65. THE METHYLATION OF WOOL WITH METHYL SULPHATE AND METHYL HALIDES

By STANLEY BLACKBURN, ERIC GEORGE HEMPHILL CARTER
AND HENRY PHILLIPS

From the Wool Industries Research Association, Headingley, Leeds, 6

(Received 19 April 1941)

ALL the recognized methods of *O*- or *N*-methylation have been applied to proteins. Casein, for example, was boiled with CH_3I and KOH by Skraup & Krause [1909] and later treated with diazomethane by Geake & Nierenstein [1914]. Herzig & Landsteiner [1914] also used diazomethane to methylate casein, edestin and silk fibroin. The same authors [1918] later studied the methylation of wool with diazomethane and after eighteen treatments with this reagent obtained wool with a methoxyl content of 5.89 and 6.28% of CH_3 linked to *N*. Treatment of wool with methyl alcoholic hydrochloric acid gave a methoxyl content of 3.80 and 1.49% CH_3 as *N*-methyl. Edlbacher [1919] methylated a number of protamines with methyl sulphate and NaOH and determined the number of *N*-methyl but not of *O*-methyl groups. The conclusions drawn from these investigations were rendered doubtful by the discovery of methionine by Mueller [1921]. The *S*-methyl group of this acid yields CH_3I when boiled with HI and thus contributed to the CH_3 content of some of the methylated proteins prepared before 1921. More recently, Felix & Rauch [1931] and Felix & Reindl [1932] have prepared methyl esters of proteins by the action of dry HCl on their suspensions in CH_3OH . They found that the products could be further methylated by either methyl sulphate at pH 8 or 9, or by diazomethane. These more fully methylated proteins did not give the Pauly diazo reaction for the free iminazole ring or the Millon reaction for tyrosine. Haurowitz [1938] methylated egg albumin and horse haemoglobin with methyl sulphate. Rutherford *et al.* [1940] have recently made an extensive investigation of the methylation of silk fibroin with diazomethane.

Whilst attempting to methylate wool to obtain evidence of the existence of free thiol groups, we have found that it is possible to introduce CH_3 groups into wool keratin by using methyl sulphate, CH_3I or CH_3Br at ordinary temperatures. Evidence is given below that this reaction is largely, but not entirely, an esterification and appears to take place only when the wool is within its isoelectric region. Some evidence has been obtained that *N*-alkylation occurs at the same time.

EXPERIMENTAL

Unless otherwise stated, the wool used was in the form of a loosely woven flannel, similar to that described in previous investigations [Elsworth & Phillips, 1938].

Determination of CH_3 linked to O and S. All the methylated wools were analysed by Baernstein's method [1932; 1936] in which CH_3 groups linked to *O* and *S*, but not to *N*, are removed as CH_3I . Baernstein [1932] found that eleven different amino-acids and also glucose, when tested by this method, did not yield

any volatile iodide. A similar result was obtained, during the present work, with methylamine. In addition, Barritt [1934] and Bailey [1937], using the method to determine the methionine in wool, were able to show that the sum of the cystine- and methionine-S was almost equal to the total S, suggesting that the method, when applied to wool, does not yield volatile iodide from unexpected sources.

Methylation of wool with methyl sulphate

(a) *Influence of pH on the reaction.* The wool used in these experiments was in the form of a worsted serge and had been scoured commercially. It was extracted with light petroleum and methylated by shaking for 90 min. with solutions of methyl sulphate in various buffers; 20 ml. of buffer containing 0.2 ml. of methyl sulphate per g. of conditioned¹ wool were used. At the end of each methylation the wool was rinsed in six changes of water, passed through a wringer after each rinse, dried and conditioned. The CH₃ content of the unmethylated wool was 0.06%. The CH₃ contents of the methylated wools, together with the pH of the methylating solutions at the beginning and at the end of each methylation are given in Table 1. The experimental conditions chosen for these methylations were not ideal, since the pH of some of the solutions changed too much. The results suggest, however, that the maximum degree of methylation is attained when the pH of the methylating solution at the end of the treatment is within the range 2.5–8.5.

Table 1. *The influence of pH on the methylation of wool with methyl sulphate*

Buffer	Initial pH	Final pH	CH ₃ %	Total CH ₃ , less methionine- CH ₃ %
0.02N H ₂ SO ₄	—	—	0.13	0.07
Phosphate	6.0	2.0	0.29	0.23
"	7.0	2.5	0.37	0.31
"	8.0	2.8	0.34	0.28
Borate	9.0	8.1	0.34	0.28
"	10.0	8.6	0.35	0.29
"	12.0	7.0	0.37	0.31
Acetate	12.0	9.0	0.13	0.07
"	12.0	11.8	0.06	0.00

(b) *Repeated methylation with methyl sulphate.* The above experiments established optimum pH conditions for methylating wool, using a limited amount of methyl sulphate. Repetitions of these treatments were now made to see whether an upper limit existed to the amount of methylation which could be produced in this way. Wool was shaken for 90 min. with phosphate buffer, 0.15M with respect to H₃PO₄ (20 ml./g.) and methyl sulphate (0.2 ml./g.). The pH of the solution fell during this time from 7.6 to 6.3. A sample of the wool (3–4 g.) was then removed, rinsed with water, as previously described, dried, conditioned and analysed. The remaining wool was methylated again for the same length of time with fresh buffer and methyl sulphate. The CH₃ contents of wool methylated up to twenty times in this manner are plotted against the

¹ All the wools described in this paper as conditioned had been exposed to a controlled atmosphere of 21° and 70% R.H. until they attained a constant weight. It was found that methylation did not alter the moisture content (14%), which was reached in this atmosphere at equilibrium. Although all the analyses were made on conditioned wool, the results are recorded as percentages of the anhydrous wool.

number of methylations in Fig. 1. This figure also contains a curve summarizing the results obtained when another sample of wool was treated with methyl sulphate (0.2 ml./g.) and sodium acetate (0.35 g./g. in 15 ml. water), these conditions being somewhat more drastic than when phosphate buffer was used. At 90 min. intervals small samples of wool were withdrawn for analysis and a further 0.2 ml. methyl sulphate and 0.35 g. sodium acetate added to the original

Fig. 1. The methylation of wool with methyl sulphate in phosphate and acetate buffers. +—+ Acetate buffer. •—• Phosphate buffer.

solution. This was repeated nine times and then, after the wool had been washed and dried, for a further eight times. In the phosphate buffer, the initial rate of reaction was fairly rapid; in the acetate buffer, the rate of reaction was more uniform. In both buffers, however, the methylation appears to be reaching a limiting value of 1.9–2.0 % CH_3 .

Methylation of wool with methyl iodide and methyl bromide

(a) *Methylation of wool with CH_3I .* For reasons that will become apparent later, it was thought that if wool reacted at ordinary temperatures with methyl sulphate it should also react with methyl iodide. Conditioned wool (1 part) was placed in a bottle containing CH_3I (6 parts), and kept at ordinary temperatures in the dark. At intervals samples of the wool were withdrawn, washed with benzene and then extracted with benzene in a Soxhlet apparatus, quinoline being placed in the distillation flask to retain the extracted CH_3I . The CH_3 content of the extracted wool was determined. A portion of the benzene-extracted wool was boiled with water through which steam was passed for 1 hr., dried, conditioned, and the CH_3 content redetermined. Another portion of the benzene-extracted wool (1.5 g.) was shaken with phosphate buffer (pH 6.0, 75 ml.) for $3\frac{1}{2}$ hr., rinsed in water, dried, conditioned and analysed. In Table 2, the CH_3 contents of wools that had been methylated with CH_3I for increasing periods of time and then extracted with benzene are recorded, together with the CH_3 contents of the extracted wools after distillation in steam and also after extraction with phosphate buffer. From these results it is apparent that after the methylated wool has been extracted with benzene, subsequent distillation in steam or extraction with phosphate buffer causes only small decreases in its CH_3 content.

Table 2. *Percentage CH₃ content of wool attained at ordinary temperatures with CH₃I*

Time of exposure to CH ₃ I, hr. ...	2	6	17	24	90	144	336
(i) After extraction with benzene	0.38	0.30	0.38	0.43	0.53	0.41	0.45
(ii) After extraction with benzene followed by steam distillation for 1 hr.	0.36	0.24	—	0.36	0.49	0.35	0.44
(iii) After extraction with benzene followed by extraction with buffer pH 6.0	0.36	0.23	0.36	0.39	0.52	0.40	—

It appears that wool reacts fairly rapidly with CH₃I, and that prolonged periods of exposure cause only a slight further reaction. The irregular variation in the analyses after different times of exposure is most likely due to slight variation in the samples of wool taken for each experiment, and the CH₃ figures after, say, 2 days may be taken to be sensibly constant. Another irregularity that has been observed is that the rate of reaction may vary within wide limits. For example, another sample of wool brought to equilibrium with a phosphate buffer of pH 5.9, and then dried and conditioned, at first reacted much more slowly; after 2 hr. exposure to CH₃I its CH₃ content was 0.09 %, after 24 hr. 0.19 %, but after 144 hr. 0.45 %. The reaction appears to depend on the physico-chemical condition of the wool. Thus when conditioned wool was heated under reflux with CH₃I for 2 hr., it contained 0.30 % CH₃ after benzene extraction. On the other hand, when the CH₃I was diluted with an equal volume of ether, and the wool heated under reflux for 2 hr., its CH₃ content, after benzene extraction, was 0.14 %. Dried wool reacted with CH₃I very slowly; after 72 hr. exposure, the wool contained only 0.09 %, i.e. only slightly more than that due to methionine (0.06 %).

Presumably when wool is methylated with CH₃I, for each CH₃ group introduced, one atom of anionic iodine is liberated. The determination of this anionic iodine thus provides an additional measure of the degree of methylation that occurs. The samples (1.5 g.) of methylated and benzene-extracted wools, the analyses of which are given in Table 2, were shaken for 3½ hr. with 75 ml. of buffer, pH 6.0. These conditions were chosen as the result of separate experiments which showed that this method gave the maximum extraction of iodide. The filtered buffer and washings from the wool were acidified with dilute HNO₃, the iodide precipitated with silver nitrate and the excess silver nitrate determined with potassium thiocyanate. The results are recorded in Table 3, in which CH₃ equivalent to the iodide is also given. By subtracting the methionine-CH₃ (0.06 %) from the figures given for the total CH₃ in Table 2, the figure for the total *O*-methyl is obtained. This is seen to be less than the CH₃ equivalent of the anionic iodide found. The extra methylation that occurs, as revealed by the

Table 3. *Anionic iodine contents of wool exposed to CH₃I for increasing periods of time and then extracted with benzene*

Time of exposure hr.	Anionic iodine %	CH ₃ , equivalent to anionic I found %	Observed CH ₃ , less methionine-CH ₃	Excess CH ₃ (calc.) %
2	3.68	0.43	0.32	0.11
6	3.46	0.41	0.24	0.17
17	3.83	0.45	0.32	0.13
24	3.98	0.47	0.37	0.10
90	5.30	0.63	0.47	0.16
144	3.83	0.45	0.35	0.10

iodide figures, probably represents *N*-methylation which is not included in the CH_3 as determined by the Baernstein method. This excess CH_3 is given in the fifth column of Table 3.

(b) *Repeated methylation of wool with CH_3I* . The wool (30 g.) was exposed to CH_3I , as in the previous experiments, except that the operation was repeated four times at intervals, recorded in Table 4. Before each exposure, the wool was buffered to pH 7.9 with borate buffer.

Table 4. *The CH_3 contents of wool after repeated exposures to CH_3I*

Time of exposure days	CH_3 %	I %
14	0.52	4.77
28	0.80	—
46	1.02	—
88	1.06	—

After hydrolysis for 24 hr. with 5*N* HCl and evaporation *in vacuo*, the CH_3 content of the hydrolysate was 0.10 %.

(c) *Methylation of wool with CH_3Br* . Conditioned wool (1 part), buffered to pH 7.7 in borate, was treated with CH_3Br (1.5 parts) in a tightly stoppered bottle at room temperature. At intervals, the pressure was released, the CH_3Br allowed to evaporate and the wool conditioned and analysed. The results obtained are given in Table 5.

When wool, methylated in this manner and having 1.30 % CH_3 , was subjected to complete hydrolysis with HCl, the CH_3 content of the hydrolysate was 0.08 %. It is noteworthy that the 'excess' CH_3 introduced by the action of CH_3Br is greater than that obtained using CH_3I .

Table 5. *Methylation of wool with CH_3Br*

Total time of exposure, days	CH_3 %	Br %	'Excess' CH_3 (calc.), %
1	0.29	—	—
4	0.58	4.98	0.42
(CH_3Br renewed)			
6	0.65	—	—
18	0.92	7.53	0.55
(CH_3Br renewed)			
44	1.30	—	—
(Re-buffered and CH_3Br renewed)			

(d) *Methylation of silk*. Because of the difference in chemical constitution between wool and silk, it was of interest to study the methylation of silk in a

Table 6. *Methylation of silk*

(a) With methyl sulphate (phosphate buffer).

No. of methylations	CH_3 , %
2	0.20
5	0.40
9	0.45
11	0.46
13	0.50
14	0.45

(b) With CH_3I

Time of exposure days	CH_3 %	I %	CH_3 , equivalent to I found %	'Excess' CH_3 (calc.) %
19	0.37	4.72	0.56	0.19

similar manner. Silk, in the form of an unweighted fabric, was methylated with methyl sulphate in a phosphate buffer, and with CH_3I , using the conditions previously described for wool. The results are given in Table 6.

Stability of the methoxyl groups introduced

The CH_3 content found by analysis after these treatments might conceivably have been due to retention of the methylating reagent in the wool without any definite chemical reaction having occurred. Experiments were made to determine whether the CH_3 content could be reduced by certain mild treatments.

(i) After heating in a strong stream of air at 115° for 2 hr., samples of wool methylated with methyl sulphate and containing 0.30 and 1.62 % CH_3 were found to give unchanged analyses, although the same treatment for 10 min. would remove 14 % of water from conditioned wool. Further, wool methylated with CH_3I and then extracted with benzene contained 0.30 % CH_3 after heating in a current of air at 115° for 30 min. and 0.29 % after 60 min.

(ii) In order to obtain evidence of the degree of resistance to hydrolysis of the methyl groups which had been introduced, the wool, methylated with methyl sulphate, was steam distilled. Wool (2 g.) containing 0.36 % CH_3 was covered with water (20 ml. pH 8) and steam was blown into the water for the times given in Table 7. At the end of each distillation, a sample of the wool was dried, conditioned and then analysed. The analyses show that the CH_3 is held tenaciously by the wool, although it is gradually lost as the time of distillation increases. The results of a similar series of experiments on methylated wools of higher CH_3 contents are also given in Table 7. In these experiments, before the steam was blown into the flask, the methylated wool was covered with 5 ml. of a borate buffer (pH 7) and 15 ml. water, in order to prevent a large alteration in pH during the distillation. A rapid drop in the CH_3 content of these wools is observed at the beginning of the distillation, followed by a smaller drop as distillation is continued. For a 'fully methylated' wool, the CH_3 groups introduced above a certain value (about 0.6–0.7 % CH_3) are much more readily removed by distillation than those below this value. This may indicate that there are two centres which are being methylated, from one of which the CH_3 groups are readily removed, whilst the CH_3 groups attached to the other resist hydrolysis under the conditions employed. This point is discussed more fully later.

Table 7. *The effect of exposing wool, methylated with methyl sulphate, to a current of steam*

		Duration of steam distillation, min.								
		0	5	10	20	40	60	120	180	240
CH ₃ , %	{	—	0.28	0.28	0.24	0.23	0.27	0.22	—	0.16
		0.31	—	—	—	—	0.23	0.20	0.19	0.16
		0.66	—	—	—	—	0.49	0.39	0.38	0.31
		1.01	—	—	—	—	0.64	0.59	0.58	0.46
		1.07	—	—	—	—	0.75	0.67	0.65	0.58
		1.63	—	—	—	—	0.72	0.71	0.57	0.55

(iii) When wool (CH_3 , 0.37 %) that had been methylated once with methyl sulphate and buffer was placed in a 0.5 % solution of sodium sesquicarbonate (pH 10.0) or in 0.1N NaOH, and steam was blown through the water, the CH_3 was quickly removed and in 30 min. the CH_3 content of the wool returned to its pre-methylation value of 0.06 %. This experiment provided clear evidence that the CH_3 was linked to the wool by a readily hydrolysable linkage and was

not, therefore, attached either to the phenolic group of tyrosine or to the S of cysteine.

Similar results were obtained with more highly methylated wools. A wool containing 1.62 % CH_3 gave a value of 0.21 % CH_3 after distillation with 1 % sodium sesquicarbonate (pH 10) for 1 hr. When the wool was distilled in a current of steam for 1 hr. and then treated with sodium sesquicarbonate, the final CH_3 content was 0.10 %. It is evident that even after repeated treatment with methyl sulphate the number of methoxyl groups which are attached to the wool by non-hydrolysable linkages is extremely small. When the wool was subjected to complete hydrolysis (24 hr.) with HCl , the CH_3 content of the hydrolysate, determined after evaporation *in vacuo*, corresponded to the methionine content of the original wool.

(iv) Experiments were made to determine the ease with which the CH_3 of methylated wool could be removed by extraction with solutions of different pH . Samples of wool (2.0 g., CH_3 , 1.66 %) methylated with methyl sulphate and buffer were treated with 100 ml. of 'Universal' buffer of different pH (containing acetic, phosphoric and boric acids and NaOH) at room temperature in closed bottles. After standing overnight, the wools were washed once with water, dried, conditioned and analysed. The pH of the solutions from which the wools had been taken was determined with a glass electrode. The results are recorded in Table 8.

Table 8. *The effect of extracting methylated wool with buffers of different pH*

Initial pH	0.46	1.80	2.82	4.59	6.27	8.38	9.46	10.45
Final pH	0.75	1.86	2.99	4.64	6.32	8.10	9.03	9.44
Residual CH_3 , %	1.09	1.44	1.40	1.32	1.24	1.15	0.82	0.87

Titration curve of wool after methylation

(a) *Wool methylated with methyl sulphate.* A large sample of wool fabric was methylated in buffer at pH 6 and on analysis contained 0.32 % CH_3 and 0.21 % after steam distillation for 60 min. The wool was remethylated and then contained 0.30 % CH_3 after distillation in steam. The disulphide contents of the wool before and after methylation were 2.93 and 2.88 %, respectively. Samples of the methylated and unmethylated wool fabrics were washed with distilled water and finally brought to equilibrium with water at pH 5. Each fabric was cut up into about 150 pieces, and samples (1–2 g.) of each weighed and put into bottles containing either solutions of H_2SO_4 or NaOH in which they were kept with occasional shaking for 6 days. The pH of the initial and final solutions was determined with the glass electrode. The more concentrated solutions were titrated, using bromocresol purple as indicator. Some of the more alkaline solutions developed an odour resembling that of the methylamines. The results obtained are plotted as ml. N acid or alkali absorbed by 100 g. of anhydrous wool in Fig. 2.

In order to detect any possible alteration in the water-holding capacity of the wool caused by methylation, which would have affected the calculations used to construct the curves of Fig. 2, samples of the wools were squeezed, dried at 65° , and then conditioned. The moisture content of each sample was determined by the method of Barritt & King [1926] by drying in a slow current of air at 110° . No appreciable difference was found between the moisture contents of the methylated and unmethylated wools.

(b) *Wool methylated with methyl iodide.* A further sample of wool, after buffering to pH 8 in borate buffer, was treated with CH_3I . After 11 days the wool was extracted with benzene and found to contain 0.59% CH_3 . The methylated and unmethylated wools were washed with distilled water, and brought into equilibrium with water at pH 5, the subsequent procedure being as described in (a) above. The results are plotted in Fig. 2.

Fig. 2. The titration curves of wool before and after methylation with methyl sulphate and with methyl iodide. $\times-\times$ Untreated. $\bullet-\bullet-\bullet$ Methylated with CH_3I (0.59% CH_3). $\blacksquare-\blacksquare-\blacksquare$ Methylated with methyl sulphate (0.25% CH_3).

In agreement with the hypothesis that the wool is esterified by both methylating agents, less acid is required to suppress the ionization of the carboxyl groups of the wool after methylation, being equivalent, at pH 1.5, to 11.3 ml. *N* acid (i.e. 0.17% CH_3) in the wool methylated with methyl sulphate, and to 23 ml. *N* acid (i.e. 0.32% CH_3) in the wool methylated with methyl iodide. No quantitative conclusions can, however, be drawn from these curves since some of the CH_3 may have been lost when the methylated wools were purified. On the alkaline side of pH 5, it is important to notice that the two methylated wools behave differently. Although both wools neutralize an appreciable amount of alkali from pH 5 to 7, the wool methylated with methyl sulphate neutralizes more than the wool methylated with CH_3I . This alkali is neutralized during the back-titration of some feebly basic group. In the more alkaline solutions, the wool methylated with CH_3I neutralizes more alkali than the wool methylated with methyl sulphate. It is probable that this alkali is neutralized during the hydrolysis of carbomethoxyl groups. On this basis, whereas methylation with CH_3I favours esterification of carboxyl groups, methylation with methyl sulphate favours the production of the feebly basic groups.

DISCUSSION

There can be little doubt that the CH_3 groups found in the methylated wools described are chemically combined, since they persist after the wool has been extracted with benzene and water, subjected to steam distillation and exposed to a current of hot air. The clearest indication of their state of combination is given by the ease with which they can be removed from the wool by acid hydrolysis and by boiling it with buffer solutions higher than pH 9. In addition, when

methylated wools are steam distilled in the presence of alkali, methyl alcohol can be detected in the distillate by Schryver & Wood's method [1920]. The removal of all the CH_3 , with the exception of the methionine- CH_3 , by this method indicates clearly that the added CH_3 is not derived from methylated thiol groups or from methylated tyrosine. Further, since the method used in the determinations of CH_3 is unlikely to return CH_3 attached to nitrogen, the CH_3 is not derived from methylamide groups. Some *N*-methylation occurs, however, when wool is methylated with CH_3I and CH_3Br , since the amount of anionic halide introduced is more than equivalent to the CH_3 as estimated by the Baernstein method.

When *O*-methylation takes place, the most probable reaction is the esterification of carboxyl groups. This conclusion is of particular interest since carboxylic acids are not esterified readily under the conditions used in the present experiments, by either methyl sulphate or methyl iodide. On the other hand, both these substances esterify the zwitterions of amino-acids [Taylor & Baker, 1937].

Engeland [1910], for example, found that phenylalanine was methylated on boiling with methyl alcoholic KOH and CH_3I , and Novak [1912] employed methyl sulphate and NaOH with a variety of amino-acids.

Free amino-acids do not, however, exist in wool in any appreciable quantities and therefore the methyl sulphate, iodide and bromide must esterify combined amino-acids. If these acids are monoaminomonocarboxy-acids, they may terminate the main polypeptide chains to which they must be linked through their amino groups. More probably they are monoaminodicarboxy-acids which are built into the polypeptide chain and esterify because their carboxyl groups terminating the side-chains form zwitterions with the amino groups terminating the side-chains derived from the monocarboxydiamino-acids. In other words, esterification occurs at salt linkages. Evidence for the existence of linkages of this nature in keratin has been accumulated by Speakman & Hirst [1931; 1933].

There are several indications that wool keratin will only esterify when within its isoelectric region. For example, the data given in Table 1 show that the highest degree of methylation was obtained when wool was methylated with solutions of methyl sulphate of *pH* 2.5–8.5. Further, anhydrous wool did not methylate during 75 hr. exposure to CH_3I , nor would conditioned wool methylate when boiled with an ethereal solution of CH_3I , although it underwent limited methylation when boiled with CH_3I . Moreover, when wool with a high CH_3 content is extracted with solutions of different *pH*, the CH_3 groups show the greatest stability in the range *pH* 2–8, solutions with *pH* values outside this range causing a much larger fall in the CH_3 content (Table 8).

The hypothesis that methylation of carboxyl groups occurs owing to the influence of the adjacent basic groups of histidine, lysine and arginine seems to be qualitatively adequate to explain the results already obtained. Amino-acid analyses of wool keratin are available from which it is possible to see the extent of the quantitative agreement between the CH_3 content of methylated wool and the CH_3 content that might be expected. In Table 9 the percentages of the amino-acids in wool likely, on the above hypothesis, to be involved in zwitterion formation are given, together with the equivalent amount of CH_3 to which they might give rise assuming that they form salt linkages.

The CH_3 content of wool should reach a maximum value equal to the free carboxyl groups, plus the CH_3 due to the *S*-methyl of the methionine present and to its probable conversion into a sulphonium group. The free carboxyl

Table 9. *The composition of wool*

Amino-acid		% of wool	Equivalent CH ₃ , % of wool
Arginine	Vickery [1940]	10.4	0.90
	Marston [1928]	10.2	0.88
Lysine	Vickery & Block [1930]	2.3	0.24
	Block [1939]	2.5	0.26
	Marston [1928]	2.8	0.29
Histidine	Vickery & Block [1930]	0.7	0.07
Glutamic acid	Abderhalden & Voitinovici [1907]	12.9	1.32
	Speakman & Townend [1937]	15.3	1.56
Aspartic acid	Abderhalden & Voitinovici [1907]	2.3	0.26
	Speakman & Townend [1937]	7.3	0.82
Methionine	This paper	0.6	0.12*
Amide-N	Speakman & Townend [1937]	1.37	1.47

* Assuming methionine combines with the methylating agent to give a sulphonium compound.

groups of wool are limited to the carboxyl groups of glutamic and aspartic acids which are not linked in the main chain or combined with ammonia as amide groups. Using the figures of Abderhalden & Voitinovici [1907] for glutamic and aspartic acids and Speakman & Townend's [1937] figure for amide-N, the CH₃ content of fully methylated wool might have a possible value of $1.58 - 1.47 + 0.06 = 0.17\%$. If the later figures of Speakman & Townend for glutamic and aspartic acids are utilized, the CH₃ content would be 0.96% . Assuming that CH₃I (or sulphate) adds on to the sulphur atom of methionine [Toennies, 1940, 1, 2], these values would be increased by 0.06% . The number of CH₃ groups that wool can take up is much larger than even the higher of these figures.

It would seem to be improbable that appreciable hydrolysis of the peptide links of the main chains, liberating further carboxyl groups which might then undergo methylation, would occur during even prolonged treatment at the pH employed in these experiments. Moreover, hydrolysis of this type could hardly occur during treatment of wool with CH₃I or CH₃Br, and yet one wool methylated with CH₃Br contained more CH₃ (1.30%) than could be accommodated by the free carboxyl groups.

The results obtained on methylating silk fibroin are of importance in this connexion. It can be seen from Table 6 that when the methods used for wool are applied to silk a maximum value of 0.45% of CH₃ is reached. The greater part of this CH₃ is removed on treatment with alkali. Rutherford *et al.* [1940] have recently obtained similar results after methylating silk with diazomethane. These authors found that the free phenolic groups of tyrosine were readily methylated, but in addition the total methoxyl content was found to include some 'extra' methoxyl groups, corresponding to 0.26 millimols/g. (or 0.39% CH₃) which were removed as CH₃OH on alkaline hydrolysis and were not attached to primary amino or serine hydroxyl groups. These authors tentatively assumed that the 'extra' groups were located on carboxyl groups. Since, as far as we are aware, no one has succeeded in isolating dicarboxylic amino-acids from silk fibroin, the amounts of dicarboxylic acids present in silk are likely to be extremely small. The diamino-acid content of silk is also very low: Vickery & Block [1931] found 0.74% arginine, 0.25% lysine and 0.076% histidine. Salt linkage formation between the basic side-chains of these acids and free carboxyl groups derived from other acids could therefore only give rise to zwitterions capable of being combined with 0.10% CH₃.

The esterification of carboxyl groups cannot account for all the hydrolysable CH_3 with which wools and silk will combine. In methylated wool as much as 0.7%, and in methylated silk as much as 0.4% CH_3 , are probably linked to oxygen atoms which are not present in carboxyl groups. We are therefore forced to entertain the possibility that the oxygen atoms to which this CH_3 is attached are the oxygen atoms of the peptide linkages. Several workers have discussed the possibility of an enolization in peptides and proteins, which can theoretically occur in two ways:

Enolization of the first type involving loss of asymmetry in the carbon atom was postulated by Dakin [1912-13] to explain the racemization of certain amino-acids in proteins by the action of alkali at low temperatures. The idea was later elaborated by Levene and co-workers [Levene *et al.* 1927; Levene *et al.* 1931; Levene & Yang, 1933] in a study of the racemization and hydrolysis of peptides under the influence of alkali. Levene also expressed the opinion that enolization of the second type, involving the nitrogen atom, could occur in polypeptides and proteins.

Moreover, it is possible to obtain stable isomers of compounds containing the peptide linkage. This isomerism has been shown to be due to enolization. Thus Leuchs & Manasse [1907] demonstrated the existence of two isomeric forms of glycylglycine carboxylic acid, the ' α ' form having the lactam formula, the ' β ' having the lactim formula:

In accordance with expectation, when the enolizable hydrogen atom is replaced by a phenyl group, only the ' α ' form is obtained. Leuchs & La Forge [1908] have prepared similar isomers of *N*-carboxydiglycylglycine.

If one or more of the peptide links in wool or silk can readily enolize, methylation might be expected to occur. It is not, however, necessary to assume that the imido-hydrogen atom migrates. More probably the methylating agent adds on to a peptide linkage which has become a resonance hybrid of the two structures (I) and (II) and as such, since it approaches a zwitterion in constitution, might conceivably methylate in a similar manner.

In support of this view, urea which undergoes methylation on the oxygen atom when treated with methyl sulphate has been considered to be a resonance-hybrid of the two forms (IV) and (V) [Taylor & Baker, 1937].

On the chemical side, two pieces of evidence are in agreement with the presence of methylated peptide linkages possibly possessing the structure indicated in (III). The first is the lability of some of the CH_3 in the highly methylated wools, and secondly, the neutralization of alkali by methylated wools

from pH 5 to 7 (Fig. 2). An imido-group, which had undergone salt formation as indicated in (III), would be expected to hydrolyse freely and to lose HI within this pH range.

Physical evidence supporting the possible occurrence of 'activated' peptide linkages is also available. From the results of an X-ray examination of the crystal structure of diketopiperazine, Corey [1938] concluded that the diketopiperazine molecule was a resonance hybrid of four possible structures, three of which contain one or more zwitterionic peptide linkages.

Ellis & Bath [1939] found that the infra-red absorption spectrum of diketopiperazine also supported Corey's idea of electron resonance. Karrer *et al.* [1923] obtained a derivative of a tautomeric form of diketopiperazine in which the hydrogen atom is enolized in the above sense. These authors found that when the silver derivative of diketopiperazine reacted with benzyl chloride, a benzyl group was attached to the oxygen atom of the peptide bond, 2:5-dihydroxy-dihydropyrazine dibenzyl ether being produced. This compound was readily hydrolysed by dilute acids to benzyl alcohol and glycine. Methylation would not be expected to occur at all the peptide linkages in wool and silk, but only at certain specific links which can assume the appropriate electronic state, or, some state approaching it electronically, due to the presence of adjacent activating groups.

The total CH_3 content of methylated wool may thus include two types of *O*-methyl groups: one attached to the free carboxyl groups of glutamic and aspartic acids, the other attached to peptide linkages. On this hypothesis, a large proportion of the CH_3 groups in methylated silk would be attached to peptide linkages. Further work is in progress to enable a clearer distinction to be drawn between methylation at these two centres.

SUMMARY

1. Wool can be methylated with methyl sulphate, iodide and bromide, at ordinary temperatures.
2. With each reagent, both *O*- and *N*-methylation takes place.
3. The experimental results indicate that the *O*-methylation consists partly of an esterification of the free carboxyl groups.
4. Evidence is presented which suggests that *O*-methylation also takes place at peptide linkages.

Thanks are due to the Council of the Wool Industries Research Association for permission to publish this paper, to Dr L. F. Story for determining the titration curves given in Fig. 2, and to Mr G. Lindley and Mr A. B. Gibson for assistance with the experimental work.

REFERENCES

- Abderhalden & Voitinovici (1907). *Hoppe-Seyl. Z.* **52**, 348.
Baernstein (1932). *J. biol. Chem.* **97**, 663.
—— (1936). *J. biol. Chem.* **115**, 25.
Bailey (1937). *Biochem. J.* **31**, 1396.
Barritt (1934). *Biochem. J.* **28**, 1.
—— & King (1926). *J. Text. Inst. Manchr*, **17**, 386.
Block (1939). *J. biol. Chem.* **128**, 181.
Corey (1938). *J. Amer. chem. Soc.* **60**, 1598.
Dakin (1912–13). *J. biol. Chem.* **13**, 362.
Edlbacher (1919). *Hoppe-Seyl. Z.* **107**, 52.
Ellis & Bath (1939). *J. chem. phys.* **7**, 862.
Elsworth & Phillips (1938). *Biochem. J.* **32**, 837.
Engeland (1910). *Ber. dtsh. chem. Ges.* **43**, 2662.
Felix & Rauch (1931). *Hoppe-Seyl. Z.* **200**, 27.
—— & Reindl (1932). *Hoppe-Seyl. Z.* **205**, 11.
Geake & Nierenstein (1914). *Biochem. J.* **8**, 287.
Haurowitz (1938). *Hoppe-Seyl. Z.* **256**, 28.
Herzig & Landsteiner (1914). *Biochem. Z.* **61**, 458.
—— — (1918). *Mh. chem.* **39**, 369.
Karrer, Granacher & Schlosser (1923). *Helv. chim. Acta*, **6**, 1108.
Leuchs & La Forge (1908). *Ber. dtsh. chem. Ges.* **41**, 2586.
—— & Manasse (1907). *Ber. dtsh. chem. Ges.* **40**, 3235.
Levene, Bass, Steiger & Bencowitz (1927). *J. biol. Chem.* **72**, 815.
—— Steiger & Marker (1931). *J. biol. Chem.* **93**, 605.
—— & Yang (1933). *J. biol. Chem.* **99**, 405.
Marston (1928). *Bull. Coun. sci. industr. Res., Aust.*, **38**.
Mueller (1921). *Proc. Soc. exp. Biol., N.Y.*, **18**, 14.
Novak (1912). *Ber. dtsh. chem. Ges.* **45**, 834.
Rutherford, Patterson & Harris (1940). *Amer. Dyest. Rep.* **29**, 583.
Schryver & Wood (1920). *Analyst*, **45**, 164.
Skraup & Krause (1909). *Mh. Chem.* **30**, 447.
Speakman & Hirst (1931). *Nature, Lond.*, **128**, 1073.
—— — (1933). *Trans. Faraday Soc.* **29**, 148.
—— & Townend (1937). *Nature, Lond.*, **139**, 411.
Taylor & Baker (1937). Sidgwick's Organic Chemistry of Nitrogen, p. 284. Oxford.
Toennies (1940, 1). *Proc. Amer. Soc. biol. Chem.* **34**, cii (*J. biol. Chem.* **133**).
—— (1940, 2). *J. biol. Chem.* **132**, 455.
Vickery (1940). *J. biol. Chem.* **132**, 325.
—— & Block (1930). *J. biol. Chem.* **86**, 107.
—— — (1931). *J. biol. Chem.* **93**, 105.