Postsynthetic Modifications of Mammalian Epidermal α-Keratin†

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ABSTRACT: The solubility and properties of the α-keratin polypeptides of the living cell layers and of serial slices through the stratum corneum of bovine and human epidermis, which reflect tissue of different ages after keratinization, have been compared. The yields and properties of the α-keratin polypeptides extracted from the living cell layers and inner stratum corneum slice were similar, except the former contained thiol groups and the latter intrachain disulfide bonds, as was shown by Steinert [Steinert, P. M. (1975) *Biochem. J. 149*, 39–48]. In contrast, in progression to the outer slice of stratum corneum, the polypeptides became less soluble, were cross-linked mostly by interchain disulfide bonds, had higher contents of α helix, contained significant amounts of free amino termini, and polymerized into native-type epidermal keratin filaments in vitro with diminished efficiency, and the relative molar amounts of the individual polypeptides were altered and increasing amounts of the high molecular weight aggregates appeared. These aggregates contained the amino acid citrulline and were cross-linked by unusual compounds, three of which were identified as lanthionine, ε-(γ-glutamyl)lysine and lysinoalanine and were present in total amounts approaching 1 mol/mol. These observations suggest that the α-keratin polypeptides in the epidermis undergo modifications after synthesis, such as initial oxidation to form intrachain and then rearrangement to form interchain disulfide bonds and the formation of ε-(γ-glutamyl)lysine cross-links. When the stratum corneum approaches the outer surface of the epidermis, however, the intracellular proteins undergo further modifications such as partial degradation and formation of cross-links under alkaline conditions that are presumably affected by exposure of the tissue to the rigors of the environment.

The epidermis forms the superficial aspect of mammals and functions by providing a tough yet flexible barrier against the environment. These functions reside primarily in the stratum corneum, the fully differentiated, dead, keratinized cell layers of the epidermis. The stratum corneum cells are filled with an insoluble protein complex, keratin, the two major components of which are keratin filaments 70–80 Å in diameter and an amorphous histidine-rich interfilamentous matrix protein (Dale et al., 1978). The insolubility of the proteins has classically been ascribed to the formation of interchain disulfide bonds during keratinization which cross-link the proteins (Fraser et al., 1972) since it has generally been thought necessary to use denaturing solvents with reducing reagents to release the proteins. This view was recently confirmed by Baynes & Levine (1976) and Sun & Green (1978a) but questioned by Steinert (1975) and Baden et al. (1976), who demonstrated that substantial amounts of protein could be released by denaturing solvents without reducing reagents, indicating that the protein contained intrachain disulfide bonds. The characteristic insolubility of the tissue was thought to be due more to the cell membrane (Steinert, 1975; Matoltsy, 1975). In each study, however, the major species solubilized from the stratum corneum was a group of α-helix-rich polypeptides (α-keratin) which constitute the subunits of the intracellular keratin filaments. In addition, the recent detection of the isopeptide cross-link ε-(γ-glutamyl)lysine in epidermal α-keratin has raised the possibility that it might also be involved in the insolubility of the proteins (Abernathy et al., 1977).

The present experiments were designed to reexamine the apparent controversies concerning the insolubility of the α-keratin proteins of the epidermis. We show that distinct changes in their solubility and properties take place following synthesis in the living cell layers of the epidermis. Certain of these postsynthetic modifications are controlled, but others reflect degradation upon exposure to the environment.

Experimental Section

Materials

Bovine skin was obtained from a local abattoir. Human skin (heel callus) was obtained from a normal subject. The following chemicals were obtained from the indicated sources: L-citrulline, L-ornithine, and DL-lanthionine, Sigma Chemical Co., St. Louis, MO; N^{ϵ}-(2-amino-2-carboxyethyl)lysine (lysinoalanine),[1] Miles Biochemicals, Elkhart, IN; ε-(γ-glutamyl)lysine, Vega Biochemicals, Tucson, AZ; iodo[2-^{3}H]acetic acid (specific activity reduced to 370 cpm/nmol) and [^{3}H]methyldansyl chloride (specific activity reduced to 410 cpm/pmol), New England Nuclear Corp., Boston MA.

Methods

Extraction of α-Keratin Polypeptides. Bovine epidermis was dissected from skin proximal to the hoof. Strips (1 mm wide) of epidermis including the living cell layers and five levels of stratum corneum were removed (Figure 1). Unpigmented epidermis from only one hoof was used for each experiment; for example, the data of Tables I–III were each obtained from one piece of tissue. Human epidermis (heel callus) was 0.5–0.6 mm thick. Three successive slices of 0.2-mm thickness were removed with a keratatome. The upper slices were shown by histological section to consist only of stratum corneum. The third slice contained some stratum corneum (<10%), the entire living cell region, and some dermis (<20%). Proteins were then extracted from the tissue with a buffer of 8 M urea and 0.05 M Tris-HCl (pH 9.0) with stirring in an atmosphere of N_2 or in this buffer containing 25 mM 2-mercaptoethanol (Steinert, 1975; Steinert & Idler, 1975). Extractions were terminated by centrifugation at 2000*g* for 5 min and then at 30000*g* for 30 min. Pellets were washed by resuspension with the urea buffer used and the supernatants pooled. Pellets were

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[1] Abbreviations used: Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; $NadSO_4$, sodium decyl sulfate; $NaDodSO_4$, sodium dodecyl sulfate; lysinoalanine, N^{ϵ}-(2-amino-2-carboxyethyl)lysine.

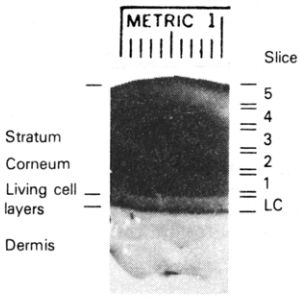

FIGURE 1: Slices of bovine epidermis used in this study.

Table I: Yields[a] of Epidermal α-Keratin Extracted from Different Levels of Bovine Epidermis

extraction conditions	living cell layer	stratum corneum (slice no.) 1 (inner)	2	3	4	5
(1) 8 M urea without 2-ME[b] for 4 h	0.60	0.14	0.08	0.05	0.02	0.02
(2) line 1; then homogenized for 4 h and extracted for a further 4 h	0.05	0.46	0.42	0.30	0.15	0.06
(3) sum of lines 1 + 2	0.65	0.60	0.50	0.35	0.17	0.08
(4) 8 M urea with 25 2-ME for 4 h	0.63	0.67	0.65	0.60	0.51	0.44

[a] Gram per gram dry weight. [b] 2-ME, 2-mercaptoethanol.

then reextracted in the original volume of buffer following homogenization in a Polytron (Steinert, 1975).

Analytical Procedures. Protein was estimated by the method of Bramhall et al. (1969) using bovine epidermal polypeptide 1 as the standard which has $E_{1cm}^{1\%} = 4.8$. Polyacrylamide gel electrophoresis was performed by using the multiphasic buffer system with 0.5% (w/v) sodium decyl sulfate ($NadSO_4$) (Steinert & Idler, 1975). Stained gels were scanned at 610 nm with an Isco Model 659 gel scanner. The N-acylated amino-terminal amino acid (*N*-acetylserine) was isolated from 0.1–0.5-mg samples of protein as before (Steinert & Idler, 1975). Free amino-terminal amino acids were identified and quantified with [^{3}H]methyldansyl chloride (Steinert, 1978). Only those amino acids which were most easily identifiable were quantified (see Tables II and III). Free thiol groups were estimated with iodo[2-^{3}H]acetic acid (Steinert, 1975). α-Helix contents were estimated by use of circular dichroism (Greenfield & Fasman, 1969). Filaments were polymerized and repolymerized in vitro as described previously (Steinert & Gullino, 1976). Proteins were hydrolyzed in 5.7 M HCl for 22 h or by total enzymic digestion (Harding & Rogers, 1976; Rice & Green, 1977). In both procedures, the resulting mixtures of amino acids were analyzed on a Beckman 119CL analyzer equipped with a Model 126 data reduction system. To effect separation of some uncommon amino acids and compounds from the usual amino acids, we used lithium citrate buffers with the following modifications: the pH of buffer B was lowered by 0.35; buffer C was 1.0 N, pH 6.40. Accurate measurement of less than 200 pmol was routinely possible.

Column Chromatography. A column (40 × 2.5 cm) of Sepharose 6B was equilibrated with a buffer of 0.05 M Tris-HCl (pH 7.6), 1 mM dithiothreitol, and 1 mM EDTA containing 0.1% (w/v) $NaDodSO_4$. Protein samples for separation were equilibrated in this buffer by dialysis and heated at 95 °C for 2 min to effect complete disruption of random aggregates and disulfide bonds.

Electron Microscopy. Tissue debris in the 2000*g* pellets following extraction with the urea buffer containing 2-mercaptoethanol (Table I, line 4) was washed 3 times with water to remove soluble protein and urea, fixed in phosphate-buffered glutaraldehyde, postfixed in OsO_4, and embedded, and thin sections were further stained on the grid with uranyl acetate.

Results

Extraction of α-Keratin Polypeptides from Different Levels of Bovine Epidermis. The yields and properties of α-keratin extracted from the six levels of bovine epidermis are presented in Tables I and II, respectively. In the absence of 2-mercaptoethanol, the yields of protein extracted from the slices diminished in progression to the outermost level of the stratum corneum, even after vigorous homogenization (Table I, lines 1–3); up to 0.65 g of protein/g of tissue (dry weight) was extracted in the absence of 2-mercaptoethanol from the slice of living epidermal tissue and the inner slice of the stratum corneum, but less than 0.1 g/g was extracted from the outermost slice. Extraction in the presence of 2-mercaptoethanol afforded higher yields of protein, but the amounts released from the outer slices (slices 4 and 5) were lower (Table I, line 4). Electron microscopy of thin sections through the 2000*g* pellets of urea extracts revealed that debris from the stratum corneum of slice 1 consisted mostly of cell membranes (parts a and b of Figure 2), whereas that from slice 5 also contained a large amount of granular material suggestive of denatured filaments (Figure 2c).

Polyacrylamide gels of the extracted polypeptides showed prominent differences (Figure 3). The band patterns of polypeptides extracted from slices 1 and 2 of the stratum corneum were comparable to those from the living cell layers, but in-

Table II: Properties of Epidermal α-Keratin Extracted from Different Levels of Bovine Epidermis with 8 M Urea Containing 25 mM 2-Mercaptoethanol

property	living cell layer	stratum corneum (slice no.) 1 (inner)	2	3	4	5
(1) protein on $NaDodSO_4$ gels; % protein as monomer polypeptides	98	95	88	74	53	37
(2) $NaDodSO_4$ gels of line 1						
rel molar amt of polypeptide 1	0.32	0.34	0.35	0.41	0.46	0.53
rel molar amt of polypeptide 5	0.33	0.31	0.30	0.26	0.24	0.19
(3) available SH (mol/54 000 g)[a]	6.05	<0.05	<0.05	0.05	0.25	0.85
(4) total α helix (% ± SD)	46 ± 3	45 ± 3	45 ± 3	48 ± 3	51 ± 3	57 ± 4
(5) % filament yields						
initial	83	80	76	69	54	45
repolymerized	95	85	71	51	42	27
(6) free amino-terminal amino acids; sum of DTSEGAVILFYR (mol/54 000 g)	0.0	<0.05	0.05	0.05	0.15	0.45

[a] Protein extracted in the absence of 2-mercaptoethanol as in Table I, line 2.

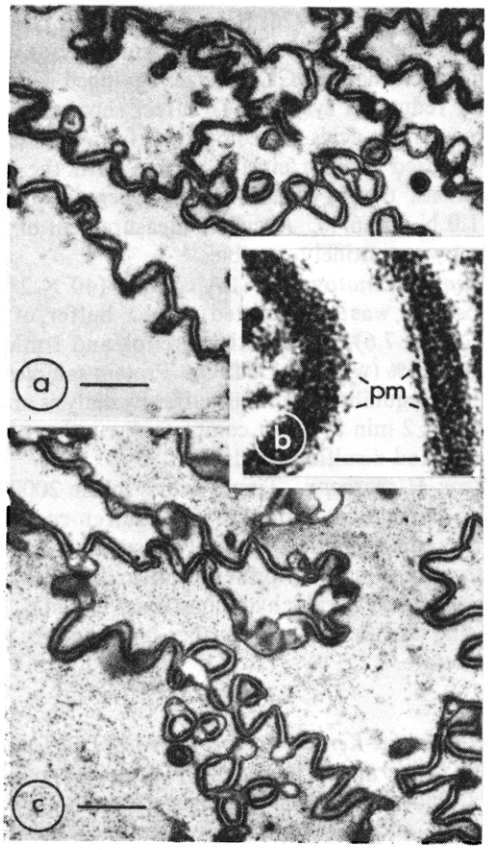

FIGURE 2: Electron microscopy of sections through the 2000*g* pellets of the insoluble debris remaining after extraction with 8 M urea buffer containing 2-mercaptoethanol of the stratum corneum slices 1 (a and b) and 5 (c) of bovine epidermis. The thickened inner surface of the plasma membrane (pm) which is characteristic of the cell membranes of keratinized epidermal cells (Matoltsy, 1975) is evident in (b). (a and c) Magnification ×18 000; the bar is 0.375 μm. (b) Magnification ×115 500; the bar is 0.075 μm.

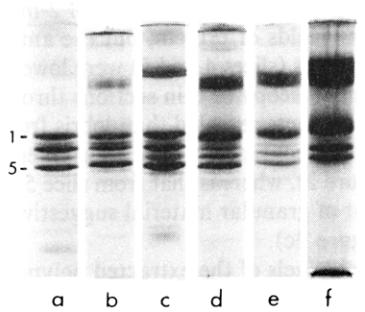

FIGURE 3: Polyacrylamide gel electrophoresis with $NadSO_4$ of polypeptides extracted from six slices of bovine epidermis. (a) Polypeptides from the living cell layers. The numbers indicate the polypeptide numbers referred to in the text. (b–f) Polypeptides extracted from the stratum corneum slices 1–5, respectively.

creasing amounts of proteins of molecular weights 100 000–200 000 were evident in extracts of the outer slices (Figure 3; Table II, line 1). The relative molar amounts of the polypeptides also changed; there was more polypeptide 1 in the extracts of the outer slices in comparison to polypeptide 5 (Figure 3; Table II, line 2), and additional components of lower molecular weight were also present in small amounts. These high molecular weight species could not be dispersed by further boiling in buffer with $NaDodSO_4$ and 2-mercaptoethanol, which suggests that they do not arise through disulfide bonds or noncovalent interactions.

The polypeptides extracted from the living cell layers in the absence of 2-mercaptoethanol contained an average of 6 mol of free thiol groups per average mol (Table II, line 3), as

Table III: Yields[a] and Properties of Epidermal α-Keratin Extracted from Different Levels of Human Epidermis

extraction conditions of property	stratum corneum (slice no.) 1 (outer)	stratum corneum (slice no.) 2 (inner)	living cell layer
(1) 8 M urea without 2-ME for 4 h	0.01	0.08	0.55
(2) line 1; then homogenized for 4 h and extracted for a further 4 h	0.03	0.11	0.02
(3) sum of lines 1 + 2	0.04	0.19	0.57
(4) 8 M urea with 25 mM 2-ME for 4 h	0.39	0.48	0.61
(5) protein from line 1; available SH (mol/58 000 g)	0.45	0.10	5.35
(6) protein from line 4; % filament yields			
initial	42	59	82
repolymerized	35	61	92
(7) protein from line 4; free amino-terminal amino acids; sum of DTSEGAVILFYR (mol/58 000 g)	0.34	0.06	0.0

[a] Gram per gram dry weight.

expected (Steinert, 1975). The polypeptides from the inner stratum corneum slices contained only traces of thiol groups, but those from the outermost slices contained significant amounts (Table II, line 3). Likewise, the polypeptides from the outer slices of stratum corneum progressively differed from those of the inner slices and living cell layers in having higher α-helix contents (line 4), diminished facilities for polymerization into filaments in vitro (line 5), and significant amounts of free amino termini (line 6).

Human Epidermis. The yields and properties of the polypeptides extracted from the three slices of human epidermal callus are shown in Table III. Significant amounts could be extracted from the inner slice of stratum corneum in the absence of 2-mercaptoethanol, but only traces were extracted from the outer slice. This observation is more similar to those of Baynes & Levine (1976) and Sun & Green (1978a). As found with bovine epidermis, the polypeptides extracted from the stratum corneum contained detectable amounts of thiol groups (Table III, line 5). Even when 2-mercaptoethanol was utilized, significantly less protein was extracted from the stratum corneum slices, and that which was contained more free amino-terminal amino acids and polymerized into filaments in vitro with lower efficiency than that from the living cell layers (Table III, lines 6 and 7). On polyacrylamide gels with $NadSO_4$, the polypeptides from the living cell layers were resolved into three major and several minor bands (Figure 4). Their molecular weights were within the range of 50 000–65 000 when compared with the mobilities of fully reduced proteins of known molecular weight. These findings are similar to those of Skerrow (1977), Skerrow & Hunter (1978), Baden et al. (1976), and Sun & Green (1978a) for normal human epidermis. In contrast, the polypeptides extracted from the stratum corneum slices were different: additional bands of higher and lower molecular weight were apparent and the relative amounts of the polypeptides were different from those of the living cell layers. Boiling of the extracted polypeptides with $NaDodSO_4$ and 2-mercaptoethanol did not reduce the amounts of the high molecular weight species.

Characterization of the High Molecular Weight Proteins of Bovine and Human Epidermal α-Keratin. Since it seemed possible that the high molecular weight proteins identified in Figures 3 and 4 contained cross-links other than disulfide bonds, they were separated from the monomer α-keratin polypeptides by chromatography on Sepharose 6B (see Methods). Complete resolution of the components was confirmed by polyacrylamide gel electrophoresis. The properties of these

Table IV: Amounts[a] of Citrulline and Cross-Links Released by Total Enzymic Digestion of Aggregates of α-Keratin and Insoluble Cell Membranes

protein or tissue slice	citrulline	lanthionine	ε-(γ-glutamyl)lysine	lysinoalanine
bovine serum albumin	0.0	0.0	0.0	0.0
polypeptide 1	0.0	0.0	0.0	0.0
enzymes alone	0.0	0.0	0.0	0.0
polypeptide 1 treated with alkali[b]	trace[c]	0.8[d]	trace	2.2[d]
aggregates from bovine epidermis				
living cell layer	0.0	0.0	trace	0.0
stratum corneum slice 1 (inner)	0.0	0.0	8.6	0.0
2	0.0	0.0	9.4	trace
3	trace	trace	10.4	0.2
4	1.1	0.4	10.1	0.7
5 (outer)	3.9	1.2	9.7	1.5
insol debris from bovine epidermis				
living cell layer	0.0	0.0	1.8	0.0
stratum corneum slice 1	0.0	0.0	29.4	0.0
aggregates from human epidermis				
living cell layer	0.0	0.0	trace	0.0
stratum corneum slice 2 (inner)	trace	trace	7.7	0.2
1 (outer)	1.7	1.5	8.9	1.3

[a] Nanomoles per milligram. [b] A sample of bovine polypeptide 1 was treated with 0.2 N NaOH (Bohak, 1964). [c] "Trace" is defined as <0.2 nmol/mg. [d] Mole per mole.

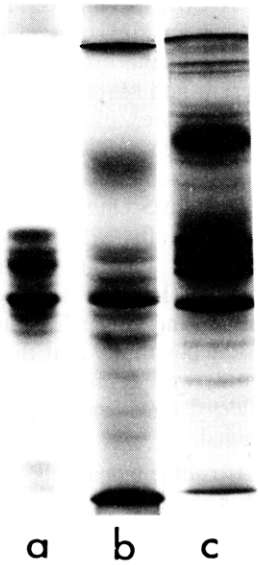

FIGURE 4: Polyacrylamide gel electrophoresis with $NadSO_4$ of polypeptides extracted from the three slices of human (callus) epidermis. (a–c) Polypeptides from slice 3 (mostly living cell layers), 2 (inner stratum corneum), and 1 (outer stratum corneum).

proteins from the various levels of the stratum corneum of both species were investigated as in Tables II and III. Increases in α-helix content, available thiol groups, and free amino-terminal amino acids were observed in these proteins in progression toward the outermost slices. Most significantly, they retained the capacity for polymerization into native-type keratin filaments in vitro with yields ranging from 75 (inner slices) to 20% (outer slice). This strongly suggests that these proteins are aggregates (dimers and trimers) of the monomer polypeptide chains. In addition, the amino acid compositions of the aggregated proteins determined after acid hydrolysis were the same as those of the monomer polypeptides within the limits of measurement, except that several other substances in small amounts were also present. Two of these were identified by chromatography of authentic compounds as citrulline and its acid-hydrolytic product, ornithine. Two other minor components were identified as the cross-link compounds lanthionine and N^{ϵ}-(2-amino-2-carboxyethyl)lysine (lysinoalanine). When the proteins were subjected to total enzymic digestion, the cross-link ε-(γ-glutamyl)lysine was also found. The amounts of each of these compounds were determined following complete enzymic hydrolysis (Table IV). The citrulline and cross-links were not present in detectable amounts in the enzymes or standard proteins used as controls, even though the latter were treated with urea buffers in the same way as the extracted proteins. On treatment with alkali, however, large amounts of lanthionine and lysinoalanine were produced in polypeptide 1 (Table IV) as expected (Bohak, 1964; Mirő & Jaume, 1971). The citrulline and cross-links were not present in the high molecular weight proteins obtained from the living cell layers in both species (Table IV). Except for ε-(γ-glutamyl)lysine, the citrulline and other cross-links were concentrated in the aggregates extracted from the outer slices of stratum corneum. In both species, ε-(γ-glutamyl)-lysine was the major cross-link, and interestingly the amount per milligram of aggregates was approximately constant through all levels of the stratum corneum (Table IV), although the amount per milligram of total protein increased markedly (compare with Table II, line 1). The total amount of all cross-links of 11–13 nmol/mg of aggregates in the outer slice of both species corresponds to ~1 mol/61 000–74 000 daltons, as compared with an average monomer molecular weight of about 54 000 (bovine) and 58 000 (human). Up to 4 nmol of citrulline per mg of aggregates or 0.3 mol/average mol of monomer polypeptides was detected (Table IV). Acid and enzymic digests of the monomer polypeptides separated on Sepharose 6B contained either none or only traces of the cross-links and citrulline (data not shown).

The 2000*g* pellets remaining after extraction of bovine epidermis with urea and 2-mercaptoethanol (Table I, line 4), which contained cell membrane material (Figure 2), were also investigated for their possible citrulline and cross-link contents (Table IV). Following acid hydrolysis, the membrane proteins of the inner slice of stratum corneum contained markedly higher amounts of half-cystine (38 residues/1000 residues) and proline (72 residues/1000 residues) than the urea-soluble α-keratin polypeptides (12 and 16 residues/1000 residues, respectively); the amino acid composition was more similar to that reported by Matoltsy & Matoltsy (1966). After total enzymic digestion, the membrane proteins contained 29.4 nmol of ε-(γ-glutamyl)lysine per mg (Table IV); that is, 21% of the lysine residues of the membrane proteins were involved in this

cross-link. This confirms the observations of Rice & Green (1977), who showed that 18% of the lysine in the cell envelope proteins of human stratum corneum participates in this cross-link. The cell membrane proteins of the outer slice of stratum corneum contained lower amounts of ϵ-(γ-glutamyl)lysine but also contained citrulline, lanthionine, and lysinoalanine, although in diminished amounts (Table IV). These observations indicate that this fraction contained some urea-insoluble α-keratin polypeptides, as suggested by electron microscopy (Figure 2c).

Discussion

Steinert (1975) reported that the α-keratin polypeptides (the subunits of the intracellular keratin filaments) could be extracted from a slice of the stratum corneum of bovine epidermis in the absence of reducing reagents. As these polypeptides did not contain thiol groups, it was concluded that they contained intrachain disulfide bonds. Since that time, several investigators have reported that the α-keratin polypeptides of bovine (Lee et al., 1976) or human (Baynes & Levine, 1976; Sun & Green, 1978a; Baden & Lee, 1978) epidermis can only be extracted in the presence of reducing reagents; they suggested that the polypeptides are cross-linked in the tissue by interchain disulfide bonds (Matoltsy, 1975). In addition, the numbers and properties of the polypeptides of stratum corneum have been reported to be different from those of the living cell layers (Baden et al., 1976; Baden & Lee, 1978). The present work attempts to reconcile the different reports. Using bovine epidermis proximal to the hoof which is unusually thickened (Ugel & Idler, 1970), it has been possible to remove serial slices which reflect tissue of various ages after synthesis of the proteins and then differentiation (keratinization). The findings reveal that there are progressive changes in the solubility and properties of the polypeptides. In the inner regions of the stratum corneum (slices 1 and 2, Figure 1), the polypeptides are very similar to those of the living cell layer, except that the former contain intrachain disulfide bonds (Table II). By way of comparison, the stratum corneum used by Steinert (1975) corresponded to slices 1 and 2 used here. The polypeptides from the outer slices of stratum corneum, however, were different in virtually all properties investigated. They were more difficult to extract; a reducing reagent was necessary, establishing that the polypeptides were cross-linked predominantly by interchain disulfide bonds in the tissue. This means that with increasing age after keratinization, the orientation of the disulfide bonds alters, possibly due to oxidation and/or the action of a disulfide exchange enzyme suspected of being present but not yet detected in keratinizing tissues (Fraser et al., 1972). In addition, the yields of protein extracted were diminished in the outer slices of stratum corneum. The protein which was extracted contained significant amounts of thiol groups and free amino termini and polymerized into keratin filaments in vitro with lowered efficiency, unlike the polypeptides extracted from the inner slices of stratum corneum or the living cell layers. Furthermore, the relative molar amounts of the polypeptides were altered, additional protein species were observed, and large amounts of high molecular weight polypeptides were present that were cross-linked by unusual bonds. These altered properties of the polypeptides are similar to some of the changes reported by Baden et al. (1976) and Baden & Lee (1978).

The same general changes in solubility and properties in the α-keratin polypeptides of human callus epidermis were apparent.

In each of the studies of other investigators, the epidermis of the species used was much thinner, and as serial sections through the stratum corneum were not studied, marked alterations in properties rather than the progressive changes observed here were detected. Tissues representing whole-thickness stratum corneum (Baynes & Levine, 1976; Baden et al., 1976) or scrapings of the outer regions of the stratum corneum (Rice & Green, 1977; Baden & Lee, 1978; Baden et al., 1976; Sun & Green, 1978a) were used for comparison with the polypeptides of the living cell layers.

The presence of cross-links other than disulfide bonds in epidermal α-keratin was first suspected by Goldsmith et al. (1974). Later, Abernathy et al. (1977) detected up to 9 nmol of ϵ-(γ-glutamyl)lysine per mg of α-keratin extracted from the stratum corneum of abnormally keratinizing human epidermis. Slightly larger amounts of this cross-link were detected here in aggregates of α-keratin extracted from the stratum corneum of normal human and bovine epidermis. This cross-link is the product of an epidermal transglutaminase (Goldsmith & Martin, 1975; Hanigan & Goldsmith, 1978; Buxman & Wuepper, 1976, 1978) which appears to predominantly cross-link cell envelope protein (Rice & Green, 1977). However, the urea-soluble proteins containing this compound in this work were clearly α-keratin proteins, since they possessed the ability to polymerize into keratin filaments in vitro and they contained low amounts of proline and cystine which are typically very abundant in epidermal cell membrane proteins [present results and Matoltsy & Matoltsy (1966) and Matoltsy (1975)]. This observation therefore differs from the findings of Rice & Green (1977), who reported that the urea-soluble proteins of human callus stratum corneum contained virtually no cross-link. On the other hand, the insoluble cell membranes remaining after extraction of the tissue (Figure 2) contained substantial amounts of ϵ-(γ-glutamyl)lysine (Table IV), which is the first report of its presence in bovine epidermis and confirms the earlier findings of Rice & Green (1977). However, the possibility that the α-keratin proteins extracted in this study were cross-linked by ϵ-(γ-glutamyl)-lysine to small peptides derived from the cell membrane cannot be rigorously excluded.

The presence of citrulline in extracts of epidermal stratum corneum of a number of species has been reported by Kubilus & Baden (1977), although the amount they detected (2–4 residues/1000 residues or 1–2 mol/average mol of polypeptides) is larger than the 0.2–0.3 mol/average mol observed here. The presence of citrulline in proteins has been ascribed to an enzyme system capable of desimidation of peptide-bound arginine (Rogers et al., 1977). However, the fact that the citrulline was detected only in the outer regions of the stratum corneum suggests that it may not have been formed by an endogenous enzyme system but perhaps by microorganisms which inhabit the outer layers of the stratum corneum.

Two other cross-links, lanthionine and lysinoalanine, were detected in significant amounts in the α-keratin polypeptides of the outer regions of the stratum corneum, which have hitherto not been found in epidermal tissues. Lanthionine and lysinoalanine form in proteins as a result of alkali-catalyzed β-elimination reactions within cystine residues and between adjacent cyst(e)ine and lysine residues, respectively. The cross-links have been identified in hydrolysates of wool proteins, but their occurrence in wool was attributed to the alkaline conditions of extraction employed (Crewther et al., 1965; Mirô & Jaume, 1971; Garcia-Dominguez et al., 1971). In this work, these compounds were not detected in the polypeptides isolated from the living cell layers and the inner stratum corneum slices or the proteins used as controls, suggesting that their occurrence cannot be accounted for by the extraction conditions

used. Nevertheless, treatment of proteins with alkali produced significant amounts of both cross-links, as expected (Table IV).

The presence of a novel series of cross-links in total amounts approaching 1 mol/mol (Table IV) accounts for the marked insolubility of the tissue and the diminished yields of protein that can be extracted from the outer regions of the stratum corneum (Tables I and II; Figure 2c).

The present experiments have demonstrated that the α-keratin polypeptides of the epidermis undergo numerous changes subsequent to synthesis and deposition in the tissue as keratin filaments. At least two of these postsynthetic modifications seem to be controlled functions in the tissue: the oxidation of thiol groups to disulfide bonds and the formation of the cross-link ε-(γ-glutamyl)lysine. In addition, it is now known that the α-keratin polypeptides are phosphorylated (Sun & Green, 1978a; Gilmartin & Freedberg, 1979; P. M. Steinert and W. W. Idler, unpublished experiments), which represents a third form of controlled postsynthetic modification of the proteins. The other changes in the α-keratin polypeptides reported in this paper occurred primarily in the superficial aspects of the epidermis. The simplest explanation of the altered chemical properties, the presence of the unusual cross-links, and the lowered efficiency of in vitro filament polymerization is that the proteins have been partially degraded. The formidable microbiological environment in which cattle, and to a lesser extent humans, live and the proclivity of humans to wash with soap solutions of high pH may be favorable to the formation of the cross-links identified and may also permit partial degradation of the α-keratin polypeptides in the outer regions of the stratum corneum.

The series of changes observed here correlate with the progressive deterioration of the anatomical structure and cohesiveness of the stratum corneum cells observed by King et al. (1979). Thus, the structural and biochemical modifications that occur on exposure to the environment may be inherent characteristics of the desquamation process of the epidermis. While the mildly altered stratum corneum proteins retain many of the properties of the proteins of the inner living cell layers, such as the ability to polymerize into native-type keratin filaments in vitro and their immunological identities (Lee et al., 1976; Sun & Green, 1978b; Steinert et al., 1979), it is self-evident that general comparisons of the properties of the α-keratin of the inner living cell layers with those of the stratum corneum should be made with caution.

Acknowledgments

We thank Dr. Marisa Gullino for skilled assistance with the electron microscopy.

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