THE MECHANISM OF THE REACTION BETWEEN CYSTINE IN KERATIN AND SULPHITE-BISULPHITE SOLUTIONS AT 50°

PART II

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INTRODUCTION

A keratin fibre—wool, human hair, etc.—can be extended and then treated in some way so that, if it is immersed without tension in boiling water after the treatment, its final length differs from its length before extension. If the final length is greater than the initial length the phenomenon is known as permanent set; if it is shorter, supercontraction. The generally accepted interpretation[1] of the experiment is that during the treatment of the extended fibre, cross-linkages between the protein chains are broken, and the degree of permanent set or supercontraction is related to the number of cross-linkages stable in boiling distilled water which are re-formed between the protein chains in their extended form.

In Part I of this paper[2] suggestions were made for the reactions in which the cystine disulphide cross-linkages in keratin are broken in sulphite–bisulphite solutions in 10% EtOH at 50°. The set acquired by a fibre was divided into two parts: (1) the part which remained when the treatment with the sulphite–bisulphite solution was followed by immersion without tension for 1 h in boiling distilled water but which was unstable when this was followed by immersion without tension for 1 h in boiling 5% $NaHSO_3$, and (2) the "bisulphite-stable" set which remained after immersion without tension in boiling 5% $NaHSO_3$. In Part I it was suggested that the set unstable in boiling 5% $NaHSO_3$ is acquired by the re-formation of cystine disulphide cross-linkages in the reverse reactions (on rinsing the fibres and on immersing them in boiling distilled water) of the equilibria

$$RSSR + H_2SO_3 \rightleftharpoons RSSO_3H + RSH \quad (1)$$
$$RSSR + HSO_3^- \rightleftharpoons RSSO_3^- + RSH \quad (2)$$
$$RSSR + SO_3^{--} \rightleftharpoons RSSO_3^- + RS^- \quad (3)$$
$$RSSR + OH^- \rightleftharpoons RSOH + RS^- \quad (4)$$

where RSSR, $RSSO_3^-$, RSH and RSOH stand for cystine, S-cysteine sulphonate, cysteine and the sulphenic acid respectively, incorporated in the protein chain. It was also shown in Part I that in the sulphite-bisulphite reaction mixtures the forward reactions of (3) and (4) compete for the cystine in keratin and that the forward reactions of (1) and (2) are probably negligible. S-cysteine sulphonate was shown to be one of the side-chains involved in the formation of cross-linkages stable in boiling 5% $NaHSO_3$.

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In this Part it will be shown that the bisulphite-stable cross-linkages, which are not formed unless the fibres are extended before immersion in the sulphite–bisulphite reaction mixtures, may be formed in the previously inaccessible region which has been made accessible by extension in a process analogous to mechanical denaturation of soluble proteins. If this is so, the available evidence about the amino-acid composition of the accessible and inaccessible regions of keratin shows that lanthionine, RSR, is most likely to be one of the bisulphite-stable cross-linkages formed in these experiments.

$$RSSO_3^- + RSH \longrightarrow RSR + HSSO_3^-$$

EXPERIMENTAL

Setting experiments in the presence of lithium bromide

(*a*) *45% extension.* ALEXANDER[3] has shown that LiBr is an effective swelling agent for keratin fibres, and he suggests that it separates the protein chains and breaks hydrogen bonds between them. The effect depends on the incomplete hydration of Li^+ and Br^- ions so that very concentrated solutions are required. LiBr is less soluble in the sulphite–bisulphite reaction mixtures than in water, and in the present experiments 25 g commercial LiBr were dissolved in 100 ml of reaction mixture. Volumetric estimation showed the LiBr concentration to be 20.8 g/100 ml, and thus approximately 4.2 ml of water were added with the deliquescent salt. Apart from this slight dilution, the same concentrations of Na_2SO_3 ($M/2$) and SO_4^{--} ($M/4$) in 10% EtOH were used in the reaction mixture as in the experiments described in Part I of this paper, and the pH values were adjusted in the same way—by varying the ratio of H_2SO_4 to Na_2SO_4 while maintaining $[SO_4^{--}]$ constant. All solutions were made up in 10% EtOH.

The same experimental sequence was used. (i) Approximately 4 cm is cut from the root end of a purified human hair, mounted in a setting frame and its dry length measured with a travelling microscope. (ii) It is extended 45% of this original length in approximately 3 min in distilled water at room temperature. (iii) The fibre is transferred to a sulphite–bisulphite–LiBr reaction mixture in a thermostat at 50°. (iv) After 1 h in the reaction mixture, the fibre is removed, rinsed twice in distilled water for 1 ¼ min in all, and then (v) immersed without tension for 1 h in boiling distilled water which has previously been boiled vigorously for 20 min to expel dissolved oxygen. (vi) The fibre is removed from the boiling water, allowed to dry and its length measured so that the permanent set or supercontraction can be calculated.

The results are shown in Fig. 1. Curve A shows the permanent set when the sulphite–bisulphite–LiBr treatment is followed by immersion without tension for 1 h in boiling distilled water. Curve A′ shows the permanent set remaining when this is followed by immersion without tension for 1 h in boiling 5% $NaHSO_3$. For comparison, results obtained in experiments which were identical except for the absence of LiBr from the reaction mixtures are given (curves B and B′).

The pH value of the reaction mixture containing 20.8% LiBr in which a fibre acquires the greatest bisulphite-stable set is approximately 5.1 compared with 5.7 in the absence of LiBr. A titration curve, determined at room temperature with the same concentrations of LiBr, Na_2SO_3, SO_4^{--}, etc. in 10% EtOH is shown in Fig. 2. The pH value where $[HSO_3^-]$ is greatest is approximately 3.9. Therefore, as in the

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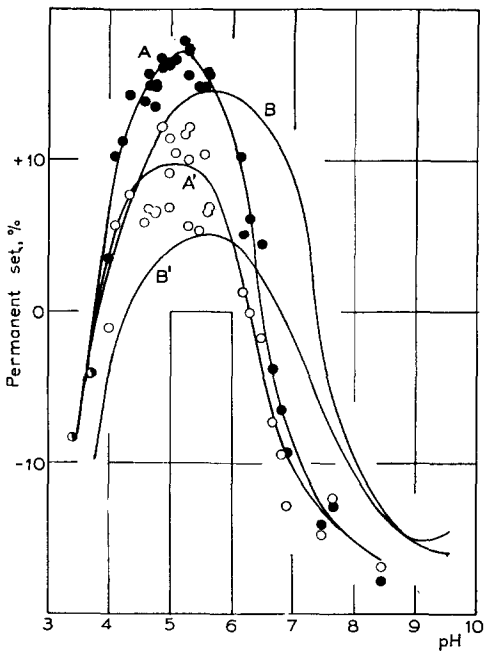

Fig. 1. Permanent set and supercontraction after initial extension of 45%. Fibres extended 45% and immersed 1 h in sulphite–bisulphite–LiBr mixture followed by 1 h without tension in boiling water (A); followed by 1 h without tension in boiling 5% $NaHSO_3$ (A'). Curves B and B' illustrate the results of identical experiments carried out in the absence of LiBr (see Part I of this paper[2]).

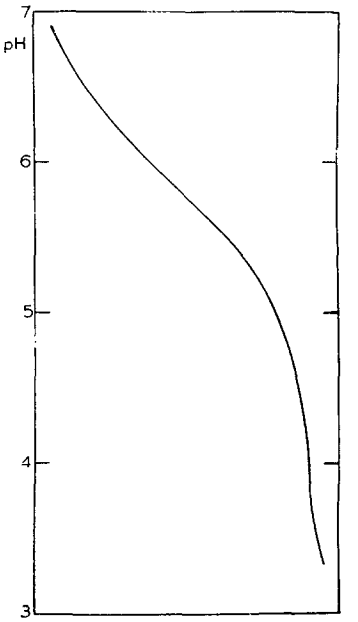

Fig. 2. Titration curve of Na_2SO_3 in 10% EtOH against H_2SO_4 in 10% EtOH in the presence of LiBr. The same concentrations of Na_2SO_3, H_2SO_4 and LiBr were used as in the setting experiments whose results are shown in Fig. 1, curves A and A'.

case (described in Part I) where LiBr is absent, the pH value of a reaction mixture where the bisulphite-stable set acquired by a fibre is greatest, differs by approximately 1.2 pH units from the pH value of a reaction mixture where $[HSO_3^-]$ is a maximum. Thus the experiment supports the explanation of the position of the pH value where bisulphite-stable set is greatest which was suggested in Part I of this paper.

(*b*) *Zero extension.* Four experiments were carried out using the same sequence as before, (i) to (vi) described above, omitting step (ii)—the fibres were immersed without tension in the sulphite–bisulphite–LiBr solutions. As in similar experiments without LiBr which will be described later, an unextended fibre treated in such a reaction mixture at 50° at pH values between 4.93 and 7.77 is indistinguishable in the subsequent treatments from an untreated fibre. Untreated fibres showed the same small contraction of the order of 0.2% after immersion without tension in boiling distilled water for 1 h. When this was followed by immersion without tension in boiling 5% $NaHSO_3$ for 1 h, the average supercontraction of seven otherwise untreated fibres was 24.13% compared with 23.89%, the average of the supercontractions of four fibres treated unextended in sulphite–bisulphite–LiBr solutions at pH values of 4.93, 5.94, 7.02, and 7.77 at 50°, followed by 1 h immersed without tension in boiling distilled water, and 1 h without tension in boiling 5% $NaHSO_3$ solution.

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Setting experiments in the absence of lithium bromide

In Part I of this paper, setting experiments were described in which fibres were extended 45% of their length before immersion in sulphite–bisulphite reaction mixtures at 50°. Experiments have also been carried out in which fibres were extended 20% before immersion, or immersed without tension, in the reaction mixtures.

(*a*) *20% extension.* Fibres were treated according to the usual experimental sequence—in step (ii) they were extended 20%. Curve C in Fig. 3 shows the permanent set or supercontraction when the treatment in the sulphite–bisulphite reaction mixture is followed by immersion without tension for 1 h in boiling distilled water. Curve C′ shows the permanent set or supercontraction when the treatment in boiling water is followed by immersion for 1 h in boiling 5% $NaHSO_3$. In Fig. 1 each point represents the set or supercontraction of one fibre. If the fibres are extended 20% instead of, as in Fig. 1, 45% before treatment with the sulphite–bisulphite reaction mixtures, the results become less reproducible, and each point on curves C and C′, Fig. 3 is therefore the average of the permanent set or supercontraction of four fibres after identical treatments. In some further exploratory experiments, the same irreproducibility was found when fibres were extended $33^1/_3$% before sulphite–bisulphite treatment at 50°. Other experiments showed that 45% extension before

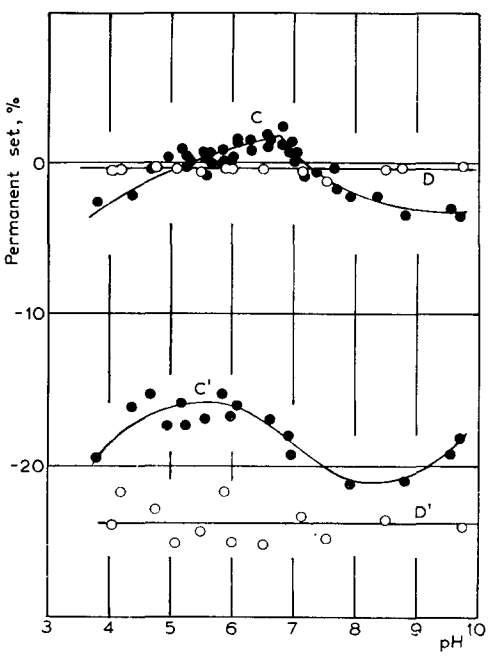

Fig. 3. Permanent set and supercontraction after initial extension of 20% and after zero initial extension. Fibres extended 20% and immersed 1 h in sulphite–bisulphite mixture, followed by 1 h without tension in boiling water (C); followed by 1 h without tension in boiling 5% $NaHSO_3$ (C′). Fibres immersed without tension 1 h in sulphite–bisulphite mixture, followed by 1 h without tension in boiling water (D); followed by 1 h without tension in boiling 5% $NaHSO_3$ (D′). Each point on curves C and C′ is the average of the results of identical experiments with 4 fibres.

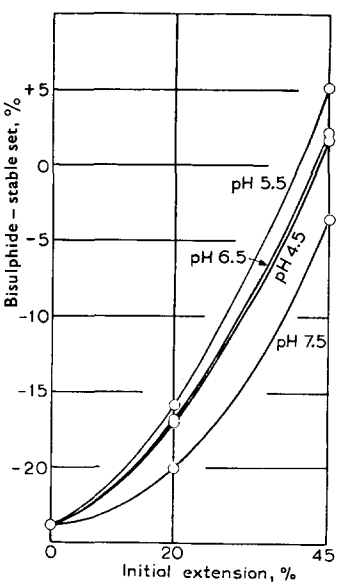

Fig. 4. Curves of bisulphite-stable set against initial extension, using sulphite–bisulphite mixtures at pH 4.5, 5.5, 6.5 and 7.5.

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treatment does not necessarily ensure reproducible results. If the strength of the sulphite–bisulphite reaction mixtures is reduced to ¼ its original value ($M/8$ Na_2SO_3, $M/16$ SO_4^{--}) then the results with different fibres from the same sample treated in the same way show considerably increased variation.

In experiments where fibres were extended 20% of their original length before immersion in the sulphite–bisulphite solutions, the pH value where the bisulphite-stable set is greatest is approximately 5.7, as in the experiments described in Part I where the extension before treatment was 45%.

(*b*) *Zero extension.* Again the same experimental sequence was used, omitting step (ii). After their dry lengths had been measured, the fibres were immersed without tension in the sulphite–bisulphite reaction mixtures at 50° for 1 h. They were rinsed and immersed without tension in boiling distilled water for 1 h, dried and measured, (line D, Fig. 3), immersed without tension for 1 h in boiling 5% $NaHSO_3$, rinsed, dried and measured (line D'). As in the case where the sulphite–bisulphite reaction mixtures contain LiBr, an unextended fibre treated in a sulphite–bisulphite mixture is indistinguishable from an untreated fibre in its behaviour in the subsequent treatments with boiling distilled water and boiling 5% $NaHSO_3$. A similar small contraction of the order of 0.2% was observed when the treated fibres were immersed without tension for 1 h in boiling distilled water. When this was followed by immersion without tension for 1 h in boiling 5% $NaHSO_3$ the average of the supercontractions (line D', Fig. 3) of twelve treated fibres is 23.77% compared with 24.13%, the average of the supercontractions of 7 fibres in boiling 5% $NaHSO_3$ for 1 h which had previously been immersed without tension for 1 h in boiling distilled water but were otherwise untreated.

Relation of extension before treatment to the bisulphite-stable set acquired during the treatment

Fig. 4 shows the relation between the extension of the fibre before treatment to the amount of bisulphite-stable set it acquires after treatment at various pH values. Only three points are available on each curve (at 0%, 20% and 45% initial extension) and it is possible that more points on these curves would show a sharper discontinuity, as has been observed in some similar experiments with other setting reagents[4].

Experimental details

The technique of the setting experiments has been given in detail in Part I of this paper. Commercial $NaHSO_3$ and LiBr were used, otherwise all chemicals were AnalaR.

DISCUSSION

(A + B) and (C + D) fractions of cystine in keratin

PHILLIPS[5], who first noticed that the cystine in wool could be divided into two approximately equal parts with different reactivities, showed that what he called the (A + B) fraction reacts at room temperature with $NaHSO_3$ to give $RSSO_3^-$ and RSH, and that the reaction can be completely reversed[6] by sufficiently prolonged rinsing at pH 7, with the re-formation of cystine. Drastic conditions are needed to make the rest of the cystine react – the (C + D) fraction. A more complicated reaction than the simple equilibria (1) to (4) occurs under these conditions, and it cannot be reversed by rinsing.

It is possible that the accessible region of keratin contains the reactive (A + B)

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fraction of the cystine, and the inaccessible region the less reactive (C + D) fraction. PHILLIPS thought that the differences between the two fractions were due to different amino-acid environments in the protein chains. These hypotheses are not mutually exclusive: if the accessible and inaccessible regions have different compositions then one follows from the other.

Composition of the accessible and inaccessible regions in keratin

The cystine in wool can thus be divided into reactive and unreactive fractions. In contrast, MIDDLEBROOK[7] found that all the ε-amino groups of lysine will react under mild conditions (40°, 48 h, pH 7.0) with 1-fluoro-2,4-dinitrobenzene. Also, PETERS[8] has found, using the most likely amino-acid analyses of wool, that all the carboxyl side-chains can be accounted for in the titration curves of wool against HCl. All appear to be accessible, and there is no evidence for the "masking" of groups. RICHARDS AND SPEAKMAN[9] found that the tyrosine in wool is fully accessible to alcoholic iodine at 25° (96% reaction). Lastly, FRASER[10] has obtained evidence from the infra-red spectrum of wool that the hydrogen atoms of all the side-chain amide groups are exchangeable in D_2O.

This experimental evidence allows a tentative suggestion that the accessible region of keratin contains the (A + B) fraction of cystine and the amino-acids with carboxyl, amide and amine side-chains; and the inaccessible region contains the (C + D) fraction of cystine, the amino-acids with non-polar side-chains, and perhaps serine and threonine which LINDLEY[11] found to be associated with the (C + D) fraction of cystine together with glycine and proline.

Experiments with silk fibroin[12] have been interpreted to show a similar state of affairs in silk. Enzymic hydrolysis of a solution of the fibroin caused the precipitation of a highly oriented protein which contained only amino-acids with small side-chains (glycine, alanine and serine) while the larger side-chains were left in solution. The precipitate was believed to be the original crystalline region of silk.

Some calculations made by VALENTINE[13] using data from BURLEY, NICHOLLS AND SPEAKMAN[14], confirm this theory of the composition of the accessible and inaccessible regions of keratin. The latter authors found the percentage increase in weight of wool when it was allowed to exchange with deuterium from heavy water. The increase in weight, if the wool were completely accessible to D_2O, was calculated from the best available amino-acid analyses, and the fraction accessible was calculated from the actual increase in weight divided by the estimated possible increase in weight if the wool were completely accessible. Values of 82.4 to 87.3 were obtained for the accessible percentage of various types of wool. The authors realised that this calculation involved the assumption that the composition of the two regions is the same, and that this was probably not justified.

If it is assumed that the accessible region contains all the polar side-chains and the inaccessible region only amino-acids with non-polar side-chains, it follows that the only exchangeable hydrogen atoms inaccessible to D_2O are those of the –NH- groups of the main protein chains in the inaccessible region. Valentine has recalculated BURLEY, NICHOLLS AND SPEAKMAN's results on this assumption and obtained values near 70% for the accessible proportion of the various types of wool, which is closer to the values from infra-red and water-absorption measurements, and histological evidence[10].

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To explain the different reactivities of the two fractions of cystine, PHILLIPS[5] originally suggested that the (A + B) fraction is associated in the protein chains with amino-acids with amine or carboxyl side-chains, and that the (C + D) fraction is associated with amino-acids with non-polar side-chains. This is consistent with the present theory of the composition of the accessible and inaccessible regions of keratin if the former region contains the (A + B) fraction of cystine and the latter the (C + D) fraction.

Orthocortex and paracortex in wool

Differential staining experiments have shown[15] that the wool fibre is made up of two hemicylinders of different reactivity. If the less reactive paracortex contains a higher proportion of the unreactive inaccessible region, and the more reactive orthocortex a higher proportion of the reactive, accessible region, then these differences should be shown in the amino-acid analyses of the paracortex and orthocortex. Paracortical and orthocortical cells have been separated, and although analyses of their amino-acid compositions have shown some inconsistency, the orthocortex, for example, has been found to be richer in aspartic and glutamic acids[16].

Physical mechanism of setting reactions

When a keratin fibre is extended in water at room temperature the load–extension curve shows a yield point when the fibre is extended approximately 2% of its original length, and a smooth shoulder at approximately 25% extension. The β X-ray diffraction pattern begins to appear (in place of the α pattern of the unstretched fibre) only after this shoulder has been reached. These observations have been interpreted as showing[17] that up to 2% extension, interchain hydrogen bonds are broken; as the fibre is extended about 25% of its original length, $\alpha \longrightarrow \beta$ transformation occurs in the amorphous region of the keratin. Extension beyond 25% causes the crystalline region of the keratin to change from the α form to the β form.

A parallel phenomenon has been observed in some setting experiments. SPEAKMAN AND STOVES[4] characterised the cross-linkages formed in fibres after different initial extensions (0–50%) and treatment in various setting mediums. The nature of the cross-linkages formed was inferred from the contractions of the set fibres when they were immersed in turn, without tension, in boiling 5% $NaHSO_3$ (which breaks disulphide cross-linkages) and boiling 0.1 *N* HCl (which breaks –CH:N– linkages). Several of the component curves of set *versus* extension before treatment which were obtained in this way showed discontinuities at approximately 25% extension.

The quantitative interpretation of these component set *versus* extension curves is not clear, but if the separation of the component curves is taken as a measure of the number of cross-linkages of each type, then it can be shown that in every case where cross-linkages involve nitrogen atoms, the number of these cross-linkages may increase (or decrease) as the extension of the fibres before treatment is increased up to approximately 25% of their initial length, but thereafter the number remains constant[18]. Only cross-linkages involving sulphur and not nitrogen—the lanthionine, RSR, cross-linkage—increase in number as the extension of the fibres before treatment is increased above 25%. (It has been suggested[18] that after setting in $NaHSO_3$ the cross-linkages which are stable in boiling 5% $NaHSO_3$ and boiling 0.1 *N* HCl are not lanthionine but –SNH– linkages, since lanthionine is not found among the

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products of hydrolysis of bisulphited wool; and yet the number of these cross-linkages continues to increase as the fibres are extended beyond 25% before treatment. However, wool is not extended before it is bisulphited, and SPEAKMAN AND STOVES's results show that the bisulphite-stable cross-linkage is not formed when unextended fibres are treated in a $NaHSO_3$ setting medium. Therefore, lanthionine cannot be ruled out as the source of set stable in boiling 5% $NaHSO_3$ until it has been shown to be absent from the products of hydrolysis of wool which has been extended and then bisulphited.)

At this point the assumptions are made that in an unextended fibre the crystalline region is inaccessible to the sulphite–bisulphite reaction mixture, and the amorphous region is accessible. It is not necessary, however, to postulate that the crystalline region is quantitatively identical with the inaccessible region or that the amorphous region is quantitatively identical with the accessible region.

If it is true that cross-linkages not involving a nitrogen atom increase in number as the extension of the fibres before treatment is increased beyond 25%, and linkages involving a nitrogen atom remain constant in number beyond 25% extension, then the above theory of the amino-acid composition of the accessible and inaccessible regions is consistent with the physical mechanism of keratin extension and the chemical nature of the cross-linkages. As the extension of the fibres before treatment is increased beyond 25%, the X-ray diffraction pattern shows that the crystalline region of the keratin is extended. New cross-linkages formed in the crystalline region (the inaccessible region of the unextended fibre) should not involve a nitrogen atom since all the lysine (the source of the nitrogen atom) is contained in the accessible, amorphous region.

Application to the present results

In the present experiments, when the set fibres are immersed without tension in boiling 5% $NaHSO_3$, the cystine disulphide cross-linkages which were re-formed during the setting treatment are broken, and therefore –CH:N– and lanthionine, –S–, are the possible sources of bisulphite-stable set. Fig. 4 shows that as the extension of the fibres before treatment increases beyond 25% the amount of bisulphite-stable set increases even more sharply, and therefore cross-linkages are formed in the originally inaccessible region of the fibres which has been made accessible by extension. Since the inaccessible region contains no lysine, lanthionine appears to be one source of bisulphite-stable set.

The results when different fibres from the same sample are extended 45% in water and then treated in a sulphite–bisulphite reaction mixture ($M/2$ Na_2SO_3, etc.) at 50° are reproducible (Fig. 1, curves B and B'). If the extension before treatment is only 20% or $33\frac{1}{3}$%, the results show considerably increased variation, and in curves C and C', Fig. 3, each point is the average of the results from four identically treated fibres. A possible explanation is that after small extensions the crystalline region may remain almost completely inaccessible to the reaction mixture, so that the number of cross-linkages formed between the protein chains is not sufficient to stabilise the structure. If the accessibility were not altered by extension, the number of cross-linkages formed in a fibre in a given reaction mixture should be independent of the extension of the fibre unless some other steric factor is involved. If the fibres are extended 45% and treated with sulphite–bisulphite mixtures at $\frac{1}{4}$ the previous

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concentrations ($M/8$ Na_2SO_3, etc.) in 10% EtOH at 50°, the results again show wide variation, confirming that a certain minimum number of cross-linkages is required to give a stable structure. This evidence again suggests that some at least of the bisulphite-stable cross-linkages must be formed in the originally inaccessible region as it is made accessible by extension, and therefore that the cross-linkages must be lanthionine linkages.

When a fibre is immersed without tension in a sulphite–bisulphite–LiBr solution at 50° for 1 h, it is indistinguishable from an untreated fibre in the subsequent treatments (without tension) in boiling distilled water and boiling 5% $NaHSO_3$. No bisulphite-stable cross-linkages are formed, and thus it appears that 20.8% LiBr solutions at 50° do not permit the penetration of SO_3^- ions, etc., into the inaccessible region of the fibre. If the fibres are extended 45% before treatment, the set acquired is greater than in the absence of LiBr. Penetration by LiBr probably permits a greater rearrangement of the protein chains by breaking remaining hydrogen bonds, and thus it allows a greater set to be acquired by the fibre.

Alternative theories

SPEAKMAN[19] pointed out that the pH value of the sulphite–bisulphite solution in which the set acquired by a fibre is greatest is probably close to the isoelectric point of the keratin. At this pH value the salt-linkages are most stable, and thus the strain on the disulphide bonds is greatest there when a fibre is extended. The disulphide bonds are therefore most reactive at the isoelectric point, and the greatest amount of set is acquired in a sulphite–bisulphite solution at this pH value. The objection to this explanation is the same as the objection to PHILLIPS's theory (see Part I). These suggestions will explain maximum breakdown of disulphide bonds at a given pH value, but it seems likely that in the present experiments breakdown is practically complete over the whole pH range used, and therefore any theory must explain, rather, maximum formation of cross-linkages at the pH value where the greatest set is acquired. Also, keratin fibres can be set in alkaline solutions far from the isoelectric point.

CECIL AND MCPHEE[20], working with cystine itself and related disulphides in solution, have shown that other, ionisable, groups in the molecule affect the reactivity of the disulphide bonds towards SO_3^-, etc. It is possible, therefore, that the reactivity of cystine incorporated in a protein chains may be influenced by neighbouring groups. The ionisation of amine and carboxylic side-chains is controlled by the pH value of the reaction mixture and it may be that at the pH value where the bisulphite-stable set is greatest, the charges on the ionisable amino-acid side-chains in the keratin are most favourable for the formation of bisulphite-stable cross-linkages.

If the environment of the side-chains giving rise to bisulphite-stable set changes as the fibre is extended, then it is possible to explain some conflicting experimental results: PHILLIPS[21] observed that maximum formation of $RSSO_3^-$ and RSH in un-extended fibres occurred in sulphite–bisulphite solutions at approximately pH 5. In the present work with fibres extended 20% and 45%, the bisulphite-stable set is greatest after sulphite–bisulphite treatment at pH 5.7. The difference between the pH values of the sulphite–bisulphite solutions where the bisulphite-stable set acquired by a fibre is greatest and where the formation of $RSSO_3^-$ and RSH is greatest can be explained by postulating a change in environment of the side-chains giving rise

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to bisulphite-stable cross-linkages as the fibre is extended from 0 to 20%. However, as shown in Part I of this paper, the pH value of maximum set (5.7) is compatible with PHILLIPS's result if the reaction in which bisulphite-stable cross-linkages are formed is alkali-catalysed. The theory of bisulphite-stable setting which was advanced in Part I is consistent with the experimental facts without any assumptions about the effect of amino-acid environment on the reactivity of the cystine disulphide cross-linkage or on the reactivity of its reduction products.

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SUMMARY

Further experiments are consistent with the mechanism of bisulphite-stable cross-linkage formation in keratin treated with sulphite–bisulphite solutions in 10% EtOH at 50° for 1 h which was advanced[2] in Part I of this paper.

Load–extension curves of keratin fibres and data from the changes in X-ray diffraction pattern as they are extended, when taken with the curves of set *versus* extension-before-treatment, suggest that some at least of the cross-linkages are formed within the originally inaccessible region of the fibre which has been made accessible to the reactive ions in the solution when the fibre is extended.

There is no evidence from the present experiments of the formation of bisulphite-stable cross-linkages in unextended fibres either in the presence or absence of a swelling agent, LiBr.

Recent work tends to show that the accessible and inaccessible regions of keratin have different amino-acid compositions. Lysine, the source of the nitrogen atom in the –CH:N– linkage, seems to be present only in the accessible region, so that lanthionine, RSR, is the most likely bisulphite-stable cross-linkage in the inaccessible region.

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