The Action of Organic Acids on some Fibrous Proteins: the Oxidation of Wool Keratin

BY S. BLACKBURN AND A. G. LOWTHER

Wool Industries Research Association, Torridon, Headingley, Leeds

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Information as to the arrangement of amino-acid residues along the peptide chains of wool has been obtained from the study of the peptides present in partial acid hydrolysates (Consden, Gordon & Martin, 1949; Consden & Gordon, 1950). The present observations arose out of experiments designed to study in what way the individual peptide chains of wool differ from one another in amino-acid composition. The chains were separated by oxidizing the cystine cross-linkages to cysteic acid with performic acid, a technique originally applied by Sanger (1949) to insulin.

The action of formic acid and acetic acid on a number of fibrous proteins, including wool, has also been studied. Toennies (1942) has previously pointed out that many proteins are soluble in formic acid, which has been shown to cause wool fibres to increase in diameter by 50% (Speakman, 1931). Meyer & Mark (1930) showed that formic acid caused marked swelling of silk and a change in the X-ray diagram; these could be reversed on washing with water. From the present results it seems likely that the extent of solution of fibrous proteins in formic acid can be correlated with the molecular configuration of the peptide chains.

Alexander & Earland (1950*a*) found that, when wool which had been oxidized with peracetic acid (Alexander, Hudson & Fox, 1950) was treated with dilute aqueous ammonia, a fraction amounting to 7–10% by weight of the original wool remained insoluble. They considered this to be the 'sub-cuticle membrane' of the wool fibre and found it, on hydrolysis, to yield all the amino-acids obtainable from wool, but in different proportions. Mercer, Lindberg & Philip (1949) have contested the view that such a 'sub-cuticle membrane' or 'subcutis' exists, considering the preparations to consist of the thin 'epi-cuticle' membrane, thickened by the adhesion of other layers of the cuticle. Experiments bearing on the structure of this 'sub-cuticle membrane' are described by the present authors, the general conclusion being that the membrane does not as such exist in the original fibre.

The paper also records further observations on the action of alkaline hydrogen peroxide on wool (cf. Consden & Gordon, 1950).

MATERIALS AND EXPERIMENTAL PROCEDURES

Analytical methods

N determination, micro-Kjeldahl; total S, Barritt (1934); cystine and cysteine S, Shinohara (1935, 1936); partition chromatography on paper, Consden, Gordon & Martin (1944). Hydrolysis of proteins was effected by refluxing in 5*N*-HCl for 24 hr. and removing excess acid by repeated evaporation *in vacuo*. In certain cases hydrolysed protein was oxidized with H₂O₂ before running on a phenol chromatogram. Cystine originally present is thus oxidized to cysteic acid, which is then readily detected on the chromatogram (Dent, 1948).

Protein preparations studied

A 70's quality wool, commercially scoured and made into dry-combed top. (Found: total S, had 3.77%; (cystine + cysteine) S, 3.28%.) This wool was used throughout the present experiments unless otherwise stated. The silk fibroin was an unweighted fabric. Before use both were extracted with light petroleum (b.p. 40–60°), then with ethanol, rinsed in several changes of distilled water and dried in air. Collagen was an acetone-dehydrated, untanned hide.

Performic acid oxidation of wool

Wool (1 g.) was treated at room temperature with a mixture of 36 ml. of formic acid (98–100%) and 4 ml. of 30% (w/w) H₂O₂ for a given time (cf. Sanger, 1949), filtered off on sintered glass, rinsed well in water and dried. The dissolved protein was isolated either by evaporation *in vacuo* below 40° or by precipitation on pouring into water. (The dissolved material is described as protein, even though it may differ from the original protein.)

Treatment of proteins with formic and acetic acids

The protein (1 g.) was treated at room temperature with either formic acid (40 ml.; 30 ml. in the case of silk fibroin) or acetic acid (40 ml.) for a suitable time and filtered off. The protein in solution was examined after evaporating the acid *in vacuo* below 40°. Its amino-acid composition was examined on a two-dimensional collidine-phenol-NH₃ chromatogram after HCl hydrolysis.

A number of chemically modified wools were also treated with formic acid. Samples of wool were boiled under reflux for 1 hr. with 100 times their weight of solutions of different pH value, rinsed in water and dried. They were then treated with formic acid for 2 hr., the dissolved protein being estimated by determining N in the filtrate.

Modification of the surface of wool

Some experiments, described below, were carried out with wool whose surface layer had been modified chemically in one of two ways:

(a) Treatment by gaseous chlorination (King & Galley, 1933), in which 1.3% of Cl_2 was found to have reacted with the wool; only the surface layer of the wool is affected by the Cl_2 under these conditions (Phillips, 1942).

(b) Treatment by papain following the gaseous chlorination (Phillips, Middlebrook & Higgins, 1942).

The chlorinated wool was treated for 1 hr. at 60° with a solution containing 1% (w/v) of NaHSO_3 and 0.025% of papain, and adjusted to pH 5; 30 ml. of solution were used to each gram of wool. The treated wool was then milled in the stocks. Under these conditions the scales of the wool are removed almost completely. Both wools were extracted with solvents and rinsed in water before use.

RESULTS

Performic acid oxidation of wool

Wool treated with performic acid swells greatly and partially dissolves. Table 1 records the cystine sulphur of the undissolved protein when the wool had been treated for different times. Oxidation of the cystine was essentially complete in 30 min.; Sanger (1949) found that the cystine of insulin was completely oxidized in 15 min. The amount of protein going into solution, however, determined by weighing the undissolved residue, increased with increasing time of treatment (Table 1). The rate at which protein went into solution varied somewhat from experiment to experiment.

Table 1. *Oxidation of wool with performic acid*

(For conditions of experiment see text.)

Time of treatment (hr.)	Loss in weight (%)	Cystine S of undissolved wool*
0.25	—	0.22
0.5	—	0.15
1	7	0.15
2	16	0.08
3	27	0.05
5	27	0.04
18	39	—

* The cystine S was in every case less than 0.03%.

It appears that after oxidation of the cystine residues the wool keratin will dissolve in the large excess of formic acid which is present in the reaction mixture. From the data in Table 1, however, it is seen that the rate of solution decreases markedly after 5–6 hr. Wool which had been oxidized with performic acid for 2 hr., rinsed in water and air dried dissolved only slowly in formic acid at room temperature, protein equivalent to 9 and 6% of the original weight of the wool being dissolved during two successive treatments with fresh formic acid for 2 and 17 hr. respectively.

The X-ray diffraction patterns of the dissolved protein (from wool oxidized with performic acid for 0.5 hr., isolated by pouring into water) and of the undissolved wool were examined by Dr T. C. Tranter. The dissolved protein showed rings corresponding to spacings of 11.3 and 4.6 Å., probably corresponding to the 'side chain' and 'backbone' spacings of a disordered β -keratin structure. The undissolved wool gave a weak photograph with two rings corresponding to spacings of 9.9 and 4.3 Å. The spacings could only be considered as approximate in view of the weakness of the photograph, but they probably also correspond to a disordered β -keratin structure. The undissolved wool, therefore, has a similar X-ray structure to the dissolved protein as isolated. It does not, however, necessarily follow that the dissolved protein has the β -keratin structure while in solution.

The relatively slow dispersion of the oxidized wool in formic acid may be correlated with the assumption of the β -configuration. When the cystine cross-linkages have been broken by the action of the performic acid, the polypeptide chains are freed from restraint and can assume new configurations. Elongation of the chains takes place and the β -configuration is attained. Proteins in the β -configuration often offer a greater resistance to dispersion than those in the α -configuration (Blackburn, 1948).

The action of organic acids on fibrous proteins

The amount of wool keratin or silk fibroin dissolved by glacial acetic acid at room temperature is inappreciable, but some 6.6% of collagen dissolves

Table 2. *Action of formic acid on fibrous proteins*

Protein	Time of treatment (hr.)	Protein dissolved by formic acid (%)
Wool (70's top)	2	0.34
	16	0.42
	48	0.45
Blackface wool	2	0.43
	24	0.56
Porcupine quill	72	7.0
Silk fibroin	0.5	10.6
	1	11.2
	2	11.3
	5	11.8
	24	12.8
Collagen	48	14.4
	72	23.1

during 48 hr. Formic acid has a more drastic action: silk fibroin and collagen swell greatly in it, much more than wool, and dissolve to an appreciable extent (Table 2). That formic acid has a greater solvent power for proteins than has acetic acid may also be seen from the appreciable solution of wool

which occurs during performic acid oxidation, as compared with that occurring during peracetic acid oxidation. Table 3 shows the amount of protein dissolved when silk fibroin was treated with formic acid for 3 days, removed and treated with a further amount of formic acid, the process being repeated thrice in all. The experiment was terminated at this point as the formic acid solution acquired a purple colour, possibly caused by degradation of tryptophan residues. The extent of the colour formation when collagen and wool were similarly treated was much less, but their tryptophan content is lower. It appears that formic acid will not readily dissolve all the silk fibroin, only some 20% dissolving rapidly.

Table 3. *Repeated treatment of silk fibroin with formic acid*

(For experimental conditions see text.)

	Time of treatment (hr.)	Total protein dissolved by formic acid (%)
First treatment	72	14.6
Second treatment	48	17.5
Third treatment	48	21.2

In contrast, only relatively small amounts of wool keratin are dissolved by formic acid (Table 2). It seemed probable that the low solubility of wool keratin in formic acid might be due to the presence of the cystine cross-linkages. Wool in which the cystine has been reduced to cysteine shows a relatively high solubility in formic acid. When wool (1 g.) was reduced with 20 ml. of a 5.5% (w/v) solution of thiolacetic acid at 39° for 17 hr. (cf. Patterson, Geiger, Mizell & Harris, 1941), rinsed in water, pressed between filter papers and treated with formic acid for 16 hr., a loss in weight of 25% was

Table 4. *Action of formic acid on chemically modified wool*

pH of buffer solution	Wool dissolved by formic acid (%)
1.2 (Approx. 0.1 N-HCl)	0.72
3.0 (Table 62)*	0.40
4.8 (Table 70)	0.26
6.6 (Table 73)	0.23
8.2 (Table 79)	0.23
8.9 (Table 79)	0.29
10.0 (Table 85)	0.37
10.8 (Table 86)	0.49

* Buffers are cited in tables by Britton (1942).

observed. The action of formic acid on a number of keratins containing medulla, which has a low cystine content (Bekker & King, 1931; Blackburn, 1948), was also investigated. Only a slightly larger amount of protein was dissolved from a medullated Blackface wool than from the 70's top wool, but a considerably larger amount of porcupine quill was dissolved.

Chemical modification of wool in which the cystine cross-linkages are not broken does not appear to alter its solubility in formic acid to any great extent. Thus, when wool is boiled in either acid or alkaline solution, the solubility in formic acid shows only a small increase (Table 4), which is probably to be attributed to some hydrolysis of the peptide chains.

The structure of the 'sub-cuticle membrane'

The introduction of the strongly acidic cysteic acid groups into wool keratin brings about a greatly increased solubility in alkali. The protein dissolved by performic acid and the insoluble wool residue are almost completely dissolved by dilute sodium carbonate or sodium hydroxide solutions at room temperature, and reprecipitated when the solution is acidified. When wool oxidized with performic acid for 0.5 hr. is treated with dilute ammonia (0.1–1.0 N) a greater proportion of the wool remains undissolved. Under the microscope this undissolved residue appears to be similar to the material described by Alexander & Earland (1950a, b). Chromatograms which were run on hydrolysates of the material insoluble in N- or 0.1 N-ammonia showed no marked differences from those of ordinary wool, except that there was a strong cysteic acid spot.

If, as Mercer *et al.* (1949) contend, the 'sub-cuticle membrane' preparations consist primarily of epi-cuticle with adhering layers of cuticle, modification or removal of the cuticle should make it difficult or impossible to isolate material with the same properties as the 'sub-cuticle membrane' from this treated wool. On the other hand, if the sub-cuticle membrane is a layer below the scales, removal or modification of the scales might have little effect on the isolation of the membrane. Experiments of this nature were therefore made to obtain further evidence on the question. Two treated wools, i.e. wool which had been chlorinated in order to modify the surface layer and wool which had been chlorinated and then treated with papain as described above to remove the scales together with the corresponding intact wool, were treated with peracetic acid followed by 0.1 N-ammonia under the conditions of Alexander & Earland (1950a). An insoluble residue, similar in appearance under the microscope to the preparation described by Alexander & Earland (1950a), was obtained in each case.

The amino-acid composition of the dissolved proteins

The protein dissolved by the performic acid treatment (0.25 or 0.5 hr.) of wool was examined on a two-dimensional collidine-phenol-ammonia chromatogram after hydrolysis. The chromatogram differed from that of normal wool in that a very strong cysteic acid spot was present and no tyrosine spot was observed. The dissolved protein from

samples of human hair, 56's top wool and the root ends of Cotswold wool fibres which had been oxidized with performic acid for 0.5 hr. was also isolated, hydrolysed and examined chromatographically. The chromatograms were similar to those of the intact proteins, except that once again cysteic acid was present and tyrosine absent.

The dissolved protein from the 3 hr. treatment of wool gave a result similar to that from the shorter treatment, but two additional spots were observed which ran fast in collidine and had R_f about 0.5 in phenol. The absence of tyrosine suggested that these additional spots might arise from the oxidation of tyrosine residues; tyrosine oxidized with performic acid gave rise to several spots in the same region of the chromatogram. The changes in colour of the wool which remained undissolved by the performic acid may also be correlated with changes in the tyrosine, though degradation of tryptophan residues may also play a part. After 0.5 hr. treatment the residue had darkened considerably, but after longer treatment it again became white in colour. Sanger (1949) found that certain of his fractions from oxidized insulin tended to darken on standing, suggesting the presence of oxidation products of tyrosine (cf. also Sanger & Tuppy, 1951). The possibility of some oxidation of threonine residues occurring cannot be completely ruled out.

The amino-acid composition of the soluble protein obtained when wool which had been reduced with thiolacetic acid was treated with formic acid did not differ greatly from that of normal wool, although the cystine (or cysteine) concentration was somewhat greater (peroxide test).

The dissolved protein obtained when collagen was treated with either acetic acid or formic acid, or when silk fibroin was treated with formic acid, appeared in each case to have a similar amino-acid composition to the corresponding intact protein.

The amino-acid composition of the small amount of wool dissolved by formic acid differed from that of intact wool, however, in that the amount of threonine in it was very low. Small amounts of cysteic acid were present, which did not increase appreciably after peroxide oxidation, indicating that very little cystine was present. The dissolved protein may, therefore, consist of peptide chains which have been liberated from the keratin structure by the splitting of cystine cross-linkages during the time the wool was exposed on the sheep's back (Race, Rowe, Speakman & Vickerstaff, 1938). Cysteic acid is known to be present in the tips of wool fibres which have suffered damage in this way (Consdon, Gordon & Martin, 1946). The amino-acid composition of the dissolved protein from a medullated Blackface wool was similar to that obtained from 70's top, cystine and threonine being present in low concentration only. Cysteic acid was present.

When a lock of Lincoln wool was cut into three parts, degreased and treated with formic acid for 2 hr., the amounts of protein dissolved from the root, middle and tip portion were 0.26, 0.31 and 0.20 % respectively. It might have been expected that more protein would be dissolved from the tip than from the root portions of the fibre, the former suffering greater damage during exposure to the weather. A possible complicating factor, however, is the presence of a certain amount of medulla in this Lincoln wool.

No peptides giving a colour with ninhydrin were revealed by running chromatograms on the (unhydrolysed) dissolved protein.

The dissolved protein from porcupine quills differed in amino-acid composition from that of the cortex, the concentration of glutamic acid, glycine, leucine and tyrosine being higher and that of the threonine lower. The dissolved protein was probably derived principally from the medulla, a similar chromatogram being obtained from medulla dissected out of the quill and treated with formic acid.

When wool was oxidized with hydrogen peroxide in alkaline solution, a polypeptide material went into solution. In this experiment 1 g. samples of wool were treated at 50° for times of 0.5, 1, 1.5, 2, 3, 4 and 5 hr., with 50 ml. of 15 % (w/w) aqueous hydrogen peroxide brought to pH 9.17 by the addition of ammonia. The solutions were then decanted from the bleached wool and acidified with dilute hydrochloric acid. The white precipitate of polypeptide nature which settled out increased in amount with increasing time of treatment, and after 5 hr. amounted to 18.7 mg.

A collidine-phenol-ammonia chromatogram of the hydrolysed precipitate showed the usual amino-acids present in a wool hydrolysate, but the threonine spot was very weak and a strong cysteic acid spot was present. No cystine spot could be seen. Cysteic acid as well as cystine was present in the undissolved part of the wool.

DISCUSSION

Wool keratin, like insulin, is readily oxidized by performic acid. The oxidized keratin, however, shows no sharp separation on the basis of solubility into two fractions differing in amino-acid composition as did oxidized insulin (Sanger, 1949). The evidence from the present experiments, therefore, suggests that the peptide chains of wool do not differ markedly from one another in amino-acid composition. Some evidence that certain of the peptide chains have a lower threonine content than others is derived from the experiments in which wool was treated with alkaline hydrogen peroxide or with formic acid. However, only a small percentage of the wool was dissolved in these experiments. That there is some difference in position of the amino-acids

along the different chains is also made probable by the fact that the chains carry many different end groups (Blackburn, 1950; Middlebrook, 1949).

When proteins were treated with formic acid no marked differences were observed between the protein which went into solution and that which remained undissolved, and it is therefore improbable that the observed differences in solubility can be attributed to the presence of different fractions in the fibre. The results probably provide an instance of the chemical reactivity of a protein depending on its molecular configuration. Recent work has shown, for example, that the solubility of a long-chain polypeptide is dependent on whether it is in the α - or β -configuration (Bamford, Hanby & Happey, 1950).

Proteins in the β -configuration seem generally to disperse only slowly in the reagents used, e.g. silk fibroin in formic acid. On the other hand, wool keratin resists solution due to the presence of the cystine cross-linkages. After these have been broken the solubility increases, but is then dependent on the configuration of the peptide chains. The initial rate of solution of the protein having the α -configuration is fairly high, but as swelling of the remaining undissolved protein occurs the peptide chains elongate and when they have attained the β -configuration, in which they can lie closer together, the rate of solution diminishes. An example is the treatment of wool with performic acid. Another example may be the solution of wool in alkaline thiolacetic acid (Goddard & Michaelis, 1934). In this reaction, according to observations in these laboratories, some 40–50% of the wool generally remains undissolved. A further instance may be the solution of keratins in solutions of urea and sodium bisulphite (Jones & Mecham, 1943). The soluble protein in both these reactions resembles the intact wool in amino-acid composition.

The action of aqueous ammonia on wool oxidized with peracetic acid may well be another example of the same phenomenon. Alexander & Earland (1950b) and Happey (1950) found that the oxidized wool gave an α X-ray diagram, whereas the 'sub-cuticle membrane' prepared from it by treatment with aqueous ammonia had a β X-ray diagram. They considered the 'membrane's' resistance to attack to be due to its β form, where more intermolecular hydrogen bonds were present than in the α form. It is possible, however, that the β form arises during the alkali treatment of the oxidized wool and is not present as such in the original fibre.

The present experiments lend little support to the idea that the membrane forms a particular structural component present as such in the original fibre. The formation of similar structures from chlorinated and 'chlorzymed' (chlorinated and papain-treated) wool

shows that the presence of the 'epi-cuticle' on the fibre is not essential to the isolation of the 'sub-cuticle' membrane (cf. Mercer *et al.* 1949). On the other hand, if the present explanation of the formation of this insoluble residue is correct, we should expect to, and indeed do, obtain a residue from chlorinated and papain-treated wool in the same way as from initially intact wool.

Peracetic acid oxidizes wool more slowly than performic acid, probably due to a slower rate of diffusion into the fibre occasioned by the smaller extent of swelling (cf. Alexander *et al.* 1950; Speakman, 1931). The still slower rate of oxidation by hydrogen peroxide in aqueous solution may similarly be related to the still smaller degree of swelling.

The product of the reaction in aqueous solution embodies oxidation products of the cystine residues at the disulphoxide or sulphinic acid stages (Harris & Smith, 1937; Consden & Gordon, 1950). The peptide material found in solution after the alkaline peroxide treatment of wool probably represents those peptide chains which have been set free from the structure by the dismutation of the residues of disulphoxide in the alkaline solution. The results of the chromatographic analysis of this peptide material after hydrolysis with hydrochloric acid are in accordance with this interpretation. Cysteic acid was shown to be present, but neither the disulphoxide nor the sulphinic acid from cystine-cysteine could be detected.

SUMMARY

1. The cystine of wool is rapidly oxidized by treatment with performic acid. The oxidized wool subsequently dissolves slowly and incompletely in the reagent solution. When wool is oxidized with alkaline hydrogen peroxide a small amount of long-chain peptide material goes into solution.

2. Fibrous proteins partially dissolve in formic acid at room temperature; the extent of solution has been correlated with the molecular configuration of the peptide chains of the protein. The small amount of wool keratin which is dissolved by formic acid probably consists of peptide chains which have been liberated from the structure by the action of weather on the raw wool.

3. These results are interpreted in terms of changes from the α -keratin to the β -keratin configuration of peptide chains present.

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Cytochrome *b* and Bacterial Succinic Dehydrogenase

By A. T. JOHNS

*Plant Chemistry Laboratory, Department of Scientific and Industrial Research,
 Palmerston North, New Zealand*

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Bach, Dixon & Zerfas (1946) pointed out that the dehydrogenases fall into distinct groups according to the intermediate hydrogen acceptors through which they react with oxygen. Succinic dehydrogenase and lactic dehydrogenase of yeast come into the category of 'cytochrome-reducing dehydrogenases' because they readily reduce cytochrome *c*, and do not depend on coenzyme I or II for the reduction of methylene blue (Mb). None of the enzymes of this group of 'cytochrome-reducing dehydrogenases' has been isolated. Bach *et al.* (1946) purified the lactic dehydrogenase of yeast to a considerable extent and found that a new cytochrome, *b*₂, was concentrated in the enzyme fractions. There was an apparent correlation between the strength of the absorption band and the enzyme activity. They presented evidence that cytochrome *b*₂ forms an essential part of the enzyme system, but were not able to determine whether cytochrome *b*₂ is the dehydrogenase itself or an essential intermediate carrier between lactate and methylene blue. They also pointed out that in the heart muscle, cytochrome *b* is intimately associated with succinic dehydrogenase as shown by Keilin & Hartree (1939),

and therefore it is possible that enzymes of this group are of the *b* type.

Ball, Anfinsen & Cooper (1947) state that the function of cytochrome *b* is unknown, and consider that it may be identical with that of succinic dehydrogenase.

In a study of the relationship between the iron enzymes of *Corynebacterium diphtheriae* and diphtheria toxin, Pappenheimer & Hendee (1947) obtained evidence that cytochrome *b* was directly concerned in the oxidation of succinate. They were unable to separate succinic dehydrogenase from cytochrome *b* by differential centrifugation. Pappenheimer & Hendee (1949) made a comparison between the diphtherial succinoxidase system and that of ox-heart muscle, and concluded that succin-dehydrogenase may well be identical with cytochrome *b* both in diphtherial extracts and in ox-heart preparations.

Slater (1949), working with heart-muscle and kidney tissue, found that cytochrome *b* was involved in the reduction of methylene blue by succinate. Slater was not able to decide whether cytochrome *b* is identical with succinic dehydrogenase or whether