The Action of Papain on Wool Keratin

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Among the criteria which have been given for distinguishing keratins from other proteins is their resistance to proteolytic enzymes (Block & Vickery, 1931). Under suitable conditions, however, keratins are attacked by enzymes, e.g. by trypsin and pancreatin. Middlebrook & Phillips (1941) showed that in the presence of sodium bisulphite solution at pH 6.7 wool was rapidly attacked by papain, and this reaction has since assumed considerable technical importance in the manufacture of felting-resistant wool fabrics.

The present paper describes the results of an investigation, including results obtained by paper chromatographic methods, of the peptides formed by the action of papain on wool. The free amino groups of these peptides have been investigated by Sanger's (1945) technique. The action of papain on wool, first treated with alkali to convert some of the cystine to lanthionine, was also studied and an attempt was made to assess the role played by natural activators, which may be present in crude papain, in the digestion of wool.

In spite of the considerable amount of work which has been done on the digestion of proteins with papain, only limited information is available about the size and nature of the peptides formed or the points of attack. Annetts (1936) found that cyanide-activated papain split crystalline egg albumin into two factions differing greatly in molecular weight, and Petermann (1942, 1946) studied the papain digests of a number of proteins by electrophoretic and ultracentrifugal methods.

Bergmann and co-workers (Bergmann, Zervas & Fruton, 1935, 1936; Bergmann, Zervas & Ross, 1935) made extensive studies of the action of papain on various synthetic polypeptides and their derivatives. They concluded that hydrolysis was not restricted to the ends of polypeptide chains, and that the most varied types of peptide linkages were split, though at widely different velocities. As a result certain peptide linkages were split preferentially. However, the results obtained from studies of synthetic peptides of low molecular weight may not necessarily apply to large polypeptides and proteins. Bergmann & Niemann (1937) found that prolonged digestion of cattle fibrin with papain yielded considerable amounts of free amino-acids, such as

tyrosine, phenylalanine, tryptophan, leucine and isoleucine. However, the methods they employed would only isolate certain of the amino-acids.

EXPERIMENTAL

Materials

Two wools were used in these experiments. (i) A 56's quality wool which had been scoured, dry combed and made into top commercially, which was extracted before use with light petroleum (b.p. 40–60°) and rinsed in water. It had a total S content of 3.40% and a cystine + cysteine-S content of 2.94%. (ii) A New Zealand 56's quality virgin wool from which the tips had been removed (Blackburn, Consden & Phillips, 1944).

The analytical methods employed were as follows: cystine and cysteine, Shinohara (1935, 1936); total sulphur, Barritt (1934); partition chromatography, Consden, Gordon & Martin (1944); total N, micro-Kjeldahl; amino N, Van Slyke method (Peters & Van Slyke, 1932).

Analytical results are expressed as a percentage of the dry weight of protein.

A sample of White Tanganyika papain was used throughout. The wool was treated at 65° with the desired concentration of papain dissolved in 1% NaHSO₃ solution the pH of which had been brought to 6.7 by the addition of NaOH, 30 ml. of solution being used for each g. of wool. Papain concentrations throughout are expressed as a percentage of the weight of wool taken. The above conditions are optimal for the action of papain on wool (Middlebrook & Phillips, 1941), but are not the same as those employed commercially.

The preparation of dinitrophenyl-(DNP)-keratin and DNP-peptides

The dinitrophenyl (DNP) derivatives studied were prepared essentially according to Sanger (1945). Wool (1 g.) was shaken mechanically with 1 g. NaHCO₃, 1 ml. of 2:4-fluorodinitrobenzene (FDNB) and a mixture of 20 ml. water and 40 ml. ethanol. After a given time the solution was decanted, the wool rinsed successively with ethanol, ether, dilute acetic acid, and finally distilled water and air dried. Before weighing for hydrolysis it was dried *in vacuo*. The DNP derivatives of cortical cells and the polypeptide material A (p. 445) were prepared similarly.

Complete reaction of wool or other insoluble protein with FDNB may be difficult to obtain if the reagent only penetrates the fibres slowly. An attempt was made to determine the extent to which the ϵ -amino group of lysine had reacted as follows. Small samples of New Zealand wool were treated under the above conditions for 2, 4, 5.25, 24, 48 and

72 hr., but for the longer periods of treatment shaking was only carried out intermittently. Each sample was then hydrolysed for 24 hr. in 5.7N-HCl and the hydrolysate examined on a one-dimensional collidine chromatogram after removal of HCl *in vacuo*. ϵ -DNP-lysine formed a yellow spot which moved fast in this solvent and turned purple after spraying with ninhydrin and heating. The size and intensity of the free lysine spot, which was taken as a measure of the amount of lysine which had not reacted with FDNB, decreased with increasing time of treatment, but even after 72 hr. a small but definite spot was still present, indicating that reaction was not quite complete. It was possible that further prolongation of the time of treatment might lead to some hydrolysis of peptide linkages, so a routine time of 72 hr. was chosen for the treatment of keratin and solid peptides.

The DNP derivatives of the soluble peptides *B* (p. 446) were prepared as follows. The papain hydrolysate of 20 g. of 56's virgin wool (2% papain (preparation *b*, p. 445), 3.5 hr. at 65°) after removal of cortical cells and polypeptide material *A* was concentrated to dryness *in vacuo* below 40°. The residue was made up to 100 ml. in distilled water and 1 ml. taken for treatment with FDNB. After making alkaline with solid NaHCO₃, a further 0.1 g., of NaHCO₃ was added, followed by 2 ml. of ethanol and 0.1 ml. of FDNB, and the solution shaken mechanically for 3 hr. After dilution with water the solution was then extracted with ether until the ether layer was no longer coloured. The aqueous solution was acidified with dilute HCl and taken to dryness *in vacuo* before hydrolysis. According to the data of Porter (1948) and Porter & Sanger (1948), reaction of the soluble peptide fraction with FDNB should be complete.

Chromatographic procedure

The DNP-amino-acids isolated by ether extraction of the hydrolysates of DNP-peptides were separated on buffered columns of silica (Blackburn, 1949). Buffer *B*-ether columns were used for the first separation, the fast-moving band containing the majority of the DNP-monoamino-acids being refractionated on buffer *B*-5% propanol-cyclohexane columns. An alternative method was to fractionate these DNP-amino-acids on a buffer *B*-ether-ligroin (b.p. 40–60°) column. A mixture of DNP-isoleucine, DNP-valine, DNP-alanine and DNP-glycine separates into four bands on a 66% ether-ligroin column. The separation of DNP-isoleucine from DNP-valine, whose bands are somewhat close together on a 66% ether-ligroin column, is conveniently carried out on a 33% ether-ligroin column.

The first band from the buffer *B*-ether column was sometimes found to be only partially soluble in the second solvent, presumably because the DNP-amino-acids formed salts with traces of buffer eluted from the column. When this occurred the solvent was removed *in vacuo*, a few drops of ether and 1 drop of HCl added and the solution again taken to dryness. It was then found to be soluble in the appropriate solvent. DNP-glutamic acid and DNP-aspartic acids may be separated on buffer *F*-ether columns.

The DNP-amino-acids thus isolated were estimated colorimetrically, using a photoelectric absorptiometer, after solution in 1% NaHCO₃, standard curves being drawn with known DNP-amino-acids. ϵ -DNP-lysine was estimated after solution in N-HCl. The concentration of the 'DNP-

leucine' band, consisting of DNP-leucine and DNP-isoleucine, was read off from a curve drawn with DNP-DL-isoleucine. (DNP-leucine has not up to the present been separated chromatographically from DNP-isoleucine, and throughout this paper the term 'leucine' refers to both leucine isomers.) Leucine predominates over isoleucine in wool, the amounts present being respectively 8.1 and 4.5% (Graham, Waitkoff & Hier, 1949).

The DNP-peptides were hydrolysed for 24 hr. with 5.7N-HCl (100 ml./g.). The identity of the various DNP-amino-acids finally isolated was confirmed by running mixed chromatograms with authentic specimens on suitable columns. A suitable amount of the hydrolysate after ether extraction was examined for the presence of acid-soluble DNP-amino-acids (Sanger, 1945; Blackburn, 1949).

Under the above hydrolytic conditions no appreciable amounts of peptides were detected on the columns. Bands which could not be assigned to any particular DNP-amino-acid and which might be DNP-peptides were hydrolysed for a further period and ether extracted, the ether extract being examined on silica gel columns and the aqueous solution examined for the presence of amino-acids by partition chromatography on paper. No correction for the destruction of DNP-amino-acids during hydrolysis has been applied to the results (cf. Porter & Sanger, 1948), the extent of the necessary correction probably varying with the worker and the details of the technique (Desnuelle, Röver & Bonjour, 1950). In addition, the breakdown of terminal DNP-amino-acid residues in peptides may be greater than the breakdown of free DNP-amino-acids under similar conditions (Woolley, 1949). DNP-glycine, DNP-tryptophan and especially DNP-proline suffer extensive decomposition on prolonged hydrolysis (Sanger, 1945; Desnuelle *et al.* 1950).

'Artifact' bands. During the chromatographic separation a number of coloured bands were encountered which could not be attributed to DNP-amino-acids or DNP-peptides. They comprised: (a) A band travelling fast on buffer *B*-CHCl₃ columns, probably 2,4-dinitroaniline. (b) A faint brownish-yellow band travelling fast on ethyl methyl ketone-ether columns or 17% butanol-CHCl₃ columns when the acid-soluble DNP-amino-acids were examined. This band was almost certainly not due to di-DNP-histidine, since it ran fast on a buffer *A*-CHCl₃ column, whereas di-DNP-histidine is insoluble in this solvent. (c) A band which travelled fast with the longer chain DNP-monoamino-acids on buffer *B*-ether columns and moved slowly on buffer *B*-CHCl₃ columns. 'Artifact' bands have been noted by other workers, some of which are probably similar to those observed in the present experiments (Desnuelle *et al.* 1950; Middlebrook, 1949*a*; Woolley, 1949; Sanger, 1945; Porter & Sanger, 1948; Butler, Phillips, Stephen & Creeth, 1950).

It was found that many of these 'artifact' bands could be greatly reduced or eliminated by the following procedure, which was adopted in the later stages of this work. The hydrolysate of the DNP-peptide was extracted with ether in the usual way, and the combined ether solutions extracted repeatedly with small amounts of 5% (w/v) KHCO₃ solution. The combined KHCO₃ solutions were acidified with HCl until acid to congo red and the DNP-amino-acids again extracted into ether, the ether solution being taken to dryness for chromatography on a buffer *B*-ether column.

RESULTS

The presence of 'activators' in crude papain

As a preliminary to the study of the products formed during the papain hydrolysis of wool, an examination of the effect of other substances present in the crude papain on the digestion of wool was made. The enzyme is often accompanied in nature by other compounds which act as 'natural activators', the activity of the enzyme towards a given substrate depending on the presence or absence of these activators (Grassman, 1935; Balls & Lineweaver, 1939; Irving, Fruton & Bergmann, 1941).

Experiments were carried out to determine the effect of these natural activators on the hydrolysis of wool. As the present work was concerned with the action of papain in the presence of bisulphite, it was considered desirable to avoid the possible complicating effects of other activators such as cyanide or cysteine. The papain was fractionated in several ways and the activity of the various preparations compared with that of the original.

Fractionation of papain. (a) (Cf. Grassman, 1935; Greenberg & Winnick, 1940.) A solution of 5 g. of papain in 50 ml. of water was centrifuged to remove insoluble material and precipitated with 200 ml. of ethanol. After standing for 30 min. at room temperature the precipitated papain was centrifuged and dried *in vacuo*.

(b) (Cf. Balls & Lineweaver, 1939.) Papain (18 g.) was dissolved in 100 ml. of distilled water and the solution centrifuged and adjusted to pH 9 with *N*-NaOH (green to thymol blue). After centrifuging and decanting the mother liquor, $(\text{NH}_4)_2\text{SO}_4$ was added to give a final saturation value of 70%, and the solution cooled. The precipitate which was removed by centrifuging was then dissolved in 60 ml. of distilled water and 6 g. of NaCl added. After cooling in the refrigerator overnight the papain was centrifuged off and dried.

(c) A 20% solution of papain in distilled water was centrifuged and then dialysed against distilled water for 20 hr. The papain was then precipitated by the addition of 4 vol. of ethanol, collected by centrifuging and dried *in vacuo*.

All these papain preparations were found to split wool into cortical cells in the presence of NaHSO_3 at pH 6.7 and 65°. The activity of the various preparations was compared by determining the loss in weight of wool when incubated with 0.3% of the papain at pH 6.7 (1% NaHSO_3 solution) and 65° for 1 hr. The relative activities were 0.81, 0.75 and 0.96 respectively, expressing that of the crude papain as unity. Further work has shown that papain treated according to method (b), but precipitated again from NaCl solution at low temperature, is still highly active in splitting wool in presence of NaHSO_3 .

These various preparations were examined on collidine-phenol- NH_3 chromatograms, 10 μl . of a 20% aqueous solution of the papain being applied to the paper. On spraying with ninhydrin the crude papain preparation showed some eight or nine different 'spots', a very similar type of diagram being given by preparation (a). Preparations (b) and (c), in contrast, gave only one or two faint

'spots' and evidently contained fewer ninhydrin-reacting substances than crude papain. No attempt was made to identify the peptides or other substances giving rise to these 'spots' in the crude papain.

These results show that papain after fractionation in three different ways is still highly active in splitting wool keratin. It therefore seems that the 'natural activators' do not play an important role in the action of papain and NaHSO_3 on wool, or else they are so closely associated with the protein as to be not readily separable from it. The differences in activity observed by other workers between papain preparations which did or did not contain 'natural activators' are much larger. Differences in activity of the present order of magnitude may not be dependent on the presence of activators at all (cf. Balls & Lineweaver, 1939; Jansen & Balls, 1941).

It was considered desirable to have some idea of the amino-acid composition of papain, since published information on this point is scanty (Kassell & Brand, 1938; Fruton & Lavin, 1939). A collidine-phenol- NH_3 two-dimensional chromatogram on a 24 hr. HCl hydrolysate of the papain preparation (b) showed the presence of aspartic acid, glutamic acid, glycine (strong), alanine, valine, leucine, phenylalanine, serine, threonine, tyrosine (strong), proline (strong), histidine, lysine (strong) and arginine. Amino-acids marked 'strong' showed up as pronounced spots compared with the remaining amino-acids.

The products of the papain hydrolysis of wool

Under the influence of papain and sodium bisulphite, wool fibres are finally broken down into cortical cells, while simultaneously part of the wool goes into solution. If the cortical cells are removed by centrifuging, an opalescent solution results. When this solution is acidified with acetic acid to pH 4 a voluminous gelatinous precipitate is thrown down in the course of a few minutes which after being centrifuged, washed and dried forms a hard horn-like mass. This precipitated polypeptide *A* comprises some 10–20% of the soluble material by weight. Table 1 shows the yield of cortical cells and polypeptide obtained in typical experiments.

Table 1. *The yield of cortical cells and acid precipitated polypeptide A from papain digests of wool*

Conditions of papain treatment	Wt. of cortical cells (as % of original wool)	Wt. of acid-precipitated polypeptide (as % of original wool)
2% papain for 3.5 hr.	73.3	2.7
2% papain for 24 hr.	43.7	7.7

Experiments were made to determine the pH range over which this polypeptide was precipitated. The maximum yield of polypeptide was obtained at about pH 4. No significant precipitation occurs in alkaline solution, and at pH values lower than 4 the weight of polypeptide precipitated decreases.

A two-dimensional collidine-phenol-ammonia chromatogram on a 24 hr. hydrolysate in 5*N*-

hydrochloric acid of a specimen of this precipitate bore a close resemblance to the chromatogram of a hydrolysate of intact wool, showing the presence of the following amino-acids: cystine, glutamic acid, aspartic acid, glycine, alanine, valine, leucine, serine, threonine, tyrosine, proline, phenylalanine, lysine and arginine. The precipitate is evidently a polypeptide or mixture of peptides of considerable complexity.

The polypeptide *A* from New Zealand virgin wool was examined by X-rays by Dr T. C. Tranter, who found that it gave spacings agreeing closely with those of disoriented β -keratin (Astbury & Lomax, 1935; Astbury, Dickinson & Bailey, 1935). The spacings are compared in Table 2. The relative intensities of the various reflexions also agreed with those given by Astbury, with 200 the strongest and sharpest reflexion.

Table 2. *The X-ray spacings of polypeptide from papain digests of New Zealand 56's virgin wool*

(Spacings in Ångström units.)

	Side-chain spacing (001)	Backbone spacing (200)	Diagonal spacing (210)	Meridian reflexion (020)
Polypeptide <i>A</i> from papain digest of wool	9.7	4.75	3.7	3.1
Disoriented β -keratin (Astbury <i>et al.</i> 1935)	9.8	4.65	3.7	3.4

Table 3 records the analysis of specimens of cortical cells and polypeptide *A* from papain digests (3.5 hr., papain 2% of weight of wool) prepared in one experiment. In each case the (cystine + cysteine) sulphur

Table 3. *The cystine and cysteine sulphur contents of cortical cells and polypeptide A from papain digests of wool*

Exp.	Cortical cells	Polypeptide <i>A</i>
(a) 56's top wool		
Cystine S (%)	2.28	0.61
Cysteine S (%)	0.39	0.35
(b) 56's top wool		
Cystine S (%)	2.40	1.18
Cysteine S (%)	0.15	0.17
(c) New Zealand 56's virgin wool		
Cystine S (%)	1.97	1.17
Cysteine S (%)	0.44	0.17

content of the precipitated polypeptide is strikingly lower than that of the cortical cells. In the presence of sodium bisulphite part of the cystine of wool reacts to give thiol and *S*-cysteine sulphonate groups, while part is converted to a form which on analysis is returned neither as cystine or cysteine, the so-called non-disulphide-sulphur (Elsworth & Phillips, 1938, 1941). Both cortical cells and precipitate will contain non-disulphide-sulphur derived from cystine under the action of bisulphite, but the small amount present will not affect the general conclusion that the

cystine content of the cortical cells is greater than that of the polypeptide *A*.

The solution (*B*) from which this polypeptide had been precipitated still contained the greater part of the soluble nitrogen. If the solution is dialysed against distilled water, no further precipitation occurs, and no insoluble material separates out in trichloroacetic acid. Saturation with sodium sulphate precipitated a small amount of material, as did addition of ammonium sulphate to 40% saturation. Addition of more ammonium sulphate produced a fairly large precipitate.

An examination of the peptides and amino-acids still present in solution after separation of the precipitated polypeptide was also made chromatographically. For this purpose a 3.5 hr. papain hydrolysate of New Zealand virgin wool using 2% of papain, after removal of cortical cells and then of

polypeptide *A* by acidification with acetic acid, was brought to pH 2 with dilute sulphuric acid and concentrated *in vacuo* below 40° to remove acetic acid. The solution was then 'desalted' (Consden, Gordon & Martin, 1947), concentrated *in vacuo* below 40° and examined chromatographically on paper. A two-dimensional collidine-phenol-ammonia chromatogram showed a very strong glycine 'spot' with weaker serine and glutamic acid spots and doubtful alanine and threonine spots. No other free amino-acids were detected. Broad, diffuse bands, which were probably due to mixtures of peptides, were seen to cover a large part of the sheet.

A papain-sodium bisulphite solution of the same concentration as that used to digest the wool was maintained at 65° and treated in the same way as the papain hydrolysate of the wool. The chromatogram showed no coloured 'spots' or bands indicating that at the concentration at which it was employed the papain present in the enzyme digest of wool made no significant contribution to the 'spots' observed on the chromatogram.

The separation of the peptides on one-dimensional chromatograms was also examined in a number of other solvents including *n*-butanol, *p*-cresol-ammonia, *o*-cresol-ammonia and *n*-amyl alcohol. On the basis of these results, a two-dimensional chromatogram was run in *p*-cresol-ammonia overnight and in *n*-butanol for 2 days, giving the chromatogram illustrated in Fig. 1. The 'spots' labelled,

1, 2, 3 and 4 were eluted from the paper, hydrolysed and the hydrolysates examined on paper chromatograms. Results are given in Table 4.

Fig. 1.

The most striking result is the almost complete absence of serine and threonine from these peptides. The material from the area marked 5 contained all the amino-acids commonly found in a wool hydrolysate. The material represented by spots 1, 2, 3 and 4 evidently only represented a small proportion of the total hydrolysate.

Table 4. *Analysis of peptides in papain hydrolysates of wool*

(Amino-acids formed on hydrolysis of spots 1-4 (Fig. 1) eluted from two-dimensional chromatogram. Intensities of the amino-acids formed are indicated by x's.)

Spot	...	1	2	3	4
Cystine	—	?	x x	?	?
Aspartic acid	—	—	x	x x x	x x x x
Glutamic acid	—	—	x x	x x x x	x x x x
Serine	—	—	—	—	x
Glycine	x x	x x x	x x	x x	x x x
Threonine	—	—	—	—	—
Alanine	x x	x	x x	x x	x x x
Valine	—	x	x x	x x	x x
Leucine	x	x x	x x	x x	x x x
Lysine	x	—	x	—	x
Arginine	?	—	?	?	?

Information as to the size of the peptides *B* remaining in solution after acidification was obtained as follows. Samples (1 g.) of New Zealand 56's virgin wool were treated at 65° with 2 % papain for different times. Cortical cells were removed by centrifuging, polypeptide *A* by acidification with acetic acid, and the solution concentrated *in vacuo* below 40° to small volume and made up to 50 ml. with distilled water. Portions of the solution were analysed for total nitrogen and amino nitrogen. The results are shown in Table 5, the values being corrected for the 'blank' of a papain solution of the same concentration as that used to digest the wool which was similarly incubated

and treated. Although the total amount of nitrogen remaining in solution after acidification increases as the time of digestion is increased, the ratio of amino nitrogen to total nitrogen remains approximately constant. These results are similar to those obtained

Table 5. *The amino nitrogen of the soluble peptides B of a papain hydrolysate of wool*

Time of treatment (hr.)	Total N after acidification (mg.)	Amino N after acidification (mg.)	Amino N as % total N
3.5	22.9	2.76	12.1
5.5	28.2	4.37	15.5
24	42.2	6.28	14.9

during the enzymic digestion of other proteins, e.g. that of lactoglobulin by pepsin (Haugard & Roberts, 1942). The amino nitrogen of a 24 hr. hydrochloric acid hydrolysate of the same wool corresponded to 74.9 % of the total nitrogen. The soluble peptides remaining in solution after acidification are thus some five amino-acid residues in length.

These peptides were also examined after conversion to their DNP derivatives as described on p. 444. Ether extracted only a very small amount of the coloured DNP derivatives, confirming that few DNP-amino-acids were present. A considerable proportion of the DNP derivatives were, however, soluble in ethyl acetate, and by fractionation on ethyl acetate columns were separated into four or five bands. The DNP-peptides present in these bands were hydrolysed, the ether-soluble DNP-amino-acids being examined on columns and the amino-acids on paper. Each of the bands was found to be very complex.

The action of papain on alkali-treated wool

Whilst intact keratins are resistant to proteolytic enzymes, keratins which have been reduced with thiolacetic acid and contain thiol groups are readily attacked, re-oxidation of the thiol groups to disulphide linkages restoring the original resistance of the keratin (Goddard & Michaelis, 1934, 1935; Geiger, Patterson, Mizell & Harris, 1941). The thiol groups produced by the reaction of the cystine with the bisulphite might therefore be a major factor in accelerating the action of papain on wool keratin. Unpublished experiments by the author showed that at pH 6 or 7 sodium bisulphite markedly accelerates the action of trypsin on keratin. It was therefore of interest to study the action of papain on wool which had been treated with alkali to convert some of the cystine to lanthionine, thus reducing the number of thiol groups that could be formed by reaction with bisulphite.

Table 6 compares the rate of loss in weight of alkali-treated New Zealand 56's wool with that of the corresponding normal wool when treated with papain and sodium bisulphite under similar conditions. As measured by the loss in weight, wool which

had been treated with alkali was attacked by papain more rapidly than untreated wool, although it did not disintegrate into cortical cells as rapidly. If the thiol groups produced in wool by sodium bisulphite are instrumental in bringing about an increased attack by papain, these results may indicate that only certain of the disulphide groups which might be reduced are concerned in the reaction. It has been found that only a few of the thiol groups of urease are essential for its activity (Hellerman, Chinard & Deitz, 1943).

Table 6. *The percentage loss in weight of alkali-treated wool on papain treatment*

Time of papain treatment (hr.)	Loss in weight on papain treatment (as % of original)		
	Normal wool	Wool boiled at pH 8 for 4 hr.*	Wool boiled at pH 10 for 1 hr.*
0.5	1.8	2.9	4.7
1	2.7	5.3	5.7
2	8.2	11.2	9.5
Total-S†	3.22	2.56	2.26
Cystine + cysteine-S†	2.66	1.81	0.92

* Under the conditions given by Lindley & Phillips (1945).

† Percentage of wool before papain treatment.

The optimum pH for the action of papain and sodium bisulphite on wool which had been boiled in pH 10 buffer for 1 hr., determined by the method used by Middlebrook & Phillips (1941), was found to be 6.9, a value which is not greatly different from that of 6.7 found for normal wool.

The papain hydrolysate (3.5 hr. with 2% papain) of alkali-treated wool (wool boiled in pH 10 buffer for 0.5 hr.) on acidification with acetic acid gave a precipitate of polypeptide nature. A two-dimensional collidine-phenol-ammonia chromatogram showed the presence of the amino-acids found in wool and in the corresponding precipitate obtained from a papain hydrolysate of normal wool. A very strong lanthionine spot was present, as well as cystine and cysteic acid spots.

The solution remaining after acidification and adjustment of the pH to below 2 was concentrated *in vacuo* to remove acetic acid and examined chromatographically on paper. The only free amino-acid produced in appreciable amount was glycine.

The terminal amino groups of the peptides

The free amino groups of the intact wool, cortical cells and other peptides are given in Table 7. The analytical results are expressed as a percentage of the dry weight of the DNP keratin or peptide, no attempt having been made to correct the values for the DNP groups present. For the soluble peptides the results are given as a percentage of the nitrogen in the solution before treatment with FDNB. The free amino groups of the intact keratin given in Table 7 are similar to those found by Middlebrook (1949*a, b*) for a specimen of virgin wool. As no correction has been applied to the present results for destruction of the DNP-amino-acids during hydrolysis, a quantitative comparison has not been attempted. The differences may well be due to differences in the characteristics of the two wools.

The free α -amino groups of the cortical cells and other peptides formed during the papain hydrolysis of wool will include those originally terminating the peptide chains of the wool in addition to those liberated by the hydrolysis of peptide linkages. The peptide chains of the cortical cells are long, as are those of the original wool, and their free amino groups will most probably include a fairly large proportion of those originally present in the wool. The peptide chains of the insoluble peptide *A* are shorter than those of the cortical cells.

The free α -amino groups of the soluble peptides will only consist to a small extent of those originally present in the wool, and they will therefore indicate the points at which enzyme attack along the chain occurs. The terminal glycine residues found among the soluble peptides will be derived, at least in part, from the free glycine which is present in the hydrolysates.

In order to ascertain whether the papain itself contributed an appreciable number of free amino

Table 7. *The free amino groups of wool, cortical cells, insoluble peptide A and soluble peptides B*

Free amino groups	Protein or peptide			
	Wool ($\mu\text{mol./g.}$)	Cortical cells ($\mu\text{mol./g.}$)	Insoluble peptide <i>A</i> ($\mu\text{mol./g.}$)	Soluble peptides <i>B</i> (amino N as % of total N)
Aspartic acid	0.3	0.3	2.4	0.15
Glutamic acid	0.8	1.6	4.3	0.43
Serine	0.2	5.1	5.8	0.74
Threonine	2.2	5.1	5.4	0.46
Glycine	0.5	2.4	6.2	0.29
Alanine	1.4	2.6	3.2	0.45
Valine	4.0	5.5	11.9	0.57
Leucine	—	0.7	7.8	0.56
Lysine ϵ -amino groups	133	114	162	2.05

groups to those found among the soluble peptides, a 'blank' determination was carried out in which the papain solution used to digest the wool was treated with FDNB. Only very small amounts of DNP-amino-acids were found after hydrolysis, the majority of the bands being only just visible on the columns. Contamination of cortical cells and insoluble peptide *A* with papain is unlikely, since they were repeatedly rinsed in distilled water.

In addition to the free α -amino groups there are the ϵ -amino groups of lysine, and ϵ -DNP-lysine was encountered in all the DNP-peptide fractions examined, i.e. in cortical cells, precipitated peptides *A* and soluble peptides *B*.

The cortical cells contain smaller amounts of ϵ -DNP-lysine than the wool. Examination by the method described on p. 443 showed that the reaction of the ϵ -amino group of lysine of the cortical cells with FDNB was less complete than in the case of wool, possibly due to resistance offered to the penetration of the reagent by the compact mass which was formed on drying. This may not necessarily indicate that reaction of the α -amino groups is incomplete (cf. Porter, 1948).

The free α -amino groups of the cortical cells and peptides are those of the monoamino-acids, leucine, valine, alanine and glycine, the hydroxy amino-acids serine and threonine, and glutamic and aspartic acids. Among the end groups of the monoamino-acids, those of valine are predominant. No free α -amino groups belonging to the basic amino-acids seem to be present, no DNP-arginine or di-DNP-histidine being found among the acid-soluble DNP-amino-acids and no bis-DNP-lysine among the ether-soluble DNP-amino-acids. Thus papain exhibits a certain degree of specificity in attack on the peptide linkages in the keratin structure. Hydrolysates of DNP-soluble peptides *B* on examination by paper chromatography showed the presence of all the amino-acids normally observed on a two-dimensional chromatogram of wool, indicating that splitting of peptide linkages by the enzyme does not occur in such a way as to liberate the amino groups of any one amino-acid residue completely.

DISCUSSION

The undissolved residue obtained when papain and sodium bisulphite or trypsin digest wool has a definite histological structure and constitutes the cortical cells. The sulphur, or cystine, content of this resistant residue is higher than that of the intact wool from which it is derived (Speakman & McMahon, 1938; Elsworth & Phillips, 1941). The present results indicate that the cystine content of the cortical cells is considerably higher than that of some of the polypeptides forming the soluble fraction of a papain digest of wool. No attempt was made to

determine the cystine content of the entire soluble fraction. Similarly, when pepsin or chymotrypsin act on insulin a resistant 'core' of the protein of high cystine content is obtained (Butler, Dodds, Phillips & Stephen, 1948*a*, *b*). The high sulphur content of cortical cells as isolated by enzyme action has led to the view that the 'intercellular' phase of the fibre contains less sulphur than the cortical cells (Speakman & McMahon, 1938). The similarity of these findings and those obtained when enzymes act on insulin (Butler *et al.* 1948*a*, *b*), whalebone (Block, 1937) and hoof proteins (Olcott, 1943) renders it probable that the high cystine content of isolated cortical cells is due to the preferential removal of peptides of low cystine content from the polypeptide chains by the enzyme. A two-dimensional collidine-phenol-ammonia chromatogram of a hydrochloric acid hydrolysate of cortical cells shows the presence of all the amino-acids usually seen on the chromatogram of a hydrolysate of intact wool. When again treated with enzyme, cortical cells undergo further hydrolysis and show a further loss in weight.

The soluble products of the action of papain on wool seem to be a mixture of peptides of varying chain length which fall into two classes, those which are precipitated on acidification of the solution (*A*) and those which remain in solution (*B*). The solubility characteristics, end groups, the wide variety of amino-acids contained in the polypeptide *A* and its β -keratin X-ray diagram are consistent with the view that its polypeptide chains are of considerable length. The phenomena involved in its precipitation may be similar to those which take place in peptic digests of insulin, where a complex insoluble material separates out on heating or adding trichloroacetic acid (Butler *et al.* 1948*b*; cf. Strain & Linderström-Lang, 1939).

The evidence so far available suggests that the smaller peptides which remain in solution on acidification are complex, and together contain a large number of amino-acids. Only a partial separation of these low molecular weight peptides has been effected so far, but the absence of serine and threonine from some of them is noteworthy. The difference between these peptides and those precipitated on acidification may only be one of degree, e.g. of chain length. Conceivably all the peptides which go into solution may represent parts from the ends of polypeptide chains which are removed by enzyme action, a residue or 'core' being left in which the chains are united by disulphide linkages and which, therefore, has a high cystine content.

The present results are in accordance with the general picture of protein decomposition by enzymes, including papain, according to which relatively few protein molecules are attacked in each time interval, but these are quite rapidly broken down to the end products, consisting of molecules of

low molecular weight (four to eight amino-acid residues on the average) and a 'core' of high molecular weight (cf. Annetts, 1936; Petermann, 1942, 1946; Winnick, 1944; Haugard & Roberts, 1942; Butler *et al.* 1948*a, b*; Tiselius & Eriksson-Quensell, 1939).

During the relatively short time (3.5 hr.) of papain hydrolysis of wool only small amounts of free amino-acids are liberated, of which glycine forms by far the largest proportion. Other workers have obtained small amounts of free amino-acids in the enzymic digests of proteins when the digestion was of limited duration and the major part of the digest consisted of relatively large polypeptides (Haugard & Roberts, 1942; Winnick, 1944; Butler *et al.* 1948*a*). The preferential liberation of glycine is probably of some significance. It may indicate that glycine occupies some particular position in the wool keratin structure. Glycine, in fact, terminates some of the peptide chains of wool keratin (Table 7; Middlebrook, 1949*a, b*). Neurath (1943) has discussed the important role that glycine plays in protein structure because of the small size of its side chain. Alternatively, it may indicate that papain splits preferentially those peptide linkages adjacent to glycine residues. Bergmann *et al.* (1936) observed that papain split a number of synthetic peptides at the peptide linkage adjacent to the glycine rather than that adjacent to a leucine, glutamic acid or lysine residue. They considered it possible that glycine-containing substrates were split readily because glycine had no side chain in such a spacial arrangement that it could hinder the approach of the enzyme (Bergmann & Fruton, 1937).

The liberation of a wide variety of different amino-acid residues from peptide linkage during papain hydrolysis contrasts with the preferential liberation of serine and threonine residues during the hydrolysis of several proteins, probably including wool, by concentrated hydrochloric acid at 37° (Desnuelle & Casal, 1948; Gordon, Martin & Synge, 1941; Consden, Gordon & Martin, 1949).

The observations of Harington & Pitt-Rivers (1944) suggest that free amino groups belonging to cysteine residues may be liberated readily during the papain hydrolysis of wool. It is unfortunate that the present techniques do not afford information on this point. The compounds *N*-DNP-cysteine and *NS*-bis-DNP-cysteine which might be found in hydrolysates of peptides treated with FDNB if such splitting occurred do not appear to have been prepared. No band corresponding to bis-DNP-cystine was encountered among the DNP-peptides of a papain digest of wool. From the relative instability of bis-DNP-cystine to acid hydrolysis (Porter & Sanger, 1948), it is possible that they would decompose on acid hydrolysis, and some of the 2:4-dinitroaniline found in hydrolysates of the DNP

peptides may be derived from a source of this kind. *S*-DNP-cysteine could arise in the papain hydrolysis products of wool by condensation of FDNB with the thiol groups of cysteine residues. No *S*-DNP-cysteine band (Sanger, 1945) was found in the hydrolysate of wool or peptide treated with FDNB. It is possible that in the alkaline water-ethanol solution used the thiol and *S*-cysteine sulphonate groups revert to disulphide groups before coupling with the FDNB occurs, as is found in the similar treatment of bisulphite-treated wool with methyl sulphate in aqueous solution (Blackburn *et al.* 1944).

Papain shows some degree of specificity in its attack on the peptide linkages of wool, in that no α -amino groups belonging to basic amino-acids are liberated.

Bergmann found that peptides containing mono-amino-monocarboxylic acids were split by papain, as were those containing lysine and glutamic and aspartic acids. Papain also split benzoylarginine amide, but its action on peptides of serine and threonine does not appear to have been studied (Bergmann, Zervas & Ross, 1935; Fruton & Bergmann, 1940). Comparison of the present results with those of Bergmann is of limited value, partly due to the fact that, although Bergmann has studied the action of papain on several peptides, their number is still insufficient to enable a satisfactory picture to be drawn of the bonds which are preferentially split. In the present state of our knowledge it would seem that evidence as to the points at which enzymes attack proteins will be obtained more rapidly by a direct study of the end products with recently developed techniques.

The validity of the above arguments depends on the assumption that no reformation of peptide bonds occurs under the influence of the papain. Bergmann & Fruton (1941), using comparatively simple substrates, have recently shown that such a synthesis of peptide linkages can occur when certain special conditions are fulfilled. Even if such a synthesis does occur in the present case it is still of interest to know the free amino groups of the final products.

SUMMARY

1. An examination of the products formed when wool is hydrolysed by papain and sodium bisulphite has been made. Acidification of the digest to pH 4 precipitates a complex polypeptide material, the cystine content of which is lower than that of the cortical cells which are liberated during the enzyme digestion. The material remaining in solution consists largely of polypeptides, although small amounts of free glycine are produced.

2. Papain fractionated in a number of different ways to remove natural 'activators' still digests

wool readily in the presence of bisulphite. Wool which has been treated with alkali to convert some of the disulphide linkages to lanthionine linkages is attacked by papain and bisulphite at an increased rate.

3. The amino end groups of the peptides produced in the reaction have been estimated by Sanger's technique. Those of the soluble peptides consist of

leucine-isoleucine, valine, alanine, glycine, threonine, serine, glutamic acid and aspartic acid.

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