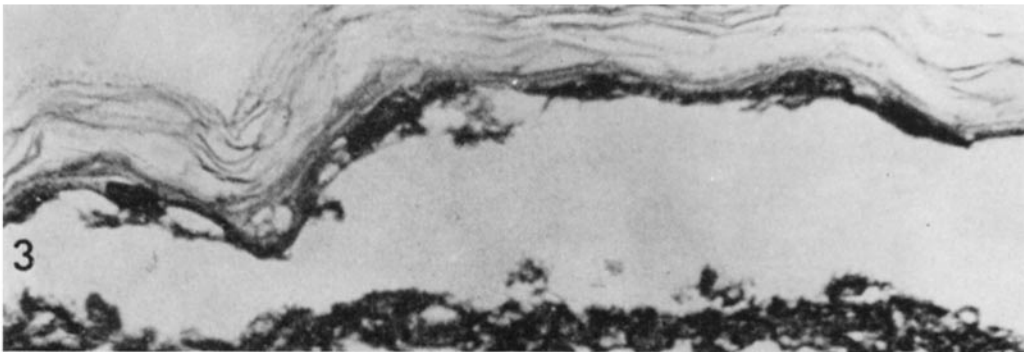
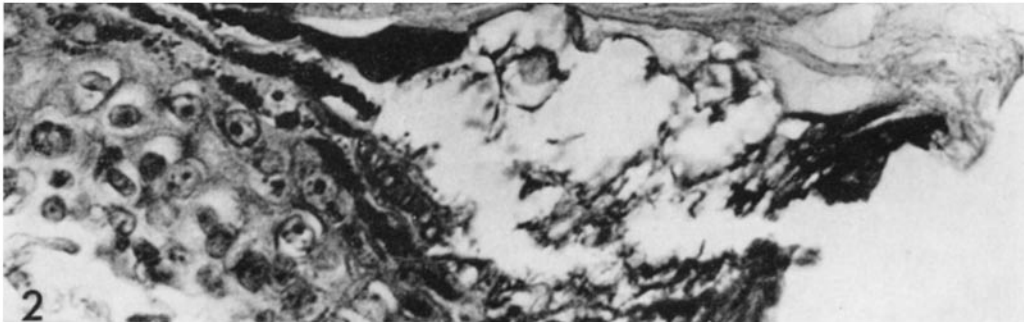
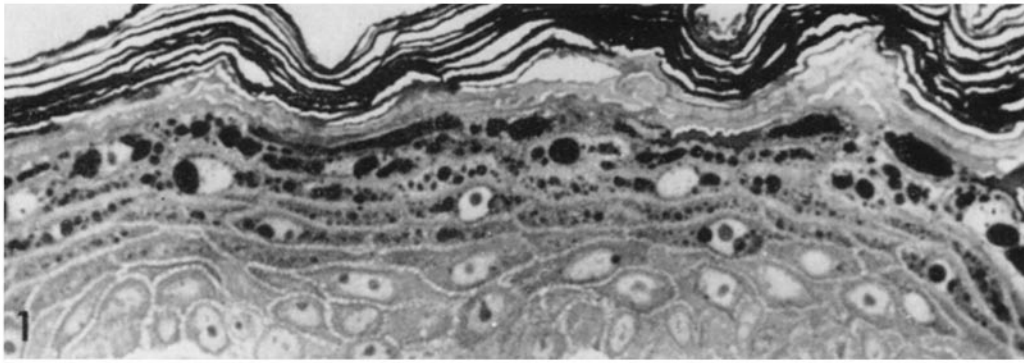


FIGURE 1 Photomicrograph of the newborn rat epidermis. Note multiple layers of granular cells containing relatively large and numerous keratohyalin granules. $\times 800$.

FIGURE 2 The solubilizing effect of citric acid is demonstrated after 10 min treatment of the newborn rat skin. Note dissociation of the granular layer. $\times 800$.

FIGURE 3 Remnants of the epidermis, including the stratum corneum and debris of the basal layer, are shown after 1 hr treatment of the newborn rat skin with citric acid. $\times 800$.

FIGURE 4 Photomicrograph of a fresh preparation of isolated keratohyalin granules. Preparation was dispersed in saline under a cover glass. $\times 1600$.

Citric acid in 2% solution decomposes the non-cornified part of the epidermis as revealed by skin sections prepared from specimens after 10, 20, 30, and 60 min treatment. After 10 min, the horny layer is separated from the rest of the skin at places where citric acid has attacked the granular layer (Fig. 2). After 10–60 min, large areas of the granular layer, as well as portions of both basal and spinous layers, are removed whereas the horny layer and the dermis are not affected (Fig. 3).

Keratohyalin granules, isolated by the use of citric acid, are seen singly or in groups in smear preparations (Fig. 4). The granules are light refractile and appear isotropic in the polarizing microscope. When isolated granules are placed in a drop of 1.0 M phosphate buffer of pH 7, ethanol, pyridine, or dimethyl sulfoxide, they reveal no structural changes. Dissolution, however, occurs in strong acid and alkali solutions.

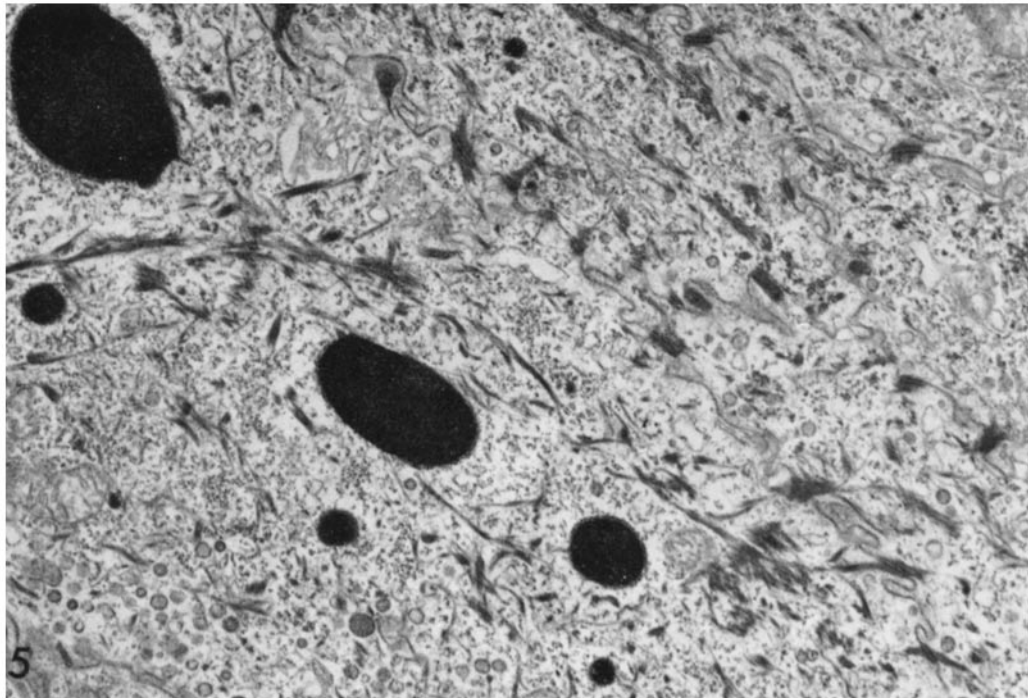


FIGURE 5 Part of the granular layer of the newborn rat epidermis. Note variable size of keratohyalin granules and associations with ribosomes and filaments. $\times 12,200$.

FIGURE 6 Coalescing keratohyalin granules. Note granular substructure, close association with cytoplasmic filaments and ribosomes, and the absence of a limiting membrane. $\times 66,600$.

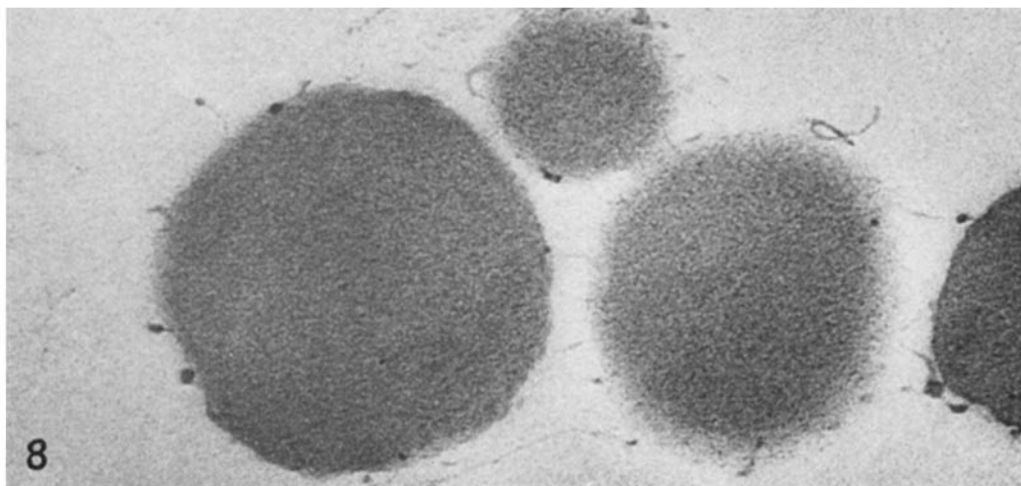
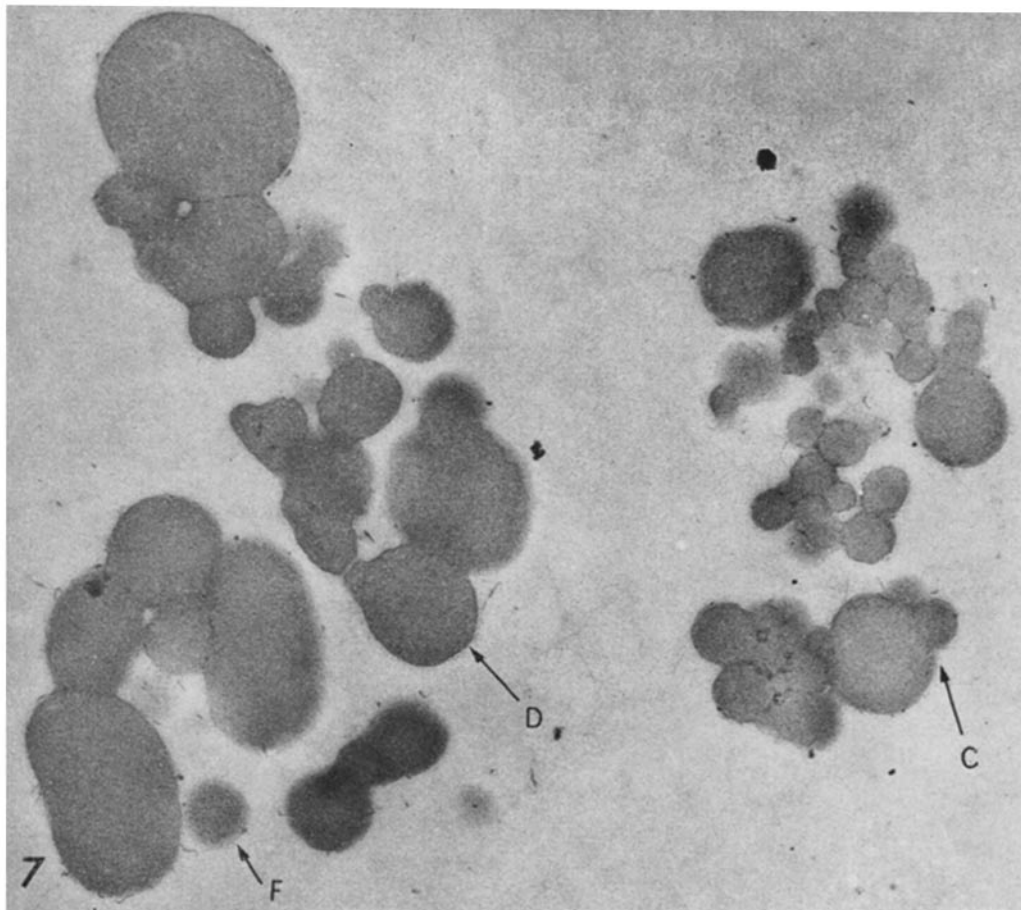


FIGURE 7 Representative field of the preparation obtained by the use of citric acid. Isolated keratohyalin granules appear singly or clumped. Note frayed surfaces (*F*), thin and dense covering layer (*D*), and clumping (*C*) resembling coalescence *in situ*. Cell debris adheres to frayed surfaces or is scattered among the granules. $\times 36,000$.

FIGURE 8 The granular substructure of isolated keratohyalin granules is shown at high magnification. Note the absence of a limiting membrane and the presence of frayed surfaces to which cell debris is adhered. $\times 83,300$.

Electron Microscopy

In thin sections of the newborn rat epidermis, keratohyalin granules vary in size from about 100 Å to several micra (Fig. 5). The small granules are round, the larger ones ovoid or irregular. Most granules are seen singly, some coalescing with one another (Fig. 6). None of the keratohyalin granules are limited by a membrane; their surfaces are in direct contact with the cytoplasmic matrix. Ribosomes and filaments often appear in their vicinity or attached to their surface. At low magnification, the keratohyalin granules reveal an amorphous structure (Fig. 5), whereas at high magnification a densely packed granular substructure can be resolved (Fig. 6).

A representative field of the preparation, obtained by the use of citric acid, is shown in Fig. 7. It can be seen that isolated keratohyalin granules appear singly or in clumps in thin sections of pellets. Ribosomes or filaments are not seen attached or blended into the peripheral part of the granules. The surface of most granules is frayed, revealing separation from filaments and other cytoplasmic components. Cell debris adheres to frayed surfaces and may appear as a thin layer around the granules. Neighboring granules either

fuse or are separated by a thin layer of debris. The isolated keratohyalin granules reveal a granular substructure at high magnification (Fig. 8) similar to that seen *in situ* (Fig. 6).

Amino Acid Analysis

The amino acid composition of the protein of isolated keratohyalin granules is shown in Table I. The amino acids are listed in order of emergence from the column for 24-, 48-, and 72-hr hydrolyzates. It can be seen that threonine, serine, and tyrosine are degraded during longer periods of hydrolysis whereas other amino acids are liberated in increasing amounts as expected. The highest value is obtained for proline, corresponding to 133 residues; half-cystine is represented by 108 residues, i.e. a relatively large amount, and histidine by only 18 residues, i.e., the smallest amount.

DISCUSSION

In the mammalian skin, keratohyalin granules are present in a small amount, and are not readily accessible for isolation, as they are located between two highly insoluble layers such as the stratum corneum and the dermis. The newborn rat is preferable for the isolation of these granules because keratohyalin is formed in this animal throughout the entire trunk epidermis in unusually large amounts as revealed by multiple layers of granular cells and numerous large keratohyalin granules. Skin appendages, such as hair follicles and sebaceous glands, have not yet fully developed, and sweat glands are absent. Consequently, the epidermis of newborn rats can be readily separated from the dermis and obtained in "clean," keratohyalin-rich sheets. Reagents easily penetrate into epidermal sheets through the base of the epidermis; thus the keratohyalin granules are more accessible for isolation than from whole skin specimens. Although dissection of the skin is time consuming, and separation of the epidermis from the dermis is tedious work, it is worth the effort because approximately 100 mg in wet weight of keratohyalin granules can be obtained from 150 newborn rats, an adequate amount for chemical analysis.

Citric acid is more suitable for isolation of keratohyalin granules than the urea-trypsin mixture used in our previous studies (22). This reagent rapidly sets free the granules and does not attack their structure. Release of the granules is primarily due to effective solubilization of filament bundles which stabilize the cytoplasm of epidermal cells.

TABLE I
Amino Acid Composition of Isolated
Keratohyalin Granules

Amino acids	Hydrolysis		
	24 hr	48 hr	72 hr
Cysteic acid	37.90	103.51	108.02
Aspartic acid	48.30	51.12	55.53
Threonine	46.25	40.94	32.49
Serine	130.02	90.08	60.39
Glutamic acid	102.03	103.14	115.95
Proline	112.62	132.56	126.46
Glycine	123.53	129.90	125.99
Alanine	38.41	38.17	40.08
Valine	40.94	43.64	46.58
Cystine	23.09	none	none
Methionine	none	none	none
Isoleucine	24.47	25.45	26.89
Leucine	88.76	92.18	101.01
Tyrosine	50.76	12.91	6.97
Phenylalanine	33.10	35.03	38.05
Lysine	24.98	26.35	28.37
Histidine	12.35	12.57	17.74
Arginine	62.49	62.46	69.50

Residues per 1000 residues.

The remarkable solubilizing effect of citric acid on filaments was already noted in a previous study when the noncornified part of the epidermis was extracted with citric acid—sodium-citrate buffer of pH 2.6, and the fibrous α -protein of filaments was recovered in the native form (23). The use of a detergent, such as Brij 35, is advantageous because it prevents adherence of isolated keratohyalin granules to tissue fragments. As a result, most granules pass through the pores of the filter paper; relatively few are withheld by adhering to nonfilterable tissue fragments.

The isolated keratohyalin granules appear as translucent and light refractile bodies in the light microscope. Their rounded appearance and light refraction resemble properties of lipid droplets; thus it is understandable that in early studies they were related to eleidin (31). On the other hand, their opacity approximates that of hyalin; thus it is comprehensible that these granules were considered to be composed of protein (38). The surface of isolated granules is "sticky," revealing that their content has a glutinous character. Hence, filament bundles abutting the surface, and ribosomes appearing to adhere to keratohyalin granules *in situ* (6, 26, 34), can be interpreted as resulting from adherence to a glutinous mass of keratohyalin *in vivo*. It may be further presumed that glutinous keratohyalin granules attach permanently after contact and coalesce *in vivo* as seen in pellets of isolated granules.

Resistance toward the solubilizing action of citric acid indicates that the main part of keratohyalin granules is formed by highly insoluble protein(s). The amino acid analyses show that the protein, as a whole, is rich in sulfur-containing amino acids and poor in histidine. This result is contradictory to the view that keratohyalin is composed of sulfur-poor and histidine-rich protein based on *in situ* histochemical (3, 7, 24, 27, 35) and radioautographic (4, 11–15) studies. Experiments conducted for clarification of this problem suggest thus far that the color intensity of histochemical tests does not accurately estimate the quantities of amino acids present in keratohyalin granules. Isolated keratohyalin granules spread on a slide show a strong orange-red color after treatment with the Pauly reagent (32), revealing that the intensity of the color is not in proportion with the small amount of histidine. The DDD (2) and ferricyanide methods (10) for —SH give very weak color reaction whereas the aldehyde-fuchsin test for

cystic acid (19) produces a very strong red coloration of isolated keratohyalin granules. Furthermore, studies of Lavker, in this laboratory, indicate that light or electron microscopic radio autography does not provide conclusive results insofar as estimating the amount of the incorporation of tritiated cystine and histidine in keratohyalin granules.

At present, the protein in keratohyalin granules may be best characterized by comparing it with other structural proteins of the epidermis. Significant differences are observed in amino acid composition when it is compared with the fibrous α -protein of filaments (23) (Table II, columns I and 2). The most striking difference noted is that the number of proline residues is high in keratohyalin protein, and is low in filament protein. Since proline is incompatible with α -helical structure, it may be concluded that the protein present in

TABLE II
Amino Acid Composition of Structural Proteins
of the Epidermis

Amino acids	Protein of keratohyalin granules X	Protein of filaments (23)	Protein of the envelope of horny cells (25)	Histidine-rich protein of Bernstein (18)
Proline	133	16	137	none
Cystine 1/2*	104‡	11§	49§	traces
Histidine	13	9	21	64
Aspartic acid	51	88	58	53
Threonine	41‡	39§	37§	60
Serine	90‡	112§	74§	156
Glutamic acid	103	142	140	206
Glycine	130	165	141	136
Alanine	38	66	52	108
Valine	44	51	52	12
Methionine	none	2	none	traces
Isoleucine	25	41	33	18
Leucine	92	89	58	10
Tyrosine	13‡	22§	11§	7
Phenylalanine	35	38	23	4
Lysine	26	51	66	16
Arginine	62	59	46	116
Tryptophane	?	33	?	?

Residues per 1000 residues.

X values of 48-hr hydrolyzate are shown from Table I.

* Determined as cysteic acid.

‡ Uncorrected value.

§ Corrected value.

|| Determined by spectroscopic method.

keratohyalin granules is not fibrous, but amorphous in character. It is also noteworthy that the number of half-cystine residues is about nine times higher in the keratohyalin protein than in the filament protein. This suggests that the former protein is more firmly stabilized by disulfide bonds than the latter, and such bonds are primarily responsible for the insoluble nature of keratohyalin granules. When the keratohyalin protein is compared with the horny cell envelope protein (25) (Table II, columns 1 and 3), it should be noted that both contain a large proportion of proline residues, and also possess half-cystine residues in a relatively large number. The envelope protein is also highly insoluble; thus it is possible that these proteins are of common origin. The presence of few histidine residues distinguishes the protein of keratohyalin granules from Bernstein's protein (18) which has a relatively high histidine content (Table II, columns 1 and 4). The absence of proline and the presence of traces of sulfur-containing amino acids suggest that Bernstein et al. obtained their protein from fibrous components of the newborn rat epidermis rather than from amorphous structures such as keratohyalin granules.

The role of keratohyalin in the keratinization process has been differently interpreted in the past. Histochemists were reluctant to accept the view that keratohyalin represented a precursor of the protective substance contained by horny cells, because negative tests were obtained for sulfur-containing amino acids (3). Electron microscopists made observations which led to the consideration of keratohyalin as a precursor substance (9, 21, 26, 30, 34). Ultrastructural studies have shown that horny cells are encased by a thickened plasma membrane, and are filled with a fibrous-amorphous complex. It was further demonstrated that the content of keratohyalin granules coalesces and infiltrates the spaces between filaments prior to formation of horny cells, revealing that the amorphous matrix is mainly derived from keratohyalin (21). Thus, prior to this study, a correlation of histochemical and electron microscopic studies indicated that the bulk of the horny cell content, i.e. the filament-amorphous matrix complex, consisted of a sulfur-poor fibrous protein derived from cytoplasmic filaments, and amorphous keratohyalin containing no sulfur. The weakness of this view has been that it did not explain adequately stabilization of the structure of the stratum corneum by —S—S— bonds. The sulfur present in

filaments did not account satisfactorily for the total sulfur (2%) of the horny cell; calculations indicated that an unknown component, presumably present in the horny matrix, contained at least 10 times more sulfur than the fibrous component (26).

The properties of keratohyalin granules, as seen in this study, indicate that differentiating epidermal cells synthesize highly insoluble amorphous protein, rich in proline and sulfur-containing amino acids. This protein accumulates in the cytoplasm as tightly packed amorphous particles, and forms viscid inclusions varying in size from a few to several micra. Association of keratohyalin with filaments in the horny cell enveloped by a resistant membrane results in a complex structure that can be held responsible for the high structural stability and chemical resistance exhibited by the stratum corneum of the epidermis. Flexibility and elastic recovery of the content of horny cells is assured by filaments, and cohesion by the amorphous matrix. Chemical resistance is provided primarily by sulfur-rich matrix protein, derived from keratohyalin granules, containing about nine times more sulfur than the fibrous α -protein of filaments (see Table II). The integrity of the filament-matrix complex is ultimately governed by the highly resistant envelope of horny cells. Thus, the amorphous protein of keratohyalin granules significantly contributes to structural stability and chemical resistance of the protective horny layer.

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