J Mol Evol (1982) 18:121–129

Journal of
Molecular Evolution
© Springer-Verlag 1982

A Comparison of Lizard Claw Keratin Proteins with those of Avian Beak and Claw

J.M. Gillespie, R.C. Marshall, and E.F. Woods

CSIRO, Division of Protein Chemistry, 343 Royal Parade, Parkville, Victoria 3052, Australia

Summary. The outer shell of translucent keratin has been dissected from the claws of the lizard, *Varanus gouldii*. It is free of calcium and hydroxyproline, in contrast to the fibrous support, and contains proteins rich in glycine (28 residues %) and half-cystine (13%). These proteins have been obtained in soluble form by treatment with 2-mercaptoethanol in 8M urea at pH 11 followed by alkylation with iodoacetate to give *S*-carboxymethyl kerateines. The three major components resolved by SDS polyacrylamide gel electrophoresis have been isolated by fractional precipitation with ammonium sulfate followed by chromatography on DEAE-cellulose or Sephadex. Two of the components, low in tryptophan content, appear to be homologous and are relatively homogeneous with respect to both size and charge whereas the third, a tryptophan-rich material, appears to contain about 20 different molecular species as judged by gel electrophoresis in urea at pH 8.9. The molecular weights of two of the isolated omponents (the tryptophan-rich and the major of the two tryptophan-poor components) are about 13000 as determined by equilibrium ultracentrifugation studies.

The major lizard claw proteins are therefore similar in size and glycine content to the proteins of avian beak and claw but differ in containing more cystine and less tyrosine. On the other hand, the reptilian proteins resemble the mammalian high-tyrosine proteins (Type II) in cystine content and overall amino acid composition, but differ in size with the lizard proteins being larger. It is suggested however that they are unlikely to be homologous.

Key words: Varanus – Lizard – Claw – Keratin – Electrophoresis – Protein – Fractionation – Glycine – Tryptophan

Offprint requests to: Dr. J.M. Gillespie

Introduction

There is still great uncertainty concerning the evolutionary origins of the mammalian hard keratins – hair, claw, horn, hoof, quill and baleen and their relationship to the hard keratins of birds and reptiles. Characteristically the mammalian keratins are composed of low-cystine proteins organised in 70 Å diameter filaments, responsible for the α X-ray diffraction pattern, embedded in a non-filamentous matrix of very heterogeneous sulfur-rich and glycine-tyrosine-rich proteins. In contrast the reptilian and avian hard keratins contain smaller filaments (about 30 Å in diameter), give β-type X-ray patterns and, in the case of the avian keratins, are built of essentially only one type of protein. No homology would be expected between these proteins if the view expressed by Sengel et al. (1980) is correct and ancestral anapsid reptiles alone acquired the ability to produce hard keratins of the β-type, in contrast to therapsid stock which developed the α-type hard keratins characteristic of mammals. There is evidence which suggests that the situation might not be as clear cut as this for there are striking similarities in composition between feather protein and certain mammalian high-sulfur proteins and between some high-tyrosine proteins and those of avian beak (Fraser et al. 1972; Frenkel and Gillespie 1976). One purpose of the present study is a search for homologies between the hard keratin proteins from reptiles and mammals.

Although there has been considerable interest in the structure and proteins of the reptilian soft keratins of the epidermis (Baden and Maderson 1970; Baden et al. 1974; Wyld 1979; Wyld and Brush 1979) there have been few investigations of the hard keratins of the claw and scute. X-ray diffraction measurements of lizard claws (*Varanus niloticus*, Rudall (1974); *V. varius*, Fraser et al. (1972)) suggest that claw is basically similar in molecular architecture to feather in being built of

0022–2844/82/0018/0121/$ 01.80

small filaments with a framework of twisted β-sheets. However, the claw patterns are not identical with feather possibly due to differences in the non-β sections of the molecules and in the helical parameters of the filaments (Fraser et al. 1972). Amino acid analyses of lizard claw keratin (Fraser et al. 1972) and keratin proteins isolated from turtle scute and lizard claw (Frenkel and Gillespie 1976) show major differences in composition compared with feather and some similarities with avian beak, claw and scale, particularly in their high content of glycine.

Avian and reptilian claws are strikingly different from mammalian claws in structure and composition and yet are apparently functionally equivalent. A second purpose of the present study, as part of a program designed to study the proteins of keratins and their relation to structure-function of the parent tissue, is an examination in some detail of the hard keratin component of lizard claw. It will ultimately be of considerable interest to determine the relative importance of protein type, arrangement, and cross-linking in the functioning of a keratin.

In this present paper we are reporting studies of the keratin and the major constituent proteins of the claws of the lizard, *V. gouldii*. This species was selected because it is plentiful, dead specimens are readily available and the claws are sufficiently large to provide adequate amounts of keratin. Studies on the minor protein constituents have been reported elsewhere (Marshall and Gillespie 1982).

Materials and Methods

Origin and Preparation of Goanna Claw Keratin

Sources of the claws are given at the end of this paper. The claws were received from collectors in the field in an air-dried form, and before use were stirred for several hours in large volumes of 0.15M NaCl-0.05% sodium azide. During this period any attached skin and flesh was dissected off. In some cases the fibrous bony core could be pulled out leaving an intact shell of keratin but usually the claws were slit lengthwise along the groove and the core dissected from the keratin. The keratin shell was scraped free of soft material, washed again in NaCl-sodium azide, then in water and ethanol before air drying. The inner side of the dried shell was covered with a firmly adherent white membrane and before further preparation this was ground off with a dental burr. The now translucent shells were ground to 20 mesh in a micro-Wiley mill and extracted successively with 0.15M NaCl, water, ethanol, petroleum ether, ethanol and then air-dried.

Preparation of Soluble Proteins

The soluble proteins were obtained by extraction of the keratins with 0.2M 2-mercaptoethanol in 8M urea at pH 11 for 2 h at 40°C. Generally about 90% of the keratin was solubilised. For electrophoresis in the reduced form the proteins were dialysed against and stored in 0.1% 2-mercaptoethanol. For all other experiments the proteins were alkylated with iodoacetate and thoroughly dialysed before being stored in the frozen state. Further experimental details are given by Frenkel and Gillespie (1976). The various fractionation techniques used are described in the text.

For the two-dimensional electrophoresis experiments, proteins were radiolabelled by the following procedure. Keratin samples (0.5 mg) were extracted for 18 h at room temperature with 50 µl of a solution containing 8M urea, 0.05M dithiothreitol and 0.05M Tris (pH 9.3). The dissolved proteins were S-carboxymethylated by adding 5 µl of an aqueous solution containing 6 µCi of iodo(2–^{14}C)acetic acid and then 0.5 µl of 3M Tris followed 10 min later by 25 µl of a solution containing 1M iodoacetic acid and 2.3M Tris (pH 8.5). After 10 min room temperature, excess iodoacetate was reacted with 5 µl of 2-mercaptoethanol.

Amino Acid Analysis

The various keratins and protein preparations were hydrolysed for 22 h in vacuo at 108°C with constant boiling HCl containing 2 mM phenol, and then freeze-dried. In order to oxidise any cysteine produced by reaction between phenol and cystine the hydrolysates were dissolved in 1 ml of 0.1M borate and shaken in contact with air before being freeze-dried and analysed.

In some cases tryptophan was estimated separately by hydrolysing the protein with methanesulfonic acid in the presence of tryptamine (Inglis et al. 1976). Surprisingly, on average 74% of the tryptophan in the claw proteins survived hydrolysis with HCl, accordingly tryptophan values for these hydrolysates have received the appropriate correction.

Polyacrylamide Gel Electrophoresis

The proteins were examined by polyacrylamide gel electrophoresis in slab gels measuring 140 × 100 × 1.5 mm (Bio-Rad apparatus Model 220). At pH 8.9, the proteins were examined in polyacrylamide gels prepared by the procedure of Davis (1964) except that 8M urea was incorporated. Polyacrylamide gel electrophoresis was also carried out in the presence of sodium dodecyl sulfate (SDS) using the method of Laemmli (1970) at pH 8.9 and that of Weber and Osborn (1969) at pH 7. In these procedures, electrophoresis was performed until the bromophenol blue tracking dye had almost reached the bottom of the separating gel. Following electrophoresis, the proteins were located by staining with Coomassie Blue G250. For complete experimental details see Marshall and Gillespie (1976).

Table 1. Amino acid compositions[a] of morphological fractions isolated from claws of *Varanus gouldii* and for comparison one mammalian and three avian hard keratin proteins

	Lizard claw Outer Part		Inner Part	Avian keratin proteins[c]			
	Keratin	White membrane	Fibrous core	Feather	Beak	Claw	Echidna quill[d] high-tyrosine protein
Lysine	1.1	1.7	3.8	0.2	0.2	0.3	0.3
Histidine	2.3	1.9	1.5	0.3	1.1	1.1	0.2
Arginine	3.2	4.1	5.8	3.7	3.3	3.4	3.8
Tryptophan[b]	1.3	0.9	0.4	0.7	NE	NE	NE
SCMC	NP	NP	NP	8.3	4.6	4.9	9.1
Aspartic acid	4.8	5.8	7.1	5.9	3.7	4.1	2.9
Threonine	3.4	3.5	3.2	4.5	3.6	3.8	1.2
Serine	7.5	7.6	6.7	10.7	6.3	7.1	5.3
Glutamic acid	3.4	5.3	10.4	7.0	3.2	3.8	0.7
Proline	9.1	9.3	8.1	11.1	9.6	9.4	3.9
Glycine	28.2	24.2	19.3	15.6	33.9	30.8	40.1
Alanine	4.8	4.6	7.1	7.0	6.1	6.1	0.8
Half-cystine	13.0	10.2	2.3	NP	NP	NP	NP
Valine	4.9	4.2	3.6	7.4	4.0	4.7	0.9
Methionine	0.8	1.0	1.4	0.0	0.3	0.5	0.0
Isoleucine	2.4	2.9	3.6	3.0	1.5	1.7	0.1
Leucine	3.6	4.1	5.8	7.8	5.4	5.8	5.3
Tyrosine	4.1	6.1	5.0	3.9	9.4	9.0	18.9
Phenylalanine	2.3	2.5	2.2	3.0	3.7	3.6	5.0
Hydroxyproline	0.0	0.0	2.4	NE	NE	NE	NE
Calcium	< 0.5%	NE	50%	NE	NE	NE	NE

NE not estimated ; SCMC = S. carboxymethyl cysteine; NP = Not Present

[a]Expressed as residues % [b]Uncorrected for hydrolysis losses

[c]Kookaburra *Dacelo novaeguinae* – beak and claw analyses from Frenkel and Gillespie 1976

[d]*Tachyglossus aculeatus* (Gillespie 1976)

Protein mixtures were also characterised by two-dimensional polyacrylamide gel electrophoresis. In this procedure the proteins (10 μl of unfractionated extract) were initially separated at pH 8.9 on a 110 x 3.5 mm polyacrylamide gel rod (1 cm 4% stacking gel, 10 cm 10% separation gel) containing 8M urea followed by SDS electrophoresis at the same pH in a 15% polyacrylamide gel slab (140 x 95 x 1.5 mm). The gel rod was held in place above the 2 cm stacking gel (4.5% acrylamide) by agarose. Following electrophoresis, the polyacrylamide gel was impregnated with the scintillant 2,5-diphenyloxazole (Bonner and Laskey 1974), dried, and exposed to Kodak X-Omat R film for various times.

Molecular Weight Measurements

Sedimentation equilibrium measurements were carried out at 20°C in a Beckman Model E ultracentrifuge equipped with Rayleigh interference optics at speeds from 36 000 to 52 000 rpm. The meniscus depletion method of Yphantis (1964) was employed with column heights of 3 mm, and the computer program of Roark and Yphantis (1969) was used to calculate the point-average molecular weights along the column. The required partial specific volumes were calculated from the amino acid compositions. Measurements were made in 0.05 I imidazole-HCl (pH 7.2) containing either 8M urea or 0.1M NaCl.

Results

Composition of the Morphological Constituents of Claw

The three morphological components of claw, namely the fibrous internal support, the outer shell of translucent keratin and the white membranous lining, were subjected to amino acid and mineral analysis. The fibrous core (Table 1) contains both calcium and hydroxyproline suggesting that it is bone, although no X-ray diffraction pattern similar to that of collagen could be obtained (T.P. MacRea, personal communication). The high content of tyrosine and the comparatively low contents of hydroxyproline, glycine and alanine suggest the presence of either a very unusual type of collagen (Eastoe 1967) or a collagen-like protein such as elastoidin (Sastry and Ramachandran 1965). The white membranous lining of the keratin shell is similar in overall composition to the shell itself (Table 1) and is presumed to be part of the keratin complex although it contains 50% more tyrosine and differs significantly in a number of other amino acids. As the outer keratin shell contains no detectable

amount of hydroxyproline and less than 0.5% Ca it is free of contamination with bone and does not appear to be mineralised.

The claw keratin has a very characteristic amino acid composition (Table 1) being rich in glycine (28 residues %) and half-cystine (13%), moderately rich in serine and proline, and poor in the content of the basic amino acids and glutamic acid. Preparations from two animals (compare first columns in Tables 1 and 2) were very similar in overall composition although there were small but significant differences in a few amino acids, notably tyrosine, possibly reflecting membrane protein contamination. The composition of lizard claw keratin is more like that of avian beak and claw than that of feather (Table 1) because of its high glycine content but differs from beak and claw by having a higher content of half-cystine and lower content of tyrosine.

Preparation of the Claw Proteins

Approximately 90% of dissected claw keratin is solubilized by treatment with urea-mercaptoethanol and it is not surprising therefore that the amino acid composition of the extracted proteins, after making allowance for the conversion of cysteinyl to *S*-carboxymethylcysteinyl (SCMC) residues, is very similar to that of the parent keratin (Table 2). As expected from the comparatively high content of aromatic amino acids (Table 2), the whole claw protein has an extinction coefficient ($E^{1\%}_{1cm}$ 280 nm) of about 16. This has been determined for only one claw sample and its constancy from sample to sample is not known.

Table 2. Amino acid compositions[a] of keratin and fractions isolated from claws of *V. gouldii*

	Keratin	Soluble protein	Insoluble fraction
Lysine	1.1	0.9	3.6
Histidine	2.3	2.2	1.3
Arginine	3.4	2.9	6.0
Tryptophan[b]	1.3	1.4	0.6
SCMC	NP	13.6	6.8
Aspartic acid	4.8	4.7	7.3
Threonine	3.3	3.7	3.0
Serine	7.2	7.9	9.0
Glutamic acid	3.4	3.1	9.8
Proline	8.8	8.5	10.5
Glycine	28.4	29.1	11.8
Alanine	5.2	4.8	5.1
1/2 Cystine	12.7	NP	NP
Valine	4.5	4.6	3.7
Methionine	0.9	0.7	1.0
Isoleucine	2.5	2.4	3.7
Leucine	3.5	3.4	6.1
Tyrosine	4.6	4.0	8.3
Phenylalanine	2.3	2.2	2.3
Hydroxyproline	0.0	0.0	0.0

[a]Expressed as residues %
[b]Uncorrected for hydrolysis losses

SCMC = S carboxymethylcysteine; NP Not present

The insoluble residue is however very different in composition to both the keratin and the soluble protein, being much richer in lysine, arginine, aspartic acid, glutamic acid, leucine and tyrosine and having a much lower content of SCMC (cystine in the keratin) and glycine. Minor high-molecular weight components of similar composition have been found amongst the soluble fractions (Marshall and Gillespie 1982) suggesting that the residue may not be completely refractory to solubilisation. It is of interest that the insoluble portion of feather differs in a similar way from the soluble feather proteins (Harrap and Woods 1964). Material of somewhat similar amino acid composition particularly with respect to glutamic acid, glycine and tyrosine has also been found associated with the resistant membranes of wool (Bradbury et al. 1966).

The claws of some species of *Varanus* are too small to be easily dissected and it is therefore necessary for the proteins to be extracted from the whole claw (keratin plus bone) rather than from the isolated keratin. For this to be a valid procedure there must be an adequate level of extraction of keratin proteins from the complete, preferably unground claw and at the same time a near zero level of extraction of non-keratinous constituents. The procedure was tested in the following way. A set of claws from one animal was divided into three lots, one lot was left intact, a second ground to 20 mesh, whilst keratin shells were prepared from the third. The three samples were extracted with urea-mercaptoethanol in the usual way, alkylated and dialysed. In each case about 90% of the keratin was solubilised and no differences could be detected between the preparations, either in the number of components or their relative proportions, by polyacrylamide gel electrophoresis at pH 8.9. The lack of hydroxyproline in hydrolysates of extracts from whole claw and the inability of urea-mercaptoethanol to solubilise protein from dissected samples of bony core all support the safety of preparing proteins from whole claw. Where the claws are sufficiently large we prefer to isolate the keratin as it simplifies handling during solubilisation.

Solubility Properties of the S-carboxymethyl Proteins

The effect of pH on the solubility of the claw proteins was investigated at an ionic strength of 0.1. Most of the proteins are soluble above pH 5.5 and as the pH is lowered below 5.5 there is progressive precipitation which is complete by pH 2.8 (Fig. 1a). There was evidence of differential precipitation of components at various points on the solubility curve but there was no clear separation of components as occurs with mammalian keratin proteins (Gillespie 1960). A minor component which provides an exception in that it is insoluble at pH 7, was

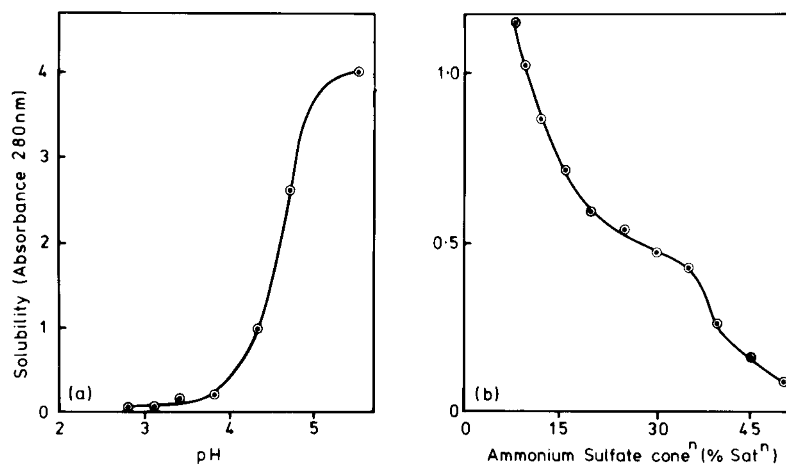

Fig. 1. a Solubility at 20°C of claw proteins (SCM form) as a function of pH. Protein concentration 0.25% at ionic strength of 0.1. b Solubility at 20°C of claw proteins (SCM form) as a function of ammonium sulfate concentration at pH 6 in 0.1 I sodium acetate-acetic acid buffer with a protein concentration of ~0.08%

Table 3. Amino acid compositions[a] of *V. gouldii* claw protein fractions

	Insoluble pH 7.0	Ammonium sulfate Fractions		Zinc acetate Fractions		SDS components	
		Precipitate	Supernatant	Precipitate	Supernatant	1	2
Lysine	1.6	0.9	0.8	0.9	0.9	1.1	1.1
Histidine	2.4	2.2	2.5	2.3	2.1	2.7	1.8
Arginine	5.3	3.2	2.8	3.2	2.9	2.6	3.7
Tryptophan	3.8[c]	4.5[b]	0.5[b]	4.2[b]	0.5[c]	0.5[c]	0.5[c]
SCMC	7.7	11.8	14.8	12.4	15.2	14.5	13.0
Aspartic acid	5.0	4.8	4.5	4.8	4.5	4.7	4.9
Threonine	2.0	3.2	4.0	3.1	4.3	3.9	4.9
Serine	7.3	6.4	8.7	7.0	8.7	8.9	8.0
Glutamic acid	4.9	3.4	2.4	3.4	2.6	2.9	2.8
Proline	9.5	8.4	8.9	8.2	8.6	8.9	9.5
Glycine	23.5	30.9	27.3	30.7	27.1	26.0	25.0
Alanine	3.5	4.1	5.3	4.2	5.7	5.4	6.1
Valine	2.4	3.2	6.0	3.6	6.1	6.1	6.9
Methionine	0.5	0.9	0.5	0.7	0.6	0.5	0.4
Isoleucine	2.0	2.3	2.4	2.4	2.2	2.5	2.2
Leucine	2.6	2.0	4.2	1.6	4.2	4.4	4.6
Tyrosine	13.9	5.2	2.7	5.1	2.7	2.3	3.4
Phenylalanine	2.1	2.6	1.7	2.2	1.7	2.2	1.2

[a]Expressed as residues % [b]Separate estimation [c]Corrected value

SCMC = S carboxymethylcysteine

purified by repeated precipitation at pH 7 following solubilisation at pH 10.5. It differs quite significantly from the bulk of the proteins (Table 3) in amino acid composition particularly in its high content of tyrosine (14%) and low content of SCMC (8%). In these respects and in its solubility characteristics, N-terminal amino acid (threonine) and molecular size (9–10,000) it resembles the Type I high-tyrosine proteins of mammalian keratins (Gillespie 1972).

A solubility curve of the claw proteins as a function of ammonium sulfate concentration at pH 6 and 20°C (Fig. 1b) showed that these proteins are relatively insoluble, with precipitation commencing at 8% of saturation and being complete by 50%. There is a very pronounced change in slope near 30% saturation, indicative of the presence of groups of proteins of markedly different solubility properties. At 30% saturation the precipitated protein contained 2/3 of the optical density but only 1/3 of the mass of protein, a reflection of the very different extinction coefficients ($E_{1cm}^{1\%}$ 280 nm) of the proteins in the precipitate (35) and supernatant (7.9). As expected these fractions showed large differences in aromatic amino acid content (Table 3) but there were also differences in many other amino acids, especially SCMC, glutamic acid, valine and leucine. A comparison (Fig. 2) by SDS polyacrylamide gel electrophoresis showed that the precipitate fraction contained some minor constituents together with the fastest moving component found in the unfractionated material (SDS Component 3) and the supernatant fraction, SDS Components 1 and 2, with remarkably little cross-contamination between them. The supernatant fraction was com-

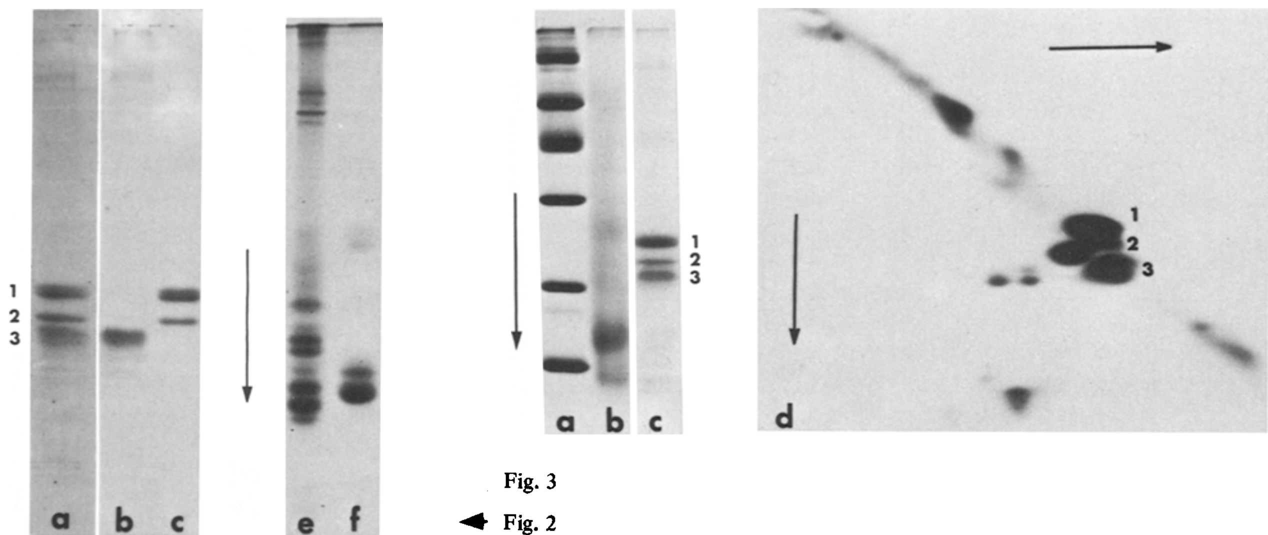

Fig. 2. Electrophoresis of claw proteins (SCM form) and ammonium sulfate fractions in polyacrylamide gels. a-c In presence of SDS at pH 8.9. 15% gel. a Unfractionated claw protein. b Ammonium sulfate precipitate fraction. c Ammonium sulfate supernatant fraction. e-f In urea at pH 8.9. 10% gel. e Precipitate fraction. f Supernatant fraction

Fig. 3. Electrophoresis of claw proteins in polyacrylamide gels. a-c In presence of SDS at pH 7. 10% gel. a Molecular weight standards x 10^3. Reading upwards: 14.4, 20, 30, 43, 67, 94. b Claw proteins in –SH form. c Claw proteins in –SCM form. d Two-dimensional electrophoresis of proteins in –SCM form. First dimension (horizontal). In 8M urea at pH 8.9. Second dimension (vertical). In SDS at pH 8.9

paratively free of minor components and continued to show only two major bands when run in polyacrylamide gel in urea at pH 8.9 (Fig. 2f). In contrast, the precipitate fraction under these conditions showed the presence of about 20 components (Fig. 2e).

The proteins were also fractionated by the addition of zinc acetate to a final concentration of 0.02M at pH 6.0 (Gillespie 1960). Amino acid analysis (Table 3) and polyacrylamide gel electrophoresis of the precipitate and supernatant fractions showed them to be similar to the 30% ammonium sulfate precipitate and supernatant fractions respectively.

Molecular Size

The molecular sizes of the claw proteins, both in the reduced and *S*-carboxymethyl forms were measured by electrophoresis in SDS polyacrylamide gels which had been calibrated by running proteins of known molecular weights (Fig. 3a). It was found that in the reduced form the major components (Fig. 3b) had apparent molecular weights between 11 000 and 14 000, slightly smaller than but comparable in size with the proteins of avian beak and claw (Frenkel and Gillespie 1976). Six minor components ranged in molecular weights from about 12 000 to 70 000 and some material was too large to enter the gel. Conversion of the reduced proteins to the *S*-carboxymethyl form increased the apparent molecular weights, with respect to the standard proteins, to between 20 000 and 30 000, a similar change to that observed following the alkylation of reduced feather and beak proteins (Frenkel and Gillespie 1976) (Fig. 3c). With the information available from these single dimension gels it is not possible to make a certain identification between the bands in the reduced protein and those of the *S*-carboxymethylated proteins. It can be concluded that the major claw proteins show only a moderate degree of heterogeneity of size.

The molecular weights of two of the three major components which are resolved in SDS gel electrophoresis have also been measured by ultracentrifugation. The slowest moving of the three components (SDS Component 1) was prepared from the 30% ammonium sulfate supernatant fraction by chromatography on DEAE-cellulose in the absence of urea at pH 7.5 under a gradient of 0–1M NaCl (Fig. 4a). Four peak tubes (81–82, Fig. 4a) containing relatively pure SDS Component 1 were concentrated and rechromatographed on DEAE-cellulose under a shallower gradient of NaCl. Protein in the peak tube (101, Fig. 4b) was essentially free of charge heterogeneity at pH 8.9 (Fig. 4c). In several equilibrium ultracentrifuge runs at speeds from 36 000 to 44 000 rpm it was found to be predominantly one species with a molecular weight close to 12 900.

The fastest moving component (SDS Component 3) was isolated from the 30% ammonium sulfate precipitate fraction by chromatography on DEAE-cellulose in 8M urea ap pH 7.5 under a gradient of 0.2–0.5M NaCl (Fig. 5a). Unless urea was included in the buffer less than 10% of this protein eluted from the column. The contents of the peak tubes (35–38, Fig. 5a) were dialysed, freeze-dried and chromatographed on G100

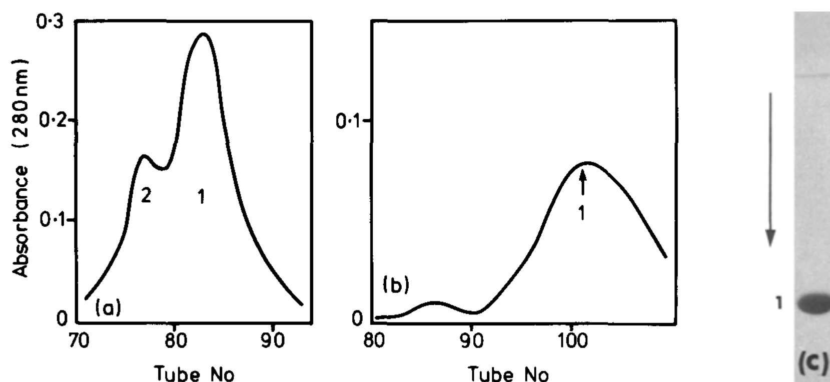

Fig. 4. a–c

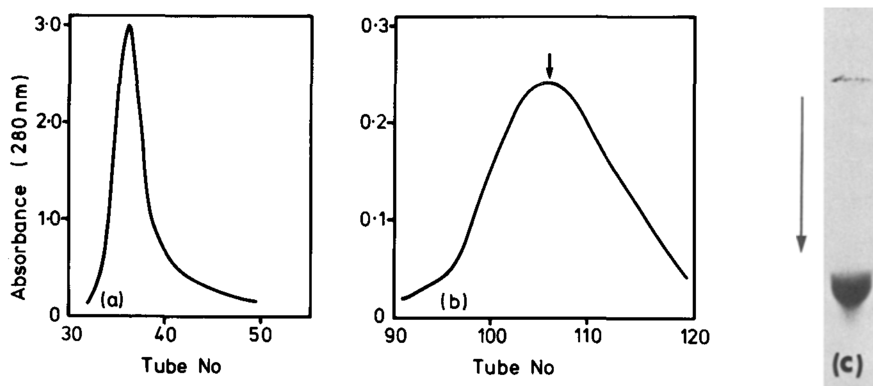

Fig. 5. a–c

Fig. 4. **a** Chromatography of 30% ammonium sulfate supernatant proteins on DEAE-cellulose at pH 7 (no urea) under a gradient of 0-1M NaCl in 0.05 I imidazole-HCl buffer. **b** Chromatography of SDS-Component 1 (tubes 81–84: Fig. 4a) at pH 7 (no urea) under a gradient of 0.2 to 0.5M NaCl in 0.05 I imidazole-HCl buffer. **c** Electrophoresis of purified Component 1 (tube 101: Fig. 4b) on polyacrylamide gel (10%) in 8M urea at pH 8.9

Fig. 5. **a** Chromatography of 30% ammonium sulfate precipitate fraction at pH 7.5 in 8M urea on DEAE-cellulose under a gradient of 0.2–0.5M NaCl in 0.05 I imidazole-HCl buffer. **b** Chromatography of SDS-Component 3 (tubes 35–38: Fig. 5a) on G100 Sephadex in 8M urea–0.05 I imidazole-HCl buffer at pH 7.5. Tube 106 used for molecular weight determination. **c** Electrophoresis of purified SDS Component 3 (tube 105: Fig. 5b) on polyacrylamide gel (15%) in presence of SDS at pH 8.9

Sephadex. The elution profile (Fig. 5b) consisted of one large peak centered on tube 106 and several smaller peaks (not shown) representing species of higher and lower molecular weights. The proteins in tube 105, after dialysis and concentration, was shown by SDS polyacrylamide gel electrophoresis to contain only SDS Component 3 (Fig. 5c). The contents of the adjacent tube (106) were used, without concentration but after dialysis against an 8M urea buffer, for an equilibrium ultracentrifuge determination of molecular weight, using a speed of 52 000 rpm. Some molecular weight heterogeneity was evident in the ultracentrifuge with values ranging from 11 500 to 14 500 from the top of the solution column to the base of the centrifuge cell. The average value over the whole cell was 13 000 and since only one band is evident in the grossly overloaded SDS gel (Fig. 5c) this value of molecular weight should be representative of the main species.

Charge Heterogeneity

The chromatographic procedure outlined in Figure 4a was used to prepare SDS Component 1 (tubes 81–84) and SDS Component 2 (tubes 76–78), both preparations were relatively homogeneous both with respect to size (Fig. 6a,b) and charge (Fig. 6d,e). Amino acid analysis showed (Table 3) that they are both low-tryptophan proteins with very similar compositions probably differing by no more than one residue/mole each of threonine, serine, tyrosine, phenylalanine, histidine and arginine and two residues of SCMC. Both have a blocked N-terminal amino acid. The faster mobility of Component 1 compared with Component 2 at pH 8.9 is probably due to its lower content of arginine and higher content of SCMC, assuming of course that the amide content of the two proteins are the same. The lower mobility of Component 1 in SDS gel electrophoresis is proba-

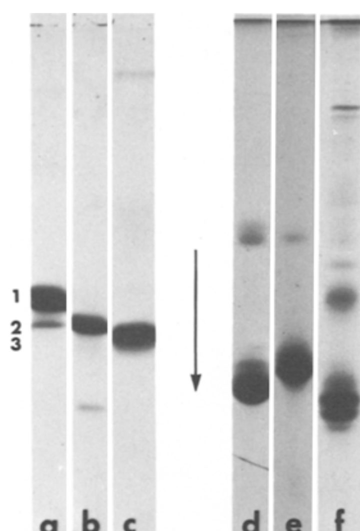

Fig. 6. Electrophoresis of claw protein fractions on polyacrylamide gels. a-c In presence of SDS at pH 8.9. 15% gel. d-f In 8M urea at pH 8.9. 10% gel. a,d SDS Component 1. (Tubes 81–84: Fig. 4a). b,e SDS Component 2. (Tubes 76–78: Fig. 4a). c,f SDS Component 3. (Tubes 35–38: Fig. 5a)

bly also due to its higher content of SCMC, a residue known to influence the movement of sulfur-rich proteins in SDS gels.

We have already seen (Fig. 2e,f) that the greater part of the charge heterogeneity observed in electrophoresis at pH 8.9 of the claw proteins is in the tryptophan-rich SDS Component 3, there being about 5 major and perhaps 15 minor components (Fig. 6f). The nature of this heterogeneity will be the subject of a later investigation.

The proteins were also separated by two-dimensional polyacrylamide gel electrophoresis, the initial separation in 8M urea at pH 8.9 being followed by SDS electrophoresis (Fig. 3d). Many of the 20 resolved components fall upon a diagonal but the molecular basis of this phenomenon is unknown. In the figure the three major spots are numbered according to the nomenclature of Figure 3d and it can be seen that the relative mobilities of SDS Components 1 and 2 are reversed at pH 8.9. In the two-dimensional system, the proteins were labelled with ^{14}C and located by fluorography hence it is not surprising the relative proportions of proteins are quite different to those expected from the one-dimensional electrophoresis where the proteins are stained by Coomassie dye.

Discussion

Two of the three major claw proteins (Components 1 and 3) of *V. gouldii* claw have similar molecular weights, around 13 000, as determined in the ultracentrifuge yet have quite different mobilities, and hence apparent molecular weights, in SDS polyacrylamide gel electrophoresis. More reliance should be placed on the ultracentrifuge molecular weights since it is well known that small differences in amino acid composition may have large effects on SDS electrophoresis mobilities and apparent molecular weight. SDS Components 1 and 2 are almost certainly homologous proteins, probably differing by amino acid substitutions at only 4 positions, and the question may be asked as to the likelihood that the proteins which comprise Component 3 form part of the same homologous series. Although only isolation and characterisation of purified components will enable this question to be settled it is unlikely that SDS Components (1, 2) and 3 form a homologous series because of their large differences in amino acid composition (at least 30 residues) and their quite striking dissimilarities in solubility and chromatographic properties.

The major lizard claw proteins are similar in size to those of avian beak and somewhat larger than those of feather. The claw proteins are notable for their high content of glycine (*ca.* 30 residues %) and comparatively high content of half-cystine (12–14%), characteristics which also distinguish them from feather. Although they are similar to avian beak and claw in glycine content they contain significantly more half-cystine and less tyrosine (Frenkel and Gillespie 1976). Glycine-cystine-rich proteins of similar composition and size have also been found in claw keratin from 10 other varanid lizards, one agamid lizard (*Chlamydosaurus kingii*) and one scincoid lizard (*Tiliqua scincoides*), (unpublished observations). It is likely therefore that such proteins are general constituents of lizard claw keratin. A comparison with published amino acid compositions of reptilian epidermal keratins reveals no apparent similarities (Baden et al. 1974; Wyld 1979; Wyld and Brush 1979).

The modified feather-type X-ray diffraction pattern places the major lizard claw proteins within the avian-reptilian group of hard keratins but also indicates a lack of identity with feather (Fraser et al. 1972). The size of the proteins and their high content of glycine suggests that they are more closely allied to avian scale, beak and claw and in the discussion which follows we will assume that they have similar features of molecular architecture to scale, i.e. a section of polypeptide chain of feather-like sequence which provides the X-ray diffraction pattern and other sections of chain which contain much of the glycine in the repeating sequence gly-gly-X (X being tyrosine, phenylalanine or leucine) (Walker and Bridgen 1976). The presence of this repeating sequence should be sought in lizard claw proteins not only because it would firmly establish a close relationship between this keratin and scale and thus provide an insight into its evolutionary origins but it would clearly distinguish it from the mammalian glycine-rich keratin proteins which have the repeating sequence gly-X (X being tyrosine, phenylalanine, serine) and which have only rare occurrences of the sequence gly-gly (Marshall et al. 1980). This reasoning suggests that in spite of their similar contents of gly-

cine and cystine, lizard claw proteins are unlikely to be related to the mammalian Type II high-tyrosine proteins. Further support for this conclusion comes from the smaller size of the latter proteins (M^r 8000), their very high content of aromatic amino acids and the lack of a feather-type X-ray diffraction pattern in keratins such as the quill of the echidna (*Tachyglossus aculeatus*) where they are a major constituent (30–40%) (Gillespie 1972).

The minor pH 7 insoluble component is quite unlike the bulk of the claw proteins, particularly in its lower content of cystine and higher content of tyrosine. In its size, amino acid composition and solubility characteristics it has many similarities with the cystine-poor (Type I) high-tyrosine proteins of mammalian hard keratins. Furthermore like all wool high-tyrosine proteins so far sequenced it has a hydroxyamino acid in its N-terminal position. We have found a number of other minor constituents which in size and composition resemble mammalian keratin proteins (Marshall and Gillespie 1982) and which possibly originate from the minor α-type keratin observed by Rudall (1947) in the predominantly β X-ray diffraction pattern of lizard claw. These results suggest that anapsid reptiles have retained the ability to synthesise α-type hard keratins.

Acknowledgements. The authors wish to thank Dr. J. Wombey, CSIRO Division of Wildlife Research, Canberra and Dr. P.H. Springell, Wildlife Section, Department of the Chief Secretary, Northern Territory for their generous gifts of *V. gouldii* claw, Dr. C.M. Roxburgh for the amino acid analyses, Mr. A.S. Inglis for the end group determination and Miss M.J. Davis for capable technical assistance.

References

Baden HP, Maderson PFA (1970) J Exp Zool 174:225–232
Baden H, Sviokla S, Roth I (1974) J Exp Zool 187:287–294
Bonner WM, Laskey RA (1974) Eur J Biochem 46:83–88
Bradbury JH, Chapman GV, King NLR (1966) Proc Third Int Wool Text Res Conf Paris I:359–364
Davis BJ (1964) Ann NY Acad Sci 121:404–427
Eastoe JE (1967) Composition of collagen and allied proteins. In: Ramachandran GN (ed) Treatise on collagen. Vol I. Academic Press Inc, London: pp 1–72
Fraser RDB, MacRea TP, Rogers GE (1972) Keratins, their composition structure and biosynthesis. Charles C. Thomas, Springfield, Ill
Frenkel MJ, Gillespie JM (1976) Aust J Biol Sci 29:467–479
Gillespie JM (1960) Aust J Biol Sci 13:81–103
Gillespie JM (1972) Comp Biochem Physiol 41B:723–734
Harrap BS, Woods EF (1964) Biochem J 92:8–18
Inglis AS, McMahon DTW, Roxburgh CM, Takayanagi H (1976) Anal Biochem 72:86–94
Laemmli UK (1970) Nature 227:680–685
Marshall RC, Gillespie JM (1976) Aust J Biol Sci 29:1–10
Marshall RC, Gillespie JM (1982) Comp Biochem Physiol (in press)
Marshall RC, Gillespie JM, Inglis AS, Frenkel MJ (1980) Proc 6th Int Wool Text Res Conf Pretoria II:147–158
Roark DE, Yphantis DA (1969) Ann NY Acad Sci 164:245–278
Rudall KM (1947) Biochim Biophys Acta 1:549–562
Sastry LVS, Ramachandran LK (1965) Biochim Biophys Acta 97:281–287
Sengel D, Dhouailly D, Mauger A (1980) Region-specific determination of epidermal differentiation in amniotes. In: Spearman RIC, Riley RA (eds) The skin of vertebrates. Linnean Society Symposium Series No. 9. Academic Press Inc, London, pp185–197
Walker ID, Bridgen J (1976) Eur J Biochem 67:283–293
Weber K, Osborn M (1969) J Biol Chem 244:4406–4412
Wyld JA (1979) The characteristics of reptilian keratins: an analysis of the molecular events associated with the evolution of the vertebrate epidermis. Thesis, Univ Conn, Storrs
Wyld JA, Brush AH (1979) J Mol Evol 12:331–347
Yphantis DA (1964) Biochemistry 3:297–317

Received July 15, 1981/Revised November 20, 1981