Intraspecies Heterogeneity of Epidermal Keratins Isolated from Bovine Hoof and Snout

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The α -keratins, the principal components of the tonofilaments, were extracted, characterized and compared in bovine hoof and snout epidermis. The α -fibrous proteins of these tissues are similar with respect to their molecular weights, amino acid composition and percentage of helical structure. However, distinct differences in the polypeptides comprising these proteins were observed. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of these proteins consistently showed that the polypeptide chain in snout, designated as band B (mol.wt. 67000), was completely absent from hoof preparations. This was confirmed with several alternative preparative procedures. The peptides produced by digestion of the intact keratins from hoof and snout with CNBr were distinctly different. Finally, digestion of keratins from hoof and snout with trypsin yielded products that differed in size and resistance to further digestion. Thus, in addition to the interspecies polypeptide heterogeneity documented in the literature, this report establishes the intraspecies heterogeneity of keratins and suggests that these differences are due to either the expression of different gene products or differences in post-translational modifications in these two tissues.

Early studies of epidermis and epidermal appendages showed the presence of α -fibrous proteins, designated keratins, which Rudall showed could be extracted from bovine snout by buffers containing urea. Matoltsy (1965) subsequently isolated an α -fibrous protein, which he designated prekeratin, from the living layers of bovine snout epidermis by extraction with 0.1 M-citrate buffer, pH 2.65, and further purified it by isoelectric precipitation. He reported that prekeratin was an α -helical rod-like molecule with dimensions of 105 nm $\times$ 3.7 nm and a mol.wt. of 640000. Prekeratin has subsequently been shown to be the principal component of the tonofilaments in the lower viable layers of epidermis. As the epidermal cells of the lower layers differentiate and form the outer keratinized layers of epidermis, known as the stratum corneum, the cells become filled with α -fibrous proteins or keratins that are only extractable with Tris buffers containing 8 M-urea or 8 M-urea plus a reducing agent. These

proteins are referred to as stratum corneum proteins. Prekeratin is generally freer of contaminating proteins than the corresponding stratum corneum protein and we have consistently chosen this protein for structural work. Since Baden *et al.* (1973) showed that this protein was composed of several different polypeptide chains, a number of studies (Skerrow, 1974; Lee *et al.*, 1975; Huang *et al.*, 1975; Steinert & Idler, 1975; Culbertson & Freedberg, 1977; Inoue *et al.*, 1976) have appeared on the characterization of the polypeptide chains of either prekeratin or the stratum corneum proteins. Although the investigators have used different species and various isolation techniques, their data show that keratins are composed of anywhere from two to seven polypeptide chains in the mol.wt. range of 45000–70000. These polypeptides are arranged in a triple-chain coiled-coil conformation (Lee & Baden, 1976; Steinert *et al.*, 1976), and have a relatively low amount of sulphur-containing amino acids and high amounts of glycine, glutamic acid, serine and aspartic acid. Although the keratins that have been described show many similarities, the polypeptide compositions of these proteins vary between species. This report shows that the keratins isolated from different areas of the same species also have distinct differences in polypeptide composition and substantiates these differences by a number of different experiments. Specifically, we have defined com-

Abbreviations used: SDS, sodium dodecyl sulphate; R_f , relative mobility; Y_0 , free electrophoretic mobility; K_r , retardation coefficient; r_e , effective radius; SCTU protein, α -fibrous protein found in the stratum corneum and solubilized with 8 M-urea containing 0.1 M-Tris, pH 9.0; SCTUM protein, α -fibrous protein found in the stratum corneum and solubilized with 8 M-urea containing 0.1 M-Tris, pH 9.0, and 0.1 M-mercaptoethanol.

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positional differences between the keratins from bovine hoof and snout. These results agree with our results in human epidermis, showing variation with the tissue used, but disagree with the studies of Steinert (1975) and Steinert & Idler (1975), which report that the keratins from bovine hoof and snout are identical.

Methods

Tissue sectioning

Snout epidermis is approx. 2–3 mm thick. It is comprised of an outer layer of anucleate cornified cells known as stratum corneum, which is 0.2–0.5 mm thick, and the viable Malpighian area consisting of basal, spinous and granular layers. The epidermis was removed with the combined use of a razor blade and hand magnifying lens, which allowed visual detection of the white dermal papillae. Sections were taken for histology to confirm that the slices were free of dermis.

Epidermis about the posterior aspect of the hoof is 4–8 mm thick (Ugel & Idler, 1970), considerably thicker than that of snout; moreover, the stratum corneum is proportionally thicker than in snout, comprising a layer 1–2 mm thick with the remainder being Malpighian layer. Epidermis was removed in the same manner as with snout, with care taken not to include dermis. Sections were also taken for histology, to confirm that slices used for extractions of keratins were free of dermis.

Preparation of prekeratin

Epidermis from the hoof and snout of the same animal was finely minced, suspended in 0.25 M-sucrose (100 ml/18 g wet wt. of tissue) and ground in a VirTis homogenizer for 4 min at 4°C. This extraction removed approximately 90% of the soluble proteins. The resulting suspension was centrifuged for 20 min at 4°C and 35000g ($r_{av.}=10.8$ cm). The pellet from the first centrifugation was suspended in 0.1 M-citrate buffer, pH 2.65 (150 ml/18 g wet wt. of tissue), and ground in the VirTis homogenizer for an additional 4 min, and the resulting mixture stirred at 4°C for 1 h. The sample was then centrifuged for a second time at 4°C and 35000g ($r_{av.}=10.8$ cm) for 20 min and the prekeratin in the supernatant purified from remaining soluble proteins by a series of precipitations at pH 7.0, 6.0, 5.0 and 4.5, as described by Matoltsy (1965).

Preparation of stratum corneum keratins

The pellet from the second centrifugation was suspended in 8 M-urea containing 0.1 M-Tris/HCl, pH 9.0, rehomogenized and stirred under N₂ at room temperature (20°C) for 18 h. The resulting

suspension was centrifuged a third time at 4°C at 35000g ($r_{av.}=10.8$ cm). Although the supernatant contained 90% of the stratum corneum protein, two additional washes with Tris/urea were done to ensure complete removal of this protein. The protein extracted by the above procedure we refer to as SCTU protein (stratum corneum Tris/urea-solubilized protein). The pellet was then extracted with 8 M-urea containing 0.1 M-Tris/HCl, pH 9.0, and 0.1 M-2-mercaptoethanol for 25 h at room temperature under N₂ and the suspension centrifuged at 4°C and 35000g ($r_{av.}=10.8$ cm) for 20 min. The resulting supernatant contained the SCTUM protein (stratum corneum Tris/urea/mercaptoethanol-solubilized protein).

Alternative procedure of keratin preparation

The epidermal mince of either hoof or snout from the same animal was ground in the VirTis homogenizer in 8 M-urea containing 0.05 M-Tris/HCl, pH 9.0, and 0.025 M-2-mercaptoethanol (25 ml/g wet wt.) and then stirred for 3–4 h at 37°C. After centrifugation, the supernatant was dialysed against 2 × 100 vol. of 10 mM-Tris, pH 8.0, and then precipitated at pH 4.5 by the addition of an equal volume of 0.2 M-sodium acetate. The precipitate was collected by centrifugation at 30000g for 10 min at 4°C. The precipitate was then redissolved in 6 M-urea containing 0.1 M-Tris and 0.1 M-2-mercaptoethanol at pH 8.0 and used for further studies.

Polyacrylamide-gel electrophoresis

Discontinuous polyacrylamide-gel electrophoresis in the presence of SDS was done by published procedures (Neville, 1971). Samples were routinely equilibrated in 8 M-urea containing 1% SDS and 1% 2-mercaptoethanol for 1 min at 100°C followed by 1 h at 50°C and then dialysed against 2 × 1000 vol. of cathode buffer, which contained 0.1% SDS. Samples (10–50 µl) were applied to gels (0.5 cm × 7.5 cm) and electrophoresis was performed at 2 mA/gel until the front was approx. 0.5 cm from the bottom of the gel (average running time approx. 45 min). Relative mobilities were calculated relative to the borate sulphate front, which stacked the marker dye, Bromophenol Blue, at the concentrations of acrylamide used in this study (6–10%). Other marker dyes such as Pyronin Y were investigated, but we concluded, as did Neville (1971), that very little unstacking occurs for these percentages of acrylamide and that Bromophenol Blue was the best marker. After electrophoresis at 20°C, gels were immediately cut at the marker dye, stained in 0.25% Coomassie Brilliant Blue dissolved in 50% (v/v) methanol/10% (v/v) acetic acid, destained in 7.5% acetic acid/5% methanol and stored in water. Densitometer tracings of these gels were done using a Canalco model K recording densitometer. Mole-

cular weights were calculated by previously outlined methods (Ferguson, 1964; Hedrick & Smith, 1968) in which the R_f of standards and unknowns are measured as a function of total acrylamide concentration. For all runs the acrylamide concentrations used were 6, 7, 8, 9 and 10%. The following proteins were used for the standards (mol.wts. in parentheses): creatine kinase (40000); ovalbumin (45000); L-glutamate dehydrogenase (50000); rabbit γ -globulin heavy chain (50000); catalase (57500); bovine serum albumin (67000); urease (83000); phosphorylase (94000). When R_f is plotted against acrylamide concentration, the Y intercept yields Y_0 , the free electrophoretic mobility. Proteins that yield a set of lines intersecting at a common value of Y_0 and at an acrylamide concentration of 0 are said to have uniform charge densities and this justifies their use as standards for determination of unknown molecular weights, provided that the unknowns have similar values of Y_0 . The slope of each line K_r , the retardation coefficient, is directly proportional to the molecular weights of the proteins under investigation (Rodbard & Chrambach, 1970, 1974). Fig. 1 shows K_r versus molecular weight for the standards used in these experiments. The least-

squares linear-regression equation describing this line is shown below. After obtaining the K_r value for each polypeptide chain of hoof, this equation was used for the calculation of their molecular weights:

$$\text{Mol.wt.} = [(25 \pm 1.25) \times 10^3] K_r - (175.85 \pm 8.79) \times 10^3$$

Polyacrylamide-gel electrophoresis in the presence of urea was done by the procedure of Davis (1964), except that 12% acrylamide and 3% bisacrylamide were used.

Digestion with CNBr

Prekeratin (10mg) was dissolved in 2.5ml of aq. 70% formic acid and treated with 75mg of CNBr for 25h at room temperature with constant stirring. The digest was roto-evaporated repeatedly with water, and the residue dissolved in 6M-urea containing 0.1M-Tris/HCl, pH8.3, and 0.01M-dithiothreitol immediately before disc electrophoresis (Davis, 1964) in the presence of urea.

Digestion with trypsin

Prekeratin (4 mg/ml) from either hoof or snout was dissolved in 2.5ml of 2M-urea containing 0.2M- NH_4HCO_3 and 0.02M-Tris/HCl, pH8.5. The digestion was initiated by the addition of 50 μg of trypsin dissolved in 0.05M- NH_4HCO_3 . The reaction mixture was maintained at 4°C for 15 min, and then a sample pipetted into a mixture of SDS, 2-mercaptoethanol and upper gel from the SDS/polyacrylamide-gel electrophoresis system used (Neville, 1971) so that the final mixture was 1% SDS, 1% 2-mercaptoethanol and at an appropriate pH for optimum resolution of bands. All samples were heated immediately at 100°C for 5min followed by 1h at 50°C, after which 10 μl samples were applied to gels. Gels were run, stained, destained and subjected to densitometry as described above.

Amino acid analysis

Samples for amino acid analysis were hydrolysed in 6M-HCl for 24h under vacuum at 110°C and run in duplicate on a Beckman 116 amino acid analyser.

X-ray diffraction

X-ray diffraction analysis was done on regenerated filaments as previously described (Lee & Baden, 1976).

Circular dichroism

Circular dichroism measurements were done on a Cary model 61 recording polarimeter in 1cm-path-length cells. The solvent used for all measurements was 0.1M-citric acid/dioxan (1:3, v/v).

Fig. 1. Standard plot of retardation coefficient (K_r) versus molecular weight for various standard proteins used for determining molecular weights of the polypeptide chains for snout and hoof prekeratin on polyacrylamide gels run in the presence of sodium dodecyl sulphate

The standard proteins used are as follows: a, creatine kinase; b, ovalbumin; c, L-glutamate dehydrogenase; d, γ -globulin heavy chain; e, catalase; f, bovine serum albumin; g, urease; h, phosphorylase. These proteins were equilibrated and run on gel electrophoresis at various concentrations of acrylamide as described in the text. Relative mobility versus total acrylamide was plotted for each protein at the various total percentages of acrylamide used (6, 7, 8, 9 and 10%). The slope of such a line is the retardation coefficient, K_r , for the various protein standards and this is shown plotted against molecular weight above. K_r values were also calculated for the keratin polypeptide chains and the above line was used to calculate their molecular weights.

Antibody production and monitoring

Snout prekeratin (2mg/ml in 0.05M-citric acid) was mixed with an equal volume of Freund's adjuvant to which had been added 9mg/ml of killed tuberculin bacilli and injected intradermally into multiple sites in New Zealand White rabbits obtained from Charles River Breeders (Boston, MA, U.S.A.). The animals had booster intramuscular injections at 2, 3 and 4 weeks and monthly thereafter. The course of antibody production was followed in the weekly bleedings by a modification of the Ouchterlony gel-diffusion technique (Lee & Baden, 1977). S-Carboxymethyl-prekeratin dissolved in 8M-urea containing 0.01M-Tris/HCl buffer, pH8.3, was used in the antigen well and a γ -globulin fraction (Thurston, 1957) in the antibody well. The non-alkylated protein also reacted, but the lines were less sharp. Control experiments indicated that the urea necessary to solubilize prekeratin did not interfere with the antibody-antigen reaction.

Results

Histology

Histological sections showing the entire epidermis of both hoof and snout are shown in Plate 1. Epidermis from various body areas has been shown by others to vary in thickness; moreover, the proportion of cell layers in the outer cornified area (stratum corneum) versus the inner living Malpighian layers also varies. This type of variation is also seen clearly in a comparison of the hoof and snout sections shown in Plate 1. The hoof stratum corneum is considerably thicker and represents a larger portion of the epidermis than in snout. This is analogous to humans, where palms and soles are the areas with the thickest keratinized layers and facial epidermis has the thinnest stratum corneum. However, examinations of these sections shows several other differences. Bovine snout stratum corneum is highly organized and the stacking of the individual cells is clearly visible, whereas hoof stratum corneum cells appear random and disorganized, similar to human palm stratum corneum. The granular layer is considerably more pronounced in the hoof epidermis. The hoof also apparently contains a higher concentration of keratohyalin granules than snout.

α -Fibrous proteins and their preparation

Matoltsy (1965) showed that citric acid buffer, pH2.65, attacked the lower viable layers of bovine snout epidermis, solubilizing the α -fibrous proteins, prekeratin, whereas urea or urea containing a disulphide reducing agent was necessary to break down the outer cornified layer and release the keratins or stratum corneum proteins. Table 1

Table 1. Yields of various α -fibrous proteins from bovine tissues

	Yield (% of the total yield of α -fibrous protein)	
	Snout	Hoof
Prekeratin (citric acid-extractable)	33	2
Stratum corneum proteins	67	98

shows the difference in the amount of protein extracted with citrate buffer and the denaturing solvents from snout and hoof epidermis. Alternatively, one can extract the keratins listed in Table 1 together by doing only one extraction with 8M-urea/0.05M-Tris/0.025M-mercaptoethanol, and then subsequently purifying the keratins by isoelectric precipitation at pH4.5. This approach (Steinert, 1975), which initially extracts a large number of soluble and difficult-to-solubilize proteins, including the keratins, has not been used as a routine by us because of the difficulty in getting rid of the contaminating non-keratin proteins. Either type of extraction yields between 0.45 and 0.55 g of α -fibrous protein/g of dry tissue. The major part of this study was done with citric acid-extractable protein, since sodium dodecyl sulphate/polyacrylamide-gel electrophoresis shows the presence of only a few non-keratin contaminants, which are readily eliminated by a series of isoelectric precipitations, and disulphide reducing agents are not required to maintain solubility as with the SCTUM protein. Moreover, since our studies (Baden *et al.*, 1976b) indicate that post-translational changes occur in the stratum corneum proteins during differentiation, we felt working with an unmodified form was desirable.

Physical properties of hoof and snout keratins

Although the purpose of our report is to demonstrate significant differences in the keratins isolated from these two sources, there are many similarities. Amino acid analysis of both proteins showed no major differences in amino acid composition. Both have the characteristically high values for aspartic acid and glutamic acid, serine and glycine and relatively low amounts of proline and cystine and cysteine generally seen in epidermal keratins. Our c.d. studies confirm results showing both keratins contain 45–50% helix (Steinert & Idler, 1975; Lee & Baden, 1974). Additionally filaments prepared from protein extracted from both tissues gave the same X-ray-diffraction pattern with 0.51 nm meridional arc and an 0.95 nm equatorial reflection. We tested both hoof and snout prekeratin by Ouchterlony double-diffusion and Laurell 'rocket' electro-immunoassay (Lee *et al.*, 1976) with various modi-

EXPLANATION OF PLATE I

Light-micrographs of bovine hoof (a) and snout (b) epidermis

The tissues were fixed in Karnovsky's (1965) cacodylate-buffered glutaraldehyde/paraformaldehyde mixture and then post-fixed in 2% OsO₄. The fixatives were all made in 0.1 M-cacodylate buffer, pH 7.4. Fixed tissues were dehydrated by ethanol solution, mounted in epoxy resins and ultrasectioned by an LKB 4804 Ultratome. Sections are shown at a 25-fold magnification.

EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis of (a) snout prekeratin and (b) hoof prekeratin and (c) co-electrophoresis of snout and hoof prekeratin

Protein samples were prepared by first extracting both snout and hoof epidermis obtained from the same animal with sucrose to remove soluble proteins, followed by extraction of the prekeratin with 0.1M-citrate buffer. The prekeratin protein was then purified by isoelectric precipitation and prepared for SDS/polyacrylamide gel electrophoresis. All protein samples were prepared by dissolving in upper-gel buffer, pH6.1 (Neville, 1971), containing 8M-urea/1% SDS/1% ethanol, heating for 1 min at 100°C followed by 1 h at 50°C, and dialysing against 1000 vol. of upper-gel buffer, pH6.1, containing 0.1% SDS and 1% mercaptoethanol. Gels (0.5 cm × 7.5 cm) containing 7% total acrylamide with 3% cross-link were loaded with 20 µg of protein in 10 µl volumes and run at 2 mA/gel for approx. 45 min at pH9.5. The direction of migration is downward. After electrophoresis gels were stained with Coomassie Blue and destained. Subsequent preparations from four additional animals all showed banding patterns identical with those shown here.

fications that have been developed for insoluble proteins (Lee & Baden, 1977). These studies showed that both proteins were immunologically identical and reacted quantitatively the same toward the antibody.

SDS/polyacrylamide-gel electrophoresis of epidermal keratins

Prekeratin. Hoof and snout prekeratin obtained from the same animal were run on SDS/polyacrylamide-gel electrophoresis by the method of Neville (1971). Each protein was run separately (Plates 2a and 2b) and co-electrophoresed (Plate 2c). The weights shown in Plate 2 were calculated as outlined in the Methods section. The snout prekeratin polypeptide designated B, with a mol.wt. of 67000, is clearly absent from the hoof preparation in which the highest-molecular-weight chains (bands 1 and 2) are 60000. Bands 1 and 2 of hoof prekeratin are shown here as co-migrating, but under conditions of low protein concentration and longer electrophoresis time they can be resolved into two distinct bands. Snout prekeratin band B' (mol.wt. 58000) corresponds to band 3 of the hoof, and A and A' co-electrophorese with bands 4 and 5 of the hoof.

Since the molecular weights shown here are different from those reported by Steinert (1975), several procedural and theoretical aspects of our methods were investigated. These included: (1) ascertaining that samples were fully equilibrated with SDS; (2) showing that the Y_0 values obtained from Ferguson (1964) plots were the same for both unknown and standards; (3) showing that a constant dependency of effective radius (r_e) on molecular weight existed through the range of molecular weights being used.

To ascertain that our samples (both standards and unknowns) were fully equilibrated with SDS, we tried a number of procedures previously outlined by different investigators (Weber & Osborn, 1969; Neville, 1971; Steinert, 1975). In addition, a full study of the variables involved in SDS equilibration was done. We found no measurable effect on the R_f or free electrophoretic mobility of the polypeptides or molecular weight markers, upon comparison with our standard procedure when testing the following variables: SDS concentration (1–5%, w/v); 2-mercaptoethanol concentration (0.1–5%, w/v); time of initial heating at 100°C (1–5 min); temperature of subsequent heating (50–90°C); and time of subsequent heating (30 min–1 h). We concluded from these studies that our procedures produced the maximum amount of binding of SDS to the proteins under study. Additionally, it should be emphasized that these studies indicate that we have no difficulty with fully equilibrating bovine serum albumin. Similarly, many other workers (Nelson,

1971; Dunker & Reuckert, 1969; Steinhart & Reynolds, 1969; Reynolds & Tanford, 1970a) have also reported that bovine serum albumin binds its full complement of SDS without any apparent difficulty. In contrast, Steinert (1975) indicates that in his hands bovine serum albumin does not bind its full complement of SDS, and therefore cannot be used as a standard. The reliability of bovine serum albumin is particularly important, since it has the same molecular weight (67000) as the B chain of bovine snout keratin, and, in fact, co electrophoreses with it and significantly behind the highest-molecular-weight polypeptide in hoof.

Many investigators (Neville, 1971; Rodbard & Chrambach, 1974) have discussed the fact that it is essential that the standards and unknowns used for determining molecular weights should have identical values of Y_0 . Y_0 values were calculated for all proteins used in this study and the values were shown to lie within 2–5% of each other, which was the same as the variation between different electrophoretic experiments. Therefore, since all of the proteins used share a uniform charge density, these standards can be used for determining the molecular weights of the keratin polypeptides. When proteins share a uniform charge density, they behave under free electrophoresis (i.e. at 0% acrylamide) as free draining structures, which means that the mobility of the whole molecule is identical with the mobility of segments of the parent molecule, that is, each protein used here is fully extended and has bound its maximum amount of SDS.

Having shown that each protein bound a maximum amount of SDS, and that they had identical free electrophoretic mobilities, it is also necessary (Neville, 1971) to show that the effective radii, r_e , of the proteins being studied have a constant dependency on molecular weight through the range of molecular weights being used. Drawing on theoretical work of a number of other people (Ogston, 1958; Rodbard & Chrambach, 1970; Reynolds & Tanford, 1970b; Fish *et al.*, 1970), Neville (1971) has shown that a plot of $\log K_r$ versus $\log$ (mol.wt.) will be linear when there is a constant dependency of r_e on molecular weight and that alterations in such a constant relationship will be apparent by a change in slope. When such a plot was made of our data, it was apparent that there was a constant relationship between r_e and molecular weight over the range used (40000–83000) for determining the molecular weight of the α -fibrous proteins of epidermis.

Stratum corneum proteins

Extraction of epidermis with 8M-urea containing 0.1M-Tris/HCl, pH 9.0, followed by 8M-urea containing 0.1M-Tris and 0.1M-mercaptoethanol, produces two proteins, designated SCTU and SCTUM,

as described in the Methods section. We have compared the two proteins from stratum corneum with the prekeratin extracted with citrate buffer, and the three proteins appear very similar by several criteria. We have shown them to be immunologically identical (Lee *et al.*, 1976), to have similar amino acid compositions and resemble each other closely on SDS/polyacrylamide-gel electrophoresis. Thus, these proteins are thought to be closely related; however, SCTUM protein requires a disulphide reducing agent to be liberated from the tissue, implying that disulphide links have been formed during keratinization (Baden *et al.*, 1976b). SCTUM proteins prepared from both hoof and snout were electrophoresed on SDS/polyacrylamide gels, and the densitometer traces shown in Fig. 2 indicate that the SCTUM protein from hoof also lacks the polypeptide chain with a mol.wt. of 67000 which clearly is present in the corresponding SCTUM protein from snout.

Alternative preparations of hoof and snout keratin

An alternative procedure for extracting keratins that has been used (Steinert, 1975) involves an initial extraction with 8M-urea containing 0.025M-2-mercaptoethanol and 0.05M-Tris/HCl, pH9.0. This

Fig. 2. Polyacrylamide-gel electrophoresis, run in the presence of SDS, of the stratum corneum proteins of snout (-----) and hoof (—)

Samples were prepared by extracting both hoof and snout epidermis from the same animal first with sucrose and subsequently with citrate buffer, pH2.5, which removed the α -fibrous proteins shown in Plate 2. A final extraction with 8M-urea containing 0.1M-Tris, pH9.0, and 0.1M-2-mercaptoethanol produced the SCTUM protein displayed here. Conditions of sample preparation and electrophoresis are identical with those described in the legend to Plate 2, except that, after staining, gels were traced with a Canalco model densitometer, and the direction of migration is from left to right. Samples obtained from four subsequent preparations were identical with those shown here.

method liberates most epidermal proteins, except for those comprising the cell envelope. SDS/polyacrylamide-gel electrophoresis of extracts prepared by this procedure showed that the 67000 mol.wt. polypeptide chain present in snout is also absent from hoof even when this alternative procedure of extraction was used. Other types of extractions with various denaturants dissolved in 0.01M-Tris buffer (e.g. SDS, deoxycholic acid guanidine hydrochloride) also failed to liberate a keratin-like polypeptide with a mol.wt. of 67000 from bovine snout.

CNBr fragments of the α -fibrous proteins of hoof and snout

Prekeratin from hoof and snout was digested with CNBr and the resulting peptides were electrophoresed on 12% polyacrylamide gels in a discontinuous system (Davis, 1964). Densitometer traces of the resulting gels are shown in Fig. 3. Some 13 peptides with distinctly different charge/mass ratios were resolved with hoof prekeratin and 11 with snout prekeratin. Digests of hoof prekeratin contain four peptides (4', 5', 10', 11') that are not present in the digests of snout prekeratin. The mobilities of the remaining hoof peptides (1', 2', 3', 6', 7', 8', 9', 12', 13') are similar to the correspondingly labelled snout peptides; however, the relative proportions of some of these peptides (6, 7, 8, 9) are quite different in the two tissues. Moreover, the snout peptides are not as well resolved into sharp discrete bands as with hoof, although several unsuccessful attempts were made to increase resolution of these peptides by varying the gel pore size and the length of the electrophoresis.

Reactivity to trypsin

Hoof and snout prekeratins were treated with trypsin at 4°C. The reaction was stopped at various times by the rapid addition of 2-mercaptoethanol and 1% SDS and heating for 1min at 100°C. After further heating for 1h at 50°C, the reaction mixtures were subjected to SDS/polyacrylamide-gel electrophoresis (Neville, 1971), stained and traced on a densitometer. Tracings for samples taken at 0 and 15min are shown in Fig. 4. Samples taken at 15min show the snout prekeratin is much more resistant to trypsin treatment and yields high-molecular-weight peptides with mol.wts. of approx. 34000 and 39000; further, these pieces are stable even after exposure to trypsin for up to 15min. High-molecular-weight peptides are also detectable early in the digestion (0–5min) of hoof prekeratin, but unlike snout prekeratin they are rapidly digested. Between 15 and 60min of digestion, both fibrous proteins show the presence of peptides in the range of 14000 to 17000 mol.wt. which are similar to the

Fig. 3. Polyacrylamide-gel electrophoresis of CNBr fragments obtained from snout (----) and hoof (—) prekeratin. Prekeratin from both hoof and snout was digested with CNBr and the fragments produced were dissolved in 6M-urea containing 0.1M-Tris, pH8.3, and 0.01M-dithiothreitol. Approx. 40 μ g of fragments was applied to each gel and electrophoresis was carried out at 2mA/gel for approx. 3 h. After staining with Coomassie Brilliant Blue, gels were traced in a Canalco densitometer and the traces shown above with direction of migration being from left to right.

Fig. 4. Polyacrylamide-gel electrophoresis of prekeratin from snout (----) and hoof (—) after digestion with trypsin. Prekeratin was digested with trypsin at a protein/enzyme ratio of 200:1 at 4°C. The reaction was stopped and the products obtained were prepared for electrophoresis by pipetting a 100 μ l sample of the reaction mixture into a mixture of 25 μ l of 5% SDS and 2 drops of mercaptoethanol. Samples were heated for 1 min at 100°C, followed by 1 h at 50°C, and approx. 10–20 μ g of protein was applied to each gel. Electrophoresis was done at 2mA/gel for approx. 45 min, gels were stained with Coomassie Brilliant Blue and traced in a Canalco densitometer with the direction of migration being from left to right. Densitometer traces are shown of samples obtained at 0 min (a) and 15 min (b).

trypsin digestion products reported by other investigators (Skerrow *et al.*, 1973) and represent highly helical fragments of the parent molecule.

Discussion

A review of the polypeptide composition and molecular weights of previously reported epidermal keratins from a number of sources is shown in Table 2. In addition we have published an immunological study of keratins (Lee *et al.*, 1976) in which the SDS/polyacrylamide-gel electrophoretic patterns of epidermal keratins from several species including pig, mouse, hamster, guinea pig, frog and turtle were shown. These patterns showed anywhere from two to seven protein bands in the mol.wt. range of 45000 to 67000 and the number, position and intensity of the bands were quite distinctive for each animal. The polypeptide composition of hair

and nail keratins is different from the epidermal keratins, but has also been shown to vary from animal to animal (Hrdy & Baden, 1973). Thus unlike other structural proteins such as myosin or collagen, which sometimes contain polypeptide chains with slightly altered sequences, there is tremendous variability in the molecular weights, number and intensity of the polypeptides comprising keratins that have been studied to date.

In addition to this interspecies heterogeneity documented in the literature, we report that the polypeptide compositions of the epidermal keratins from bovine hoof and snout are, in contradiction to a previous report (Steinert, 1975), distinctively different from each other. These differences could be attributable to either the expression of different gene products or the presence of different types of post-translational modification mechanisms. The

Table 2. *Summary of molecular weights reported for epidermal keratins*
Abbreviation: m, minor bands that comprise less than 10% of the total protein.

<i>M</i>	Lee & Baden (1976)	Skerrow (1974)	The present report	Steinert (1975)
Bovine snout	67000	72000	67000	58000
	60000 (m)	60000	58000 (m)	56000 (m)
	47000	60000	52000	54000
	45000		48000	51000 (m)
				48000
				47000 (m)
Bovine hoof	The present report	Steinert (1975)		
	60000	58000		
	58000	56000 (m)		
	52000 (m)	54000		
	48000	51000 (m)		
		48000		
		47000 (m)		
Newborn rat	Culbertson & Freedberg (1977)	Inoue <i>et al.</i> (1976)	Huang <i>et al.</i> (1975)	
	68000	66000	67000	
	60000	60000	59000	
Human palm	Inoue <i>et al.</i> (1976)			
	64000			
	60000			
Human breast	Baden & Lee (1978)			
	67000			
	59000			
	51000			
	45000			
Human body	Abernathy <i>et al.</i> (1977)			
	67000			
	57000			

fact that the hoof and snout keratins have distinct differences has been demonstrated by three types of experiments. (1) SDS/polyacrylamide-gel electrophoresis of the α -fibrous proteins of hoof and snout prepared several different ways showed that B, one of the three principal polypeptide chains of snout keratin (mol.wt. 67000), is completely absent from hoof preparations. (2) Digestion of both hoof and snout prekeratin with trypsin showed that the hoof prekeratin is more susceptible to enzymic attack. (3) Finally, digestion of these two proteins with CNBr showed that the peptides produced are significantly different from each other. These differences exist even though both proteins have been shown to be similar chemically and immunologically. Moreover, the absence of the 67000-mol.wt. component in the hoof keratins apparently does not affect its ability to form filaments, give an α -fibrous X-ray diffraction pattern, or the overall percentage of helix in the molecule. In all tissues we have studied to date the lower-molecular-weight polypeptide chains (designated A and A') appear invariable, but the higher-molecular-weight chains (designated B and B') are hypervariable both with respect to the number of bands present and their intensity.

We have shown that the absence of a B polypeptide chain (mol.wt. 67000) from hoof preparations cannot be ascribed to the preparative procedures used. All experiments used freshly prepared samples (i.e. no freeze-dried protein was used) and repeated preparations gave identical results. Moreover, several alternative procedures of preparing keratins from hoof and snout gave identical results. Further, additional types of extractions containing other types of denaturants (e.g. SDS) were tried to see if any keratin-like polypeptide chain with a mol.wt. of 67000 was extractable from hoof. No evidence of such a chain was found. We have thoroughly investigated the possibility that the electrophoretic differences described above are the result of any trivial explanation such as incomplete binding of SDS, improper electrophoretic technique, or sample preparation and handling. Moreover, the theoretical and practical basis of the Neville (1971) electrophoretic method, which is used as a routine in this laboratory, has been completely ascertained as recommended by Rodbard & Chrambach (1974). The electrophoretic differences discussed here have also been demonstrated in the gel system described in the initial isolation, characterization, and description of hoof epidermal proteins (Steinert, 1975).

The validity of molecular-weight measurements is primarily dependent on accurate measurements of R_t , and Wyckoff *et al.* (1977) studied in detail a number of factors that can lead to considerable indeterminacy in R_t even for samples run at the

same time in the same apparatus. Although neither Steinert (1975) nor our electrophoretic procedures follow exactly these optimum conditions, we have eliminated or greatly minimized the errors that Wyckoff *et al.* (1977) suggest occur in R_t measurements by co-electrophoresing these proteins.

Our previous work shows that all the polypeptide chains of snout prekeratin (Plate 2a) have similar amino acid compositions and digests of the purified A and B chains (Lee *et al.*, 1975). We have also shown that these chains are not generated from each other by any epidermal proteolytic activity and that all chains apparently participate in forming the three-stranded coiled-coil ropes that are the basic structures seen in tonofilaments (Lee & Baden, 1976). Similarly, previously published work on hoof shows that all the polypeptides shown in Plate 2 are components of hoof prekeratin and originate from the tonofilaments and participate in filament formation (Steinert *et al.*, 1976). Therefore none of the chains of hoof or snout prekeratin can be explained as being extraneous, non-keratin-like proteins.

The differences we have described in the present report between the hoof and snout keratins could be the result of the expression of different gene products (i.e. production of substantially different polypeptide chains). Since the keratins of hair and nail have been shown to be subject to genetic variation in the types of polypeptide chains produced in at least three different instances (Baden *et al.*, 1975, 1976a; Lee *et al.*, 1978), this seems a viable possibility. Another explanation would be the presence of different types of post-translational mechanisms in these two tissues. The diversity in structural proteins of various keratinizing epithelia such as hair, nail and epidermis is such that it is possible that the mechanisms of keratinization are not the same in every tissue and thus altered products are produced. Another indication that keratins may be subject to many as yet undefined post-translational modifications is the considerable charge heterogeneity shown by the B and B' polypeptides. This charge heterogeneity is seen when keratins are chromatographed on ion exchangers such as DEAE-cellulose, and the B-like (i.e. those with mol.wts. greater than 52000) chains are eluted at a variety of salt concentrations, implying a number of proteins with identical molecular weights, but with different charges. No such heterogeneity has been shown with the lower-molecular-weight chains (A and A').

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