The Extraction and Characterization of Bovine Epidermal α -Keratin

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(Received 9 December 1974)

1. The α -fibrous protein (α -keratin) component of bovine epidermis has been extracted and characterized. 2. Prekeratin, a multichain unit of the epidermal tonofilaments, was shown to consist of six different polypeptide chains on polyacrylamide-gel systems containing sodium dodecyl sulphate or sodium decyl sulphate with discontinuous gel buffers, but only three chains were seen when a gel system containing sodium dodecyl sulphate with a continuous gel buffer was used. 3. Extraction of the 'keratinized' stratum corneum and the living part of the epidermis with urea buffers at pH 7.6 or 9.0 released 60% of the total dry weight of the tissues in the form of α -helical polypeptides. 4. The numbers, relative amounts and properties of the extracted polypeptides were the same as the subunits of prekeratin and thus are derived from the tonofilaments *in situ*. 5. The subunits of prekeratin and the polypeptides extracted from the living cell layers contained an average of six cysteine residues, but those from the stratum corneum contained an average of three intrachain disulphide bonds. 6. The polypeptide chains aggregated through non-covalent interactions *in vitro* into filaments that were similar to the tonofilaments. 7. Since the polypeptides could be released from the stratum corneum without breaking covalent bonds, it is concluded that such bonds do not cross-link the tonofilaments and non-fibrous keratohyalin. It is suggested that the tonofilaments and keratohyalin of bovine epidermis are associated by secondary bonding forces.

The inner living cell layers of mammalian epidermis synthesize large quantities of an α -type fibrous protein which is deposited intracellularly in the form of tonofilaments. A non-fibrous protein, keratohyalin, is also produced. These two classes of protein eventually form the bulk of the outer dead ('keratinized') cell layer of the epidermis, the stratum corneum (Rudall, 1968; Fraser *et al.*, 1972).

Both proteins have been isolated from the living part of mammalian epidermis. Prekeratin, a multichain α -helical protein derived from the tonofilaments, was isolated by Matoltsy (1965). The nature of keratohyalin, however, is still equivocal since it has variously been described as a cysteine-rich protein (Matoltsy & Matoltsy, 1970), a histidine-rich protein (Sibrack *et al.*, 1974) and a histidine-rich ribonucleoprotein (Ugel, 1971).

The stratum corneum is insoluble in normal solvents for protein, and by analogy with other keratin-containing tissues such as wool and hair, this insolubility has been ascribed to cross-linking of the proteins by disulphide bonds (Rudall, 1968; Fraser *et al.*, 1972). In order to extract the keratins (or more accurately, their constituent polypeptide chains), workers have used several procedures designed to cleave disulphide or other types of covalent bonds, including alkaline urea solutions containing 2-mercaptoethanol (Baden & Bonar, 1968; Baden *et al.*, 1973b), 0.02–0.04M-NaOH (Rothberg, 1960;

Crounse, 1963, 1966) and formic acid (O'Donnell, 1971). In all cases, the major species extracted was a group of α -helical polypeptides derived from the tonofilaments. A second group which could have derived from the keratohyalin has not been described. However, in other studies, substantial amounts of α -helical polypeptides could be released from the stratum corneum tissue under denaturing conditions without the use of reagents that cleaved disulphide bonds (Rudall, 1952; Carruthers *et al.*, 1955; Roe, 1956; Baden & Bonar, 1968). The implication of these findings is that bonding forces, other than or additional to disulphide bonds, may perhaps be prominent in the mammalian epidermis. The present paper reports an investigation of these possibilities. The stratum corneum of bovine epidermis is extracted with urea buffers and it is shown that α -helical polypeptides derived from the tonofilaments can be released without cleavage of covalent bonds. The properties of the polypeptides are described and compared with the subunits of prekeratin. The findings are discussed in terms of the structure of bovine epidermal keratin.

Experimental

Materials

Bovine snouts and skin about 1 cm above the hooves were obtained from a local abattoir within 2 h of slaughter. Sodium decyl sulphate, sodium dodecyl

sulphate, acrylamide and methylenebisacrylamide (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) were recrystallized twice from ethanol before use. Coomassie Brilliant Blue G-250 was obtained from Serva Feinbiochemica, Heidelberg, West Germany. All other reagents were of the highest available purity. Iodo[2-³H]acetic acid (122Ci/mol) was obtained from Amersham/Searle, Arlington Heights, Ill., U.S.A.

Methods

Preparation of tissue. Bovine epidermis about the posterior aspect of the hoof is as much as 10mm thick (Ugel & Idler, 1970). The basal, spinous and granular layers which together comprise the living part of the epidermis, form a distinct band 1–2mm thick. A 1mm-thick slice of it was removed in the present work. Histological examination showed that it was free of superficial 'keratinized' stratum corneum and contained only small amounts of interdigitating dermis (Ugel & Idler, 1970). The stratum corneum is more than 5mm thick and a 1mm-thick slice was removed 2mm above the living cell layer of the epidermis. Examination of this slice indicated a complete absence of living cell layers (Ugel & Idler, 1970). In contrast, the epidermis of the snout is about 1mm thick and shows prominent interdigitation of cell layers (Rudall, 1952; Matoltsy, 1965). Two slices, 0.2mm thick, were removed with a keratome. Histological examination of the upper slice showed that it was mostly stratum corneum tissue, but it also contained some living cells (Rudall, 1952; Matoltsy, 1965) and was discarded in the present work. The lower slice was comprised mostly of the living cell layers, but substantial amounts of dermis and stratum corneum were also present (Rudall, 1952; Matoltsy, 1965). This slice was used in the present work only for the preparation of prekeratin. Each of the tissues was chopped into pieces of 0.5mm³ or less before extraction.

Preparation of prekeratin. This was prepared from the living tissue of the snout and hoof epidermis with 0.1M-sodium citrate buffer (pH 2.6) as described by Matoltsy (1965) except that all steps were carried out at 0–2°C. Prekeratin was serially precipitated at pH values of 7.0, 6.0, 5.0 and 4.5 to remove contaminants (Matoltsy, 1965) and was used within 2h of preparation.

Extraction of tissues with urea buffers. The tissues were suspended (25ml/g wet wt.) in a buffer containing 8M-urea and 0.05M-Tris-HCl at pH values of either 7.6 or 9.0. 2-Mercaptoethanol (0.05M) was added in some experiments. The extractions were done by stirring at either 37° or 4°C and were terminated by centrifugation at 30000g for 30min at 4°C. The 8M-urea solutions were deionized before the addition of the other components and Tris

base was used for the buffer to serve as a scavenger for cyanate ions (Hirs, 1967; Steinert & Rogers, 1973).

Polyacrylamide-gel electrophoresis. Polyacrylamide gels (10%T) with 0.1% (w/v) sodium dodecyl sulphate were used for molecular-weight estimations as described by Weber & Osborn (1969). A system containing discontinuous buffers adapted by Ugel *et al.* (1971) from the procedures of Davis (1964) was used, in which 0.5% (w/v) sodium decyl sulphate or 0.1% (w/v) sodium dodecyl sulphate was included in the cathode buffer. Protein samples were prepared for electrophoresis as described in the explanation of the Plates. The gels were stained with Coomassie Brilliant Blue G-250 in 12.5% (w/v) trichloroacetic acid (Diezel *et al.*, 1971) and destained by rinsing in 7.5% (v/v) acetic acid. Fast Green was also used for staining (Gorovsky *et al.*, 1969) and the gels were destained either electrophoretically or with Rexyn deionizing beads (Fisher Scientific Co., Fair Lawn, N.J., U.S.A.). In the Weber & Osborn (1969) system, a calibration line of log (molecular weight) against relative mobility was constructed by using creatine kinase (mol.wt. 40000), ovalbumin (45000), glutamate dehydrogenase (50000), catalase (57500), bovine serum albumin (68000) and urease (83000) as standards. Expressed as a least-squares linear regression $\pm$ S.D., the line used for molecular-weight estimation was:

$$\log_{10}(\text{molecular weight}) = (5.41 \pm 0.22) + [-(1.25 \pm 0.04)] \times R_F$$

Molecular weights were estimated in the discontinuous system with 0.5% (w/v) sodium decyl sulphate with the use of the precautions described in detail by Rodbard & Crambach (1971) and Ugel *et al.* (1971). The standard proteins and the polypeptide samples were electrophoresed on gels of total acrylamide concentration of 7.0, 7.5, 8.0, 8.5 and 9.0% with 3% cross-link. All samples were run simultaneously at one gel concentration in one electrophoresis apparatus and all gel solutions were made on the same day with the same batch of reagents. After electrophoresis, the gels were cut off at the pyronin-Y band which marked the Tris-phosphate boundary and the mobilities of the stained bands were measured relative to the remaining gel length. In some experiments, mobilities were measured relative to the second stacked boundary formed by the decyl sulphate-glycinate micelle complex which migrated behind the Tris-phosphate boundary. Semilogarithmic plots of mobilities of the same six standard proteins, used above, against gel concentration (Ferguson, 1964) yielded a series of straight lines which met at a common point (Ugel *et al.*, 1971) (Fig. 1a). This means that each protein had bound an amount of detergent which was directly related to its molecular size, and thus a

Fig. 1. Molecular-weight estimations on polyacrylamide gels containing discontinuous gel buffers

The standard proteins used were: 1, creatine kinase; 2, ovalbumin; 3, glutamate dehydrogenase; 4, catalase; 5, bovine serum albumin; 6, urease. These proteins were converted into their thiol form by incubation at 37°C for 16h in a buffer containing 8M-urea, 0.1M-Tris-HCl (pH9.0) and 0.1M-2-mercaptoethanol. The proteins were then dialysed into the cathode buffer of the discontinuous buffer system (Ugel *et al.*, 1971) containing 0.5% (w/v) sodium decyl sulphate and heated at 95–100°C for 3min immediately before loading. For other details, see the text. (a) Semilogarithmic plots of the mobilities of the standard proteins against total acrylamide concentration. (b) Plots of the slopes of these lines against molecular weight. A linear regression equation (see the text) was calculated from this line and was used for the estimation of the molecular weights of keratin polypeptides run simultaneously on other gels.

subsequent plot of the slope (K_R) of the lines against protein molecular weights was linear (Fig. 1b). The latter was used for molecular-weight estimation only if all the lines of the 'Ferguson plot' of the standard proteins and keratin polypeptides met at the common point. These considerations are implicit in the method of Weber & Osborn (1969) for molecular-weight estimation. The line described in Fig. 1(b)

when expressed as a least-squares linear regression $\pm$ s.d. was:

$$\text{molecular weight} = [-(3.75 \pm 0.02) \times 10^4] + [(1.09 \pm 0.03) \times 10^6] \times K_R$$

Sephadex G-200 chromatography. A column (90cm $\times$ 2.0cm) was prepared, equilibrated with a buffer consisting of 8M-urea, 0.05M-Tris-HCl (pH7.6), 0.2M-KCl, 1mM-EDTA and 25mM-2-mercaptoethanol and operated as described previously (Steinert & Rogers, 1973).

Measurement of protein concentrations. These were determined spectrophotometrically by using an $E_{277}^{1\%}$ value of 5.6. This was the same for prekeratin as well as for the polypeptides extracted from both layers of the epidermis.

Optical rotatory dispersion and circular dichroism (c.d.). These were measured at 23°C on a Cary model 60 spectropolarimeter equipped with a model 6001 c.d. accessory with the use of a 1cm path length. In optical-rotatory-dispersion studies, the α -helix content was estimated on protein solutions of 0.2–0.5mg/ml from the mean residue rotation observed at 233nm by using values of -12000° for 100% α -helix and -1800° for 0% α -helix, with presumption of linear interpolation (Simons *et al.*, 1961). In addition, values for b_0 were calculated from Moffit plots of $\lambda_0 = 212\text{nm}$, and corrected to α -helix contents by the formula $b_0/(-630 \times 100^\circ)$ (Blout, 1960). In c.d. studies, the α -helix content was estimated on protein solutions of 0.08–0.1mg/ml by measurement of the mean molar ellipticity observed at 208nm and by using values of $-33000^\circ \cdot \text{cm}^2/\text{decimol}$ for 100% α -helix and $4000^\circ \cdot \text{cm}^2/\text{decimol}$ for random coil (Greenfield & Fasman, 1969). Samples for measurement were dialysed into a buffer of 10mM-Tris-HCl (pH7.6) except for prekeratin which was measured in 10mM-sodium citrate buffer (pH2.6). Prekeratin was denatured by the addition of solid urea to a concentration of 8M and then dialysed into the 10mM-Tris-HCl buffer.

Amino acid analysis. Proteins were thoroughly desalted by dialysis and samples (2–3mg) were hydrolysed *in vacuo* in 5.7M-HCl for 22, 48 and 72h, and analysed on a Beckman 121 amino acid analyser. Appropriate corrections were made for the slow release or destruction of some amino acids. Tryptophan was estimated colorimetrically (Gaitonde & Dovey, 1970).

Estimation of thiol groups with iodo[2- ^3H]acetic acid. This was done by using the same techniques as described for two other thiol reagents (Steinert *et al.*, 1974). The specific radioactivity of the iodo[2- ^3H]acetic acid was decreased by the addition of unlabelled iodoacetic acid and the exact specific radioactivity was then determined by titration against yeast alcohol dehydrogenase which has 8.0mol of thiol groups/151000g (Krakow & Goolsby, 1971).

Reaction mixtures contained, in 1.0 ml of 8.0 M-urea and 0.1 M-Tris-HCl buffer (pH 8.5), protein containing up to 10 nmol of thiol groups and 1 μ mol of iodo[2-³H]acetic acid, and were incubated at 37°C. Samples (0.1 ml) were withdrawn to follow the time-course of the reaction, and were filtered on to nitrocellulose membranes (Millipore Corp., Bedford, Mass., U.S.A.; 0.45 μ m pore size). They were washed with an excess of 2-mercaptoethanol to remove unchanged iodoacetic acid, and counted for radioactivity in a toluene-based fluid containing 0.3% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene. The reaction was considered complete when a stable plateau of radioactivity had bound to the protein. Blank values (no added thiol) were about 50 d.p.m. above background, which represented less than 1% of the assay activity. Samples of prekeratin or of the extracted polypeptides were dialysed into the above urea buffer for 1 h and assayed immediately. All steps including preparation of the samples were carried out in an atmosphere of N₂ to prevent the formation of random disulphide bonds.

Results

Polypeptide composition of prekeratin

When prekeratin was dissociated, and examined on polyacrylamide gels by the method of Weber & Osborn (1969), Baden *et al.* (1973a) found three polypeptide chains with molecular weights of 47 000, 58 000 and 98 000, but Skerrow (1974) found only two chains, with molecular weights 60 000 and 72 000. Since the properties of the prekeratin subunits will be compared with those of the polypeptides extracted with urea buffers, attempts were made here to verify these studies by using the same and additional techniques.

Prekeratin was prepared from the living cell layer of bovine epidermis of both the hoof and snout, with yields of 0.45 and 0.37 g/g dry wt. of tissue respectively.

Prekeratin was treated with sodium dodecyl sulphate and 2-mercaptoethanol and prepared for electrophoresis as described by Skerrow (1974) and Weber & Osborn (1969). The resulting gel is shown in Plate 1(a). Two major bands and one minor band are evident, which appear similar to those described by Skerrow (1974). Plates 1(b) and 1(c) show gels of the same preparation of prekeratin which had been treated with 2-mercaptoethanol and respectively sodium dodecyl sulphate and sodium decyl sulphate and prepared for electrophoresis in the discontinuous gel-buffer system. A more complex band pattern is seen, with four major and two minor bands as well as a minor band of much lower mobility. The possibility that the increased numbers of bands were due to electrophoretic arti-

facts or proteolytic degradation was examined. Solutions of prekeratin were treated with sodium dodecyl sulphate, or sodium decyl sulphate, and 2-mercaptoethanol as above, but heated at 95–100°C for 3 min before or after dialysis into the cathode buffer. In a second experiment, prekeratin was denatured with urea, reduced and alkylated with iodoacetic acid as described by Weber *et al.* (1972) before electrophoresis. Both of these controls were shown to destroy the contaminating proteinases which might cause limited cleavage of proteins during preparation for electrophoresis, and moreover, the former procedures ensure the complete reaction of protein with the sodium dodecyl sulphate or sodium decyl sulphate (Weber *et al.*, 1972). The gels obtained by these different methods were the same as those shown in Plate 1, except that the minor band of low mobility on gels (b) and (c) disappeared, which suggests that it is an aggregate of the chains. It is concluded that the band patterns are valid and reproducible.

To explore the differences between the two gel systems, the bands of gel (a) were cut out, eluted into buffer containing sodium dodecyl sulphate as described by Weber *et al.* (1972) and subjected to electrophoresis in the discontinuous system as in gel (b). The results (not shown) indicated that the band of highest molecular weight on gel (a) co-electrophoresed with the major band of highest molecular weight of gel (b), but the two bands of lower molecular weight on gel (a) were resolved into the five additional bands seen on gel (b). The improved resolution of the bands is presumably due to the isotachophoretic effect produced in the 'stacking' gel by the discontinuous buffers. Maizel (1970) and Weber *et al.* (1972) have also indicated that similar systems containing discontinuous buffers with a 'stacking' gel afford superior resolution to continuous-buffers systems.

In other experiments, 2-mercaptoethanol was omitted during the preparation for electrophoresis, and the resultant band patterns were the same as in Plate 1. Skerrow (1974) make a similar observation and concluded that prekeratin does not contain interchain disulphide bonds.

The relative amounts of each band in the gels of Plate 1 were quantified by densitometry and were, in decreasing order of molecular weights; gel (a), 0.33, 0.65 and 0.02; gel (b), 0.35, 0.01, 0.21, 0.11, 0.31 and 0.01; and gel (c), 0.33, 0.03, 0.21, 0.10, 0.32 and 0.01.

The above experiments were done with prekeratin prepared from both the hoof and snout epidermis with the same results.

Extraction of epidermis with urea buffers

In initial studies, unbuffered 6 M-urea solutions were used (Rudall, 1952) but the amounts of poly-

EXPLANATION OF PLATE I

Polyacrylamide gels of dissociated prekeratin

In each gel, the same preparation of prekeratin was used (1 mg/ml in 0.1 M-sodium citrate buffer, pH 2.6). (a) The solution was made to 1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol, adjusted to pH 7, heated for 3 h at 37°C and dialysed into the gel buffer of Weber & Osborn (1969); 40 µg of protein was loaded on to the gel. Electrophoresis was carried out for 5 h at 8 mA/gel; 10% T gels (0.6 cm × 10 cm) were used. The gel was fixed and stained with Coomassie G-250 in 12.5% (w/v) trichloroacetic acid as described under 'Methods'. (b) The prekeratin was treated with 1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol, adjusted to pH 9, heated for 3 h at 37°C and dialysed into the Tris-glycinate cathode buffer containing 0.1% (w/v) sodium dodecyl sulphate and 0.1% (v/v) 2-mercaptoethanol; 50 µg of protein was loaded on to the gel. Electrophoresis was carried out for 3 h at 2 mA/gel; 9% T gels (0.6 cm × 6.5 cm) were used. The gel was fixed and stained with Fast Green in 7.5% (v/v) acetic acid. The band B marks the boundary formed by the dodecyl sulphate-glycinate micelle complex which is not completely eluted from the gel. (c) Same as (b) except that the cathode buffer contained 0.5% (w/v) sodium decyl sulphate. Control experiments were carried out to check the band patterns. In one, samples were treated with 1% (w/v) sodium dodecyl sulphate or sodium sulphate and 1% (v/v) 2-mercaptoethanol, adjusted to pH 7 or 9 and heated for 3 min at 95–100°C. Samples were then dialysed into the appropriate cathode buffer for electrophoresis. Alternatively, the samples in the cathode buffer were heated immediately before application to the gels. In a second experiment, the prekeratin was made to 8 M-urea, 0.1 M-2-mercaptoethanol, reduced and alkylated with iodoacetic acid as described by Weber *et al.* (1972). Samples were then treated as above for electrophoresis.

EXPLANATION OF PLATE 2

Polyacrylamide gels of polypeptides extracted from the epidermis

After extraction, the samples were centrifuged at 30000g for 30 min at 4°C. Preparation of the samples for, and the performance of, electrophoresis was as described in Plate 1(c) except that 2-mercaptoethanol was omitted at all steps. Other details are given under 'Methods'. (a) Prekeratin from hoof epidermis was dissociated as in Plate 1(c). (b) The living portion of the epidermis was extracted with the buffer of 8 M-urea, 0.05 M-Tris-HCl (pH 9.0) and 0.05 M-2-mercaptoethanol for 3 h at 37°C. (c) The stratum corneum was extracted with the buffer of 8 M-urea and 0.05 M-Tris-HCl (pH 7.6) at 37°C, disrupted at 4 h with the Polytron device (Fig. 2b) and extracted for a further 3 h. (d) The same sample as in (c) was used. The 30000g supernatant was diluted to 2 mg/ml with the same urea buffer and dialysed into 10 mM-Tris-HCl buffer (pH 7.6) with two changes of 100 vol. each. The pH of the clear solution was lowered to 5.5 with 1 M-HCl and the coarse precipitate was collected by centrifugation at 10000g for 10 min at 4°C. The pellet was redissolved in the urea buffer, dialysed and the precipitation was repeated a second time. The pH 5.5 supernatant was made to pH 4.5 by addition of more 1 M-HCl and the fine precipitate was collected and treated as above.

EXPLANATION OF PLATE 3

Electron micrograph of filaments

Polypeptides were extracted from the stratum corneum with a buffer of 8M-urea–0.05M-Tris–HCl (pH9.0) at 37°C for 7h including a disruption step at 4h (Fig. 2b). After centrifugation at 30000g for 30min at 4°C, the supernatant was recentrifuged at 220000g for 1h at 2°C to remove any filamentous material which may have survived extraction. The polypeptides in the supernatant were diluted to about 3mg/ml with the urea buffer and dialysed into 100vol. of 10mM-Tris–HCl (pH7.6) buffer for 24h at 4°C. A drop of the diffusate which by this time contained filaments was stained on a holey grid with 2% uranyl acetate and examined in a Siemens Elmiskop 1 electron microscope. Magnification $\times 114000$; the bar represents 0.2 μ m.

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Fig. 2. Extraction of epidermis with urea buffers

Extractions were carried out with a urea buffer containing 8M-urea and 0.05M-Tris-HCl at 37°C with gentle stirring in the presence (●) or absence (○) of 0.05M-2-mercaptoethanol by using (a) the living part of the epidermis at pH9.0 and (b) the stratum corneum at pH7.6. Samples were withdrawn at the indicated times and centrifuged at 30000g for 30min at 4°C. The supernatant was dialysed exhaustively against water and freeze-dried. Part of the stratum-corneum extract with no added 2-mercaptoethanol was subjected at 4h to disruption with a Polytron device for 2min at 23°C and further extraction was continued as before (Δ). The amounts of polypeptides released were corrected to g/g dry wt. of the original tissues.

peptides released between different experiments were variable. Accordingly, 8M-urea solutions were used and were buffered with Tris-HCl near physiological pH (7.6) and at an alkaline pH (9.0), similar to that of Baden & Bonar (1968).

Fig. 2(a) shows a time-course of the extraction at pH9.0 of the living part of the epidermis in the presence and absence of 2-mercaptoethanol. The reducing agent was not required for the release of polypeptides and about 0.55g were extracted/g dry wt. of tissue within 2h at 37°C. Similar amounts were extracted at pH7.6 within the same time, but about 4h was required when the extractions were done at 4°C.

When the stratum corneum was extracted at pH7.6 at 37°C with 2-mercaptoethanol (Fig. 2b), about

0.65g of polypeptides/g dry wt. of tissue was released in 4h. At pH9.0, the same time-course was found, but about 8h was required at 4°C at both pH values. However, if 2-mercaptoethanol was omitted, little polypeptide material was released at 37°C and pH 7.6 by 4h (Fig. 2b). Examination of this extract in the light-microscope showed the presence of clumps of swollen cells that had lost their birefringence. This suggested that the tonofilaments of the cells had been dissociated but the polypeptides had not been released. When this tissue was subjected to vigorous shearing action by a Polytron device (Brinkman Instruments Inc., Westbury, N.Y., U.S.A.), the cells were broken open and by 3-5h after disruption, the amount of polypeptides extracted in the absence of 2-mercaptoethanol was the same as the amount extracted in its presence (Fig. 2b). The same results were obtained at pH9.0. At 4°C similar amounts of polypeptides were released at both pH values on disruption at 12h. As reported by Rudall (1952), prolonged treatment with urea solutions alone, without 2-mercaptoethanol or disruption, extracted substantial amounts of polypeptide material (Fig. 2b).

The polypeptides extracted by these procedures from the living and stratum corneum layers of the epidermis were examined on polyacrylamide gels. Electrophoresis was performed in the absence of 2-mercaptoethanol [Plate 2, gels (b) and (c) respectively]. The band patterns and the relative amounts of each band were very similar to each other and to prekeratin [Plate 2, gel (a)]. Samples were also examined on the Weber & Osborn (1969) system and the bands were very similar to those in Plate 1 gel (a). Since the polypeptide chains can be dissociated by urea, sodium dodecyl sulphate or sodium decyl sulphate alone, without use of 2-mercaptoethanol, these findings mean that the polypeptide chains are not cross-linked by interchain disulphide bonds or other types of covalent bonds.

In earlier studies, extracts were precipitated at various acid pH values and the fractions showed variations in certain properties. For example, Rudall (1952) isolated a minor fraction insoluble at pH4.5 which was 'non-fibrous' and which contained more sulphur than the main fibrous fraction insoluble at pH5.5. In the present study, fractions insoluble at pH5.5 (about 90-95% of the total protein) and pH4.5 ($\leq 2\%$) were prepared. Irrespective of which layer of the epidermis was used, or how the extractions were done, polyacrylamide gels of the pH fractions were the same as Plate 2 gel (d) and the same as the unfractionated samples [Plate 2, gels (b) and (c)]. Proteins soluble at pH4.5 (3-7% of the total protein) and proteins soluble at pH7 from a prekeratin preparation contained large numbers of protein bands which were most probably derived from the cytoplasmic enzymes present in the tissue (Matoltsy, 1965; Ugel, 1971).

Fig. 3. Molecular-weight estimation by chromatography on Sephadex G-200

(a) Calibration line prepared with the same standard proteins used in Fig. 1. (b) Chromatography of the polypeptides extracted from hoof epidermis as described in Plate 2(c). The polypeptides were applied to the column immediately after isolation. Other details are given under 'Methods'.

Properties of the polypeptide chains extracted from the epidermis, and of prekeratin

Molecular weights. These were determined by both a column-chromatographic and two gel-electrophoretic procedures. Whereas each of the standard proteins chromatographed as a single sharp peak on Sephadex G-200, the polypeptide chains obtained from prekeratin, or from the urea extracts of the epidermal cell layers, chromatographed as a broad peak, corresponding to molecular weights within the range 50000–60000 (Fig. 3). The high-molecular-weight material eluted at V_0 (≥ 90000) could be readily dissociated with sodium dodecyl sulphate or sodium decyl sulphate into polypeptide chains and therefore consisted of non-covalently bound aggregates of the chains.

On the basis of the mobilities of the standard proteins on the polyacrylamide-gel system of Weber & Osborn (1969), the two major and one minor band

observed for prekeratin [Plate 1, gel (a)] had molecular weights of 58000, 49000 and 48000 respectively. When samples of the polypeptides extracted from the two epidermal cell layers were examined on this system, similar estimates were obtained (with a variation of $\pm 6\%$).

When the polypeptides extracted from both layers of the epidermis, as well as those of dissociated prekeratin, were examined on 7–9% T gels of the discontinuous-buffer system [containing 0.5% (w/v) sodium decyl sulphate], a family of straight lines was obtained which met at the same point as the standard proteins examined simultaneously on other gels. The molecular weights of the polypeptides were estimated from the slopes of these lines by use of a linear regression equation derived from the standard proteins as described in Fig. 1(b). For example, for the polypeptide chains of hoof prekeratin, the molecular weights were 58000, 56000, 54000, 51000, 48000 and 47000. The polypeptides extracted with urea buffers had the same molecular-weight values (with a variation of $\pm 3\%$). The molecular weights of polypeptides that had been converted into their *S*-carboxymethyl derivatives (Weber *et al.*, 1972) were within the same range. The minor band of lower mobility than the main polypeptide chains had a molecular weight of 100000, and was probably, therefore, a dimer of the chains.

The three methods have provided very similar molecular-weight estimates. Whereas Baden *et al.* (1973a) obtained similar values, Skerrow's (1974) values are substantially higher. The reason for this difference is not clear, but if the standard proteins used in his work were not fully reduced, higher-molecular-weight estimates would be expected.

α -Helix contents. These were determined by optical rotatory dispersion and c.d. The b_0 values were difficult to measure accurately as the polypeptides displayed little rotation above 350 nm and are not given. However, α -helix contents were calculated from the mean residue rotation $[\alpha]_D$ at 233 nm (Simons *et al.*, 1961) and the mean molar ellipticity $[\theta]$ at 208 nm (Greenfield & Fasman, 1969) (Table 1). The α -helix contents of polypeptide chains derived from the two epidermal cell layers, irrespective of their mode of extraction, of prekeratin and denatured prekeratin were within the range of 38–45%. Analyses by the c.d. mode yielded as a routine somewhat higher values. The α -helix content of prekeratin is similar to that found by Matoltsy (1965) and Baden *et al.* (1973a) who used bovine snout epidermis.

Amino acid analyses. These were done on prekeratin and polypeptides extracted from hoof epidermis and are the same within the limits of experimental error (Table 2). The analysis of prekeratin is similar to that obtained in other studies with snout epidermis (Skerrow *et al.*, 1973; Baden *et al.*, 1973a).

Table 1. α -Helix contents of prekeratin and the polypeptides extracted from the epidermis calculated from optical-rotatory-dispersion and c.d. measurements

For the data presented, polypeptides were extracted from the two epidermal cell layers of hoof epidermis in a buffer of 8M-urea, 0.05M-Tris-HCl (pH9.0) and 0.05M-2-mercaptoethanol for 3h at 37°C. Other details including denaturation of prekeratin and calculation of the optical-rotatory-dispersion and c.d. data are described under 'Methods'.

Source of polypeptides	% α -Helix contents calculated from	
	Optical rotatory [m] ₂₃₃	C.d. [θ] ₂₀₈
Prekeratin	38	45
Denatured prekeratin	39	43
Living cell layer	40	45
Stratum corneum	38	44

Table 2. Amino acid compositions (residues/100 residues) of prekeratin and the polypeptides extracted from the hoof epidermis

For the data presented, the polypeptides were extracted at 37°C in a buffer of 8M-urea and 0.05M-Tris-HCl (pH9.0) for 3h for the living cell layer, and a total of 7h for the stratum corneum, including the disruption step at 4h (Fig. 2b). Other details are given under 'Methods'.

Amino acid	Prekeratin	Polypeptides from epidermal	
		Living layer	Stratum corneum
Aspartic acid	9.15	9.05	8.90
Threonine	3.90	3.95	4.00
Serine	9.35	9.40	9.70
Glutamic acid	14.70	14.45	14.50
Proline	1.55	1.50	1.65
Glycine	15.50	15.20	15.25
Alanine	6.45	6.30	6.15
Half-cystine	1.15	1.10	1.15
Valine	5.15	5.00	5.05
Methionine	1.75	1.75	1.85
Isoleucine	4.00	3.80	3.85
Leucine	8.75	9.05	8.80
Tyrosine	3.00	3.10	3.10
Phenylalanine	3.60	3.50	3.40
Lysine	5.00	5.50	5.30
Histidine	1.10	1.00	1.20
Arginine	6.10	6.20	6.05
Tryptophan	0.39	0.35	0.38

Estimation of the thiol-group content of the extracted polypeptides and prekeratin

The above results indicate that 2-mercaptoethanol is not required for the dissociation of prekeratin or for the dissociation or release of the α -helical polypeptides from the epidermal cells. Therefore the cysteine residues of the polypeptides exist either in

Fig. 4. Estimation of the thiol content of prekeratin

The reaction mixture contained 96μg of prekeratin prepared from hoof epidermis. Other details are given under 'Methods'. The specific radioactivity of the iodo[2-³H]acetic acid used was measured in an identical experiment (Steinert *et al.*, 1974) and was 9420 d.p.m./nmol.

Table 3. Estimation of the thiol-group content of prekeratin and the polypeptides extracted from hoof epidermis

Prekeratin and polypeptides were treated with iodo[2-³H]acetic acid (see under 'Methods') to give a time-course as in Fig. 4. The data show the average $\pm$ S.D. values for three preparations of prekeratin and for three preparations of polypeptides extracted at both pH and temperature values in the absence of 2-mercaptoethanol. The average molecular-weight value used was calculated from the molecular weight and relative amount of each band determined on gels as in Plate 1(c).

Source of polypeptides	Thiol-group content	
	(μmol/g of polypeptides)	(mol/54000 g of polypeptides)
Prekeratin	108 $\pm$ 3	5.9 $\pm$ 0.2
Living cell layer	110 $\pm$ 3	6.0 $\pm$ 0.2
Stratum corneum	3 $\pm$ 2	0.2 $\pm$ 0.1

the thiol form or are linked in intrachain disulphide bonds. These possibilities were tested by the reaction of iodo[2-³H]acetic acid with prekeratin, and a time-course of the reaction is illustrated (Fig. 4). A stable plateau of bound radioactivity was obtained within 15 min of incubation, which signifies that all thiol groups had undergone complete reaction and that little if any reaction had occurred with other amino acids. The plateau value obtained in Fig. 4 is 109 μmol of thiol groups/g of prekeratin and a similar value was obtained for polypeptides extracted from the living cell layer of the epidermis (Table 3). These data

Fig. 5. Disappearance of thiol groups from filaments

The living portion of hoof epidermis was extracted with a buffer of 8M-urea and 0.05M-Tris-HCl (pH9.0) for 3 h at 37°C in an atmosphere of N₂. The extract was then centrifuged and dialysed as in Plate 3 except that dialysis was continued for 6 days with daily changes of buffer. Samples were removed daily for assay with the iodo[2-³H]-acetic acid to give a time-course as in Fig. 4. In this experiment, the initial thiol content was 111 μmol/g of polypeptides.

correspond to 5.9 and 6.0 mol of thiol groups/average mol of all polypeptide chains (molecular weight about 54000). In addition, calculations show that the half-cystine contents determined by amino acid analysis after acid hydrolysis (Table 2) are about 6 residues/average mol. Therefore all cysteine residues of prekeratin, and the polypeptides extracted from the living cell layer, exist in their thiol form. On the other hand, polypeptides extracted from the stratum corneum contained 3 μmol of thiol groups/g (0.2 residues/average mol) which suggests that the cysteine residues of these polypeptides are linked in an average of three intrachain disulphide bonds.

Aggregation of the polypeptide chains into filaments

Urea extracts of the living and stratum corneum epidermal cell layers formed opalescent viscid gels on removal of the urea by dialysis. The gel was due to the formation of filaments. Filaments formed at polypeptide concentrations as low as 0.1 mg/ml and the gel formed at concentrations above about 10 mg/ml. When examined in the electron microscope after negative staining with uranyl acetate, the filaments were about 8 nm wide (with a variation of ±1 nm) and 0.3–0.8 μm long (Plate 3). Prekeratin also formed filaments when the citrate buffer was removed by dialysis, as found by Matoltz (1965). In comparison, the tonofilaments *in situ* are 7–8 nm wide and are of indeterminate length (Brody, 1960) since they are not well aligned in the plane of the section. Thus although

probably shorter, the aggregated filaments are distinctly similar to the tonofilaments *in situ*.

Aspects of the assembly of the filaments were examined *in vitro* by using electron microscopy to monitor experiments.

The gel could not be dispersed in aqueous salt solutions, but solutions of 4–8M-urea or 0.1% (w/v) sodium decyl sulphate or sodium dodecyl sulphate dispersed the gel and filaments into polypeptide chains within 1 h at 23°C. At similar concentrations, these reagents prevented filament and gel formation during dialysis. This suggests that the polypeptide chains aggregate through non-covalent interactions and that the structure of the filaments is sustained by secondary bonding forces. The presence of 2-mercaptoethanol up to 0.1M did not interfere with filament or gel formation. As the chains extracted from the stratum corneum contained intrachain disulphide bonds, as described above, and also formed filaments (Plate 3), this shows that filament assembly is independent of disulphide bond formation. This aspect was further examined with reaction of iodo-[2-³H]acetic acid. The gradual disappearance of reacting cysteine residues in assembled filaments over several days (Fig. 5) reflects formation of disulphide bonds within the filaments. Similar findings were made when prekeratin aggregated into filaments. On the other hand, after chemical modification of the cysteine residues by conversion into the S-carboxymethyl derivatives with iodoacetic acid (Weber *et al.*, 1972), filament formation did not occur on dialysis, possibly because of steric interference.

Discussion

Bovine epidermal prekeratin is a multichain particle consisting of regions of triple-chain α-helix and random coil, and presumably forms a repeating structural unit of the tonofilaments (Matoltz, 1965; Skerrow *et al.*, 1973; Skerrow, 1974). By the use of polyacrylamide-gel systems containing sodium dodecyl sulphate or sodium decyl sulphate with discontinuous gel buffers, prekeratin from bovine snout and hoof epidermis was shown in the present work to contain six polypeptide chains, which is more than was found when the continuous gel-buffer system with sodium dodecyl sulphate of Weber & Osborn (1969) was used here or in other studies (Baden *et al.*, 1973a; Skerrow, 1974). A number of control experiments showed that the differences were not due to electrophoretic artifacts or selective degradation of the polypeptide chains in the discontinuous system. Improved resolution of protein bands on gel systems by the use of a 'stacking' gel and discontinuous buffers has been reported elsewhere (Maizel, 1970; Weber *et al.*, 1972).

The close similarity of properties such as numbers and relative amounts of polypeptide chains, molecular

weights, α -helix contents and amino acid compositions, showed that the polypeptides extracted with urea buffers from the 'keratinized' portion of the epidermis, the stratum corneum, were identical with the subunits of prekeratin. Therefore the polypeptides originated from the tonofilaments or the α -keratin component. Experiments involving extraction at different pH and temperature values, and fractionation at acid pH values, showed that the observed numbers of bands were also not due to isolation artifacts, random degradation by proteinases or to the presence of non-keratin proteins. In a preliminary report Lee & Baden (1974) observed that prekeratin and polypeptides extracted from bovine snout epidermal tissue 'have similar patterns on SDS electrophoresis and consist of several polypeptides' of which four were described. Although no experimental details were provided, these findings appear similar to those described in the present paper. In other studies (P. M. Steinert unpublished work), each of the chains has been isolated and characterized. Although the chains have generally similar properties, such as common terminal amino acid residues, significant differences in the α -helix contents and amino acid compositions were evident. Thus it seems that α -keratin of bovine epidermis consists of a heterogeneous group of polypeptides. This is perhaps not surprising since polypeptide chain heterogeneity is also apparent in the fibrous keratin proteins of wool (Fraser *et al.*, 1972), hair (Steinert & Rogers, 1973) and feather (Kemp & Rogers, 1972).

The yields of the extracted polypeptides indicate that the α -keratin component forms at least 60% of the total dry weight of the stratum corneum tissue, but it is not known whether quantitative extraction was achieved in these experiments. Nevertheless, it is unlikely that any of the chains was selectively withheld from extraction, since the relative amounts and numbers of the extracted polypeptide chains were the same as the subunits of prekeratin. Of major significance was the observation that the polypeptides could be released from the stratum corneum with simple urea buffers and without use of additional reagents that cleave disulphide or other types of covalent bonds. Exposure of proteins to urea solutions at pH 7.6–9.0 is not considered to break covalent bonds, or under the controlled conditions used here, chemically modify or damage proteins (Hirs, 1967). It may be concluded therefore that covalent bonds do not cross-link the tonofilaments with other intracellular components in the stratum corneum of bovine epidermis, and moreover, that the tonofilaments are not in themselves stabilized by covalent cross-links between their constituent subunits. However, an average of three intrachain disulphide bonds formed within each subunit subsequent to initial deposition in the cells.

These findings are different from the situation in the keratin proteins of epidermal appendages. The fibrous and non-fibrous proteins of wool and hair, for example, are cross-linked by disulphide bonds, and in the inner root sheath, ϵ -(γ -glutamyl)-lysine cross-links are present (Harding & Rogers, 1971). It has not been possible to release significant amounts of polypeptides from these 'keratinized' tissues without the use of reagents that cleave the cross-links or peptide bonds (Fraser *et al.*, 1972).

The polypeptides containing thiol groups are synthesized in the living epidermal cells, and in all likelihood, are assembled into tonofilaments at the site of synthesis (Munro & Steinert, 1975). By the time the cells become 'keratinized' units of the stratum corneum, the thiol groups are fully oxidized to disulphide bonds. To an extent, these events were duplicated in the experiments *in vitro*. It was shown that prekeratin and the polypeptides readily aggregated into native-type filaments through non-covalent interactions. Formation of intrachain disulphide bonds occurred independently of filament assembly. The filaments were stabilized by secondary bonding forces since reagents that interfered with these bonds either inhibited filament formation *in vitro* or dissociated preformed filaments. Because of the ease with which the tonofilaments *in situ* could be dissociated by urea solutions, it is clear that they are also stabilized only by secondary bonding forces. The isolation of triple-chain α -helical segments from prekeratin has provided direct evidence for the existence of these forces (Skerrow *et al.*, 1973).

The only apparent limitation on the release of polypeptides from the stratum corneum was the cell membrane. As physical disruption of the cells after swelling in urea permitted release of polypeptides, it would appear that the characteristic insolubility of the stratum corneum is mostly due to the cell membrane rather than the intracellular contents. However, extraction in urea with 2-mercaptoethanol released the polypeptides more readily, which implies that disulphide bonds are involved in the cell-membrane structure. This is consistent with the known high content of half-cystine in the isolated stratum-corneum membrane (Matoltsy & Matoltsy, 1966). As the membrane accounts for only 5% of the total mass of the stratum-corneum cell (Matoltsy & Matoltsy, 1966), most of the 30–40% of the tissue remaining after extraction must have been keratohyalin. Since no other polypeptide fraction was extracted in these studies, the keratohyalin presumably remained as large insoluble fragments that sedimented at 30000g. Keratohyalin is thought to form an interfibrillar matrix with the tonofilaments and to stabilize the complex with disulphide bonds (Rudall, 1968; Matoltsy & Matoltsy, 1970; Fraser *et al.*, 1972). The present experiments do not support this view. It seems more probable that the

filament-matrix complex of the bovine epidermis is stabilized by secondary bonding forces than by the covalent bonds that are found in other keratins.

I thank Dr. Peter McPhie for advice with the optical-rotatory-dispersion and c.d. measurements, Mrs. Marisa Gullino for the electron microscopy and Mr. William Idler for skilled assistance with the polyacrylamide-gel-electrophoresis experiments.

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