I. 4. THE VARYING REACTIVITY OF THE CYSTINE OF WOOL

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I. Introduction

Middlebrook and Phillips published a paper in 1942 in which they showed that the disulfide bonds of wool displayed marked differences in chemical reactivity. They suggested that the combined cystine of wool could be considered as behaving as two distinct fractions (A + B) and (C + D) of very differing reactivity and that each of these fractions in turn could be considered as being made up of two subfractions (A and B, and C and D respectively) which were much more similar in their behavior. The purpose of this paper is to review some of this earlier work in the light of more recent knowledge of wool structure. Additionally it was found possible to reinterpret some of the data and this reinterpretation has confirmed and extended some of the earlier conclusions.

II. The (A + B) Fraction

The experimental basis of the subdivision of wool cystine into different fractions by Middlebrook and Phillips (*1*) came from a study of the interaction of wool and bisulfite solutions. Elsworth and Phillips (*2*) have earlier produced very convincing evidence that the reaction formulated by Clarke (*3*) for the action of sulfite on cystine applied also to wool viz:

$$WSSW' + NaHSO_3 \rightleftharpoons WSH + W'SSO_3Na \qquad (1)$$

On acid hydrolysis the *S*-cysteinesulfonate breaks down to give a further

molecule of thiol

$$W'SSO_3Na + H_2O \xrightarrow{HCl} W'SH + NaHSO_4 \quad (2)$$

Equation 1 thus represents the reaction with intact wool, but after hydrolysis of the wool the over-all reaction can be formulated as the sum of Eqs. 1 and 2.

$$WSSW' + NaHSO_3 + H_2O \rightarrow WSH + W'SH + NaHSO_4 \quad (3)$$

Since all analyses were carried out on hydrolyzates of wool Eq. 3 represents the reaction from an analytical standpoint and is formally analogous to a reduction.

The reaction of wool with bisulfite differs markedly from that of cystine and other simple disulfides (*4*) in that even under optimum conditions it cannot be induced to go to completion at room temperature, and only 50–60% of the cystine can be made to react. The upper curve in Fig. 1

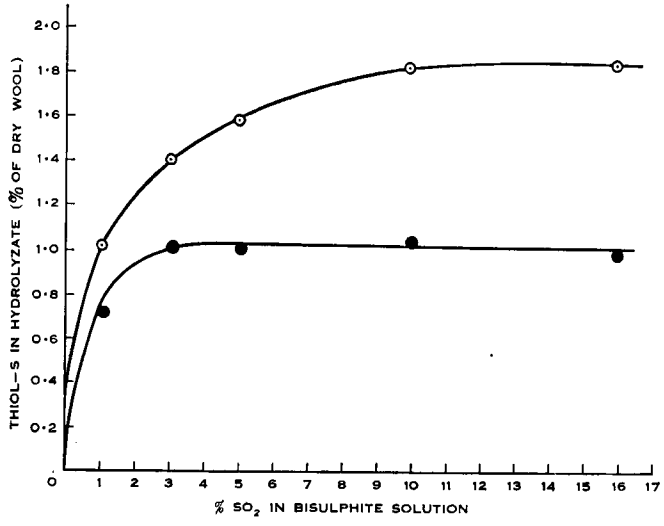

FIG. 1. KEY: ⊙——⊙, unrinsed bisulfited wool; ●——●, rinsed bisulfited wool. The effect of bisulfite concentration on the extent of the reaction with wool. (Reproduced with permission from *Biochem. J.*)

(taken from the paper by Middlebrook and Phillips) shows the relationship between the concentration of sodium bisulfite and the thiol-S in the hydrolyzate of the wool after reaction for 18 hours in the bisulfite solution. Fraction (A + B) of the wool cystine was defined by Middlebrook and Phillips as the maximum amount of cystine which could react with sodium bisulfite, according to Eq. 1 and from Fig. 1, can accordingly be assumed to amount to 1.8% (compared to a total cystine-S of 3.6%).

Various lines of evidence have been quoted to show that the (A + B) fraction has a real significance and represents a division of the cystine into fractions of differing reactivity rather than representing an equilibrium value. Thus the limit of the reaction with bisulfite (1.8% S) is also the limit for the extent of reduction of the wool by various reducing agents at pH 5. Both thioglycolate (1) and mercaptoethanol (*5*) reduce the cystine of wool to the same extent as bisulfite solutions, and wool reduced first with thioglycolate at pH 5 does not react further with bisulfite solutions (*1*). Moreover, if the reaction with bisulfite is carried out in the presence of methylating agents, *S*-methylcysteine residues are formed in the wool instead of cysteine residues (*6*). The reaction then becomes irreversible but Blackburn and co-workers found no evidence that the reaction proceeded beyond the (A + B) fraction. However there was definite evidence that the (C + D) fraction reacts to some extent under the prolonged conditions of bisulfite and methylation treatment used, but the reaction followed a different chemical pathway and led to unidentified products.

This same conclusion can actually be derived from the data in Fig. 1. From Eq. 1

$$K = \frac{[WSH][W'SSO_3Na]}{[WSSW'][NaHSO_3]}$$

and since

$$[WSH] = [W'SSO_3Na]$$

$$K = \frac{[WSH]^2}{[WSSW'][NaHSO_3]} \tag{4}$$

The data embodied in Fig. 1 can be used in conjunction with Eq. 4 to derive a value for [WSSW'], i.e., the total amount of cystine-S of the wool potentially capable of reacting with sodium bisulfite under these conditions. Whilst the mathematical form of this equation does not lend itself to an estimate of [WSSW'] to which standard errors can readily be fitted, it becomes obvious on substituting the experimental data that certainly no more than 2.2% cystine-S and probably not more than 2.0% cystine-S is involved in the reaction with bisulfite. From a chemical standpoint the evidence is thus quite clear and straightforward that some of the cystine [i.e., the (A + B) fraction] of wool reacts with bisulfite solutions at pH 5 to give thiol and *S*-cysteinesulfonate groups while the remainder, the (C + D) fraction, is inert. The observation by Farnworth (*7*) that some of the initially inert (C + D) fraction can be induced to react in chemically modified wools does not affect this conclusion.

III. Subfractions A and B

The evidence on which Middlebrook and Phillips divided the (A + B) fraction into subfractions A and B was based on the ease of reversal of the bisulfite reaction (Eq. 1) on washing the bisulfited wool with water. The upper curve in Fig. 2 shows the thiol content of fully bisulfited wools

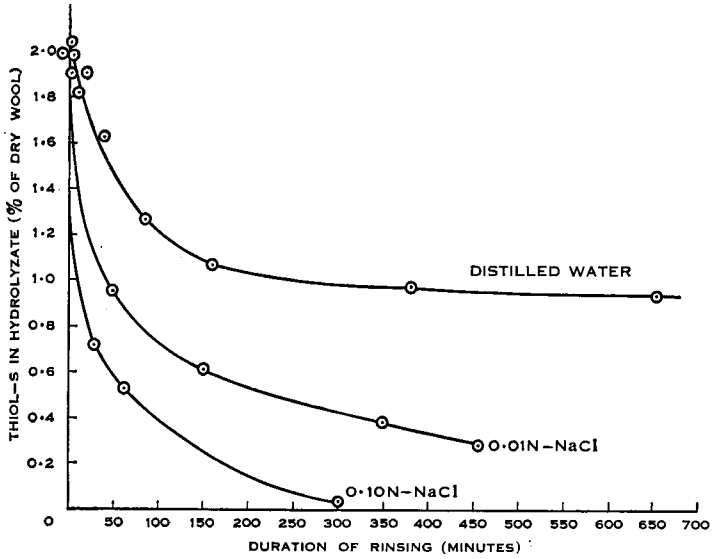

FIG. 2. The influence of salt concentration of the rinsing solution on the magnitude of subfractions A and B. (Reproduced with permission from *J. Soc. Dyers Colourists.*)

which had been subsequently soaked in distilled water for the times stated. The loss of thiol-S on continued rinsing was compensated for in each case by an equivalent increase in disulfide-S, showing that a reversal of the original reaction with bisulfite was occurring. Consideration of the curve of Fig. 2 suggests that the reversal only goes part way and that reversal of the remainder is extremely slow. Middlebrook and Phillips designated the readily reversible fraction, A, and the fraction stable to rinsing, B. The lower curve of Fig. 1 shows how subfraction B varies with bisulfite concentration.

This subdivision has been criticized as being vague and indefinite, and it has been pointed out (*7*) that the precise values found experimentally for subfraction B depend very much on the conditions of rinsing. An extreme example of this is shown in Fig. 2 which is from a paper by Carter *et al.* (*8*) and which compares the effect of washing bisulfited wool in distilled water and dilute salt solutions. Obviously very differing values for

subfraction B would be obtained as a result of washing in dilute salt solutions compared with distilled water. This however, is merely looking at the data from an equilibrium standpoint. If we replot the data for the NaCl solutions of Fig. 2 as for a first order reaction we get the curves shown in Fig. 3. These quite obviously do not fit a simple first order plot but are

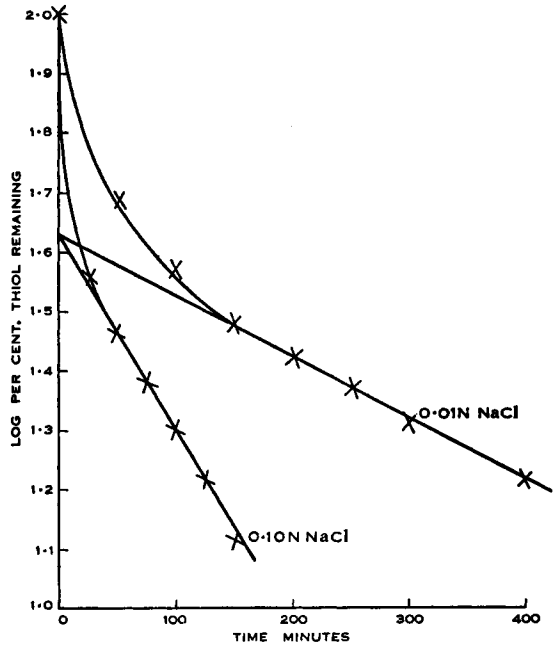

FIG. 3. The data of Fig. 2 replotted as for a first order reaction.

typical of the curves obtained for two first order reactions occurring simultaneously. Analyzed in this way the curves can be very closely approximated to

$$\log (SH_t/SH_o) = Ae^{-k_1 t} + Be^{-k_2 t}$$

where SH_t = % thiol-S remaining in the wool after rinsing for time, t; SH_o = % thiol-S initially present in wool, i.e., fraction (A + B); A and B refer to the magnitudes of subfractions A and B, and k_1 and k_2 are the rate constants for the reaction for subfractions A and B respectively.

From Fig. 3 it appears that the data on rinsing bisulfited wool in sodium chloride can be explained on the basis of subfraction A = 1.03% S and subfraction B = 0.81% S reacting at differing rates. Only the rates and not the magnitude of the fractions are dependent on the sodium chloride concentration. The curve for the wool rinsed in distilled water

can be approximated very closely to a simple first order reaction reaching completion at final SH = 0.8%. Thus the data agree well with the assumption that fraction (A + B) of the cystine is made up of two subfractions of differing stability. In particular there is no need to postulate a whole range of slightly differing reactivities as has been suggested (*9*).

Another set of observations which have never been completely reconciled with the concept of definite A and B subfractions is illustrated in Fig. 4, again taken from the paper by Carter *et al.* (*8*). This shows the

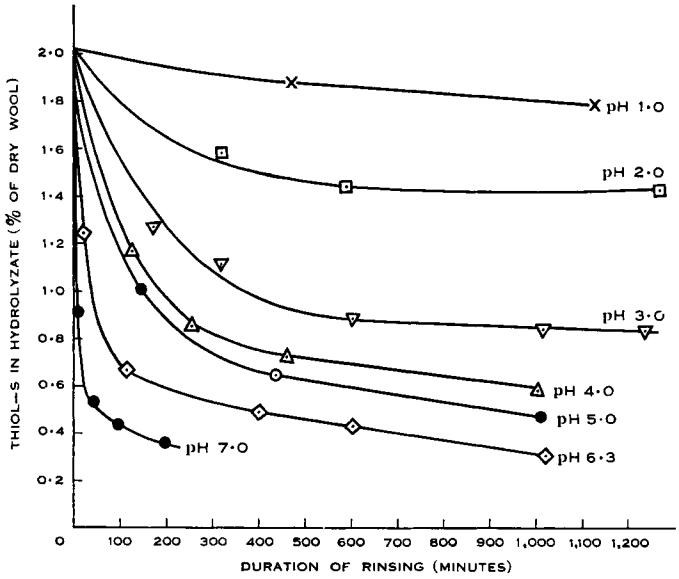

FIG. 4. The influence of the pH of the rinsing solution on the magnitude of subfractions A and B. (Reproduced with permission from *J. Soc. Dyers Colourists.*)

effect of varying the pH of rinsing the bisulfited wool on the rate of reversal of the bisulfite reaction. From these figures it is obvious that the size of subfraction B is dependent on the pH at which the wool is rinsed. If we use the same technique as was used in the previous case and plot the results as for a first order reaction we get Fig. 5. Again it appears that the data can be fitted as the sum of two first order reactions and we can arrive at an estimate of subfraction B for each pH of rinsing. Fig. 6 shows the way in which these values for subfraction B vary with the pH; for convenience the values have been expressed as a fraction of the total thiol. Consideration of this curve suggests that from pH 4 to 5.5, i.e., the pH of water rinsing, the subfraction B is a fairly constant fraction of the total (A + B) fraction. This evidently corresponds to what we may call the

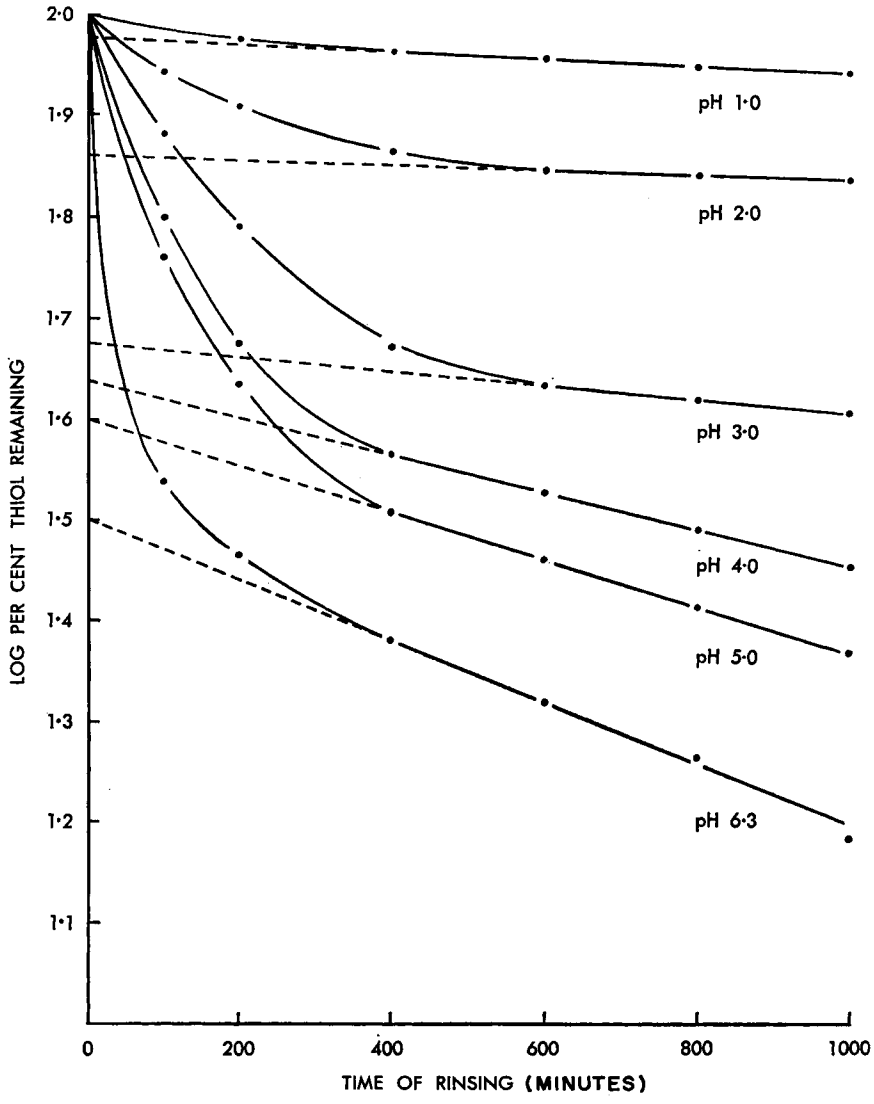

FIG. 5. The data of Fig. 4 replotted as for a first order reaction.

"constitutional" subfraction B. The most striking feature of the curve, however, is its sigmoid form at pH values below 4. This obviously approximates very closely to that of a titration curve of a group with a pK $\doteq$ 2. The simplest interpretation of this result is that the cystine designated subfraction A by Middlebrook and Phillips owes its special reactivity to ionized carboxyl groups in its vicinity and when these are converted to

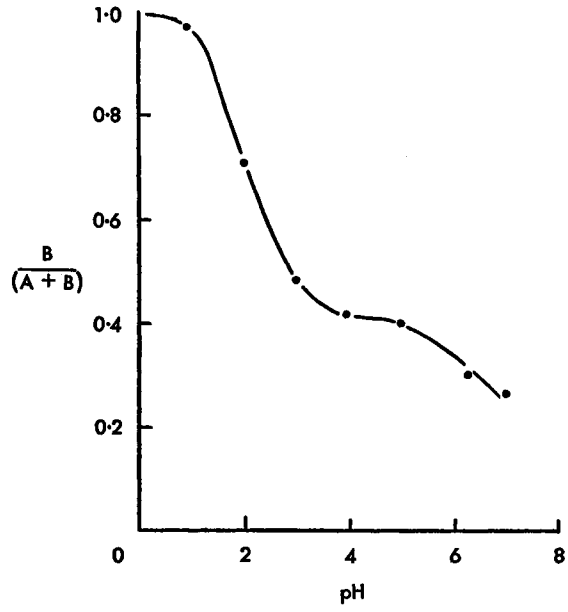

FIG. 6. The apparent variation of subfraction B with pH.

un-ionized —COOH groups then its behavior becomes indistinguishable from that of the "constitutional" subfraction B.

This interpretation is in fact identical with earlier conclusions of Lindley and Phillips drawn from the behavior of esterified wool (*10*). They found that if wool was esterified prior to bisulfite treatment then the extent of reversal of the bisulfite reaction on subsequent washing was greatly reduced, i.e., subfraction B apparently increased. A linear relationship was found between the magnitude of subfraction B and the extent of esterification of the carboxyl groups of the wool. Figure 7, taken from their original paper, shows the results graphically. The upper of the two curves relates to wools methylated by methyl bromide and dimethyl sulphate and the intercept on the ordinate corresponds to the value for untreated wool. The lower curve relates to results on wools treated with a mixture of methyl alcohol and acetic anhydride. This technique causes acetylation as well as esterification and hence the intercept on the ordinates is the experimental value of subfraction B on acetylated wool.

Figure 8 shows the effect of pH on the rate of reversal of subfraction B; this again is data derived from Figs. 4 and 5. It appears that this is related to the ionization of a group of pK 4. However this conclusion must be regarded as tentative since it is clear from Fig. 3 that the rate of reversal of the bisulfite reaction is dependent on ionic strength in some way.

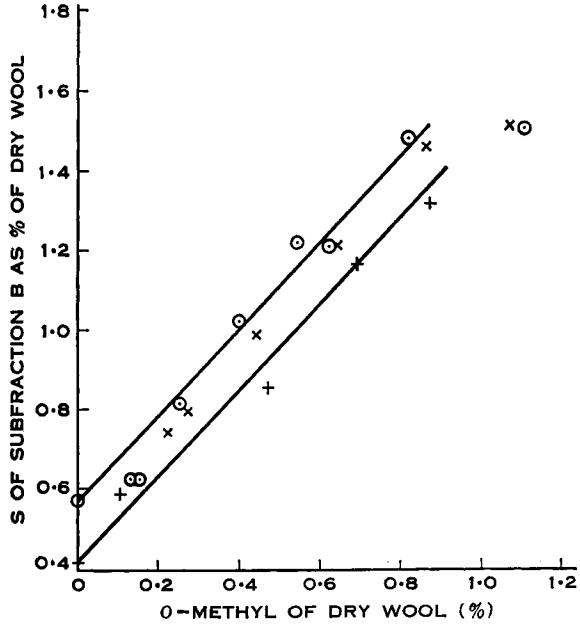

FIG. 7. The effect of esterification of the carboxyl groups of wool on the magnitude of subfraction B. (Reproduced with permission from *Biochem. J.*)

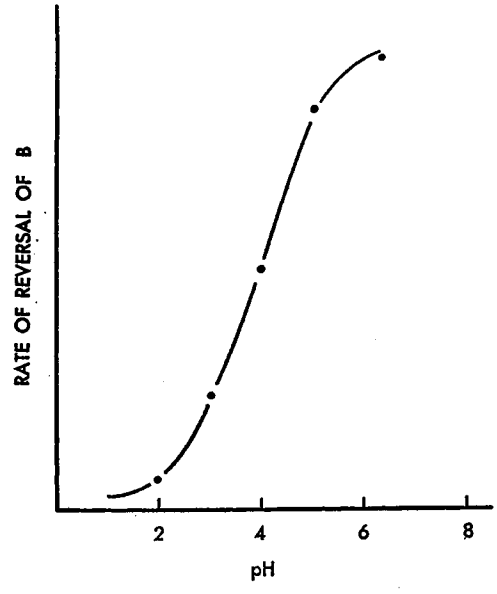

FIG. 8. The effect of pH on the rate of reversal of the bisulfite reaction for subfraction B.

While Carter and associates were aware of this and made some attempt to control the ionic strength, it is not clear from their paper exactly what was the composition of the buffers used to rinse the wools. This criticism does not apply to the extrapolations used in Fig. 5 to obtain the magnitude of subfraction B, since the curves of Fig. 3 show that this is independent of ionic strength.

Taken together all these results lead to a simple explanation of the properties of subfractions A and B, which is illustrated in a purely diagrammatic fashion in Fig. 9. In this, (1) represents a possible situation

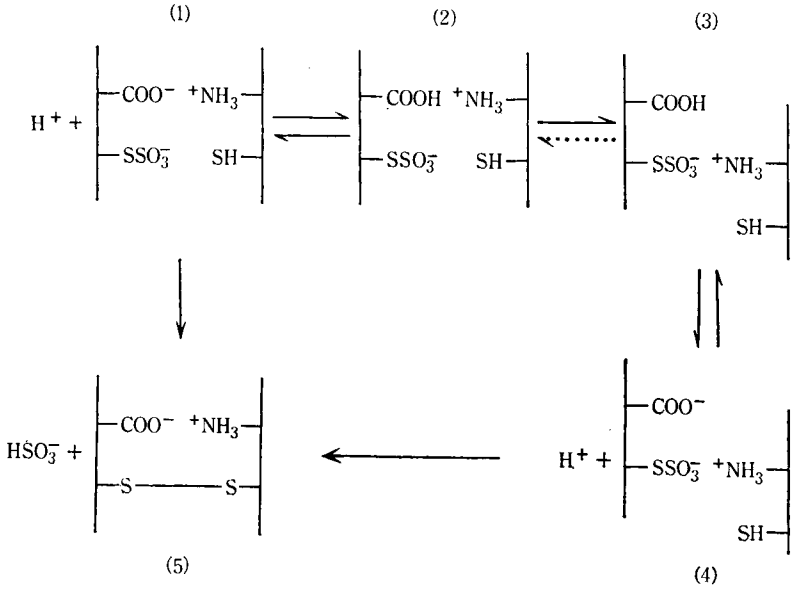

FIG. 9. Schematic representation of behavior of subfraction A.

in the wool molecule after treatment with bisulfite. In acid solution we can visualize the situation represented by (2) where a previously ionized carboxyl group is now discharged. This of course could upset the tertiary structure perhaps as suggested in Fig. 9 by rupture of a salt linkage, but it could equally be any other secondary type of bond involving a carboxyl group. In any case if in the immediate neighborhood of the carboxyl group there is a *S*-cysteinesulfonate group which has a very low pK and is therefore still ionized the situation represented by (3) is likely to arise. Here, at the expense of some configurational stability, the charged *S*-cysteinesulfonate group has taken over the function previously performed by the carboxyl group.

However ionization of the carboxyl group as shown in (4) need not

imply reversal to (1). Conceivably the pK of the carboxyl group could be raised as a result of this change in its environment. This is a possible explanation of the pK of 4 deduced from Fig. 8. Reversal of the bisulfite reactions to (5) would obviously require extra activation energy in the case of (4) → (5) compared to (1) → (5) because of the necessity in the former case of breaking the salt link between $—SSO_3^-$ and $—NH_3^+$. (1) can thus be taken as exemplifying Middlebrook and Phillips' subfraction A; the "constitutional" subfraction B can be visualized as having no carboxyl group in its vicinity but still having the acceptor group we have taken as $—NH_3^+$. Consequently on treatment with bisulfite subfraction B is immediately stabilized by formation of a salt link between the $—NH_3^+$ and the *S*-cysteinesulfonate group. Whatever the precise nature of the interaction involved it would seem that the scheme mentioned above can satisfactorily explain the general properties of the A and B subfractions of the cystine. It will be seen that fundamentally it implies that the cystine links are situated in polar parts of the molecule and that the peptide chains have sufficient freedom to be able to move apart if the disulfide bonds are broken.

IV. The (C + D) Fraction

The (C + D) fraction of the cystine was defined by Middlebrook and Phillips as the cystine which did not react with bisulfite according to Eq. 1. When attempts were made to induce it to react with bisulfite by raising the temperature or greatly prolonging reaction times it was found that reaction took place by some other mechanism and did not lead to the formation of thiol and *S*-cysteinesulfonate groups. Experimentally all that was observed was a disappearance of cystine without any corresponding increase in the amount of thiol-S. This reaction appeared to slow down considerably when about half of the (C + D) fraction had reacted and the differentiation into subfractions C and D was made on this basis.

The most significant chemical observations since then are those of Farnworth (*7*) and O'Donnell (*11*). O'Donnell extended earlier work of Geiger and associates (*12*) and showed that a number of successive reductions of wool with thioglycolate at pH 5, each reduction being followed by alkylation with iodoacetamide, eventually produced a modified wool containing only traces of cystine. Thus by this procedure the (C + D) fraction has been induced to react with thioglycolate under conditions where it is normally inert. Farnworth examined this type of reaction specifically from the standpoint of the (A + B) and (C + D) fractions. He found that if fraction (A + B) was broken by reduction and the thiol groups masked by alkylation then the (C + D) fraction would now react

partially either with thioglycolate at pH 5 or sodium bisulfite. In the latter case the reaction could be formulated as in Eq. 1 and led to the formation of thiol and *S*-cysteinesulfonate groups. Moreover this reactivity of the (C + D) fraction was found to extend even to wools in which the (A + B) fraction had been cross-linked by alkylene dihalides except in the case of agents introducing a methylene group, i.e., the shortest possible cross-link other than the disulfide bond itself. Farnworth concluded from this that the difference between the (A + B) and (C + D) fractions is merely one of accessibility.

This explanation is almost certainly correct in so far as it goes, but begs the question when considered in terms of wool structure. The important question is: at what level of structure does the accessibility factor operate? Is it at the level of the immediate environment of the disulfide bond and hence to be regarded as steric hindrance, or is it at the molecular or even histological level? The balance of evidence suggests that probably all these levels play some part in the case of wool. From the limited extent of the bisulfite reaction with some soluble proteins (*13*, *14*) and also evidence adduced by Farnworth from the behavior of wool treated with phenyl isocyanate there can be little doubt that steric hindrance can affect the reactivity of disulfide bonds. Farnworth was able to suppress the reactivity of (A + B) fraction of the cystine of wool towards bisulfite almost completely by a prior treatment with phenyl isocyanate. This reagent did not react with the cystine but with other polar groups and in consequence hindered the approach of reagents to the disulfide bond. The combined cystine of the modified wool was also very stable towards alkali so that even the approach of a hydroxyl ion was strongly hindered.

An investigation of the elastic properties of chemically modified fibers has shown important differences between the (A + B) and (C + D) fractions from the viewpoint of molecular architecture (*15*). Fig. 10 and Table I summarize some of the results. From these results, one may observe the following:

(1) Reduction of (A + B) increases the ultimate extensibility of a wool fiber from 70% to 100% [cf. (i) and (iv)].

(2) Cross-linking of fraction (A + B) with ethylene dibromide after reduction almost restores the original elastic properties [cf. (i) and (v)]. Hence either the same sulphur atoms are cross-linked or it is mechanically unimportant to replicate the original structure.

(3) Breakage of (C + D) causes great reduction in fiber-breaking strength irrespective of whether (A + B) is intact or not [cf. (vi) and (vii)].

(4) Cross-linking of (C + D) after breakage at pH 11 has little or no

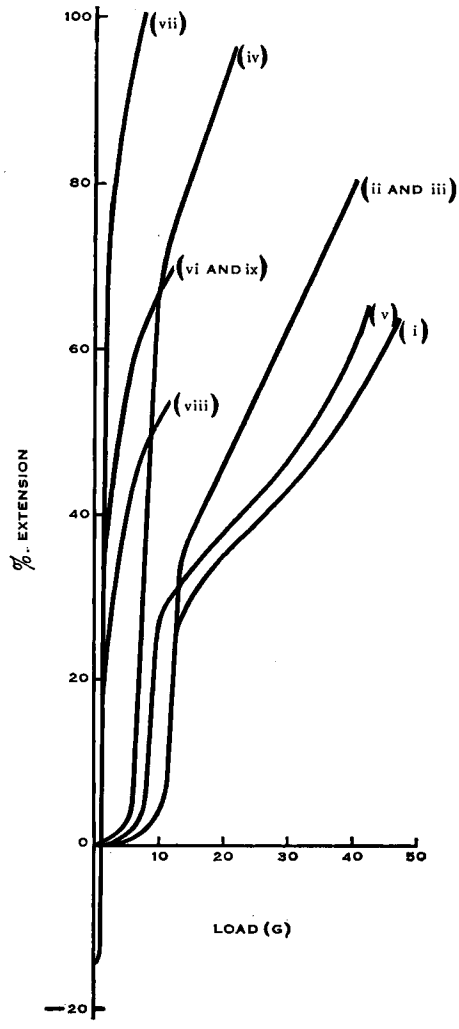

FIG. 10. Load *vs.* elongation (to break) of wool fibers treated as indicated in Table I. (Reproduced with permission from *Textile Research J.*)

effect [cf. (vi) and (viii)]. Hence, when (C + D) is broken cross-linking does not regenerate the original bonds.

To explain these results it has been postulated that some at least of the (C + D) fraction is incorporated in the crystalline phase of the wool and joins cyclic units end-to-end in the fiber structure. These are key linkages on which the strength of the fiber depends. The remainder of the (C + D)

TABLE I

CHEMICAL MODIFICATION OF WOOL FIBERS

Treatment of fiber	Effect on cystine bonds
(i) Untreated	All intact (SS = 2.59% S)
(ii) 20% $NaHSO_3$ solution 24 hours; washed in distilled water overnight	Subfraction B broken; A, C, and D intact (SH = 0.49% S)
(iii) Reduced 0.5 *M* sodium thioglycolate pH 5 overnight, cross-linked methylene iodide pH 8.5 buffer 24 hours, washed in water	Subfraction B as SH, subfraction A cross-linked as djenkolic acid. (C + D) intact
(iv) Reduced 0.5 *M* sodium thioglycolate pH 5 overnight, washed in water	(A + B) broken (as SH); (C + D) intact (SH = 1.26%)
(v) As (iv) but subsequently cross-linked with ethylene dibromide pH 8.5 overnight	(A + B) cross-linked as dimethylene-*SS'*-biscysteine (C + D) intact
(vi) As (v) but subsequently oxidized with 1.6% peracetic acid overnight	(C + D) broken (as cysteic acid); (A + B), presumably sulfone of dimethylene-*SS'*-biscysteine
(vii) Untreated wool oxidized with 1.6% peracetic acid overnight	90% broken by conversion to cysteic acid
(viii) As (v) but subsequently reduced 0.5 *M* thioglycolate pH 10.7 and re-cross-linked with ethylene dibromide	(A + B) and large proportion of (C + D) converted to dimethylene-*SS'*-biscysteine (SS = 0.44% S)
(ix) As (viii) then oxidized with 1.6% peracetic acid overnight	Mainly cross-linked as sulfone of dimethylene-*SS'*-biscysteine

fractions may well be incorporated in the crystalline phase as conventional cross-linkages; being in the crystalline phase it would necessarily be less accessible.

In collaboration with Dr. R. B. Fraser and Mr. T. P. MacRae of these laboratories, an attempt has been made to obtain direct evidence by X-ray diffraction techniques for some of these ideas. Lincoln wool was reduced at pH 5 with thioglycolate to convert the (A + B) cystine to cysteine residues and the thiol groups were then coupled with methylmercury iodide. The effect of this on the X-ray diffraction photograph was to intensify greatly the low angle equatorial spots corresponding to spacings of 27 Å, 45 Å, and 80 Å, whilst leaving other features unchanged. Fraser and MacRae (*16*) have produced impressive evidence to suggest that these particular diffraction maxima are best explained as being due to the packing arrangement of microfibrils in matrix material, so that the conclusion to be drawn from the methylmercury-treated fibers is that the (A + B) fraction of the cystine is associated mainly with the interfibrillar matrix material. Fibers have also been prepared in which the (A + B) cystine links have been replaced by bismethylene thioether links so that the

(C + D) cystine bonds could be broken by oxidation or reduction and then coupled with heavy atoms for X-ray diffraction work. It was thought that possibly enhancement of meridional reflections might occur giving some indication of the distribution of cystine residues along the peptide chain, but no evidence of this has been found. Instead it has been found that any treatment causing fission of the (C + D) cystine bonds, also causes the loss of fine low angle detail from the X-ray diffraction photographs of wool. It would seem therefore that we cannot draw any specific conclusions about the mode of incorporation of the (C + D) cystine into the wool molecule; however the general conclusion seems quite inescapable that the crystallinity revealed by the X-ray diffraction photograph of wool is dependent on the existence of an intact (C + D) fraction of the cystine. An interesting finding in this respect is the recent one that some of the (C + D) cystine becomes accessible to reducing agents at high extensions of the fiber, i.e., when the crystalline phase is broken up mechanically (*17*). This parallels very closely the finding for crystalline soluble proteins that all the cystine only becomes available for reduction when the protein is denatured by urea, guanidine hydrochloride, or detergents (*13, 14, 18*).

V. Summary and Conclusion

The over-all conclusion emerging from this study is that the reactivity of the cystine disulfide bonds of wool is influenced by structural factors at all levels of molecular organization. It is now well recognized that a division of sulfur bond reactivity similar to the (A + B) and (C + D) fractions of wool occurs in soluble crystalline proteins, and that this variation in reactivity can be abolished if the tertiary structure of the protein is destroyed by denaturing agents such as urea. In wool, this picture is complicated by the presence of histological structure which probably contributes in part to the varying activity of cystine. The finer differences in reactivity, such as those between subfractions A and B, reflect more subtle environmental differences of the cystine bonds and offer a possible approach to the study of some of the details of tertiary structure.

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Discussion

LORAND: I presume you studied these elongation curves in a hydrated medium?

LINDLEY: In water.

LORAND: Would there be any difference in the hydration of these variously treated fibers?

LINDLEY: If I remember the work of Carter, Middlebrook, and Phillips correctly, there is a very great difference. The picture of water uptake by wool which we have at the moment is that water is absorbed preferentially into the amorphous regions of the fiber where it causes considerable lateral swelling. The fact that water acts as an internal lubricant for fiber stretching is therefore understandable, and any treatment which would assist swelling of the amorphous region might be expected to produce an enhanced effect. Since according to Carter *et al.* (*J. Soc. Dyers Colourists* **62**, 203–303, 1946) the splitting of disulfide bonds causes an increase in hydration of the fiber, the effects can be interpreted in this general way.

HARRIS: I would like to comment on some of these observations. There are some complicating features here. It is easy to recognize the possibility of alternate regions of more or less order because in all of the natural fibrous polymer systems we find such structures. They occur in cellulosic materials, etc. You do not have to invoke, however, ordered and less ordered regions to account for a lateral swelling, because this will occur in any polymer system where you have oriented polymers.

The interesting thing that you brought out is why, when you stretch this thing, it becomes less crystalline. If this is correct, I think it needs further explanation. When you get to elongations of the order of 80 to 90%, you are almost at the breaking point. This now means that if you look at a fibrous molecule as just a series of overlapping molecules—let us forget the cross-linking for the moment, and think in terms of the straight linear polymers—when you get near a breaking point, it means statistically a great many ends are beginning to appear in a cross section and that is when a fiber will rupture. This means then that as you get to 80 or 90%, these molecule ends are being freed and they can start coiling up and give you a reversal from the fibrous to the so-called globular state. That is the only way you would now start getting less crystallinity rather than more.

LINDLEY: Couldn't it be that it is one matter to crystallize nylon by stretching but a very different matter where you have polypeptide chains with side groups of varying sizes which do not pack together readily?

HARRIS: I agree. We all know that perhaps more than half of the weight of the wool polymer is in the side chains.

LINDLEY: I think one has to regard crystallization as a rate process, i.e., when a wool fiber is stretched a true β-configuration is only produced at a rate which is very temperature dependent.