The Action of Sulphites on the Cystine Disulphide Linkages of Wool

4. METHYLATION OF THE THIOL GROUPS OF BISULPHITED WOOLS

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Elsworth & Phillips (1938 *a, b*, 1941) showed that some of the disulphide-S of wool reacted with NaHSO_3 according to the equation

where R' and R'' represent polypeptide chains. Middlebrook & Phillips (1942) subdivided the disulphide-S of wool into four fractions, which differed in their reactivity towards NaHSO_3 as follows: *Fraction A* reacted as in equation (1) giving SH and $\text{NaOSO}_2\cdot\text{S}$ groups which were labile, the reaction being easily reversed by water. *Fraction B* gave SH and $\text{NaOSO}_2\cdot\text{S}$ groups which were more stable to water. *Fraction C* was less reactive. *Fraction D* was changed to non-disulphide-S.* Each fraction roughly corresponded to one-quarter of the total disulphide-S of the wool, but fractions A and C were each somewhat smaller than fractions B and D.

In the present paper the action of methyl iodide, methyl bromide and dimethyl sulphate on bisulphited wool has been studied to see whether the thiol groups, produced by reaction (1), could be methylated. The methylation of these groups would destroy the reversibility of the bisulphite reaction and hence might cause the remaining disulphide-S (fractions C and D) to react with NaHSO_3 . In addition, a determination of the amount of S-methylcysteine produced in the wool would provide additional evidence as to the extent of the bisulphite reaction.

METHODS

Two wools have been used: a New Zealand wool of 56's quality and a Cape wool of 64's quality. The tips of the locks were cut off before the wools were cleaned by methods previously described (Elsworth & Phillips, 1941). During the cleansing and subsequent carding, discoloured and damaged fibres were removed by hand. Before analysis, the untreated and treated wools were conditioned by exposure to an atmosphere of 21° and 70% relative humidity until they attained constant weight. Total-S was determined by Barritt's modification (1934) of the Benedict-Denis method; thiol-S and disulphide-S by Shinohara's

modification (1935 *a, b*) of the Folin-Marenzi method (1929), using a Spekker absorptiometer. Methionine-S was calculated from a determination of methionine by Baernstein's method (1936). All analytical results are calculated on the weight of anhydrous wool taken for analysis. In the experiments described below the New Zealand wool was used unless otherwise stated. It contained total-S, 3.22%; thiol-S and disulphide-S, 2.66%; and methionine-S, 0.14%; the corresponding values for the Cape wool were 3.79, 2.99 and 0.09%.

Methylation of bisulphited wool

When bisulphited wool was treated with methyl iodide, methyl bromide or dimethyl sulphate in buffer at pH 8, the main reaction which occurred was the reformation of disulphide-S from the thiol and S-cysteine-sulphonate groups (Elsworth & Phillips, 1938*a*; Middlebrook & Phillips, 1942). This reversal of the bisulphite reaction did not take place when the wool was methylated by the following methods:

(a) *Methylation of unrinsed, bisulphited wool with methyl iodide.* The wool was immersed in a 20% solution of NaHSO_3 (50 ml./g. of wool) for 24 hr. at room temperature, these conditions having been shown to give a wool containing the maximum number of thiol and S-cysteine-sulphonate groups (Middlebrook & Phillips, 1942). It was then squeezed and put in a stoppered bottle with a visible excess of methyl iodide. The bottle was placed in a dark cupboard for 3 days, during which it was occasionally shaken. The methylated wool was squeezed, washed three times in benzene, air-dried, allowed to stand for 18 hr. in distilled water which was changed frequently, dried, conditioned and analyzed. When the methylated wool was not extracted with water, it was stained brown during the initial stages of hydrolysis (possibly due to the liberation of iodine) and the thiol-S content of its hydrolysate was very low. During methylation, the wool sometimes acquired a deep yellow colour, which was removed by benzene. This colour may have been due to the formation of a loose addition compound between SO_2 and reactive groups of the wool; it was not observed when rinsed bisulphited wool was methylated, or when methyl bromide was used as the methylating agent.

(b) *Methylation of rinsed, bisulphited wool with methyl iodide.* The wool, bisulphited as described above, was squeezed, rinsed in distilled water (Middlebrook & Phillips, 1942), dried and conditioned. It was methylated with methyl iodide, a few ml. of water being added to the bottle.

(c) *Methylation of rinsed and unrinsed bisulphited wools with methyl bromide.* The wools were methylated with methyl bromide in a tightly stoppered bottle under conditions similar to those described in (a) and (b). At the end

* The term 'non-disulphide-S' is used throughout this paper to denote S not present as either disulphide-S, thiol-S or methionine-S.

of the methylation, the methyl bromide was allowed to evaporate, and before analysis the methylated wool was extracted with water.

Simultaneous treatment of wool with NaHSO₃ and dimethyl sulphate

The wool was shaken for 90 min. with 5% NaHSO₃ (30 ml./g.) and dimethyl sulphate (0.2 ml./g.). A small amount of the wool was then removed, rinsed six times in water, squeezed, dried and conditioned. The treatment was repeated on the remaining wool.

The action of buffer solutions on wool which had been bisulphited and methylated

The wool (1.0 g.) was soaked at room temperature in borate buffer of pH 8 (0.4M. with respect to H₂BO₃), and squeezed and immersed for 1 hr. in 200 ml. of the same buffer maintained at 50°. It was then rinsed twice with water, dried and conditioned.

The isolation of dimethyl sulphide from bisulphited and methylated wool

Wool which has been bisulphited and methylated often has a marked odour resembling that of dimethyl sulphide, which becomes more pronounced when the wool is hydrolyzed. Dimethyl sulphide was identified among the hydrolysis products of Cape wool (3.5 g.; thiol-S, 0.92%; disulphide-S, 0.41%) which had been treated simultaneously with NaHSO₃ and dimethyl sulphate. This wool was boiled for 7 hr. with 120 ml. 5N-HCl, through which air was drawn and then passed through solutions of Hg(CN)₂ and HgCl₂ (Blackburn & Challenger, 1938). After recrystallization from benzene, the small amount of white crystals formed in the HgCl₂ solution had m.p. 153–4° alone and when mixed with an authentic specimen of the mercuri-chloride of dimethyl sulphide (Blackburn & Challenger, 1938; Challenger, Taylor & Taylor, 1942). Methyl mercaptan was not liberated in appreciable amounts.

Preparation of acetyl derivatives of S-methylcysteine

L-N-Acetyl-S-methylcysteine. *S*-Methylcysteine (du Vigneaud, Loring & Craft, 1934) was acetylated with acetic anhydride and NaOH (Synge, 1939). After acidification with 6N-H₂SO₄ and evaporation to dryness *in vacuo*, the acetyl derivative was extracted with hot ethyl acetate. Removal of the solvent *in vacuo* left a gum which slowly solidified in a vacuum desiccator. It then had m.p. 60–70°, $[\alpha]_D^{18} - 33.6^\circ$ in water (*l*, 2; *c*, 1.22). This substance was very soluble in water, ethanol, acetone and ethyl acetate, and insoluble in benzene and light petroleum. It could not be induced to crystallize from any of these solvents. After extraction from aqueous solution by 20% butanol-chloroform mixture, removal of the solvent and drying in a vacuum desiccator, the *L-N*-acetyl-*S*-methylcysteine had m.p. 73–80°, $[\alpha]_D^{18} - 37.8^\circ$ in water (*l*, 1; *c*, 0.74). Found: C, 41.3; H, 6.4; N, 8.1%; acid equiv. wt. 179. C₆H₁₁O₂NS requires C, 40.7; H, 6.2; N, 7.9%; acid equiv. wt. 177.

DL-N-Acetyl-S-methylcysteine. *L*-*S*-Methylcysteine was acetylated under racemizing conditions (du Vigneaud & Meyer, 1932). After addition of the calculated amount of 6N-H₂SO₄ and evaporation to dryness *in vacuo*, the acetyl derivative was extracted with 20% butanol-chloroform.

After recrystallization from ethanol it had m.p. 153–155°, and after further recrystallization from either ethanol or acetone, m.p. 155–156°, $[\alpha]_D^{18} 0^\circ$. Found: C, 40.8; H, 6.5; N, 8.0%; acid equiv. wt. 174. C₆H₁₁O₂NS requires C, 40.7; H, 6.2; N, 7.9%; acid equiv. wt. 177. The same compound (m.p. and mixed m.p.) was obtained by racemization of the sodium salt of the *L*-acetyl derivative with acetic anhydride (du Vigneaud & Meyer, 1932).

Racemization of L-N-acetyl-S-methylcysteine by heat

The *L*-acid, m.p. 73–80° (0.1015 g.) was heated *in vacuo* at 100–110°. Soon after heating was started, the melt began to solidify. At the end of 4 hr., the weight of the acid became constant (0.0997 g.). It had m.p. 140–152°, $[\alpha]_D^{18} - 10^\circ$ in water (*l*, 1; *c*, 0.80), acid equiv. wt. 180, but when recrystallized from acetone it gave the pure *dl*-acid, m.p. 153–155°, alone or mixed with an authentic specimen.

Partition chromatography of N-acetyl-S-methylcysteine: estimation of S-methylcysteine in bisulphited and methylated wool

With the procedure of Martin & Synge (1941) and Gordon, Martin & Synge (1943), *L-N*-acetyl-*S*-methylcysteine was found to have a rate $R=0.13$ on a 1% butanol-chloroform column. It would thus be included in the (proline + valine + methionine) fraction in an amino-acid analysis. These amino-acids can be separated from one another by employing 30% propanol-cyclohexane. *L-N*-Acetyl-*S*-methylcysteine moved with $R=0.51$ in this solvent, and a mixture of this compound and *N*-acetyl-methionine readily separated into two bands. The separation of *N*-acetyl-*S*-methylcysteine from a hydrolysate of bisulphited and methylated wool was therefore carried out according to the scheme given in Table 1.

Table 1. *Chromatographic analysis of bisulphited and methylated wool*

The procedure of Gordon *et al.* (1943) was employed to estimate the *S*-methylcysteine in the treated wools, an amount of the acetylamino-acids corresponding to 20 mg. wool being used in each determination. The band 3.3 corresponding to *N*-acetyl-*S*-methylcysteine was titrated with 0.01N-Ba(OH)₂ with bromothymol blue as internal indicator. No band corresponding to 3.3 was obtained from untreated wool. In a control experiment, *S*-methylcysteine was added to untreated wool before hydrolysis. Titration of the band 3.3 indicated 1.04 mg. of *S*-methylcysteine, the actual amount in the portion taken being 1.00 mg. (20 mg. wool).

In an experiment carried out to isolate the acetyl derivative, the acetylamino-acids from a hydrolysate of 2 g. wool (Cape, methylated 27 times with NaHSO₃ and methyl sulphate; thiol-S, 0.92%; disulphide-S, 0.41%) were extracted with 17% butanol-chloroform. The solvent was

removed *in vacuo* and 3% butanol-chloroform used as fractionating solvent on a 60 g. column. Band 1 (phenylalanine) and band 2 (leucine) were run out and band 3 collected. A fourth band appeared above this which had not been noticed previously on the small columns. It probably corresponded to one of the 'artefact bands' noted by Gordon *et al.* (1943). Band 3 was evaporated to dryness *in vacuo* and run on a second 60 g. column with 30% propanol-cyclohexane. It was resolved into five bands: band 1 (valine), band 2 (methionine), band 3 (*S*-methylcysteine), band 4 (unknown) and band 5 (proline). The band corresponding to *S*-methylcysteine on evaporation to dryness *in vacuo* gave rosettes of gummy needles (85 mg.) similar in appearance to synthetic *L*-*N*-acetyl-*S*-methylcysteine. This material was racemized by treating the sodium salt with acetic anhydride (du Vigneaud & Meyer, 1932). Crystallization from acetone then gave needles m.p. 153°, alone or mixed with synthetic *DL*-*N*-acetyl-*S*-methylcysteine. Found: N, 8.1%; acid equiv. wt. 175. No attempt was made to characterize the unknown material from band 4, which may possibly have been the acetyl derivative of an *N*-methylamino-acid.

RESULTS

When bisulphited wool is treated with methylating agents we might expect the thiol groups to be converted to *S*-methyl groups (2).

Clarke (1932) observed that cystine with Na_2SO_3 and benzyl chloride gave a 60–80% yield of *S*-benzylcysteine calculated on the supposition that only the thiol groups, produced according to equation (1), react. Patterson, Geiger, Mizell & Harris (1941) found that the thiol groups in wool reduced by thiolacetic acid were methylated readily by methyl iodide.

The thiol-S found after acid hydrolysis of bisulphited wool methylated with methyl iodide or methyl bromide (Table 2), or wool which has been treated repeatedly with NaHSO_3 and dimethyl sulphate (Table 3), could be derived from the *S*-cysteinesulphonate groups, which are converted to thiol groups on hydrolysis with hydrochloric acid (Elsworth & Phillips, 1938*a*). We have found that *S*-methylcysteine does not interfere with the determination of these groups by Shinohara's method (1935*a*).

Remethylation, with either methyl bromide or methyl iodide, of bisulphited and methylated wools, after they had been rinsed with borate buffer of pH 8 to facilitate methylation, did not decrease the thiol-S content of their hydrolysates. These also remained unchanged when the bisulphited and methylated wools were shaken with phosphate buffer containing methyl bromide or iodide (Patterson *et al.* 1941). The failure to remove thiol-S by

Table 2. *The S-distributions of hydrolysates of unrinsed and rinsed bisulphited wools before and after methylation with methyl iodide or methyl bromide*

Before methylation			After methylation		
Thiol-S (%)	Disulphide-S (%)	Non-disulphide-S (%)	Thiol-S (%)	Disulphide-S (%)	Non-disulphide-S (%)
Unrinsed bisulphited wool					
(a) Methylated with methyl iodide					
1.59	0.75	0.74	0.81	0.37	1.90
1.38	1.00	0.70	0.88	0.49	1.71
1.44	0.97	0.67	0.80	0.45	1.83
(b) Methylated with methyl bromide					
1.56	0.85	0.67	0.93	0.23	1.92
—	—	—	0.87	0.28	1.93
—	—	—	0.84	0.25	1.99
Rinsed bisulphited wool					
(a) Methylated with methyl iodide					
0.84	1.67	0.57	0.50	1.53	1.05
0.72	1.97	0.39	0.30	1.93	0.85
0.77	1.80	0.51	0.21	1.86	1.01
(b) Methylated with methyl bromide					
0.67	2.03	0.38	0.34	1.60	1.14

Table 3. *The S-distribution of the hydrolysates of wools that have been simultaneously bisulphited and methylated with NaHSO₃ and dimethyl sulphate*

No. of methylations	Thiol-S (%)	Disulphide-S (%)	Non-disulphide-S (%)
2	0.68	1.45	0.95
4	0.75	0.94	1.39
6	0.71	0.92	1.45
8	0.71	1.14	1.23
10	0.72	1.08	1.28
12	0.80	0.68	1.60
14	0.81	0.52	1.75
16	0.77	0.82	1.49
18	0.77	0.55	1.76

remethylation provides strong evidence that the bisulphited and methylated wools did not contain thiol groups and supports the conclusion that the thiol-S found in their hydrolysates arose by the acid hydrolysis of *S*-cysteinesulphonate groups. This was confirmed by the presence of SO_4^- in the hydrolysates of bisulphited and methylated wools, which, before hydrolysis, had been extracted with water until free from SO_4^- .

Attempts to determine the *S*-methyl groups directly by the methyl iodide evolved on distillation of the methylated wools with HI were unsuccessful. During 5 hr. heating with HI, *S*-methylcysteine yielded only 1.5–4.3% CH_3 as compared with the theoretical value of 11.1% (see also Lacourt, 1935; Arndt, Loewe & Ozansoy, 1939).

By partition chromatography, *S*-methylcysteine was isolated from the hydrolysates of unrinsed,

bisulphited and methylated wools in amounts approximately equivalent to the thiol-S contents of the hydrolysates (Table 4).

Table 4. *The S-methylcysteine contents, as determined by partition chromatography, of wools which had been bisulphited and methylated*

Treatment of wool (Cape)	Thiol-S in hydro- lysate (%)	Disul- phide-S in hydro- lysate (%)	S-methyl- cysteine (as S) (%)
Bisulphited, unrinsed wool	1.74	1.04	0.0
Bisulphited, unrinsed wool, methylated with methyl iodide	0.97	0.48	0.75
Wool simultaneously bisulphited and methylated with NaHSO ₃ and di- methyl sulphate	0.92	0.41	0.89

DISCUSSION

By methylation of unrinsed and rinsed bisulphited wools with methyl iodide or bromide, the thiol-S in their hydrolysates can be approximately halved. This may be due either to the methylation of all the thiol groups or to the methylation of half the thiol groups and the conversion of their opposed *S*-cysteinesulphonate groups into *SCH*₃ groups. The following experiments showed that the methylation was restricted to the thiol groups.

When fully bisulphited wool is rinsed with water approximately half the thiol and *S*-cysteinesulphonate groups revert to disulphide groups (see Table 2 and Middlebrook & Phillips (1942)). All the bisulphited and methylated wools described in the present paper were rinsed in water before analysis, but the analyses given in Table 2 show that such rinsing did not lead to any increase in disulphide-S. Similarly, it was found that the bisulphite reaction (1) could be reversed completely by treating bisulphited wool for 1 hr. at 50° in pH 8 buffer. For example, a bisulphited wool (giving 1.56% thiol-S on hydrolysis) gave, after treatment with buffer, a hydrolysate containing 2.68% disulphide-S but only 0.09% thiol-S. On the other hand, when unrinsed or rinsed bisulphited wools were treated with buffer in this way after methylation, no appreciable alteration was caused in the thiol-S contents of their hydrolysates: an unrinsed bisulphited wool methylated with methyl iodide, gave a hydrolysate containing 0.80% thiol-S and 0.45% disulphide-S before treatment with buffer, whilst after treatment its hydrolysate contained 0.79% thiol-S and 0.49% disulphide-S. Similar results were obtained with unrinsed, bisulphited wool after methylation with methyl bromide and with a wool methylated with NaHSO₃ and dimethyl sulphate.

Elsworth & Phillips (1938b) showed that it was possible to determine the *S*-cysteinesulphonate groups in bisulphited wool by distilling the wool with dilute phosphoric acid, when one molecule of SO₂ was liberated for each *S*-cysteinesulphonate group decomposed. Under similar conditions, the bisulphited and methylated wools did not yield SO₂. Either the *S*-cysteinesulphonate groups they contained did not decompose, or alternatively they decomposed to yield NaHSO₄ and cysteine, as they do when decomposed with hydrochloric acid.

Wool cannot be fully bisulphited with cold 5% NaHSO₃ (Middlebrook & Phillips, 1942). In spite of this, when wool is simultaneously bisulphited and methylated with 5% NaHSO₃ and dimethyl sulphate (Table 3), the reaction proceeds to the usual limit and the analyses show that the wool finally contains as many *SCH*₃ and *S*-cysteinesulphonate groups as wool which was first bisulphited with 20% NaHSO₃ and then methylated. It thus appears that the methylation of the thiol groups produced when the disulphide-S fractions A and B react with bisulphite does not enable the disulphide-S fractions C and D to yield thiol and *S*-cysteinesulphonate groups with NaHSO₃. The analyses show, however, that fractions C and D are largely converted into non-disulphide-S. The state of chemical combination of this S has not been elucidated, but may be similar to that of the non-disulphide-S produced from fraction D, when wool is bisulphited (Middlebrook & Phillips, 1942).

The small amount of dimethyl sulphide evolved when bisulphited wool is methylated may arise from the decomposition of sulphonium groups, formed by the addition of the methylating agents to *S*-methylcysteine side-chains. The S so removed from the wool would be returned in our analyses as non-disulphide-S.

In addition to the methylation of thiol groups, some *O*- and *N*-methylation takes place when bisulphited wool is methylated with methyl halides or when wool is simultaneously bisulphited and methylated (Blackburn, Carter & Phillips, 1941). For example, an unrinsed bisulphited wool (thiol-S 0.78%, disulphide-S 0.39%), methylated with methyl iodide, was found by Baernstein's method (1936) to contain 0.60% CH₃ linked to *O*.

The physical properties of bisulphited and methylated wools are still under investigation. It may be said, however, that when they are wet, the resistance of the fibres to extension is much less than that of normal wool fibres. In this respect they are similar to the *S*-methylated wools of Harris, Mizell & Fourn (1942), prepared by the action of methyl iodide on wools reduced by thiolacetic acid.

SUMMARY

1. The thiol groups produced when wool is treated with NaHSO_3 can be methylated by methyl iodide or methyl bromide; the *S*-cysteinesulphonate groups are unaffected. A similar reaction occurs when wool is treated simultaneously with NaHSO_3 and dimethyl sulphate.

2. A technique for the isolation of *S*-methylcysteine from hydrolysates of *S*-methylated wools by partition chromatography of the *N*-acetylated amino-acids is described.

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Human-milk Fat

1. COMPONENT FATTY ACIDS

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It has long been known that human-milk fat has marked differences in analytical characteristics from cow-milk fat. Thus Halden & Grün (1929), surveying the data then available, quoted ranges of observed values as follows: Reichert-Meissl 1·4–3·4, Polenske 1·5–2·2, iodine value 36–54, and saponification equivalent 268–273; they suggested that the chief fatty acids are oleic (52–54%) and palmitic (25–35%), with myristic, lauric and probably decanoic acids, and possibly small quantities of octanoic, hexanoic and butyric acids. Elsdon (1928) has recorded the following characteristics for a mixed sample of human-milk fats at very early stages of lactation: Reichert-Meissl 3·4, Polenske 1·9, Kirschner 2·0, i.v. 35·9, and has also observed the following average figures for carbohydrate,

protein and fat in human milk, based upon over 500 specimens:

Stage of lactation	Lactose (g./100 ml.)	Protein (g./100 ml.)	Fat (g./100 ml.)
1–7 days	6·8	2·1	2·86
8–28 days	6·8	1·6	3·58
1–9 months	6·9	1·3	3·44

According to these figures the proportions of lactose, protein and fat in human milk do not differ greatly from those of cow's milk.

Bosworth (1934) separated the acids from about 3 lb. of human-milk fat, by fractional distillation of the methyl esters, into 30 fractions. The original fat had sap. equiv. 273·4, i.v. 56·2, Reichert-Meissl value 2·5, Polenske value 0·1, and contained 1·1%