TERMINAL AMINO GROUPS IN WOOL AND *S*-CARBOXYMETHYL KERATEINE 2

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Summary

The *N*-terminal residues of Merino 64's quality wool and of a purified protein derivative extracted from it, *S*-carboxymethyl kerateine 2, have been determined. A similar total *N*-terminal residue content is present in both wool and *S*-carboxymethyl kerateine 2 comprising glycine, serine, threonine, aspartic acid, glutamic acid, and alanine. These occur in different proportions in the two materials and valine is an additional *N*-terminal amino acid present only in wool.

It is concluded from the quantitative data that these *N*-terminal residues are not stoichiometrically significant in either case and that cyclic structures are present.

I. Introduction

By treating wool with alkaline thioglycollate solutions a soluble protein representing at least 20 per cent. of the wool has been isolated, which behaves as a relatively pure substance during electrophoresis (Gillespie and Lennox 1955*a*, 1955*b*). This protein has been converted to the *S*-carboxymethyl derivative (Gillespie 1956) and further fractionated by precipitation with zinc ions or ammonium sulphate (Gillespie 1957).

The 1,2,4-fluorodinitrobenzene (FDNB) (Sanger 1945) and phenyl*iso*thiocyanate methods (Edman 1950) of *N*-terminal group analysis have been applied to *S*-carboxymethyl kerateine 2 (SCMK2) to investigate whether any of the known *N*-terminal residues of wool (Middlebrook 1951) have been lost during extraction of the soluble protein, or alternatively if any new *N*-terminal residues indicative of random hydrolysis have appeared, and also to compare quantitatively the *N*-terminal residues with those present in wool. It was also hoped that end-group studies would be of some value in following the course of fractionation of SCMK2.

In contrast to soluble proteins, which react quantitatively with FDNB in aqueous solution during 2 hr at 40°C, wool requires at least 48 hr for adequate penetration of the reagent (Middlebrook 1951). In the present studies on wool the method of Fritze and Zahn (1956) for the exhaustive dinitrophenylation of wool has been used to ensure maximum reaction with the *N*-terminal residues.

II. Materials and Methods

Virgin Merino wool, 64's quality, from one fleece, was supplied by Dr. F. G. Lennox after light scouring in the following manner: the tips were removed and the wool was washed three times successively with ether and six times with water; after squeezing out excess water the wool was dried in a forced air draught at 40°C; the wool was then treated once more with ether and four times with water before

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drying for 2 hr at 40°C; finally, the wool was hand-combed and conditioned at 20°C and 63 per cent. relative humidity.

A further sample of Merino 64's wool in the form of dry combed top was also studied. This was extracted four times with light petroleum, four times with ethanol, and repeatedly with distilled water before being dried and conditioned. Samples of SCMK2 were prepared by Mr. J. M. Gillespie according to the published procedures (Gillespie 1956, 1957).

III. Experimental

(*a*) *Dinitrophenylation*

The standard conditions of Fritze and Zahn (1956) were used to dinitrophenylate wool: 1·66 per cent. sodium bicarbonate solution, 50 per cent. FDNB on the weight of wool (liquor : wool ratio of 60 : 1) were incubated at 60°C for 7 hr with changes of the reaction medium after $2\frac{1}{2}$ and 5 hr. Washing of the dinitrophenylated wool was carried out according to the directions of these workers and after drying in warm air the DNP-wool was equilibrated at 63 per cent. relative humidity and 20°C.

Similar conditions were used for the dinitrophenylation of SCMK2. To 100 ml of a 1 per cent. solution was added 1·5 g $NaHCO_3$ and, after bringing to 60°C, FDNB (0·35 ml) was added and the mixture vigorously stirred for $2\frac{1}{2}$ hr. Further amounts of FDNB were added after $2\frac{1}{2}$ and 5 hr. After 7 hr the solution was extracted three times with ether in a centrifuge tube, acidified, and the suspension again extracted with ether. The precipitated DNP-protein was separated by centrifugation and washed three times with water, acetone, and ether before conditioning at 63 per cent. relative humidity and 20°C.

Since these conditions are more severe than those used with other proteins, control experiments were carried out on insulin. DNP-glycine (1·07 moles) and DNP-phenylalanine (0·91 mole) were the only DNP-amino acids detectable in hydrolysates of the DNP-insulin (Sanger 1945) showing that with this protein, at least, no splitting of peptide bonds had taken place during the dinitrophenylation.

The percentage of protein in the DNP-protein was calculated from the weight increase in the case of wool and from the amino acid composition (Simmonds 1955), assuming one DNP group was attached to each imidazole, phenolic, and amino group in the case of SCMK2. In both cases this was equal to 91 per cent. Calculation of the percentage of wool in DNP-wool from the analytical data of Simmonds (1954) also gives 91 per cent.

(*b*) *Hydrolysis of DNP-proteins and Extraction of DNP-amino Acids*

About 0·5 g of DNP-wool or 0·1–0·3 g of DNP-SCMK2 was hydrolysed in a sealed tube at 105°C for 16 hr with 6N hydrochloric acid (10 ml) which had been distilled three times in glass.

The hydrolysates were filtered free of humin and diluted to 50 ml. The DNP-derivatives were extracted four times with 50 ml portions of peroxide-free ether. The combined extracts were washed three times with 10 ml N HCl.

(*c*) *Chromatography*

Chromatography on buffered "Celite 545" (Perrone 1951) was used to isolate the DNP-amino acids from hydrolysates of DNP-wool with the techniques previously described (Thompson 1954). All the DNP-amino acids derived from wool could be separated on two columns. However, the characteristics of the "Celite 545" were somewhat different from those of the previous batch and the following method of separation was employed.

The extracted DNP-derivatives, after sublimation (Mills 1952) at 60°C to remove dinitrophenol, were taken up in water-saturated chloroform and transferred to a chloroform column of pH 3·7 (stationary phase 1 ml 2M Na_2HPO_4). The fast band was collected and when it had run off the column (2 g) the solvent was changed to a solution of 5 per cent. ether in purified chloroform which was saturated with water (CE5), when the DNP-threonine moved ahead of the DNP-glutamic acid, DNP-serine, and DNP-aspartic acid. Once the DNP-threonine was separated from the slower band the solvent was changed to CE10 which gave four distinct bands in the column with the following R values:

DNP-threonine	0·9–1·0	DNP-serine	0·2–0·25
DNP-glutamic acid	0·5–0·7	DNP-aspartic acid	0·1–0·15

These were collected separately for spectrophotometric estimation in 1 per cent. $NaHCO_3$ (Sanger 1949). The fast band from the pH 3·7 column was taken to dryness, dissolved in a small quantity of water-saturated chloroform, and transferred to a pH 6·5 chloroform column. The dinitroaniline and dinitrophenol ($R = 1$) moved rapidly through the column and DNP-valine ($R = 0·2$) was the fastest of the DNP-amino acids. Development with chloroform was continued until the DNP-valine was near the bottom of the column since a faint yellow artefact band preceded it. Then the proportion of ether in the chloroform was increased through CE10, CE25, and CE50 to water-saturated ether to separate the DNP-alanine from DNP-glycine. The DNP-glycine could be finally eluted from the column by adding ethyl acetate to the ether. The DNP-amino acids from hydrolysates of DNP-SCMK2 were estimated after two-dimensional paper chromatography using *tert.*-pentanol–pH 6 phthalate buffer as solvent in the first dimension on Whatman No. 1 paper which had been sprayed with 0·05M phthalate buffer of pH 6 (Blackburn and Lowther 1951) and 1·5M phosphate buffer (pH 6) as solvent (Levy 1954) for the second dimension. For quantitative estimation the spots were cut out and extracted with 1 per cent $NaHCO_3$ (Levy 1954). The aqueous phase after extraction of the ether-soluble DNP-amino acids was examined for DNP-arginine by paper chromatography in the *tert.*-pentanol–pH 6 phthalate system but none was detected in either DNP-wool or DNP-SCMK2.

(*d*) *Reaction with Phenylisothiocyanate*

The procedure used for the coupling was that of Fraenkel-Conrat and Singer (1954) using reagents purified as described by Fraenkel-Conrat, Harris, and Levy (1955). To 5 ml of 1·5 per cent. SCMK2 solution in a 30-ml beaker were added 5 ml of purified dioxan. The solution was brought to 40°C, stirred magnetically, and

the pH was adjusted to 8·5 with 0·1N KOH using an external glass-electrode assembly. Phenyl*iso*thiocyanate (0·1 ml) was added and the mixture was stirred at 40°C for 1½ hr maintaining the pH constant by addition of 0·1N KOH. The solution was brought to pH 9, transferred to a centrifuge tube made from a "Quickfit" B24 socket of 40-ml capacity with several washes of water, and extracted with equal volumes of *cyclo*hexane (2×) and benzene (5×). The aqueous solution was then freeze-dried in the same tube. Preliminary experiments on the cyclization of phenylthiocarbamyl (PTC)-insulin, prepared in a similar way, at temperatures of 100, 70, and 40°C using 1N and 4N HCl for periods of time ranging from ½ to 24 hr showed that the maximum yield of phenylthiohydantoins (PTH) was obtained using 4N HCl at 70°C for 30 min or 40°C for 18 hr. At 100°C 2N HCl gave better yields of PTH after 30 min than did 1N or 4N HCl. With longer heating at 100°C yields decreased in all cases but most sharply at the 4N concentration.

For cyclization of PTC–SCMK2, heating in 4N HCl at 70°C for 30 min was therefore employed. The residue was dissolved in 1·33 ml water and 2·67 ml redistilled 6N HCl added to make a final concentration of 4N HCl. The mixture was heated in the B24 tube fitted with a condenser for 30 min at 70°C with periodic shaking. The solution was then cooled, diluted with 4 ml water, and extracted with 8 ml ethyl actetate (3×). The combined ethyl acetate extracts were washed with 2 ml water (2×) and made up to 25 ml. Aliquots were taken, the ethyl acetate removed *in vacuo*, and the residue dissolved in optically-pure ethanol for spectrophotometric measurements in the range 240–290 mμ. The solutions were then concentrated to dryness and hydrolysed with 6N HCl at 150°C for 18 hr to regenerate the free amino acids.

IV. Results

The results of replicate determinations of the *N*-terminal amino acids in two samples of the 64's virgin wool dinitrophenylated several months apart are shown in Table 1. In Table 2 the results obtained on 64's virgin wool and 64's top together with the results on samples of SCMK2 are presented. The values for 64's top are from a single determination and for the SCMK2 samples the results are the mean of duplicate determinations.

In view of the correction factors which have been applied to allow for the breakdown of DNP-amino acids during hydrolysis of DNP-proteins the values in Table 2 do not have a high order of accuracy. The correction factors used have been based on the recoveries of the DNP-amino acids reported by Porter and Sanger (1948) and Middlebrook (1951). No attempt has been made to determine them under the conditions of the experiments in view of the difficulty of getting an adequate control, as emphasized by the recent difficulties reported by Geschwind and Li (1956) with α-corticotropin. With SCMK2 the results obtained with the phenyl*iso*thiocyanate procedure suggest that these correction factors are of the correct order.

Table 2 shows the values obtained for the *N*-terminal residues of several SCMK2 preparations. Fractionation of the SCMK2 preparations by Zn^{++} precipitation has reduced the number of *N*-terminal residues to a level similar to that

present in wool. In unfractionated SCMK2 small quantities of *N*-terminal leucine were detected which is not present as an *N*-terminal residue in wool. This could

TABLE 1

N-TERMINAL AMINO ACIDS IN VIRGIN 64's WOOL

Values in μmoles/g DNP-protein

Terminal Amino Acid	Sample 1				Sample 2		Mean ± 95% Confidence Limits
	1	2	3	4	1	2	
Aspartic acid	0·39	0·36	0·41	0·32	0·28	0·27	0·34 ± 0·04
Glutamic acid	0·57	0·61	0·87	0·72	0·60	0·58	0·66 ± 0·13
Serine	1·32	1·28	1·22	1·14	1·19	1·23	1·23 ± 0·08
Threonine	4·54	(4·91)*	4·35	4·33	4·34	4·50	4·41 ± 0·16
Glycine	1·00	1·04	0·90	1·10	1·21	1·16	1·07 ± 0·09
Alanine	0·85	0·96	0·93	(1·38)*	0·70	0·80	0·85 ± 0·09
Valine	0·97	1·05	0·93	1·08	1·03	0·91	1·00 ± 0·08

* The values in parenthesis were omitted in the statistical analysis.

imply a small degree of peptide bond hydrolysis during the isolation of SCMK2 from wool.

TABLE 2

N-TERMINAL AMINO ACIDS IN WOOL AND S-CARBOXYMETHYL KERATEINE 2 (SCMK2)

Values in μmoles/g protein

Terminal Amino Acid	Recovery Assumed (%)	Virgin 64's ± 95% Confidence Limits	64's Top	Unfractionated SCMK2*	Fractionated SCMK2	
					Zn Ppt.†	Zn Ppt. Washed Twice‡
Aspartic acid	70	0·5 ± 0·1	0·5	3·6	1·2	0·8
Glutamic acid	67	1·1 ± 0·2	0·7		3·1	1·6
Serine	80	1·7 ± 0·1	1·2	6·1	3·7	5·0
Threonine	86	5·6 ± 0·2	4·1	3·5	2·6	2·2
Glycine	15	7·8 ± 0·7	8·6	7·1	8·2	5·7
Alanine	64	1·5 ± 0·2	1·3	1·1	0·7	0·9
Valine	64	1·7 ± 0·1	1·6	0·6	0	0
Leucine	64	0	0	0·7	0	0
Total		19·9	18·0	22·7	19·5	16·2

* Preparation No. Z2F. † Preparation No. Z4F. ‡ Preparation No. Z8F.

Recent work by Harrap (unpublished data) has shown that kerateine 2 may be extracted from wool during 18 hr at pH 10·5 and 4°C, conditions which are probably milder than those used by Gillespie and Lennox (1955*a*) for the isolation of the preparations used in the present work.

Figure 1 shows the absorption curves of the PTH derived from a preparation of PTC-insulin and PTC–SCMK2. The PTC–SCMK2 preparation showed only small absorption in the region 265–270 mμ where PTH show their maximum. The concentration of the PTH in the ethanol solution was calculated from the absorption at 267·5 mμ using a value of 16,000 as the average molar extinction coefficient (Fraenkel-Conrat, Harris, and Levy 1955). This gave a value of 19·9 μmoles/g SCMK2 for the number of *N*-terminal residues, in good agreement with the figures obtained by the DNP-technique. The amino acids formed by hydrolysis of the PTH were identified by paper chromatography with 75 per cent. (w/w) phenol–water in an atmosphere

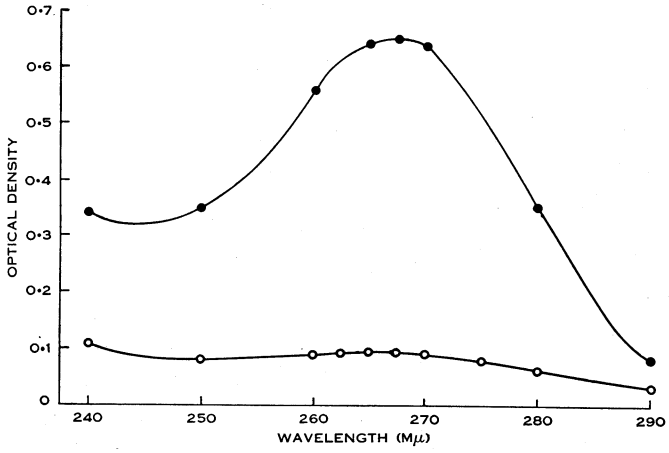

Fig. 1.—Absorption curves of the phenylthiohydantoins derived from phenylthiocarbamyl-insulin (●) and phenylthiocarbamyl-SCMK2. (○). The optical densities are for the extract from 0·25 μmole of insulin and 3 mg of SCMK2 in 10 ml of ethanol.

of NH_3 and HCN. Only small amounts of glutamic acid and glycine together with traces of aspartic acid, alanine, and α-aminobutyric acid were detected. Dr. J. H. Bradbury (unpublished data) has studied this SCMK2 preparation and wool for *C*-terminal residues by the hydrazinolysis technique. A similar low content of *C*-terminal residues was found for both wool and SCMK2 of the same order as for the *N*-terminal residues.

V. Discussion

Qualitatively the results on wool agree with those of previous workers (Blackburn 1950; Middlebrook 1951; Alexander and Smith 1956). The quantitative estimates of the total *N*-terminal residues of the samples of wool used in this study (18–20 μmoles/g protein) are in reasonable agreement with those of Middlebrook (1951) who used Lincoln fleece wool (16·8 μmoles/g) and for New Zealand Romney wool and Australian Merino wool (16·2 μmoles/g) quoted by Speakman (1955). In view of the known heterogeneity and variability of wool fibres, and the difficulty of obtaining a suitable correction factor for the losses of DNP-amino acids that occur during peptide bond hydrolysis and the different techniques used for the preparation

and hydrolysis of the DNP-proteins, differences such as these are not suprising. However, the results of Alexander and Smith (1956) using a Botany yarn are considerably lower (11·6 μmoles/g).

Attempts to reduce the number of *N*-terminal residues in wool by electrodialysis in water (3 days) or in 0·2N acetic acid (4 days) or by Soxhlet extraction with water at 90°C for 24 hr did not significantly alter the values on virgin 64's wool. Moreover, when dinitrophenylation was carried out in 6M guanidine hydrochloride solution at 60°C for 7 hr or at pH 13 and 40°C for 3 hr the *N*-terminal residues were not significantly increased.

Compared with the arginine (602 μmoles/g) and lysine (193 μmoles/g) residues (Simmonds 1954) the *N*-terminal residues represent only a minor proportion of the basic groups in wool.

It is doubtful whether the observed *N*-terminal residues in wool can be interpreted in terms of a molecular model along the lines suggested by Middlebrook (1951). The lack of a well-defined molecule makes interpretation difficult but it would seem more profitable to regard the *N*-terminal residues as representing impurities or breakage points in a collection of cyclic molecules held together by disulphide linkages rather than representing discrete polypeptide chains. Woodin (1956) has recently examined feather keratin in some detail and has proposed cyclic structures for this keratin molecule which appears to have a definite molecular weight. The *N*-terminal residues in feather keratin are qualitatively similar to those in wool keratin and were attributed to impurities or hydrolysis of peptide bonds during the coupling reaction.

If wool protein is composed of a series of different polypeptide chains held together by disulphide linkages, then it might reasonably be expected that methods of fractionation applied subsequently to disulphide bond rupture would give fractions substantially free of some of the end-groups of wool. In fact, however, oxidation of the disulphide linkages followed by fractionation into α, β, and γ keratoses, representing 60, 10, and 30 per cent. of the wool respectively, has given proteins with exactly the same *N*-terminal groups as the original wool although the proportions do vary (Alexander and Smith 1956).

Reduction followed by fractionation has produced similar results as outlined in this paper. SCMK2 represents only about 20 per cent. of the wool yet contains the same *N*-terminal residues with the exception of valine. In view of the fact that no pure protein has given more than three different *N*-terminal residues, as in the case of α-chymotrypsin (Bettelheim 1955), while most have one or two terminal residues (Sanger 1955), it seems probable that the *N*-terminal residues observed in SCMK2 and wool are not stoichiometrically significant and represent slightly degraded cyclic molecules. For SCMK2, estimates of the molecular weight have been made in this Laboratory. Harrap (1956) found a value of about 8000 for the molecule unfolded at the surface of a solution of low ionic strength, whereas, when dissolved in a solution of high ionic strength at pH 7, O'Donnell and Woods (1956) found aggregation occurred to give values of the order of 200,000. In urea or sodium dodecyl sulphate solution disaggregation to particle sizes of 45,000–50,000 occurred and lower values of 20,000–30,000 were estimated for solutions of pH 13. For

none of these observed molecular weights would the observed N-terminal residues be stoichiometrically significant.

It is of some interest that collagen, which has no detectable N-terminal residues (Bowes and Moss 1953; Grassman and Hörmann 1953), on conversion to gelatin gives rise to glycine, serine, threonine, aspartic acid, glutamic acid, and alanine as the principal N-terminal residues (Courts 1954) regardless of whether acid- or alkali-processing is used and irrespective of the origin of the collagenous precursor. These are the same N-terminal residues as are detectable in wool and wool proteins.

VI. Acknowledgment

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VII. References

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