# THE REACTION OF WOOL KERATIN WITH ALKALI

by

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It has been known for many years that when hot alkaline solutions are allowed to act on wool keratin, degradation of the cystine residues takes place. A most important step in the elucidation of the course of the reaction was the isolation of lanthionine by HORN, JONES AND RINGEL[1,2] from wool boiled with sodium carbonate solution. Later work by PHILLIPS and colleagues[3,4] suggested that other reactions might occur with the loss of two atoms of sulphur from cystine, giving residues of α-amino acrylic acid. The evidence on which this suggestion was principally based was a series of analyses of the wool for total sulphur and cystine sulphur from which the lanthionine content was deduced indirectly. The present paper represents a re-examination of the question using, among other techniques, a recently devised method for the determination of lanthionine in aminoacid mixtures and protein hydrolysates[5]. A comparison of the amino-acid composition of alkali-treated and virgin wool has also been made.

## EXPERIMENTAL

The wool used in this investigation was an Australian 64's A quality from which the tips had been cut off. It was degreased with light petroleum, b.p. 40–60°, in a Soxhlet apparatus, rinsed in water and air-dried.

*Amino-acid analyses.* These were made by the methods of MOORE AND STEIN[6] after hydrolysis of the keratin with 5 *N* HCl for 24 h, followed by removal of HCl by distillation *in vacuo* with successive additions of water. Good recoveries were obtained when a known mixture of amino-acids was applied to the column. As proline gives much less colour than other amino-acids and sometimes overlapped the glutamic acid peak, it was determined in a separate experiment in which the fractions from the 100 × 0.9 cm column were treated with ninhydrin at about pH 1 according to CHINARD[7] (*cf.* HARFENIST[8]). The amount of proline was then read off from a standard curve prepared using proline under similar experimental conditions. Lanthionine determinations were carried out as described previously[5] and total sulphur analyses were made by BARRITT's method[9].

*Cystine analysis.* Cystine was estimated (a) by SHINOHARA's method[10], *cf.* CUTHBERTSON AND PHILLIPS[3] and (b) by conversion to cysteic acid. The wool was oxidised with performic acid under controlled conditions (*cf.* BLACKBURN AND LOWTHER[11], SANGER AND THOMPSON[12]) when cystine is converted to cysteic acid, which was estimated after hydrolysis on columns of Dowex-50 The wool (1 g) was treated with a mixture of 9 ml formic acid and 1 ml of 100 vol. hydrogen peroxide for 1 h, removed, rinsed well in distilled water and air-dried. Cystine analyses on the treated wools by the Shinohara method indicated that oxidation of the cystine was essentially complete, values of less than 0.1 % of cystine sulphur being found. The oxidised wool was hydrolysed with HCl as described above and an aliquot of the solution applied to a 100 × 0.9 cm column of Dowex-50, the fractions which emerged being analysed with ninhydrin. Cysteic acid is the first amino-acid to emerge; its "colour-yield" was determined in separate experiments (*cf.* SCHRAM, MOORE AND BIGWOOD[13]). Known amounts of cysteic acid applied to the column could be quantitatively accounted for.

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*"Bromine-oxidisable sulphur" determination.* "Bromine-oxidisable sulphur" was determined on the hydrolysate of the wool by the method of BLUMENTHAL AND CLARK[14]. 1 g of wool was hydrolysed for 4 h with 20 ml of 5 *N* HCl, a few drops of bromine added and the mixture allowed to stand for 1 h at room temperature with occasional stirring. Excess bromine was removed by evaporation on the water bath, the solution neutralised to Congo Red with ammonium hydroxide, and sulphate precipitated at the boil with barium chloride. Only a small part of the sulphate thus estimated is present as sulphate before oxidation.

*Buffer treatment of wool.* The wool was boiled under reflux with 150 times its weight of buffer solution of the desired pH for the time given in Table I. It was then repeatedly rinsed in distilled water and air-dried. The buffer solutions were made up according to LINDLEY AND PHILLIPS[4].

In a further experiment the severity of the alkaline treatment was drastically increased. Wool (10 g) was boiled with 500 ml of 1 % sodium carbonate solution for 1 hour. The gelatinous residue was centrifuged off, washed with water, dilute acetic acid, and again with water and dried with acetone. Only 18 % of the original wool remained undissolved and the alkaline solution on acidification gave a dense precipitate of degraded protein material which was not further examined.

## RESULTS

### *The lanthionine content of treated wools*

The results of these analyses on a number of wools treated with alkali under different conditions are given in Table I.

TABLE I

THE LANTHIONINE SULPHUR, CYSTINE SULPHUR AND TOTAL SULPHUR CONTENT OF ALKALI-TREATED WOOLS

| *Wool* | *Lanthionine –S%* | *Cystine–S% (Cysteic)* | *Cystine–S% (SHINOHARA)* | *Total–S%* | *Bromine-oxidisable–S%* |
|---|---|---|---|---|---|
| Untreated | 0.01 | 2.68 | 3.05 | 3.53 | 0.12 |
| pH 7 for 2 h | 0.30 | 1.85 | 2.13 | 3.46 | 0.42 |
| pH 8 for 1 h | 0.40 | 1.82 | 2.11 | 3.38 | 0.49 |
| pH 9 for ½ h | 0.56 | 1.37 | 1.67 | 3.13 | 0.74 |
| pH 10 for ½ h | 0.79 | 1.00 | 1.10 | 2.10 | 0.50 |
| $Na_2CO_3$ for 1 h | 1.32 | — | 0.35 | 2.01 | 0.13 |

Methionine-S = 0.12 % by BAERNSTEIN's method[29].

Untreated wool has a negligible content of lanthionine, the amount present in treated wools increasing as the severity of the alkaline treatment is increased. The lanthionine present in the hydrolysates was shown on analysis to be a mixture of the lanthionine isomers, *i.e.*, of *meso-* and *dl* (or *l-*) lanthionine, two peaks being observed in the eluate from the chromatogram. HORN, JONES AND RINGEL[1,2] earlier isolated both these forms from wool boiled with sodium carbonate solution. The amounts of the isomers in the wools treated under the more strongly alkaline conditions appeared to be approximately equal, but no precise quantitative estimates of each isomer were made, as in some cases the peaks overlapped each other. The lanthionine values in Table I thus represent the sum of all the isomers present in the hydrolysates.

The racemisation of amino-acid residues in proteins which have undergone alkali treatment is a fairly common phenomenon. It was originally ascribed by DAKIN[15] to enolisation of the carboxyl group of the peptide linkage, but is now considered to be caused by ionisation of the α-carbon atom of the amino-acid residue[16].

If racemisation occurred equally readily at both the asymmetric carbon atoms of the lanthionine (or cystine from which it is derived) during the alkaline treatment, the equilibrium mixture would contain equal amounts of *meso-* and *dl*-lanthionine,

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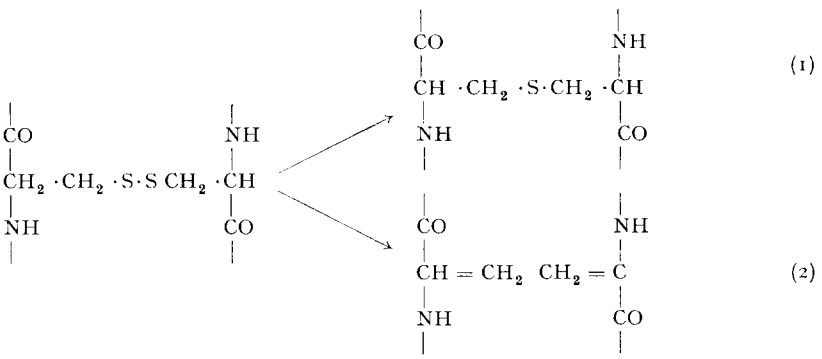
in agreement with the experimental findings. It can be seen (Table I) that as the severity of the alkaline treatment is increased, the amount of lanthionine in the wool increases. Even under a fairly drastic treatment with pH 10 buffer for 0.5 h a substantial proportion of the cystine has not reacted. A much more drastic treatment with $Na_2CO_3$ at the boil for 1 h, under which much of the wool goes into solution, still leaves some of the cystine unattacked. These results are in accordance with those of earlier workers in that part of the cystine of wool is less reactive to alkalis than others. They are perhaps more in agreement with the idea of a steady gradation of reactivity of the cystine residues than of the presence of definite fractions or sub-fractions.

*The mode of decomposition of cystine*

The question as to whether certain of the cystine residues behave in a different manner to others may now be considered. The principal mode of decomposition of cystine in proteins under alkaline conditions is that leading to the formation of lanthionine (equation 1); the other suggested mode leads to the formation of α-amino acrylic acid residues and no lanthionine (equation 2).



(1)

(2)

The two modes of decomposition can be distinguished by a comparison of the loss of cystine sulphur from the wool with the amount of lanthionine formed; equation 1 requiring the loss of two atoms of cystine sulphur with the formation of one atom of lanthionine sulphur, while equation 2 leads to the loss of two atoms of cystine sulphur with no lanthionine formation (*cf* LINDLEY AND PHILLIPS[4]). The accuracy with which this comparison can be made however is dependent on the accuracy of the various analytical figures involved. These may now be briefly considered. The accurate determination of the cystine content of proteins is notoriously difficult. The cystine sulphur content of the present sample of intact wool as measured by the SHINOHARA method is 10.5% lower than the total sulphur, making allowance for the small amount of methionine sulphur. Many workers have obtained a similar result[17,18,19,20]. The cystine sulphur determined by the chromatographic cysteic acid method is considerably lower than either that determined by the SHINOHARA method or the total sulphur. Only about 50% of the total sulphur of papain and carboxypeptidase[21,22] and less than 75% of that of ribonuclease[23], can be accounted for as, cysteic acid using similar method of analysis to the above. Analysis of wool keratin for cystine sulphur by the MOORE AND STEIN procedures also gives results appreciably lower than the total sulphur. The cause of this difference is at present obscure, but

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it is possible that some destruction of cystine occurs during the performic acid oxidation. SCHRAM, MOORE AND BIGWOOD[13] obtained only a 90% yield of cysteic acid on oxidising cystine with performic acid. The higher values given by the SHINOHARA method may be attributed to the presence of reducing substances which are not cysteine or cystine in the hydrolysate of wool. It seems clear that both these methods may give low values for the cystine content of proteins; the chromatographic cysteic acid method however is regarded as giving the more reliable values. Nevertheless, they are useful for yielding comparative values for the cystine content in a series of similar proteins, such as alkali-treated keratins.

A comparison of the lanthionine content of the wools after alkali treatment with the loss of cystine sulphur as determined chromatographically (Table II) shows that two atoms of cystine sulphur are destroyed for every atom of lanthionine sulphur formed. This result means that the reaction of the crystine with alkali takes place principally according to equation 1; the extent to which reaction 2 takes place is evidently small. Previous evidence for the occurence of this reaction was based on an indirect determination of lanthionine and on the presence of pyruvic acid in the hydrolysates of alkali treated wool. This pyruvic acid was supposed to be derived from the decomposition on acid hydrolysis of $\alpha$-amino acrylic acid residues produced according to equation 2, however, it can equally well be derived from the breakdown of $\alpha$-amino acrylic acid residues produced by the alkaline decomposition of serine residues (*cf.*p) or from the acid decomposition of cystine. Untreated wool gives some pyruvic acid on hydrolysis. When lathionine was boiled with 5*N* HCl for 24 h no ammonia was formed and no appreciable destruction of lanthionine, as measured colorimetrically, occurred.

TABLE II

A COMPARISON OF THE LANTHIONINE-SULPHUR OF ALKALI TREATED WOOL AND THE LOSS OF CYSTINE SULPHUR

| *Conditions of alkali treatment* | *Lanthionine-S of wool %* | *Loss of cystine-S % by* | |
|---|---|---|---|
| | | *(a) cysteic acid method* | *(b)* SHINOHARA |
| — | 0.01 | — | — |
| pH 7 for 2 h | 0.30 | 0.73 | 0.92 |
| pH 8 for 1 h | 0.40 | 0.86 | 0.94 |
| pH 9 for ½ h | 0.56 | 1.31 | 1.38 |
| pH 10 for ½ h | 0.79 | 1.68 | 1.95 |
| $Na_2CO_3$ for 1 h | 1.32 | — | 2.70 |

*Bromine-oxidisable sulphur.* Hydrolysates of virgin or alkali-treated wools contain a sulphur compound which gives rise to sulphuric acid on oxidation with bromine. In view of its possible importance, a number of experiments were made to determine the nature of this sulphur in alkali-treated wool. The hydrolysate of 1g of wool boiled in buffer at pH 9 for 1 h was distilled *in vacuo* with addition of water to remove HCl, and the residue dissolved in 25 ml of water. When dialysed against 200 ml distilled water for 48 h 87% of the sulphur giving sulphate on oxidation passed through the cellophan membrane. Only 3% of this sulphur was removed from a similar solution by extracting twice with *n*-butanol. A large part of the sulphur was removed by shaking with 50–100 mesh beads of Dowex-50 with 8% cross-linking agent.

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Cystine gave no appreciable amount of sulphate on oxidation alone or in admixture with other amino-acids simulating a wool hydrolysate in composition, or when boiled with this mixture in 5*N* HCl for 24 hours. Lanthionine however gave sulphate equivalent to 15.5% of its sulphur content. CUTHBERTSON AND PHILLIPS[3] suggested that the bromine-oxidisable sulphur of alkali-treated wool might consist of free sulphur firmly held in the fibre, or of complex inorganic compounds. The observation that the sulphur will dialyse through cellophan demonstrates that it is not present in the hydrolysate as collodial sulphur. Some at least of this sulphur is derived from lanthionine, but some is also probably derived from complex inorganic sulphur compounds present in the alkali-treated fibres (*cf.* LINDLEY AND PHILLIPS[4]). In addition, these observations mean that it is impossible accurately to deduce the cystine sulphur content of treated wools from the difference between their total sulphur content and the sum of the lanthionine sulphur, methionine sulphur and bromine-oxidisable sulphur + sulphate as was done by earlier workers (*cf.* LINDLEY AND PHILLIPS[4]).

*Changes in composition of wool on alkali treatment with respect to non-sulphur-containing amino-acids*

Table III compares the amino-acid analyses of virgin and alkali treated wools; the analyses for the sulphur containing amino-acids are given in Table I. With the amount of protein hydrolysate used in these experiments the smallness of the methionine peak precluded its accurate determination so no value is recorded. No value is given for tryptophan, the insolubility of keratin making it difficult to obtain reliable analytical results in this instance. The results for untreated (virgin) wool are in good agreement with those of earlier workers (*cf.* SIMMONDS[20]) and are not further discussed in this connection; the differences for some amino-acids are probably due to real differences in amino-acid composition between the different wools.

TABLE III

THE AMINO-ACID COMPOSITION OF VIRGIN WOOL AND WOOL BOILED IN pH 10 BUFFER

| | *Wt from 100 g virgin wool* | *Wt from 100 g alkali-treated wool* |
|---|---|---|
| Amino-acid | | |
| Alanine | 4.48 | 4.29 |
| Arginine | 9.30 | 9.67 |
| Aspartic acid | 7.73 | 7.68 |
| Amide-N | 1.18 | 1.30 |
| Glutamic acid | 14.22 | 15.39* |
| Glycine | 5.53 | 5.33 |
| Histidine | 0.76 | 0.70 |
| *Iso*leucine | 3.61 | 4.06 |
| Leucine | 8.33 | 8.83 |
| Lysine | 2.93 | 3.27 |
| Phenylalanine | 4.48 | 3.96 |
| Proline | 4.83 | 4.95 |
| Serine | 10.68 | 8.38 |
| Threonine | 6.87 | 6.22 |
| Tyrosine | 6.34 | 6.28 |
| Valine | 5.72 | 5.81 |

* Glutamic acid overlapped lanthionine and graphical estimation was necessary; this value may therefore be somewhat in error.

*References p. 512.*

For the majority of the amino-acids no change in the amount present in the wool is observed after alkali treatment; the differences being no larger than those to be expected between single analyses for each individual amino-acid employing only a single time of hydrolysis. Some errors in both this and the previous calculations will be caused if appreciable dissolution of wool takes place during the alkaline treatment, particularly if preferential dissolution of some amino-acids occurs. Except in the more alkaline solutions however, the loss in weight of wool during such treatment is small. The value for serine however shows a decrease which is much greater than experimental error, that for threonine shows a similar but smaller decrease. Decomposition of the serine side chains probably occurs giving α-amino acrylic acid residues, which are subsequently converted to pyruvic acid and ammonia on acid hydrolysis. NICOLET, SHINN AND SAIDEL[24] found that the serine and threonine residues in silk fibroin and sericin were much more labile than the corresponding free amino-acids when boiled with 0.1 *N* sodium hydroxide. They postulated the formation of dehydroamino-acids and showed that additional ammonia, quantitatively correlated with the amount of serine and threonine destroyed, was liberated during acid hydrolysis of the treated proteins. In the present experiments the alkali-treated wool shows a similar increase in "amide-nitrogen" content (Table III). The alkaline degradation of the arginine residues could produce ornithine. However, this amino-acid was not detected when the hydrolysate of wool boiled in pH 10 buffer was applied to a Dowex-50 column 15 × 0.9 cm and the eluate fractions tested with ninhydrin at pH 1.

## DISCUSSION

The present results establish that when wool is treated with hot alkaline solutions the principal amino-acid residues to undergo degradation are those of threonine and serine, and especially cystine. As regards cystine, the main interest of the results, apart from providing the first direct measurements of the amount of lanthionine produced when wool is treated with hot alkaline solutions, probably lies in their bearing on the general question of the relative reactivity of cystine residues in proteins, particularly keratin. It is a matter of general observation that certain residues of a particular amino-acid in a protein are often more reactive than the other residues; this behaviour has been observed particularly for cysteine, cystine and tyrosine. The cystine of wool, and indeed of other keratins and proteins, has been divided into two main fractions A + B and C + D, or four subfractions, A, B, C and D, fraction A + B generally reacting more readily than fraction C + D towards a number of reagents including sodium bisulphite, alkalis, formaldehyde and thioglycollic acid[25].

The reaction of wool with sodium bisulphite giving thiol and S-cysteine sulphonate groups, *viz.*

$$—CH_2SS\cdot SCH_2— + NaHSO_3 \rightleftharpoons —CH_2SH + NaO\cdot SO_2\cdot CH_2—$$

is reversible under appropriate conditions; the results may be taken as typical of those with the other reagents. Of the total cystine sulphur of wool, the subfraction called A gave thiol and S-cysteine sulphonate groups labile to rinsing with water, subfraction B gave water-stable groups of a similar kind, subfraction C was inert and subfraction C decomposed to give combined α-amino acrylic acid. There is

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evidence for a definite difference in reactivity of the cystine residues in the fact that when wool is treated with sodium bisulphite in the presence of methyl sulphate, which makes the reaction irreversible by methylating the thiol groups, the reaction only proceeds slowly when about half of the cystine has reacted. Towards alkali, fraction A + B gave lanthionine, subfraction A changing more rapidly than subfraction B, fraction C + D was supposed to give α-amino acrylic acid, subfraction C slowly. The present analyses do not substantiate this conclusion, they show a difference in reactivity between the cystine residues in wool towards alkali, but provide no evidence that two different types of reaction occur as the conditions of treatment are varied. A gradual gradation in reactivity towards alkali rather than a definite break is indicated.

This difference in reactivity is probably caused by the differences in the groups neighbouring on the cystine residues concerned. LINDLEY AND PHILLIPS[26] have, in fact, shown that chemical modification of wool, as for example by methylating the carboxyl groups, alters its reactivity towards sodium bisulphite. Similar differences in the reactivity of a number of synthetic cystine-containing peptides towards sodium bisulphite have been observed, and are dependent on the nature of the amino-acid residues attached to the cystine residues[27].

The formation of lanthionine from the cystine of wool on treatment with alkali is presumably due to the fact that after the initial fission of the disulphide group, the two "halves" of the cystine residue are held in close proximity by the restraint of the peptide chains until decomposition of one "half" and recombination is complete. Presumably because a similar restraint is not exercised in solution, cystine does not form appreciable amounts of lanthionine on treating with alkali, indeed no report of this reaction taking place has appeared in the literature. Lanthionine formation however can occur from cystine residues embodied in a single peptide chain. Lysozyme, which consists of a single polypeptide chain internally cross-linked by cystine[28] forms an insoluble precipitate of protein-like material when boiled in sodium carbonate solution. Paper chromatography of a hydrolysate of this precipitate shows that it contains appreciable amounts of lanthionine.

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*Note added in proof*

Recent work has shown that alkali treatment of cystine and cystine peptides can give lanthionine[30,31]. The present paper principally describes the final products of the reaction of wool cystine with alkali, and does not give information as to the detailed mechanism by which lanthionine is formed from cystine. This probably involves the intermediate formation of cysteine residues and α-amino acrylic acid residues, which then combine to form lanthionine residues in a manner analogous to the synthesis of lanthionine described by SCHÖBERL AND WAGNER[32].

## SUMMARY

Amino-acid analyses have been made by the MOORE AND STEIN method on normal wool and wool treated with boiling alkaline buffers: the only amino-acid to undergo appreciable destruction is cystine, serine and threonine being destroyed to a small extent.

Determination of lanthionine in a number of wools treated with alkali under differing conditions shows that for every cystine residue attacked during the alkaline treatment one residue of lanthionine is produced. During the degradation of cystine, therefore, reactions other than lanthionine formation do not take place to any appreciable extent.

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## RÉSUMÉ

Les auteurs ont effectué par la méthode de MOORE ET STEIN, l'analyse en aminoacides de la laine normale et de la laine traitée à l'ébullition par des tampons alcalins: le seul aminoacide, qui subisse une destruction appréciable, est la cystine; la sérine et la thréonine sont légèrement détruites.

Le dosage de la lanthionine dans plusieurs échantillons de laine traitée par les alcalis dans différentes conditions montre que, pour chaque résidu de cystine attaqué au cours du traitement alcalin, il apparaît un résidu de lanthionine. Donc, au cours de la dégradation de la cystine, des réactions autres que la formation de lanthionine n'ont pas lieu de fa on appréciable.

## ZUSAMMENFASSUNG

Normale Wolle und mit siedenden alkalischen Puffern behandelte Wolle wurde mit Hilfe der MOORE UND STEIN-Methode Aminosäurenanalysen unterworfen. Die einzige Aminosäure, welche unter diesen Bedingungen erheblichen Schaden erlitt, war Cystin, während Serin und Threonin nur in geringem Masse zerstört wurden.

Eine Anzahl von Wollen wurden unter verschiedenen Bedingungen mit Alkali behandelt und der Lanthioningehalt bestimmt; es konnte dabei festgestellt werden, dass ein Lanthioninrest für jeden, während der Alkalibehandlung angegriffenen Cystinrest produziert wurde. Während des Cystinabbaus finden daher, ausser der Lanthioninbildung, keine anderen Reaktionen in wesentlichem Ausmasse statt.


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