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The Polypeptide Composition of Bovine Epidermal α-Keratin

By PETER M. STEINERT and WILLIAM W. IDLER
Dermatology Branch, National Cancer Institute, Bethesda, Md. 20014, *U.S.A.*

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1. The polypeptide chains that comprise the subunits of the tonofilaments, or the α-keratin component, of bovine epidermis were fractionated by a combination of chromatography on DEAE-cellulose and preparative polyacrylamide-gel electrophoresis. 2. The seven polypeptide chains investigated had generally similar properties; all contained two residues per molecule of tryptophan and *N*-acetylserine was the common *N*-terminal amino acid residue. 3. On the basis of close similarities in α-helix content and amino acid composition, the polypeptide chains were classified into three distinct groups. Each group contained approximately one-third of the total polypeptides on a molar basis. The groups and designated polypeptide chain numbers were: group one, polypeptides 1a and 1b, which had molecular weights of 58000, contained about 25% α-helix, 86 glutamic acid and 8 cysteine residues per molecule, but which differed in net charge, extinction coefficients and tyrosine contents; group two, polypeptides 2, 3 and 4, which had molecular weights within the range of 52000–56000, contained about 48% α-helix, 54 glutamic acid and 6 cysteine residues per molecule, but which differed in extinction coefficients and tyrosine contents; and group three, polypeptides 5 and 6, which had molecular weights of 47000–48000, contained about 56% α-helix, 64 glutamic acid and 4 cysteine residues per molecule, but which differed in extinction coefficients and tyrosine contents. 4. From the differences in glutamic acid and tyrosine contents, it is suggested that none of the chains is a precursor or a degradation product of other polypeptide chains. 5. It is concluded that bovine epidermal α-keratin consists of a heterogeneous group of similar polypeptide chains.

The living cells of mammalian epidermis synthesize large amounts of an α-fibrous protein, which is deposited intracellularly in the form of tonofilaments. This component, referred to as α-keratin, eventually forms the bulk of the dead outermost layer of the epidermis, the stratum corneum (Fraser *et al.*, 1972).

Extraction of the α-keratin component can be accomplished by treating the living epidermal cells with a citrate buffer that releases the protein in a multichain state termed prekeratin (Matoltsy, 1965). Alternatively, denaturing solvents such as 8M-urea solutions can be used either with the living cells or with the stratum corneum. This dissociates the tonofilaments and their constituent polypeptide chains are released (Baden & Bonar, 1968; Baden *et al.*, 1973*b*; Steinert, 1975).

A notable feature of both prekeratin and the extracted polypeptides is their marked propensity for aggregation. Indeed, in the absence of denaturing reagents, they assemble *in vitro* into filaments that appear very similar to the tonofilaments seen *in situ* (Steinert, 1975). This property offers an opportunity for a detailed study of the process of assembly and structure of the tonofilaments. Before useful experiments can be performed, it is necessary to obtain basic information on the chemistry and structure of the individual polypeptides. The present paper describes the fractionation of the polypeptides and characterization of some of their properties.

Experimental

Materials

Bovine skin was obtained from a local abattoir within 2h of slaughter. Chicken lysozyme (grade III, crystalline), bovine trypsin (pancreas, type III), Pronase (*Streptomyces griseus*, type VI), carboxypeptidase A (bovine pancreas, di-isopropylfluorophosphate-form) and mercuripapain (papaya latex, twice-crystallized) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Yeast alcohol dehydrogenase was obtained from Schwarz–Mann, Orangeburg, N.J., U.S.A. All other reagents were of the highest standard of purity. Iodo[2-^{3}H]acetic acid (148.7Ci/mol) and sodium dodecyl [^{35}S]sulphate (4.72Ci/mol) were obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

Methods

Extraction of the α-keratin polypeptides. Strips of epidermis (1–2mm thick) were dissected from the

skin about the hooves of freshly slaughtered cattle as described by Steinert (1975). In most experiments, the viable part of the epidermis was used, but the stratum corneum portion was also used occasionally. The polypeptides extracted from either layer are indistinguishable (Steinert, 1975). The tissue was extracted in a buffer of 8M-urea–0.05M-Tris–HCl (pH 9.0)–0.025M-2-mercaptoethanol (25ml/g wet wt.) with stirring at 37°C for 3–4h. Tissue debris was removed by centrifugation at 30000*g* for 30min at 4°C. Both the thiol forms and *S*-carboxymethyl derivatives of the polypeptides were used in this work. The latter were prepared by the addition to the supernatant of iodoacetic acid (dissolved in 3M-Tris base, 200mg/ml) to make a final concentration of iodoacetic acid of 0.05M. The use of Tris base enabled simultaneous correction of the pH to 8.5, which is the optimum pH for selective modification of thiol groups by iodoacetic acid (Hirs, 1967). The alkylation reaction was continued at 23°C for 15min and stopped by the addition of an excess of 2-mercaptoethanol. Both forms of the polypeptides were dialysed against two changes of 100vol. of 10mM-Tris–HCl (pH 8.0) and then precipitated at pH 4.5 by the addition of an equal volume of 0.2M-sodium acetate buffer. The precipitate was collected by centrifugation at 30000*g* for 10min at 4°C, redissolved and equilibrated in the urea buffer used for chromatography on DEAE-cellulose. Steinert (1975) showed that this acid-precipitation step removes the contaminating non-keratin proteins.

Column chromatography. A column (90cm × 2.0cm) of Sephadex G-200 was equilibrated with a buffer of 8M-urea–0.05M-Tris–HCl (pH 7.6)–0.2M-KCl–1mM-EDTA–0.025M-2-mercaptoethanol and was operated exactly as described previously (Steinert & Rogers, 1973*a*; Steinert, 1975). Samples for loading were concentrated to about 10mg/ml by use of the acid-precipitation procedure described above. DEAE-cellulose (DE-52) was equilibrated at 23°C in a buffer of 8M-urea–0.02M-Tris–HCl (pH 7.6)–1mM-EDTA–2% (v/v) propan-1-ol. When the *S*-carboxymethyl derivatives of the polypeptides were examined, the buffer also contained 0.02M-KCl. When the thiol forms of the polypeptides were examined, the KCl was omitted but 0.025M-2-mercaptoethanol was added. Development of the column chromatogram was by stepwise elution with increasing concentrations of KCl. Precautions were taken to remove cyanate from the urea buffers (Steinert & Rogers, 1973*a*; Steinert, 1975).

Protein analysis. Polypeptides were determined by the method of Bramhall *et al.* (1969) by using the stain Xylene Brilliant Cyanin G. The total mixture of polypeptides was used as a standard, and has $E^{1\%}_{277} = 5.6$.

Polyacrylamide-gel electrophoresis. This was performed by using a discontinuous gel buffer system (Ugel *et al.*, 1971) with the inclusion of either 6M-urea in the gel or 0.1% (w/v) sodium dodecyl sulphate in the cathode buffer. The rigorously controlled conditions delineated in detail by Rodbard & Chrambach (1971, 1974) were used throughout. The gels (6.5cm × 0.6cm) were maintained at 20.0 ± 0.5°C during preparation and electrophoresis. Samples were dialysed against 1000vol. of the cathode buffer before loading. For urea gels, the cathode buffer for equilibration of the sample contained 10M-urea. When the thiol forms of the polypeptides were examined, 0.025M-2-mercaptoethanol was added to prevent aggregation through random disulphide-bond formation. A current of 2mA/gel was used and was continued until the π–λ boundary (formed by the Tris–phosphate boundary and marked by Bromophenol Blue in urea gels and pyronin Y in the gels with sodium dodecyl sulphate) had migrated to within 5mm of the end of the gel. Gels with urea were fixed in 12.5% (w/v) trichloroacetic acid, stained with 0.05% (w/w) Xylene Brilliant Cyanin G and rinsed and stored in 7.5% (v/v) acetic acid. Gels with sodium dodecyl sulphate were fixed and stained simultaneously with 0.05% (w/v) Fast Green in 7.5% (v/v) acetic acid and destained overnight with Dowex AG1W-X8 beads (50-mesh size) in 7.5% (v/v) acetic acid. Occasionally, Coomassie Brilliant Blue R-250 was used for staining (Weber & Osborn, 1969) and the gels were destained electrophoretically.

The two gel systems were used for molecular-weight estimations of the α-keratin polypeptide chains. These estimates were based on the fact that a linear relationship exists between the logarithm of the relative mobility of a protein and the total acrylamide concentration (%T) of a gel (the 'Ferguson plot'; Ferguson, 1964). The slope of this line, or the retardation coefficient (K_R), is in turn related to protein molecular weight (Rodbard & Chrambach, 1971, 1974; Banker & Cotman, 1972). In initial experiments, the range of %T and %C (concentration of cross-linker) over which a linear relationship with protein mobility existed was determined. For studies with sodium dodecyl sulphate, gels of 3%C with 7.0, 7.5, 8.0, 8.5 and 9.0%T were used. In the urea system, 3%C with 5.0, 6.0, 7.0, 8.0 and 9.0%T gels were used. In both cases, the gels were cut off at the π–λ boundary after electrophoresis and the mobilities of the stained bands were measured relative to the remaining gel length. Calculations of the data were afforded by the published computer programs (Rodbard & Chrambach, 1971). Proteins of known molecular weight were converted into their thiol forms (Steinert, 1975) or *S*-carboxymethyl derivatives (Steinert & Rogers, 1973*a*) and were used to establish the standard line only if electrophoretically homogeneous on both gel systems. The use of globular proteins as standards for molecular-weight estimates of the fibrous keratin polypeptides is valid only

if all disulphide bonds are broken and all species are converted into rigid structures by the sodium dodecyl sulphate (Reynolds & Tanford, 1970*a*) or are fully denatured to random coils by urea (Tanford, 1968; Poole *et al.*, 1974).

The same gel system with sodium dodecyl sulphate was used for preparative polyacrylamide-gel electrophoresis by using a Uniphor 7900 apparatus (LKB Instruments Inc., Rockville, Md., U.S.A.). A 7% T, 3% C gel (15cm×2.4cm) was used. The load that could be fractionated satisfactorily was usually 2–10mg or 20–60 times the amount separable on the above analytical gels. Electrophoresis was at 20mA at 20.0±0.5°C for 20–24h. Material that electrophoresed off the end of the gel was eluted with anode buffer containing 0.1% (w/v) sodium dodecyl sulphate at 12ml/h and was collected into fractions of 1.5ml. Initial localization of the fractionated polypeptide chains was done by measuring the rate of migration of bands made visible as refractive-index discontinuities; this permitted accurate prediction of the position of elution of each band. Usually, analytical gels were done on fractions about the predicted region, and less frequently, the necessary fractions were assayed for protein (Bramhall *et al.*, 1969).

Binding of sodium dodecyl sulphate. This was done to check the validity of using sodium dodecyl sulphate for molecular-weight estimations of the α-helical polypeptides. Solutions of standard proteins or polypeptides (0.5–1.0mg/ml) were equilibrated in a buffer of 10mM-Tris–HCl (pH8.0). To a sample was added 0.1vol. of aq. 1% (w/v) sodium dodecyl sulphate and the mixture was dialysed against 1000vol. of the Tris–glycine cathode buffer containing 0.1% (w/v) sodium dodecyl sulphate for 1–48h. The ionic strength of the buffer was 0.10mol/l and the equilibrium critical micelle concentration of the sodium dodecyl sulphate was 1.45mM. The amount bound was measured colorimetrically by the method of Reynolds & Tanford (1970*b*). The binding experiments were also done with sodium dodecyl [^{35}S]-sulphate and the amount bound was determined from the difference between the radioactivity of an equal volume of the protein solution and dialysis buffer. This method is valid only if equilibrium between monomer and micelle forms of the dodecyl sulphate is rapidly achieved during dialysis. Control experiments with both the unlabelled and ^{35}S-labelled material indicated that the equilibrium was reached within 1h in this experimental system. The two methods gave very similar results, although the latter provided superior sensitivity.

Removal of bound sodium dodecyl sulphate. Polypeptides that were equilibrated with sodium dodecyl sulphate were soluble at pH4.5. However, when solutions were dialysed against two changes of 100vol. of 10mM-Tris–HCl (pH8.0), the polypeptides could then be precipitated at pH4.5 by the sodium acetate buffer. By use of the sodium dodecyl [^{35}S]sulphate, such polypeptides had about 0.03–0.1g of sodium dodecyl sulphate bound/g. To effect virtually complete removal, the dialysed solutions were made to 8M-urea and 0.1M-NH_4HCO_3 by the addition of solid reagents, incubated at 23°C for 4h and chromatographed on a small column of Dowex AG1W-X2 as described by Weber & Kuter (1971). The amount still bound was less than 0.003g/g or less than 1mol/mol of polypeptides.

Estimation of α-helix content. This was measured on a Cary model 60 spectropolarimeter with a model 6001 circular-dichroism accessory. By using optical rotatory dispersion, the α-helix content was estimated from the mean residue rotation observed at 233nm from values of −12000 degrees for 100% α-helix and −1800 degrees for 0% α-helix, with presumption of linear interpolation (Simons *et al.*, 1961). In circular-dichroism studies the α-helix content was estimated from the mean molar ellipticity observed at 208nm from values of −33000 degree·cm^2/decimol for 100% α-helix and 4000 degree·cm^2/decimol for random coil (Greenfield & Fasman, 1969). The thiol forms and *S*-carboxymethyl derivatives of the polypeptides, which were demonstrated to have less than 1mol of sodium dodecyl sulphate bound/mol, were used. This aspect is important, since it has been shown that complexes of 0.4g or more of sodium dodecyl sulphate/g of protein have highly ordered α-helical structures (Reynolds & Tanford, 1970*a*). Samples for measurement were equilibrated at a concentration of 0.05–0.2mg/ml in a buffer of 10mM-Tris–HCl (pH7.6).

Amino acid analysis. After removal of the sodium dodecyl sulphate, where applicable, the polypeptides were thoroughly desalted by dialysis. Samples (1–2mg) 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. Most samples obtained by preparative gel electrophoresis contained traces of acrylamide (less than 2% by wt.) which did not interfere with the hydrolyses or analyses. Duplicate analyses on the same or different batches of polypeptides gave variations of about 4–5%. Tryptophan was measured colorimetrically by two procedures (Opieńska-Blauth *et al.*, 1963; Gaitonde & Dovey, 1970), which yielded values that were within about 4% of each other. On the basis of these tryptophan values, the tyrosine contents of the intact polypeptides were also determined spectrophotometrically (Edelhoch, 1967). As *S*-carboxymethylcysteine is partially destroyed on acid hydrolysis, more reliable estimates were obtained by reaction of the fully reduced polypeptides with iodo[2-^{3}H]acetic acid. The specific radioactivity of the iodo[2-^{3}H]acetic acid was determined

as described by Steinert (1975), by titration against native yeast alcohol dehydrogenase, which has 8.0 mol of thiol groups per 151 000 g (Krakow & Goolsby, 1971), and fully reduced chicken lysozyme, which has 8.0 mol of thiol groups per 14 300 g (Dayhoff, 1972). A freshly prepared extract of the polypeptides was alkylated with the iodo[2-^{3}H]acetic acid in the routine way described for the unlabelled iodoacetic acid. Each of the radioactively labelled polypeptides was isolated as described in the present paper for subsequent calculation of the *S*-carboxymethylcysteine content.

Isolation of N-terminal-blocked amino acids. The procedures used were modified from those described (Steinert & Rogers, 1973*b*). The *S*-carboxymethyl derivatives of the polypeptides were dissolved in 0.2 M-*N*-ethylmorpholine acetate buffer (5 mg/ml) and were digested with: day 1, 1% (w/w) trypsin and 10% (w/w) Pronase; day 2 and day 3, 10% (w/w) Pronase; day 4 and day 5, 1% (w/w) carboxypeptidase A and 0.1% (w/w) mercuripapain. Subsequently, the digestions were deproteinized with aq. 1% (w/v) picric acid and chromatographed on a column of Dowex AG50W-X8 to obtain the *N*-terminal-blocked acidic species (Steinert & Rogers, 1973*b*). This was then chromatographed at 30 ml/h and 23°C on a column (25 cm × 0.8 cm) of Dowex AG1W-X8 (formate form) by using a gradient of increasing formic acid concentration, which consisted of five chambers containing 70 ml of 0.1, 0.25, 1.0, 2.0 and 5.0 M-formic acid. Samples of the 2.0 ml fractions were assayed with ninhydrin after alkaline hydrolysis (Hirs *et al.*, 1956). The nature of the amino acids of the *N*-terminal-blocked species was determined after acid hydrolysis followed by amino acid analysis or modification to the dansyl derivatives (Woods & Wang, 1966). The nature of the acyl group was determined by g.l.c. of the trimethylsilyl esters of the *N*-terminal-blocked species (Gehrke *et al.*, 1969) on a Barber–Coleman instrument with a column (120 cm × 0.2 cm) of Chromasorb W (100–200 mesh) coated with 1% (w/w) OV-275. The initial temperature of the column was 100°C, which was held for 8 min after sample application and then increased at a rate of 7.5°C/min.

Results

Nomenclature of the α-keratin polypeptides

The thiol forms and *S*-carboxymethyl derivatives of the polypeptides are resolved on polyacrylamide gels containing urea into two broad bands of low electrophoretic mobility and two bands of greater electrophoretic mobility (Plate 1*a*, gel L). When the polypeptides are chromatographed on a column of Sephadex G-200, the former bands are excluded (eluted at V_0), whereas the latter are retarded and well separated (Steinert, 1975). The bands of high molecular weight and low electrophoretic mobility are termed the dimer bands and the other bands are termed the monomer bands (Plate 1*a*, gel L). (The justification of adopting this terminology is set out below.)

Both the thiol and *S*-carboxymethyl forms of the polypeptides are resolved into the same six chains on polyacrylamide gels containing sodium dodecyl sulphate (Steinert, 1975). In the present paper, these are enumerated 1–6 in order of increasing electrophoretic mobility (Plate 1*b*, gel L).

Fractionation of the polypeptides

Chromatography on DEAE-cellulose. An elution profile of the *S*-carboxymethyl derivatives of the polypeptides on DEAE-cellulose with a urea buffer (Fig. 1) and urea gels of samples of the peaks obtained (Plate 1*a*) show that a degree of fractionation is obtained. Most of the dimer material appeared in peaks A and B, but smaller amounts were also present in peaks C–E (Plate 1*a*). The dimer and monomer bands of peaks C–E were separated by chromatography on Sephadex G-200 (Steinert, 1975). On gels containing sodium dodecyl sulphate, the dimer band of peak A was resolved into polypeptides 1 and 3, and that of peak B was resolved into polypeptides 2 (trace), 3, 5 and 6 (Plate 1*b*). The dimer bands of peaks C–E were resolved into polypeptides 2, 3, 4, 5 and 6 (not shown). Therefore the dimer bands consist of non-covalently bound aggregates of the polypeptide chains, since they can be dispersed by the sodium dodecyl sulphate. The monomer bands of peaks D

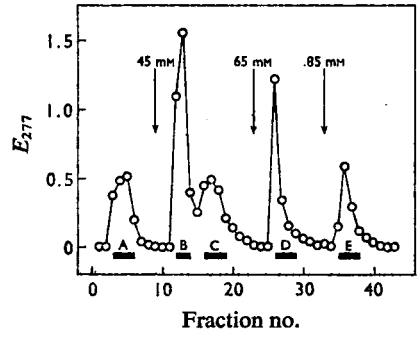
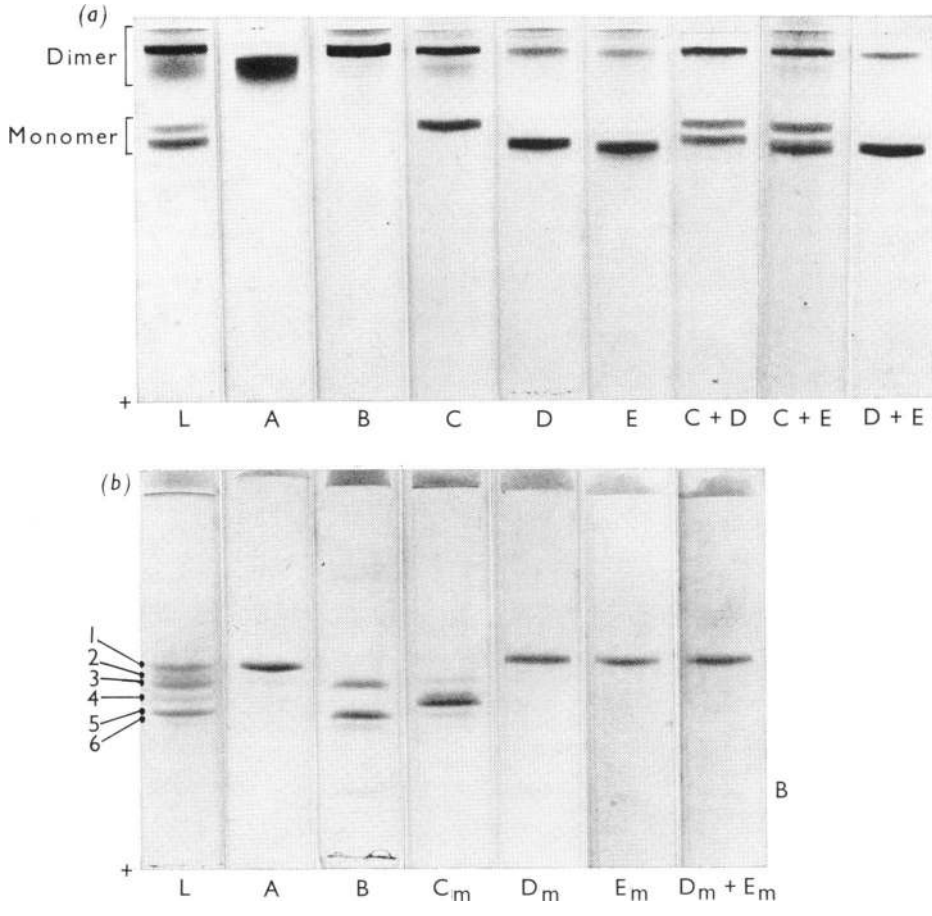


Fig. 1. *Chromatography of the S-carboxymethyl derivatives of the polypeptides on DEAE-cellulose*

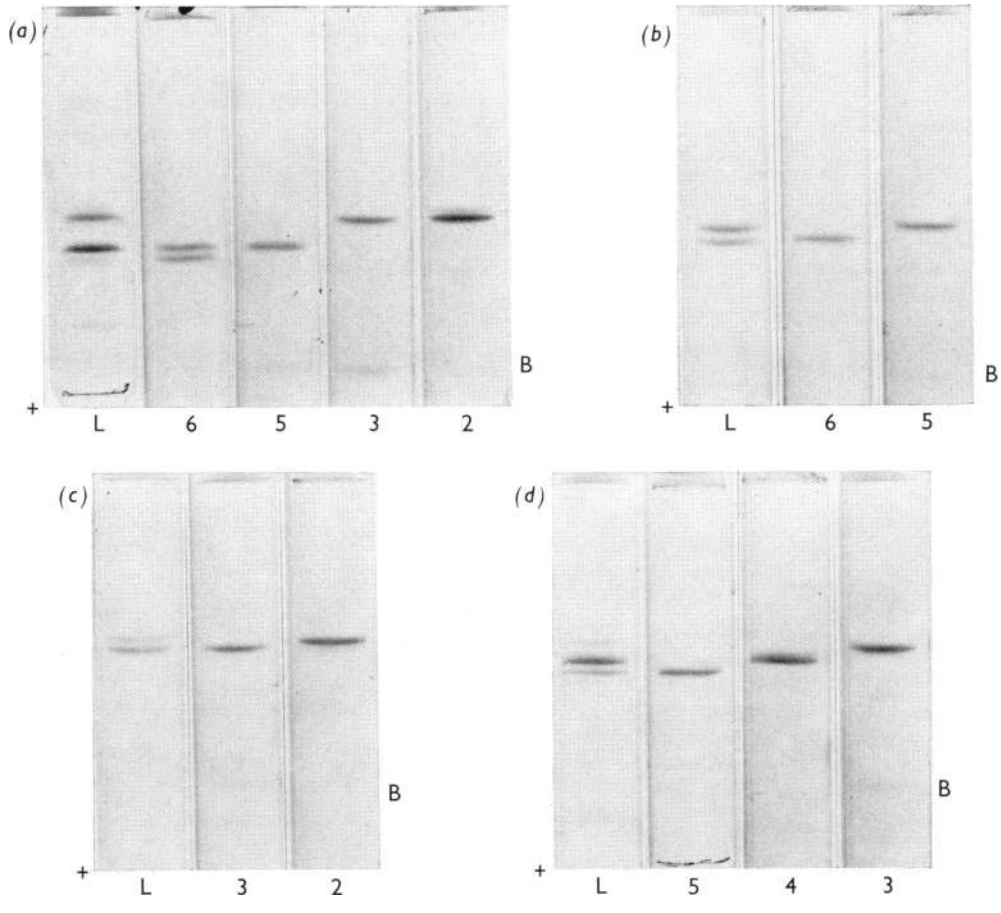
A column (15 cm × 2.0 cm) was equilibrated with the urea buffer containing 20 mM-KCl as described under 'Methods'. The flow rate was 50 ml/h and the fraction size was 20 ml. About 0.5 g of polypeptide mixture was applied. Buffers of the same composition but with the increased KCl concentrations of 45, 65 and 85 mM were applied at tubes 9, 23 and 33 respectively. The bars A–E refer to the tubes that were pooled for further study.



EXPLANATION OF PLATE 1

Polyacrylamide-gels of fractions obtained from the DEAE-cellulose column of Fig. 1

(*a*) Gels with urea. Samples of the pooled fractions of Fig. 1 containing 50–75 μg of protein were applied directly to the gels and were electrophoresed for 4 h. Other details are given under 'Methods'. Gel L, load of polypeptides applied to the DEAE-cellulose column. The bands are operationally divided into the monomer and dimer bands as described in the text. Gels A–E are from the pooled fractions of Fig. 1. Co-electrophoresis of a sample of peak C with samples of peaks D and E show that the polypeptides of the monomer bands of peaks D and E have slightly different mobilities, although they co-migrate as a broad band when mixed together. (*b*) Gels with sodium dodecyl sulphate. The proteins fractionated in peaks C, D and E of the DEAE-cellulose column were chromatographed on a column of Sephadex G-200 (Steinert, 1975) to separate the monomer and dimer bands. The resulting monomer bands were respectively denoted C_m, D_m and E_m. Samples of the pooled fractions of peaks A and B and of the C_m, D_m and E_m fractions were dialysed against the Tris–glycine cathode buffer containing 0.1% (w/v) sodium dodecyl sulphate for 16 h at 23°C and heated at 90–95°C for 2 min to ensure complete binding. Samples (20–30 μg) were loaded and electrophoresed for 2.5 h. Other details are given under 'Methods'. The numbers 1–6 on the gel L refer to the polypeptide chain numbers described in the text. The band B marks the position of the dodecyl sulphate–glycine micelle complex. The polypeptides of samples D_m and E_m co-electrophoresed, which indicates that they have the same apparent molecular weight.



EXPLANATION OF PLATE 2

Polyacrylamide-gels of fractions obtained from the preparative electrophoresis experiments of Fig. 2

In all cases, samples of the pooled fractions were equilibrated in the Tris–glycine cathode buffer containing 0.1% (w/v) sodium dodecyl sulphate and electrophoresed as described in Plate 1(*b*). Gel L, load of polypeptide mixture applied to the preparative gel. The numbers refer to the polypeptide chains of the same number (see Plate 1). (*a*) is from Fig. 2(*a*); (*b*) is from Fig. 2(*b*); (*c*) is from Fig. 2(*c*); (*d*) is from Fig. 2(*d*).

and E consisted of polypeptide 1 and had the same mobility on gels containing sodium dodecyl sulphate (Plate 1*b*, gel D_m+E_m), indicating that they have the same apparent molecular weight. When examined on the urea gels, the monomer bands of these two peaks possessed slightly different electrophoretic mobilities relative to the monomer band of peak C, although they co-migrated as a broad band (Plate 1*a*, gels C+D, C+E and D+E). This suggests that the polypeptides of the monomer bands of peaks D and E have different charges. The monomer band of peak D will hereafter be called polypeptide 1a and the monomer band of peak E will be called polypeptide 1b. The monomer band of peak C was resolved into polypeptides 3, 4 and 5 on gels containing sodium dodecyl sulphate (Plate 1*b*). When seen in the more concentrated form, polypeptide 4 as a routine appeared as a pair of closely migrating species (Plate 1*b*, cf. gel C_m and gel L). Attempts to separate the mixture of polypeptides of the peak C monomer band by further chromatography on DEAE-cellulose were unsuccessful, as the polypeptides became aggregated during preparation for re-chromatography.

When the thiol forms of the polypeptides were chromatographed on DEAE-cellulose, the initial buffer contained 0.025M-2-mercaptoethanol and no KCl. Buffers containing KCl concentrations of 20, 40 and 60mM were applied to elute the peaks B–E. A similar degree of fractionation was obtained to that shown in Fig. 1, but the resolution of the peaks B and C was inferior.

Preparative polyacrylamide-gel electrophoresis. Except for the minor amounts present in the monomer component of DEAE-cellulose peak C, polypeptides 2, 3, 4, 5 and 6 always existed as aggregates even in the presence of urea. As electrophoresis on gels containing sodium dodecyl sulphate provided the only means of resolution of these components, separation of them by preparative gel electrophoresis was attempted and proved successful. The analytical procedure was scaled up to the preparative level with only minor variations (see under 'Methods'). In Fig. 2(*a*), the fractionation of the DEAE-cellulose peak B polypeptide mixture is shown. Analytical gels of the peaks obtained show that the polypeptides 3 and 5 were homogeneous (Plate 2*a*). Polypeptides 6 and 2 were concentrated and were subsequently purified by re-electrophoresis as shown in Fig. 2(*b*) and Plate 2(*b*), and Fig. 2(*c*) and Plate 2(*c*), respectively. Similarly, preparative electrophoresis provided the most satisfactory method for the purification of polypeptide 4 from the DEAE-cellulose peak C monomer fraction (Fig. 2*d* and Plate 2*d*).

Properties of the polypeptide chains

Molecular weights. Skerrow (1974) found that the polypeptides of denatured prekeratin had molecular weights of 60000 and 72000, but Baden *et al.* (1973*a*) reported values of 47000, 58000 and 98000. We have used three different techniques and reported that the polypeptides of denatured prekeratin or those extracted with urea have molecular weights within the range of 47000–58000 (Steinert, 1975). However, in other reports, Lee & Baden (1974*a*) have cited values of 60000–70000 for the polypeptides of prekeratin or those extracted with urea buffers, but later changed these to 45000–70000 (Lee & Baden, 1974*b*). Because of these discrepancies, we have considered it necessary to re-examine the molecular-weight estimations. One possible source of the differences might be inadequate binding of sodium dodecyl sulphate. To check this, binding experiments with sodium dodecyl sulphate were performed in the Tris–glycine cathode buffer used for molecular-weight estimations on polyacrylamide gels. The thiol forms and *S*-carboxymethyl derivatives of all six polypeptide chains and the standard proteins creatine kinase, ovalbumin, catalase and urease bound 1.35 ± 0.05g of sodium dodecyl sulphate/g within 2–6h at 23°C (Fig. 3). Samples that were heated at 90–95°C for 2min before dialysis achieved this degree of binding within 1h. The binding of about 1.4g of sodium dodecyl sulphate/g of protein has been found with many other proteins and is considered an optimum value for molecular-weight estimations (Reynolds & Tanford, 1970*a*,*b*; Weber *et al.*, 1972). Bovine serum albumin did not bind this amount of sodium dodecyl sulphate even on heating, which means that this protein may not be a reliable standard for molecular-weight estimations in this gel system. When samples of the polypeptides or standard proteins were freeze-dried and then examined, the sodium dodecyl sulphate bound with difficulty, and over a period of 2 days, still did not reach the value of about 1.4g/g after heating. Fig. 3 shows such an experiment with polypeptide 5. It is noteworthy that Baden *et al.* (1973*a*,*b*) as a routine introduced a freeze-drying step during the preparation of the polypeptides.

The gel system with discontinuous buffers and sodium dodecyl sulphate was used in the present work for molecular-weight estimations. Semi-logarithmic plots of the relative mobilities of the thiol forms of the standard proteins and the α-keratin polypeptides against % T yielded a series of straight lines which shared a common *y*-intercept except for bovine serum albumin (Fig. 4*a*). For the purposes of clarity, only polypeptides 1a, 3 and 5 are depicted, but similar lines were obtained for each of the other polypeptides. As the *y*-intercept value, the electrophoretic free mobility, is related to net charge (Rodbard & Chrambach, 1971), the data suggest that each protein (except bovine serum albumin) and polypeptide had bound an amount of sodium dodecyl sulphate that was directly related to its molecular size. This conclusion was demonstrated independently

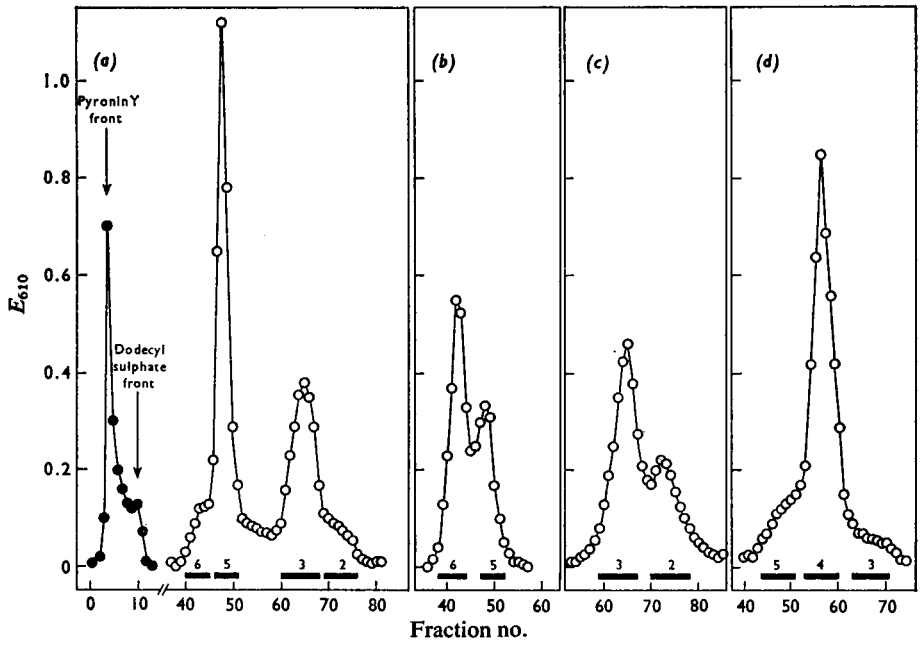


Fig. 2. *Preparative polyacrylamide-gel electrophoresis*

(*a*) Fractionation of the DEAE-cellulose peak B polypeptide mixture. A 10mg sample was dialysed into the Tris–glycine cathode buffer containing 0.1% (w/v) sodium dodecyl sulphate for 16h at 23°C, heated at 90–95°C for 2min and electrophoresed on the preparative gel as described under 'Methods'. ●, Absorbance due to pyronin Y, which marks the π–λ boundary. In this system, the dodecyl sulphate–glycine micelle complex migrates as a stacked boundary just behind the π–λ boundary and some pyronin Y diffuses between the two. (This second boundary is marked by the band B on the analytical gels.) ○, Absorbance due to protein (Bramhall *et al.*, 1969). The bars numbered 2–6 refer to the fractions that were pooled which contained the polypeptide chains of the same numbers. Samples of these were removed for analytical gels (Plate 2*a*). (*b*) Re-electrophoresis of the concentrated polypeptide 6. The fractions containing polypeptide 6 from several experiments identical with that above were dialysed to remove most of the bound sodium dodecyl sulphate and concentrated by acid precipitation as described under 'Methods'. After equilibration in the cathode buffer, 2–4mg was applied to the preparative column. Analytical gels (Plate 2*b*) show that polypeptide 6 was recovered in homogeneous form. (*c*) Re-electrophoresis of the concentrated polypeptide 2. This was done as for polypeptide 6 above. As judged by quantitative densitometry on the gels (Plate 2*c*), polypeptide 2 was recovered 95–97% pure. (*d*) Purification of polypeptide 4. About 3–5mg of the DEAE-cellulose peak C monomer polypeptide mixture was electrophoresed exactly as in (*a*) above, and polypeptide 4 was obtained in pure form (Plate 2*d*).

in the binding experiments described above. Moreover, it is apparent from Fig. 4(*a*) that none of the chains has demonstrated unusual or anomalous behaviour or is an electrophoretic artifact of the gel system (Rodbard & Chrambach, 1971, 1974; Banker & Cotman, 1972). The retardation coefficient (K_R) derived from the line of each protein and polypeptide was thus directly proportional to molecular weight (Fig. 4*b*). A least-squares linear regression of molecular weight on K_R based on the data for the standard proteins with 95% confidence limits is:

$$\text{Molecular weight} = [-(3.60 \pm 0.18) \times 10^4] + [(8.68 \pm 0.44) \times 10^5] \times K_R.$$

This equation was used for the estimation of the molecular weights of the α-keratin polypeptides, and for the experiment shown, the values for the polypeptides were: 1a and 1b, 60000; 2, 58000; 3, 56000; 4, 54000; 5, 49000; 6, 47000. Both the thiol forms and *S*-carboxymethyl derivatives of the standard proteins and polypeptides were used with this gel system and the molecular-weight estimates were the same (with a variation of ±5% between different experments). This is as expected, since the two forms of each species co-electrophoresed on the gels containing sodium dodecyl sulphate.

The same relationship between relative mobility, retardation coefficient and molecular weight exists for the urea gels (Rodbard & Chrambach, 1971, 1974). Thus semilogarithmic plots of the relative

mobilities of the *S*-carboxymethyl derivatives of the standard proteins and α-keratin polypeptides also yielded a series of straight lines (not shown). Since they have different charges at the pH of the gel system, the lines for each species do not share a common intercept, but a linear relationship existed between the retardation coefficient and molecular weight of the standard proteins (Fig. 5). This finding implies that each standard protein and polypeptide had been converted into a random-coil configuration by the urea, as is necessary for the use of gels for molecular-weight estimations with urea (Tanford, 1968; Poole *et al.*, 1974). A least-squares linear regression of molecular weight on K_R based on the standard proteins with 95% confidence limits is:

$$\text{Molecular weight} = [-(3.28 \pm 0.16) \times 10^4] + [(1.06 \pm 0.04) \times 10^6] \times K_R,$$

from which the molecular weights were estimated to be: peak C monomer band, 52000; peak D monomer band (polypeptide 1a), 57000; peak E monomer band (polypeptide 1b), 59000. The bands of peaks A and B had molecular weights within the ranges of 111000–118000 and 96000–103000 respectively, which therefore provides the evidence that these bands constitute dimers of the main polypeptide chains. Similar values (with a variation of ±4%) were obtained in other experiments.

Amino acid compositions. Table 1 shows that the seven polypeptides have similar contents of amino acids. Comparisons of the contents of such amino acids as *S*-carboxymethylcysteine, serine, glutamic

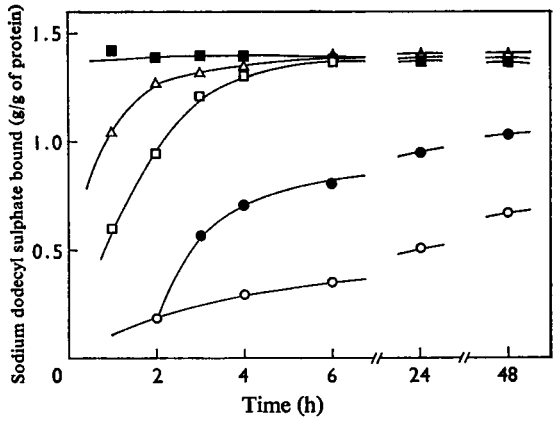


Fig. 3. *Binding of sodium dodecyl sulphate*

The standard protein and polypeptide samples were dialysed into the Tris–glycine cathode buffer containing unlabelled sodium dodecyl sulphate as described under 'Methods'. The amount that bound to the samples was determined by the colorimetric procedure (Reynolds & Tanford, 1970*b*). △, *S*-Carboxymethyl derivative of polypeptide 1a; □, thiol form of polypeptide 4; ■, same, but the solution was heated at 90–95°C for 2min before dialysis. Other polypeptides and standard proteins gave very similar results. ○, *S*-carboxymethyl derivative of polypeptide 5 that had been freeze-dried; ●, same, but a sample of the solution at 2h was heated for 2min at 90–95°C. Other freeze-dried polypeptides and standard proteins also bound sodium dodecyl sulphate with difficulty. The same results were obtained when sodium dodecyl [^{35}S]-sulphate was used.

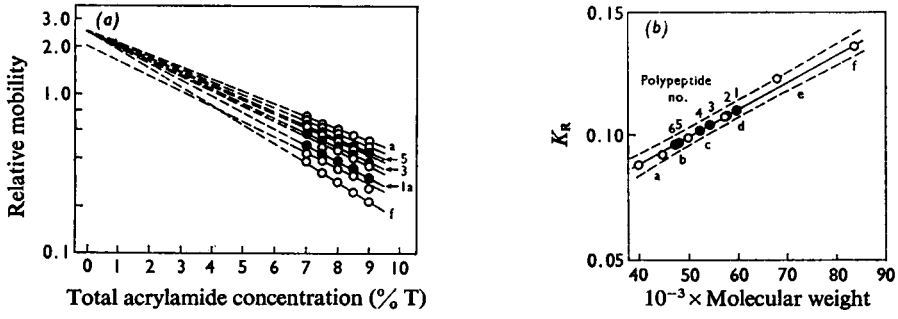


Fig. 4. *Molecular-weight estimations on polyacrylamide gels containing sodium dodecyl sulphate*

(*a*) Logarithmic plot of the relative mobility of each standard protein and three α-keratin polypeptides against % T. Samples of the thiol forms of the standard proteins and unfractionated polypeptides or mixtures of polypeptides fractionated by the DEAE-cellulose chromatography experiment (Fig. 1) were treated for electrophoresis as described under 'Methods'. ○, Standard proteins; ●, α-keratin polypeptides as marked. The standard proteins used were creatine kinase (a, upper line, mol.wt. 40000), ovalbumin (b, 45000), glutamate dehydrogenase (c, 50000), catalase (d, 57500), bovine serum albumin (e, 68000) and urease (f, lower line, 83000). ----, Extrapolation to 0% T, where all lines except that of bovine serum albumin share a common *y*-intercept. (*b*) Plots of the slope of these lines (the K_R values) against molecular weight. ○, K_R values for the standard proteins a–f as marked; ●, K_R values of the α-keratin polypeptides as marked; ----, 95% confidence limits for a single observation about the line for the standard proteins. The *S*-carboxymethyl derivatives of the standard proteins and polypeptides gave very similar K_R values in different experiments.

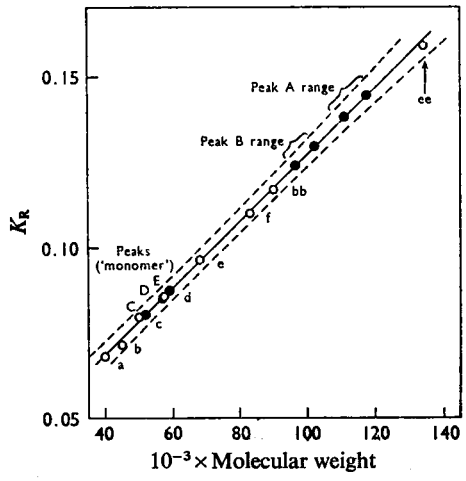


Fig. 5. *Molecular-weight estimations on polyacrylamide gels containing urea*

Samples of the *S*-carboxymethyl derivatives of the standard proteins and of the five fractions from the DEAE-cellulose experiment of Fig. 1 were equilibrated in the Tris–glycine cathode buffer containing 10M-urea (see under 'Methods'). About 20–50μg of protein was loaded and electrophoresis was for 5h. Other details are given under 'Methods'. The same standard proteins a–f used in Fig. 4 as well as the dimers of ovalbumin (bb) and bovine serum albumin (ee) were used. K_R values were calculated from plots of the relative mobilities against %T as described in Fig. 4(*a*). K_R values are plotted against protein molecular weight. ○, K_R values of standard proteins as marked; ●, K_R values of the α-keratin polypeptide fractions as marked; ----, 95% confidence limits for the standard proteins.

acid, glycine and leucine suggest that the polypeptides may be divided into three groups. Group one contains polypeptides 1a and 1b; group two, polypeptides 2, 3 and 4; and group three, polypeptides 5 and 6. Within these groups, however, the polypeptides display marked differences in extinction coefficients (Table 1). Since each polypeptide contains about 2 residues of tryptophan per molecule, the major variation exists in the tyrosine contents. Thus polypeptide 1b contains less tyrosine than polypeptide 1a, although both have the same apparent molecular weight (Table 1). Of the polypeptides of group two, the smallest, polypeptide 4, contains the most tyrosine residues/molecule; the larger and major polypeptide 3 contains fewer tyrosine residues/molecule; and the largest, polypeptide 2, contains the least tyrosine residues/molecule (Table 1). Similarly, in the third group, polypeptide 5 contains fewer tyrosine residues/molecule than does the smallest and minor polypeptide 6. Therefore it is apparent that the polypeptides are distinctly different. The possibilities that some of the polypeptide chains are degradation products or precursors of other polypeptides may be excluded by further consideration of the contents of glutamic acid and tyrosine. The tyrosine contents of polypeptides 2, 3, 4, 5 and 6 (18–24 residues/molecule) significantly exceed those of polypeptides 1a and 1b (11–14 residues/molecule) (Table 1). Thus it is unlikely that polypeptides 2–6 are degraded from polypeptides 1a and 1b, and conversely, it is unlikely that polypeptides 1a and 1b are precursors of polypeptides 2–6. The glutamic acid contents of polypeptides 5 and 6 (each about 64 residues/molecule) significantly exceed those of polypeptides 2, 3 and 4 (each about 54 residues/molecule) (Table 1). Accordingly, it is unlikely that polypeptides 5 and 6 are related to polypeptides 2, 3 and 4.

Included in Table 1 is the relative molar amount of each polypeptide chain quantified by densitometry of a gel of the unfractionated polypeptides (Steinert, 1975). The amounts of polypeptides 1a and 1b were estimated from the amount of each obtained in the DEAE-cellulose experiment (Fig. 1). Corrections were made from the densitometry data for the molecular weights of the different polypeptides (also presented in Table 1) and it is seen that each of the groups contains approximately equal numbers of polypeptide molecules.

α-Helix contents. These were determined from both optical-rotatory-dispersion and circular-dichroism measurements (Table 2). On the basis of the values obtained, it is possible to classify the polypeptides into the same three groups described above; polypeptides 1a and 1b have about 25% α-helix content; polypeptides 2, 3 and 4 have about 48% α-helix content; and polypeptides 5 and 6 have about 56% α-helix content. The *S*-carboxymethyl derivatives and the thiol forms of the polypeptide chains yielded very similar results. The weighted average value for the fractionated mixture, calculated from the relative amount of each polypeptide chain (Table 1), is about 40%. Interestingly, the unfractionated mixture of polypeptides that had not been exposed to the same experimental manipulations or sodium dodecyl sulphate also had an average α-helix content of 40–45% (Steinert, 1975). This implies that the polypeptides have resumed their native structural configurations after removal of the denaturing reagents. In contrast, polypeptides that had been freeze-dried displayed significantly decreased α-helix contents as judged by both techniques (Table 2). It should be noted that Baden *et al.* (1973*a*,*b*) freeze-dried their polypeptides and reported that the individual chains did not contain α-helix.

N-Terminal residues. Reactions of samples of all of the purified *S*-carboxymethyl derivatives of the polypeptides with dansyl chloride did not yield α-Dns derivatives. Therefore, a search was made for *N*-

Table 1. *Amino acid compositions (residues/100 residues) and related properties*

All data are the averages of analyses on at least three different preparations of polypeptides. The S.D. values given for each amino acid were determined by multiple analyses of samples. The numbers in parentheses show the number of residues per molecule, based on the residue molecular weights of the amino acids. The *S*-carboxymethylcysteine content was estimated by the radiochemical procedure and the second tyrosine value was estimated spectrophotometrically after estimation of the tryptophan content (see under 'Methods'). The extinction coefficient was calculated from the absorption of a solution of the polypeptides of known concentration.

		Polypeptide chain number						
Amino acid or property	S.D. (%)	1a	1b	2	3	4	5	6
Lysine	2.6	5.10	5.05	4.75	5.20	4.95	4.60	4.35
Histidine	3.7	1.25	1.35	1.00	0.95	1.15	1.15	1.15
Arginine	3.5	5.40	5.70	6.15	6.80	6.95	6.45	6.40
S-carboxymethylcysteine	4.3	1.55 (8.2)	1.49 (8.0)	1.21 (6.1)	1.19 (6.0)	1.28 (6.1)	0.91 (4.0)	0.95 (4.0)
Aspartic acid	2.1	8.80	9.00	8.10	8.35	8.35	9.65	9.50
Threonine	4.9	4.40	4.55	4.50	4.20	3.90	3.85	3.70
Serine	5.8	13.90	13.75	10.60	10.20	9.75	10.50	10.15
Glutamic acid	2.7	16.10 (86.2)	16.00 (86.1)	10.55 (53.6)	10.95 (53.9)	11.25 (53.8)	14.75 (64.4)	15.10 (64.0)
Proline	6.0	0.65	0.60	1.15	1.35	1.25	1.25	1.35
Glycine	2.6	16.75	15.20	16.75	16.85	17.15	12.85	12.45
Alanine	3.1	3.75	4.40	6.60	6.65	6.10	6.55	6.35
Valine	3.8	4.65	4.85	6.05	5.35	5.40	5.65	6.40
Methionine	4.7	2.00	2.15	1.10	1.30	1.45	2.00	1.90
Isoleucine	5.0	3.90	3.70	4.20	4.30	4.35	3.75	3.70
Leucine	5.2	7.70	7.85	8.35	8.65	8.60	10.15	9.70
Phenylalanine	2.5	2.20	2.85	3.40	3.50	3.65	2.40	2.70
Tyrosine	3.8	2.60 (13.6)	2.10 (11.3)	3.55 (18.0)	4.25 (21.3)	5.05 (24.0)	3.65 (15.9)	4.30 (18.2)
Tryptophan	4.1	0.38 (2.0)	0.37 (2.0)	0.41 (2.1)	0.40 (2.0)	0.40 (1.9)	0.44 (1.9)	0.47 (2.0)
Tyrosine	4.6	2.68 (14.4)	1.97 (10.6)	3.57 (18.1)	4.35 (21.9)	5.20 (24.7)	3.80 (16.6)	4.49 (19.0)
$E^{1\%}_{277}$		5.2	4.0	6.0	6.5	7.2	6.1	6.9
Relative molar amount		0.22	0.11	0.02	0.21	0.11	0.31	0.02
Average molecular weight		58000	58000	56000	54000	52000	48000	47000

Table 2. *α-Helix contents of the polypeptide chains*

The data are averages±S.D. of estimates on at least two batches of both the thiol forms and *S*-carboxymethyl derivatives of the polypeptide chains. The polypeptides were freed of sodium dodecyl sulphate as described under 'Methods'.

	α-Helix (%) estimated by:	
Polypeptide	Optical rotatory dispersion $[m']_{233}$	Circular dichroism $[\theta]'_{208}$
1a	24±2	24±2
1a freeze-dried	10±6	15±7
1b	26±3	25±2
2	45±3	48±2
3	47±4	49±3
3 freeze-dried	17±5	15±6
4	47±2	50±3
5	55±3	57±2
5 freeze-dried	13±6	12±8
6	55±3	58±3

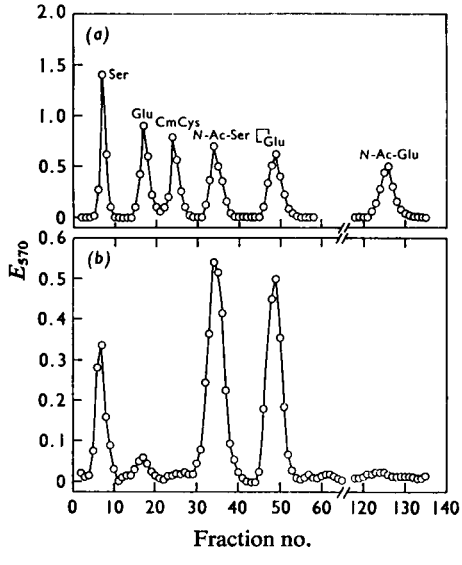


Fig. 6. *Isolation of N-acetylserine*

(*a*) The Dowex AG1W-X8 column used for the fractionation of the *N*-terminal-blocked species (see under 'Methods') was calibrated with about 3.0μmol each of serine (Ser), glutamic acid (Glu), *S*-carboxymethylcysteine (CmCys), *N*-acetylserine (*N*-Ac-Ser), pyrrolidine carboxylic acid (Glu) and *N*-acetylglutamic acid (*N*-Ac-Glu). A 0.5ml sample of the fractions was used for the ninhydrin assay. (*b*) Chromatography of the *N*-terminal-blocked species obtained from 275mg of the unfractionated polypeptides. Details of the digestion and subsequent chromatographic procedures are given under 'Methods'. About 4.0μmol of *N*-acetylserine was recovered.

terminal-blocked amino acids (see under 'Methods'). The chromatographic system used for the separation of these compounds was initially calibrated with amino acids and suitable derivatives (Fig. 6*a*). The enzymic digestion and chromatographic procedures were also checked with *S*-carboxymethyl lysozyme and *S*-carboxymethyl bovine serum albumin, both of which do not contain *N*-terminal-blocked amino acids. Small amounts of pyrrolidone carboxylic acid (less than 0.3mol/mol of protein) were found in each case. This most probably arose by cyclization of free glutamine liberated during enzymic digestion of the proteins (Press *et al.*, 1966). The results of an experiment with 275mg (about 5.0μmol, assuming an average molecular weight of 54000) of the unfractionated polypeptides is shown in Fig. 6(*b*). The peak eluted at tube 7 contained material possessing free α-amino groups and that eluted at tube 48 contained pyrrolidone carboxylic acid. The peak eluted at tube 35 co-chromatographed with *N*-acetylserine and 91% of the amino acid material was serine, with traces of glutamic acid and glycine. If it can be assumed that the serine was due only to *N*-blocked serine and not due in part to other short peptides containing serine, then the total yield of *N*-blocked serine was 0.80mol/mol. Samples of this were converted into the trimethylsilyl ester and examined by g.l.c. (see under 'Methods'). The derivative and that of authentic *N*-acetylserine were co-eluted at about 3.0min, but those of *N*-formylserine and *N,O*-diacetylserine were eluted at about 1.0min and 4.5min respectively. This identifies the *N*-terminal species as *N*-acetylserine. Similar experiments were done with smaller amounts (5–20mg) of each of the purified polypeptides, and the yields of *N*-acetylserine obtained were: polypeptide 1a, 0.79mol/mol; 1b, 0.76mol/mol; 2, 0.90mol/mol; 3, 0.71mol/mol; 4, 0.80mol/mol; 5, 0.72mol/mol; 6, 0.71mol/mol. These high yields of *N*-acetylserine in each case suggest the existence of other types of *N*-terminal-blocked derivatives is unlikely. However, the pyrrolidone carboxylic acid present in all samples (Fig. 6*b*) cannot be rigorously excluded as a possible *N*-terminal residue.

Discussion

In Steinert (1975), methods for the extraction of the α-keratin polypeptide chains from the bovine hoof and snout epidermis were examined. Irrespective of the method used for extraction, it was found that the chains formed two major and one minor band when electrophoresed in a gel system containing a continuous buffer with sodium dodecyl sulphate. However, when examined in a gel system containing discontinuous buffers with sodium decyl or dodecyl sulphate, six bands of polypeptides were obtained.

This increased number was thought not to be due to electrophoretic artifacts or random degradation, but to be due to improved resolution caused by the isotachophoretic effect of the discontinuous buffers. In the present work, each of the six polypeptide chains has been isolated by a combination of ion-exchange chromatography and preparative polyacrylamide-gel electrophoresis. Characterization of their properties indicates that there are significant differences between them. From the *S*-carboxymethylcysteine, glutamic acid and α-helix contents especially, it seems possible to classify the polypeptides into three groups of approximately equal numbers of polypeptides. These comprise polypeptide 1, polypeptides 2, 3 and 4, and polypeptides 5 and 6. Polypeptide 1 was shown to contain two components of slightly different charge, but significantly different extinction coefficients and tyrosine contents. In the second and third groups, the polypeptides could be distinguished primarily by differences in their extinction coefficients and tyrosine contents. Further evaluation of the glutamic acid and tyrosine contents of all of the polypeptides indicated that the polypeptides are neither precursors of nor degradation products of other polypeptide species. No evidence was found in these experiments that could support a recent claim (Lee & Baden, 1975) that precursors exist for the epidermal α-keratin polypeptide chains.

The major differences between the findings of the present work and those of other investigators are the numbers of the polypeptide chains and the estimates of their molecular weights. Steinert (1975) obtained the same gel patterns as those found by Skerrow (1974), but it has not been possible to reproducibly duplicate any of the gel patterns variously reported by Baden *et al.* (1973*a*,*b*) or Lee & Baden (1974*a*,*b*). We have now obtained molecular-weight estimates of 47000–58000 (both values ±5%) by using several techniques, including chromatography on Sephadex G-200, gel electrophoresis in the continuous-buffer system with sodium dodecyl sulphate and in the discontinuous-buffer system with sodium dodecyl sulphate, sodium decyl sulphate or urea. Independent confirmation of our molecular-weight values is possible by examination of the contents of *S*-carboxymethylcysteine and tryptophan (Table 1). All polypeptides contained integral amounts of these two amino acids (with a variation of ±5%) when the average molecular-weight values summarized in Table 1 were used. The variable and usually higher molecular weights obtained in the studies of other investigators could be explained by incomplete binding of sodium dodecyl sulphate as a result of freeze-drying or inadequate equilibration of the standard proteins and polypeptides with sodium dodecyl sulphate. As the theoretical and practical bases for the molecular-weight estimations have been more thoroughly ascertained in our studies, as is recommended (Rodbard & Chrambach, 1971, 1974; Banker & Cotman, 1972; Weber *et al.*, 1972; Poole *et al.*, 1974), we conclude that our values may be more reliable.

The findings of the present work confirm the conclusion (Steinert, 1975) that the α-keratin polypeptides consist of a heterogeneous group of similar polypeptides. A comparison of the epidermal α-keratin with the α-keratin extracted from wool and hair may be instructive. Although there are differences between the two keratins, especially in the cysteine contents, there are marked resemblances, such as high α-helix contents and the common *N*-terminal residue, *N*-acetylserine. Moreover, polypeptide heterogeneity has also been established (Fraser *et al.*, 1972; O'Donnell & Thompson, 1968; O'Donnell, 1969; Steinert & Rogers, 1973*a*). Sequence analysis of one component of wool α-keratin has shown a number of single amino acid replacements along the polypeptide chain, of which the tyrosine–phenylalanine replacement was most prominent (O'Donnell & Thompson, 1968; O'Donnell, 1969). The variations in tyrosine contents found in the present work may reflect the presence of these replacements in the epidermal α-keratin polypeptides as well. In fact, polypeptide-chain heterogeneity is a common feature of all proteins extracted from keratinized tissues. The β-fibrous proteins of feather (Kemp & Rogers, 1972) and the non-fibrous proteins of hair (Steinert & Rogers, 1973*a*) and wool (Fraser *et al.*, 1972) also consist of families of similar polypeptides that often display only minor differences in amino acid sequence.

The molecular structure of epidermal keratin has been the subject of study for many years. On the basis of X-ray diffraction data, it was suggested (Crick, 1952; Pauling & Corey, 1953) that the α-helix of epidermal α-keratin is arranged in a three-stranded coiled-coil configuration. This view has been verified by the isolation of triple-stranded α-helical segments from bovine epidermal prekeratin (Skerrow *et al.*, 1973). Presumably therefore, the epidermal tonofilament is a multichain structure that contains coherent lengths of coiled-coil triple α-helix interspersed by non-α-helical regions. Further delineation of the structure of the tonofilament will require detailed information on the chemistry, structure and amino acid sequence of the individual polypeptide-chain subunits. Some of this information is provided in the present work. A complementary approach to analysing the structure of the tonofilament exists in the recent finding that the thiol forms or intrachain-disulphide-bond forms of the polypeptide chains readily associate *in vitro* into tonofilament-like filaments (Steinert, 1975). Therefore studies on the mechanism of assembly of the tonofilaments as well as studies on the packing of the polypeptide chains within the tonofilaments should now be possible.

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