The Reaction of Oxidizing Agents with Wool

5. THE OXIDATION PRODUCTS OF THE DISULPHIDE BOND AND THE FORMATION OF A SULPHONAMIDE IN THE PEPTIDE CHAIN

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It has long been recognized that the reaction between wool keratin and oxidizing agents is extremely complex (see Stoves, 1946). Although most oxidizing agents act on the disulphide bond, other groups are also oxidized and considerable general degradation is found. As the disruption of the disulphide bond alone causes a great reduction in the strength of the fibres, it is difficult to detect limited main chain breakdown. In the work described below, one of the reagents used was peracetic acid which acts specifically on the disulphide bonds and tryptophan side groups and undesirable reactions are eliminated (Alexander, Hudson & Fox, 1950).

It has hitherto been customary to interpret the reaction of the combined cystine in keratin in the same way as the reactions of cystine or similar disulphides. Cystine is oxidized by perbenzoic acid in chloroform to a disulphoxide which is readily converted to the corresponding sulphonic and sulphinic acids by 95 % methanol and hydrochloric acid (Lavine, Toennis & Wagner, 1934).

$$\mathrm{RSSR} \rightarrow \mathrm{R.\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}.\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}.R} \rightarrow \mathrm{RSO_3H + RSO_2H}$$

In this reaction an intermediate R.SO.SO.R was isolated which was found to be relatively stable in dilute acids (Lavine, 1936), but which reacts with ammonia to form a complex mixture including cystine and the sulphinic acid corresponding to cysteine. Speakman & Elliott (1940) showed that dibenzyl disulphide is oxidized by sulphuryl chloride to the sulphur chloride in non-aqueous media, and for this reason Speakman (1945) has postulated the formation of sulphur chlorides to be the primary reaction between wool and chlorine. Harris & Smith (1937) assumed that cysteic acid is the main product in the reaction with hydrogen peroxide, although some evidence for the formation of an intermediate sulphoxide was advanced. They also deduced that bond fission occurs only when one of the sulphur atoms is converted to cysteic acid and that both sulphur atoms are not oxidized simultaneously. Consden, Gordon & Martin (1946) found cysteic acid in hydrolysates of wool treated with bromine. Consden & Gordon (1950) showed the presence of intermediary oxidation products, possibly the sulphoxide and the sulphinic acid, in wool oxidized with hydrogen peroxide.

Alexander *et al.* (1950) found that the combined cystine in wool could be divided into two fractions which are recognized by their behaviour towards oxidizing agents. Chlorine in acid solution and peracetic acid oxidize all the cystine, whereas potassium permanganate in acid solution and sodium hypochlorite oxidize only 25 %. The results presented in the present paper show that the nature of the products formed depends upon the cystine fraction reacting. It is shown that the reactions of the cystine in wool differ considerably from the corresponding reaction of free cystine, and this is not surprising in view of the stereochemical restrictions which must exist in the three-dimensional network of cross-linked polypeptide chains. In the reactions with peracetic acid and chlorine at pH 2, it was found that the disulphide bond was broken and that subsequent hydrolysis gave cysteic acid quantitatively. Cation-exchange experiments showed that free sulphonic acid groups were not present in the wool fibres after oxidation so that an intermediate compound must be formed. Further, after treatment of the oxidized fibres with dilute alkali a product was obtained which did not yield cysteic acid on hydrolysis by the normal method. Acidic compounds were extracted from such hydrolysates, which on drastic hydrolysis gave cysteic acid. Evidence is presented which indicates that the oxidized sulphur is present as a cyclic sulphocarboxylic acid imide, which gives rise to a sulphonamide on treatment with alkali.

EXPERIMENTAL

The wool fabric used throughout this work was loosely knitted from a high quality botany yarn and had received no prior treatment other than mild scouring with NH_3 to remove wool grease and dirt. It was further purified by thorough extraction with ether and ethanol.

Oxidations. These were performed as described by Alexander *et al.* (1950).

Cystine determinations. Wool samples were hydrolysed with 5N-HCl at 120° for 5 hr. in sealed tubes. The cystine content of the hydrolysate was determined colorimetrically with phosphotungstic acid (Shinohara, 1935).

Cysteic acid determinations. Wool hydrolysates were chromatographed on Whatman no. 4 paper in phenol-0·3N-NH_3 (Consden, Gordon & Martin, 1944). Cysteic acid was estimated on these chromatograms by the spot dilution method (Polson, Mosley & Wyckoff, 1947).

Two-dimensional paper chromatography. The chromatogram was run in one direction with phenol (8-hydroxyquinoline addition) and then at right angles with *s*-collidine.

Total sulphur. The wool S was oxidized to sulphate by a modification of the method of Kahane & Kahane (Jones, 1943). Frequent control analyses gave an average value of 3·50% S for whole wool. The reproducibility of the results was better than ±3%.

Cation exchange. Oxidized wool samples (1 g.) were shaken successively with six lots of 30 ml. of N-NH_4Cl solution. They were washed with distilled water till free of Cl^-. The samples were then shaken successively with six lots of 30 ml. of N-KCl. All KCl solutions were bulked after shaking and tested for NH_4^+ qualitatively with Nessler's solution and quantitatively by boiling with NaOH and absorbing the NH_3 in standard acid (see Kressman & Kitchener, 1949). Alternatively, oxidized wool samples which had been equilibrated by successive shakings with distilled water were shaken with six lots of 30 ml. of N-KCl and the liquors bulked and titrated against standard alkali.

Acid-binding capacity of wool. Wool samples (1 g.) were equilibrated to pH 6 by successive shakings with N-KCl. The samples were freed from excess water by squeezing and shaken for 30 min. in a flask shaker with 30 ml. of 0·05N-HCl containing N-KCl. Samples of the solution were then back titrated with standard alkali.

Load-extension of fibres. Tensile measurements on fibres were carried out as described by Speakman (1947).

RESULTS

The difference in products on acid and alkaline oxidation

Chlorination at pH 2, 4 *and* 8. The yields of cysteic acid obtained in the hydrolysates of wool which had been chlorinated at pH 2, 4 and 8 are recorded in Table 1. All the cystine which reacted was converted quantitatively to the sulphonic acid when 100 g. of wool were reacted with up to 20 g. of chlorine; but with greater quantities of chlorine, loss of cysteic acid occurred. This is supported by analyses for total sulphur, as the sum of the disulphide and cysteic acid sulphur remains constant for the various treatments.

The relatively large amount of non-disulphide sulphur in wool has been investigated by Cuthbertson & Phillips (1945) who conclude that all the sulphur in virgin wool can be accounted for as disulphide sulphur and methionine. At pH 8, oxidation did not give cysteic acid quantitatively, as the solution contained an appreciable concentration of hypochlorite ions. At that pH the reaction proceeds partly with hypochlorous acid and partly with hypochlorite ions (Alexander *et al.* 1950) and a quantitative conversion to cysteic acid cannot be expected.

Table 1. *Disulphide sulphur, cysteic acid and total sulphur analyses of wool treated with various concentrations of chlorine at* pH 2, 4, 8 *at* 25°

		g./100 g. wool		
pH	Chlorine reduced (g./100 g. of wool)	Disulphide sulphur	Cysteic acid sulphur	Total sulphur
2·0	Nil	2·66	—	3·50
	5	2·05	0·6	3·57
	10	1·57	1·3	3·35
	20	0·72	1·9	3·40
	50	0·27	1·4	2·50
4·0	20	0·98	1·7	3·54
	50	0·16	1·7	2·80
8·0	20	2·17	0·1	3·33
	50	1·40	0·4	2·69

Chlorine solutions at pH 2–8, though reacting preferentially with the cystine and tyrosine in wool (Alexander & Gough, 1951), do not confine their attack to these amino-acids. In so far as can be ascertained from paper chromatograms of chlorinated wools there is no difference between these and untreated wool except for the disappearance of tyrosine and cystine and the appearance of cysteic acid. It appears, therefore, as if a general attack on all the other amino-acids occurs and it seems likely that this proceeds via the terminal amino-acids. Tryptophan may be oxidized preferentially together with tyrosine and cystine, but since it does form a significant part of the wool it was not determined.

Chlorination at pH 10. Chlorine in solution at pH 10 consists almost entirely of hypochlorite ions which are found to yield a different reaction product. No cysteic acid was detected in the hydrolysates of wool treated with chlorine at pH 10,* and two-dimensional chromatograms revealed lanthionine. Cuthbertson & Phillips (1945) have shown this amino-acid, which was first isolated by Horn, Jones & Ringel (1941) from alkali-treated wool, to be present in wool boiled with pH 10 buffer. Unlike cysteic acid, the lanthionine could not be estimated quantitatively, but its presence was confirmed by the technique of Dent (1947), using hydrogen peroxide.

The value for the lanthionine sulphur (Table 2) was not obtained directly, but was calculated on the assumption, made by Cuthbertson & Phillips (1945), for evaluating the lanthionine in alkali-treated wool, that all the sulphur can be accounted for as lanthionine, disulphide and non-disulphide sulphur

* The reaction mixture was found to contain a small amount of cysteic acid at the end of the reaction.

(0·88 %) of untreated wool. The slight loss in total sulphur compared with that of untreated wool is in agreement with the formation of lanthionine in the alkaline degradation of wool (Cuthbertson & Phillips, 1945). The eliminated sulphur which is seen to correspond to approximately one-half of the disulphide sulphur lost could not, however, be detected as sulphate in the reaction liquor. Cuthbertson & Phillips (1945) also were not able to isolate part of the sulphur-containing reaction products formed during the alkaline degradation of wool.

Table 2. *Analyses of disulphide sulphur and total sulphur in wool treated with various amounts of chlorine at* pH 10 *at* 25°

Chlorine reduced (g./100 g. of wool)	g./100 g. wool		
	Disulphide sulphur	Lanthionine sulphur*	Total sulphur
5	2·08	0·34	3·31
20†	1·86	0·58	3·32
50	1·86	0·48	3·22

* Calculated as the difference between the total sulphur and the sum of the cysteic acid and original non-disulphide sulphur (0·88 %).

† Reaction performed at 80°.

The formation of cross links in wool fibres treated with sodium hypochlorite at pH 10 is confirmed by the load-extension data given in Table 3. The work

Table 3. *The reduction in work* (R.W.) *obtained on stretching the fibres to a constant extension, after treatment with chlorine solutions at* pH 2 *and* 10 *at room temperature*

pH	Chlorine reduced (g./100 g. of wool)	Percentage cystine oxidized (percentage of that originally present)	R.W. at pH 7
2·0	4	16·5	21
	6	25·5	35
10·0	4	20·0	3·3
	6	22·2	4·6
	8	—	10·4
	10	22·4	14·4

required to stretch the fibre to a constant extension was greatly reduced by chlorination at pH 2. After chlorination at pH 10, however, the reduction in work (R.W.) was small, suggesting that new cross links (i.e. lanthionine) were formed after the oxidation of the disulphide bonds. Furthermore, a peracetic acid treatment sufficient to oxidize all the disulphide bonds does not render wool which had been chlorinated at pH 10 soluble in dilute ammonia (cf. Fig. 1).

Effect of acid permanganate. In the treatments with acid permanganate solutions, analyses for cystine and cysteic acid showed that approximately one-half the cystine oxidized was converted to sulphonic acid (Table 4). Most of the sulphur which is removed from the wool could be recovered from the reaction mixture as sulphate (e.g. 0·18 % sulphur was found as sulphate after a potassium permanganate treatment, 25 g./100 g. wool).

Table 4. *Analyses for disulphide, cysteic acid and total sulphur in wool treated at* 25° *with* $KMnO_4$ *in* 0·5 N-H_2SO_4

(Total sulphur of untreated wool = 3·5 %.)

$KMnO_4$ reduced (g./100 g. of wool)	g./100 g. wool		
	Disulphide sulphur	Cysteic acid sulphur	Total sulphur
12·5	2·10	0·3	3·22
25·0	1·87	0·3	3·20

Alkaline permanganate treatment. The cystine in wool is only attacked by alkaline permanganate solutions when a large excess of the oxidizing agent is used, causing severe degradation (Alexander *et al.* 1950). The high sulphur content of the treated wool suggests that the reaction is completely non-specific for cystine and it appears that other residues are preferentially attacked. Thus, a sample which had received treatment with 25 g. permanganate/100 g. wool had a sulphur content of 3·88 % compared with a normal value of 3·50 % showing that other residues had reacted and passed into solution leaving the cystine mainly unattacked. No cysteic acid was observed on acid hydrolysis of the treated wools, but two-dimensional paper chromatography revealed some lanthionine.

Table 5. *Analyses for disulphide, cysteic acid and total sulphur on wool treated with* 0·25 N-*peracetic acid* (270 % *on wt. of wool*)

Time of reaction in 0·25 N-peracetic acid	g./100 g. wool		
	Disulphide sulphur	Cysteic acid sulphur	Total sulphur
5 min.	2·10	0·5	3·60
30 min.	1·44	1·2	3·50
1 hr.	0·66	2·0	3·46
25 hr.	0·27	2·3	3·56

Peracetic acid. The hydrolysates of wool samples which had been treated with peracetic acid were estimated for cysteic acid. Within the experimental error of the method quantitative conversion of the combined cystine to the sulphonic acid was observed (Table 5). All the sulphur is accounted for as cysteic acid even when 90 % of the cystine has reacted in contrast to chlorination in an acidic medium. The reason for this is that the peracetic acid, unlike chlorine, reacts specifically with the cystine and no side reactions involving oxidized cystine take place.

After complete oxidation of the cystine with peracetic acid 90 % of the wool became soluble in dilute alkali. Fig. 1 shows the relation between the solubility of wool in cold 3N-ammonia and the proportion of cystine oxidized by peracetic acid and chlorine at pH 2. The greater solubility of chlorinated wool when less than 80 % of the cystine was oxidized also indicates the non-specific nature of the reaction with chlorine.

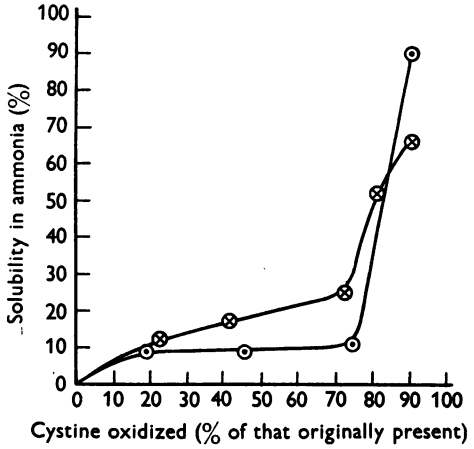


Fig. 1. Relationship between solubility of wool in 3N-ammonia and the proportion of cystine oxidized by chlorine solutions at pH 2, —⊗—; and peracetic acid, —⊙—.

After chlorination in acid the tensile strength of single fibres was greatly reduced (Table 3). Load-extension data on fibres which had been oxidized with 0·25N-peracetic acid showed that a similar reduction in work (R.W.) occurs. This reduction increased with the extent of disulphide bond oxidation (Table 6).

It was also observed that the difference in R.W. at pH 1 and 7 found with untreated fibres disappeared on extensive oxidation of the disulphide bond. According to Speakman (1947), the increased tensile strength of untreated fibres at pH 7 is due to the formation of salt linkages which are broken at pH 1. Sookne & Harris (1937), however, attributed the difference in strength to increased swelling in acid solution. The above results support this latter view, as Harris, Mizell & Fourt (1942) showed that swelling of the fibre increased with increasing disulphide bond breakdown. It appears, therefore, that swelling is the operative factor responsible for the difference in elasticity of wool fibres in acid and neutral solutions, the breaking of salt linkages being only of secondary importance.

Table 6. *The reduction in work* (R.W.) *on stretching the fibres to a constant extension, after treatment with* 0·25N-*peracetic acid at room temperature*

Time of treatment in 0·25N-peracetic acid (min.)	Cystine oxidized (percentage of that originally present)	R.W. at pH 1	R.W. at pH 7
—	0	38	2
5	19·8	48	28
30	46·3	70	67
60	71·1	82	84
120	75	93	97

It is clear that oxidation with peracetic acid or chlorine solution at pH 2 disrupts the disulphide bond in such a way as to yield cysteic acid on subsequent acid hydrolysis. In order to determine whether combined cysteic acid formed within the fibre or only resulted on acid hydrolysis, experiments were made in order to exchange the H^+ or NH_4^+ ions initially absorbed on any free sulphonic acid groups within the fibre by potassium ions using the procedure already described. No exchange could be detected in any of the experiments performed, showing that free cysteic acid does not exist within the fibres.

The nature of the oxidized disulphide bond

Ammonia extraction of wool oxidized in acid solution. Wool samples which had been oxidized with peracetic acid or chlorine at pH 2 were allowed to stand overnight in cold 3N-ammonia. On hydrolysis with 15 % HCl at 125° for 5 hr. very little cysteic acid could be detected, although the sulphur content of the wool had not decreased appreciably (Table 7).

Table 7. *Analyses for cysteic acid sulphur and total sulphur contents of wool oxidized with peracetic acid and chlorine at* pH 2, *before and after alkaline extraction with* 3N-NH_3

Treatment	g. S/100 g. wool: Cysteic acid sulphur, Before extraction	Cysteic acid sulphur, After extraction	Total sulphur, Before extraction	Total sulphur, After extraction
Cl_2 (5 g./100 g. wool)	0·6	Nil	3·57	3·40
Cl_2 (10 g./100 g. wool)	1·3	0·1	3·35	3·40
Cl_2 (20 g./100 g. wool)	1·9	0·2	3·40	3·12
5 min. in 0·25N-peracetic acid	0·5	0·1	3·50	3·35
30 min. in 0·25N-peracetic acid	1·2	0·3	3·50	3·16
1 hr. in 0·25N-peracetic acid	2·0	0·2	3·46	2·97

The loss in weight on extraction with 3N-ammonia (see Fig. 1) was small until more than 80% of the cystine had been oxidized.

Similar results were obtained when 0·1N-NaOH was used instead of 3N-NH_3. Wool, for example, which had been treated for 1 hr. with excess peracetic acid and subsequently shaken with 0·1N-NaOH for 0·5 hr. had a total S content of 2·95%, although the hydrolysate contained a negligible quantity of cysteic acid. Experiments with peptides containing cysteic acid showed that such treatment with dilute alkali did not produce a loss of cysteic acid. The soluble fraction obtained when wool treated at pH 2 with 20 g. chlorine/100 g. of wool was extracted with dilute NH_3 solution, consisted of material with a total S content of 3·37%. Hence cysteic acid is not preferentially extracted, as untreated wool contains about 3·50% sulphur. It was found that the peptide hydrolysate contained 2·2% cysteic acid sulphur in contrast to the low cysteic acid content of the larger insoluble fraction.

Acid-combining capacity of alkaline-extracted oxidized wool. The fibres produced by oxidation in acid followed by extraction with alkali showed an increase in acid-combining capacity. Except for the 18 hr. treatment (see Table 8) the wool was shaken for the times indicated in each case. The results show that the product of this dual treatment contains approximately 45% more acidic groups than the untreated fibres. No increase in acid-binding capacity was observed with untreated fibres or fibres which had been oxidized with peracetic acid or chlorine in acid, and not treated further with alkali (see also Lemin & Vickerstaff, 1946).

Table 8. *Maximum acid-combining capacity of wool oxidized for 1 hr. with 0·25N-peracetic acid and extracted with 3N-NH_3 for various times*

Time of treatment with NH_3	Maximum acid combined* (g.equiv./1000 g. wool)
Nil	0·80
3 min.	0·98
5 min.	0·95
15 min.	1·11
18 hr.	1·15
30 min. (0·1N-NaOH)	1·04

* The value for untreated wool was 0·82.

That the increased titration is not due to the liberation of free carboxyl groups resulting from peptide chain breakdown during the mild alkaline treatment, is shown by load-extension data obtained with alkali-extracted fibres. No difference was found in tensile strength with fibres which had been treated with 3% chlorine at pH 2 before and after alkali extraction.

Hydrolysis of the product formed by oxidation in acid followed by alkali extraction. The unknown product formed after oxidation in acid and treatment with dilute alkali was found to be very stable to hydrolysis with acid and to cause the liberation of free acidic groups during its formation. That the unknown is not a lower oxidation product of cystine was shown by treating the hydrolysate with potassium permanganate solution when very little cysteic acid was produced. It was concluded that the oxidized sulphur combines with the imino nitrogen in the main polypeptide chain and gives a sulphonamide on mild alkaline hydrolysis. The latter are known to be very stable to acid and alkaline hydrolysis. In attempts to hydrolyse the suspected sulphonamides it was found that hydrolysis with concentrated hydrochloric acid for relatively short periods or prolonged hydrolysis with 25% (w/v) hydrochloric acid gave maximum yields of cysteic acid. Thus, oxidation of wool with 20% chlorine at pH 2 gave 1·9% of cysteic acid sulphur on hydrolysis with 15% (w/v) hydrochloric acid for 5 hr. at 125°. After treating the oxidized wool with dilute ammonia only 0·2% cysteic acid sulphur was obtained by hydrolysing under the same conditions, whereas hydrolysis with 25% (w/v) hydrochloric acid at 125° for 4 days gave a value of 1·0% cysteic acid sulphur, and hydrolysis with 32% (w/v) hydrochloric acid at 125° for 5 hr. resulted in a 0·8% yield. All the hydrolyses were carried out in sealed tubes. Control experiments showed that under the conditions employed, cystine was not oxidized and the destruction of cysteic acid was negligible.

Attempted separation of sulphonamides. The hydrolysate of a wool sample (15% (w/v) hydrochloric acid for 5 hr. at 125°) which had been oxidized with peracetic acid or chlorine at pH 2 to different extents and then extracted with 3N-ammonia for 20 hr. was desalted (Consden, Gordon & Martin, 1947) and the pH adjusted to 3–4. The solution was then applied to a column of Deacidite *B* (see Consden, Gordon & Martin (1948) who used Amberlite IR-4) to separate the acidic amino-acids from the remainder which passed through. The former were eluted from the column with N-hydrochloric acid. The small amount of cysteic acid originally present in the hydrolysate was not eluted by this procedure. The acidic fraction was again desalted, adjusted to pH 3–4, applied to the Deacidite column and eluted with water (Consden, Gordon & Martin, 1949). Several fractions were collected and shown by one-dimensional paper chromatography to contain aspartic and glutamic acids only. When these fractions were hydrolysed with concentrated hydrochloric acid at 125° for 5 hr. and again chromatographed in phenol-ammonia, every amino-acid including cysteic acid appeared.

Partial oxidation products

In addition to the main reaction giving a product which yields cysteic acid on acid hydrolysis, some evidence for the formation of an intermediate oxidation product of cystine after chlorination in acid

solution was obtained. The cystine content increased after alkali treatment (Table 9). The cystine produced by alkali treatment was probably derived by

Table 9. *The cystine content of wool oxidized by chlorine solution at* pH 2, *before and after extraction with* 3N-NH_3

Chlorine reduced (g./100 g. of wool)	Cystine content (%) Before extraction	After extraction
Nil	10·00	9·65
13	5·32	6·60
35	2·02	2·95
50	0·39	2·20

hydrolysis from cystine disulphoxide or some other incomplete oxidation product, e.g.

$$3RSO.SOR + 4NaOH \rightarrow RS.SR + 4RSO_2Na + 2H_2O$$

(Lavine, 1936).

After reducing with a mixture of 5N-potassium iodide and 5N-hydrochloric acid (see Lavine, 1936) the cystine content of a sample of wool which had been oxidized with 50% chlorine at pH 2 rose from 0·39 to 1·46%. Reduction under these conditions occurs only with oxidation products below the sulphonic level. No evidence for the presence of such products in wool oxidized with peracetic acid was found.

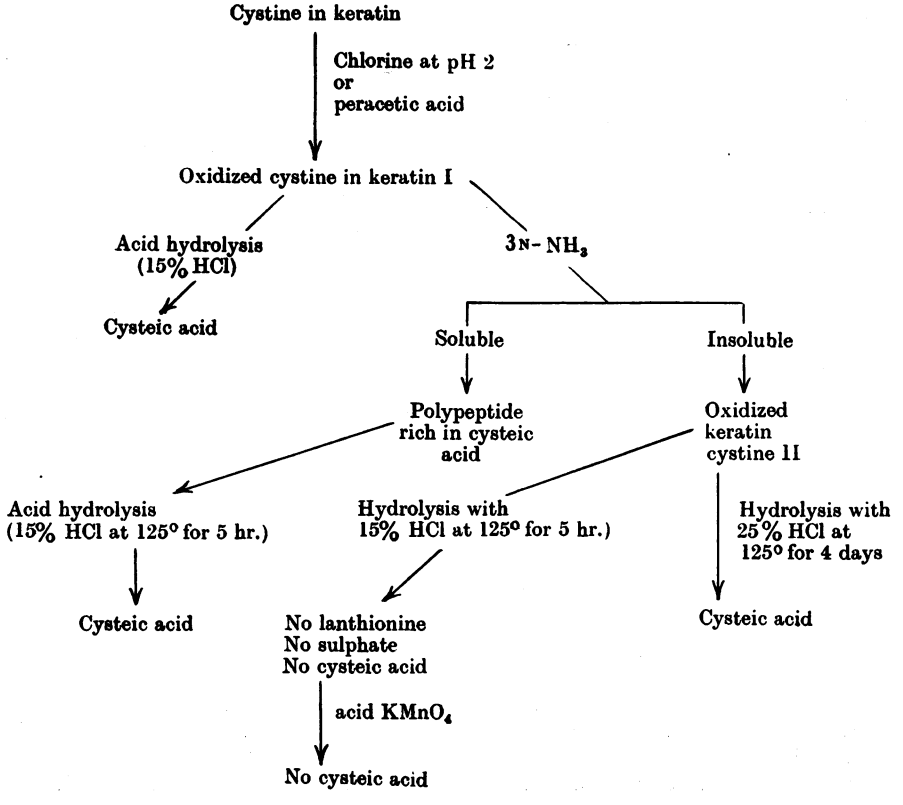
DISCUSSION

The analytical results show that the combined cystine in wool keratin may be oxidized under different conditions to give different products depending on the particular fraction which is reacting. Thus, oxidizing agents capable of reacting with all the cystine in wool gave products which were converted quantitatively to cysteic acid on subsequent acid hydrolysis. On the other hand, acid potassium permanganate which oxidized only 25% of the cystine, gave a mixture of cysteic acid and sulphate. The conversion of both sulphur atoms to cysteic acid may be prevented by steric factors so that the permanganate reacts with part of the side chains in addition, thus eliminating one sulphur atom/disulphide bond as sulphate.

Oxidation in alkaline solution

In alkaline solution, however, the 25% fraction capable of reacting is converted to lanthionine by permanganate and hypochlorite. The state of combination of the ejected sulphur atom is unknown (see Cuthbertson & Phillips, 1945).

Lanthonine has been produced by the action at 100° of alkaline buffers on wool (Cuthbertson & Phillips, 1945), although only 50% of the total



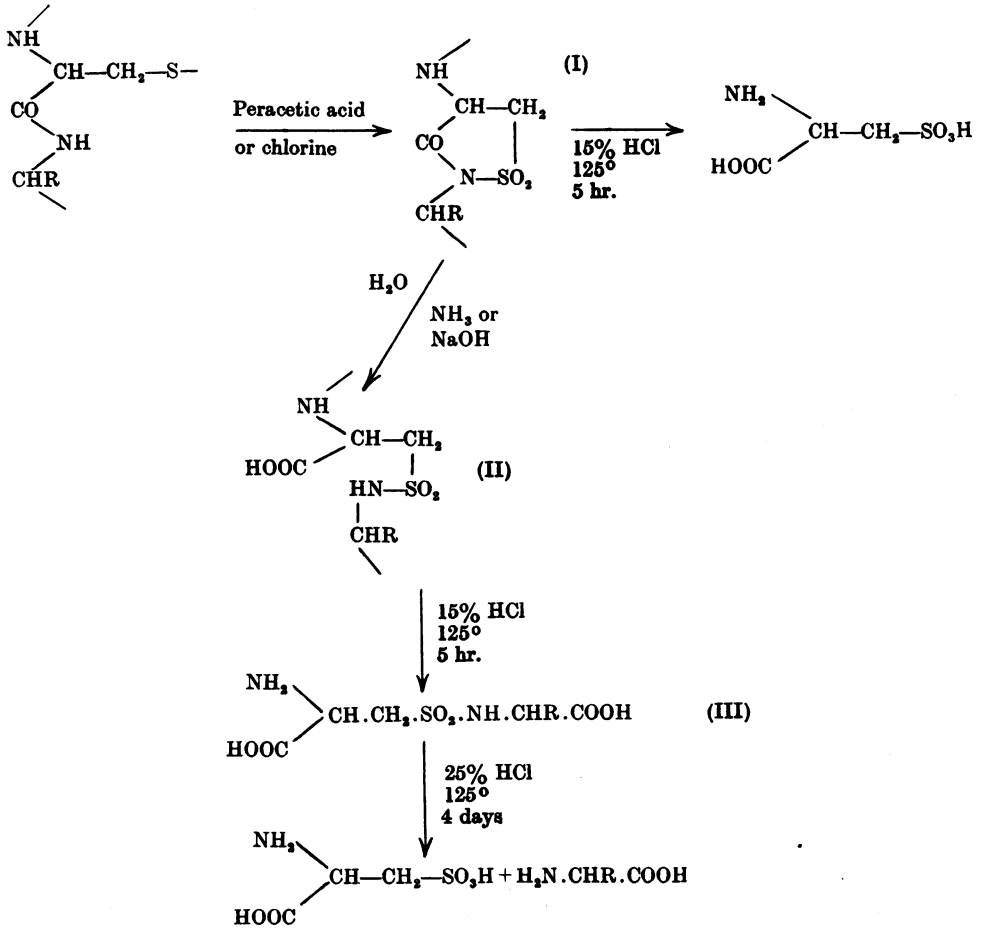
cystine reacted in this way. In the present experiments, however, the conversion to lanthionine was rapid in the cold, so that the hypochlorite ions must enter into the reaction. Cuthbertson & Phillips (1945) suppose their 50 % fraction to be linked in the molecule in such a way that the residues formed by the fission of the disulphide bond remain in close proximity and readily condense to form the monosulphide bond. Alexander & Earland (1950*b*) showed that only a small proportion of the sulphur is associated with the long polypeptide chains, the greater proportion residing in much shorter chains. It is suggested that the latter act as a cement between the micelles formed from the high molecular weight fraction. It is tempting to correlate the difference in reactivity of cystine with oxidizing agents, with the two protein components postulated in this model. There is no evidence, however, to enable the cystine fractions to be identified with either the high or low molecular weight polypeptides.

Formation of new cross-links by hypochlorite is supported by observations (Alexander, 1950) that above pH 8·5 the chlorine is not effective in reducing shrinkage. Methods of rendering wool unshrinkable depend on disulphide bond breakdown in the surface layers of the fibres (Speakman, Nilssen & Elliott, 1938). Farnworth, Neish & Speakman (1949) have shown that fibres in which lanthionine had been formed could not be rendered unshrinkable. The sharp decrease in resistance to felting above pH 8·5 is in agreement with lanthionine formation. This is also supported by the high tensile strength of such fibres (see Table 3).

Oxidation in acid solution

The relevant observations on oxidation in acid are summarized on p. 134.

On oxidation the disulphide bonds in the wool are broken, yielding the theoretical amount of cysteic acid on acid hydrolysis without further oxidation, although (see p. 132) no free sulphonic acid groups are present in the wool before hydrolysis. In addition to the evidence from cationic exchange it was found that the oxidized wool has the same affinity for dyes as the untreated wool, whereas wool containing charged



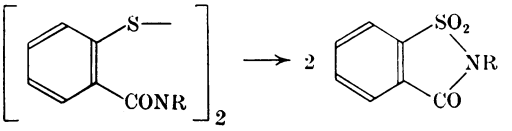
acidic groups has been found to repel dye anions (Lemin & Vickerstaff, 1946).

It was found that the soluble peptides obtained by ammonia extraction of wool oxidized with peracetic acid or chlorine were rich in cysteic acid, but the insoluble portion on hydrolysis contained little cysteic acid, no lanthionine and no sulphate. Total sulphur analyses showed, however, that no appreciable loss in sulphur had occurred during alkaline extraction (see Table 7). The elasticity measurements (Tables 3 and 6) on oxidized fibres support disulphide bond breakdown. There is a considerable reduction in work required for a given extension, but from the solubility in weak alkali (Fig. 1) it is seen that the oxidized residues are firmly held to the chains. It appears that in the keratin molecule, two adjacent cysteic acid groups cannot be formed on the disruption of the disulphide bond, probably owing to electrical repulsion. If the chains can move apart in the alkali solution after complete oxidation with peracetic acid, this restriction no longer exists. The soluble (90%) portion was shown to contain 3·7% cysteic acid sulphur after normal hydrolysis. Product II was not formed under these conditions. It is unlikely that intermediate oxidation products are formed to any extent (Harris & Smith, 1937), as these would not be quantitatively converted to cysteic acid on hydrolysis: e.g. the disulphone is hydrolysed to one mol. of sulphinic and one mol. of sulphonic acid (Lavine *et al.* 1934).

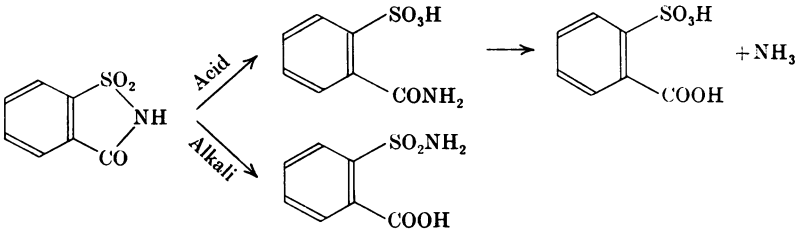
The most likely possibility when the stereochemical arrangement of the polypeptide chains is considered, appears to be intramolecular cyclization to give a compound which readily hydrolyses in acid to give cysteic acid. In alkaline solution, however, an alternative reaction occurs, leading to a very stable sulphur-containing compound. On this basis the set of reactions is suggested as probable (see p. 135).

The oxidation intermediate (I) is a mixed imide containing a sulphonic and a carboxylic acid residue with the N atom highly activated by the two negative groups $>CO$ and $>SO_2$ and is therefore so unstable that it cannot exist in solution. With acid it hydrolyses as a typical amide, the S—N bond breaking to give cysteic acid. In alkaline solution, however, hydroxyl ions attack the OC—N linkage giving a free carboxyl group (II). Hydrolysis of (II) with 15% hydrochloric acid yields a *N*-β-sulphoalanyl amino-acid complex (III) which is partially hydrolysed under vigorous conditions to give cysteic acid and the amino-acid which was originally in the wool linked to the cystine residue through its carboxyl group.

This proposed set of reactions closely follows that of the formation and hydrolysis of saccharin and its *N*-substituted derivatives. Thus, oxidation of the *N*-substituted 2:2′-dithiobenzamide gives a substituted saccharin (McLelland, Warren & Jackson, 1929).



This behaves differently towards acid and alkaline hydrolysis, S—N bond fission occurring in the former case, C—N bond fission in the latter.



In the acid hydrolysis the amide formed is easily hydrolysed unlike the imide formed in wool.

The formation of the combined sulphonamide explains the observation that the tensile strength of the fibres remains the same after initial oxidation and subsequent mild alkali treatment. The sulphonamide forms without main chain breakdown although an extra group is incorporated. After treatment with ammonia, fibres oxidized with peracetic acid, but not untreated fibres, had an increased acid-combining capacity (see Table 8). Sulphonic acid groups cannot be responsible for this increase since they would not be titrated by 0·05N-hydrochloric acid. Also, since the wool had been equilibrated with N-potassium chloride and the titration was carried out in the presence of potassium chloride, no acid could have combined by ion exchange. The increased titration of the wools can therefore be assumed to be due to the formation of free carboxyl groups. Thus, 60% of the theoretical yield of carboxyl groups formed in the conversion of the combined sulphocarboxylic imide to the sulphonamide has been detected.

The conversion of the substituted sulphonamide (III) to cysteic acid by hydrolysis is only achieved

with difficulty. Sulphonyl derivatives of amino-acids have been used for end group determinations in proteins. Thus, Gurin & Clarke (1934) found that hydrolysis with 15% hydrochloric acid for 5 days at 90–100° resulted only in a 28% yield of the amino-acid from benzenesulphonylalanine. Helferich & Grünert (1940) found methanesulphonyl derivatives of amino-acids and peptides to be extremely resistant to both acid and alkaline hydrolysis. In the present investigation it was found that short hydrolysis (5 hr.) with concentrated hydrochloric acid or prolonged hydrolysis (approx. 4 days) with 25% hydrochloric acid resulted in a considerable increase in cysteic acid (see p. 133). More drastic conditions were shown to lead to the destruction of cysteic acid so that quantitative conversion of the sulphonamide to the cysteic acid is impossible.

An attempt to isolate the stable sulphur compounds from the hydrolysate of wool which had been oxidized and extracted with alkali was made using a weakly basic ion-exchange column, since the sulphonamides postulated would be expected to be acidic. In this way a solution was obtained which contained no free amino-acids other than aspartic and glutamic acids. When this solution was hydrolysed under fairly drastic conditions one-dimensional chromatograms showed the presence of all the amino-acids found in wool, including proline, together with cysteic acid. A noticeable feature was the high proportion of aspartic acid so obtained. That the amino-acids which appeared after strong hydrolysis were not derived from cysteic acid peptides stable to normal hydrolysis is shown by the fact that all the cysteic acid can be recovered by normal hydrolysis from wool which had been oxidized but not reacted with alkali. It is also unlikely that peptides of aspartic or glutamic acid would survive normal protein hydrolysis since Partridge & Davis (1950) and Bull (1949) have shown that these amino-acids are preferentially liberated during acid hydrolysis. These results agree with the general conclusions of Consden *et al.* (1949) and Consden & Gordon (1950), except that these workers failed to detect proline and aspartic acid combined with the cystine in wool.

Apart from the main reactions which have been fully discussed above, evidence for the limited formation of an intermediate cystine oxidation compound is obtained from cystine analyses of wool which has been treated with chlorine at pH 2 (see Table 9). There is also evidence that chlorination in acid may lead to cross linking. It is shown in Fig. 1 that wool, in which 90% of the disulphide bonds have been oxidized by peracetic acid, is almost completely soluble in ammonia. The small insoluble fraction which remains is the subcuticle membrane (Alexander & Earland, 1950*a*). The fibres, however, remain largely insoluble in ammonia until more than 80% of the disulphide bonds have been broken. However, wool oxidized to the same extent with chlorine is only soluble in ammonia to the extent of 70%. Moreover, while the wool oxidized with peracetic acid supercontracts, the chlorinated wool does not (Whewell & Woods, 1946). It is suggested that on chlorination in acid solution, new cross-linkages are formed which prevent supercontraction and increase the resistance to alkali.

SUMMARY

1. Oxidation of wool with solutions of potassium permanganate at pH 9·2 and sodium hypochlorite at pH 10 led to the formation of lanthionine in the cold from the reactive cystine fraction (approx. 30% of the total cystine).

2. Acidic solutions of potassium permanganate, which also react with only 30% of the total cystine, gave cysteic acid and sulphate.

3. On oxidation with acid solutions of chlorine and peracetic acid, which are capable of reacting with all the cystine, combined cysteic acid was not produced directly within the fibre although cysteic acid was produced almost quantitatively on acid hydrolysis. To explain this and other observations the formation of a combined heterocyclic mixed imide of a carboxylic and sulphonic acid respectively within the wool is postulated.

4. In order to interpret results obtained by treatment of wool oxidized with peracetic acid or chlorine at pH 2 with dilute alkali it is postulated that alkali hydrolyses this sulphocarboxylic imide across the CO—N bond to give a sulphonamide within the fibres. The formation of the sulphonamide explains the increase in acidity on alkali extraction.

5. Hydrolysis of the postulated sulphonamides by drastic treatment with hydrochloric acid led to the production of cysteic acid together with all the amino-acids of wool. This shows that cystine is combined with all the various amino-acids within the fibre.

6. Evidence for the limited formation of a partially oxidized product formed from cystine by a side reaction was obtained. As this reverts to cystine, at least partially, on treatment with alkali, it is suggested that this intermediate may be a sulphoxide.

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The Metabolism and Glutamic Acid Content of Rat Brain in Relation to Thiopentone Anaesthesia

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It has recently been shown that the free glutamic acid content of the rat brain falls during insulin hypoglycaemia (Dawson, 1950). This observation suggests that in the physiological emergency produced by a shortage of glucose the brain oxidizes glutamic acid to gain high-energy phosphate bonds for metabolic activity. It is well known that during barbiturate anaesthesia the metabolic activity of the brain, as measured by its consumption of oxygen, slows down in parallel with the reduction of physiological activity (Etsten & Himwich, 1946; Himwich, Homburger, Maresca & Himwich, 1947). It has been suggested from the results of *in vitro* studies that this depressed metabolism is a consequence of inhibition of brain oxidations by the narcotic (Quastel & Wheatley, 1932). The present investigation was undertaken to determine whether the reduced metabolism of the brain during anaesthesia was associated with a change in the level of the free glutamic acid in the brain. It was found that in the rat during anaesthesia produced by sodium 5-ethyl-5-(1′-methylbutyl)-2-thiobarbiturate (thiopentone sodium) the free glutamic acid content of the brain was reduced by about 30%. Simultaneous observations also showed that thiopentone anaesthesia reduced the concentration of ammonia found in the brain and slightly increased the glutamine content. Observations were also made on the inhibition by thiopentone of the respiration of isolated rat-brain tissue, and the effect of the drug on some enzyme systems in brain tissue capable of producing free ammonia.