Adult amphibian epidermal proteins: biochemical characterization and developmental appearance

By O. RAYMOND REEVES

From the Medical Research Council,
Laboratory of Molecular Biology, Cambridge, U.K.

SUMMARY

The keratin-like proteins (KLPs) from the epidermis of adult frogs of the species Xenopus laevis have been isolated and biochemically characterized by means of polyacrylamide gel electrophoresis, amino acid analysis, tryptic peptide mapping, amino-terminal end-group analysis and isoelectric focusing. One particular protein fraction of rather unusual amino acid composition found only in epidermal tissue was isolated in quantity by preparative gel electrophoresis and monospecific antibodies prepared against it. Using this anti-KLP antibody preparation it was possible to show that at least one kind of keratin-like protein characteristic of the adult epidermis first appears within the larval epidermis during metamorphosis. This is the first reported biochemical characterization of a tissue-specific protein from adult amphibian skin.

INTRODUCTION

The biochemical identification and characterization of tissue-specific proteins is often a prerequisite for the qualitative and quantitative study of specific gene action during differentiation and morphogenesis. In the present paper the keratin-like proteins (KLPs) that are characteristic of the epidermis of adult skin from the amphibian Xenopus laevis have been isolated and biochemically characterized. This is the first reported analysis of the biochemical composition of these proteins from amphibian skin. Compared to the complex protein heterogeneity reported in the literature for various mammalian and avian keratins (Baden & Bonar, 1968; Frazer, MacRae & Rodgers, 1972; Kemp & Rogers, 1972; Beckingham Smith, 1973a, b), the KLPs of adult Xenopus skin present a surprisingly simple picture. Advantage has been taken of the limited heterogeneity within this class of amphibian proteins to isolate one type of adult skin keratin-like protein in quantity by preparative gel electrophoresis. Monospecific antibodies have been prepared against this tissue specific protein preparation. In the present report this antibody preparation has been used to quantify the time and rate of appearance of epidermis-specific proteins as amphibian skin cells differentiate normally in vivo during development. These

1 Author's address: Department of Zoology, University of British Columbia, Vancouver 8, Canada.
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results lay the foundation for further studies on in vitro differentiation of cultured epidermal cells reported on in the following paper (Reeves & Laskey, 1975).

MATERIALS AND METHODS

Separation of the epidermis from the dermis

The skin from animals at various stages of development from mid-metamorphosis (around stage 54, Nieuwkoop & Faber, 1967) to adulthood was manually dissected from different parts of the body and washed several times in ice cold modified Barth's saline solution (Gurdon & Laskey, 1970) lacking Ca\(^{2+}\) and Mg\(^{2+}\) (CMFB). The epidermis, which is only about 4–10 cells thick as seen in histological preparations of skin from late metamorphic and older animals, is recovered from the underlying dermis by overnight exposure to CMFB containing 3 mM-\(\text{Na}_2\) EDTA (\(\text{Na}_2\) EDTA = disodium salt of ethylene diaminetetraacetic acid) at 4 °C with continuous slow stirring. This is a modification of a procedure described by Dodson (1967) for use with chick embryo skin. After this treatment the epidermis could be easily separated from the dermis under a dissecting microscope. Histological sections showed that the epidermis and dermis were cleanly separated along the basement membrane using this method. The skin from early metamorphic tadpoles (about stage 50 of Nieuwkoop & Faber, 1967) and younger stages is only a few cell layers thick and was manually dissected from the larva after washing the tadpoles in CMFB at 4 °C. Alternatively, it was found that the skin from these early stages could be easily removed by quickly freezing the tadpoles in a minimal amount of water onto a clean microscope slide resting on a piece of dry ice and then quickly plucking the body of the tadpole off of the slide with a dissecting needle leaving the epidermis frozen to the slide. The epidermis from these younger stages was used directly for extractions without further washings.

Extraction of keratin-like proteins from the epidermis

Keratins, as a general class of proteins, have rather unusual properties. They are relatively insoluble in neutral buffered saline and also contain unusually large amount of cross-linked thiol groups usually in the form of cystine disulfide bridges (Crewther, Fraser, Lennox & Lindley, 1965; Mercer, 1961; Rudall, 1968; Fraser et al. 1972). An extraction procedure was used to selectively reduce and solubilize such proteins. After solubilization, these reduced proteins were alkylated to prevent any reoxidation of thiol groups which would lead to renewed aggregation and insolubility. This procedure is based on one described by Baden & Bonar (1968) for the selective extraction of keratins from human stratum corneum. These authors modified an original extraction method described by Harrap & Woods (1964) for feather keratins. More recently this method of extraction has been used to characterize the keratin proteins of adult
and embryonic avian scales and feathers (Kemp & Rogers, 1972) and the proteins of chick epidermis cultured in vitro (Beckingham Smith, 1974b).

The procedure is as follows: the isolated epidermis preparations were suspended in a 0.1 M phosphate buffered saline solution, pH 7.4, at a liquid to tissue dry weight ratio of 100:1 and then homogenized for one minute in a Virtis homogenizer at full speed. The homogenate was gently stirred for 1 h at 37 °C and centrifuged at 20000 g for 30 min at 4 °C. The pellet was re-extracted with the above saline for 30 min at 37 °C followed by another centrifugation. After washing the pellet several times with 95% ethanol and absolute ethanol, the pellet was defatted by two 10 min incubations at 40 °C in 1:1, ethanol: ether, and finally ether extracted and dried.

The keratin-like proteins in this pellet were reduced and solubilized by incubation for 24 h at 20 °C in a solution containing 0.1 M mercaptoethanol, 0.1 M ethanolamine buffered at pH 10.5, and 8 M urea which had been freshly deionized using 'Amberlite' MB-3 monobed resin (BDH Chemicals). The extraction volume to tissue dry weight ratio was again 100:1. Solubilized proteins were collected by centrifugation at 20000 g and the pellet re-extracted for another 12 h. The supernatant from this second extraction was combined with the first for alkylation. All extractions were carried out under a nitrogen atmosphere. Between 85 and 90% of the saline insoluble proteins were solubilized by this method.

Carboxymethylation

Alkylation of the reduced proteins was carried out essentially as described by Beckingham Smith (1973a). A fresh solution of 2.5 M iodoacetic acid (I2 free) at pH 6.0 was added to the extraction solution to give a final concentration of 0.1 M iodoacetate. This solution was maintained at pH 9.5 until the nitroprusside or 5,5'-dithiobis (2-nitrobenzoic) acid (Cavallini, Graxiani & Dupre, 1966) tests for free thiol groups were negative. The reaction was always complete in less than 15 min. To destroy any residual iodoacetic acid, the solution was made 75 mM in mercaptoethanol. This solution was then dialysed overnight against distilled water to remove side products of the reaction prior to electrophoresis. To minimize the possibility of incomplete reduction and carboxymethylation of these proteins (Beckingham Smith, 1973a) the initial extracts were subjected to two cycles of re-reduction and carboxymethylation. After dialysis against distilled water and lyophilization, the carboxymethylated proteins were stored at −20 °C until required. Amino acid analysis demonstrated that the reduction and carboxymethylation reactions had gone to completion.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis at high pH (pH 8.8) using a 13.5% running gel with a 2.5% stacking gel was performed as described by Davis
polyacrylamide gels containing 0.1% sodium dodecyl sulphate (SDS) were used according to the method described by Laemmli (1970). Molecular weights of proteins were determined by comparing their mobilities on SDS gels with those of marker proteins of known molecular weights as described by Weber & Osborne (1969). Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Isoelectric focusing of proteins

Isoelectric fractionation of proteins in 8% polyacrylamide gels using various pH range Ampholines (LKB Instruments Ltd., Stockholm) at a final concentration of 1% in the gel solution was performed as described by Fawcett (1968) except that the gel and electrode solutions contained, in addition, 5 M urea (freshly deionized).

Preparative polyacrylamide-gel electrophoresis

To obtain purified protein fractions in suitable quantities for further chemical analysis and for antibody production, protein bands were cut out from thick (6 mm) polyacrylamide slab gels after electrophoresis and the proteins eluted from the gel slices. Bands of unfixed and unstained proteins were located in these wet gels by running marker proteins labeled with 125I in slots on either side of the slab with non-labeled proteins running in a wide slot in between. After electrophoresis the radioactive bands of protein were located in the wet gels by means of autoradiography using Kodirex AP 54 film separated from the gel by a piece of Saran Wrap. Control experiments showed that the iodination of the proteins does not change their electrophoretic mobilities. Proteins were eluted from the excised gel slices by homogenization of the slices in a 0.1 M tris buffer (pH 7.4) containing 0.5 M NaCl and 5 M freshly deionized urea followed by stirring at 4 °C for 24 h with two changes of elution medium. Acrylamide fragments were removed from the pooled supernatants by high speed centrifugation (20000 g for 30 min) followed by Millipore filtration. The protein solution was then dialyzed against distilled water. Electrophoretic purification was repeated a second time and the extracts were lyophilized and stored at −20 °C. Extraction efficiency from the gels was always over 90%.

Iodination of proteins

Gentle enzymic iodination of keratin-like proteins and purified immunoglobulins was performed by the lactoperoxidase method described by Marchalonis (1969) using bovine lactoperoxidase (Sigma) and carrier-free Na125I (Radiochemical Centre, Amersham). After the enzymic reaction had reached appropriate iodination levels (within about 15 min) the reaction was terminated with 2-mercaptoethanol and the unreacted 125I removed from the iodine-labeled proteins by dialysis followed by passage through a Sephadex
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G-25 column. Control experiments indicated that labeling of immunoglobulins with radio-iodine to a specific activity of $1\times 10^6$ cpm/μg of protein reduced the titer of the antibody preparation by about 20–25% but did not interfere with the ability of the remaining active antibody fraction to form specific antigen-antibody complexes. Labeling of other types of proteins to much higher specific activity levels (of up to $2.8 \times 10^7$ cpm/μg of protein) likewise resulted in little degradation or inactivation if the proteins were used within a few days. Specific activities of labeled proteins were obtained by using the method of Lowry (1951) to determine protein concentrations and using either a Wallac Decem-GTL 300–500 Gamma Counter or a Nuclear Chicago Model 4233 Auto-γ Counter to determine radioactivity.

Amino acid analysis

Samples were hydrolysed in 6 N-HCl in vacuo at 105 °C for 16, 24 or 48 h as indicated. Hydrolysates were analyzed on a Locarte Automatic Amino Acid Analyzer.

Peptide mapping

Protein samples dissolved in 0.1 M-NH₄HCO₃ (pH 7.9) at 5–10 mg/ml were digested for 24 h at 37 °C with TPCK trypsin (Worthington) at an enzyme to substrate ratio of 1:50. After four lyophilizations, 100–300 μg of peptide fragments were dissolved in 50–100 μl of pyridine-acetate buffer (pH 6.5) and spotted on Whatman No. 1 chromatography paper for two-dimensional separations. Peptides were separated in the first dimension by electrophoresis in pyridine-acetate buffer, pH 6.5, for 60–70 min at 4kV. After drying, the relevant 4 cm strip was cut from the paper and sewn to another piece of Whatman No. 1 paper for electrophoresis in the second dimension in pyridine-acetate buffer at pH 3.5 at 3kV for 60 min. Alternatively, peptides were separated in the second dimension by descending chromatography for 18 h using a solvent mixture of butanol:acetic acid:water (3:1:1). Peptide spots were localized under ultraviolet light after dipping the paper in a dilute solution of Fluram (fluorescamine/Roche Diagnostics).

Amino terminal end-group analysis

The N-terminal amino acids of proteins were determined using the dansyl chloride method described by Gray (1967) in which the reaction solutions contained 8 M urea. The dansylated proteins were hydrolysed, after removing the urea by dialysis, at 105 °C in an evacuated tube for 4–16 h with 6 N HCl. After removal of the HCl in vacuo the dried hydrolysates were dissolved in freshly made up water-saturated ethyl acetate, applied to the corner of a thin layer (15 x 15 cm) polyamide plate and separated in two dimensions by chromatography using the solvent systems described by Percy & Buchwald (1972). Dansylated amino acids were located under ultraviolet light and
identified by comparison with known standards. The yields of dansylated amino acids from the three protein bands was high since all of the protein in each starting fraction was solubilized in the initial solvent and none precipitated out during the dansylation reaction. However, exact quantification of yields after dansylation was not attempted.

*Immunological techniques*

Antisera to keratin-like proteins (purified by electrophoresis as explained below) were prepared in rabbits using an initial injection of complete Freund's adjuvant followed by three antigen injections at weekly intervals. The rabbits were then bleed and the γ-globulin fraction of the serum isolated and purified using the methods described by Clausen (1969) and Kabat and Mayer (1961). Double immunodiffusion tests were performed by the methods of Ouchterlony (1968) using 1% agar gels in 0.01 M sodium phosphate buffer (pH 7.2). Reactivity and specificity of immunological reactions were determined according to the methods described by Clausen (1969) and Brown (1967). Iodination of the immunoglobulin fractions, as described above, resulted in a 20–25% drop in antibody titer probably owing to the tyrosyl residues known to be present in the reactive sites of some antibodies (Singer & Doolittle, 1966). However, this drop in reactivity did not interfere with the specificity of antigen/antibody complex formed by the remaining active antibody fraction.

A modified agar gel immunodiffusion technique was used to quantify the amounts of keratin-like proteins (KLPs) in carboxymethylated extracts of unknown KLP content. Various known concentrations of carboxymethylated proteins extracted from different tissues were incorporated into molten (54 °C) buffered agar (1%) and 0.2 ml aliquots placed in the bottom of the wells of plastic microtiter trays (0.4 ml capacity, Linbro Plastics) and allowed to solidify. Anti-keratin antibody, labeled to known specific activity with $^{125}$I, was then placed in the wells and diffusion of the antibody into the agar allowed to occur for 36–48 h at 4 °C. Unreacted antibody was then allowed to diffuse out of the gels into a large excess of standard phosphate buffered saline (pH 7.2) for 48–60 h and the agar discs removed from the wells and the amount of radioactivity in the $^{125}$I-antibody/antigen complex counted in a gamma counter. This amount of radioactivity was related to a standard curve based on the reaction of the same labeled antibody solution to known concentrations of electrophoretically purified antigens incorporated into the agar discs. All determinations were made under conditions of antibody excess.

*RESULTS*

*Gel electrophoresis*

To identify putative keratin-like proteins in the skin of *Xenopus laevis*, extracts were made of the soluble carboxymethylated proteins from isolated
Fig. 1. Coomassie brilliant blue stained banding pattern of the carboxymethylated proteins selectively extracted from various tissues of *Xenopus laevis* and run on high pH (8-8), 13.5% polyacrylamide disc gels containing 5 M urea. The proteins were run toward the anode (bottom of gels) for 4 h at 5 mA per gel. Gel 1: 30 μg of an extract from the isolated epidermis of an adult web. Gel 2: 35 μg of an adult dermis extract. Gel 3: 35 μg of an adult heart extract. Gel 4: 50 μg of an adult brain extract. Gel 5: 35 μg of an adult lung extract. Gel 6: 55 μg of an extract from the isolated epidermis of the foot webs of newly metamorphosed young froglets (Nieuwkoop & Faber, stage 66). Gel 7: 55 μg of an extract from the isolated epidermis of whole body skin of adult frogs.

Footnote a: Apparent molecular weights of proteins found in bands I, II and III as determined by SDS gel electrophoresis according to the method of Weber & Osborn (1969).

epidermal tissue taken from frogs varying in age from early post-metamorphosis to adulthood. However, since true KLPs should be tissue specific and confined to vertebrate epithelia and their derivatives, it was necessary to determine the selectivity of the extraction procedure described in the methods section. Therefore various control tissues, such as heart, lung, brain and dermis were also
extracted and their carboxymethylated proteins compared with those extracted from the epidermis.

Samples of carboxymethylated proteins from all of these sources were electrophoresed on high pH (8.8) polyacrylamide gels containing 5 M urea. The protein banding patterns seen after Coomassie brilliant blue staining are shown in Fig. 1. It is obvious from these gels that the extraction procedure is not entirely specific for keratins since the control tissues also contain a considerable number of protein bands isolated by the reduction and carboxymethylation procedure.

However, extracts from the epidermis of both the adult web (Fig. 1, gel 1) and from whole body skin (gel 7) do contain two protein bands with electrophoretic mobilities quite different from those of the control tissues (gels 3–5). These two protein bands, labeled I and II, have apparent molecular weights of 69,000 ± 2,000 and 49,000 ± 6,000 daltons, respectively, as determined by SDS gel electrophoresis. A third mass band, labeled III, with an apparent molecular weight of 20,000 ± 6,000 daltons is also present in adult epidermal extracts but the amount of this protein varied a great deal from preparation to preparation. This third band also migrates with proteins from other extracted control tissues.

All three adult epidermal protein bands failed to stain for glycoproteins with periodic acid-Schiff's reagent and have quite different electrophoretic mobilities from those of mucus or collagen run on parallel gels. Bands I and II ran as single, but often rather diffuse, mass bands on both urea and SDS gels at all acrylamide gel concentrations tested (7.5–18%) even after prolonged electrophoresis. The diffuse nature of the protein bands seen in the extracts from isolated epidermis (gels 1 and 7, Fig. 1) seems unlikely to be a result of incomplete reduction of disulfide bonds by the extraction procedure (Beckingham Smith, 1973b) since amino acid analysis shows that all of the cystine residues present have been completely converted to carboxymethylcysteine (see below). Rather, it seems more likely that the diffuse banding is a result of microheterogeneity of several different proteins, with similar electrophoretic mobilities being present within a given band. This explanation is supported by peptide end group analysis (see below) and by the fact that band III can be resolved into a doublet of diffuse bands after prolonged electrophoresis or by separation of the proteins on higher percentage (18%) acrylamide gels.

The adult epidermal keratin-like proteins also have other unusual characteristics. For example, they stain less intensely, on a relative weight basis, with Coomassie brilliant blue than do carboxymethylated proteins from other control tissues (Fig. 1, gels 2–5). They are also much less soluble than extracts from control tissues. For example, over 75% of the KLPs will precipitate from solution with the addition of 10% (w/v) NH₄SO₄ and over 90% will precipitate at a concentration of 15% NH₄SO₄. This relative insolubility of the epidermal proteins may explain why there is always some protein left at the interface of
Fig. 2 (A) A thick (6 mm) polyacrylamide slab gel containing 13.5 % acrylamide and 5 M urea used for electrophoretic separation and bulk preparation of purified protein bands as described in the Methods section. In the wide centre slot, 1-2 mg of adult epidermal S-carboxymethylated proteins was electrophoresed. In each of the two corner slots, on either side of the centre slot, epidermal proteins iodinated with \( ^{125} \text{I} \) were electrophoresed (at 100 µg/slot). The gel was then stained with Coomassie brilliant blue. Note that the iodination did not change the electrophoretic mobilities of the various epidermal protein bands. The separated bands were cut from the gel and the proteins eluted as described.

(B) A Coomassie brilliant blue stained SDS disc gel showing a single protein band when 50 µg of purified band I protein was electrophoresed for 4 h at 5 mA on a 7.5 % acrylamide gel containing 0.1 % sodium dodecyl sulfate. The purified band I protein fraction ran as a single mass band on all concentrations of SDS acrylamide gels tested (7.5–18 %).

It thus appears that at least two carboxymethylated protein bands extracted from adult epidermis are unique to this tissue and have different electrophoretic, staining and solubility properties from the carboxymethylated proteins of other tissues. These bands were therefore tentatively identified as keratin-like proteins (KLPs) and analysed further. In particular, large amounts of the protein(s) in Band I were purified by preparative electrophoresis and subjected to extensive chemical analysis and finally antibodies were prepared against them.
Table 1. *Amino acid analysis of keratin-like proteins from adult Xenopus skin epidermis (residues per 100 residues)*

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Unfractionated*</th>
<th>Band I*</th>
<th>Band II†</th>
<th>Band III†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>10.75</td>
<td>10.71</td>
<td>6.70</td>
<td>10.76</td>
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<tr>
<td>Arginine</td>
<td>1.48</td>
<td>0.16</td>
<td>2.03</td>
<td>0.22</td>
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<tr>
<td>Aspartic acid</td>
<td>4.95</td>
<td>1.60</td>
<td>3.90</td>
<td>12.27</td>
</tr>
<tr>
<td>CM-cysteine</td>
<td>4.04</td>
<td>4.70</td>
<td>3.27</td>
<td>0.51</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>8.44</td>
<td>3.54</td>
<td>7.20</td>
<td>14.53</td>
</tr>
<tr>
<td>Glycine</td>
<td>32.30</td>
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<td>3.23</td>
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<td>3.59</td>
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<tr>
<td>Hydroxyproline</td>
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<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.69</td>
<td>4.90</td>
<td>4.20</td>
<td>3.22</td>
</tr>
<tr>
<td>Leucine</td>
<td>3.96</td>
<td>3.54</td>
<td>6.34</td>
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<tr>
<td>Lysine</td>
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<td>2.66</td>
<td>2.20</td>
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<tr>
<td>Methionine</td>
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<td>0.72</td>
<td>0.00</td>
<td>0.59</td>
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<tr>
<td>Phenylalanine</td>
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<td>4.75</td>
<td>3.20</td>
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<tr>
<td>Proline</td>
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<td>6.68</td>
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<td>1.77</td>
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</table>

* The average of two 16 h and two 48 h hydrolysates.
† The average of two 24 h and one 48 h hydrolysates.
‡ Includes the asparagine concentration.
§ Includes the glutamine concentration.

In Fig. 2A a urea slab gel used for the preparative isolation of electrophoretically separated keratin-like protein bands as described in the Methods section is shown. Fig. 2B pictures a 7.5% polyacrylamide SDS gel on which a sample of purified band I protein was electrophoresed. Purified band I protein electrophoresed as a single band on all SDS gels regardless of the acrylamide concentration used (7.5–18%).

Amino acid analysis

From the amino acid composition of the unfractionated carboxymethylated epidermal proteins, and of the various electrophoretically purified subfractions, listed in Table 1, it is apparent that these are unusual proteins. In particular, the extremely high content of glycine found in the unfractionated proteins and accentuated in the subfractions of purified bands I and II, has no known biological equivalent except in collagen (Dayhoff, 1973) and various silk fibroins (Rudall, 1968; Dayhoff, 1973). The absence of hydroxyproline and the low levels of proline found in these proteins shows, moreover, that they are not derivatives of collagen.

In addition to their high content of glycine (35.4% in I, and 37.2% in II), purified bands I and II are characterized by their relatively high contents of
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CM-cysteine (4.7% in I, and 3.27% in II), alanine (10.71% in I and 6.7% in II), and valine (6.68% in I and 4.7% in II). Together glycine, alanine and CM-cysteine make up about 50% of the residues in both purified bands I and II. The high content of cysteine, in particular, would, when compared to keratinous and non-keratinous proteins from other vertebrates, place both bands I and II into the keratin-like class of proteins (Seifter & Gallop, 1966; Rudall, 1968; Fraser et al. 1972). It should also be mentioned that Kemp & Rogers (1972), in an amino acid analysis of the keratin proteins of chick-scale tissues, both embryonic and adult, have shown that these proteins, like those of bands I and II reported here, contained very high levels of glycine. The CM-cysteine, serine, alanine and methionine contents of these two groups of proteins also appear to be quite similar. It is therefore concluded that purified bands I and II from adult Xenopus epidermis are keratin-like in overall amino acid composition.

Electrophoretically prepared band III, the protein(s) that migrates with protein bands from other tissues on urea gels, has, on the other hand, an amino acid composition quite different from that of the other two epidermal protein bands. It contains only moderate amounts of glycine compared to I and II (16%) but has relatively high concentrations of glutamic acid, aspartic acid, alanine, leucine, threonine, phenylalanine and serine. It has only trace amounts of CM-cysteine. By its overall residue composition, band III would not necessarily be placed in the category of KL-proteins without further biochemical characterization (cf. Fraser et al. 1972).

Amino end-group analysis

The heterogeneity of proteins and polypeptide chains within electrophoretically purified subfractions was investigated by identifying the amino end-group residues using the dansyl chloride procedure described in the Methods section. Microheterogeneity within the subfractions of bands II and III was confirmed. Electrophoretically prepared band II proteins contained two different amino terminal residues, the major residue being glycine with a minor amount of alanine also being present. Electrophoretically prepared band III had at least three recognizable end-groups, the two major species being alanine and serine but with a minor glycine component always being present. Isolated band I proteins, on the other hand, had only one amino terminal residue, glycine, and was therefore analysed further. It should be pointed out, however, that even though the recovery of dansylated amino acids was quite high in these determinations no attempt was made to eliminate the possibility of blocked amino terminal end groups in these protein fractions (cf. Partington, Kemp & Rogers, 1973).
Peptide mapping

Fig. 3 shows a two-dimensional electrophoretic, tryptic peptide map of the KL-proteins found in electrophoretically purified band I preparations. The 15 tryptic peptide fragments vary greatly in the amount of material within each spot (as shown by their Fluram staining intensities) and also in their relative electrophoretic mobilities. The peptide map has about the tryptic peptide complexity which one would expect from a single protein species of about 69000 daltons molecular weight containing 2.66% lysine and 0.16% arginine residues.

Isoelectric focusing

As a final check on the homogeneity of band I proteins, samples were subjected to isoelectric focusing in 8% polyacrylamide gels containing 8 M urea as described in the Methods section. If urea was omitted from the gels, all of the
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Fig. 4. Photograph of an Ouchterlony double immunodiffusion plate stained with Amido black 10B. The gel is of 1 % agar containing 0.9 % (w/v) NaCl and 0.01 M phosphate buffer, pH 7.2. The centre well contained 2.5 μg of anti-KLP antibody (against purified band I proteins) in 5 μl of saline buffer. Various carboxymethylated protein extracts from different tissues were dissolved in saline phosphate buffer at 2.0 mg/ml and 5 μl of these solutions placed in the wells around the antibody-containing well. Immunodiffusion was allowed to occur for 30 h at 4 °C and then unreacted antigen and antibody removed by soaking the gel in saline buffer for 2 days before staining. The contents of the wells were as follows: D, adult dermal extract; ME, extract from the isolated epidermis of newly metamorphosed froglets; AE, extracts from the isolated epidermis of adult skin; LE, larval epidermis extract (stage 42); H, adult heart extract; and L, adult lung extract.

protein precipitated at the origin and did not enter the gel. Using a narrow ampholine gradient (pH 3.0–5.0) it was determined that band I contained proteins that formed a single precipitant band in the gels with an iso-electric point of about pH 4.32.

Anti-KLP antibodies

Antibodies against electrophoretically purified band I protein were produced in rabbits as described in the Methods section. The immunoglobulin fraction
of the serum was purified and treated with acetone-extracted liver powder to remove non-specific reacting materials (Kabat & Mayer, 1961). The immunological specificity of the anti-KLP antibody preparations was then tested by the standard antibody-blocking tests to determine whether the carboxymethyl groups attached to various other proteins would inhibit the formation of specific antigen/antibody complexes between band I proteins and the anti-KLP antibodies. Even though the carboxymethyl group is a potential hapten, it was found that this group had no inhibitory effect on the specific binding of antibody preparations.

The antibodies were thus directed against the protein moiety and not the carboxymethyl moiety of the antigen. Likewise, carboxymethylated protein extracts from various other tissues of adult or embryonic *Xenopus* also failed to block the antibody reaction.

Double immunodiffusion tests (Ouchterlony, 1968) showed that the anti-KLP antibodies were specific for band I proteins and would not cross-react with any other tissue extracts. Fig. 4 shows an Ouchterlony plate where the anti-KLP antibody preparation was placed in the centre well with various protein extracts placed in wells around it. It is evident that the antibody formed a single antigen-antibody precipitation band with proteins extracted from adult epidermis (AE) and, to a much lesser extent, with proteins from the epidermis of a newly metamorphosed froglet (ME). The antibody did not react with proteins extracted from larval epidermis (LE), or adult heart (H), lung (L) or dermis (D), nor with keratin proteins extracted from embryonic chick skin or human hair (unpublished observations). The antibody preparation was thus monospecific for keratin-like proteins confined to the epidermis of frogs from the stages of metamorphosis to adulthood.

**Appearance of KLP's in the epidermis at metamorphosis**

Fig. 5 plots the reaction of $^{125}$I-labeled antibody against skin and epidermal carboxymethylated protein extracts from animals at various developmental stages before and after metamorphosis. As described in the Methods section, protein extracts were immobilized in agar; the anti-band I KLP antibody preparation labeled with $^{125}$I was allowed to diffuse into the agar; and the amount of radioactivity in the resulting antigen-antibody complex was counted after washing away the unreacted antibodies. Before metamorphosis there is no immunoprecipitation above the background level. Starting around mid-metamorphosis, however, and continuing on through metamorphosis and up to maturity, the absolute amount of antibody precipitated KL-proteins per µg of extracted protein increases greatly. Between stage 55 and the end of metamorphosis (stage 66), the increase in relative percentage of band I keratin-like proteins in the epidermis is almost linear. From early postmetamorphosis to maturity the amount of these KL-proteins within the epidermis increases a further threefold in absolute concentration.
Fig. 5. Graph of the amount of $^{125}$I-labeled anti-KLP antibody (against band I proteins) that reacts with proteins extracted from the epidermis of animals at different developmental stages. The specific activity of the antibody preparation was $1.48 \times 10^6$ cpm/µg of protein. The ordinate is a logarithmic scale plot of the cpm of antibody/antigen complexes formed with epidermal extracts (S-carboxymethylated or SCM-proteins) made from the various developmental stages shown on the abscissa. The developmental stages are not drawn to scale. The break in the abscissa indicates an unknown length of time between stages. The standard error of the mean (S.E.M.), based on between four and six determinations per point, is indicated (approximately) by the cross-bars.

*Nieuwkoop & Faber (1968; stages not to scale).*
Table 2. The data from which Fig. 4 was plotted

<table>
<thead>
<tr>
<th>Stage*</th>
<th>Av. $^{125}$I (cpm per µg SCM-Proteins)$^\dagger$</th>
<th>ng KLP$^\ddagger$ per µg SCM-Proteins</th>
<th>KLP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank§</td>
<td>3</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>BSA</td>
<td></td>
<td></td>
<td>46</td>
</tr>
<tr>
<td>25</td>
<td>$64 \pm 5$</td>
<td>0.350</td>
<td>0.035</td>
</tr>
<tr>
<td>27/28</td>
<td>$54 \pm 7$</td>
<td>0.298</td>
<td>0.030</td>
</tr>
<tr>
<td>31</td>
<td>$43 \pm 4$</td>
<td>0.236</td>
<td>0.024</td>
</tr>
<tr>
<td>35</td>
<td>$51 \pm 9$</td>
<td>0.279</td>
<td>0.028</td>
</tr>
<tr>
<td>40</td>
<td>$57 \pm 5$</td>
<td>0.311</td>
<td>0.031</td>
</tr>
<tr>
<td>45</td>
<td>$61 \pm 8$</td>
<td>0.333</td>
<td>0.033</td>
</tr>
<tr>
<td>51</td>
<td>$105 \pm 11$</td>
<td>0.577</td>
<td>0.058</td>
</tr>
<tr>
<td>55</td>
<td>$314 \pm 79$</td>
<td>1.720</td>
<td>0.170</td>
</tr>
<tr>
<td>57</td>
<td>$1635 \pm 431$</td>
<td>8.960</td>
<td>0.900</td>
</tr>
<tr>
<td>61</td>
<td>$6278 \pm 316$</td>
<td>34.30</td>
<td>3.40</td>
</tr>
<tr>
<td>66</td>
<td>$15759 \pm 603$</td>
<td>86.40</td>
<td>8.60</td>
</tr>
<tr>
<td>Adult</td>
<td>$49831 \pm 977$</td>
<td>273.0</td>
<td>27.3</td>
</tr>
</tbody>
</table>

$^*$ The stage of development after Nieuwkoop & Faber (1967).
$^\dagger$ The average specific activity and the s.e.m. (based on three different determinations) of antigen/$^{125}$I-antibody complexes obtained for different stages as described in the Methods section.
$^\ddagger$ The conversion of cpm of bound $^{125}$I-antibody to ng of KLP in extracts from the various developmental stages based on a standard antigen/antibody complex curve obtained as described in the text.

§ The amount of $^{125}$I-antibody non-specifically retained by the agar gel when no protein was mixed in with the original liquid agar before solidification.
|| The amount of non-specifically bound $^{125}$I-antibody retained by the agar gels when the original liquid agar contained bovine serum albumin (BSA) at 1 mg/ml.

The column labeled %KLP indicates the percentage of total carboxymethylated proteins extracted from epidermis that is antibody reacting, band I type keratin-like proteins.

This appearance of KL-proteins in the epidermis of metamorphosing frogs and its subsequent increase in concentration is tabulated in Table 2 as a percentage of total epidermal carboxymethylated proteins. It is seen that from its first appearance at around stage 55 until the end of metamorphosis (stage 66) the amount of band I KLP's within the epidermis increases by some 50-fold (0.17-8.6% of the total extracted proteins). This initial appearance of KLP's within the epidermis is somewhat earlier than the cytological appearance of histologically recognizable keratinizing epithelial patches at around stage 57 as described by Nieuwkoop & Faber (1967). From post-metamorphosis until maturity the relative amount of band I KLP's in the epidermis continues to increase but at a much slower rate and only increases from about 8.6-27.3% of the total extracted proteins, an increase by about a factor of three.
DISCUSSION

Apart from the immunoglobulins, the keratin-like proteins of vertebrate epithelia and their derivatives constitute one of the most heterogeneous groups of related proteins known to occur in a single cell type (Fraser et al. 1972; Crewther et al. 1965; Mercer 1961). Thus it came as no great surprise that the initial apparent simplicity of the KLP’s extracted from adult Xenopus epidermis was deceptive and that further biochemical analysis revealed a considerable amount of microheterogeneity within at least two of the three electrophoretic bands seen on polyacrylamide gels. Only one electrophoretic band, Band I, appeared to contain a single protein.

Nevertheless, certain inferences can be drawn from the unusual amino acid compositions of the tissue specific bands I and II even though they might contain a mixture of proteins. The high concentrations of glycine and cysteine, which are known to destabilize α-helix formation in natural proteins and synthetic polymers (Ptitsyn, 1969; Chou & Fasman, 1974a,b), makes it very unlikely that the proteins reported on here are in the form of an α-helix. Most likely they occur naturally as random coils.

The de novo appearance of a new class of epidermis specific proteins during amphibian metamorphosis is consonant with the numerous other morphological and metabolic changes known to be occurring at this time (Weber, 1967; Etkin & Gilbert, 1968; Cohen, 1970; Tata, 1971). However, the keratin-like proteins described here are somewhat unusual in that the changes occurring in the skin during metamorphosis do not stop at the end of the process but continue on through to maturity. Also, as the percentage of epidermal KLP’s increases by a factor of three between the end of metamorphosis and maturity (Table 2), the complexity of the protein mixtures extracted from various post-metamorphic stages rather unexpectedly decreases (Fig. 1, gels 6, 7). The reason for this biochemical simplification of the profile of total extracted proteins with age is unknown. Perhaps cessation of synthesis of certain individual proteins, or groups of proteins, plays just as important a role in progressive cellular differentiation as does the ‘switching on’ of new types of protein synthesis.

This biochemical characterization of the tissue-specific keratin-like proteins found in the epidermis of adult Xenopus laevis lays the foundation for an investigation of the factors involved in terminal cellular differentiation studied in tissue culture reported on in the following paper (Reeves & Laskey, 1975).

I would like to express my thanks to Dr John Gurdon, in whose laboratory this work was done, for his advice and helpful criticisms. I would also like to thank Dr John Knowland for his many helpful and entertaining discussions.

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