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THE FIBROUS PROTEINS OF STRATUM CORNEUM

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The proteins of cow snout stratum corneum can be extracted in part with Tris buffer, pH 9.0, containing 6 M urea. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis shows that about half the extracted material migrates as prekeratin when the extract is treated with both SDS and mercaptoethanol; only trace amounts of the proteins migrating as prekeratin are seen when SDS alone is used. The urea extract gives precipitin lines to an antibody against prekeratin. After exhaustive extraction with the Tris-urea buffer an additional amount of protein is solubilized by extraction with buffer containing mercaptoethanol. This extract shows an electrophoretic pattern similar but not identical to prekeratin. These results suggest that the stratum corneum contains fibrous proteins with different degrees of cross-linking. An analogous system has also been observed in human stratum corneum.

Although keratins have been considered highly insoluble proteins containing large amounts of cystine, the fibrous proteins of epidermis contain only about one ½-cystine residue per 100 amino acids. We have previously shown that the fibrous proteins of stratum corneum are extracted in slightly alkaline buffers containing urea and a reducing agent [1,2]. In those same studies some protein was extracted with a urea buffer without a reducing agent but this protein was thought not to be the α fibrous protein because an α x-ray diffraction pattern could not be obtained [1].

The present study was designed to determine whether the urea-soluble proteins have a fibrous component using more recently developed techniques [3] for identifying the filamentous proteins. In addition, changes in intermolecular and intramolecular cross-linking were examined in the proteins solubilized by various denaturing solvents.

MATERIALS AND METHODS

Snouts were obtained from freshly slaughtered cows and calves from a local abbatoir. Ultrapure urea was purchased from Schwartz-Mann, Orangeburg, N. Y.; sodium dodecyl sulfate (SDS) from Matheson, Coleman, Bell Manufacturing Chemists of East Rutherford, N. J.; bisacrylamide, TEMED, riboflavin, and ammonium persulfate from Eastman Chemical Co., Rochester, N. Y.; mercaptoethanol from Sigma Chemical Co., St. Louis, Mo.; complete Freund's adjuvant and Bacto M Tuberculosis H37 RA from Difco Laboratories, Detroit, Mich.; and agarose from Biomedical Systems, Rockland, Md. All other chemicals were of reagent grade purchased from Fisher Scientific Co. and were used as provided except iodoacetic acid which was recrystallized from anhydrous ether and light petroleum.

Preparation of α-fibrous proteins (Fig. 1). Epidermis was removed from the snouts of freshly slaughtered cows by slicing with a razor blade, and only tissue which did not include dermis was used. The resulting slices were placed in 0.25 M sucrose (150 ml per 18 gm of wet tissue), ground in a Virtis homogenizer for 4 min at 4°C and the resulting suspension centrifuged at 35,000 *g* for 20 min. The pellet was homogenized by hand in 0.1 M sodium citrate-citric acid buffer at pH 2.65 (150 ml per 18 gm of wet tissue) using a conical glass homogenizer, and the resulting mixture stirred at 4°C for 1 hr. The sample was then centrifuged at 35,000 *g* for 20 min and the prekeratin in the supernatant purified by a series of precipitations at pH = 7.0, 6.0, 5.0, and 4.5. The insoluble pellet was reground for 4 min in a Virtis homogenizer at 4°C in sodium citrate-citric acid buffer, pH 2.65, and stirred overnight at 4°C. The suspension was centrifuged at 35,000 *g* for 20 min and the pellet homogenized in a Virtis homogenizer in 6 M urea containing 0.1 M Tris, pH 9.0, (Tris-urea) and stirred under nitrogen at room temperature for 18 hr. Examination of a suspension by light microscopy revealed quite complete breakage of the cells. The suspension was centrifuged at 35,000 *g* and the Tris-urea extraction procedure repeated two additional times. The pellet was then extracted in 6 M urea containing 0.1 M Tris, pH 9.0, and 0.1 M mercaptoethanol (Tris-urea-mercaptoethanol) for 25 hr at room temperature under nitrogen and the suspension centrifuged at 35,000 *g* for 20 min.

Human stratum corneum was obtained from the plantar skin of amputation specimens by heating the excised skin for 1 min at 50°C and lifting off the epidermis. The malpighian layer was removed by gently scraping the lower surface of the specimen. The tissue was then processed as described for cow epidermis.

Antibody production and monitoring. Antibodies to prekeratin were prepared in rabbits using Freund's adjuvant and additional killed tubercle bacilli as previously

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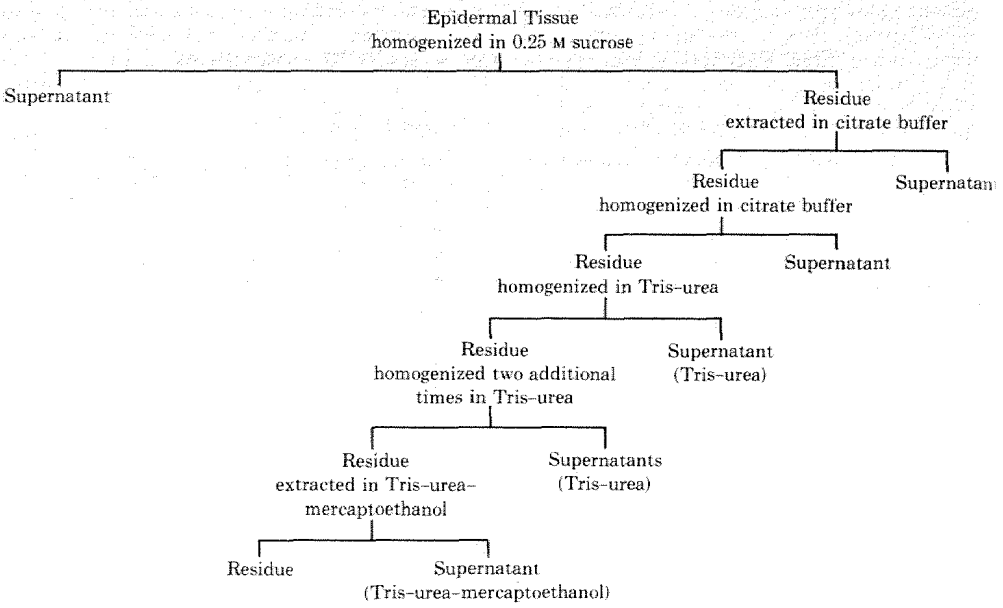

FIG. 1. Flow sheet of extraction procedure.

TABLE. *Yield of solubilized stratum corneum proteins*

The extractions were done sequentially and the amount of protein in each expressed as a percent of the sum of both extracts plus the insoluble residue.

	Yield (%)	
	Tris-urea	Tris-urea-mercaptoethanol
Cow snout epidermis	60	25
Human plantar epidermis	21	42

described [3]. Antibody-antigen reactions were followed by the Ouchterlony technique with the antigen dissolved in 8 M urea.

Protein content. Protein content was determined on the solubilized proteins with the Lowry technique, and the insoluble pellets were washed with water and dried to constant weight at 100°C.

Electrophoresis. SDS-polyacrylamide gel electrophoresis was done using the concentrating gel system described by Neville [4]. The samples were incubated at 50°C for 1 hr either in the presence of 1% SDS or 1% SDS and 1% mercaptoethanol. They were then dialyzed for 18 hr against the electrophoresis buffer.

RESULTS

The sucrose and sodium citrate-citric acid buffer extractions of cow snout epidermis remove proteins from the malpighian layer and leave the stratum corneum intact, since it is resistant to these solvents [5]. As shown in the Table, the Tris-urea buffer appears to extract a very large amount of the stratum corneum and the subsequent Tris-urea-mercaptoethanol buffer solubilizes a smaller additional amount. We found that three additional extractions with each solvent only increased the yield for that solvent by 10%, indicating our extraction techniques were adequate. In the case of human tissue the Tris-urea buffer extracts less material than the subsequent Tris-urea-mercaptoethanol buffer.

SDS-polyacrylamide gel electrophoresis of the Tris-urea-extracted cow snout proteins treated only with SDS showed that most of the protein behaved as large aggregates with very little material in the position of the α polypeptides (Fig. 2). When the Tris-urea-extracted protein was treated with SDS and mercaptoethanol, a pattern identical to prekeratin was observed and this represented about half the material on the gel (Fig. 2). The prekeratin pattern was the same whether or not mercaptoethanol was in the extracting buffer, indicating no interchain disulfide bonds. Proteins extracted in the Tris-urea-mercaptoethanol buffer require the presence of a reducing agent to insure their solubility and they can only be studied by SDS-polyacrylamide gel electrophoresis in the presence of a reducing agent (Fig. 2). The SDS electrophoretogram of these proteins is similar to that of prekeratin but with reproducible differences in the relative intensity of the bands and their mobilities.

Tris-urea extracts of human tissue which were treated only with SDS prior to SDS-polyacrylamide gel electrophoresis aggregated at the origin of the gel (Fig. 3). With the addition of mercaptoethanol to the SDS treatment, a number of distinct bands corresponding to those of the protein extracted

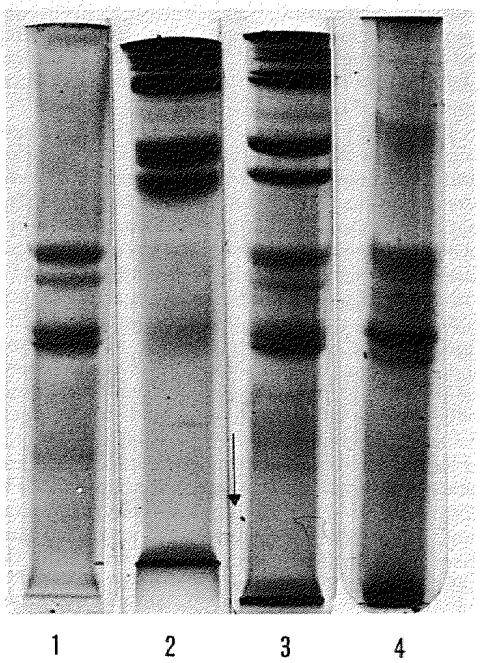

FIG. 2. SDS-polyacrylamide gel electrophoresis of various extracts from cow epidermis. *1* is prekeratin, *2* and *3* the Tris-urea extract, and *4* the Tris-urea-mercaptoethanol extract. In *1* and *2* the samples were treated only with SDS, and in *3* and *4* with SDS and mercaptoethanol.

with the Tris-urea-mercaptoethanol buffer were observed. The patterns, however, were not completely identical.

Following prolonged dialysis against distilled water, the Tris-urea extracts from both human and cow tissues formed clots which redissolved in the Tris-urea buffer. On the other hand, the Tris-urea-mercaptoethanol extracts of both tissues treated in a similar manner would only dissolve in the Tris-urea-mercaptoethanol buffer.

Both the Tris-urea-soluble and Tris-urea-mercaptoethanol-soluble proteins of cow snout epidermis gave precipitin lines with the Ouchterlony technique to antibody prepared to prekeratin and specific for epidermal fibrous protein (Fig. 4). This reactivity was identical to that observed with prekeratin, indicating the presence of both A and B families of polypeptides which have been previously described [3].

The Tris-urea and Tris-urea-mercaptoethanol extracts of human epidermis gave identical precipitin lines to an antibody prepared against Tris-urea-mercaptoethanol-soluble human epidermal proteins (Fig. 5).

DISCUSSION

In developing methodology for isolating the fibrous proteins of stratum corneum, it was decided that a urea extract without a reducing agent should be used first since the extracted proteins did not give an α pattern with x-ray diffraction analyses [6]. It was thought that these urea-soluble proteins were unrelated to the fibrous protein, representing a different component. Indeed, a small amount of material was purified which gave an unusual

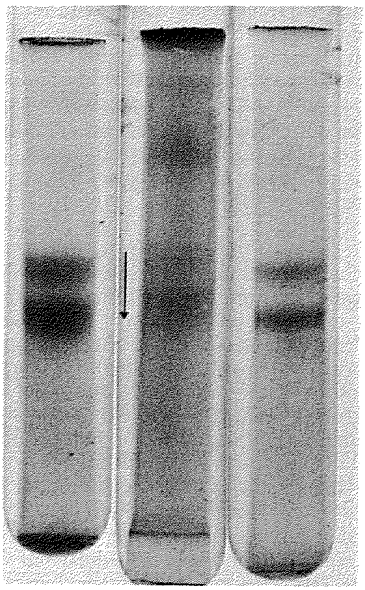

FIG. 3. SDS-polyacrylamide gel electrophoresis of human epidermal proteins. *1* and *2* are the Tris-urea extract, and *3* the Tris-urea-mercaptoethanol extract. *1* and *3* were treated with SDS and mercaptoethanol and *2* only with SDS.

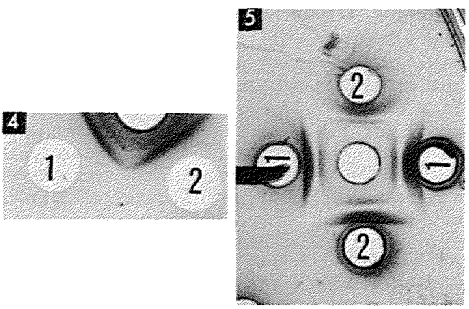

FIG. 4. (*Left*) Double diffusion with Tris-urea (*1*) and Tris-urea-mercaptoethanol (*2*) soluble cow snout epidermal proteins. An antibody to cow prekeratin is in the center well. The two bands are identical to those seen with prekeratin.

FIG. 5. (*Right*) Double diffusion with Tris-urea (*1*) and Tris-urea-mercaptoethanol (*2*) soluble human epidermal proteins. An antibody to Tris-urea-mercaptoethanol-soluble protein is in the center well. A heavy band close to the antigen wells and two weaker bands are seen with both specimens.

cross-β pattern [6]. With the development of more refined electrophoretic and immunologic techniques, it became apparent that this hypothesis was incorrect. Although some unrelated material may be present, the urea extract contains α polypeptides. The explanation for the failure to obtain an α pattern by x-ray diffraction analysis is not clear.

Steinert [7] recently has suggested that mercaptoethanol is not necessary for isolation of cow hoof stratum corneum fibrous proteins. He reported that urea alone when used with a special homogenizer gave the same yield as urea and mercaptoethanol. He stated that the urea-extracted proteins gave the same SDS-polyacrylamide gel electrophoretic pattern as prekeratin and did not require prior reduction. These data implied that interchain disulfide cross-links were not present in the stratum corneum fibrous proteins, although he did report that the cysteine was reduced and ½ cystine increased in the urea extracts of the hoof proteins.

Our results do not agree with his conclusions since electrophoresis in SDS of our cow snout crude urea extracts showed only small amounts of α polypeptides if mercaptoethanol was not used in the electrophoresis. Steinert did not look at the crude urea extract but at a dialyzed, precipitated, and redissolved fraction and this may have selected for the small amount of non-cross-linked α polypeptides. After extensive grinding and extraction with urea which caused very thorough disruption of cells, we obtained an additional fraction with the Tris-urea-mercaptoethanol buffer which, following dialysis against water, did not dissolve in Tris-urea buffer but required a reducing agent. This is evidence against the idea that the reducing agent works by attacking the cell wall. In addition, the electrophoretic pattern we obtained was not identical to that of prekeratin or the Tris-urea extract. Steinert did not extract with a urea buffer containing a reducing agent after the efficient breakdown of cells with urea buffer alone, although extraction with each of the buffers separately showed only a small difference in the amount of material solubilized. Using human stratum corneum we have also found that the Tris-urea extract contains fibrous protein and additional amounts are solubilized with the Tris-urea-mercaptoethanol buffer.

These data indicate that a series of changes occurs in the fibrous proteins during keratinization and this must be viewed as a dynamic process. Prekeratin has only cysteine while the fully differentiated stratum corneum proteins must contain a considerable amount of cystine considering that they require the presence of mercaptoethanol for solubilization. The Tris-urea-soluble extract, unlike prekeratin, needs treatment with a reducing agent before SDS electrophoresis can be done, indicating that disulfide bonds exist between polypeptide chains. The presence of very big aggregates which do not enter the gel suggest that there may be some intermolecular bonds as well. Thus, the urea-extracted proteins may represent an intermediate stage in the keratinization process. The difference in the amount of Tris-urea-extracted and Tris-urea-mercaptoethanol-extracted proteins suggests there may be some variation in the completeness of cornification in the epidermis.

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