2. As a result, a method of assay has been devised depending upon the fact that, within certain prescribed limits, the fall in the flow-time through an Ostwald viscosimeter of a buffered hyaluronate-hyaluronidase mixture incubated for a fixed time is proportional to the logarithm of the concentration of the enzyme.

3. Satisfactory assays which have standard errors

of less than 10 % can be made with a total of twelve readings.

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REFERENCES

- Bliss, C. I. & Marks, H. P. (1939). *Quart. J. Pharm.* **12**, 182.
 Madinaveitia, J. & Quibell, T. H. H. (1940). *Biochem. J.* **34**, 625.
 McClean, D. (1943). *Biochem. J.* **37**, 169.
 McClean, D. & Hale, C. W. (1941). *Biochem. J.* **35**, 159.
 McIlvaine, T. C. (1921). *J. biol. Chem.* **49**, 183.
 Robertson, W. van B., Ropes, M. W. & Bauer, W. (1940). *J. biol. Chem.* **49**, 183.

The Action of Sulphites on the Cystine Disulphide Linkages of Wool

5. THE EFFECT OF SOME CHEMICAL MODIFICATIONS OF THE WOOL ON THE MAGNITUDE OF THE BISULPHITE-REACTIVE FRACTION (*A + B*) AND THE RELATIVE MAGNITUDES OF SUBFRACTIONS *A* AND *B*

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Elsworth & Phillips (1938, 1941) showed that approximately half of the cystine disulphide-S of wool reacted with solutions of NaHSO_3 to give thiol and *S*-cysteine-sulphonate groups according to the reaction:

where *R'* and *R''* represent polypeptide chains. Middlebrook & Phillips (1942) found that this reactive fraction of the disulphide-S could be divided into two subfractions: subfraction *A* which was readily reversed to disulphide on rinsing the bisulphited wool with water, and subfraction *B* which gave thiol and *S*-cysteine-sulphonate groups which were stable to water-rinsing.

In an endeavour to elucidate the cause of this difference in the behaviour of subfractions *A* and *B*, we have examined a number of chemically modified wools. Whilst we have not discovered any method of modifying the wool which causes more of the total cystine-S to react with NaHSO_3 , we have found that any treatment which blocks the free carboxyl groups of the combined glutamic and aspartic acids, produces an increase in the number of thiol and *S*-cysteinesulphonate groups stable to water rinsing (subfraction *B*) and a corresponding decrease in the water-labile subfraction *A*.

METHODS

The chemically modified wools were prepared, as described below, from the sample of wool (64's combed merino) used by Lindley & Phillips (1945). All analyses are calculated on the weight of the anhydrous wool.

Deaminated wool. The wool (10 g.) was immersed for 2 days in a solution prepared by mixing 80% (w/v) NaNO_2 (2 vol.) with glacial acetic acid (1 vol.), washed for 2 days in running water, dried and conditioned by exposure to a controlled atmosphere of 21° and 70% relative humidity until it attained constant weight. This method deaminates the lysine ϵ -amino-N and in addition an indefinite number of terminal arginine amino-N groups (Speakman & Stott, 1934; Rutherford, Harris & Smith, 1937).

Acetylated wools. These were obtained by (a) boiling, under reflux, for 30 min. with excess acetic anhydride, (b) by immersion overnight in acetic anhydride at room temperature.

Methylated wools. These were prepared by the methods described by Blackburn, Carter & Phillips (1941), Blackburn & Phillips (1944). Prior to conditioning, all the methylated wools were rinsed thoroughly in distilled water to remove buffer salts.

O-Methyl was determined by Baernstein's method (1932, 1936). Pregl's method (1924) was used for *N*-methyl, but the methyl iodide evolved was determined as in the *O*-methyl determinations. Neither the use of gold chloride nor fuming HI was found advantageous (Edlbacher, 1918). Preliminary investigations showed that in agreement with

observations on casein by Burns (1914) and Geake & Nierenstein (1914), untreated wool gave an apparent *N*-methyl content of 0.32%. Burns (1914) attributed the apparent *N*-methyl content of unmethylated casein to the decomposition of glycine and alanine to methylamine and ethylamine on heating with HI at the elevated temperatures used in Pregl's method. We were unable to devise conditions which would give satisfactory results for the *N*-methyl content of sarcosine-HCl. The *N*-methyl contents of the methylated and untreated wools were therefore carried out under as nearly as possible identical conditions. The wools were first hydrolyzed in boiling HI under reflux for 3 hr. and the hydrolysate then heated at 290–300° for 1 hr.

Amino-N of wool. An apparatus was designed for use with the Van Slyke (1927) manometric gas analysis apparatus. Doherty & Ogg (1943) have since described a similar apparatus and technique, except that they used wool which had been ground in a ball mill, whereas we used intact wool. Our results agree with theirs.

Treatment of modified wools with sodium bisulphite. The wools were treated with 100 times their weight of 30% (w/v) NaHSO₃ for 17 hr. To wet them thoroughly, the samples were submerged in the NaHSO₃ by a glass sinker, the space above the solution was exhausted and then air was re-admitted. Fraction (*A* + *B*) of the disulphide-S was determined by hydrolyzing 0.2 g. of the bisulphited wool, after it had been squeezed to remove the excess NaHSO₃ solution, in 20 ml. of 5*N*-HCl for 4 hr., and estimating the cysteine and cystine-S liberated by Shinohara's methods (1935*a, b*) using a Spekker absorptiometer. Two methods of rinsing the wool were used before the cysteine liberated from subfraction *B* was determined. In our earlier experiments, the bisulphited wools were rinsed during $\frac{1}{2}$ hr. in four changes of tap-water of pH 9 (Lindley & Phillips, 1945) but in later experiments they were rinsed by the following procedure of Carter, Middlebrook & Phillips (1946): after rinsing the bisulphited wool (0.2 g.) for 10 min. in 250 ml. of distilled water, it was immersed overnight in 2 l. of distilled water. The rinsed wool was hydrolyzed in 10 ml. 5*N*-HCl for 4 hr. and the cysteine and cystine contents of the hydrolysate determined.

RESULTS

Fraction (A + B) and subfraction B of the combined cystine-S of methylated wools

The CH₃ contents, disulphide-S, fraction (*A* + *B*) and subfraction *B* of the cystine-S of untreated wool and of wool methylated by three different reagents are given in Table 1. The CH₃ contents were unaltered by bisulphiting, except in the case of the wool methylated with methyl sulphate, the CH₃ content falling to 0.86%.

Blackburn *et al.* (1941) have produced evidence that during methylation of wool with methyl bromide and methanol-acetic anhydride only the carboxyl groups of the combined glutamic and aspartic acids are esterified. Methyl sulphate also esterifies carboxyl groups, but in addition *O*-methylates some other centre. These additional CH₃ groups, which are possibly attached to peptide

linkages (Blackburn *et al.* 1941; Blackburn, Middlebrook & Phillips, 1942; Blackburn & Phillips, 1944) are removed easily either by steam or alkaline buffers. It now appears that NaHSO₃ is a useful reagent for removing selectively these CH₃ groups.

Table 1. *The disulphide-S of fraction (A + B) and of subfraction B of methylated wools*

Methylating agent	CH ₃ (%)	Di- sulphide-S (%)	Fraction (<i>A</i> + <i>B</i>) (%)	Subfraction <i>B</i> * B' B''	
				B' (%)	B'' (%)
Untreated	0.0	3.10	1.84	1.02	0.58
Dimethyl sulphate	1.44	2.86	1.74	1.60	1.46
Methyl bromide	0.80	2.60	1.65	1.30	1.22
Methanol-acetic anhydride	0.47	3.02	1.72	1.52	0.86

* *B'* stable to rinsing in tap-water of pH 9 (Lindley & Phillips, 1945); *B''* stable to rinsing in distilled water (Carter *et al.* 1946).

The results given in Table 1 show that esterification of the free carboxyl groups does not affect greatly the amount of disulphide-S which reacts with bisulphite, i.e. fraction (*A* + *B*) is substantially unchanged, but it does increase the water-stable subfraction *B* considerably at the expense of water-labile subfraction *A*. To pursue the investigation further, wools of increasing CH₃ contents were prepared by each method of methylation. The initial CH₃, disulphide-S and fraction (*A* + *B*) of these wools are given in Table 2, whilst Fig. 1 shows graphically the relationship between subfraction *B* and the CH₃ content of the methylated and bisulphited wools. Subfraction *B* was determined after these wools had been rinsed in distilled water (Carter *et al.* 1946). Two lines are shown in Fig. 1: both have a slope corresponding to four CH₃ groups to each disulphide bond (i.e. 2 S atoms) of subfraction *B*. The upper line is drawn through the point 0.0% CH₃ and 0.58% S, these values corresponding to those of unmethylated, bisulphited wool after rinsing in distilled water. This line provides an excellent fit for the results on the wools methylated with methyl sulphate and a fairly good approximation for the wools methylated with methyl bromide. The lower line has the same slope, but cuts the axis at 0.42% S which is the value of the subfraction *B* of wool acetylated with cold acetic anhydride.

The simple conclusion seems therefore to emerge from these experiments that the difference in stability towards water of the thiol and *S*-cysteine sulphonate groups of subfraction *A* and *B* is due to the presence of free carboxyl groups of glutamic and aspartic acids in the neighbourhood of subfraction *A*.

Table 2. *The initial CH₃, disulphide-S and fractions (A + B) of methylated wools*

Methylating agent	CH ₃ (%)	Disulphide-S (%)	Fraction (A + B) (%)
Dimethyl sulphate	0.31	3.10	1.87
	0.46	3.16	1.82
	0.73	3.04	1.80
	0.99	3.17	1.86
	1.94	2.78	1.75
Methyl bromide	0.15	3.02	1.74
	0.23	2.92	1.80
	0.34	2.94	1.75
	0.51	2.82	1.75
	0.63	2.77	1.73
	0.81	2.79	1.79
Methanol-acetic anhydride	1.29	2.72	1.74
	0.14	3.11	1.66
	0.72	3.09	1.79
	0.86	3.06	1.83

Fig. 1. The relationship between subfraction B and degree of methylation of various methylated wools. $\odot$ Wool methylated with methyl bromide. $\times$ Wool methylated with methyl sulphate. $+$ Wool methylated with methanol-acetic anhydride.*The N-methyl of methylated wools*

Besides esterifying free carboxyl groups, there is some evidence (Blackburn *et al.* 1941) that the methylating agents used to prepare the methylated wools also cause some *N*-methylation. Attempts were made to measure the degree of *N*-methylation of the wools, since it may have influenced the relative water-stabilities of the thiol and *S*-cysteinesulphonate groups derived from subfractions A and B.

(a) *Direct estimations.* The *N*-methyl contents of the untreated and methylated wools (other analyses of which are given in Table 1) as determined by Pregl's method are given in Table 3.

Table 3. *The N-methyl contents of methylated wools*

Methylating agent	<i>N</i> -methyl (%)
(Untreated)	0.32
Dimethyl sulphate	0.64
Methyl bromide	0.73
Methanol-acetic anhydride	0.74

(b) *Indirect determination by estimations of terminal NH₂ groups.* (i) ϵ -Amino-N of lysine. Rutherford *et al.* (1937) showed that it is possible to estimate the ϵ -amino-N of the combined lysine in

Fig. 2. The rate of liberation of nitrogen from methylated wools on treatment with nitrous acid. $\bullet$ Untreated wool. $+$ Wool methylated with methanol-acetic anhydride. $\odot$ Wool methylated with methyl bromide. $\times$ Wool methylated with methyl sulphate.

wool from the rate of liberation of N_2 on treating wool with nitrous acid: *N*-methylation of this amino-acid should be detectable therefore by a decrease in the amino-N.

Fig. 2 shows the rate at which N_2 was liberated, when the untreated and methylated wools of Table 1

were treated with nitrous acid. Extrapolation of the straight line portion of the curves back to zero time gives the following values for the amino-N of the wools: untreated wool, 0.31%; methyl bromide-treated wool, 0.20%; dimethyl sulphate-treated wool, 0.37%; methanol/acetic anhydride-treated wool, 0.00%.

Some methylation of the ϵ -amino-N of lysine thus occurs with methyl bromide, but no explanation can be offered for the increase in the amino-N of the wool methylated with methyl sulphate, except that some slight hydrolysis may have taken place during this rather prolonged methylation. The disappearance of amino-N from the wool when methylated with methanol/acetic anhydride is not surprising, since it is probable (Blackburn & Phillips, 1944) that this reagent methylates and acetylates respectively the carboxyl and amino-groups of opposed side-chains. *N*-Acetylation rather than *N*-methylation is therefore the more probable explanation of the disappearance of the amino-N in this instance.

(ii) *Arginine*. Arginine is the other amino-acid in wool, with a terminal amino-group which might be methylated. Arginine estimations were therefore made on untreated wool and a wool (CH_3 , 2.0%) which had been methylated with methyl sulphate. Expressed as percentage of total N, the arginine-N contents of the wools by Vickery's (1940) method were: untreated wool, 18.7; methylated wool, 18.9. Although the arginine flavanate isolated from the methylated wool appeared to be pure, it was converted into the hydrochloride (Pratt, 1926) and the purity was tested chromatographically (Consden, Gordon & Martin, 1944). It was found that whereas the arginine hydrochloride obtained from the flavanate isolated from the untreated wool behaved normally, that from the flavanate isolated from the methylated wool did not give the usual colour when heated with ninhydrin. A mixture of the two hydrochlorides also gave no colour with ninhydrin, although both gave a very strong Sakaguchi reaction. The arginine from the methylated wool thus appeared to contain an impurity (possibly a *N*-methyl derivative of arginine) which inhibited the ninhydrin reaction and could not be separated readily from arginine by partition chromatography using the filter-paper technique.

Fraction (A + B) and subfraction B of the combined cystine-S of acetylated and deaminated wools

The S distributions of the wools which were acetylated and deaminated to modify their basic side-chains and then bisulphited are given in Table 4.

From Table 4 it will be seen that cold acetylation does not affect the magnitude of fraction (*A* + *B*), although subfraction *B* is decreased slightly. On the

Table 4. *The disulphide-S and subfractions A and B of acetylated and deaminated wools*

Treatment	Di-sulphide-S (%)	Fraction (<i>A</i> + <i>B</i>) (%)	Sub-fraction <i>A</i> (%)	Sub-fraction <i>B</i> * (%)
Acetylated (cold)	3.10	1.79	1.36	0.43
Acetylated (hot)	2.70	1.15	1.13	0.02
Deaminated	2.70	1.07	0.58	0.49

* Stable to rinsing in distilled water (Carter *et al.* 1946).

other hand, acetylation with boiling acetic anhydride and deamination both affect the reactivity of the combined cystine-S: hot acetylation causes fraction (*A* + *B*) to fall from 1.84 to 1.15%, whilst deamination causes it to fall to 1.07%. To some extent both these changes are due to the destruction of disulphide-S. In addition, it is probable that hot acetylation and deamination, besides altering the terminal amino-groups, also attack other centres in the wool.

DISCUSSION

Astbury (1942) has suggested that approximately equal numbers of polar and non-polar side-chains of wool are disposed on opposite sides of the polypeptide chains. Hence successive chains in a polypeptide grid may be separated alternately by polar and non-polar side-chains. Carter *et al.* (1946) have pointed out that such a disposition of side-chains might explain why the combined cystine of wool falls into two approximately equal fractions: fraction (*A* + *B*) which may cross-link the main-chains separated by polar side-chains and fraction (*C* + *D*) which may cross-link the main-chains separated by non-polar side-chains.

The present work has demonstrated that the relative magnitudes of subfraction *A* and *B* can be changed by the esterification of the free carboxyl groups of the combined glutamic and aspartic acids. Probably the success attending these experiments has been due to the mild and selective methods by which the carboxyl groups were esterified. Although our experimental evidence is less conclusive, the methylation or acetylation of the terminal amino-groups of lysine and arginine is without systematic effect. Hot acetylation and prolonged treatment of wool with nitrous acid would appear to attack too many different side-chains to bring about simple changes in the reactivities of subfractions *A* and *B*.

Since the chemical reactivities of these two subfractions are closely similar and tend to become identical when the free carboxyl groups are converted to carbomethoxyl groups, it is possible that whilst free carboxyl groups predominate in the

environment of subfraction *A*, amide groups predominate in the environment of subfraction *B*. On Astbury's (1942) calculations there are enough dicarboxylic acid residues and amide groups to provide each disulphide group of subfraction *A* with an environment containing 6 carboxyl groups and each disulphide group of subfraction *B* with an environment containing 6 amide groups. At present, it is inadvisable to pursue this suggestion further, since the available analyses of the glutamic and aspartic acids and their amides in wool are too variable.

Attempts were made to remove the amide groups of wool by hydrolysis with boiling 0.1N-HCl and to examine the reaction of the deaminated wool with sodium bisulphite. To lower the amide-N of the wool from 1.31 to 0.80%, the wool had to be boiled for 8 hr. The wool then tended to disperse in the bisulphite and made quantitative interpretation of the results impossible. It was observed, however, that the partially deaminated wool gave qualitatively the same reaction as normal wool with sodium bisulphite: fraction (*A* + *B*) was less than the total cystine-S and was divisible into subfraction *A* and *B* by water rinsing.

The observed influence of esterification on the relative magnitudes of subfractions *A* and *B* has a bearing on the question whether the different reactivities of the cystine subfractions arise from differences in the accessibility of the subfractions to the reagents we have used. In the first place, such differences in accessibility might arise from variation in the degree of 'crystallinity' of different parts of the fibres. One would expect that the variation in chemical reactivity to which this might give rise would tend to be eliminated by increasing the severity of the treatments. Experimentally, however, when the treatments are made more severe, the additional cystine-S that is caused to react undergoes a different type of reaction. For example, the more reactive fraction (*A* + *B*) is converted into thiol and *S*-cysteinesulphonate groups by bisulphite and into

lanthionine by alkalis: fraction (*C* + *D*), on the other hand, when caused to react by increasing the severity of the treatments, is converted into combined α -aminoacrylic acid by both these reagents.

Such differences in the type of reaction which takes place may arise from variations in the restrictions imposed on the movement of groups by the interactions of neighbouring side-chains. For example, the ease of reversibility of the reaction between the combined cystine of fraction (*A* + *B*) and sodium bisulphite may depend on the ease with which the thiol and *S*-cysteinesulphonate groups that are produced can move apart. This in turn may depend on the nature of the neighbouring side-chains. A possible explanation of the difference between subfractions *A* and *B* may be that whereas the neighbouring side-chains of subfraction *A* give salt-linkages, those of subfraction *B* give cross-linkages of a less stable character. On such assumptions, the thiol and *S*-cysteinesulphonate groups of subfraction *A* might be held closer together than those derived from subfraction *B*.

SUMMARY

1. A study has been made of the reactivity of the cystine-S of chemically modified wools.

2. Esterification of the free carboxyl groups of the combined glutamic and aspartic acids, whilst it does not increase the magnitude of the bisulphite-reactive fraction (*A* + *B*), causes the thiol and *S*-cysteinesulphonate groups produced from subfraction *A* to become stable to water-rinsing like those produced from subfraction *B*.

3. Hot acetylation and deamination by prolonged treatment with nitrous acid do not cause systematic changes in the magnitudes of subfractions *A* and *B*.

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REFERENCES

- Astbury, W. T. (1942). *J. Chem. Soc.* p. 337.
 Baernstein, H. D. (1932). *J. biol. Chem.* **97**, 663.
 Baernstein, H. D. (1936). *J. biol. Chem.* **115**, 25.
 Blackburn, S., Carter, E. G. H. & Phillips, H. (1941). *Biochem. J.* **35**, 627.
 Blackburn, S., Middlebrook, W. R. & Phillips, H. (1942). *Nature, Lond.*, **150**, 57.
 Blackburn, S. & Phillips, H. (1944). *Biochem. J.* **38**, 171.
 Burns, J. H. (1914). *Biochem. J.* **8**, 154.
 Carter, E. G. H., Middlebrook, W. R. & Phillips, H. (1946). *J. Soc. Dy. Col., Bradford*, **62**, 203.
 Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
 Doherty, D. G. & Ogg, C. L. (1943). *Industr. Engng Chem. (Anal. ed.)*, **15**, 751.
 Edlbacher, S. (1918). *Hoppe-Seyl. Z.* **101**, 278.
 Elsworth, F. F. & Phillips, H. (1938). *Biochem. J.* **32**, 837.
 Elsworth, F. F. & Phillips, H. (1941). *Biochem. J.* **35**, 135.
 Geake, A. & Nierenstein, M. (1914). *Biochem. J.* **8**, 287.
 Lindley, H. & Phillips, H. (1945). *Biochem. J.* **39**, 17.
 Middlebrook, W. R. & Phillips, H. (1942). *Biochem. J.* **36**, 428.
 Pratt, A. E. (1926). *J. Biol. Chem.* **67**, 351.
 Pregl, F. (1924). *Quantitative Organic Microanalysis*, London: Churchill.
 Rutherford, H. A., Harris, M. & Smith, A. L. (1937). *Bur. Stand. J. Res., Wash.*, **19**, 467.
 Shinohara, K. (1935a). *J. biol. Chem.* **109**, 665.
 Shinohara, K. (1935b). *J. biol. Chem.* **112**, 671, 683.
 Speakman, J. B. & Stott (1934). *J. Soc. Dy. Col., Bradford*, **50**, 341.
 Van Slyke, D. D. (1927). *J. biol. Chem.* **73**, 121.
 Vickery, H. B. (1940). *J. biol. Chem.* **132**, 325.