# STUDIES ON REDUCED WOOL

## IX.* THE *N*-TERMINAL SEQUENCE OF A FRAGMENT PRODUCED BY CLEAVAGE OF COMPONENT 8 WITH CYANOGEN BROMIDE

By I. J. O'Donnell†

[*Manuscript received August* 7, 1968]

*Summary*

Component 8 is a major component extracted from reduced and carboxymethylated wool. Further study of its reaction with cyanogen bromide and of the fractions obtained under carefully controlled disaggregating conditions has revealed that while most of the methionine residues are in the same position relative to the ends of the chains, at least 30% of them appear to be in a different chemical environment from the rest. The evidence can be interpreted in terms of variations in some of the amino acids at particular points in a fundamental sequence of the 60 residues (CNBr3) at the amino terminal end. Further amino acid sequences near the acetylated terminal residue have been determined and provide examples of the amino acid variation along the chain. One sequence with variants is: *N*-acetylSer-(Tyr *or* Phe *or* Pro)-Asp-(Phe *or* Leu)-SCMCySH-Leu-Pro-Asp-Leu-Ser-Phe-Arg-. There is a region in component 8 where many of the *S*-carboxymethylcysteine residues are congregated.

### I. Introduction

Component 8 is an operationally defined protein (or protein fraction) extracted from reduced and carboxymethylated wool. It represents approximately 20% of wool, has a constant composition when isolated from different merino fleeces or top by either of two methods, has a tendency to aggregate, an $\alpha$-helix content of approximately 50%, and together with component 7 is believed to constitute the protofibrils of the wool fibre (see Thompson and O'Donnell 1967). It contains regions of differing susceptibility to enzyme attack, the readily attacked areas comprising the "tails" (Crewther and Harrap 1967). Component 8 has amino terminal groups which are acetylated (O'Donnell and Thompson 1968) and probably pyroglutamyl *N*-termini also (O'Donnell 1968). It has no detectable free carboxyl groups at its *C*-terminal end (Thompson, personal communication). In buffers containing 8M urea, component 8 has a molecular weight of 45,000 (Thompson and O'Donnell 1965; Jeffrey 1968*a*). In 0·01M borate, evidence for dissociation at low concentrations to units of molecular weight approximately 22,500 has been presented (Jeffrey 1968*b*, cf. DeDeurwaerder and Harrap 1965). It was pointed out previously (Thompson and O'Donnell 1965) that these subunits must be different from each other in their amino acid compositions and sequence. So far it has not proved possible to isolate them (nor to verify their existence by chemical means) and so the starting material

* Part VIII, *Aust. J. biol. Sci.*, 1968, **21**, 385–93.

† Division of Protein Chemistry, CSIRO Wool Research Laboratories, Parkville, Vic. 3052.

has to be the unit of 45,000 molecular weight. It is in this form that it will be considered in this paper.

Component 8 contains approximately one methionine residue per mole of 45,000 mol. wt. Cleavage with cyanogen bromide produces two main fractions, CNBr1 and CNBr3, separable on Sephadex G-75 in the disaggregating media 8M urea or 50% formic acid (Thompson and O'Donnell 1967). Their molecular weights are approximately 41,000 and 6,600 respectively and they arise from carboxyl and amino termini respectively. In the previous experiments the yield of CNBr3 was only about 50% of what it should be if the methionine residues occupied the same relative position in all molecules. By working under conditions to minimize the possibility of aggregation this yield has been substantially increased.

Optical rotatory dispersion measurements on samples of CNBr1 and CNBr3 have been carried out to see if the CNBr3 portion could be identified with a randomly organized section, the "tails" of Crewther and Harrap (1967), as its amino acid composition suggests.

Further sequences of amino acid residues in tryptic peptides from CNBr3 have provided further evidence of variations in sequence of a proportion of component 8 molecules, and supporting evidence for a family of related proteins.

## II. Materials and Methods

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

Extracts of reduced and carboxymethylated wool from single Merino fleeces, MW157 or MW158, were used. Component 8 was made by a mixed-solvent fractionation method (Crewther and Dowling, personal communication) and was used in most experiments without further purification. In some experiments it was freed from small amounts of material of lower molecular weight by passage through a Sephadex G-200 column (134 by 2·3 cm diam.) in buffer containing 8M urea at pH 10 (Thompson and O'Donnell 1967). Sometimes it was given a further passage through a similar column of Sephadex G-200 in 0·01M borate (cf. Jeffrey 1968*b*).

Buffer solutions with de-ionized urea were made as described previously (Thompson and O'Donnell 1967).

### (b) *Enzyme Digestions*

These were carried out with chymotrypsin, TPCK-trypsin, and carboxypeptidases A and B as described previously or according to the directions of Ambler (1963).

### (c) *Determination of Amino Acid Sequences*

The dansyl–Edman method was used according to the directions of Gray (1967). The dansylamino acids were identified on polyamide layers (Woods and Wang 1967). Wherever sufficient material was available samples of the remaining peptide were also taken for amino acid analysis. Where possible the phenylthiohydantoin derivatives of the amino acids were regenerated with constant boiling hydrochloric acid for 24 hr at 150°C (Van Orden and Carpenter 1964; Africa and Carpenter 1966) for identification on a Beckman amino acid analyser. In this manner it was also noticed that variable small amounts of peptides dissolved in the butyl acetate used in the extraction procedure.

### (d) *High-voltage Paper Ionophoresis, Amino Acid Analyses, Cleavage with Cyanogen Bromide, Hydrazinolysis, and Partial Acid Hydrolysis*

These were all carried out as described previously (Thompson and O'Donnell 1967; O'Donnell and Thompson 1968). In the process of cleavage of component 8 with cyanogen bromide the reagents were not removed by lyophilization but were dialysed away (see Section III) in 18/32 Visking cellulose tubing against several changes of 8M urea buffer containing ammonia before loading on a Sephadex G-75 column equilibrated with 8M urea buffer at pH 10.

Some peptides were eluted off the ninhydrin-stained paper with constant boiling hydrochloric acid for subsequent hydrolysis and amino acid analysis (Clegg, Naughton, and Weatherall 1965). No corrections have been applied for the hydrolytic breakdown of serine and threonine.

### (e) *Optical Rotatory Dispersion*

Measurements of optical rotatory dispersion were carried out on protein solutions in 0·05M sodium tetraborate with a Perkin–Elmer spectropolarimeter in the wavelength range 365–578 mμ. Values of $b_0$ were calculated using the Moffitt–Yang equation as described by Urnes and Doty (1961). A value of 212 mμ was taken for $\lambda_0$. The helix content of the protein was calculated on the basis that a value of $b_0 = -630$ represents 100% helix content.

The CNBr1 used for this determination was prepared from component 8 made by the mixed-solvent fractionation method, and separated from the reaction mixture on Sephadex G-75 in 50% formic acid.

The CNBr3 used was prepared from specially purified component 8 which had been given a passage through a column of Sephadex G-200 in 0·01M borate or buffer containing 8M urea at pH 10, or through both. The main peak was used. A small amount of material of lower molecular weight (<10%) was removed.

## III. Results

### (a) *Cyanogen Bromide Treatment of Component 8 and Yield of CNBr3*

In a previous paper (Thompson and O'Donnell 1967) a method was described whereby the cyanogen-bromide-treated component 8 was freeze-dried to remove cyanogen bromide before redissolving and separating the fragments CNBr1 and CNBr3 on Sephadex G-75 in either 50% formic acid or buffer containing 8M urea. The results showed that some homoserine (derived from the methionine) was present in CNBr1, and supported the conclusion that there were at least two types of molecules with their methionine residue in different positions relative to the ends of component 8 chains. In continued experiments, however, there was an accumulation of data that suggested that the homoserine content of CNBr1 might be due (at least predominantly) to non-covalently bound CNBr3 rather than homoserine in CNBr1 itself. These results were:

(1) There was a variability of yields (6·1, 10·8, 8·6, and 8·4%) of CNBr3 from component 8 previously reported for different preparations. On the basis of 7,700 and 45,000 for the molecular weights of CNBr3 and component 8 respectively, the yield of CNBr3, assuming all the methionine residues occupy identical positions in different molecules, should be 17%. Even if the molecular weight for CNBr3 is closer to 6,600, as later results in this paper suggest, the yield should be 14·7%.

(2) Only one kind of *N*-terminal sequence was found in CNBr1. This would correspond to that following methionine in the original component 8. This was $Glu(NH_2)$- (Thompson and O'Donnell 1967) and was further extended to $Glu(NH_2)$-Phe-Leu-Asp-Asp-Arg- (Hosken *et al.* 1968). If component 8 contained more than one type of molecule whose methionine position varied, these facts implied repeating sequences in component 8, an unlikely occurrence.

Since wool proteins aggregate readily, efforts were made to increase the yield of CNBr3 by considering this property and it was found best to use freshly prepared and freeze-dried component 8. Furthermore, the yield was much increased if the

reagents, cyanogen bromide and formic acid, were removed by dialysis against 8M urea buffer containing ammonia rather than by freeze-drying. In this way the yield of CNBr3 was substantially increased to a regular 12·4–14% for different preparations. This approximates the theoretical figure for a fragment of this size. This and the fact that the CNBr1 prepared in this manner contained homoserine at a barely detectable level makes it appear that the majority of the methionine residues in component 8 are in identical (or close) positions in different molecules relative to the ends of the chains. This corrects our previous conclusion (Thompson and O'Donnell 1967; O'Donnell, Frater, and Thompson 1968).

### (*b*) *Attempts to Dissociate CNBr1*

Recent experiments of Jeffrey (1968*b*) have shown that component 8 (mol. wt. 45,000) appears to consist of two subunits each of molecular weight approximately 22,500. This dissociation is apparent only at very low protein concentrations (< 0·05%) in 0·01M sodium tetraborate but not in buffers containing 8M urea. It had previously been attempted to dissociate component 8 using Sephadex columns in media such as phenol–acetic acid–water (1:1:1, cf. Brattsten, Synge, and Watt 1965) and 5M guanidine hydrochloride, but without success. From the methionine content of approximately one residue per 45,000 g of component 8, and from the large number of tryptic peptides (more than 40; the arginine and lysine content suggests there should be approximately 40 for a molecular weight of 45,000), it is seen that the subunit chains of mol. wt. 22,500 must be very different chemically and that only one of them can contain a methionine residue. It was reasoned that removal of approximately one-quarter of one of these subunit chains as CNBr3 may render the remaining CNBr1 (consisting of a 22,500 mol. wt. unit to which is attached a 16,000 unit) more amenable to dissociation. Accordingly, attempts were made to separate the component parts of CNBr1 on Sephadex G-200 in 8M urea buffer at pH 10, on Sephadex G-75 in 50% formic acid, and by gradient elution on DEAE-cellulose in buffers containing 8M urea. No evidence of a size separation was obtained though, as shown previously (Thompson and O'Donnell 1967), there was a gradation of chemical composition across the curve when using DEAE-cellulose. Hence, the two fragments comprising CNBr1 must be held tightly together by non-covalent forces and must be worked with as one unit.

### (*c*) *Behaviour of CNBr3 of Increased Yield on DEAE-cellulose*

Fractionation of CNBr3 by gradient elution from DEAE-cellulose in buffer containing 8M urea was done. This has previously been reported for the CNBr3 obtained in only 50% yield (Thompson and O'Donnell 1967), and that for the material obtained here in higher yield is shown in Figure 1(*a*). It is the same type of pattern as given previously (from a different single fleece, MW138), but there is a higher proportion (36%) of material now in peaks D1 plus D2 (Table 1). This approximate one-third of CNBr3 which contains 14·4% of the total homoserine, suggests that in CNBr3 there is a particular substantial fraction, D2, which is very different chemically from the closely related fractions D3–D6. It was suggested in a previous paper that this D2 fraction was probably an impurity derived from the high-glycine class of proteins which exist in wool (cf. Crewther *et al.* 1965). In one experiment designed to test

this idea, CNBr3 was prepared from component 8 which had been specially purified by successive passages through Sephadex G-200 in 0·01M borate and in buffer containing 8M urea. There was still 17% of D2 (containing 7% of the homoserine) in this CNBr3. If this can be interpreted to mean that some of fraction D2 arises from component 8 (i.e. not physically adsorbed) it points to differing chemical sequences around some of the methionine residues in different molecules of component 8.

TABLE 1

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

Fractions were isolated as in Figure 1, from single fleece MW157. Amino acid nitrogen values 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 amino acid nitrogen)* recovered from the column

| Amino Acid | Composition of Fraction | | | | |
|---|---|---|---|---|---|
| | D1+D2 | D3 | D4 | D5 | D6 |
| Lysine | 3·43 | 2·83 | 2·93 | 2·65 | 2·45 |
| Histidine | 2·07 | Trace | Trace | Trace | Trace |
| Ammonia | 11·10 | 11·10 | 11·10 | 11·10 | 11·10 |
| Arginine | 18·49 | 14·77 | 12·61 | 11·16 | 10·68 |
| Aspartic acid | 5·06 | 7·43 | 8·22 | 8·26 | 7·93 |
| Threonine | 3·54 | 4·00 | 4·13 | 4·09 | 4·40 |
| Serine | 7·85 | 9·51 | 11·32 | 11·66 | 11·33 |
| Glutamic acid | 7·20 | 6·75 | 5·52 | 4·90 | 5·11 |
| Proline | 3·92 | 5·19 | 6·35 | 6·73 | 7·01 |
| Glycine | 9·59 | 7·78 | 7·32 | 7·15 | 7·74 |
| Alanine | 3·52 | 3·33 | 2·99 | 2·89 | 2·86 |
| Valine | 2·05 | 3·75 | 2·02 | 2·93 | 3·06 |
| Methionine | 0·06 | 0 | 0 | 0 | 0 |
| Isoleucine | 2·19 | 1·83 | 1·66 | 1·49 | 1·61 |
| Leucine | 5·58 | 5·39 | 4·56 | 4·11 | 3·89 |
| Tyrosine | 4·14 | 2·24 | 1·33 | 0·94 | 0·71 |
| Phenylalanine | 3·38 | 3·39 | 4·77 | 5·38 | 5·16 |
| Homoserine | 0·41 | 1·02 | 1·24 | 1·53 | 1·47 |
| SCM-cysteine | 6·39 | 9·68 | 11·89 | 13·00 | 13·45 |
| Yield† | 36·0 | 6·8 | 22·0 | 27·5 | 7·7 |
| Homoserine‡ | 14·4 | 6·7 | 26·6 | 41·1 | 11·0 |

* Solutions were de-ammoniated with potassium carbonate before being put on the amino acid analyser. Homoserine lactone was thereby converted to homoserine.

† Given as percentage by weight of original CNBr3.

‡ As percentage of total homoserine.

Similar trends in amino acid composition of D4–D6, although not commented on previously (in table 4, Thompson and O'Donnell 1967), are apparent again in the present work. In going from D4 to D6 there is an increase in proline and *S*-carboxymethylcysteine (SCM-cysteine) and a decrease in arginine, leucine, and tyrosine. This trend emphasizes a degree of heterogeneity.

Rechromatography of peak D5 on DEAE-cellulose [Fig. 1(*b*)] shows that it emerges predominantly as a single peak and hence the original pattern is in no way artefactual.

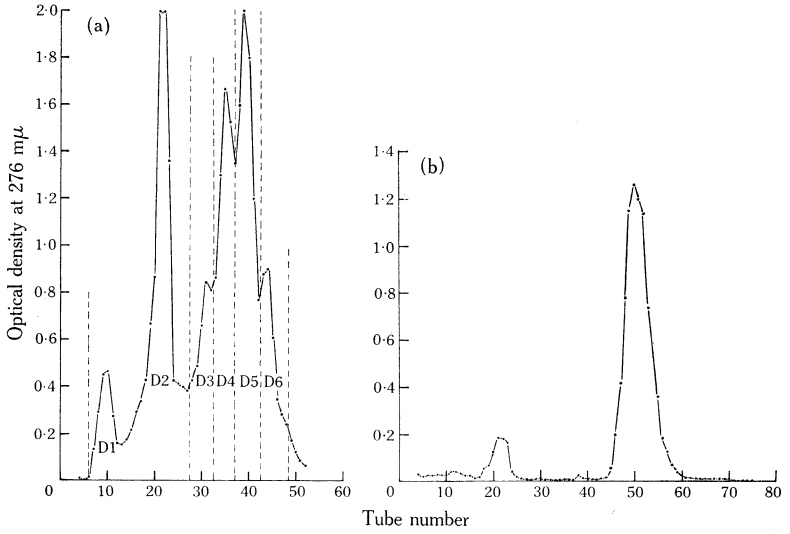


Fig. 1.—(*a*) Gradient elution at 25°C of "high-yielding" CNBr3 (approx. 400 mg) from DEAE-cellulose (Serva) (23 by 2 cm diam.). Starting buffer 8M urea–0·01M Tris–0·001M Versene at pH 7·4. Final buffer contained an additional 0·55M KCl. Linear gradient device had approximately 360 ml on each side. Fraction size approximately 8 ml; flow rate approximately 80 ml/hr. (*b*) Rechromatography of peak D5 under the same conditions.

### (*d*) *Size of CNBr3*

In an earlier paper the molecular weight of CNBr3 was estimated to be 7,700 by use of calibrated Sephadex columns. The accuracy of this figure is ±12% (Andrews *et al.* 1964). Present considerations suggest that its true value is nearer 6,600 for the following reasons:

(1) the homoserine contents of different batches of CNBr3 suggest the range 5,900–7,200;

(2) the sum of the probable number of residues in the fragments TS1, TS2, and TS3 produced by trypsin digestion of CNBr3 suggests it contains approximately 60 residues (see later).

### (*e*) *Tryptic Digestion of a Single Peak, D5, from CNBr3 and Separation of Fragments from It*

Peak D5 was selected for tryptic digestion and after freeze-drying the digest was fractionated on Sephadex G-25 in 50% formic acid as described previously (Thompson and O'Donnell 1967) for "purified" CNBr3 (i.e. peaks D4, D5, and D6 combined). The elution patterns were similar. The peaks TS1, TS2, and TS3 were

obtained. The numbers of residues they contain are given in Table 2, taking 6,600 as the molecular weight of CNBr3. It can be seen from Table 2 that the percentage

TABLE 2

AMINO ACID COMPOSITIONS OF FRACTIONS TS1, TS2, AND TS3 OF A TRYPTIC DIGEST OF PEAK D5 [FIG. 1(*a*)] FROM CNBr3

Results are given as residues per mole assuming D5 (from single fleece MW157) to have a molecular weight of 6,600 (*c.* 60 residues) and TS1, TS2, and TS3 to consist approximately of 45, 12, and 3 residues respectively

| Amino Acid | Composition of: | | | |
|---|---|---|---|---|
| | Unfractionated D5 | Peak TS1 | Peak TS2 | Peak TS3 |
| Lysine | 1·1 | 0·9 | | |
| Arginine | 2·1 | 1·0 | 1·0 | |
| Aspartic acid | 6·4 | 4·4 | 1·9 | |
| Threonine | 3·1 | 2·4 | 0·1 | 0·9 |
| Serine | 9·3 | 7·4 | 1·8 | 0·3 |
| Glutamic acid | 3·6 | 2·8 | 0·3 | 0·9 |
| Proline | 5·2 | 4·1 | 1·0 | |
| Glycine | 5·5 | 5·0 | 0·3 | |
| Alanine | 2·2 | 1·9 | | |
| Valine | 2·3 | 2·0 | | |
| Isoleucine | 1·1 | 1·0 | | |
| Leucine | 3·0 | 1·2 | 1·8 | |
| Tyrosine | 0·7 | 0·3 | 0·4 | |
| Phenylalanine | 4·0 | 1·9 | 2·1 | |
| SCM-cysteine | 9·2 | 8·2 | 1·1 | |
| Homoserine | 1·1 | 0·5 | | 1·0 |
| Total | 59·9 | 45·0 | 11·8 | 3·1 |
| Percentage of material in each peak* | 100 | 83·6 | 19·9 | 2·8 |
| Percentage expected yield† | 100 | 75 | 20 | 5 |

* Based on amino acid analysis.

† Assuming that these three fragments occurred in every molecule and accounted completely for the composition of CNBr3.

of material in peaks TS1, TS2, and TS3 was 83·6, 19·9, and 2·8 respectively. A corresponding analysis from different materials gave the following results:

| | Percentage of Material in: | | |
|---|---|---|---|
| | Peak TS1 | Peak TS2 | Peak TS3 |
| Tryptic digest of D4+D5+D6 from single fleece MW138 (Thompson and O'Donnell 1967) | 69·3 | 29·0 | 4·4 |
| Tryptic digest of D4 from mixture of top MW129 and single fleece MW138 | 55·1 | 30·7 | 3·2 |
| Tryptic digest of D3+D4+D5+D6 from top MW129 | 75·4 | 21·1 | 3·1 |

The amount of homoserine remaining in peak TS1 was 48% from the values in Table 2, and 31, 24, and 46% respectively from the above three analyses.

The values given in Table 2 may be compared with those in table 5 of Thompson and O'Donnell (1967) where there was an undetected error in the isoleucine value of peak TS2. It is now apparent that TS2 is a 12-residue fragment (see later). TS3 is the tripeptide Glu-Thr-Homoser and TS1 contains about 45 residues. The disposition of the tryptic fragments is TS2-TS1-TS3.

### (*f*) *Search for Reason Why Some Homoserine is in Peak TS1*

The values given above show that the middle fragment, TS1, contains some 24–48% of the homoserine of D5 (or D4+D5+D6 when referring to values from the previous paper). Attempts were made to release more homoserine-containing TS3 (or similar peptide) from TS1 by (1) repeated and prolonged tryptic digestion; (2) heating before digestion to ensure denaturation; and (3) solution of trypsin plus TS1 in 8M urea immediately before dilution to 4M urea (Harris 1956; Kimmel, Rogers, and Smith 1965) in an attempt to attack any inaccessible bonds. All were unsuccessful. Likewise gel filtration on Sephadex G-25 and G-75 in 50% formic acid and Sephadex G-75 in 8M urea buffer at pH 10 failed to remove any TS3 or other homoserine-containing peptide from TS1. Gradient elution of TS1 from DEAE-cellulose in buffers containing 8M urea at pH 7·4 released 12·7% of material of quite different chemical constitution to TS1, but it contained no homoserine. Its origin is not yet known.

TABLE 3

ANALYSIS OF FRACTIONS OF CNBr3-D5-TS2 SEPARATED ON DEAE-CELLULOSE (FIG. 2)

Values are moles of amino acid per mole of peptide assuming the arginine value = 1·0

| Amino Acid | Composition of Fraction | | Amino Acid | Composition of Fraction | |
|---|---|---|---|---|---|
| | d1 | d2 | | d1 | d2 |
| Lysine | | 0·26 | Valine | | 0·34 |
| Arginine | 1·00 | 1·00 | Isoleucine | | |
| Aspartic acid | 1·87 | 1·44 | Leucine | 1·86 | 1·63 |
| Threonine | | 0·47 | Tyrosine | 0·36* | 0·76 |
| Serine | 1·65 | 1·87 | Phenylalanine | 2·40* | 1·23 |
| Glutamic acid | | 0·81 | SCM-cysteine | 0·92 | 1·23 |
| Proline | 0·87 | 0·87 | | | |
| Glycine | | 0·84 | Yield as percentage of | | |
| Alanine | | 0·29 | total recovered (total | | |
| | | | recovery = 101%) | 78·3 | 21·7 |

* Mixture of two peptides, one containing a tyrosine substituted for a phenylalanine residue.

Nor could the resistance to tryptic digestion of any lysyl bond in the neighbourhood of the homoserine be attributed to partial carbamylation of the $\epsilon$-amino group by cyanate present in urea used in the preparation of CNBr3, since no homocitrulline was detected in acid hydrolysates of TS1.

The most probable explanation for some homoserine remaining in TS1 is that there is a variation in amino acid sequence in some molecules in D5 so that a trypsin-susceptible amino acid residue is not close to the homoserine residue or else the bond is rendered stable by a preceding amino acid such as proline or an acidic amino acid. In either case it points to a substitution of amino acid residues in a good percentage of the D5 molecules in this area.

### (*g*) *Sequence Studies on CNBr3-D5-TS2*

The tryptic peptide fraction CNBr3-D5-TS2 was insoluble in the absence of urea. It was purified using gradient elution from DEAE-cellulose in buffers containing 8M urea. Two peaks, d1 and d2, were obtained, d2 being 21·7% of the total (Fig. 2 and Table 3). The urea was removed using a Sephadex G-10 column (130 cm

Fig. 2

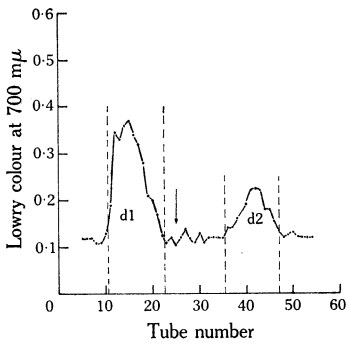


Fig. 3

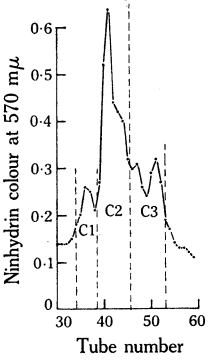


Fig. 2.—Gradient elution of tryptic fraction (25 mg) CNBr3-D5-TS2 from DEAE-cellulose (21 by 2·3 cm diam.). Fraction size approximately 6·5 ml; flow rate *c.* 96 ml/hr. Starting buffer 8M urea–0·01M Tris–0·001M Versene at pH 7·4. Final buffer contained an additional 0·5M KCl. 360 ml on each side of linear gradient device. Gradient applied at tube 25 (indicated by arrow). Peptide d1 = 78·4% (w/w) of total.
Fig. 3.—Fractionation of chymotryptic digest (16 hr) of peptide d1 (2·3 mg) (Fig. 2) on Sephadex G-25 (140 by 0·9 cm diam.) in 50% formic acid. Fraction size = 2·0 ml. 100 μl aliquots taken for alkaline hydrolysis and development of ninhydrin colour.

by 2·3 cm diam.) in 50% formic acid. Peak d1 probably contains 12 residues and it seems that d2, even though it is not pure, is related to d1 and in addition probably contains a glycine and glutamic acid residue and lacks a phenylalanine residue which points to substitutions or additions, since this is an *N*-terminal tryptic peptide (with its *N*- and *C*-terminal amino acids fixed). Fraction d1 was chosen initially for further study and was digested with chymotrypsin and passed through Sephadex G-25 or G-15 (Fig. 3). The analyses of the resulting fractions are shown in Table 4.

#### (i) *Sequence of Peptide CNBr3-D5-TS2-d1-C3*

This is the mixed tetrapeptide *N*-acetylSer-(Tyr *or* Phe)-Asp-Phe at the amino terminal end of component 8 and isolated previously (O'Donnell and Thompson 1968).

(ii) *Sequences of Peptides from CNBr3-D5-TS2-d1-C2*

This fraction consists of a mixture of peptides which were purified by paper ionophoresis (Table 5). Peptide δ contained (Ser, Phe)-Arg and a subtractive Edman reaction removed serine. Therefore its sequence is Ser-Phe-Arg. The sequence of peptide γ is then Leu-Ser-Phe-Arg. Hydrazinolysis showed peptide β to have a *C*-terminal leucine. The subtractive Edman method on peptide α showed the sequence to be SCMCySH-Leu-Pro-Asp. (The yields of the peptide after each step were 70–80% of previous step.) Therefore, the sequence of the whole peptide is: SCMCySH-Leu-Pro-Asp-Leu-Ser-Phe-Arg.

TABLE 4

AMINO ACID COMPOSITION OF CHYMOTRYPTIC FRAGMENTS OF PEPTIDE CNBr3-D5-TS2-d1 SEPARATED ON SEPHADEX (FIG. 3)

Amino acid content is expressed as residues per mole, assuming the peptides C1, C2, and C3 to contain 12, 8, and 4 residues respectively

| Amino Acid | Composition of Peptide(s) | | |
|---|---|---|---|
| | C1 | C2 | C3 |
| Lysine | (0·36)* | | |
| Arginine | 0·8 | 1·1 | |
| Aspartic acid | 2·0 | 1·0 | 1·0 |
| Threonine | (0·2)* | | |
| Serine | 1·7 | 1·1 | 1·0 |
| Glutamic acid | | | |
| Proline | 1·7 | 0·9 | |
| Glycine | (0·5)* | | |
| Alanine | (0·3)* | | |
| Leucine | 3·1 | 1·7 | |
| Tyrosine | | | 0·3 |
| Phenylalanine | 1·1 | 1·1 | 1·7 |
| SCM-cysteine | 1·5 | 0·8 | |
| Total residues | 11·9 | 7·7 | 4·0 |
| Percentage by weight of total† | 23 | 47 | 30 |

* These are probably from contaminant peptides and are neglected. Other preparations did not contain so much of these but this is the preparation on which quantitative yields were done.

† Based on amino acid analysis.

Hence the sequence of one of the peptides in CNBr3-D5-TS2 is *N*-acetylSer-(Phe *or* Tyr)-Asp-Phe-SCMCySH-Leu-Pro-Asp-Leu-Ser-Phe-Arg.

(iii) *Sequence of Peptide CNBr3-D5-TS2-d1-C1*

This peptide (23% of the total material) from its position on Sephadex (Fig. 3) is bigger than C2 and is resistant to attack by chymotrypsin. If C2 and C3 are added together it is seen that the amino acid composition is similar to C1 except for a probable Phe/Pro and Phe/Leu substitution which could account for its resistance to chymotryptic attack.

From a partial acid hydrolysate peptides were separated by paper ionophoresis at pH 1·9 and gave the following approximate compositions: (SCMCySH *or* Asp, Leu, Pro), (SCMCySH, $Leu_2$, $Pro_2$, $Asp_2$), and (SCMCySH, $Leu_2$, $Pro_2$, Asp), which suggests the sequence for C1 to be *N*-acetylSer-Pro-Asp-Leu-SCMCySH-Leu-Pro-Asp-Leu-Ser-Phe-Arg.

TABLE 5

ANALYSIS OF COMPONENT PEPTIDES OF CHYMOTRYPTIC PEPTIDE FRACTION CNBr3-D5-TS2-d1-C2 (FIG. 3)

Peptides were eluted from ninhydrin-stained paper with constant boiling HCl. SCM-cysteine values are sometimes low after elution of peptides from paper. The mobility of peptides α, β, γ, δ, and ε on paper electrophoresis at pH 1·9 relative to lysine = +1·0 and origin = 0 were 0·29, 0·34, 0·66, 0·78, and 0·92 respectively. Peptide β was not completely free from α but contained considerably less serine and phenylalanine. Values are expressed as moles of amino acid per mole of peptide

| Amino Acid | Composition of Peptide | | | | |
|---|---|---|---|---|---|
| | α | β | γ | δ | ε |
| SCM-cysteine | 0·7 | 0·7 | | | |
| Leucine | 2·0 | 2·0 | 0·8 | | |
| Phenylalanine | 1·0 | 0·3 | 1·3 | 1·0 | |
| Proline | 1·0 | 1·0 | | | |
| Aspartic acid | 1·1 | 1·2 | | | |
| Serine | 1·0 | 0·5 | 1·6 | 1·0 | |
| Arginine | 0 | 0·2 | 1·1 | 1·0 | 1·0 |

The results show that the first 12 residues of some of the chains of component 8 contain several substitutions and fraction d2 (Fig. 2) probably contains peptide sequences with further changes.

### (*h*) *Sequence Studies on CNBr3-D5-TS1*

A mixture of carboxypeptidases A and B on the tryptic peptide fraction CNBr3-D5-TS1 released lysine and a smaller amount of glutamic acid (or glutamine). No amino terminal group could be detected by the dansyl technique on the whole material nor after successive steps of the Edman degradation procedure. Hence the residue must be either a glutamine which has ring-closed to a pyroglutamyl residue, or possibly an SCM-cysteine residue which has cyclized (cf. Smyth and Utsumi 1967). Both these cyclizations would be encouraged by the gel filtration in 50% formic acid during the preparation of TS1.

The fractionation on Sephadex of TS1 (10 μmoles) after digestion with chymotrypsin is shown in Figure 4. Peak 5 contained no amino-acid-containing material. The composition of peaks 1–4 are given in Table 6.

### (*i*) *Sequences in CNBr3-D5-TS1-C4*

The number of residues in C4 is probably 13 since it contains homoserine, but this amino acid was not determined quantitatively. C4 must come from the *C*-terminal

end of the homoserine-containing chains and could include some molecules with -Glu-Thr-Homoser as *C*-terminal sequences which have not been completely liberated by trypsin.

The separation and composition of component peptides in C4 are given in Table 7. It is obvious that the peptides are related to each other, even $\gamma$, which is not of the same degree of purity as the others. It appears in $\delta$, $\epsilon$, and $\phi$ that there is a partial substitution of threonine for serine. Peptide $\epsilon$ must arise from those molecules of TS1 which contain homoserine (see above) and must terminate in homoserine. The cause of electrophoretic separation of $\delta$ and $\phi$ is not known.

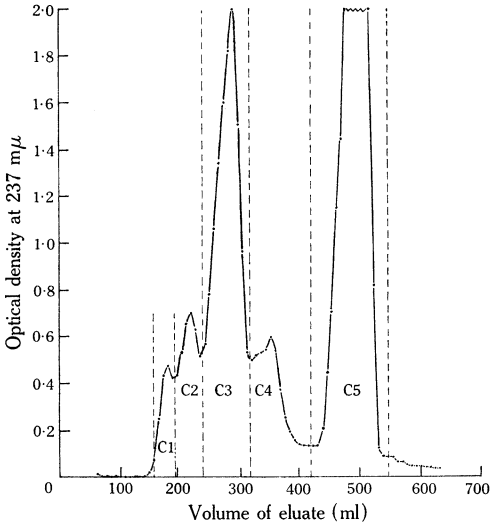


Fig. 4.—Elution diagram of a chymotryptic digest of peptide CNBr3-D5-TS1 (10 $\mu$moles, 7 hr) on Sephadex G-50 (120 by 2·3 cm diam.) in 0·05M ammonia. Fraction size 8 ml; flow rate 16 ml/hr.

The significance of the fact that $\delta$, $\epsilon$, and $\phi$ each appear to contain 13 residues is difficult to grasp. Some molecules of TS1 terminate in lysine. These arise from the majority of the CNBr3 molecules when Glu-Thr-Homoser (fraction TS3) has been removed by trypsin. Peptides $\delta$ and $\phi$ could arise from these. Peptide $\epsilon$ shows that there are other molecules of TS1 which contain in their last 13 residues a lysine residue, and yet must terminate in a homoserine residue. The lysyl bond in these molecules is resistant to tryptic attack. These are presumably the molecules discussed earlier as the reason for homoserine being in TS1. Since $\delta$, $\epsilon$, and $\phi$ each contain 13 residues it is seen that the TS1 molecules giving rise to $\epsilon$ cannot be considered as the same as those of the TS1 molecules giving rise to $\delta$ and $\phi$ (ignoring for the present the reason for the resistance of the lysyl bond). If $\delta$ and $\phi$ were each extended to contain homoserine by the addition of the tripeptide Glu-Thr-Homoser they would be 16 residues long. The overall conclusion is that variations in sequence is the explanation.

The dansyl–Edman method suggests that the probable sequence of CNBr3-D5-TS1-C4-$\epsilon$ is Phe-SCMCySH-(Gly *or* Glux)-Gly-Phe-(Ser *or* Thr, $Aspx_2$, $Glux_2$, Gly, Lys, Thr)-Homoser.*

* Aspx and Glux are used to indicate that aspartic and glutamic acids cannot be differentiated from asparagine and glutamine respectively.

TABLE 6

AMINO ACID COMPOSITIONS OF COMPONENT FRACTIONS OF CHYMOTRYPTIC DIGEST OF CNBr3-D5-TS1 ISOLATED ON SEPHADEX (FIG. 4)

Composition expressed as moles of amino acid residues relative to the italicized one in each fraction which has been fixed arbitrarily so that the numbers of residues in C3 and C4 are approx. 33 and 12 respectively

| | Composition of Fraction | | | |
|---|---|---|---|---|
| | C1 | C2 | C3 | C4 |
| Sephadex G-50 (Fig. 4) elution volume* | 1·1 | 1·4 | 1·7 | 2·1 |
| Percentage by weight (of total) of material† | 3·2 | 9·6 | 60·4 | 26·7 |
| Amino acid | | | | |
| Lysine | | *c.* 0·5 | 0·2 | 0·9 |
| Arginine | | *c.* 0·3 | 0·6 | (0·1) |
| Aspartic acid | 2·6 | 3·8 | 2·6 | 2·1 |
| Threonine | 1·5 | 2·0 | 2·2 | 0·5 |
| Serine | 5·0 | 6·2 | 5·9 | 1·4 |
| Glutamic acid | | 1·7 | 0·9 | 1·9 |
| Proline | 5·0 | 4·8 | 3·1 | 0·5 |
| Glycine | 3·5 | 3·5 | 3·4 | 1·8 |
| Alanine | 1·8 | 1·7 | 2·0 | (0·3) |
| Valine | 1·2 | 2·2 | 2·2 | (0·2) |
| Isoleucine | *1·0* | *1·0* | *1·0* | (0·2) |
| Leucine | 0·7 | 1·2 | 1·2 | (0·3) |
| Tyrosine | | | | |
| Phenylalanine | 0·7 | 1·5 | 0·6 | *1·3* |
| SCM-cysteine | 3·2 | 5·8 | 7·0 | 1·3 |
| Homoserine | | | | ‡ |
| Total residues§ | | | 32·9 | 11·6 |
| Yield (%)‖ | | | 82 | 92 |

* The elution volume of the column is expressed as the ratio of the column volume at which the sample is eluted to the void volume of the column (measured with component 8).

† Computed from amino acid analyses.

‡ Homoserine was present but it was not quantitatively determined.

§ Fractions are not single peptides but the numbers for C3 and C4 are indicative enough to warrant expression of the values in this manner.

‖ Yields calculated as percentage of expected recovery of a homogeneous peptide with the number of residues shown.

### (*j*) *Examination by Optical Rotatory Dispersion*

Measurements showed that CNBr3 had an α-helix content of zero while that of CNBr1 was 52%. Component 8 itself had an α-helix content of 63% and it can therefore be seen that fission of component 8 at the methionine residue has caused the helix content of the whole system to decrease. This implies that the *N*-terminal

fragment which becomes CNBr3 stabilizes some helical parts of the original component 8.

TABLE 7

AMINO ACID COMPOSITIONS OF SIGNIFICANT CHYMOTRYPTIC PEPTIDES IN FRACTION C4 OF FRAGMENT CNBr3-D5-TS1 (FIG. 4)

Amino acid contents expressed as moles per mole of peptide assuming lysine = 1·0. Mobility of peptides $\gamma$, $\delta$, $\epsilon$, and $\phi$ on paper electrophoresis at pH 6·5 relative to lysine = +1·0, aspartic acid = −1·0, and neutral amino acids = 0 was −0·36, −0·54, −0·61, and −0·70 respectively

| Amino Acid | Composition of Peptide | | | |
|---|---|---|---|---|
| | C4$\gamma$ | C4$\delta$ | C4$\epsilon$ | C4$\phi$ |
| Lysine | 1·0 | 1·0 | 1·0 | 1·0 |
| Arginine | 0·5 | | | |
| Aspartic acid | 2·4 | 2·0 | 2·1 | 2·2 |
| Threonine | 0·4 | 0·3 | 0·6 | 0·5 |
| Serine | 1·9 | 1·3 | 1·2 | 1·3 |
| Glutamic acid | 1·2 | 2·5 | 2·8 | 2·7 |
| Proline | 0·6 | | | |
| Glycine | 1·9 | 2·3 | 2·1 | 2·3 |
| Alanine | 0·5 | | | |
| Valine | 0·6 | | | |
| Isoleucine | 0·3 | | | |
| Leucine | 0·3 | | | |
| Phenylalanine | 0·4 | 2·1 | 1·5 | 1·7 |
| SCM-cysteine | 1·2 | 1·2 | 1·1 | 1·1 |
| Homoserine lactone | | 0·08* | 0·42* | |
| Total residues | 13·2 | 12·8 | 12·8 | 12·8 |
| Percentage of each peptide† | 10·5 | 40·0 | 25·7 | 23·7 |

* The homoserine present was not determined so these numbers could be far too low (for the value of total homoserine+homoserine lactone), especially as homoserine lactone was not well resolved from ammonia.

† Of total of the four peptides recovered.

### (*k*) *Extinction Coefficient of CNBr3*

A sample of CNBr3 was prepared from component 8 especially purified by passage through a column of Sephadex G-200 in 8M urea at pH 10 and subsequently through a column of Sephadex G-200 in 0·05M sodium tetraborate. Its $E^{1\%}_{1\,cm}$ at 276 m$\mu$ in glacial acetic acid was 11·8, the protein concentration being measured by refractive index increment of protein solution versus its dialysate in 0·05M borate (the specific refractive increment, d$n$/d$c$, for the protein was taken as $1{\cdot}86\times10^{-3}$ at 546 m$\mu$). Component 8 itself has an $E^{1\%}_{1\,cm}$ of 7·5 (Thompson and O'Donnell 1965) and, since CNBr1 and CNBr3 have approximately the same tyrosine content, the tryptophan content of CNBr3 must be higher than that of CNBr1.

## IV. Discussion

Two main points emerge from the experimental work presented here. The first is the additional evidence to that presented previously, that component 8 is heterogeneous and consists of a family of proteins. The second is that there appears to be an underlying basic sequence in the group of proteins and that the heterogeneity is formed by variations superimposed on this sequence. In this respect the system is somewhat analogous to the heavy chains of normal rabbit immunoglobulin IgG (Cebra, Steiner, and Porter 1968).

Pointers towards further heterogeneity are given below.

(1) In the gradient elution of CNBr3 from DEAE-cellulose there is a peak, D2, which has very different amino acid composition from D3 to D6 and still contains some 7% of the homoserine derived from the one methionine residue per 45,000 g of component 8. This indicates that the methionine residue in component 8 is not in a unique chemical environment. However, most of the methionine residues do seem to be in a similar position relative to the ends of the chain. This is shown by the almost theoretical yield of CNBr3 when precautions are taken during its preparation to prevent any irreversible adsorption of it onto CNBr1. The possibility that D2 represents protein impurities physically adsorbed on the original component 8 cannot be definitely excluded.

(2) When the D5 fraction of CNBr3 (Fig. 1) is digested with trypsin and the resulting mixture separated on Sephadex into fractions TS1, TS2, and TS3, the homoserine is distributed between the largest (TS1) and smallest (TS3) fragments instead of being contained in one of them exclusively. This again indicates a change in the susceptibility, and hence chemical environment, of a lysine bond closest to the methionine of component 8. It should be pointed out that in these experiments only one of the peaks (D5) given by gradient elution of whole CNBr3 from DEAE-cellulose was used.

These two facts suggest that at least 30% of the molecules have their methionine residues in a different chemical environment to the rest.

(3) The trends in contents of the amino acids proline, SCM-cysteine, arginine, and tyrosine in fractions obtained when CNBr3 is fractionated by chromatography on DEAE-cellulose (Fig. 1) points to the presence of different groups of molecules in CNBr3.

(4) The probable starting sequence of some of the molecules of component 8 can be deduced from that of CNBr3 and extended further from that of CNBr1 (Hosken *et al.* 1968) and the work of Corfield, Fletcher, and Robson (1968). It is *N*-acetylSer-(Pro *or* Phe *or* Tyr)-Asp-(Phe *or* Leu)-SCMCySH-Leu-Pro-Asp-Leu-Ser-Phe-Arg-(Lys *or* Arg, $Aspx_3$, $Thr_2$, $Ser_6$, Glux, $Pro_3$, $Gly_{3.4}$, $Ala_2$, $Val_2$, Ile, Leu, $Phe_{0.6}$, $SCMCySH_{7.0}$)-Phe-SCMCySH-(Glu *or* Gly)-Gly-Phe-(Thr *or* Ser, $Aspx_2$, Gly, Glux)-Lys-Glu-Thr-Met-Glu($NH_2$)-Phe-Leu-Asp-Asp-Arg-Leu-Ala-Ser-Tyr-Leu-Glu-Lys-Val-Arg-Glu-Leu. The presence of several substitutions of one amino acid for another in the few sequences which have been determined points to a complex mixture of proteins in component 8.

Considering the second main point it is seen that several peptides have been obtained from component 8 in high yield and those that have been studied conform

to a pattern, if not a unique sequence. It is possible to place them in position in the parent molecule. Corfield, Fletcher, and Robson (1967, 1968), using a somewhat analogous protein preparation from oxidized wool, obtained many pure peptides, some in high yield, but have not so far been able to place them in position. Because of the complex nature of proteins comprising component 8, sequence determinations on every type of chain present is not yet possible. However, by choosing only one or two major peptides from each section of the molecule, it should be possible to construct a basic sequence to show what types of amino acid sequences occupy the various sections of the molecule.

It is seen that CNBr3, comprising approximately 60 residues from the *N*-terminal section of component 8, contains no $\alpha$-helix in agreement with what might be expected from its high content of serine, proline, glycine, and *S*-carboxymethylcysteine (cf. Crewther *et al.* 1968). It thus corresponds to part of the "tails" section of these types of molecules extracted from reduced and carboxymethylated wool (Crewther and Harrap 1967; Crewther *et al.* 1968). However, the trend in the aspartic acid values are in opposite directions (Table 8). Its high value for $E^{1\%}_{1\,cm}$ of 11·8 is in accord with the correspondingly low value of 5·9–6·5 found for the helix-rich fractions obtained for analogous preparations of wool proteins (Crewther and Harrap 1967).

The cystine residues are congregated in CNBr3 which is analogous to the "tails" (i.e. non-helical) fraction of Crewther and Harrap (1967). Whereas in CNBr1 there is approximately 1 half-cystine residue per 16–20 residues, in CNBr3 there is 1 per 6 residues (Table 2). Thus areas of the less-ordered regions of component 8 are "sulphur rich" to a degree similar to that in the matrix "high-sulphur" proteins (1 SCM-cysteine residue in 4–6 residues, Gillespie 1967). Further similarity of the tails section and the high-sulphur matrix proteins are seen in the relatively high values for proline, serine, and glycine (Table 8). Corfield, Fletcher, and Robson (1968) have also postulated high-sulphur regions in the same molecules in which the low-sulphur helical regions occur.

Since it has not been possible to obtain component 8 or its cyanogen bromide fragments, CNBr1 and CNBr3, in a homogeneous state the values listed in Table 8 have been taken to be representative analyses of these materials. The molecular weights of component 8 and CNBr3 have been taken as 45,000 and 6,600 respectively; the molecular weight for CNBr1 is therefore taken as 38,400. The balances are only approximate but it can be seen that 10 residues of *S*-carboxymethylcysteine, i.e. half the total content of component 8, reside in CNBr3. Of these 10, 7 reside in the peptide CNBr3-D5-TS1-C3 of 33 residues (Table 6), i.e. there is 1 SCM-cysteine residue per 4–5 total residues. Thus not only are cystine–cystine sequences common in wool (see Lindley and Haylett 1967) but there are regions in component 8 where the SCM-cysteine residues are concentrated. It seems probable from the composition of the helical section (Crewther *et al.* 1968) that another similar length of peptide will be found in CNBr1 to account for the majority of the remaining SCM-cysteine residues.

In view of the similarity between the amino acid compositions of CNBr3 and the high-sulphur fraction (matrix proteins from wool), the idea of some novel method of polypeptide chain synthesis or assembly whereby high-sulphur proteins may either remain in the matrix fraction or be incorporated into the helical (i.e. low-

sulphur) fraction is attractive. Gillespie (1965) has postulated the possibility of stepwise addition of sulphur-rich peptides to high-sulphur proteins. The outstanding role of sulphur in keratin chemistry and biosynthesis (cf. Gillespie and Reis 1966)

TABLE 8

REPRESENTATIVE AMINO ACID COMPOSITIONS OF COMPONENT 8 AND ITS CYANOGEN BROMIDE FRAGMENTS

Values are given either as moles per mole of component 8 or fragment (M/M) or as amino acid nitrogen values (N/N) which are expressed as a percentage of the total nitrogen content of the amino acids plus ammonia recovered from the column, or in both ways. Ammonia was taken arbitrarily as one-eighth of the sum of the amino acid nitrogen

| Amino Acid | Component 8* | | CNBr1† | | CNBr3‡ | | High-sulphur Fraction§ (N/N) | Non-helical Sections‖ (N/N) |
|---|---|---|---|---|---|---|---|---|
| | N/N | M/M | N/N | M/M | N/N | M/M | | |
| Lysine | 4·37 | 11·6 | 3·82 | 8·7 | 2·65 | 1·0 | 1·0 | |
| Histidine | 1·24 | 2·2 | 1·90 | 2·9 | Trace | | 0·8 | 0·66 |
| Ammonia | 11·10 | | 11·10 | | 11·10 | | 9·7 | 8·71 |
| Arginine | 22·0 | 29·3 | 23·0 | 26·2 | 11·16 | 2·1 | 17·6 | 24·7 |
| Aspartic acid | 7·51 | 40·1 | 7·65 | 34·8 | 8·26 | 6·5 | 1·6 | 3·05 |
| Threonine | 3·61 | 19·3 | 3·47 | 15·8 | 4·09 | 3·2 | 7·2 | 4·69 |
| Serine | 5·47 | 29·2 | 4·57 | 20·8 | 11·66 | 9·1 | 9·7 | 9·98 |
| Glutamic acid | 12·22 | 65·2 | 14·28 | 65·0 | 4·90 | 3·8 | 8·1 | 2·30 |
| Proline | 2·53 | 13·5 | 1·41 | 6·41 | 6·73 | 5·3 | 8·0 | 5·96 |
| Glycine | 3·10 | 16·6 | 2·08 | 9·48 | 7·15 | 5·6 | 5·8 | 12·8 |
| Alanine | 4·30 | 22·9 | 4·01 | 18·3 | 2·89 | 2·3 | 2·8 | 2·98 |
| Valine | 4·45 | 23·8 | 5·03 | 22·9 | 2·93 | 2·3 | 3·7 | 2·98 |
| Methionine | 0·24 | 1·3 | | | | | | 0·74 |
| Isoleucine | 2·55 | 13·6 | 2·48 | 11·3 | 1·49 | 1·2 | 2·4 | 1·63 |
| Leucine | 8·00 | 42·7 | 8·75 | 39·8 | 4·11 | 3·2 | 2·5 | 2·68 |
| Tyrosine | 1·80 | 9·6 | 1·63 | 7·41 | 0·94 | 0·7 | 2·0 | 3·87 |
| Phenylalanine | 1·50 | 8·0 | 1·02 | 4·6 | 5·38 | 4·2 | 1·1 | 3·0 |
| SCM-cysteine | 3·96 | 21·1 | 3·42¶ | 15·4¶ | 13·0 | 10·2 | 15·8 | 9·16 |
| Homoserine | | | Trace | | 1·53 | 1·2 | | |
| Total | | 370 | | 310 | | 62 | | |

* Molecular weight taken as 45,000. Amino acid values taken from column 1 of table 6 of Thompson and O'Donnell (1967).

† Molecular weight taken as 38,400. Amino acid values are averages of fraction 2 and fraction 3 of table 3 of Thompson and O'Donnell (1967).

‡ Molecular weight taken as 6,600. Amino acid values are those of D5 of Table 1 of this paper.

§ Values taken from table 1, fraction G, of O'Donnell and Thompson (1964).

‖ Values from table 1, i.e. extrapolated values of non-helical sections, of Crewther *et al.* (1968).

¶ Average values. Duplicate runs gave the following results: N/N, 2·96 and 3·89; M/M, 13·5 and 17·7.

is exemplified by the fact that the heterogeneity of component 8 or CNBr1 is most clearly defined in the overall analyses (Thompson and O'Donnell 1967) by the variation of SCM-cysteine content across the profiles of these materials on chromato-

graphy. It would thus be surprising if the sulphur content of the low-sulphur components of wool, such as component 8, were not affected by the sulphur content of the diet, as has been found for the matrix high-sulphur proteins.

## V. Acknowledgments

I wish to thank Professor E. O. P. Thompson for helpful advice and discussion of this work.

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