Two Polypeptide Chain Constituents of the Major Protein of the Cornified Layer of Newborn Rat Epidermis†

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ABSTRACT: The insoluble component of stratum corneum of rat epidermis yields two major bands after extraction with 8 *M* urea–mercaptoethanol–dithiothreitol. The ratio of these two bands is about 1:1 in terms of protein stain intensity and *S*-[^{14}C]carboxymethyl label. Both polypeptides were purified to homogeneity by DE-52-cellulose, sodium dodecyl sulfate hydroxylapatite C column chromatography, and preparative $DodSO_4^-$ polyacrylamide gel electrophoresis. The heavier polypeptide contains 30% α helix and the lighter contains 27% α helix as determined by circular dichroism studies. Both are sensitive to Pronase and resistant to trypsin, collagenase, and elastase. The lighter chain is stable to pepsin but the heavier can be partially degraded to a smaller polypeptide with a molecular weight similar to that of light chain. Amino acid analysis shows that the light chain contains 12 more tyrosine residues than does the heavy chain, suggesting that the light chain is not generated from the heavy chain. However, the two chains may have a common peptide region. Antiserum prepared against the heavier polypeptide can be completely absorbed by purified lighter polypeptide and vice versa indicating that both chains have some common antigenic determinants. Antibody against either chain can cross-react with the stratum corneum and keratohyalin granules in the epidermis of newborn rat as indicated by fluorescent microscopic observation. Similarly, this antibody also cross-reacts with the cell surface or the contents of spinous and granular cells, and very weakly with basal cells, indicating that the two proteins may be present in the lower strata as well as the stratum corneum.

The stratum corneum is a histologic term which refers to the upper-most layer of epidermis and other cornifying epithelia. It is keratinized and the two terms, cornified and keratinized, are used interchangeably. However, keratinization, the process by which a keratinized stratum is formed, is still primarily described on a morphologic basis. The precise sequence of biochemical events which occur during keratinization is still unresolved, as is the precise description of epidermal keratin. Keratin classically is described either in terms of insolubility in dilute acids and alkalis, water, and organic solvents and resistance to digestion by trypsin and pepsin, or on the basis of an α X-ray diffraction pattern and numerous S–S bonds (Mercer and Matoltsy, 1967). On the other hand, keratin is heterogeneous containing fibrous and nonfibrous components (Mercer and Matoltsy, 1967). The fibrous proteins of epidermis have been studied extensively after extraction from the stratum corneum or the lower cell layers where it is referred to as prekeratin or as tonofilament protein. Prekeratin or tonofilamentous protein are precursors to keratin, but their interrelationship remains to be demonstrated. The extraction procedures for either involve solubilization in urea or citric acid and fractionation by pH precipitation (Rudall, 1952, 1968; Carruthers et al., 1955; Carruthers, 1970; Crounse, 1963; Matoltsy, 1965; Rothberg et al., 1965; Baden et al., 1968; Bernstein et al., 1970; Skerrow, 1972; Tezuka and Freedberg, 1972).

More recently, further purification of solubilized keratinous proteins has employed *S*-carboxymethylation with iodoacetic acid (O'Donnell, 1971; Baden and Goldsmith, 1972; Shimizu et al., 1974). Shimizu et al. (1974) report the presence of two fibrous protein components of molecular weight 68,000 and 61,000 in cornified cells of newborn rat epidermis.

Solubilized epidermal fibrous proteins can be fractionated by pH precipitation (Rudall, 1952; Carruthers et al., 1955; Bernstein et al., 1970). The different pH precipitates of an 8 *M* urea extract of epidermal protein are immunologically related (Carruthers and Bhattacharya, 1972). It is conceivable that all epidermis contains a common subunit (Bauer, 1972) but keratin itself is composed of heterogeneous subunits (Baden et al., 1971).

Earlier studies in this laboratory indicate the presence of two major bands in dodecyl sulfate gels of urea-HSEtOH[1] extracted protein obtained from the stratum corneum of newborn rat epidermis (Dale and Stern, 1975). These protein derivatives are purified and characterized in the course of the present study and their relationship to keratinization is discussed.

Materials and Methods

Preparation of Cornified Tissue from Newborn Rat Skin. The dorsal skin of 10–30 Sprague-Dawley strain rats was taken from 1 to 3 litters ranging in age from newborn to 1 day.

Three different methods were used to obtain cornified tissue from newborn rat skin. These all gave equivalent results and subsequently only method 1 was employed. (1) Trypsin and EDTA digestion (Stern and Sekeri-Pataryas, 1972). (2) Heat separation. The skin was layered on the surface of

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[1] Abbreviations used are: SCM, *S*-carboxymethylated; EDTA, Ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; HSEtOH, 2-mercaptoethanol.

the petri dish and heated for 30 min at 60°. The epidermis was lifted from the dermis with tissue forceps. (3) Sodium bromide separation. The skin was suspended for 2 hr in 2 *M* sodium bromide, at which time the epidermis was lifted from the dermis with tissue forceps.

The epidermis was then stirred for 30 min in 0.1 *M* phosphate buffer (pH 7.0) containing 1% sodium dodecyl sulfate. The residues which are cornified material were collected by centrifugation and washed twice with 0.1 *M* phosphate buffer (pH 7.0). The cornified material was further purified by repeating this procedure. The cornified tissue separated by method 1 was used directly without dodecyl sulfate treatment.

Extraction of the Protein from Cornified Tissue. The tissue obtained above was washed three times with 0.05 *M* Tris-HCl buffer (pH 8.5) containing 4 *M* urea to remove easily solubilized proteins and contaminating trypsin. The residue was suspended in 0.10 *M* Tris-HCl (pH 8.5) containing 8 *M* urea, 10^{-3} *M* dithiothreitol, and 0.1 *M* HSEtOH in a sealed flask, stirred for 2 hr, homogenized, and stirred for another 6 hr. This tissue was spun down (25,000 *g* for 10 min), and reextracted as described above. The combined supernatants were used for *S*-carboxymethylation.

S-Carboxymethylation of the Reduced Protein. Small quantities of the combined supernatants (0.5 ml) obtained above were dialyzed for 3 hr against 0.1 *M* Tris-HCl (pH 8.5) containing 6 *M* urea, 10^{-3} *M* dithiothreitol; 1 *M* Tris-HCl (pH 8.5) and 0.5 *M* [^{14}C]iodacetic acid were added to the dialyzed extract to adjust the concentrations to 0.15 *M* and 0.01 *M* (2.5 μCi/ml), respectively. After standing for 2 hr at room temperature in a sealed and aluminum foil wrapped flask, additional 0.5 *M* nonradioactive iodacetic acid was added to a final concentration of 0.06 *M*. After an additional 4 hr at room temperature, the reaction was stopped by adding excess HSEtOH. The reaction mixture was dialyzed overnight against three changes of 0.05 *M* Tris-HCl (pH 8.5) containing 4 *M* urea and 10^{-3} *M* dithiothreitol.

For the preparation of large quantities of [^{14}C]SCM fibrous protein, 100 ml of cold acetone–HCl [40 ml of acetone:1 ml of 0.1 *N* HCl; modified procedures of White (1964)] was added to 20 ml of combined supernatants obtained above and stirred at 4°. The precipitates were spun down, washed three times with 0.1 *M* Tris-HCl (pH 8.5) containing 10^{-3} *M* dithiothreitol, and dissolved in 0.15 *M* Tris-HCl (pH 8.5) containing 8 *M* urea and 10^{-3} *M* dithiothreitol. This supernatant was then *S*-carboxymethylated as described above.

DE-52-Cellulose Chromatography. The dialyzed proteins were passed through a DE-52-cellulose column (0.8 × 28 cm) preequilibrated with 0.05 *M* Tris-HCl (pH 8.5) containing 4 *M* urea and 10^{-3} *M* dithiothreitol in the cold. The protein was eluted with 600 ml of a linear gradient from 0 to 0.12 *M* KCl in 0.05 *M* Tris-HCl (pH 8.2), 4 *M* urea, and 10^{-3} *M* dithiothreitol. Fractions of 3.3 ml were collected. The ^{14}C-radioactive fractions were pooled and concentrated by negative pressure.

The concentrate was dialyzed against two changes of buffer containing 0.01 *M* sodium phosphate (pH 7.0), 0.1 *M* dodecyl sulfate, 1 m*M* dithiothreitol, and 10 μg/ml of $PhCH_2SO_2F$. The concentration of dodecyl sulfate and HSEtOH in the dialysate was adjusted to 1%, then incubated at 37° for 30 min to prevent protein aggregates and diluted with 5 volumes of 0.01 *M* sodium phosphate buffer (pH 7.0) containing 1 m*M* dithiothreitol and 10 μg/ml of protease inhibitor ($PhCH_2SO_2F$).

Hydroxylapatite C Column Chromatography. The diluted protein solution was passed through a column containing hydroxylapatite C (2.5 × 30 cm preequilibrated with 0.01 *M* phosphate buffer (pH 7.0) containing 1% dodecyl sulfate). The protein was eluted with three linear gradients containing 1 m*M* dithiothreitol, 10 μg/ml of $PhCH_2SO_2F$, 0.1% dodecyl sulfate, and 200 ml each of sodium phosphate buffer (pH 7.0) (from 0.14 to 0.24 *M*, from 0.23 to 0.33 *M*, and from 0.32 to 0.42 *M*). Fractions of 3 ml were collected.

The first radioactive peak fractions were checked by dodecyl sulfate polyacrylamide gel electrophoresis for purity. The fractions which showed only the light chain with a minor polypeptide band were pooled and used for a second DE-52-cellulose column after removing dodecyl sulfate with a Bio-Rad resin column (2 × 10 cm) as described by Lenard (1971). The second radioactive peak fractions containing a major heavy chain plus a minor amount of light chain were pooled, concentrated, and used for preparative dodecyl sulfate polyacrylamide gel slicing.

Total Nitrogen Determination. The method of Stegeman and Loeschke (1962) was used to determine the total nitrogen content of the 8 *M* urea–HSEtOH–dithiothreitol extract and tissue residue. The stratum corneum was extracted twice as described above. The combined extract and tissue residue were dialyzed separately against 4 l. of distilled water at 4° with three changes and homogenized. Duplicate samples of 0.5 ml for each homogenate and dialysate were hydrolyzed and nitrogen was determined. The dialysate was used as a control.

Protein Determination. Protein concentration was determined either by the method of Lowry et al. (1951) or Bramhall et al. (1969). Bovine serum albumin was used as the standard. For amino acid analysis and circular dichroism study, the proteins were measured by the method of Groves et al. (1968). Purified light SCM polypeptide was used as the standard.

Dodecyl Sulfate Acrylamide Gel Electrophoresis. Dodecyl sulfate polyacrylamide gels and buffers were prepared as described by Dunker and Rueckert (1969). Samples to be run on these gels were dissolved in 4 *M* urea containing 1% dodecyl sulfate and 1% HSEtOH, and denatured in boiling water for 1 min. Staining and destaining of the gels were carried out in mixtures of 2-propanol, acetic acid, and Coomassie Brilliant Blue as described by Fairbanks et al. (1971). When proteins were checked for radioactivity, dodecyl sulfate gels were removed from gel tubes, frozen, and sliced with a homemade razor blade slicer. Slices were placed in scintillation counter vials, dissolved by incubating overnight at 37° with 0.1 ml of 30% H_2O_2; 10 ml of Aquasol (New England Nuclear Corp.) was added, and the vials were shaken and assayed for radioactivity in a liquid scintillation counter (Packard Model 3320).

Molecular Weight Estimation by Electrophoresis on Polyacrylamide Gels Containing Dodecyl Sulfate. The denatured SCM protein was applied to one side of a 7.4% polyacrylamide split gel containing dodecyl sulfate; a denatured standard protein calibration mixture of bovine serum albumin, bovine γ-globulin, ovalbumin, and cytochrome *c* was applied to the other side. The molecular weight of the protein was calculated from its mobility relative to cytochrome *c* compared to a semilog plot of the molecular weights of the standard proteins vs. their mobilities relative to cytochrome *c*.

Preparative Gel Electrophoresis. Dodecyl sulfate polyacrylamide gels and buffer were prepared as described above. Samples were dissolved in 10% gylcerol containing dodecyl sulfate, HSEtOH, and 10^{-3} *M* dithiothreitol with a protein concentration of ca. 3 mg/ml, and denatured in boiling water for 1 min; 40 μl were loaded on each of 12 identical gels. Cytochrome *c* (2 mg/ml) was added as a marker. Electrophoresis was terminated when cytochrome *c* migrated to the bottom of the gel. The location of proteins was calculated from its previously determined R_f value. Each gel was frozen and sliced with a homemade razor blade apparatus into 12 slices in the protein region. A total of 12 corresponding slices obtained from the 12 gels were put into a vial. They were frozen and extracted by shaking overnight at 37° with 5 ml of 0.005 *M* sodium bicarbonate buffer containing 0.05% dodecyl sulfate, 10^{-3} *M* dithiothreitol, and 10 μg/ml of $PhCH_2SO_2F$. The supernatants were centrifuged, divided into two fractions (0.5 and 4.5 ml), and lyophilized. The 0.5-ml sample was rerun on a dodecyl sulfate polyacrylamide gel. The 4.5-ml samples containing identical protein as determined from gels of the 0.5-ml fraction were pooled and used directly or dialyzed against phosphate buffer (pH 7.0) containing Bio-Rad resin (anion exchange AG 2-X, 200–400 mesh) suspension to remove dodecyl sulfate in presence of $PhCH_2SO_2F$.

Amino Acid Analysis. Amino acid analyses were carried out on a Spinco Model M.S. amino acid analyzer. Protein samples were hydrolyzed in constant boiling HCl at 105° under nitrogen for 24 hr.

COOH-Terminal Amino Acid Determination. Determinations of the C-terminal amino acid of both the heavy and light chain were made as described by Ambler (1967). Carboxypeptidase (diisopropyl fluorophosphate treated) was used to release the COOH-terminal of the peptide chain. Protein samples were dissolved in 0.2 *M* NH_4HCO_3 plus 0.1% dodecyl sulfate. The enzyme was prepared as described by Ambler (1967). The ratio of substrate to enzyme was about 40:1. The released amino acid was analyzed by thin-layer chromatography as described by Clark (1968).

Measurement of α-Helix Content. The α-helix content of both the heavy and light chain proteins was estimated by circular dichroism (Adler et al., 1973). A Cary 60 recording spectropolarimeter was used to record the CD spectrum. Dodecyl sulfate was removed by dialysing in phosphate buffer (pH 7.0) containing Bio-Rad resin (Ag 2-X, 200–400 mesh) for heavy chain and by passing through a Bio-Rad resin column as described by Lenard (1971) for light chain. The samples were dialyzed against distilled water, centrifuged, and flushed with nitrogen before using for circular dichroism (CD) recording. Double distilled water was used as solvent and control. The concentration of heavy and light chain used was 18 and 60 μg/ml, respectively.

Enzyme Digestion. Trypsin, Pronase, pepsin, collagenase, and elastase were used to digest partially purified heavy and light chain proteins. Enzyme incubation was carried out at 37° for 1 hr. With the exception of pepsin, all enzyme digestions were run in pH 8.0 $NaHCO_3$ buffer containing 0.1% dodecyl sulfate. Pepsin digestions were run in pH 2.0 glycine-HCl buffer. After digestion samples were lyophilized and checked by dodecyl sulfate polyacrylamide gel electrophoresis as described above.

Preparation of Antiserum against SCM Proteins. Light chain judged to be homogeneous by dodecyl sulfate polyacrylamide gel electrophoresis was used as an antigen. The anti-SCM protein (light chain) antiserum was prepared by repeated injection of a goat with this preparation of light chain (50 μg/ml) in Freund's complete adjuvant. This antiserum was used to detect the SCM protein by Ouchterlony analysis. Antiserum absorbed with an excess of the heavy chain protein was prepared by incubating antiserum with heavy chain at 37° for 1 hr. The precipitate was spun down; the supernatant was incubated again with heavy chain protein until no more precipitation could be observed.

The anti-SCM protein (heavy chain) antiserum was prepared by repeated injection of a rabbit with the preparation of purified heavy chain (50 μg/ml) in Freund's complete adjuvant. Antiserum absorbed with an excess of the light SCM chain was prepared as above.

Preparation of Fluorescent Antibody against Light SCM Protein Conjugate. Antibody was purified by passing antiserum through a Sepharose-light chain SCM protein column which was prepared by coupling pure light SCM protein to cyanogen bromide activated Sepharose (5 mg of SCM protein/5 ml of swollen Sepharose) as described by Cuatrecasas (1970). The columns were washed extensively with PBS and the pure antibody was eluted by 0.1 *M* sodium phosphate buffer (pH 7.0) containing 3 *M* sodium thiocyanate. The eluates were dialyzed against 0.05 *M* phosphate buffer (pH 7.0) overnight with two changes and concentrated by negative pressure. The concentrated antibody was tested for activity by Ouchterlony analysis. Fluorescein isothiocyanate (isomer 1) was stirred with the active antibody at room temperature for 4 hr (40 μg/mg of antibody). Excess fluorescein was removed by passing through a Sephadex G-25 column.

Fluorescent Microscopic Observation. Rats, 1–2 days old, were used as a source of skin for the experiment. The skin was cleaned, and frozen sections were prepared; the slides were immersed in PBS for 5 min. One slide was covered with normal goat serum and the other was covered with antiserum against light SCM protein plus 0.5 mg/ml of active purified antibody as a blocked control. The slides were then incubated in a 37° water bath for 0.5 hr. The serum or antibody was removed and the procedure repeated twice at room temperature. The slides were immersed in PBS and then covered with fluorescent antibody conjugate (1:128 dilution in PBS containing 4 mg/ml of bovine serum albumin). After 0.5-hr incubation at room temperature, the slides were washed and immersed in PBS for 10 min. The fluorescent patterns of skin were observed with a fluorescent microscope (Zeiss, Model 1106) and photographed with a Polaroid camera.

Electron Microscopic Observations of Stratum Corneum. The stratum corneum was separated from epidermis by trypsin and EDTA digestion as described above. One half of the stratum corneum was fixed without urea treatment in glutaraldehyde plus 1% paraformaldehyde in 0.1 *M* sodium cacodylate at pH 7.4 and the other half of the stratum corneum was fixed after being pretreated overnight in 8 *M* urea containing 0.1 *M* HSEtOH and 10^{-3} *M* dithiothreitol. The tissue was washed three times, post-fixed in 1% OsO_4, sectioned, and observed with an electron microscope (AEI Model EM6B).

Chemicals. Urea, obtained from Fisher Scientific Co., was further purified by passing it through a mixed bed resin column (AG 501-X8D, 20–50 mesh, Bio-Rad Laboratories). [^{14}C]Iodoacetic acid was obtained from New England Nuclear Corporation. Fluorescein isothiocyanate was obtained from Dickinson Company. All other chemicals were of reagent grade. With the exception of Pronase, the en-

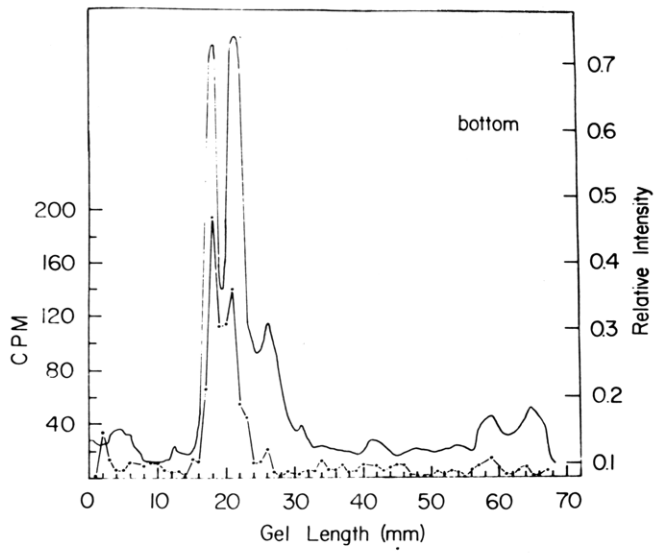
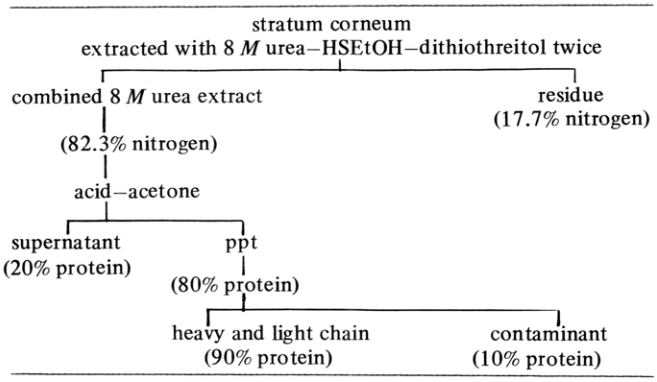


FIGURE 1: SCM derivatives of the insoluble protein extracted from the stratum corneum of newborn rats. Each gel was loaded with about 50 μg of the SCM fibrous proteins with specific radioactivity of 25,000 cpm/mg. (—) Gel stain intensity; (·--·) cpm of ^{14}C.

Table I: Protein Distribution[a] of Various Fractions.



[a] Calculated as percent of each previous yield.

zymes used for digestion of fibrous proteins were crystalline products.

Results

Demonstration of Two Insoluble Subunits. The results of dodecyl sulfate polyacrylamide gel electrophoresis of washed acetone-HCl precipitates of extracts of stratum corneum are presented in Figure 1 (see Materials and Methods). Two major radioactive peaks can be detected. They coincide with the two major protein bands demonstrated by staining. The ratio of radioactivity of these two peaks is about 1:1. As determined by total nitrogen analysis (Table I), greater than 80% of the total protein in stratum corneum is solubilized by urea–HSEtOH–dithiothreitol and based on gel staining intensity, about 60% of the protein is found in the two bands under investigation. No differences can be detected in the two SCM protein bands obtained from tissue separated by the trypsin–EDTA, heat, or sodium bromide methods indicating trypsin digestion of these bands has not occurred.

Electron Microscopic Observations of Stratum Corneum from Newborn Rat Epidermis. Electron microscopic observations of stratum corneum tissue with and without treatment by urea and reducing agent are shown in Figure 2. The filamentous structures are altered or solubilized by urea as indicated by comparing the bottom with the middle electron micrograph. The top micrograph shows a general field of one stratum corneum cell to indicate that the lower micrographs are taken from similar fields and represent intracellular material.

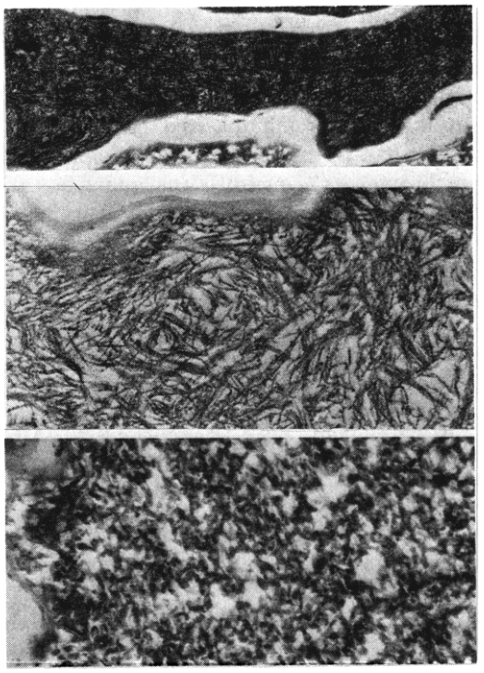


FIGURE 2: Electron micrograph of stratum corneum of newborn rat. (Top) General field of one cornified cell, ×8750 magnification; (middle) area of the above ×22,500 magnification; (bottom) area of stratum corneum after standing overnight in 0.1 *M* Tris-HCl (pH 8.4) containing 8 *M* urea, 0.1 *M* 2-mercaptoethanol, and 10^{-3} *M* dithiothreitol ×22,500 magnification.

Purification of SCM-Fibrous Protein. The total purification of protein is presented in Table II. The final yield in terms of protein is about 1.1% for heavy chain (specific radioactivity of 47,600 cpm/mg) and 5.2% for light chain SCM protein (specific radioactivity of 46,000 cpm/mg). The decrease of radiospecificity of both chains in the early purification steps apparently is due to the contamination of the SCM proteins with free [^{14}C]iodoacetic acid. No further decrease of the specific radioactivity of both SCM proteins can be detected after dodecyl sulfate hydroxylapatite C column chromatography. Furthermore, when the whole extracted corneum is *S*-carboxymethylated with [^{14}C]iodoacetic acid, only these two ^{14}C-radioactive bands could be detected by dodecyl sulfate polyacrylamide gel electrophoresis.

Purity and Molecular Weight of Purified SCM Polypeptide Chains. The purity and molecular weight of purified heavy and light SCM polypeptide chains as determined by dodecyl sulfate polyacrylamide gel electrophoresis and a semilog plot of their mobility relative to cytochrome *c* are shown in Figure 3. A single band with a molecular weight of 60,000 is observable when a relatively high concentration of purified light chain is applied on the gel. A single band, molecular weight 67,000, is observed with a high concentration (40 μg) of purified heavy chain on the gel. If an overloaded sample is applied to the gel (>80 μg), contamination of one chain by the other is not observed.

COOH-Terminal Amino Acid. Glycine is detected by

Table II

Fraction	Total Protein (mg)	Total Radioactivity (cpm)	Specific Radioactivity (cpm/mg)	Recovery[c]	% Total Protein Recovery
8 *M* urea extract	440				
S-Carboxymethylated derivative	354	31,677,100	82,000	80	80
First DE-52-cellulose column chromatography	210	16,800,000	57,100	60	48
Sodium dodecyl sulfate	61 (L chain[a])	2,713,500 (L chain)	46,400	29	14
Hydroxylapatite C column chromatography	56 (H chain[b])	2,837,900 (H chain)	49,200	27	13
Preparative dodecyl sulfate polyacrylamide gel electrophoresis	4.8 (H chain)	276,080	47,600	9	1.1
Second DE-52-cellulose column chromatography	23 (L chain)	1,058,100	46,000	38	5.2

[a] L chain; light SCM fibrous protein (59,000 mol wt). [b] H chain, heavy SCM fibrous protein (67,000) mol wt). [c] % recovery was estimated from total protein recovered at each step.

Table III: Amino Acid Composition[a] of Heavy and Light SCM Polypeptide.

	H chain		L chain	
Amino Acid	g of Amino Acid/ 100 g of protein	Nearest Integer/ 67,000	g of Amino Acid/ 100 g of Protein	Nearest Integer/ 59,000
Lysine	7.1	41	4.6	22
Histidine	0.8	3	0.7	3
Arginine	74	35	5.8	23
Aspartic acid	8.9	55	8.2	43
Threonine	2.8	20	3.2	19
Serine	9.5	63	11.9	67
Glutamic acid	14.3	81	17.9	87
Proline	2.0	14	0.6	4
Glycine	11.5	126	15.1	144
Alanine	4.0	37	2.8	22
Valine	3.2	22	1.7	10
Methionine	4.9	27	2.8	13
Isoleucine	3.6	23	2.8	15
Leucine	5.9	38	7.7	42
Tyrosine	2.2	10	5.5	22
Phenylalanine	4.8	24	3.6	15
Trytophan	1.5	6	2.3	8
SCM-cysteine	0.8	3	0.8	3
Total	95.2	628	99.0	563

[a] Samples were hydrolyzed in duplicate for 24 hr. A value of 23 residues of isoleucine for heavy chain and 15 residues of isoleucine for light chain was used to calculate the number of residues of other amino acids per molecule. This gave a molecular weight of about 67,000 for heavy chain and 59,000 for light chain.

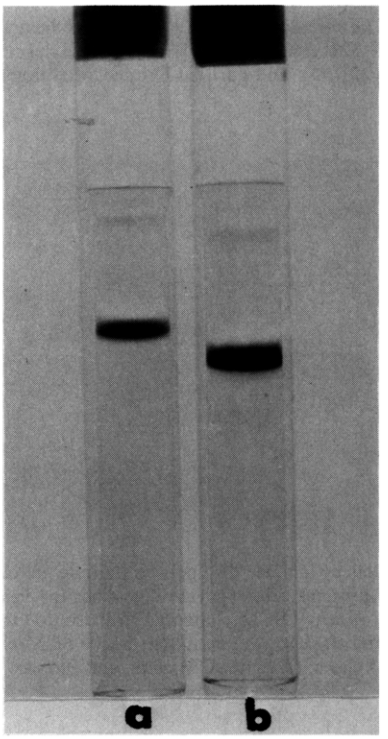


FIGURE 3: Dodecyl sulfate disc polyacrylamide gel electrophoresis of buffered SCM fibrous proteins: (a) 40 μg of purified heavy SCM chain; (b) 60 μg of purified light SCM chain.

thin-layer chromatography (MN-300-cellulose) when either purified heavy or light SCM polypeptide chain is treated with carboxypeptidase A. About 0.90 equiv of glycine is released per polypeptide chain based on the molecular weight described for heavy and light chain. No other amino acids can be detected by adding carboxypeptidase B to carboxypeptidase A. This indicates that the COOH-terminal amino acid of both heavy and light SCM polypeptide chain is glycine and suggests that both preparations are homogeneous.

Amino Acid Analysis. The amino acid compositions of both heavy and light SCM polypeptide chains are shown in Table III. The tryptophan content is derived from the ultraviolet absorption spectrum of the protein in 0.1 *M* NaOH (Goodwin and Morton, 1946). The minimal chemical molecular weight calculated from the amino acid composition is 59,000 for light chain and 67,000 for heavy chain which is close to the molecular weights obtained by dodecyl sulfate polyacrylamide gel electrophoresis. Both chains contain relatively high levels of serine and glycine. The heavy chain has about 10 proline residues more and 12 tyrosine residues less than that of light chain. Both chains contain about three residues of SCM-cysteine.

α-Helix Content of Heavy and Light SCM Polypeptide Chains. The α-helix content of heavy and light SCM proteins estimated by CD spectroscopy is shown in Figure 4. Both show approximately the same α-helix content based on residue ellipticity at 208 nm using a reference polylysine spectrum in water as 100% (Greenfield and Fasman, 1969). The α-helix content is estimated to be 30% for the heavy chain and 27% for the light chain. Attempts to measure the CD of 8 *M* urea extract of stratum corneum failed because of interference by urea. Organic solvents such as formate may also interfere with the CD spectrum (Adler et al.,

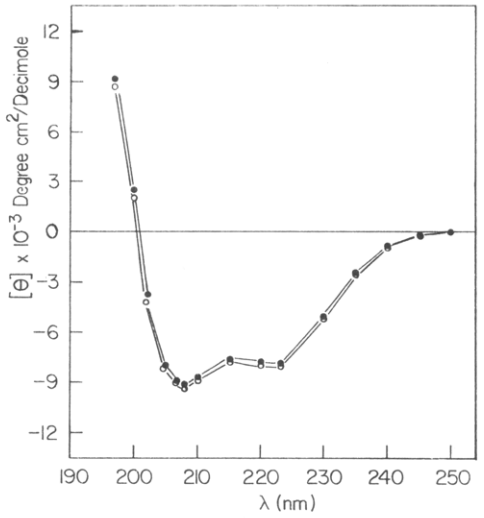


FIGURE 4: The circular dichroism of purified heavy chain SCM fibrous protein (30% α helix), calculated from polylysine reference spectra in water (25) (O), and purified light SCM fibrous protein (27% α helix) (●).

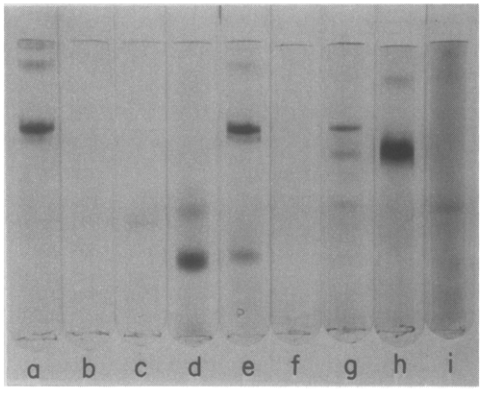


FIGURE 5: Dodecyl sulfate disc polyacrylamide gel electrophoresis of SCM fibrous proteins after enzyme digestion: (a) heavy SCM chain; (b) Pronase; (c) heavy SCM chain and Pronase; (d) trypsin; (e) heavy SCM chain and trypsin; (f) pepsin; (g) heavy SCM chain and pepsin; (h) light SCM chain; (i) light SCM chain and Pronase.

1973). The partially purified SCM proteins (from DE-52-cellulose column) which contain both heavy and light chain proteins untreated by dodecyl sulfate also show about the same α-helix content as that of purified heavy or light chain.

Enzyme Digestion. As shown in Figure 5 both heavy and light SCM polypeptide chains are resistant to trypsin, collagenase, and elastase, but susceptible to Pronase digestion. The light SCM chain is resistant to pepsin, while heavy chain can be partially degraded to a lower molecular weight similar to that of the light chain.

Immuno Analysis. Figure 6 shows the patterns of Ouchterlony analysis of heavy and light chain SCM proteins, each tested against its antibody (top right, Figure 6). The antibody against heavy chain also has a line of identity with and can be completely absorbed by light chain (Figure 6, bottom). No light chain antibody–antigen precipitation line can be observed if antiserum is absorbed with an excess of heavy chain (top left, Figure 6). This indicates that both heavy and light chain have a common antigenic determinant. Furthermore, SCM bovine serum albumin does not block or cross-react with antibody against either chain.

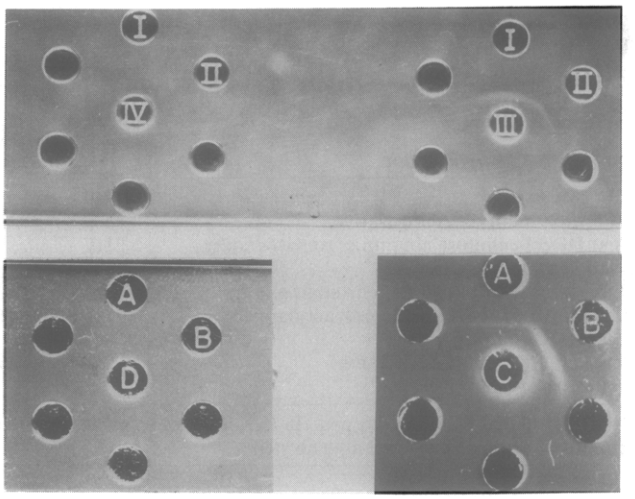


FIGURE 6: (Top) Ouchterlony analysis of heavy and light chain SCM fibrous protein with antibody prepared against purified light SCM protein. Antiserum (III) and antiserum absorbed with heavy chain (IV) were tested against (I) purified light chain (1.0 mg/ml) and (II) purified heavy chain (0.5 mg/ml). (Bottom) Ouchterlony analysis of heavy and light chain SCM fibrous protein with antibody prepared against SCM protein. Antiserum (C) and antiserum absorbed with light chain (D) were tested against (A) purified light chain (0.5 mg/ml) and (B) purified heavy chain (1.5 mg/ml).

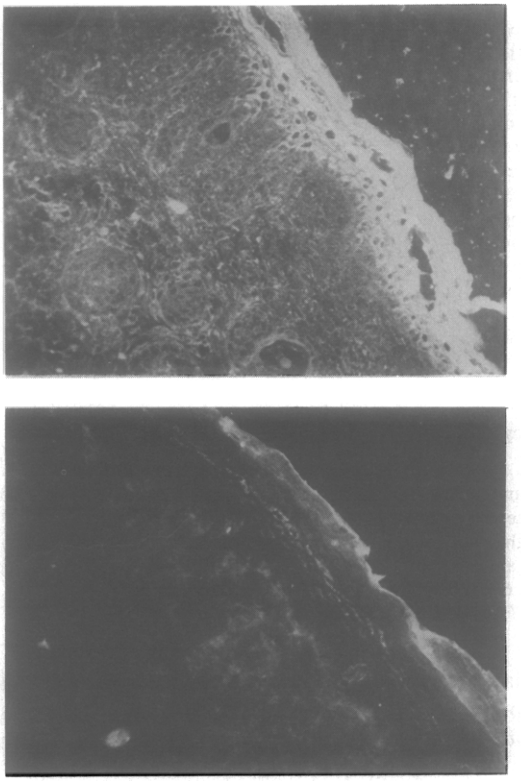


FIGURE 7: Fluorescent microscopic observation of skin from newborn rat. (Top) Skin from newborn rat preincubated with normal goat serum (45-sec exposure). (Bottom) Skin from newborn rat preincubated with antiserum against light chain (45-sec exposure).

Fluorescence Microscopy. Fluorescent microscopic observations of newborn rat skin, with and without blocking by non-fluorescent antibody, are shown in Figure 7. Fluorescence is observed in the epidermis primarily in the region of the stratum corneum and the keratohyalin granules (top of Figure 7). No fluorescence can be observed in the dermis.

The intensity of fluorescence decreases from the cornified layer to the basal layer. This fluorescence is well blocked in all regions except the keratohyalin granules, which are partially blocked, by antibody against light SCM (bottom of Figure 7) or heavy SCM protein. This indicates that the antibody against either chain cross-reacts with specific regions of the epidermis. To examine whether fluorescent antibody cross-reacts with basal, spinous, and granular cells, cell mixtures separated from rat epidermis (Stern and Sekeri-Pataryas, 1972) were tested for their ability to bind fluorescent antibody. The results show that fluorescence is observed with both cell surface and contents of spinous and granular cells and very weakly with basal cells. This fluorescence also can be blocked by pretreatment with nonfluorescent antibody against light chain.

Discussion

The present study has isolated two protein constituents of the stratum corneum in the form of SCM derivatives. These proteins are major components representing about 60% of the total protein of the stratum corneum. They have molecular weights of 67,000 and 60,000 and are referred to as heavy and light chain, respectively.

The elucidation of the nature of the fibrous protein of the epidermis present in either tonofilaments or the stratum corneum has been the goal of many investigators (Baden et al., 1968; Tezuka and Freedberg, 1972). The relationship of proteins of the stratum corneum to keratin has been discussed by Baden et al. (1968). With the presumption that the major protein of stratum corneum is keratin or keratinous in nature, this present paper presents several pieces of evidence suggesting that the light and heavy chain protein isolated in this study belong to the keratin family of structural proteins as defined by Rudall (1968). (1) These proteins are extracted from isolated and uncontaminated stratum corneum of which they are major components. (2) They possess an α-helix content. (3) They are cross-linked by disulfide bonds. (4) These proteins are insoluble in dilute acids and alkalies and are resistant to trypsin. (5) The electron micrographs indicate that the filamentous proteins are solubilized by urea containing HSEtOH and dithiothreitol. Over 80% of the protein of cornified tissue is solubilized by treatment with 8 *M* urea plus HSEtOH. (6) Antibodies produced against these proteins react strongly with the stratum corneum as well as the keratohyalin granules, a localization in the prime native components presumed to be involved in the process of keratinization. The fluorescence of the keratohyalin granules may be due to associated tonofilaments (Brody, 1959). One may also consider the possibility that there is a fibrous protein component in keratohyalin.

The data suggest that the heavy and light chain share common antigenic determinants, because antibody prepared against either is absorbed by the other chain. The argument might be made that the antigenicity of the SCM-fibrous protein is due to the SCM component. However, the reaction with stratum corneum and the keratohyalin granules in histologic sections of frozen but unfixed tissue tends to refute that argument. Furthermore, SCM bovine serum albumin does not block or cross-react with antibody against either chain. Carruthers and Bhattacharaya (1972) found that the pH 6.3, 5.5, and 4.5 precipitates of urea extracts of bovine snout epidermis have the same antigenic determinants. Similar findings with human epidermis have been reported by Bauer (1972). Tezuka and Freedberg (1972) demonstrated that alkaline urea extracts of newborn rat epidermis yield three proteins which exist in several states of polymerization. Each of the polymeric forms contains an immunologically identical component. The possibility of identifying and interrelating these various immunologically identifiable components will be necessary in future studies. The present study has made a start by purifying the two chains described above.

Two pieces of data indicate that both heavy and light SCM polypeptides have been obtained as homogeneous preparations: (1) a single band is observed for each chain upon electrophoresis in dodecyl sulfate polyacrylamide gels; and (2) only a single COOH-terminal amino acid spot can be detected by thin layer (MN-cellulose) when each chain is treated with carboxypeptidase A or carboxypeptidase A + B.

The purified proteins contain high glycine and low cysteine content which are similar to that reported for low sulfur keratin extracted from human stratum corneum (Baden et al., 1968). The molecular weights of both chains are close to those previously reported for stratum corneum by Shimizu et al. (1974) and in the range of figures reported by O'Donnell (1971) for cow's lip keratin precursor. Furthermore, both chains have shown characteristic α-helix absorption bands. Many of the published reports related to the measurement of the α helix of keratin have utilized X-ray diffraction and optical rotary dispersion (ORD) (Pauling and Corey, 1951; Crewther et al., 1966). However, most recent investigations of protein conformation have utilized CD instead of ORD (Adler et al., 1973). Polypeptides measured by the different techniques often yield differing values for α-helix content. The CD spectrum of both heavy and light SCM protein are comparable to that of polylysine with lesser amount of α-helix content. The values presented here of 27 and 30% are lower than the values of 43–50% reported by Baden and Bonar (1968) for human epidermis.

The ratio of the heavy and light chain of the SCM protein prepared from stratum corneum is about 1:1 as indicated by both the protein staining intensity and *S*-[^{14}C]carboxymethyl labeling in the dodecyl sulfate polyacrylamide gels. Furthermore, *S*-[^{14}C]carboxymethyl labeling is only found in cysteine residues as indicated by amino acid analysis. While the present findings suggest that the stratum corneum of newborn rat epidermis contains two different fibrous subunits, one a heavy and the other a light chain with an S–S bond cross-linkage, the possible presence of a soluble subunit is not excluded, particularly if non S–S cross-linked polypeptide chains are present. Heterogeneous subunits have also been found in human stratum corneum (Baden et al., 1968) and feather keratin (Kemp and Rogers, 1972). Further experiments to explore the subunit structure of fibrous protein of the stratum corneum and possible precursors are necessary.

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