A SEARCH FOR A SIMPLE KERATIN—THE PRECURSOR KERATIN PROTEINS FROM COW'S LIP EPIDERMIS

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Abstract

In searching for a material which might contain a homogeneous keratin protein as a major constituent, an examination was made of a soluble keratin precursor from cow's lip epidermis. Formic acid was used as extractant. Assessment of homogeneity was based on behaviour on DEAE-cellulose and on a consideration of the radioactively labelled peptides in a size–charge peptide map of [^{14}C]*S*-carboxymethylcysteine-containing peptides from an enzyme digest. It is concluded that there is no major homogeneous keratin precursor in cow's lip epidermis and that a family of closely related proteins is present. The situation resembles that found in another α-keratin, wool. There is evidence for the existence of a fraction, amounting to 6–8% of the total protein, of higher sulphur content than the remainder of the protein both in the soluble precursor protein and in the stratum corneum.

I. Introduction

Evidence to date suggests that the microfibrillar (low-sulphur) and the matrix (high-sulphur) proteins, the two major groups present in the α-keratin, wool, are heterogeneous and consist of families of proteins (Gillespie 1963; Thompson and O'Donnell 1967; Joubert, De Jager, and Swart 1968; O'Donnell 1969). In an attempt to understand the reason for this heterogeneity and to see if it is a general characteristic of keratins, analogous perhaps in some ways to heterogeneity of the immunoglobulins (cf. Porter 1967) a study has been commenced of keratins other than those found in wool. This has been aimed at answering the general question "Is there a major homogeneous keratin protein present in nature or do epidermis and its appendages all contain families of closely related proteins?" To prove homogeneity is difficult without determining the complete amino acid sequence and so size–charge peptide map radioautographs of labelled *S*-carboxymethylated cysteine peptides have been relied on to provide indications of heterogeneity. Cysteine peptides were chosen because of the findings of a large degree of intra- and interspecies evolutionary stability around these residues in the immunoglobulins (cf. Edelman and Gall 1969; Frangione, Milstein, and Pink 1969; Fruchter *et al.* 1970; O'Donnell, Frangione, and Porter 1970), and hopefully that a similar situation exists in the keratins, and also because the cysteine residues are readily labelled with radioactive iodoacetic acid. The findings with precursor keratin proteins from cow's lip epidermis are reported here.

Rudall (1952, 1968) gave the name epidermin to a soluble keratin precursor protein present in relatively large amount in the epidermis of cow's lip or nose and which could be dissolved from it by concentrated solutions of urea. He found that it gave an α-type X-ray diffraction pattern and therefore he classified it with the α-keratin family whose members include disulphide cross-linked materials such as wool, hair, and

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porcupine quill (cf. also Carruthers *et al.* 1955; Roe 1956). The presence of epidermin in relatively large amount in cow's lip epidermis and the fact that it can be extracted in the absence of alkaline reducing agents, necessary when one is working with hardened (disulphide cross-linked) keratins, makes it an attractive material to study some aspects of keratin biosynthesis (cf. Rudall 1952; Matoltsy 1965). Rogers (1964) and Fraser (1969) have previously worked with a keratin precursor isolated from wool roots.

II. Materials and Methods

(*a*) *Extraction of Cow's Lip*

A lip from a freshly slaughtered cow was scraped with a scalpel to remove much of the underlying collagen. 1·0 g (wet wt.) was then stood at room temperature for 3–6 hr with 6 ml 98–100% formic acid (A.R.). 1 ml of the extract (equivalent to 0·16 g of original lip and containing approximately 23 mg of protein) was then dialysed versus buffer containing 8M urea–0·01M Tris–0·001M versene at pH 7·4 using 18/32 cellulose tubing until the pH was 4–5. 50 μl of a stock solution of [2-^{14}C]iodoacetic acid (Radiochemical Centre, Amersham, England) containing 1 mCi/ml (activity 21·1 mCi/mmole) was added and the pH adjusted to 8·5 where it was kept for 15 min. To ensure complete reduction of all disulphide bonds present the material was then completely reduced (cf. O'Donnell and Thompson 1964). The pH was adjusted to 10·5 with 20 μl 5N potassium hydroxide and 10 μl mercaptoethanol and the solution allowed to stand for 1–2 hr at 37°C. This ensured complete reduction of any disulphide bonds present. Alkylation was then done with 0·15 ml 3M Tris buffer (pH 8·5) and 50 μl 3M iodoacetic acid. (Probably this reduction is not necessary, there being only –SH groups present in soluble epidermin and cold alkylation could follow the hot alkylation with [^{14}C]iodoacetic acid). After 10 min the nitroprusside test was used to ensure that a slight excess of mercaptoethanol was present. The solution was then loaded on a column of Sephadex G200 in buffer containing 8M urea at pH 10·1. The column was operated at this pH to avoid carbamylation of protein groups with cyanate present in the urea (Thompson and O'Donnell 1966). In some experiments designed to look for small peptides containing *S*-carboxymethylcysteine (SCMCySH), the extract of cow's lip in formic acid was not dialysed but freeze-dried to remove the formic acid before alkylation with radioactive iodoacetic acid. The stratum corneum remaining was extracted with fresh lots of formic acid over another 2–3 hr until no more protein dissolved. It was then freeze-dried.

(*b*) *Solubilization of Stratum Corneum*

The freshly freeze-dried stratum corneum (80 mg) was completely reduced in buffer containing 8M urea as described above and then dialysed versus 0·4N trichloroacetic acid, and finally water to remove reagents before alkylation with 100 μl of the stock [2-^{14}C]iodoacetic acid (cf. Thompson and O'Donnell 1965). It was then given another complete reduction followed by cold alkylation with iodoacetic acid as described above. Approximately 60% of the stratum corneum dissolved.

(*c*) *Reduction and Carboxymethylation of Standard Proteins*

^{14}C-labelled SCM-lysozyme and SCM-ovalbumin were made under conditions described for stratum corneum. For 25 mg of protein 25 μl of [2-^{14}C]iodoacetic acid solution [see Section II(*b*)] was used.

(*d*) *Liquid-scintillation Counting*

Liquid-scintillation counting of radioactively labelled proteins was done with a Tri-carb liquid scintillation spectrometer. The liquid scintillation solution consisted of 50–100 μl column effluent (no more than 50 μl of 8M urea buffer could be tolerated), 0·40–0·45 ml water, and 5 ml of scintillation mixture (Butler 1961). Counting was generally for a period of 1 min.

(*e*) *Digestions with Enzymes*

These were carried out for 8 or 16 hr at 37°C in 2% ammonium bicarbonate at pH 8·4. Trypsin and chymotrypsin were added to the solution at the same time. The digests were then either stored frozen or loaded straight onto a column of Sephadex G50.

(*f*) *Preparation of Size–Charge Peptide Maps*

The tryptic–chymotryptic digests of the radioactively labelled protein were fractionated on a column (130 by 0·9 cm diam.) of Sephadex G50 with 0·05M ammonium bicarbonate, which contained toluene as a preservative, at pH 8·5. Fractions of approximately 2·5 ml were collected by drop counting. These tended to vary in size from month to month as the ambient temperature underwent seasonal variation. After radioactive counting of 50- or 100-μl aliquots of these fractions, the required tubes were dried down to remove ammonium bicarbonate and then 25 μl of water was added. Fractions were run on Whatman No. 1 paper (using high-voltage paper ionophoresis at both pH 6·5 and 3·5).

(*g*) *Radioautography*

Radioautography with Kodak BB-54 X-ray film was carried out for 24–48 hr after paper electrophoresis. With starch gels radioautography was carried out for 2–3 weeks.

(*h*) *Oxidation with Performic Acid*

For oxidation of stratum corneum with performic acid 45 mg protein were reacted overnight at 0°C with 2 ml performic acid (1 vol. 30% hydrogen peroxide plus 9 vol. 98–100% formic acid and allowed to stand for 1–2 hr at room temperature). The performic acid was removed by dialysis versus water, and further dialysis against 0·1N ammonia extracted the proteins (cf. O'Donnell and Thompson 1962). Approximately 70% of the stratum corneum dissolved. The soluble proteins were lypholized, dissolved in 1 ml buffer containing 8M urea at pH 10·1, and fractionated on a column of Sephadex G200 (for conditions used see legend to Figure 1).

(*i*) *Other Methods*

Visking cellulose tubing used for dialysis was extracted with boiling water (Hughes and Klotz 1956).

High-voltage ionophoresis was carried out as described previously (Thompson and O'Donnell 1967). Varsol was used when the tank was operated with buffer at pH 3·5 while toluene was used for operation at pH 6·5.

Urea was deionized before being used in buffer solutions by passage through a bed of mixed ion-exchange resin. When buffers containing 8M urea were used for starch-gel electrophoresis, procedure was as described by O'Donnell and Thompson (1964).

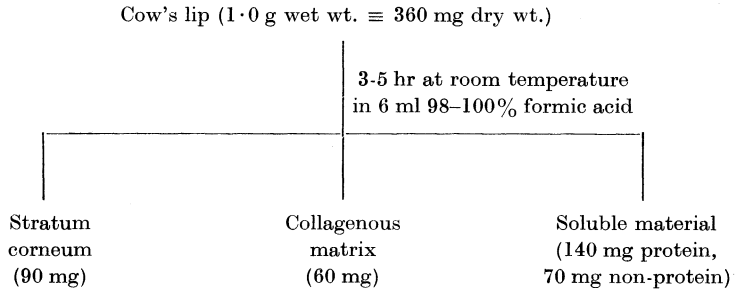
Amino acid analyses were performed with a Beckman model 120C amino acid analyser. Hydrolyses with constant boiling hydrochloric acid were carried out *in vacuo* for 24 hr at 110°C. No correction was made for hydrolytic losses of serine or threonine. Phenol or thioglycollic acid (10 μl of 0·1M solution) were added before hydrolysis to prevent destruction of tyrosine (Sanger and Thompson 1963).

III. Results

The precursor keratin (PK) from cow's lip precipitated very readily in the absence of disaggregating agents and it was not always easy to get to a molecularly dispersed state again. Therefore it was kept in disaggregating medium wherever possible. It seems as if the smearing of some of the bands during starch-gel electrophoresis, particularly during the later stages of handling, was due to this tendency to aggregate.

(a) Extraction of Proteins from Epidermis and Stratum Corneum

The extraction of cow's lip with 98–100% formic acid is summarized below (all dry weights refer to the freeze-dried condition):



Approximately two-thirds of the extracted material was protein (calculated from amino acid analysis). The underlying collagenous material and the outside layer of stratum corneum were easily separated. Mr. J. Bavington of this laboratory showed

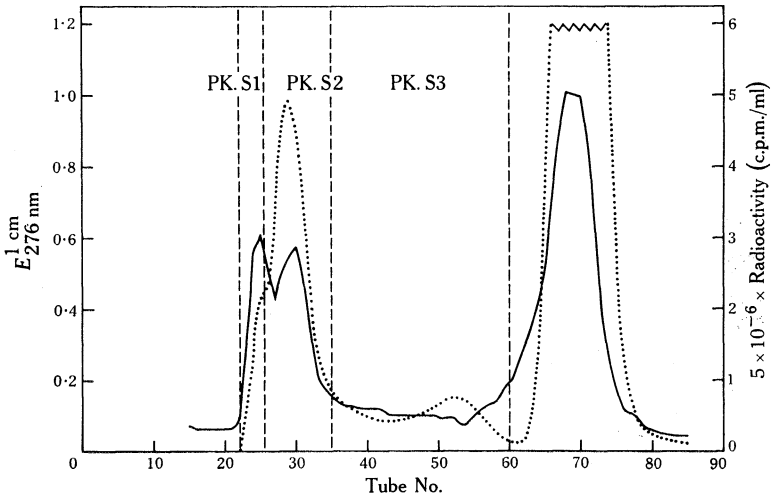


Fig. 1.—Fractionation of *S*-carboxymethylated precursor keratin proteins from cow's lip (0·16 g wet wt.) on a column of Sephadex G200 (120 by 0·9 cm diameter). Buffer was 8M urea–0·05M Tris–0·001M versene–0·1M KCl –0·1M NH_3 at pH 10·1. Fraction size approximately 1·5 ml. E_{276} (solid line) is high in fraction PK.S1 due to opalescence of these tubes. ..... Radioactivity.

that the entire malphigian layer was dissolved, leaving a preparation of stratum corneum almost free from any layers of stratum germanitivum. Repeated treatment of the collagenous matrix removed very little more soluble material. Low proline values in an amino acid analysis suggested that there was very little soluble collagen present. The soluble material thus extracted was, after carboxymethylation, dialysed against buffer containing 8M urea at pH 10·1 and passed through a column of Sephadex G200 (Fig. 1). Three major zones PK.S1, PK.S2, and PK.S3 were seen.

The amino acid compositions of fractions PK.S2 and PK.S3 are listed in Table 1, together with those of a similar material prepared by Matoltsy (1965) who extracted cow's lip with an acid citrate buffer (in an attempt to maintain the molecules in an aggregated "native" state) and fractionated the extract by acid precipitation. The

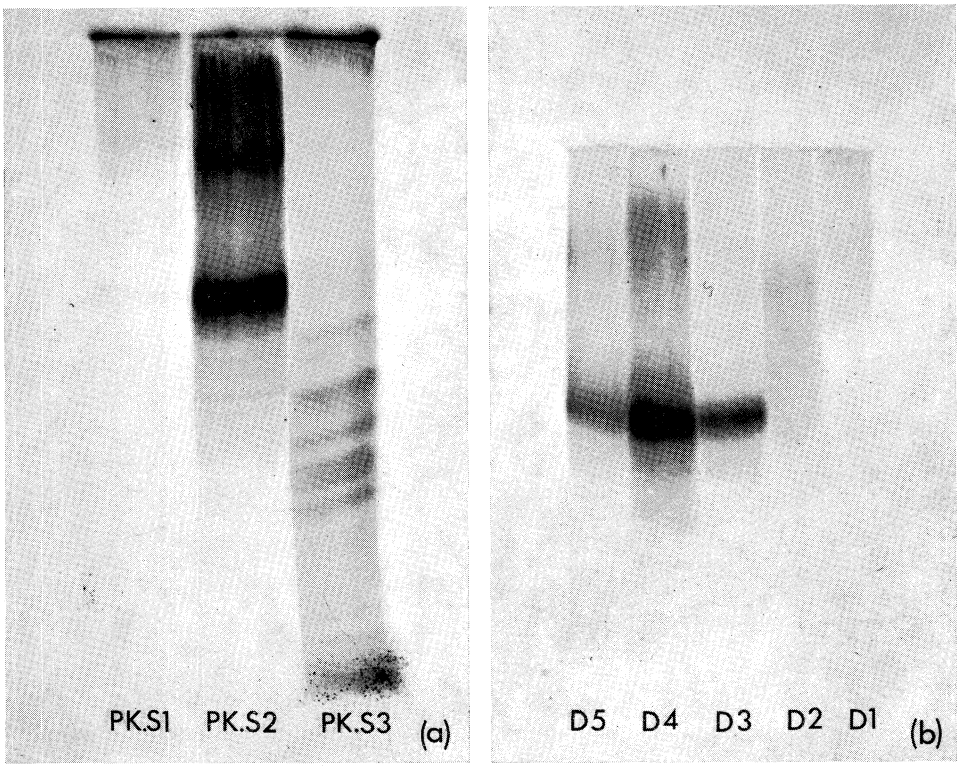
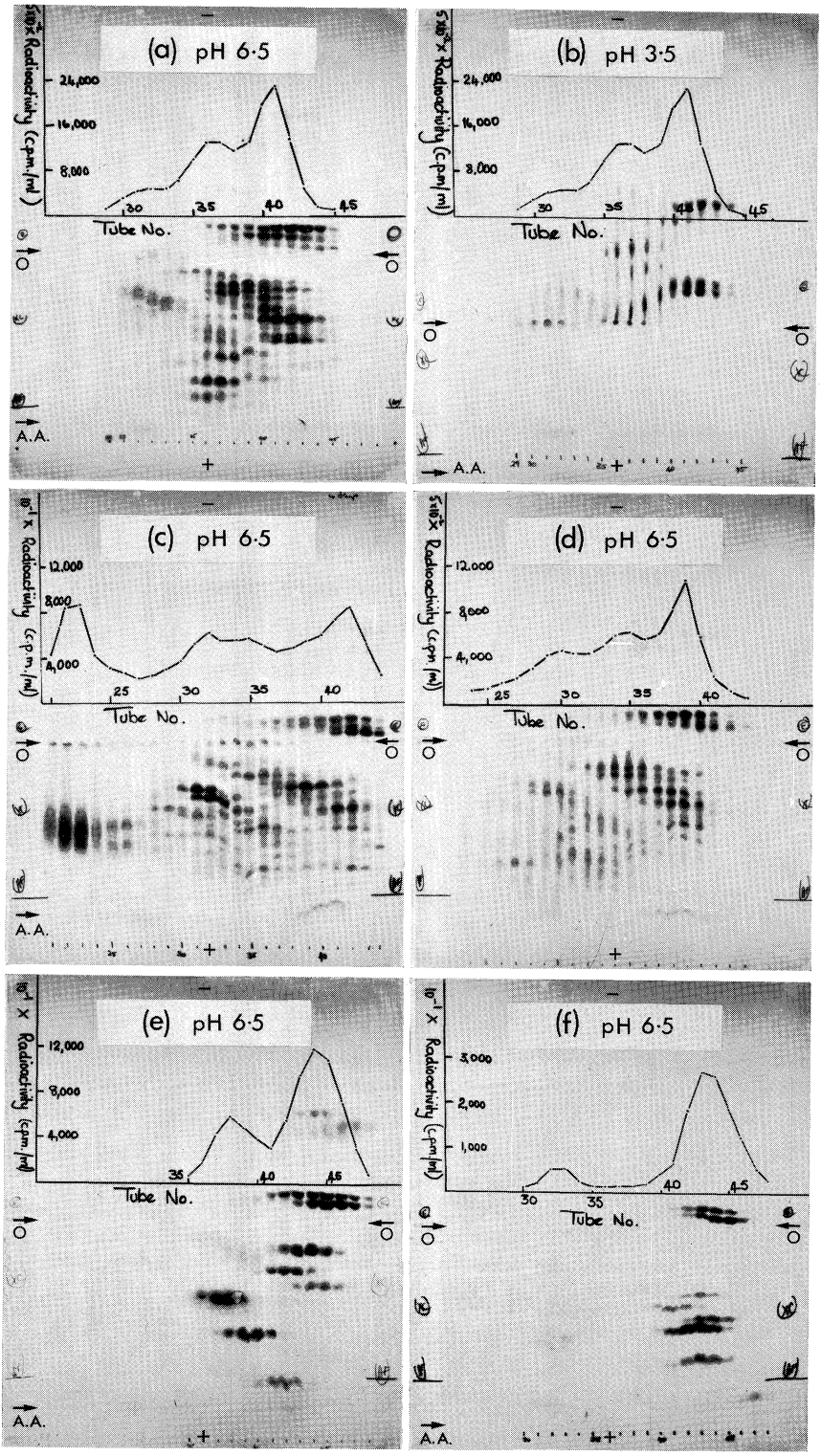


Fig. 2.—(*a*) Starch-gel electrophoresis patterns (radioautographs) of precursor keratin fractions PK.S1, PK.S2, and PK.S3 obtained by passage through Sephadex G200 (Fig. 1). Electrophoresis carried out at pH 8·4 in buffer containing 8M urea. (*b*) Starch-gel electrophoresis patterns (radioautographs) of five cuts D1–D5, separated from precursor keratin fraction PK.S2 (Fig. 1) by gradient elution from DEAE-cellulose (Fig. 4).

composition of whole stratum corneum (SC) and its carboxymethylated SC.S2 and SC.S3 fractions are listed for comparison. Approximately 60% of the stratum corneum dissolved (cf. approx. 44% found for human stratum corneum by Baden and Bonar 1968). The following points may be made:

(1) The major fraction in Figure 1 is peak PK.S2. All pigment material came through the column with fraction PK.S1. The three fractions gave different patterns when submitted to starch-gel electrophoresis [Fig. 2(*a*)]. The peptide map radioautographs of SCMCySH-containing peptides [Figs. 3(*a*) and 3(*c*)] of the fractions PK.S2 and PK.S3 revealed distinct differences. Those of fractions PK.S1 and PK.S2 were indistinguishable although the elution profiles from the Sephadex G50 column were slightly different. Furthermore, the amino acid compositions of peaks PK.S1 and PK.S2 seemed to be identical.


(a) pH 6·5
(b) pH 3·5
(c) pH 6·5
(d) pH 6·5
(e) pH 6·5
(f) pH 6·5
Radioactivity (c.p.m./ml)
Tube No.
A.A.

PK.S2 could be made to aggregate to give a peak running in the position of PK.S1 on starch-gel electrophoresis. The protein component of PK.S1 appears to be mainly an aggregated PK.S2.

(2) PK.S2 isolated from two different cows had indistinguishable peptide map radioautographs and amino acid compositions; the latter resembled Matoltsy's purified material except that the methionine value obtained in the present work was much higher and almost the same as the SCMCySH value.

(3) In the precursor keratin there was a fraction PK.S3, of higher sulphur content (1·27%) than that of the predominant material, PK.S2 (c. 0·7%). The appearance of the starch-gel electrophoresis pattern [Fig. 2(*a*)] and the estimated molecular weight range (c. 20,000–40,000) of this high-sulphur fraction, as judged from the spread and position in the Sephadex column profile (Fig. 1), were similar to those found for the high-sulphur proteins of wool. It is difficult, without further fractionation studies on fraction PK.S3, to assess the percentage of the higher-sulphur fraction in this whole extract of soluble proteins from epidermis. However, in a radioactively labelled sample, PK.S3 had some 20% of the total counts (i.e. of PK.S1+PK.S2+PK.S3), and in a large-scale experiment non-labelled PK.S3 had a protein content of approximately 6–8% of the total extracted protein as determined by amino acid analysis. It was found that the peptide map radioautographs of fractions PK.S3 from two cows were very similar.

(4) Taking a value of 8% (of the total PK.S1+PK.S2+PK.S3) for the content of the higher-sulphur proteins it is seen that the sulphur contents of fractions PK.S1, PK.S2, and PK.S3 did not account for the sulphur content of *c.* 1% of stratum corneum. No small sulphur-containing peptides could be found in the salt peak (Fig. 1) even when the soluble precursor keratins were prepared using a procedure not involving dialysis—i.e. the formic acid extract was freeze-dried and this material was dissolved in urea before carboxymethylation and application to the Sephadex G200 column.

(5) Only –SH groups were present in the precursor keratin fraction. This is suggested by the facts that the same Sephadex profile (Fig. 1) and the peptide map radioautographs [i.e. Figs. 3(*a*) and 3(*b*)] were obtained irrespective of whether the precursor keratins soluble in formic acid were alkylated as extracted or after a complete reduction with alkaline mercaptoethanol. The

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Fig. 3.—Size–charge peptide map radioautographs of the SCMCySH-containing peptides in tryptic–chymotryptic digests of SCM-precursor keratin fractions PK.S2 (*a*) and (*b*) and PK.S3 (*c*), separated on Sephadex G200 (Fig. 1), and of stratum corneum fraction SC.S3 (*d*). The pattern for fraction SC.S2 was identical with that of PK.S2 and is not shown. Similarly obtained peptide map radioautographs for chicken SCM-lysozyme (*e*), with 8 SCMCySH residues per molecule, and chicken SCM-ovalbumin (*f*), with 6 SCMCySH residues per molecule, are shown for comparison with that of fraction PK.S2 (*a*), which has 6–7 SCMCySH residues per molecule. Upper elution patterns in each case are from columns (130 by 0·9 cm diameter) of Sephadex G50 in 0·05M ammonium bicarbonate at pH 8·5. Fraction size approximately 2·5 ml. O, origin. A.A., aspartic acid.

TABLE 1

AMINO ACID ANALYSIS OF WHOLE EXTRACT OF COW'S LIP EPIDERMIS, OF THE STRATUM CORNEUM AND *S*-CARBOXYMETHYLATED SOLUBLE FRACTIONS SC.S2 AND SC.S3 FROM IT, AND OF SOLUBLE *S*-CARBOXYMETHYLATED PRECURSOR KERATIN FRACTIONS PK.S2 AND PK.S3

The fractions were obtained by passage through Sephadex G200 (Fig. 1). Values are given as residues of amino acid per 1000 residues. No correction has been made for destruction of amino acids during hydrolysis *in vacuo* for 24 hr at 110°C. Phenol or thioglycollic acid was added before hydrolysis *in vacuo* to prevent destruction of SCM-cysteine and tyrosine (Sanger and Thompson 1963). Values obtained by Matolsty (1965) for prekeratin and by Baden and Bonar (1968) for human stratum corneum extract and included for comparison. n.d., not determined. ↑ High values. ↓ Low values

Amino Acid	Whole Extract[a]	PK.S2[b]	PK.S3[b]	Prekeratin (Matolsty)	Stratum Corneum[c]	SC.S2	SC.S3	Stratum Corneum Residue[d]	Human Stratum Corneum[e]
Lysine	56·0	53·0	79·3 ↑	49·8	58·0	49·9	58·0 ↑	53·1	50·5
Histidine	11·0	11·4	24·6 ↑	8·3	11·2	9·2	16·0 ↑	10·2	10·9
Arginine	61·3	62·5	43·3 ↓	57·9	52·7	69·3	51·5 ↓	65·6	48·7
Aspartic acid	90·7	90·2	88·7	87·0	87·0	91·3	82·3	87·8	97·5
Threonine	46·4	42·6	46·8	38·1	41·9	45·0	46·5	44·0	45·6
Serine	94·5	91·2	63·8 ↓	115·7	96·5	95·4	91·6	90·5	81·1
Glutamic acid	132·2	131·4	130·3	142·1	138·6	135·9	109·5 ↓	135·2	150·0
Proline	35·0[h]	21·6	71·4 ↑	15·7	26·3	14·3	38·5 ↑	31·2	22·1
Glycine	134·9	141·1	101·9 ↓	163·4	140·8	138·1	165·2	126·6	150·0
Alanine	69·7	69·4	59·0	65·3	65·8	68·7	61·0	66·5	62·1
Valine	53·2	56·6	63·9	50·0	54·4	57·7	60·9	57·9	45·8
Methionine	16·2	16·1	15·5	2·3	15·7[i]	8·9	4·9	15·2[i]	8·5
Isoleucine	40·0	42·0	44·3	40·3	39·8	43·7	44·4	41·8	45·4
Leucine	94·0	92·6	76·7	87·2	87·0	98·5	80·7	94·1	96·9
Tyrosine	31·5	30·1	22·4 ↓	21·5	31·8	33·7	24·9 ↓	32·2	23·1
Phenylalanine	33·1	36·0	35·8	37·1	31·3	30·5	41·0 ↑	30·9	32·5
SCM-cysteine		11·8	31·8 ↑			9·4	22·6 ↑		
½Cystine + cysteine	n.d.			13·7	21·2[j]			20·3[j]	29·1
Tryptophan	n.d.	n.d.	n.d.	4·5	n.d.	n.d.	n.d.	n.d.	n.d.
Unknown amino acid[f]					+				
Sulphur content[g]		0·74	1·27	0·44	1·00	0·48	0·74	0·94	

[a] Single analysis only. [b] Average of duplicate hydrolyses of PK.S2 and PK.S3 from three and two different cows respectively. [c] Average of duplicate hydrolyses of normal and oxidized stratum corneum preparations from three different cows. The only amino acids which varied between the cows were arginine (52·6, 43·3, 62·1) and glycine (134·2, 157·2, 131·0). [d] Average duplicate hydrolyses from extraction of a single stratum corneum preparation using urea–mercaptoethanol as extractant. Approximately 50% stratum corneum dissolved. [e] Values for extract of human stratum corneum and epidermis calculated by averaging (and converting to residues per 1000 residues) the first four columns of Table III of Baden and Bonar (1968). [f] There was a small peak between glutamic acid and proline found regularly in the hydrolysates of stratum corneum. [g] Calculated from amino acid composition and an assumed nitrogen content of 16·6% (and assuming NH_3 value is one-eighth of the amino acid nitrogen total). [h] Value will be too high due to any cysteine present. [i] Determined both as methionine and methionine sulphone. [j] Determined as cysteic acid in separate experiments by the method of Moore (1963).

absence of disulphide cross-links in this precursor keratin agrees with the findings of Rudall (1952). An analogous situation occurs with soluble proteins from hair follicles (Rogers 1964).

(6) A comparison of the peptide map radioautographs of PK.S2, PK.S3, and SC.S3 [Figs. 3(*a*), 3(*c*), and 3(*d*)] suggested that there were common peptides in S2 and S3 fractions from stratum corneum and the precursor keratins.

(7) The elution pattern from Sephadex columns for the soluble proteins from reduced and carboxymethylated stratum corneum was very similar to that for the corresponding precursor keratins in Figure 1. The curve (and amino acid analyses) did not indicate any noticeable increase in the proportion of the higher-sulphur material S3 in the stratum corneum. The peptide map radioautographs from the SC.S2 fraction appeared identical with that of PK.S2, but those of the PK.S3 and SC.S3 showed several differences [Figs. 3(*c*) and 3(*d*)].

(8) The values for the amino acid composition of SC.S2 show much similarity to a related preparation from human stratum corneum and epidermis (Baden and Bonar 1968).

(9) Approximately 70% of stratum corneum oxidized with performic acid dissolved in the extractant, 0·1N ammonia. The elution profile of the soluble material from the same column of Sephadex G200 used in Figure 1 was similar to that found for the reduced and carboxymethylated proteins, three fractions SC.S1 (oxidized), SC.S2 (oxidized), and SC.S3 (oxidized) being obtained. Amino acid analyses of the various fractions are shown in Table 2. It is seen that the analyses of the SC.S1 (oxidized) and the various PK.S2 fractions (Tables 2 and 3) are very similar, but that those of SC.S3 (oxidized) was very different from those of SC.S3 and PK.S3 given in Table 1. The percentage of the total soluble material in fraction SC.S3 (oxidized) was c. 15% compared with the 6–8% for the corresponding material from epidermis and stratum corneum. This suggests that there may have been some bond cleavage in the treatment with performic acid additional to that encountered during the reduction procedure.

(*b*) *Characterization of Precursor Keratin Proteins*

(i) *Molecular Weight.*—By using reduced and carboxymethylated bovine plasma albumin and ovalbumin as calibrating standards on a column of Sephadex G200 in buffer containing 8M urea (Thompson and O'Donnell 1965), the molecular weight of PK.S2 was estimated to be 59,000. Using material from the peak tube of fraction PK.S2 from a Sephadex run (cf. Fig. 1) and the Yphantis high-speed meniscus depletion method (at speeds of 34,000 and 24,000 r.p.m.) M. Pont and E. F. Woods of this laboratory found the molecular weight of the main component to be 67,000. A small amount of aggregated material was present. A value of 0·715 was taken as the partial specific volume of PK.S2 in 8M urea solution.

TABLE 2

AMINO ACID COMPOSITION OF WHOLE STRATUM CORNEUM AND OF THE THREE OXIDIZED STRATUM CORNEUM FRACTIONS

Fractions separated from an ammonia extract of performic acid-oxidized stratum corneum by fractionation on Sephadex G200. The conditions of separation are as for precursor keratin fractions (see legend to Fig. 1) and the elution profile obtained was similar to that of Figure 1. All values are given as amino acid residues per 1000 residues. No correction has been made for any destruction of histidine or tyrosine during the oxidation with performic acid

Amino Acid	Whole Stratum Corneum*	SC.S1 (ox.)†	SC.S2 (ox.)†	SC.S3 (ox.)†	Residue (ox.)†
Lysine	57·2	51·3	51·4	31·7	56·2
Histidine	10·1	9·5	8·5	8·1	11·6
Arginine	62·1	66·2	66·1	33·8	63·1
Aspartic acid	87·9	96·2	96·1	51·6	87·6
Threonine	42·8	43·4	42·6	27·5	41·4
Serine	93·2	92·0	90·7	67·8	85·9
Glutamic acid	135·6	138·7	145·6	74·0	142·5
Proline	27·0	16·1	12·3	17·8	35·6
Glycine	131·0	135·2	130·3	136·1	121·6
Alanine	65·3	72·0	71·5	109·7	64·6
Valine	56·9	59·7	60·5	156·4	58·6
Methionine sulphone	14·7	15·2	15·2	8·4	15·3
Isoleucine	41·9	43·3	44·3	30·9	41·8
Leucine	92·4	97·6	100·8	189·2	94·3
Tyrosine	32·1	17·6	18·2	16·8	24·8
Phenylalanine	29·6	31·0	29·6	23·6	31·6
Cysteic acid	20·2	14·9	16·0	16·3	23·4
Material in each fraction (%)‡	100	23	36	11	30

* Values given are averaged from duplicate hydrolyses on oxidized (Moore 1963) and unoxidized samples of a stratum corneum preparation from one particular cow.

† Values given are averages of duplicate hydrolyses.

‡ Approximate values only, determined from amino acid analyses.

TABLE 3

AMINO ACID COMPOSITION OF CUTS A, B, AND C (SEE FIG. 4) OF PRECURSOR KERATIN FRACTION PK.S2 SEPARATED ON DEAE-CELLULOSE

PK.S2 fraction from a single cow's lip. Conditions of separation are as given in the legend to Figure 4. Values are expressed as residues per 1000 residues and are the average of duplicate hydrolyses. ↑ High values. ↓ Low values

Amino Acid	Peak A	Peak B	Peak C	Amino Acid	Peak A	Peak B	Peak C
Lysine	53·8	52·1	67·1 ↑	Alanine	68·4	66·6	67·2
Histidine	10·4	9·7	15·0	Valine	59·8	58·4	59·2
Arginine	64·2	67·3	68·6	Methionine	12·2	18·4	16·6
Aspartic acid	91·2	97·9	95·5	Isoleucine	50·0	48·8	51·4
Threonine	42·8	42·5	43·7	Leucine	90·6	101·2	100·3
Serine	86·4	98·4	91·2	Tyrosine	26·1	23·2	11·6 ↓
Glutamic acid	119·5	134·2	127·7	Phenylalanine	38·6	31·7	33·7
Proline	26·4	12·0	22·3	SCM-cysteine	11·9	11·6	15·2 ↑
Glycine	147·3	125·8	113·5 ↓				

(ii) *Extinction Coefficient.*—The extinction coefficient ($E^{1\%}_{1cm}$ at 276 nm) of PK.S2 was found to be 7·2 when measured in 50% glacial acetic acid. A value of 16·6% was taken as the nitrogen content of the protein.

(iii) *Fractionation of PK.S2 on DEAE-cellulose.*—Figure 4 shows the profile obtained when PK.S2 was subjected to gradient elution from DEAE-cellulose. [A similar curve was obtained with the corresponding S2 from stratum corneum.] Several peaks are apparent and five cuts—D1, D2, D3, D4 and D5—were taken for comparison by starch-gel electrophoresis [Fig. 2(*b*)]. No marked fractionation has been achieved.

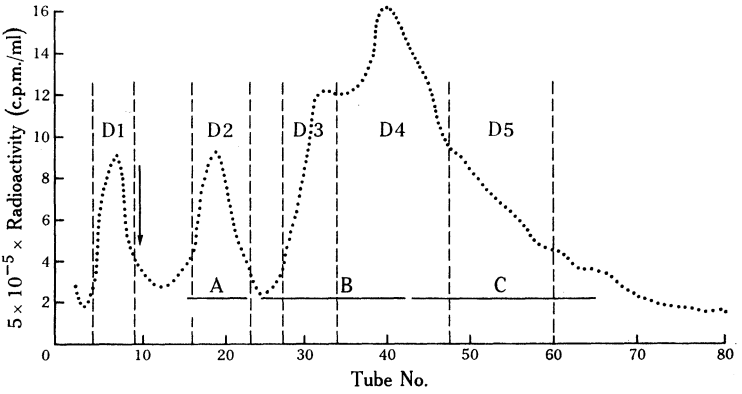

Fig. 4.—Gradient elution of 50 mg of *S*-carboxymethylated precursor keratin fraction PK.S2 (Fig. 1) from a column of Whatman DE52 DEAE-cellulose (18 by 0·9 cm diameter) at room temperature. Fraction size approximately 1·5 ml. Linear gradient with 100 ml on either side of the gradient device. Starting buffer was 8M urea–0·01M Tris–0·001M versene at pH 7·4 and the other buffer contained an additional 0·4M KCl. Gradient applied at arrow. Cuts D1–D5 refer to fractions bulked for examination by starch-gel electrophoresis [Fig. 2(*b*)], and peptide-map radioautographs. In another experiment cuts in positions A, B, and C were taken for amino acid analysis (Table 3).

The amino acid composition of three arbitrary cuts A, B, and C (Fig. 4) were very similar (Table 3), the only sizeable differences being in glycine, SCMCySH, and tyrosine. An inverse relation of SCMCySH and glycine has been suggested by Crounse (1966) for hair and epidermal proteins. The peptide map radioautographs of tryptic–chymotryptic digests of the five cuts, D1–D5, were qualitatively indistinguishable from the unfractionated PK.S2 [Fig. 3(*a*)], though they varied in relative intensity particularly in the region of tubes 30–35 as can be judged from the profiles of the radioactive peptides eluted from Sephadex G50 (Fig. 5). The possibility of unwanted carbamylation, by cyanate present in concentrated urea solutions, as an artefactual cause of heterogeneity was eliminated by minimum contact of protein with urea in the neutral pH region and by completing the DEAE-cellulose runs in 3–4 hr.

(*c*) *Comparison of Peptide Map Radioautographs of PK.S2 with those of Standard Homogeneous Proteins*

A comparison of the peptide map radioautographs of tryptic–chymotryptic digests of fraction PK.S2 (6–7 SCMCySH residues per molecule) [Fig. 3(*a*)] with those from the homogeneous proteins chicken SCM-lysozyme (8 SCMCySH residues per

molecule) and chicken SCM-ovalbumin (6 SCMCySH residues per molecule—Smith and Back 1970) [Figs. 3(*e*) and 3(*f*) respectively] shows that that of the PK.S2 fraction was more complex than the others.

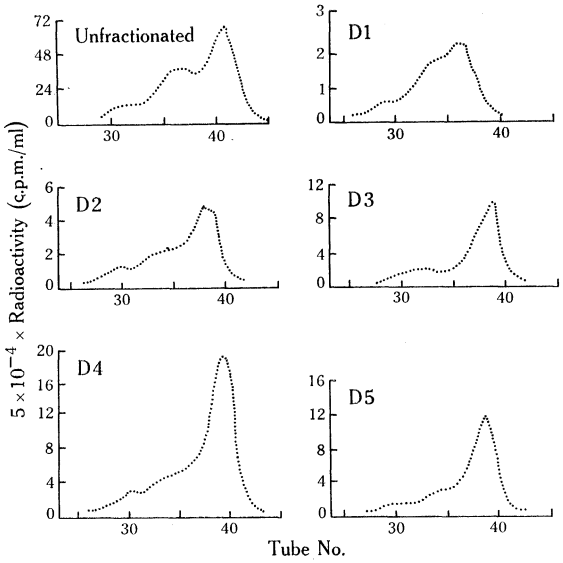

Fig. 5.—Elution profiles of tryptic–chymotryptic digests of cuts D1–D5 [Figs. 2(*b*) and 4] from fractionation of PK.S2 on DEAE-cellulose. Columns of Sephadex G50 were used as described in Figure 3.

IV. Discussion

It appears that the precursor keratin fraction, PK.S2, isolated in the present work is the same as that referred to as epidermin by Rudall (1952) and prekeratin by Matoltsy (1965). Rudall used concentrated urea as an extractant while Matoltsy used a sodium citrate buffer.

The use of 98–100% formic acid for the extraction of soluble proteins from cow's lip epidermis has some attractive features in that it is quick (2–3 hr) and the formic acid should effectively prevent any proteolysis by cell enzymes as the tissue is broken up. Thus one would expect that there would be no artificially induced heterogeneity. Furthermore disulphide interchange is prevented and the original state of the –S–S– and –SH groups should be preserved. It seems as if they are all in the –SH form in the precursor keratin. Further advantages of formic acid are that it is a good disaggregating medium and it leaves a clean stratum corneum. Any amino terminal groups are free from danger of carbamylation which is present when urea solutions at around neutral pH values are used for extraction of epidermin (cf. Rudall 1952). Formic acid probably leads to an unfolding of the protein molecules and, if it is hoped to maintain the soluble epidermin in its "native or aggregated" state, it is probably better to work along the lines of Matoltsy (1965) using acid citrate buffers.

The three bands PK.S1, PK.S2, and PK.S3, visible on starch-gel electrophoretic patterns of *S*-carboxymethylated precursor keratins and able to be isolated using Sephadex G200 and DEAE-cellulose, correspond to the aggregate, low-sulphur, and

high-sulphur fractions found when working with another α-keratin, wool (cf. O'Donnell and Thompson 1964). PK.S2 probably represents the "low-sulphur" fraction and PK.S3 the "high-sulphur" fraction, using the vocabulary developed for wool and other fibrous α-proteins (cf. Crewther *et al.* 1965). However, the sulphur contents of these fractions (Table 1) of *c.* 0·7% (for PK.S2) and *c.* 1·3% (for PK.S3) were much lower than the corresponding values of *c.* 2% and *c.* 5% for wool (cf. Gillespie 1965). The value of *c.* 0·7% for PK.S2 agrees with the values obtained by Rudall (1952) for his fibrous "fraction" of urea-extracted epidermis. However, the higher-sulphur fraction PK.S3 described here had a lower sulphur content (*c.* 1·3%) than Rudall (1952) found for his non-fibrous (high-sulphur) fraction (1·13–2·86%). No obvious reason can be seen in order to explain this discrepancy, but it may be pointed out that the present values were calculated from the methionine and SCMCySH values in hydrolysates while Rudall's values are from elementary sulphur analysis. Likewise no reason can be given for the fact that the calculated sulphur contents for fractions SC.S2 and SC.S3 (prepared from stratum corneum) are lower than the corresponding ones from epidermis; the lower methionine values are the main contributing factor.

Fraction PK.S3 showed several bands when subjected to starch-gel electrophoresis [Fig. 2(*a*)] and in this way resembles the higher-sulphur fraction from wool (O'Donnell and Thompson 1964) as also did its estimated molecular weight range of 20,000–40,000. This fraction PK.S3 (and SC.S3 from stratum corneum) represents 6–8% of the total protein extracted compared with the values of approximately 20–30% found for wool (Gillespie 1965). In the high- and low-sulphur fractions from wool the characteristic difference is that the high-sulphur fraction has higher contents of SCMCySH, proline, serine, and threonine and lower contents of lysine, histidine, aspartic acid, and the aromatic amino acids and no methionine. Such differences are not paralleled in the precursor keratins or the stratum corneum fractions; the biggest differences are shown by the vertical arrows in Table 1. Matoltsy and Parakkal (1967) have also given evidence for the existence of a high-sulphur fraction in the precursor keratin material.

The amino acid composition of PK.S2 was, except for the methionine value, very similar to a corresponding preparation made by Matoltsy (1965) using an acid citrate buffer as extractant (Table 1). It is seen (Table 4) that the amino acid composition of PK.S2 bore no obvious resemblance to component 8, a low-sulphur protein of wool nor to feather rachis extracts (Harrap and Woods 1967); relatively high glycine and methionine contents and low SCMCySH content are the characteristics of the epidermin (PK.S2). In view of the low sulphur content of PK.S2 it was thought that it may resemble the helical section of the low-sulphur proteins from wool (Crewther *et al.* 1968) which only contain a few SCMCySH residues. Inspection of Table 4 shows this not to be so.

The precursor keratin fraction present in major amount, PK.S2 (Fig. 1), had a molecular weight between 59,000 (calibrated Sephadex column) and 67,000 (ultracentrifuge). A value of 63,000 has been taken as most likely to be near the true value. This compares with 60,000 (sedimentation velocity and diffusion coefficient) obtained by Mercer and Olofsson (1951) on a urea-extract of cow's lip epidermis. These workers used sodium bisulphite to break any –S–S– bonds present in their material. The value is higher than the 49,000 unit that Crounse (1966) estimated to be in Matoltsy's "native"

pre-keratin (from Matoltsy's values for its methionine content) and the value of 50,000 obtained by Crounse for material prepared using 0·02N NaOH for 24 hr at 22°C when extracting proteins from human plantar callus keratin; similar alkaline conditions have been previously shown to increase the ninhydrin colour of protein solutions presumably by breakage of peptide bonds (O'Donnell and Thompson 1962).

There is no marked difference between the compositions of fraction S2 prepared from precursor keratins and stratum corneum. That the tryptic–chymotryptic peptide map radioautographs of PK.S2 and SC.S2 were also indistinguishable from each other

TABLE 4

COMPARISON OF AMINO ACID COMPOSITIONS OF PRECURSOR KERATIN FRACTION PK.S2 WITH SOME OTHER KERATINS

Values are expressed as residues of amino acid per 100 residues. n.d., not determined

Amino Acid	PK.S2	Feather Keratin*	Component 8†	Helical Fraction of SCMKA‡
Lysine	53	5	31	59
Histidine	11	3	6	8
Arginine	62	35	79	68
Aspartic acid	90	59	108	107
Threonine	43	34	52	36
Serine	91	129	79	66
Glutamic acid	131	49	176	218
Proline	22	107	36	11
Glycine	141	160	45	28
Alanine	69	76	62	79
Valine	57	71	64	53
Methionine	16	0·4	4	<2
Isoleucine	42	39	37	40
Leucine	93	86	115	138
Tyrosine	30	22	26	34
Phenylalanine	36	35	22	19
SCM-cysteine (or ½Cys)	12	84	57	35
Tryptophan	n.d.	4	n.d.	n.d.

* From goose rachis. Values calculated from Table 2 of Harrap and Woods (1967).

† A low-sulphur protein from wool. Values calculated from Table 6 of Thompson and O'Donnell (1967).

‡ A low-sulphur protein from wool. Values taken from Table 1 of Crewther *et al.* (1968).

suggests strongly that PK.S2 undergoes no major change, apart from conversion of –SH groups to –S–S– groups, in its passage to, and conversion into, stratum corneum. In the case of the higher-sulphur fractions, PK.S3 and SC.S3, both of these criteria indicate substantial differences between the corresponding fractions prepared from precursor keratin and stratum corneum [Table 1; Figs. 3(*c*), 3(*d*)]. It thus seems that the higher-sulphur fraction of the precursor keratin undergoes substantial changes in its passage to the stratum corneum. This picture could be complicated somewhat if

there are cytoplasmic proteins in the precursor keratin which are withdrawn as the stratum corneum is approached (as suggested by Downes *et al.* 1966) for the wool root system. However, the fact that the peptide map radioautographs are only concerned with peptides originally containing half-cystine residues indicates that this complication would probably be a minor one; furthermore, the fact that the amino acid composition of the whole extract (column 1, Table 1) is so similar to that of PK.S2 does not indicate the presence of a substantial proportion of proteins other than keratins. The similarities in low-sulphur proteins (PK.S2 and SC.S2) and differences in higher-sulphur proteins (PK.S3 and SC.S3) found among the corresponding proteins in precursor keratin material and stratum corneum from cow's lip epidermis are analogous to those found in the wool root protein–wool system (Fraser 1969).

Comparison of the peptide maps of the low-sulphur (PK.S2) and higher-sulphur (PK.S3) fractions shows [Figs. 3(*a*) and 3(*c*)] that there is a probability that some of the radioactive peptides in the two fractions are identical.

It appears that fraction PK.S2 is not a homogeneous protein preparation but probably consists of a mixture of similar proteins. The overall similarity of amino acid compositions of subfractions of PK.S2 separated on DEAE-cellulose (Fig. 4) and the qualitative similarity and quantitative differences in the peptide maps of the SCMCySH-containing peptides (cf. Fig. 5) support this hypothesis. The existence of approximately 9 methionine residues per 63,000 g of PK.S2 suggest that these could be utilized to obtain fragments to see if the differences resided more in some sections of the molecule than in others. Furthermore, there is additional complexity obvious in the peptide map radioautograph of PK.S2 [Fig. 3(*a*)] when compared with those from the corresponding homogeneous proteins SCM-lysozyme and SCM-ovalbumin with approximately the same number of SCMCySH residues per molecule [Figs. 3(*e*), 3(*f*)]. It is concluded from these three pieces of evidence that the precursor keratin fraction PK.S2 contains no major homogeneous fraction; nor is there a major homogeneous keratin protein present in the precursor keratin proteins from cow's lip epidermis. The material probably consists of a family of closely related proteins as was found previously (cf. O'Donnell 1969) for wool, which is also classified as an α-keratin. The molecular mechanism by which this heterogeneity arises is not known but micro-heterogeneity of proteins in nature is being found frequently [e.g. in the sulphydryl endopeptidases from *Ficus glabrata* latex studied by Jones and Glazer (1970)]. The current arguments for generation of such diversity in nature are given by Hood and Talmage (1970), Hood *et al.* (1970), and Gally and Edelman (1970) in their studies on immunoglobulins.

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