STUDIES ON REDUCED WOOL

VII.* THE COMPLEXITY OF ONE OF THE MAJOR COMPONENTS

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[*Manuscript received April* 19, 1967]

Summary

A major component (component 8) isolated from reduced and carboxymethylated wool has been studied chemically after digestion with trypsin and after reaction with cyanogen bromide.

The peptides in tryptic digests of component 8 are present in greater numbers and smaller yields than expected for a substantially pure protein with the same amino acid composition.

The products obtained after cyanogen bromide degradation were fractionated by gel-filtration and DEAE-cellulose chromatography. From the yield of products and the distribution of homoserine in the fractions it is concluded that component 8 is heterogeneous. The products obtained by cyanogen bromide degradation of a second major component (component 7) are different from those given by component 8. Both components are probably best described as a family of related proteins. There seems no possibility that a sizeable fraction of these components can be isolated as a single polypeptide chain of unique chemical sequence.

I. INTRODUCTION

One of the most searching tests of homogeneity of a protein involves a study of the yield of the chemical products of degradation using reagents known from experiments with other proteins to react smoothly and with good yields. Two such reagents are cyanogen bromide (Gross and Witkop 1962) and trypsin (Hirs, Moore, and Stein 1956). These methods have been applied to one of the two major components isolated from reduced and carboxymethylated wool (Thompson and O'Donnell 1965). These components gave single bands on starch-gel electrophoresis in buffers containing 8M urea and have been called components 7 and 8. Of the 80% by weight of wool extracted into solution, component 7 represents approximately 40% and component 8 some 20% of the extracted protein. They are "low-sulphur" proteins and originate from the protofibrillar portion of wool (see Crewther *et al.* 1965). They have a molecular weight of approximately 45,000 and a helix content of 50%. In previous studies of the peptides obtained from components 7 and 8 by tryptic digestion (Thompson and O'Donnell 1965) or partial acid hydrolysis (O'Donnell and Thompson 1965) similarities were apparent in the peptide maps. A further study of the number of different peptides and their yields after digestion with trypsin and trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK-

* Part VI, *Aust. J. biol. Sci.*, 1965, **18**, 1227–34.

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trypsin) under a number of different conditions is reported. The two components differ markedly in methionine content, component 8 (1 residue in 38,000 g) having a lower content than component 7 (1 residue in 19,000 g). By cleavage with cyanogen bromide it was hoped to establish whether component 8 consists of a single polypeptide chain or a family of chains such as exist in the immunoglobulins (cf. Cohen 1966; Wilkinson, Press, and Porter 1966) and whether similar degradation products are formed from both components.

II. Experimental

(*a*) *Preparation and Characterization of Proteins*

Extracts of reduced and carboxymethylated wool from either a single fleece (MW 138) or top (MW 129) were prepared as described previously. Initially component 8 was made as described before (Thompson and O'Donnell 1965) but more recently by a mixed solvent fractionation method (Crewther and Dowling, personal communication). Components 7+8 were prepared from the acid-precipitable fraction (pH 4·4, 0·5M KCl) known as *S*-carboxymethylkerateine A (SCMKA) by passage of 15 ml of a 2% solution through a column of Sephadex G-200 in buffer containing 8M urea at pH 10. The required effluent fractions were dialysed against water and freeze-dried. This enabled components 7+8 to be freed from "high-sulphur" proteins and "high glycine–high aromatic amino acid" proteins.

Buffer solutions were prepared from urea solutions which had been passed through mixed-bed ion-exchange resins and the Sephadex gel and DEAE-cellulose chromatographic columns were operated as described previously (Thompson and O'Donnell 1966). Estimations of molecular weight of protein fractions were made from the elution volumes from the Sephadex G-200 and G-75 columns using the *S*-carboxymethylated derivatives of proteins of known molecular weight (Thompson and O'Donnell 1965) and the 8M urea buffer at pH 10.

Buffers at pH 10 were used because it has been shown that there is no reaction between the cyanate in the urea and amino groups of proteins at this pH (Thompson and O'Donnell 1966).

(*b*) *Enzyme Digestions*

TPCK-trypsin was made by the method of Kostka and Carpenter (1964) and Wang and Carpenter (1965) using L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (Schoellman and Shaw 1963) kindly prepared by Mr. D. Rivett. The presence of chymotryptic activity was tested for by the method of Crestfield, Moore, and Stein (1963) and Kostka and Carpenter (1964) on the B chain of reduced and carboxymethylated insulin prepared as described by Thompson and O'Donnell (1966). There were still some slight traces of peptides indicating chymotryptic or chymotryptic-like activity in the trypsin.

Digestion with trypsin or TPCK-trypsin was carried out at 37°C on 1% solutions of protein using an enzyme to protein weight ratio of 1%. The times of digestion were varied from 3 to 24 hr and the pH was maintained at 8·7 in 1% ammonium carbonate–ammonia solution containing 0·1% phenol. After freeze-drying,

separation of tryptic peptides on Sephadex-gel columns was carried out using either 50% formic acid or 0·01N ammonia as solvent. The runs in formic acid were completed at room temperature in less than 24 hr and under these conditions there is no evidence of breakage of peptide bonds (Thompson and O'Donnell 1966). Alkaline hydrolyses of fractions prior to their determination with ninhydrin reagent were carried out in a forced-draught oven at 110°C (Fruchter and Crestfield 1965). After neutralization to pH 5 the reagent of Yemm and Cocking (1955) was used (as described by Chibnall, Mangan, and Rees 1958).

(c) *Determination of Amino Terminal Groups*

(i) *Dinitrophenyl Method.*—Protein (50 mg) was reacted for 8–16 hr with 0·1 ml of 1-fluoro-2,4-dinitrobenzene (Sanger 1945) either in 1 ml water and 2 ml ethanol containing 40 mg potassium bicarbonate, or in 2 ml 8M urea buffer containing 0·01M Tris – 0·001M Versene (ethylenediaminetetraacetic acid, disodium salt), pH 7·4, with the addition of 40 mg potassium bicarbonate. In the latter case the protein dissolved and was precipitated by the addition of 2 ml water and acidification prior to extraction with ether. The dried dinitrophenyl (DNP) proteins were hydrolysed in sealed tubes at 105°C for 16 hr and the ether-soluble and water-soluble DNP-amino acids examined quantitatively by the methods given by Fraenkel-Conrat, Harris, and Levy (1955) using two-dimensional paper chromatography (solvents were t-amyl alcohol – ammonia and 1·5M phosphate. The area containing the DNP-aspartic acid, DNP-glutamic acid, and DNP-*S*-carboxymethylcysteine was not resolved and was eluted from the paper and examined further at pH 3·7 in a Spinco hanging-curtain electrophoresis apparatus. These three DNP-amino acids also separated readily during ionophoresis on a cooled flat plate (1 hr at 80 V/cm) at pH 4·4 in a pyridine – acetate buffer (Gray and Hartley 1963*b*). The water-soluble DNP-amino acids, after extracting into butanol, drying, and dissolving in acetone, were examined for DNP-arginine by chromatography in the t-amyl alcohol – ammonia solvent and also by flat-plate electrophoresis at pH 9. Correction factors for destruction of DNP-amino acids during hydrolysis were taken from Porter (1957).

(ii) *Edman Method.*—The reaction of protein (50 mg) with phenyl isothiocyanate was carried out as described by Blömback *et al.* (1966) except that *N*-ethyl morpholine was used in place of diallylamine. The identification of each phenyl thiohydantoin (PTH) was by thin-layer chromatography (Cherbuliez, Baehler, and Rabinowitz 1964) and by hydrolysis of the PTH back to amino acids (Van Orden and Carpenter 1964; see also Africa and Carpenter 1966) and determination on a Beckman amino acid analyser. PTH-glutamine and PTH-glutamic acid were distinguished by flat-plate ionophoresis (1 hr, 70 V/cm) at pH 4·4 in a pyridine–acetate buffer (Gray and Hartley 1963*b*) and spraying with iodine – azide reagent (Sjoquist 1953).

(iii) *Dansyl Method.*—Protein or peptide (10^{-2}–10^{-3}μmole) was allowed to react with 1-dimethylaminonaphthalene-5-sulphonyl (dansyl) chloride according to Gray and Hartley (1963*a*, 1963*b*). The dansyl amino acids were identified either by flat-plate ionophoresis using the pH 4·4 buffer of Gray and Hartley (1963*b*) or else by thin-layer chromatography on silica gel G (Morse and Horecker 1966).

(iv) *Carbamylation Method.*—Proteins were carbamylated by the method of Stark and Smyth (1963). The hydantoins of acidic and neutral amino acids were separated and, after hydrolysis, determined on the amino acid analyser as described by them.

(*d*) *High-voltage Paper Ionophoresis*

The apparatus described by Michl (1951) was used with toluene as cooling solvent. The buffers were those used by Sanger and co-workers (cf. Naughton *et al.* 1960), and contained pyridine – acetic acid – water (25 : 1 : 225 v/v, pH 6·5; 1 : 10 : 189 v/v, pH 3·6) and formic acid – acetic acid – water (1 : 5 : 36 v/v, pH 1·9). The usual voltage gradient was 50 V/cm. For flat-plate ionophoresis (80 V/cm) additional buffers (1% ammonium carbonate, pH 9, and the 0·05M pyridine–0·14M acetic acid buffer, pH 4·4, of Gray and Hartley 1963*b*) were used. Peptides were stained either with ninhydrin or with chlorine – tolidine – iodide by the method of Reindel and Hoppe (1954).

(*e*) *Cleavage with Cyanogen Bromide*

Protein (300 mg) was dissolved in 10 ml 90% (v/v) formic acid, approximately 300 mg CNBr added (Gross and Witkop 1961, 1962), and the mixture allowed to react at 2–4°C for 24 hr. The reaction mixture was freeze-dried to remove CNBr, dissolved either in 50% formic acid or in 8M urea buffer at pH 10, and passed through a column of Sephadex G-75. The proteins were recovered by dialysis and freeze-drying.

(*f*) *Amino Acid and Peptide Analyses*

These were carried out using a Beckman Spinco amino acid analyser and hydrolysates prepared under vacuum (Crestfield, Moore, and Stein 1963). For peptides containing homoserine the hydrolysates, after removal of hydrochloric acid by a freeze-drying technique, were de-ammoniated by drying in a vacuum desiccator with 1 ml 0·1M potassium carbonate (Moore and Stein 1951). This completely converted any homoserine lactone to homoserine which was then determined using the long column with buffer adjusted to pH 3·20 (Ambler 1965). Under these conditions the conversion to homoserine was better than by boiling at pH 6·5 as described by Ambler (1965) and the absence of ammonia made the detection of any homoserine lactone easier. The colour yield for homoserine was taken as 87% of the average constant (cf. Hofmann 1964).

Analyses of small peptides were carried out assuming that the average amounts of the predominant amino acids were integral. Trace amounts were ignored.

For study of tryptic digests of component 8, the peptides (approximately 2–20 mg) were loaded on the short column (with Beckmann 15A resin) and were serially eluted with the pH 3·25, 4·25, and 5·28 buffers (Crestfield, Moore, and Stein 1963). It was assumed that the colour factors of the peptides were the same as that of arginine.

III. Results

(a) *Tryptic Digestion of Component 8*

The lysine+arginine content of component 8 suggests that trypsin should produce some 40 peptides per mole of 45,000 if component 8 consisted of an assembly of identical polypeptide chains. Peptide maps do show approximately this number with those peptides basic (i.e. positively charged) at pH 6·5 being much smaller in size than the acidic ones (Thompson and O'Donnell 1965). In order to determine molar yields of some of these peptides a size fractionation of a tryptic digest was first done on a column of Sephadex G-25 in 0·01N ammonia (Fig. 1). The peptide maps, using paper electrophoresis, corresponding to the five cuts taken are given in

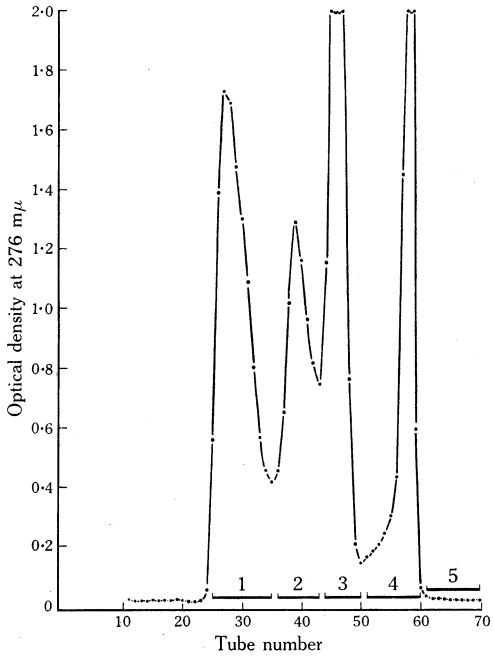

Fig. 1.—Gel-filtration of a tryptic digest of component 8 (400 mg) on a column (118 cm by 2·3 cm diameter) of Sephadex G-25 in 0·01N ammonia. Flow rate 16 ml/hr. Fraction size 8 ml. For every 100 ml of ammonia passing through an unloaded column 5 mg of residue (Sephadex) are recovered after dialysis and freeze-drying.

Plate 1, Figure 1, and corresponding comparisons of fractions 2, 3, and 4 (the largest peptides in fraction 1, the major fraction by weight, do not fractionate on this resin) on the short column of the Beckman amino acid analyser are shown in Figure 2. We are indebted to Professor S. Moore for assistance with these column fractionations of peptide mixtures which have the advantage of giving an approximate estimation of the relative yields of peptides and basic amino acids. It can be seen that the number of peaks visible on the analyser recorder charts is greater than is visible on paper-ionophoretic separations and there are more than the 40 expected from a homogeneous polypeptide chain. From fraction 4 only one peptide with a molar yield exceeding 18% occurred, the yield for the other six peaks being 2–4%. Arginine yield was 133% and lysine 7%. The high arginine value confirms the existence

of sequences of two or more consecutive basic amino acids in the proteins of wool (cf. Fell, LaFrance, and Ziegler 1960; Thompson and O'Donnell 1962; Blackburn and Lee 1965). The small yields of other peptides were not affected by using different times of digestion or TPCK-trypsin instead of commercial trypsin containing a small amount of chymotryptic activity. Nor did the small yields appear to be due to incomplete digestion by trypsin due to unavailability of some groups of the protein

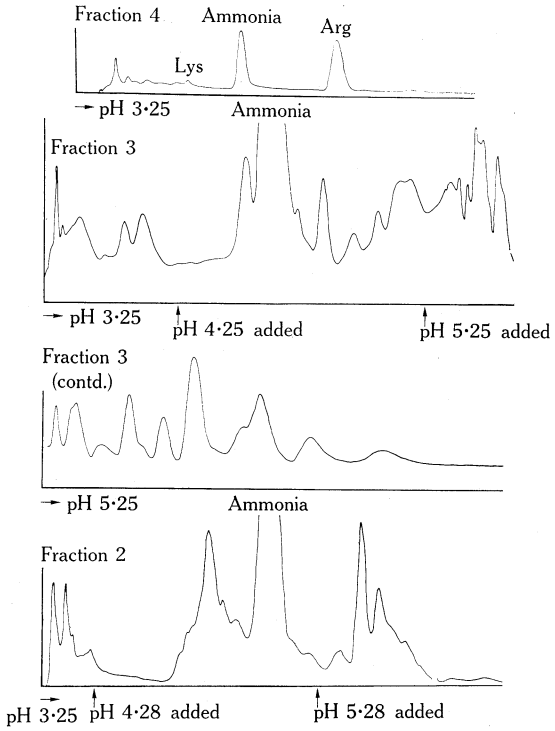

Fig. 2.—Traces from the short column of a Beckman amino acid analyser of fractions 2, 3, and 4 of the tryptic digest of component 8 separated on Sephadex G-25 (Fig. 1). Initially the column was equilibrated with standard Spinco buffer at pH 3·25 and then changed as shown on the diagram. Loads of tryptic digest put on the column for fractions 2, 3, and 4 were 20, 10, and 2 mg respectively.

to the trypsin, since the peptide map was unchanged when component 8 and trypsin were initially dissolved in buffer containing 8M urea before mixing and dilution to 4M or less to allow the enzyme to become active (Harris 1956; Kimmel, Rogers, and Smith 1965).

Fraction 2 (Fig. 2) gave at least 21 peaks, the yield of an intermediate-sized one being approximately 37%. Fraction 3 (Fig. 2) gave a minimum of 36 peaks with yields varying from 6 to 60%. The assignment of a different peptide to each peak is an assumption based on the experience of other workers with different proteins.

Although there is a possibility that some peaks may represent the same peptide sequence in amide and deamidated forms, for example, there is a higher probability of each peak containing more than one peptide component.

(b) Reaction of Component 8 with Cyanogen Bromide

(i) *Cleavage.*—Since b_0 values (Harrap 1963) indicated that reduced and carboxymethylated wool proteins retain all their helical content (50%) below about 65% formic acid concentration and progressively change to a helical content of 15% at 100% formic acid concentration, the CNBr cleavage was carried out in 90%

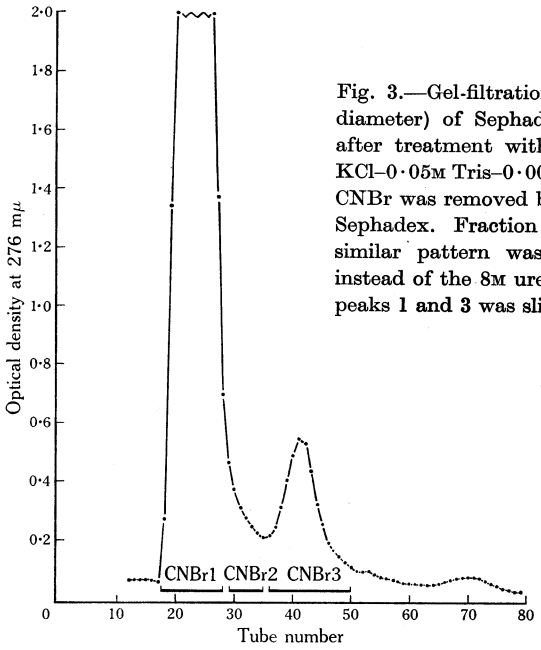

Fig. 3.—Gel-filtration on a column (127 cm by 2·3 cm diameter) of Sephadex G-75 of component 8 (300 mg) after treatment with CNBr. Buffer was 8M urea–0·1M KCl–0·05M Tris–0·001M Versene–0·2N ammonia at pH 10. CNBr was removed by freeze-drying before application to Sephadex. Fraction size 8 ml. Flow rate 16 ml/hr. A similar pattern was obtained using 50% formic acid instead of the 8M urea buffer, but the separation between peaks 1 and 3 was slightly greater.

formic acid. Some water is essential to the degradation which proceeds in the case of component 8 to give essentially the same yield at formic acid concentrations in the range 50–90%. In 90% formic acid at 2–4°C, amino acid analyses for methionine showed that component 8 had been cleaved by CNBr almost quantitatively. The effect of 50–90% formic acid concentrations on peptide bonds under these conditions should be negligible, since at higher temperatures (20–30°C) it has been reported that 50% (Thompson and O'Donnell 1966) and 70% (Steers *et al.* 1965; Brown *et al.* 1966; Press, Piggot, and Porter 1966) formic acid solutions do not break peptide bonds in 24 hr. The resolution of CNBr digests of component 8 on Sephadex G-75 in buffer containing 8M urea (Sephadex G-200 gave a similar type of pattern) at pH 10 is shown in Figure 3. The amino acid analyses of the three cuts (CNBr1, CNBr2, CNBr3) are given in Table 1 and their yields in Table 2. The resolution using 50% formic acid in place of the urea was equally as good. Approximately one-half of the

TABLE 1

AMINO ACID COMPOSITION OF FRACTIONS OF COMPONENT 8 FORMED BY CYANOGEN BROMIDE CLEAVAGE*

Amino acid nitrogen values are the mean of four experiments and are given as a percentage of the total nitrogen content of the amino acids plus ammonia (taken arbitrarily as one-eighth of the sum of amino acid nitrogen)† recovered from the column

Amino Acid	Fraction CNBr1	Fraction CNBr2	Fraction CNBr3
Lysine	4·08	4·15	3·20
Histidine	1·52	1·04	0·38
Ammonia	11·10	11·10	11·10
Arginine	21·34	18·33	12·91
Aspartic acid	7·23	5·53	7·45
Threonine	3·63	3·66	3·74
Serine	5·53	7·81	11·35
Glutamic acid	13·59	7·80	4·79
Proline	2·61	4·60	7·63
Glycine	2·52	6·96	8·32
Alanine	3·80	3·74	3·10
Valine	4·46	4·50	3·15
Methionine	0·005	0·063	0·05
Isoleucine	2·39	2·21	1·46
Leucine	8·19	5·92	4·02
Tyrosine	1·88	2·22	1·77
Phenylalanine	1·34	2·52	4·78
Homoserine	0·052	0·39	0·99
S-Carboxymethylcysteine	4·58	7·16	11·60
Cysteic acid	0·10	0·18	0·16

* Wool was MW129 top.

† Solutions were deammoniated with potassium carbonate before being put on the amino acid analyser. Homoserine lactone was thereby converted to homoserine.

TABLE 2

DISTRIBUTION OF PROTEIN AND HOMOSERINE IN FRACTIONS* CNBr1, CNBr2, AND CNBr3 OF CYANOGEN BROMIDE-TREATED COMPONENT 8

Quantitation was based on analytical values for amino acid composition of the fractions in four experiments

	Fraction CNBr1	Fraction CNBr2	Fraction CNBr3
Percentage of the total protein in each peak	88·8, 82·8, 86·5, 86·7 (mean 86·2)	5·2, 6·4, 4·9, 4·9 (mean 5·4)	6·1, 10·8, 8·6, 8·4 (mean 8·5)
Percentage of the total homoserine in each peak	48·4, 23·7, 32·5, 36·5 (mean 35·3)	13·5, 18·1, 11·5, 12·8 (mean 14·0)	38·1, 58·2, 56, 50·8 (mean 50·8)

* Separated as in Figure 3.

homoserine formed by the attack of CNBr on the methionine residue is in fraction 1 and the other half in fraction 3. If 8M urea or 50% formic acid are accepted as having

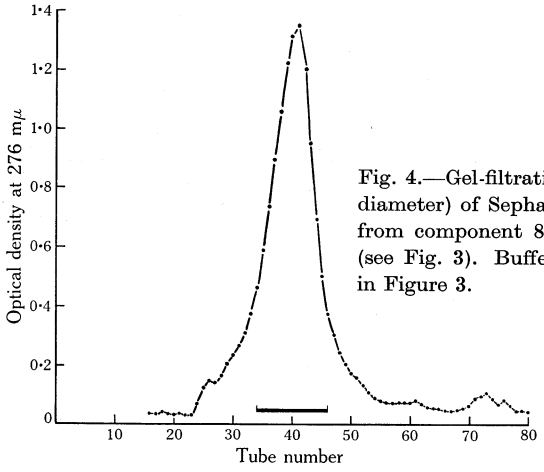

Fig. 4.—Gel-filtration using a column (127 cm by 2·3 cm diameter) of Sephadex G-200 of fraction CNBr1 obtained from component 8 (300 mg) after treatment with CNBr (see Fig. 3). Buffer, fraction size, and flow rate were as in Figure 3.

disaggregated all the fragments, this suggests that there are molecules in component 8 with their single methionine residue (molecular weight of component 8 taken to be

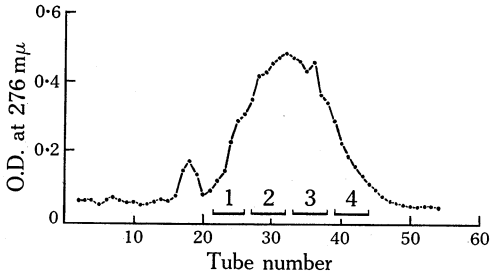

Fig. 5.—Gradient elution on DEAE-cellulose (18 cm by 0·9 cm diameter) of peak CNBr1 (*c.* 40 mg) obtained from component 8 (ex MW138) after treatment with CNBr and gel-filtration on Sephadex G-75 (see Fig. 3). It was also passed through Sephadex G-200 in 8M urea buffer (see Fig. 4) before applying to DEAE-cellulose. Fraction size approximately 2 ml. Flow rate *c.* 16 ml/hr. The fractions indicated by bars were bulked and prepared for amino acid analysis. Starting buffer 8M urea–0·01M Tris–0·001M Versene at pH 7·4. Final buffer 8M urea–0·01M Tris–0·001M Versene–0·75M potassium chloride at pH 7·4. 100 ml on each side of linear gradient device. Gradient applied after five tubes.

45,000) in different positions. In accord with this is the fact that the yield of the CNBr3 fraction was 8% by weight (average of 6·1, 10·8, 8·6, 8·4), whereas if methionine always occurred in the same position and gave a fragment of molecular

weight 7,700 (comparison of elution volumes from Sephadex with those of standard proteins) the yield would have been 17%. The largest fragment (fraction CNBr1) was found to have a molecular weight of 41,000. Andrews *et al.* (1964) give the accuracy of such values as ±12%.

TABLE 3

AMINO ACID COMPOSITION OF FRACTIONS OF CNBr1 ELUTED FROM DEAE-CELLULOSE*

Amino acid nitrogen values given are expressed as a percentage of the total nitrogen content of the amino acids plus ammonia (taken arbitrarily as one-eighth of the sum of the amino acid nitrogen)† recovered from the column

Amino Acid	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Lysine	—‡	4·04	3·60	3·39
Histidine	—‡	2·23	1·58	1·59
Ammonia	11·10	11·10	11·10	11·10
Arginine	16·91	22·52	23·49	21·36
Aspartic acid	8·79	7·63	7·67	7·76
Threonine	3·68	3·42	3·52	3·65
Serine	6·58	4·63	4·52	5·12
Glutamic acid	16·69	14·39	14·17	13·91
Proline	2·16	1·41	1·42	2·36
Glycine	3·70	2·12	2·04	2·76
Alanine	5·36	4·12	3·90	3·90
Valine	5·88	5·11	4·96	5·20
Isoleucine	3·05	2·51	2·46	2·59
Leucine	10·18	8·99	8·50	8·52
Tyrosine	1·08	1·66	1·60	1·24
Phenylalanine	1·85	1·04	1·00	1·22
S-Carboxymethylcysteine	2·98	2·96	3·89	4·39
Homoserine	—	—	0·02	0·04
Cysteic acid	0·11	—	—	—

* Separated as in Figure 5 from component 8 from MW129 top.

† Solutions were deammoniated with potassium carbonate before being put on the amino acid analyser. Homoserine lactone was thereby converted to homoserine.

‡ Data lost.

(ii) *Further Examination of Fraction CNBr1.*—Fraction CNBr1 was prepared using Sephadex G-75 and freed from small amounts of heavier and lighter material (approx. 5%) by passage through Sephadex G-200 in pH 10 buffer containing 8M urea (Fig. 4). It was then loaded on a column of DEAE-cellulose in buffer containing 8M urea at pH 7·4 and eluted with a gradient of potassium chloride from 0 to 0·75M (Fig. 5). The effluent tubes were collected into four fractions and their amino acid composition is listed in Table 3. It is seen that there are significant differences in the four fractions, suggesting that CNBr1 consists of an assembly of molecules with different amino acid compositions.

The amino terminal group of CNBr1 was found to be either Glu or Glu(NH_2) by the DNP, cyanate, and dansyl methods and it was shown to be Glu(NH_2) by identification using ionophoresis of PTH–Glu(NH_2) after Edman degradation. The yield of terminal glutamine was 0·44–0·5 mole per mole of CNBr1 (mol. wt. 41,000).

This value will be low because the acidic conditions of CNBr cleavage cannot be avoided and favour formation of pyrrolidone carboxylic acid peptides (cf. Moore and Stein 1951; Das and Roy 1962; Press, Piggot, and Porter 1966). There were traces of other amino terminal amino acids.

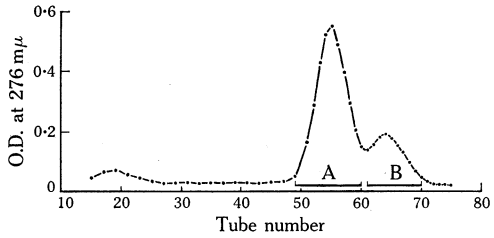

Fig. 6.—Gel-filtration on a column of Sephadex G-200 (100 by 2·3 cm diameter) of fractions 4–6 of CNBr3 (*c.* 70 mg) purified using DEAE-cellulose. Buffer for the gel-filtration was 8M urea–0·1M potassium chloride–0·05M Tris–0·001M Versene–0·2N ammonia at pH 10. Fraction size approximately 8 ml. Flow rate 16 ml/hr. Amino acid analysis of hydrolysates showed that the amino acid content of peak *B* was only 2·2% of that of peak *A* and therefore the ultraviolet absorption in peak B is not indicative of a high protein content.

(iii) *Further Examination of Fraction CNBr3.*—The small fragment (fraction CNBr3) (mol. wt. 7,700) isolated using Sephadex G-75 was run on Sephadex G-200 in buffer containing 8M urea at pH 10 (cf. Fig. 6). The appearance of the peak suggests

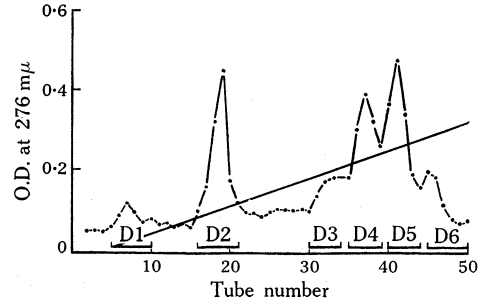

Fig. 7.—Gradient elution at 25°C of CNBr3 (approx. 24 mg) from DEAE-cellulose (18 cm by 0·9 cm diameter). Starting buffer 8M urea–0·01M Tris–0·001M Versene at pH 7·4. Final buffer contained an additional 0·75M potassium chloride. Linear gradient device had 100 ml on each side. Fraction size approximately 2 ml. Flow rate approximately 16 ml/hr. Percentages of material in peaks 2–6 (calculated from amino acid content) were 11·5, 8·4, 35·5, 35·8, and 8·7, respectively. Protein load increased three times without significant loss of resolution, and percentages in peaks 1–6 then were 4·2, 21·8, 8·4, 28·5, 26·1, and 13·2, respectively.

that it is fairly uniform with regard to molecular size. Peak *B* of Figure 6 contained only 2% as much protein as did peak *A*, as judged by amino acid analysis and, being

at the total volume of the column, must contain non-protein ultraviolet-absorbing impurities. Fraction CNBr3 when examined by gradient elution in buffer containing 8M urea (pH 7·4) from DEAE-cellulose (Fig. 7) gave six peaks and the amino acid compositions are listed in Table 4. It is seen that peaks D4, D5, and D6 are very similar in composition and these three together represent 80% (percentage weight calculated

TABLE 4

AMINO ACID COMPOSITION OF FRACTIONS OF CNBr3 OBTAINED BY GRADIENT ELUTION FROM DEAE-CELLULOSE*

Amino acid nitrogen values given are expressed as a percentage of the total nitrogen content of the amino acids plus ammonia (taken arbitrarily as one-eighth of the sum of the amino acid nitrogen)† recovered from the column

Amino Acid	Fraction D1	Fraction D2	Fraction D3	Fraction D4	Fraction D5	Fraction D6
Lysine	3·34	3·78	4·07	2·72	2·68	3·14
Histidine	2·89	2·15	0·66	Nil	Nil	Nil
Ammonia	11·10	11·10	11·10	11·10	11·10	11·10
Arginine	22·97	17·37	14·00	11·94	11·37	12·23
Aspartic acid	3·14	5·45	7·32	8·24	8·50	8·38
Threonine	2·55	2·33	3·70	3·90	3·91	4·02
Serine	7·96	7·96	10·42	11·81	12·35	11·61
Glutamic acid	4·15	6·85	5·45	4·60	4·55	4·83
Proline	2·94	3·72	5·08	6·58	6·89	6·53
Glycine	13·15	10·90	8·89	7·24	6·92	7·13
Alanine	3·42	4·88	2·99	2·69	2·71	2·88
Valine	2·56	2·98	3·67	3·16	2·92	3·11
Methionine	0·07	0·06	Trace	—	Trace	Trace
Isoleucine	1·84	1·70	1·62	1·46	1·29	1·37
Leucine	4·30	4·92	5·04	4·28	4·08	3·98
Tyrosine	2·94	3·10	0·90	0·86	0·79	0·29‡
Phenylalanine	5·85	3·18	3·77	5·18	5·56	4·98
Homoserine	0·32	0·52	0·97	1·15	1·17	1·18
S-Carboxymethylcysteine	4·47	6·89	10·13	12·82	13·14	12·86
Cysteic acid	0·02	0·12	0·20	0·20	Trace	0·24

* Isolated as in Figure 7 from single fleece MW138.

† Solutions were deammoniated with potassium carbonate before being put on the amino acid analyser. Any homoserine lactone present was thereby converted to homoserine.

‡ This value is low. In similar experiments using the lower loads the value was the same as for fraction D5. It is suspected that there was greater destruction of the tyrosine in this hydrolysis (cf. Markland *et al.* 1966).

from the amino acid content of the fractions) of CNBr3. This means that the yield of the main CNBr3 fractions from component 8 falls from 8% (from Sephadex G-75) to 6·4% of the weight of component 8. The separation of peaks D4, D5, and D6 on DEAE-cellulose is possibly due to a difference in amide content (which were not determined). However, an indirect attempt to ascertain whether this was so was by using longer exposure to acidic conditions during the cleavage with CNBr in 90% formic acid for 24 hr at room temperature. The DEAE-cellulose pattern was not substantially changed under these conditions. Hence, if the separation of peaks,

D4, D5, and D6 on DEAE-cellulose is due to amide differences, it seems that they must be different in the fibre itself and not an artefact of preparation. Peak D2 has a high content of glycine and aromatic amino acids and is probably an impurity derived from the high glycine class of proteins which exist in wool (cf. Crewther *et al.* 1965).

For bulk preparations of purified CNBr3 it was found that the load of CNBr3 (ex Sephadex G-75) could be increased three times (i.e. to 72 mg from 30 g wool) without affecting the separation, though the proportions of the peaks were somewhat different (see Fig. 7).

Peaks D4, D5, and D6 from DEAE-chromatography were those used subsequently for examination of CNBr3 by tryptic digestion.

CNBr3 has no major amino terminal groups and therefore it must be from the *N*-acetylated terminal portion of component 8 molecules.

(*c*) *Examination of the Tryptic Digestion Products of CNBr3*

Purified CNBr3, i.e. peaks D4, D5, and D6 combined from a purification of CNBr3 by passage through a column of DEAE-cellulose (Fig. 7), was digested with trypsin for 6 hr at 37°C. The digest (17 mg), after freeze-drying to remove ammonium carbonate and phenol and re-solution in 0·5 ml formic acid, was passed through a column of Sephadex G-25 in 50% formic acid and the effluent monitored by ninhydrin determinations on aliquots (100 μl) of the fractions after alkaline hydrolysis (Fig. 8). Amino acid analyses of definite fractions of the combined tubes of each peak allowed calculation of the relative amounts of material in each peak as well as the individual amino acids (Table 5). It is seen that there is a considerable fraction of the homoserine in peak S1 as well as in peak S3 and this suggests that all the molecules in this purified CNBr3 do not have their trypsin-sensitive bond closest to the *C*-terminal homoserine in the same place. Peak S3 contains the amino acids (Glu, Thr).Homoser, and analysis of the residue after an Edman degradation revealed that the glutamine had been removed. The sequence of the tripeptide is therefore Glu-Thr-Homoser or Glu-Thr-Homoser.lactone, and represents the *C*-terminal sequence of a proportion of CNBr3 molecules.

When the examination was made by high-voltage paper electrophoresis of the whole tryptic digest of purified CNBr3 (i.e. from peaks D4+D5+D6 of Fig. 7) and the individual peaks S1, S2, S3 obtained from it by gel-filtration using Sephadex G-25 (Fig. 8) the patterns in Plate 1, Figure 2, were observed. Peak S3 sometimes consisted of two bands, one corresponding to the homoserine of the tripeptide in the lactone form. In the latter form it is neutral whereas in the free acid form it is acidic. As peak S3 did not give a band moving to the negative electrode it is seen that the peptide contains glutamic acid and not glutamine. Peak S1 gives a band with some substructure and looks as though it consists of three bands. Peak S2 has one almost neutral ninhydrin-positive band and another strong neutral spot which is revealed with chlorine–tolidine reagent. This peptide therefore has its amino terminal group blocked, most probably by acetylation (cf. O'Donnell, Thompson, and Inglis 1962; O'Donnell and Thompson 1964*a*).

When the individual peaks D4, D5, and D6 from cuts of CNBr3 (Fig. 7) were examined on paper after digestion with trypsin, the patterns appear almost identical but there may be a trend in the position of the largest peptide (corresponding to S1

in Fig. 8) in each peak (Plate 1, Fig. 3). This suggests that some differences, perhaps amide, do occur among them (however, see Section IV). No further effort has been made to establish other differences between D4, D5, and D6.

(*d*) *Chromatography of Component 8 on DEAE-cellulose*

In view of the heterogeneity in component 8 as revealed by analysis of the CNBr fractions it was of interest to see if this could be detected in fractions of component 8 itself. Figure 9 shows the gradient elution pattern of component 8 from DEAE-cellulose and Table 6 the analyses of the collected fractions. It is seen that there is a trend across the main peak in the values of some of the amino acids, supporting the idea of a non-homogeneous population of molecules in component 8.

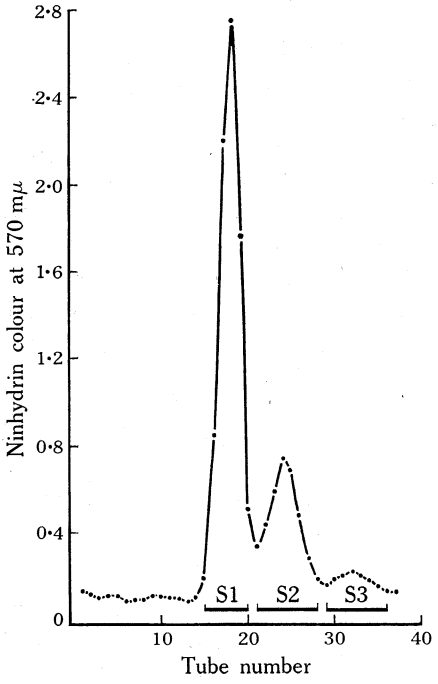

Fig. 8.—Gel-filtration on a column (133 cm by 1·07 cm diameter) of Sephadex G-25 in 50% formic acid of a tryptic digest of 25 mg of purified fragment CNBr3 of component 8 (see Figs. 3 and 6). Ninhydrin colour determined after alkaline hydrolysis. The percentages of material calculated from amino acid contents of aliquots of the tubes comprising the peaks were: peak S1, 68·0%, peak S2, 27·6%, and peak S3, 4·3%. Fraction size *c.* 2·7 ml. Flow rate *c.* 5·4 ml/hr.

(*e*) *Reaction of a Mixture of Components 7 and 8 with Cyanogen Bromide*

Figure 10 shows the pattern obtained when a mixture of components 7 and 8 is treated with CNBr and the fragments separated on Sephadex G-75. The major peak (peak 1) was further fractionated on Sephadex G-200 (Fig. 11) in buffers containing 8M urea at pH 10. Table 7 lists the amino acid composition of these four fractions (1*A*, 1*B*, 2, and 3) and comparison with corresponding values for component 8 (Table 1) suggests that peak 1*A* of Figure 11 arises mainly from component 8 but peaks 1*B* (Fig. 11) and 2 (Fig. 10) arise from component 7. Peak 3 (Fig. 10) arises mainly from component 8 but there appears to be some contribution from component 7. The amino terminal end groups in the mixture of components 7 and 8 after treatment with CNBr showed that there was 0·29 mole aspartic acid (or asparagine),

0·18 mole glutamic acid (or glutamine), and 0·11 mole alanine per mole of protein (mol. wt. 45,000). It is most probable that the terminal glutamic acid arises from component 8 and the aspartic acid and alanine must therefore arise from component 7. It is not known what are the relative proportions of components 7 and 8 in the mixture but it is of the order of 1 : 1. It is of interest that Corfield *et al.* (1965) have identified

TABLE 5

AMINO ACID COMPOSITIONS OF THE FRACTIONS OF A TRYPTIC DIGEST OF PURIFIED CNBr3*

Results are expressed as the total number of μmoles of amino acid present in the digest, and also as the relative number of amino acid residues assuming the total number of residues in peaks S1, S2, and S3 to be approximately 44, 24, and 3, respectively

Amino Acid	Unfractionated		Peak S1		Peak S2		Peak S3	
	Total No. of Residues	Relative No. of Residues	Total No. of Residues	Relative No. of Residues	Total No. of Residues	Relative No. of Residues	Total No. of Residues	Relative No. of Residues
Lysine	11·0	1·3	9·5	1·0	0·9	0·1	1·2	0·2
Histidine	Trace	—	—	—	0·4	0·1	0·3	0·0
Arginine	22·0	2·5	9·2	1·0	12·5	1·9	0·9	0·1
Aspartic acid	65·8	7·6	41·3	4·4	21·9	3·3	1·0	0·1
Threonine	30·7	3·5	21·3	2·3	1·8	0·3	6·6	1·0
Serine	92·8	10·7	70·2	7·5	23·4	3·5	1·3	0·2
Glutamic acid	36·0	4·1	23·5	2·5	3·4	0·5	7·2	1·0
Proline	56·2	6·5	37·4	4·0	10·7	1·6	—	—
Glycine	55·7	6·4	46·9	5·0	4·3	0·6	0·4	0·1
Alanine	21·2	2·4	18·6	2·0	1·3	0·2	—	—
Valine	26·0	3·0	20·1	2·1	2·1	0·3	—	—
Isoleucine	10·1	1·2	9·2	1·0	7·3	1·1	—	—
Leucine	32·4	3·7	11·2	1·2	23·0	3·4	—	—
Tyrosine	6·6	0·8	1·8	0·2	5·1	0·8	—	—
Phenylalanine	42·4	4·9	17·8	1·9	25·7	3·8	0·7	0·1
S-Carboxymethyl-cysteine	86·7	10·0	79·0	8·4	16·6	2·5	—	—
Homoserine	9·4	1·1	2·9	0·3	0·2	0	6·8	1·0
Cysteic acid	2·1	0·2	1·3	0·1	0·4	0·1	0·2	0
Total No. of residues (μmoles)	607·1		421·1		171·0		26·6	
Relative No. of residues		69·9		43·9		24·1		3·8

* Separated as in Figure 8 from a single fleece MW138.

a methionyl–alanyl sequence in a peptide isolated from US3, a protein from oxidized wool which would correspond to a mixture of components 7 and 8, together with some high-sulphur proteins.

IV. DISCUSSION

Component 8, which comprises approximately 20% of the total protein extracted from wool after reduction and carboxymethylation, gives only a single band on starch-gel electrophoresis, a single peak on Sephadex G-200, and has a

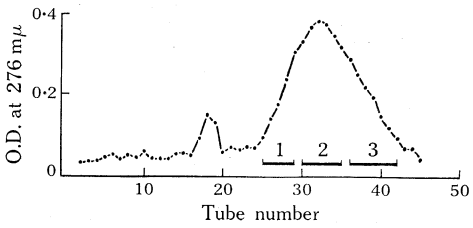

Fig. 9.—Gradient elution on DEAE-cellulose (18 cm by 0·9 cm diameter) of 35 mg of component 8 (ex MW129 top). Fraction size *c.* 2 ml (2 ml diluent added before measuring). Flow rate 16 ml/hr. The tubes indicated by bars were bulked and prepared for amino acid analysis. Starting buffer 8M urea–0·01M Tris–0·001M Versene at pH 7·5. Final buffer 8M urea–0·01M Tris–0·001M Versene–0·75M potassium chloride at pH 7. 100 ml of buffer on each side of linear gradient device. Gradient applied after five tubes.

TABLE 6

AMINO ACID COMPOSITION OF FRACTIONS OF COMPONENT 8, ELUTED FROM DEAE-CELLULOSE*

Amino acid nitrogen values are given as a percentage of the total nitrogen contents of the amino acids plus ammonia (taken arbitrarily as one-eighth of the sum of amino acid nitrogen) recovered from the column

Amino Acid	Whole Component 8†	Fraction 1	Fraction 2	Fraction 3
Lysine	4·37	5·22	4·20	3·99
Histidine	1·24	0·92	1·26	1·20
Ammonia	11·10	11·10	11·10	11·10
Arginine	21·96	20·89	22·18	21·57
Aspartic acid	7·51	7·68	7·56	7·19
Threonine	3·61	3·53	3·53	3·60
Serine	5·47	6·14	5·30	5·60
Glutamic acid	12·22	12·90	13·10	12·68
Proline	2·53	2·92	3·36	3·79
Glycine	3·10	3·38	2·43	3·20
Alanine	4·30	4·15	3·53	3·70
Valine	4·45	4·14	4·12	4·29
Methionine	0·24	0·15	0·17	0·20
Isoleucine	2·55	2·45	2·35	2·50
Leucine	8·00	8·45	8·48	8·39
Tyrosine	1·80	0·77	1·68	1·50
Phenylalanine	1·50	1·38	1·51	1·60
S-Carboxymethylcysteine	3·96	3·38	3·78	4·69
Cysteic acid		0·46	0·34	0·20

* Separated as in Figure 9 from MW129 top.

† These values were from a different wool (MW138 single fleece) and are the mean of five analyses.

methionine content of approximately one residue per 38,000 g (O'Donnell and Thompson 1964*b*). Its estimated molecular weight is 45,000 (±12%) (Thompson and O'Donnell 1965; cf. however, DeDeurwaerder and Harrap 1964, 1965).

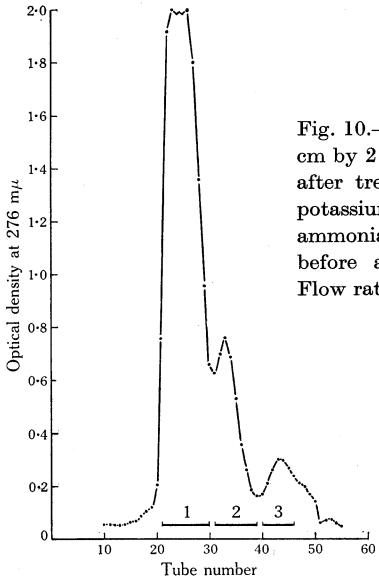

Fig. 10.—Gel-filtration on columns of G-75 Sephadex (127 cm by 2·3 cm diameter) of 300 mg of components 7 plus 8 after treatment with CNBr. Buffer was 8M urea–0·1M potassium chloride–0·05M Tris–0·001M Versene–0·2N ammonia at pH 10. CNBr was removed by freeze-drying before application to Sephadex. Fraction size 8 ml. Flow rate 16 ml/hr.

The experimental evidence in this paper arose from efforts to define the heterogeneity of component 8. Two possibilities were considered: either there is a major proportion of polypeptide chains with a unique sequence of amino acid residues

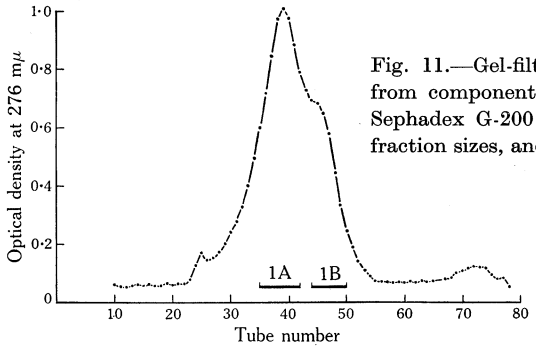

Fig. 11.—Gel-filtration of peak 1 (obtained with CNBr from components 7 plus 8; cf. Fig. 10) on a column of Sephadex G-200 (127 cm by 2·3 cm diameter). Buffer, fraction sizes, and flow rates were the same as in Figure 3.

or there is not. If not, it is important to know whether there is any similarity between the various components and how many different ones there are. The various pieces of evidence suggest a complex mixture or family of related components and are discussed in turn.

(*a*) *Basic Peptides in Tryptic Digests*

The yields of basic peptides produced in tryptic digests of component 8 are very small when calculated on a molar basis. Qualitatively they are very reproducible when examined on peptide maps (Plate 1, Fig. 1; cf. Thompson and O'Donnell 1962). It does not seem likely that this number of basic peptides in small amounts would arise from one protein impurity present in small amounts and carried

TABLE 7

AMINO ACID COMPOSITION OF THE FRACTIONS* ISOLATED FROM THE TREATMENT OF COMPONENTS 7 AND 8 WITH CYANOGEN BROMIDE

Amino acid nitrogen values are given as a percentage of the total nitrogen content of the amino acids plus ammonia (taken arbitrarily as one-eighth of the sum of the amino acid nitrogen)† recovered from the column

Amino Acid	Fraction 1*A*	Fraction 1*B*	Fraction 2	Fraction 3
Lysine	4·52	7·06	7·29	3·34
Histidine	1·59	1·52	1·29	—
Ammonia	11·10	11·10	11·10	11·10
Arginine	21·93	18·79	19·66	17·98
Aspartic acid	7·47	6·80	5·50	6·76
Threonine	3·34	2·83	2·93	3·65
Serine	4·83	4·50	5·23	9·37
Glutamic acid	14·15	13·19	11·02	6·15
Proline	1·97	2·05	1·20	4·63
Glycine	1·97	3·03	5·54	7·64
Alanine	4·28	4·82	7·54	3·22
Valine	4·80	4·85	5·34	4·16
Isoleucine	2·61	3·06	2·88	1·82
Leucine	8·52	7·88	5·76	4·60
Tyrosine	1·84	1·80	1·18	1·16
Phenylalanine	1·03	2·08	0·40	3·15
S-Carboxymethylcysteine	3·88	4·28	5·57	10·10
Homoserine	0·06	0·26	0·49	0·94
Cysteic acid	0·06	0·06	0·01	0·36

* Separated as in Figures 10 and 11 from single fleece MW138.

† Solutions were deammoniated with potassium carbonate before being put on the amino acid analyser. Homoserine lactone was thereby converted to homoserine.

along with the bulk of the protein material. Adsorption of proteins to each other is, of course, well known but we have consistently used 8M urea solutions to avoid interactions wherever possible. Only a protein with many basic amino acid residues could give the large number of small basic peptides (in slowest fractions 3 and 4 on Sephadex G-25, Fig. 1) that are present. Although we have not identified these peptides the possibility that they arise from small amounts of chymotryptic activity in the trypsin is unlikely since shorter times of digestion with TPCK-trypsin did not eliminate them. The probable structure of these peptides is that given by Fell, LaFrance, and Ziegler (1960) who found, as would be expected for tryptic peptides,

either arginine or lysine in each peptide. The most plausible explanation is that they arise from small amounts of proteins with different sequences present in component 8.

Other workers have examined yields of peptides obtained from tryptic digests of oxidized wool (Blackburn and Lee 1965) and a fraction called US3 obtained from oxidized wool (Corfield, Fletcher, and Robson 1964, cf. also 1967; Corfield *et al.* 1965; Cole *et al.* 1965). From 5 g of US3, Corfield and co-workers obtained yields of 1–60 μmoles of peptides which represents a molar yield of 1–60% if the molecular weight of US3 be taken as 45,000. These workers have also drawn attention to even lower yields of many other peptides.

(*b*) *Fractionation of Tryptic Digests*

It was previously estimated with two-dimensional paper ionophoresis that the number of spots in a tryptic digest of component 8 was consistent with that expected for a homogeneous protein of molecular weight 45,000 and its known lysine plus arginine content. Using columns of Dowex-50, however, with the amino acid analyser for separation of peptides, the number is considerably in excess of this, pointing to heterogeneity. This evidence alone can be misleading in assessing the size or homogeneity of a protein, as pointed out by Harris and Hindley (1965). In the present case, however, there is other evidence to support this interpretation.

In a tryptic digest of US3 from oxidized wool some 300–350 peptides have been reported (Corfield, Fletcher, and Robson 1964). However, further work using 76 major peptides (i.e. yield greater than 1 μmole per 5 g US3) indicated that US3 could represent a polypeptide chain(s) "of about 700 amino acid residues and is not seriously contaminated with short-chain degradation products" (Corfield *et al.* 1965).

(*c*) *Chromatography of Component 8*

When component 8 was chromatographed on DEAE-cellulose using gradient elution in buffers containing 8M urea and various fractions collected, they were found to have amino acid compositions with definite differences and trends which indicated a non-homogeneous population of molecules (Table 6). In particular the *S*-carboxymethylcysteine content showed a gradation. The trends were not all in one direction, a fact which argues against the idea of them being mixtures of different proportions of only two different proteins.

(*d*) *Cyanogen Bromide Cleavage of Component 8*

Cleavage of component 8 with CNBr at the methionine residues releases a fragment (CNBr3) of molecular weight 7,700 which is separable on Sephadex G-75 gel-filtration (Fig. 3). However, only a half of the homoserine formed in the degradation is contained in this peak, the rest being in larger-sized peptide peaks, CNBr1 and CNBr2 (Table 1). The yield of CNBr3 is only 8% and if all the molecules of component 8 had their methionine in identical positions it should be 17% (taking the molecular weight of component 8 as 45,000). Furthermore the content of methionine in component 8 is somewhat larger than would be expected for a molecular weight of 45,000 and a single component. This would again suggest heterogeneity but, alternatively could be the result of the impurities of approximately 20% which analyse so differently after fractionation of CNBr3 (Table 4; Fig. 7).

(*e*) *Elution of CNBr3 from DEAE-cellulose*

When CNBr3 is submitted to gradient elution from DEAE-cellulose, six peaks are obtained (Fig. 7). Three of these, amounting to some 20% of CNBr3, have a very different amino acid composition from the others and could be regarded as impurities (Table 4). However, the remaining three (peaks 4, 5, and 6) have almost identical amino acid compositions and are all significantly low in tyrosine for the estimated molecular weight of 7,700. It is possible that they differ in amide contents but, as pointed out earlier, this is unlikely to have arisen during the degradation since more extended exposure to acidic conditions did not alter the relative proportions of the peaks.

With other proteins (Edmundson and Hirs 1962; Atassi and Saplin 1966) it has been possible to separate components with different chromatographic and electrophoretic rates which were identical in amino acid composition and amide content. The separations were attributed to shape differences by Atassi and Saplin (1966), which is possible in the absence of urea. In the present case, although 8M urea buffer was used, it cannot be said with certainty that the separation of peaks 4, 5, and 6 of CNBr3 on DEAE-cellulose shows that they have different amino acid sequences even though this would appear to be the most easily acceptable explanation.

(*f*) *Treatment of Fraction CNBr3 with Trypsin*

The peptides obtained when purified CNBr3 is treated with trypsin can be separated into three well-resolved peaks on Sephadex G-25 (Fig. 8). Table 5 shows that, while the tripeptide glutamine–threonine–homoserine contained approximately two-thirds of the homoserine, approximately one-third of it was involved with the longer peptides. This suggests that in the molecules comprising fraction CNBr3 the trypsin-sensitive bond nearest the homoserine *C*-terminal residue is not in the same place in all molecules.

(*g*) *Elution of CNBr1 from DEAE-cellulose*

When the largest fraction, CNBr1, obtained in the cleavage of component 8 with cyanogen bromide, is eluted with a salt gradient from DEAE-cellulose the broad curve obtained (Fig. 5) has a gradation across it in amino acid composition (Table 3). This is consistent with the results for component 8 itself (Fig. 9, Table 6). The heterogeneity is not lessened if wool from a single fleece rather than from a mixed flock of similar animals is used.

(*h*) *Acetyl Peptides*

Acetyl peptides with different but related terminal sequences have been isolated from both component 8 and from CNBr3 (O'Donnell and Thompson 1968).

The fact that component 8 representing 20% of the extracted wool gives only half the amount of CNBr3 expected for a homogeneous component (of mol. wt. 45,000) brings the maximum amount of component 8 with identical molecules to 10%. This is reduced to 8% when account is taken of the presence of 20% impurities in peaks 1, 2, and 3 when CNBr3 is fractionated on DEAE-cellulose. Even this 8%

CNBr3 produces two classes of homoserine-containing peptides on tryptic digestion and if we assume that the trypsin-sensitive bonds have been completely broken, the 8% is reduced to approximately 6%. We have ignored the heterogeneity suggested by the separation of peaks 4, 5, and 6 (Fig. 7) in case there is an alternative explanation such as differences of shapes among the molecules, although the non-stoichiometric amounts of tyrosine point to heterogeneity. The two different *N*-acetyl sequences in the first four residues of component 8 and fraction CNBr3 (O'Donnell and Thompson 1968) reduce this value of 6% even lower, and it would appear that there will be no appreciable fraction of component 8 having an assembly of identical molecules. Since the peptide maps do not show an unlimited number of spots and there are obviously many peptide sequences terminating with glutamine–threonine–homoserine in fraction CNBr3, it must be concluded that there is some plan of synthesis to tailor the molecules for their biological function. It is suggested that component 8, and by analogy component 7, consist of a family of related proteins in which there may be portions of the peptide chain common to all molecules, as in the immunoglobulins (cf. Wilkinson, Press, and Porter 1966).

(*i*) *General*

Differences have already been reported between components 7 and 8 and the fact that mixtures of these two components when treated with CNBr give different products to those obtained from component 8 alone emphasizes the difference in sequences of amino acid residues in them. These differences are also reflected in the different *N*-terminal residues of glutamine in component 8, and aspartic acid and alanine in component 7, that are detected after cleavage.

The reason for the collection of non-identical protein molecules in the low-sulphur proteins of wool can be looked at from several points of view. It could reflect genetic changes in sheep which have occurred over a considerable period and which are accommodated in changes in the amino acid sequence of molecules which have less demands on their unique structure for biological activity than do some other types of protein molecules of more exacting function. However, even with proteins which one would imagine to have a more exacting role than wool there are cases where a variety of chemical sequences occur. Such cases are found in the immunoglobulins (cf. Cohen 1966) and the haemoglobins (cf. Rifkin, Rifkin, and Konigsberg 1966; Kilmartin and Clegg 1967). A particular example shows that for adult sheep there are at least three different haemoglobins, which show 7–21 amino acid sequence variations in the β-chains (Boyer *et al.* 1966). In these cases, suggestions for the cause of the heterogeneity include variable translation of a single gene for the individual chains of the haemoglobins, and somatic differentiation of the immune system as well as variable translation and mutation and selection processes on the evolutionary time scale for the immunoglobulins. Some of these factors may be a contributory cause of the heterogeneity observed in wool. Furthermore there is also evidence (Downes *et al.* 1966; Frater 1966) that the low-sulphur proteins of wool are synthesized over a region which commences in the lower parts of the follicle and extends to the zone of keratinization, where the synthesis of the high-sulphur proteins is proceeding at its maximum rate and this may help contribute to the

heterogeneity. Until this process is understood more clearly it is probably as difficult to make progress in understanding the amino acid sequence of low-sulphur wool proteins as it has been in the case of immunoglobulins.

As wool is a member of the group of fibrous proteins one wonders to what extent increasing knowledge of the structure of other members of this group will aid in the interpretation of data on keratin. In the case of collagen the evidence at present (Bornstein and Piez 1966; Piez *et al.* 1966) suggests that unique sequences may be expected. For myosin and muscle proteins there is as yet insufficient evidence available on which to base an opinion although to date only single *N*-acetyl sequences have been reported (Offer 1965; Alving, Moczar, and Laki 1966; Alving and Laki 1966; Gaetjens and Bárány 1966).

The characteristic property of fibrous proteins of forming two- or three-chain helical structures suggests that if the chains are not identical, two or three different components might be expected in approximately equal amounts. This was the case with cod-fish collagen (Piez 1965). The component chains of myosin have not yet been isolated and studied so no remarks can be made about them. In the case of the low-sulphur proteins of wool, which come from the helical coiled-coil region of the fibre, the chemical evidence is not consistent with the presence of only two or three components in equal proportions. It is possible that the critical regions responsible for the characteristic folding of the chains are accurately reproduced but that changes occur while the rest of the molecule is being synthesized to mask the pattern of chemical identity. It is now known that in the case of haemoglobins, extensive changes in their chemical sequence produced during evolution can be readily accommodated in an apparently unchanged folded structure capable of performing, in different species, their necessary biological function (Braunitzer *et al.* 1964).

V. References

Africa, B., and Carpenter, F. H. (1966).—*Biochem. biophys. Res. Commun.* **24**, 113.

Alving, R. E., and Laki, K. (1966).—*Biochemistry* **5**, 2597.

Alving, R. E., Moczar, E., and Laki, K. (1966).—*Biochem. biophys. Res. Commun.* **23**, 540.

Ambler, A. (1965).—*Biochem. J.* **96**, 32P.

Andrews, P., Bray, R. C., Edwards, P., and Shooter, K. V. (1964).—*Biochem J.* **93**, 627.

Atassi, M. Z., and Saplin, B. J. (1966).—*Biochem. J.* **98**, 82.

Blackburn, S., and Lee, G. R. (1965).—Proc. 3rd Int. Wool Text. Res. Conf., Paris. Vol. 1. p. 321.

Blombäck, B., Blombäck, M., Edman, P., and Hessel, B. (1966).—*Biochim. biophys. Acta* **115**, 371.

Bornstein, P., and Piez, K. A. (1966).—*Biochemistry* **5**, 3460.

Boyer, S. H., Hathaway, P., Pascasio, F., Orton, C., Bordley, J., and Naughton, M. (1966).—*Science, N.Y.* **153**, 1539.

Braunitzer, G., Hilse, K., Rudloff, V., and Hilschmann, N. (1964).—*Adv. Protein Chem.* **19**, 1.

Brown, J. L., Koorajian, S., Katze, J., and Zabin, I. (1966).—*J. biol. Chem.* **241**, 2826.

Cherbuliez, E., Baehler, B., and Rabinowitz, J. (1964).—*Helv. chim. Acta* **47**, 1350.

Chibnall, A. C., Mangan, J. L., and Rees, M. W. (1958).—*Biochem. J.* **68**, 111.

Cohen, S. (1966).—*Proc. R. Soc.* B **166**, 114.

Cole, M., Corfield, M. C., Fletcher, J. C., and Robson, A. (1965).—Proc. 3rd Int. Wool Text. Res. Conf., Paris. Vol. 1. p. 335.

Corfield, M. C., Fletcher, J. C., Myers, C., and Robson, A. (1965).—Proc. 3rd Int. Wool Text. Res. Conf., Paris. Vol. 1. p. 345.

CORFIELD, M. C., FLETCHER, J. C., and ROBSON, A. (1964).—Abstr. 6th Int. Congr. Biochem, New York. Vol. 32. Sect. 2. Item 36. p. 145.
CORFIELD, M. C., FLETCHER, J. C., and ROBSON, A. (1967).—*Biochem. J.* **102**, 801.
CRESTFIELD, A. M., MOORE, S., and STEIN, W. H. (1963).—*J. biol. Chem.* **238**, 622.
CREWTHER, W. G., FRASER, R. D. B., LENNOX, F. G., and LINDLEY, H. (1965).—*Adv. Protein Chem.* **20**, 191.
DAS, H. K., and ROY, S. C. (1962).—*Biochim. Biophys. Acta* **62**, 590.
DE DEURWAERDER, R., and HARRAP, B. S. (1964).—*Makromolek. Chem.* **72**, 1.
DE DEURWAERDER, R., and HARRAP, B. S. (1965).—*Makromolek. Chem.* **86**, 98.
DOWNES, A. M., FERGUSON, K. A., GILLESPIE, J. M., and HARRAP, B. S. (1966).—*Aust. J. biol. Sci.* **19**, 319.
EDMUNDSON, A. B., and HIRS, C. H. W. (1962).—*J. molec. Biol.* **5**, 663.
FELL, M., LAFRANCE, N. H., and ZIEGLER, K. (1960).—*J. Text. Inst.* **51**, 797T.
FRAENKEL-CONRAT, H., HARRIS, I., and LEVY, A. L. (1955).—In "Methods of Biochemical Analysis". (Ed. D. Glick.) Vol. 2. p. 359. (Interscience Publishers: New York and London.)
FRATER, R. (1966).—*Aust. J. biol. Sci.* **19**, 699.
FRUCHTER, R. G., and CRESTFIELD, A. M. (1965).—*J. biol. Chem.* **240**, 3868.
GAETJENS, E., and BÁRÁNY, M. (1966).—*Biochim. biophys. Acta* **117**, 176.
GRAY, W. R., and HARTLEY, B. S. (1963*a*).—*Biochem. J.* **89**, 59P.
GRAY, W. R., and HARTLEY, B. S. (1963*b*).—*Biochem. J.* **89**, 379.
GROSS, E., and WITKOP, B. (1961).—*J. Am. chem. Soc.* **83**, 1510.
GROSS, E., and WITKOP, B. (1962).—*J. biol. Chem.* **237**, 1856.
HARRAP, B. S. (1963).—*Aust. J. biol. Sci.* **16**, 231.
HARRIS, J. I. (1956).—*Nature, Lond.* **177**, 471.
HARRIS, J. I., and HINDLEY, J. (1965).—*J. molec. Biol.* **13**, 894.
HIRS, C. H. W., MOORE, S., and STEIN, W. H. (1956).—*J. biol. Chem.* **219**, 623.
HOFMANN, T. (1964).—*Biochemistry* **3**, 356.
KILMARTIN, J. V., and CLEGG, J. B. (1967).—*Nature, Lond.* **213**, 269.
KIMMEL, J. R., ROGERS, H. J., and SMITH, E. L. (1965).—*J. biol. Chem.* **240**, 266.
KOSTKA, V., and CARPENTER, F. H. (1964).—*J. biol. Chem.* **239**, 1799.
MARKLAND, F. S., KREIL, G., RIBADEAU-DUMAS, B., and SMITH, E. L.(1966).—*J. biol. Chem.* **241**, 4642.
MICHL, H. (1951).—*Mh. Chem.* **82**, 489.
MOORE, S., and STEIN, W. H. (1951).—*J. biol. Chem.* **192**, 663.
MORSE, D., and HORECKER, B. L. (1966).—*Analyt. Biochem.* **14**, 429.
NAUGHTON, M. A., SANGER, F., HARTLEY, B. S., and SHAW, D. C. (1960).—*Biochem. J.* **77**, 149.
O'DONNELL, I. J., THOMPSON, E. O. P., and INGLIS, A. S. (1962).—*Aust. J. biol. Sci.* **15**, 732.
O'DONNELL, I. J., and THOMPSON, E. O. P. (1964*a*).—*Aust. J. biol. Sci.* **17**, 271.
O'DONNELL, I. J., and THOMPSON, E. O. P. (1964*b*).—*Aust. J. biol. Sci.* **17**, 973.
O'DONNELL, I. J., and THOMPSON, E. O. P. (1965).—*Aust. J. biol. Sci.* **18**, 1227.
O'DONNELL, I. J., and THOMPSON, E. O. P. (1968).—*Aust. J. biol. Sci.* **21**, in press.
OFFER, G. W. (1965).—*Biochim. Biophys. Acta* **111**, 191.
PIEZ, K. A. (1965).—*Biochemistry* **4**, 2590.
PIEZ, K. A., MARTIN, G. R., KANG, A. H., and BORNSTEIN, P. (1966).—*Biochemistry* **5**, 3813.
PORTER, R. R. (1957).—In "Methods in Enzymology". (Eds. S. P. Colowick and N. O. Kaplan.) Vol. 4, p. 221. (Academic Press, Inc.: New York.)
PRESS, E. M., PIGOTT, P. J., and PORTER, R. R. (1966).—*Biochem. J.* **99**, 356.
REINDEL, F., and HOPPE, W. (1954).—*Chem. Ber.* **87**, 1103.
RIFKIN, D. B., RIFKIN, M. R., and KONIGSBERG, W. (1966).—*Proc. natn. Acad. Sci. U.S.A.* **55**, 586.
SANGER, F. (1945).—*Biochem. J.* **39**, 507.
SCHOELLMANN, G., and SHAW, E. (1963).—*Biochemistry* **2**, 252.
SJÖQUIST, J. (1953).—*Acta Chem. Scand.* **7**, 447.
STARK, D. G., and SMYTH, G. R. (1963).—*J. biol. Chem.* **238**, 214.
STEERS, E., CRAVEN, G. R., ANFINSEN, C. B., and BETHUNE, J. L. (1965).—*J. biol. Chem.* **240**, 2478.

THOMPSON, E. O. P., and O'DONNELL, I. J. (1962).—*Aust. J. biol. Sci.* **15**, 552.
THOMPSON, E. O. P., and O'DONNELL, I. J. (1965).—*Aust. J. biol. Sci.* **18**, 1207.
THOMPSON, E. O. P., and O'DONNELL, I. J. (1966).—*Aust. J. biol. Sci.* **19**, 1139.
VAN ORDEN, H. O., and CARPENTER, F. H. (1964).—*Biochem. biophys. Res. Commun.* **14**, 399.
WANG, S. S., and CARPENTER, F. H. (1965).—*J. biol. Chem.* **240**, 1619.
WILKINSON, J. M., PRESS, E. M., and PORTER, R. R. (1966).—*Biochem. J.* **100**, 303.
YEMM, E. W., and COCKING, E. C. (1955).—*Analyst* **80**, 209.

Aust. J. biol. Sci., 1967, **20**, 1001–24

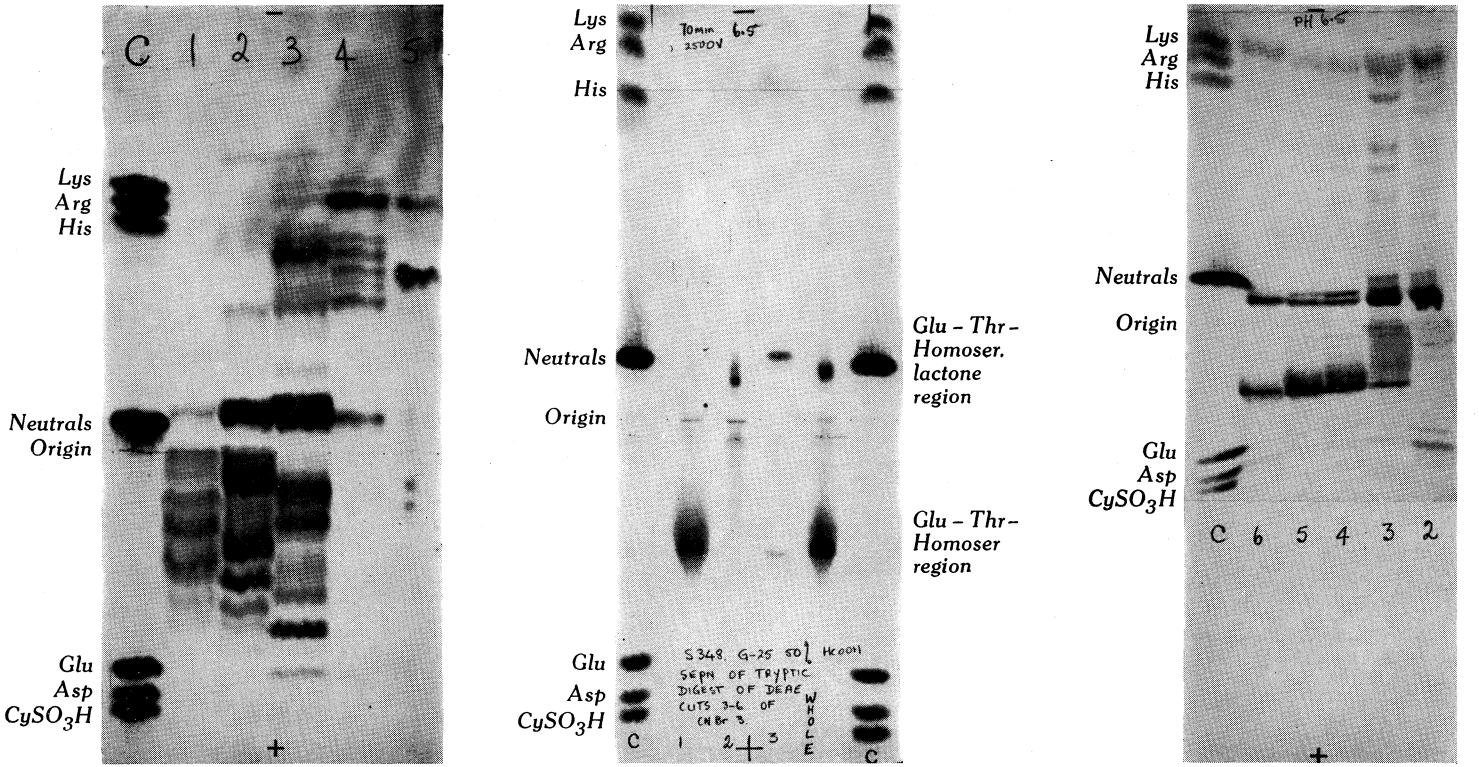

Fig. 1.—Paper electrophoresis at pH 6·5 for 50 min at 50V/cm of cuts from Sephadex G-25 runs (cf. text-fig. 1) of a tryptic digest of component 8. *C*, control.

Fig. 2.—Paper electrophoresis at pH 6·5 for 70 min at 50V/cm of fractions S1, S2, and S3 isolated from tryptic digests of fraction CNBr3 (cf. text-fig. 8). Stained with ninhydrin. An additional heavy stain appeared at the origin when fraction S2 was treated with the chlorine–tolidine–potassium iodide reagent. *C*, control.

Fig. 3.—Paper electrophoresis at pH 6·5 for 50 min at 50V/cm of tryptic digests of fractions isolated from fraction CNBr3 of component 8 by gradient elution from DEAE-cellulose (cf. text-fig. 7). Spots developed with ninhydrin. *C*, control.