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Reactions of β-Ethyl Sulphone Crosslinking Agent with Wool. Part III: Wool Fractionation and Amino Acid Analysis

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ABSTRACT

A study of the effect of the application to wool of the new substantive cross-linking agent, 2-chloro-4, 6-di(-aminobenzene-4-β-sulphato-ethylsulphone)-1,3,5-s-triazine (XLC) is reported, with special reference to determining crosslinking at individual amino acid sites.

Amino acid analysis was used to detect reactions of this compound at specific amino acid residues. Lysine and histidine residues were shown to be important sites of reaction, and indirect evidence was obtained that the compound restricts the degree of thiol–disulphide interchange which occurs when boiling wool under mildly acidic aqueous conditions (pH values 5–6).

NOTATION

Amino acids

aa Amino acid
Ala Alanine
Arg Arginine

* To whom correspondence should be addressed.

Asp	Aspartic acid
Cys	Cystine
Glu	Glutamic acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Tyrosine
Val	Valine
HPLC	High performance liquid chromatography
o.w.w.	on weight of wool
PTC	Phenylthiocarbamyl
XLC	2-chloro-4,6-di(-aminobenzene-4-β-sulphato-ethylsulphone)-1,3,5-*s*-triazine

INTRODUCTION

The application of the trifunctional agent, XLC, to wool has been reported in Parts I and II of this series.[1,2] These studies indicated that crosslinking reactions between XLC and nucleophilic sites in the wool fibre may have occurred, but the techniques employed did not give conclusive evidence for the presence of such crosslinks.

The use of solubility studies in various chemically active solutions to detect crosslinking reactions of XLC with wool is open to question, since the degree of wool dissolution can be affected by modification of charged groups and by the introduction of hydrophobic moieties. The only unambiguous proof that crosslinking occurs is to isolate and identify crosslinked pairs of amino acids from a hydrolysate of the treated wool. Where amino acid derivatives that are unstable to hydrolysis are produced, indirect evidence for crosslinks must be sought.

EXPERIMENTAL

Materials and methods

Materials

The wool used in these studies was the same wool serge fabric described in Parts I and II of this series.[1,2]

The crosslinker XLC was supplied by IWS. All other chemicals were of laboratory reagent grade.

Application of crosslinker
XLC was applied at varying concentrations and pH according to the methods employed in Part II.[2] Its uptake by the fibre following treatment for 1 h at the boil was measured by the methods described previously.[1,2]

Amino acid analysis

Amino acid analysis was performed, after acid hydrolysis of the wool samples, using the Waters Pico Tag System,[3] which involved pre-column derivatisation with phenyl-isothiocyanate (PITC), producing phenylthiocarbamoyl–amino acids which were then chromatographed using reversed-phase HPLC.

Acid hydrolysis

Wool (20 mg) samples were hydrolysed with 5·8 M HCl (5 ml) in sealed tubes under nitrogen for 20 h at 110°C. After hydrolysis, the acid was removed by rotary evaporation at 40°C, the hydrolysates diluted with distilled water and stored at −10°C prior to analysis.

PITC derivatisation of amino acids

An aliquot of the hydrolysate (40–120 μl) was placed in a 50 × 6 mm sample tube and dried down under vacuum. A redrying solution (10–20 μl) consisting of a 2:2:1 mixture (v/v) of ethanol–water–triethylamine was then added and the sample redried.

The derivatisation reagent consisted of a mixture of ethanol–triethylamine–water–phenyl-isothiocyanate (PITC) in a ratio 7:1:1:1 (v/v) and

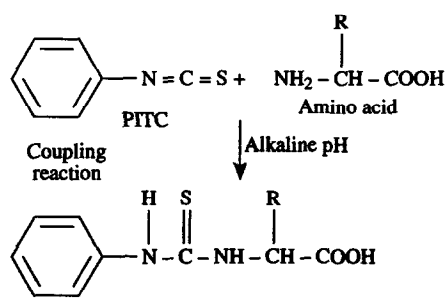


Fig. 1. Formation of PTC–amino acid.

was always freshly prepared. This reagent (10 μl) was added to the redried sample, mixed by vortexing, and the coupling reaction, illustrated in Fig. 1, was allowed to proceed for 20 min at room temperature. PITC and solvents were then removed under high vacuum over 1 h; the sample was then ready for HPLC analysis. Phenyl-isothiocyanate (PITC) reacts with the uncharged α-amino group to form a phenylthiocarbamyl (PTC)-amino acid. The ε-amino group of lysine also reacts with PITC to give a disubstituted product. PTC-amino acids have a broad UV spectrum with λ_{max} of 269 nm and are easily detected with a fixed wavelength detector operating at 254 nm.

Chromatography of PTC-amino acids

Derivatised samples were redissolved, for chromatography, in a loading buffer prepared by dissolving 710 mg of Na_2HPO_4 in 1 litre of distilled water, adjusting the pH to 7·4 with 10% H_3PO_4 and mixing with 50 ml of acetonitrile.

PTC–amino acids were then separated using a Waters Pico Tag System, consisting of two HPLC pumps, a controller and a Waters Pico Tag bonded phase column, 15 cm × 3·8 mm, housed in a column heater at 39°C. A gradient, composed of two eluents, was used for elution. Eluent A was prepared by dissolving sodium acetate trihydrate (19·0 g) in 1 litre of distilled water, adding triethylamine (0·5 ml), titrating to pH 5·7 with glacial acetic acid and finally mixing with acetonitrile (63·8 ml). Eluent B was prepared by mixing acetonitrile (600 ml) with distilled water (400 ml). Both eluents were filtered and kept under a blanket of helium. With the flow rate set at 1 ml/min, the gradient programme started with 10% B traversing in a convex fashion to 51% B in 10 min, followed by a washing step to 100% B. Samples were introduced onto the column with a WISP™ auto injector (Waters Assoc.) using injector volumes in the range 5–20 μl. Column effluent was monitored with a fixed wavelength detector set at 254 nm, whose output signal was connected to a Trivector Trio computing integrator. The chromatographic separation achieved using this system is shown in Fig. 2.

Quantitation of amino acid analysis

To calibrate the system an aliquot (10 μl, 2·5 μmol of each amino acid/ml (1·25 μmol Cys/ml)) of an amino acid standard (Sigma) was derivatised, redissolved in loading buffer (200 μl) and 5 μl (625 pmol of each amino acid (312·5 pmol of Cys)) injected onto the column. A response factor for each amino acid was found by dividing the number of

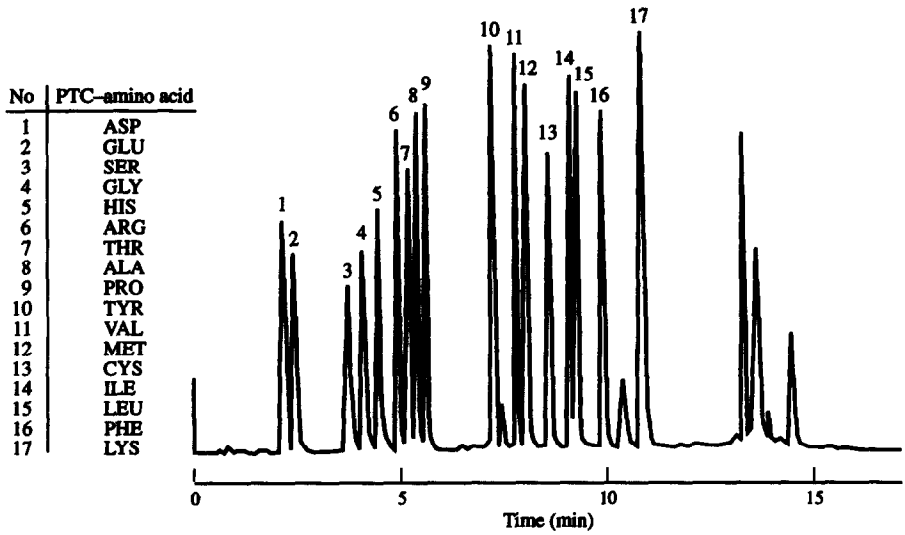


Fig. 2. Separation of phenylthiocarbamyl-amino acid derivatives of an amino acid standard mixture.

moles of the amino acid injected onto the column by the peak area, the latter being calculated by peak integration. For samples of unknowns, the computing integrator then calculated the number of moles of each amino acid injected onto the column, as the product of the computed peak area and response factor.

For wool hydrolysates the concentration of each amino acid was calculated using the following formula:

$$\text{mol of amino acid per gram wool} = \frac{\mu\text{mol on column} \times \text{vol. of hydrolysate} \times \text{vol. of derivatised sample}}{\text{Wool sample wt} \times \text{vol. dried down} \times \text{vol. injected}} \quad (1)$$

with volumes in microlitres and weight in grams.

Determination of cystine

The cystine content of the wool samples was determined by the phosphotungstic acid method using the procedure described by Leach.[4] This colorimetric method involves release of cystine by acid hydrolysis of the protein, followed by oxidative sulphitolysis with sulphite and phosphotungstic acid, producing a blue coloured reduction product:

$$RSSR + HSO_3^- \rightarrow RSH + RSSO_3^-$$

$$2RSH + 2H_6P_2O_8(WO_3)_{18} \rightarrow RSSR + [H_6P_2O_8(WO_3)_{17}]_2W_2O_5 + H_2O$$

This procedure gave the cystine content in μmol/g. The total amino acid (aa) composition of the wool hydrolysates was then reported in mol%, calculated with a computer program using the following formula:

$$\text{mol\% aa} = \frac{\mu\text{mol aa/g}}{\sum \mu\text{mol aa/g}} \times 100 \qquad (2)$$

RESULTS AND DISCUSSION

General

Amino acid analysis was carried out on acid hydrolysates of wool keratin, using a technique that involved pre-column derivatisation of the free amino acid with phenyl-isothiocyanate. The phenylthiocarbamyl–amino acid (PTC–aa) derivatives so formed were resolved by reversed-phase liquid chromatography. The separation achieved with this system, for a standard mixture of amino acid, is shown in Fig. 2. This system was, however, found to be unsuitable for the quantitative analysis of cystine from acid hydrolysates of wool keratin. The reasons for this lay in the ability of the system to resolve PTC-*meso*-cystine, a diastereoisomer of PTC-L-cystine formed by derivatisation of *meso*-cystine. The latter is known to arise from the partial racemisation of L-cystine which occurs during acid hydrolysis.[5] Racemisation is normally not detected by conventional ion-exchange chromatography, as the resultant D-, L-, and *meso* diastereoisomeric forms of cystine are eluted as a single peak. Derivatisation with PITC obviously created a chemical species in PTC-*meso*-cystine that had sufficiently different properties to its stereoisomers to permit its resolution under RPLC. It has been reported that PTC-*meso*-cystine elutes just prior to PTC-L-cystine. In the present work a substantial peak between PTC-L-methionine and PTC-L-cystine was observed in all wool hydrolysates, whose area was greatest when the apparent concentration of cystine was lowest. The result was consistent with the racemisation of L-cystine during the acid hydrolysis of wool keratin.

The recovery of *meso*-cystine plus cystine was found to be usually 70–80%. The fact that the total yields of cystine still did not approach 100%, even when allowance was made for *meso*-cystine, meant that the estimation of cystine in wool hydrolysates was not possible with this technique. An attempt was made to determine cystine as cysteic acid using the oxidation procedure of Moor.[6] Following derivatisation of the oxidised hydrolysate with PITC, chromatographic analysis (HPLC)

showed that the peaks for PTC-L-cystine and that thought to be PTC-*meso*-cystine, were absent. They were replaced by one larger peak that corresponded to PTC-cysteic acid, which eluted just before PTC-aspartic acid. This technique was, however, impractical for the determination of cystine in all the samples. Consequently, the colorimetric procedure of Shinohara[7] was used to obtain a value for cystine in wool. This technique gave reasonably consistent results, although the contribution of the small amount of cysteine present in wool (20–40 μmol/g)[5] was not measured. This could not be assessed due to the large number of samples and the unreliable nature of such estimates on hydrolysed material. The pre-column derivatisation technique was found to be satisfactory for the analysis of the other amino acids present in the standard.

The sites of reaction of XLC

The sites where reactive dyes covalently bond with wool keratin have been the subject of many studies with various different reactive systems. These have been reviewed,[8,9] and, in general, the main sites of reaction were the cysteine thiol, lysine ε-amino, histidine secondary amino and *N*-terminal residues of wool, regardless of the type of reactive system used. The hydroxyl groups of serine, threonine and tyrosine have been shown to be much less reactive than the foregoing nucleophiles[10] and generally only react at substantially higher pH values than those utilised in the reactive dyeing of wool.

The sites of reaction could be directly determined by amino acid analysis if the various bonds between XLC and the amino acid residues were stable in the conditions used to hydrolyse the protein. The vinyl sulphone group forms a carbon–nitrogen bond with lysine or histidine side chains which was stable to acid hydrolysis;[11] it would also form an acid stable carbon–sulphur bond by reaction with cysteine. Therefore reaction of the β-sulphato-ethylsulphone groups of XLC with these residues should, in principle, be detectable. In contrast, the triazine ring was ruptured by strong acid at elevated temperatures[12] and any crosslink formed between amino acid residues would therefore be cleaved during acid hydrolysis. Whether or not an amino acid residue that had reacted with the monochlorotriazine group would be released in a modified form that would not be detected by amino acid analysis, or as the free amino acid, is unknown. Due to these latter complications, it was decided to assess the wool amino acid residues involved in reaction with XLC by an indirect method, measuring the total individual amino acid concentrations before and after reaction.

The possibility was considered that unreacted β-sulphato-ethylsulphone

TABLE 1
Amino Acid Composition of Wool Treated with XLC at pH 3·0

Amino acid residue	*Blank treated*	*XLC applied (μmol/g), pH 3·0*		
		15	*40*	*140*
Aspartic acid	6·08	6·17	6·20	6·06
Glutamic acid	11·62	11·70	11·78	11·52
Serine	10·06	10·16	10·40	10·06
Glycine	8·36	8·49	8·75	8·50
Histidine	0·80	0·82	0·77	0·78
Arginine	6·52	6·36	6·11	6·47
Threonine	6·25	6·33	6·30	6·32
Alanine	5·36	5·45	5·49	5·24
Proline	7·52	7·33	7·42	7·54
Tyrosine	3·63	3·59	3·45	3·60
Valine	5·22	5·18	5·11	5·05
Methionine	0·46	0·51	0·46	0·47
1/2 Cystine	12·66	13·01	13·20	13·28
Isoleucine	2·82	2·73	2·68	2·73
Leucine	7·33	7·20	7·12	7·26
Phenylalanine	2·69	2·57	2·50	2·65
Lysine	2·62	2·40	2·26	2·47

Values given in mol% ± 8%.

groups still present in the fibre react further with the wool during acid hydrolysis. This was thought most unlikely however, as these groups rapidly hydrolyse to the hydroxyethyl sulphone on treatment with strong hot acid. Also all the potential nucleophiles in wool would be protonated at this low pH value and reaction would not be favoured. To summarise, amino acid analysis of XLC treated wool should be capable of indicating the nature of the reaction of the β-sulphato-ethylsulphone group with wool. Whether or not such analysis could also detect reaction of the monochlorotriazine group was unknown, due to uncertainties about the stabilities of the bonds formed between the amino acid residues and this reactive group.

The results for the amino acid analyses of wool treated with XLC in the pH range 3–6 are given in Tables 1–4. They are reported in mol%; that is, the number of moles of a particular amino acid per 100 moles of amino acids. These units were adopted so that the weight gains of the wool samples treated with XLC up to 10% (o.w.w.) had no effect on the amino acid concentration values reported.

Analysis of the data in Tables 1–4, using the blank treated values for comparison, showed a consistent trend for lysine and histidine reaction

TABLE 2
Amino Acid Composition of Wool Treated with XLC at pH 4·0

Amino acid residue	*Blank treated*	*XLC applied (μmol/g), pH 4·0*			
		15	*34*	*66*	*105*
Aspartic acid	6·48	6·31	6·27	6·09	6·41
Glutamic acid	12·08	11·59	11·46	11·25	11·85
Serine	10·16	9·95	10·09	9·88	10·18
Glycine	8·21	8·29	8·21	8·28	8·42
Histidine	0·74	0·80	0·75	0·73	0·68
Arginine	6·40	6·32	6·24	5·85	6·09
Threonine	6·10	6·11	5·96	6·02	5·63
Alanine	5·12	5·26	5·20	5·19	5·32
Proline	7·18	7·24	7·53	7·38	7·43
Tyrosine	3·69	3·77	3·69	3·81	3·58
Valine	4·92	5·05	5·06	5·34	5·10
Methionine	0·53	0·50	0·42	0·50	0·46
1/2 Cystine	12·86	12·55	13·01	12·78	13·66
Isoleucine	2·70	2·83	2·82	2·99	2·84
Leucine	7·15	7·58	7·51	7·83	7·23
Phenylalanine	2·75	2·90	2·92	3·09	2·84
Lysine	2·92	2·95	2·86	2·99	2·26

Values given in mol% ± 8%.

in the XLC treated wool samples. There was a consistent fall in the concentrations of these two basic amino acids which correlated with a rise in both the quantity of XLC applied, and the application pH used. Reaction of any of the other amino acids was, in general, not observed. None of the other amino acids showed a consistent reaction that could be correlated with the amount of XLC applied, or the application pH. The other amino acids monitored that could possibly have reacted with XLC were the hydroxyl amino acids serine, threonine and tyrosine, and also the highly basic amino acid, arginine. If any of the foregoing amino acid residues had reacted, they did so to a very limited extent (i.e. less than 8%). Any reaction at a low level could not be detected, as any slight changes were irresolvable from the variations in the amino acid content of the untreated samples, and experimental error.

The amino acid compositions of wool samples treated with XLC at pH 3 (Table 1) exhibited negligible changes when up to 140 μmol XLC/g of wool were applied. The only exception was lysine, whose concentration fell by a maximum of 14% at this pH. A fall of 4% in the concentration of histidine at pH 3 was within experimental error. The results for the reaction of lysine at pH 4 (Table 2) were erratic; one result, for 105 μmol

TABLE 3
Amino Acid Composition of Wool Treated with XLC at pH 5·0

Amino acid residue	*Blank treated*	*XLC applied (μmol/g), pH 5·0*			
		15	*34*	*55*	*104*
Aspartic acid	6·32	6·61	6·30	6·20	6·41
Glutamic acid	11·45	12·30	11·95	13·38	13·61
Serine	9·57	10·02	9·37	10·07	9·89
Glycine	8·48	8·47	8·50	8·48	8·58
Histidine	0·81	0·63	0·67	0·59	0·52
Arginine	7·00	6·37	6·71	5·76	5·83
Threonine	5·36	5·50	5·59	5·58	5·55
Alanine	5·31	5·25	5·40	5·38	5·43
Proline	6·88	7·01	6·92	6·65	6·62
Tyrosine	3·66	3·77	3·42	3·28	3·16
Valine	5·64	5·61	5·73	5·31	5·50
Methionine	0·40	0·40	0·42	0·48	0·46
1/2 Cystine	12·50	12·04	12·99	14·79	14·83
Isoleucine	3·21	3·06	3·36	2·85	2·81
Leucine	7·82	7·71	7·66	7·00	6·90
Phenylalanine	2·80	2·74	2·80	2·48	2·40
Lysine	2·79	2·51	2·22	1·73	1·49

Values given in mol% ± 8%.

XLC/g of wool, indicated a reaction of 23% of lysine, whereas the other results exhibited very little reaction of lysine at pH 4. Histidine appeared to undergo more reaction than lysine at this pH; at least 8% and possibly 15% (if the blank was falsely low and taken as 0·8 mol%) of histidine residues reacting when 105 μmol XLC/g of wool was applied. Overall, there was still very little reaction of the compound with wool at pH 4 which agreed with the rather low levels of crosslinking previously found for these samples.[2]

The results of amino acid composition for the pH 5 treated samples (Table 3) were more consistent than those of the previous two pH values, and demonstrated considerable reaction of both lysine and histidine with XLC. These reactions reached values of 47% for lysine and 36% for histidine with XLC when 104 μmol XLC/g were applied to wool at this pH. These values represent unambiguous proof that XLC reacts with wool, and supports the previous observations of extensive crosslinking of XLC treated wool at pH 5 and above.

The greatest degree of reaction of XLC with lysine and histidine occurred at pH 6 (Table 4), where 52% of lysine residues and 47% of histidine residues reacted when 118 μmol XLC/g of wool was applied.

TABLE 4
Amino Acid Composition of Wool Treated with XLC at pH 6·0

Amino acid residue	*Blank treated*	*XLC applied (μmol/g), pH 6·0*			
		12	*38*	*61*	*118*
Aspartic acid	6·26	6·21	6·44	6·43	6·18
Glutamic acid	11·81	11·65	11·76	11·98	11·77
Serine	9·96	9·91	10·27	10·10	10·06
Glycine	8·21	8·32	8·35	8·28	8·87
Histidine	0·79	0·66	0·56	0·54	0·42
Arginine	7·16	6·39	6·45	6·33	6·73
Threonine	5·80	5·70	5·83	5·88	6·06
Alanine	5·43	5·47	5·52	5·38	5·27
Proline	6·76	6·95	6·84	6·74	6·66
Tyrosine	3·71	3·47	3·46	3·26	3·40
Valine	5·19	5·20	5·26	5·06	5·05
Methionine	0·42	0·45	0·45	0·49	0·49
1/2 Cystine	12·30	13·46	13·84	14·65	15·37
Isoleucine	2·86	3·25	3·09	2·75	2·64
Leucine	7·65	7·65	7·42	7·12	7·15
Phenylalanine	2·86	2·70	2·60	3·37	2·51
Lysine	2·81	2·55	1·87	1·65	1·36

Values given in mol% ± 8%.

Considering the short time in which these reactions occurred (1 h at 100°C), the reaction at this pH and also at pH 5 was fairly rapid. Undoubtedly, with a longer application time and more compound applied, higher degrees of modification of these two amino acids could be achieved.

There are two main reasons why XLC reacts more extensively with wool with increasing pH. First, increasing the pH promotes the activation of XLC to the vinyl sulphone form and second, increasing the pH raises the nucleophilicity of the wool fibre.

The monochlorotriazine group reacts directly by a nucleophilic substitution mechanism and thus requires no activation, unlike the β-sulphatoethylsulphone group, which forms the vinyl sulphone reactive species by a β-elimination reaction which is promoted by increasing temperature and pH.

$$\underset{\text{SES}}{-SO_2CH_2CH_2OSO_3Na} \xrightarrow{NaOH} \underset{\text{VS}}{-SO_2CH{=}CH_2} + Na_2SO_4 + H_2O$$

Osterloh[13] found pH 6 to be the optimum for formation of the vinyl sulphone dyes from their parent β-sulphatoethylsulphone (Remazol) dyes in boiling aqueous solutions according to the above reaction. Zahn and

TABLE 5
Dissociation Constant (pK_a) of the Functional Groups in Wool at 25°C

Amino acid	*Functional group*	*pK_a at 25°C*
Histidine	—NH—	6·0
α-Amino groups	$—NH_2$	7·5–8·5
Cysteine	—SH	7·5
Lysine ε-amino	$—NH_2$	10·8
Tyrosine	—OH	11·0
Arginine	guanidino	approx. 12·5
Serine	—OH	approx. 13·6

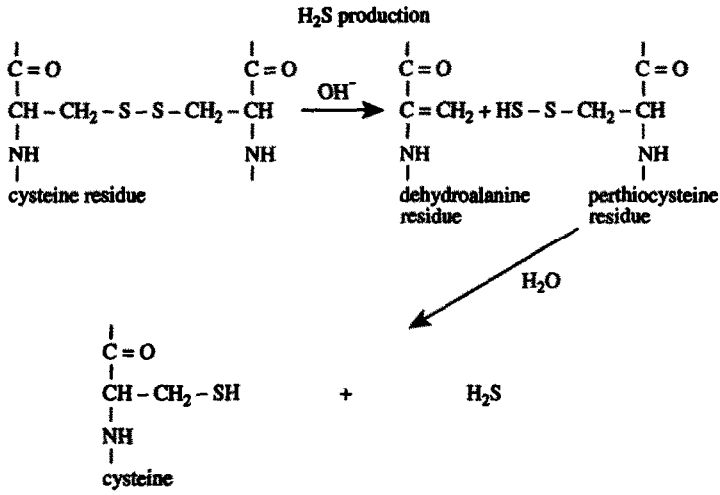
Rouette[14] also found pH 6 gave the greatest fixation of Remazol Brilliant Blue R (as SES type dye) on wool. These results support the observations made in the present work that activation of XLC to the vinyl sulphone form in boiling solutions was favoured as the pH approached neutrality.

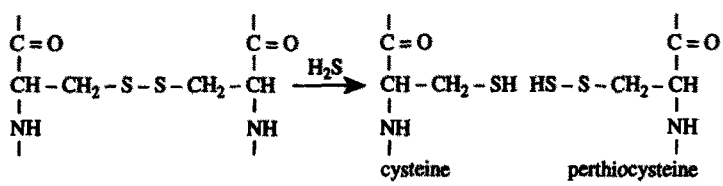
The parameters that affect the reactivity of the functional groups of wool keratin towards reactive groups such as those present in XLC have been summarised by Baumann.[15] One parameter of special importance is the pK_a value of the functional groups, which corresponds to the pH value at which half of the functional groups are protonated. The pK_a values of the important functional groups in wool[16] are given in Table 5. The unprotonated forms of these functional groups are the nucleophiles which react with vinyl sulphone and monochlorotriazine groups.

Altenhofen *et al.*,[17] have shown that these pK_a values are, on average, 2 units lower (only 1 unit lower for Tyr) at 100°C. A change in the pH of plus or minus one pH unit had the effect of increasing or decreasing the number of reactive species by a factor of 10. Increasing the pH of application of XLC to wool thus effectively increased the number of reactive nucleophilic amino acid residues. At the boil, over the pH range 3–6, the only groups present in significant amounts in the reactive form would be histidine secondary amine, cysteine thiol and the α-amino groups. Reaction of XLC with the latter two groupings was not measured; however, they were initially present in wool only in very small quantities (20–40 μmol cysteine/g and 10 μmol α-amino/g) and in the case of the α-amino residue would not therefore represent a major site of reaction for XLC.

Even though cysteine was initially present in very low amounts, previous work[9] has clearly shown that it is a significant site for reactive dye fixation; this may only be explained by hot aqueous conditions promoting β-elimination reactions, thus leading to the plentiful supply of cysteine from cystine. This cystine β-elimination reaction is also important in wool setting. Steenken and Zahn[18] have also stressed the promotional

role of hydrogen sulphide produced in the above reaction on subsequent thiol-disulphide interchange reactions:





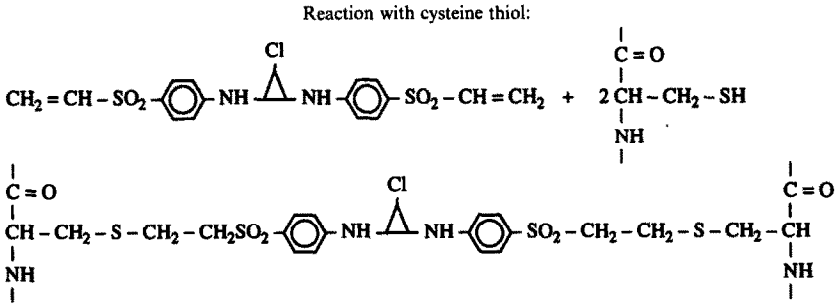
Scheme 1

Wool setting reactions are implicated in the damage which occurs in wool dyeing, and anti-setting agents which block cysteine and efficiently remove hydrogen sulphide from the system have been suggested as fibre protective agents.[19–21]

The initial reason for developing XLC was precisely in this context, i.e. as a fibre protective agent for preventing damage in dyeing.[21] Evidence that it is effective in suppressing the thiol-disulphide interchange reaction is obtained from a study of the cystine contents in wool treated with increasing amounts of XLC at different pH values (Tables 1–4); for convenience, these results are presented graphically in Fig. 3. A study of this figure reveals that, more especially at pH5 and 6, increasing the concentration of XLC applied to the wool increases the level of intact cystine, confirming that XLC somehow inhibits the hydrolytic rupture of cystine disulphide residues, thus reducing the degree of setting. It is postulated that XLC

reacts rapidly with both cysteine thiol, and with hydrogen sulphide (or hydrosulphide anion), to prevent their further involvement with cystine disulphide breakdown. These reactions may be summarised:

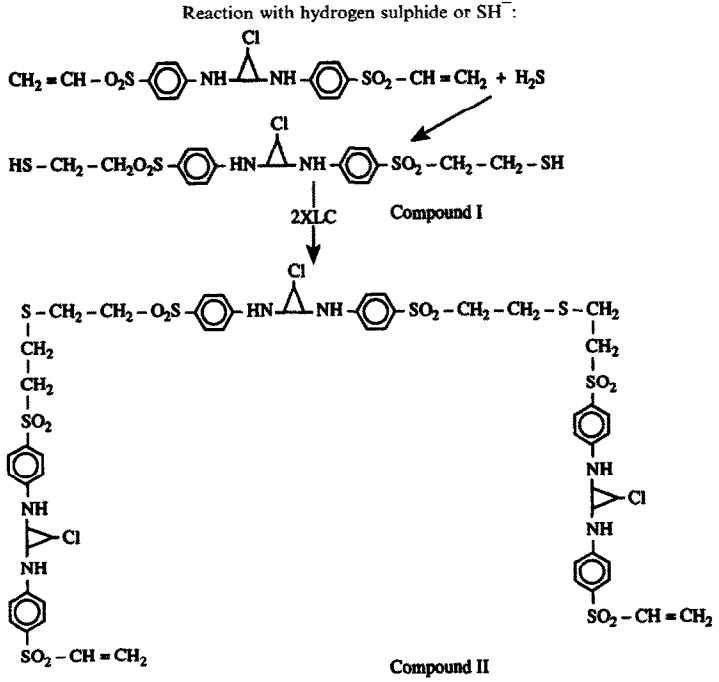
Reaction with cysteine thiol:



Scheme 2

where Δ = *s*-triazine. The thioether linkages formed are extremely stable and will contribute to crosslinking.

Reaction with hydrogen sulphide or SH^-:



The above reactions are confidently proposed, since analogous thioether species, such as **II**, have been identified by Lewis and Smith[22] following wool dyeing with monofunctional vinyl sulphone dyes. Clearly, if excess XLC was not present species **I** would predominate, and being

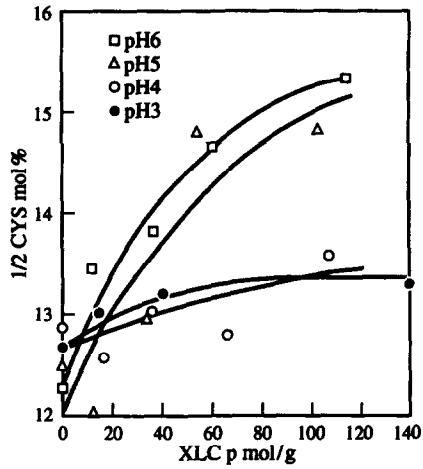


Fig. 3. Effect of pH and XLC concentration on cystine content.

thiol terminated would take part in thiol–disulphide setting reactions. This could explain the relative inefficiencies of XLC in preventing cystine breakdown at the lower pH values (3 and 4), since at these pH values, XLC remains largely in the sulphato-ethyl sulphone form, and only a small amount of reactive vinyl sulphone would be available to participate in these thiol–H_2S reactions.

In these discussions, the role of the monochloro-*s*-triazinyl part of XLC has not been considered. Work with monochloro-*s*-triazinyl dyes[12] has indicated that thiol substituted triazines undergo further nucleophilic substitution reactions with amino nucleophiles to reform the original thiol, together with the corresponding amino substituted triazine; this behaviour is reminiscent of the well-known reactivity of acyl thiols towards amines. Thus, under acidic conditions, XLC is likely to possess one main reactive group, viz. the chloro-triazine, which will initially react readily with H_2S and cysteine, but on prolonged boiling, amino substitution from histidine or lysine will occur, liberating H_2S and cysteine, which will then be available for activating thiol–disulphide interchange. This could explain the reduced cystine protecting effect of XLC observed at pH 3 and 4 in Fig. 3, in comparison to the results at pH 5 and 6 where vinyl sulphone reaction to form stable thioethers is feasible. These explanations are supported by the work of Lewis,[23] who examined the fibre-protection properties of chloro-triazine, α-bromoacrylamido and vinyl sulphone based reactive dyes; the chloro-triazines were noticeably less effective than the α-bromo-acrylamido and vinyl sulphone derivatives, these latter two types forming stable thioethers.

Despite its moderately high pK_a, the lysine ε-amino group still represents

a major reaction site, due to its high nucleophilicity. The lysine ε-amino group is 10 times more nucleophilic than the histidine seondary amino group, and lysine is present in wool at more than three times the concentration of histidine; taking these factors into consideration, it was thus not unexpected that amino acid analysis showed substantial reaction of XLC with these two residues. The lack of reaction of XLC with tyrosine, serine and threonine could be explained by their high pK_a values and the low nucleophilicity of the hydroxyl group. The highly basic arginine residue also had too high a pK_a to permit reaction with XLC in the range pH 3–6.

Evidence for crosslinking from amino acid analysis

Indirect evidence for the crosslinking of wool may be obtained from the extent of reaction of a multifunctional compound. If x moles of a multifunctional compound reacted with less than x moles of amino acid residues in the protein, then crosslinks must be formed. These crosslinks are not necessarily interchenic; however indirect evidence for this may be obtained by other tests.

The apparent functionality of the compound may be defined as the number of moles of amino acids that reacted, divided by the number of moles of the compound applied. The concentrations of lysine and histidine that had reacted, expressed in μmol/g protein and the apparent functionalities of XLC applied to wool at pH 5 and pH 6 were calculated using the following equations and are given in Table 6.

$$\mu\text{mol aa reacted/g of protein} = \mu\text{mol aa/g in blank}\left(1 - \frac{(\text{mol\% of aa in treated sample})}{(\text{mol\% of aa in blank sample})}\right) \quad (7)$$

$$\text{Apparent functionality} = \frac{\mu\text{mol aa/g reacted}}{\mu\text{mol\% XLC applied/g}} \quad (8)$$

The functionality data in Table 6 indicate that XLC reacted trifunctionally at low concentrations. Since these data were derived from amino acid hydrolysates, they indicate that both the vinyl sulphone and monochlorotriazine groups in XLC reacted with modified lysine and histidine residues in wool, producing derivatives that did not yield the free amino acids on acid hydrolysis. For 12·6 μmol XLC per gram of wool, applied at pH 5, the histidine value was much lower than expected from the trend shown by the other samples at this pH (see Table 3). As was stated earlier, the amino acid analyses of samples treated with XLC at low levels were more prone to errors. The apparent functionality of 3·49 that this

TABLE 6
The Apparent Functionality of the XLC Wool Reaction at pH 5 and 6

Application pH	*XLC applied μmol/g*	*Lys + His reacted μmol/g*	*Apparent functionality of XLC*
5	12·6	43·9	3·49
5	34·3	68·2	1·99
5	54·9	123·0	2·24
5	103·9	157·3	1·51
6	12·5	37·4	3·00
6	37·9	112·3	2·97
6	60·7	135·3	2·23
6	118·1	174·6	1·48

For pH 5 blank His = 80·5 and Lys = 275·4 μmol/g.
For pH 6 blank His = 75·9 and Lys = 269·6 μmol/g.

produced was just within experimental error. The functionality of XLC decreased with increasing amounts of XLC applied, as would be expected on a probability basis. However, the functionality was always greater than about 1·5, even at >100 μmol XLC applied/g wool, and this provided strong evidence for extensive crosslinking of wool by XLC when applied at pH 5 and pH 6.

It can thus be postulated that the following crosslinks are highly likely to be present in wool treated with XLC:

Lys–XLC–Lys
His–XLC–Lys
His–XLC–His

Low concentrations of crosslinks of the type:

Cys–XLC–Cys
Cys–XLC–Lys
Cys–XLC–His

cannot be ruled out.

CONCLUSIONS

The application to wool of 2-chloro-4,6-di(-amino benzene-4-β-sulphato-ethylsulphone)-1,3,5-*s*-triazine (XLC) has been studied. Initial work had indicated that extensive crosslinking reactions occurred within the fibre when XLC was applied from boiling baths at pH 5 and 6 using short

application times. Amino acid analysis has been used to obtain a qualitative and quantitative picture of the reaction with wool residues. Lysine and histidine reacted extensively, especially at the higher pH values studied. The general indication is that both lysine and histidine side chains are bridged by the compound; the nature of the crosslinks may be symmetrical, such as lysine–XLC–lysine or histidine–XLC–histidine derivatives, or it may be possible that asymmetrical lysine–XLX–histidine crosslinks may be formed. The involvement of cysteine thiol groups in crosslinking reactions cannot be ruled out, but further work is needed to clarify this area.

Both the XLC and the amino acid group are activated at higher pH, with the vinyl sulphone and monochlorotriazine groups being the reactive species. The reaction was trifunctional at low XLC concentrations, but became bifunctional as the concentration was increased.

Clear evidence has emerged that XLC interferes with the setting (thiol–disulphide interchange) reactions, since the amounts of intact cystine recovered from acid hydrolysates are significantly greater in those samples boiled with XLC than in the blank treated samples.

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