butylene glycol, ethanol, acetic and lactic acids, CO_2 and H_2, with traces of acetoin and succinic acid. The 2:3-butylene glycol is a mixture of the *l*- and meso-forms.

2. The products of xylose fermentation are very similar, both quantitatively and qualitatively, to those of glucose fermentation. This is regarded as evidence against a simple C_3-C_2 split of the pentose molecule.

3. The main products of the fermentation of pyruvic acid are lactic and acetic acids, CO_2 and H_2. Approximately three molecules of pyruvic acid are oxidized to acetic acid for every one reduced to lactic acid.

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Experiments on the Methylation and Acetylation of Wool, Silk Fibroin, Collagen and Gelatin

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In a previous paper (Blackburn, Carter & Phillips, 1941) it was shown that wool and silk fibroin could be *O*-methylated either by contact with methyl bromide or iodide at room temperatures, or with methyl sulphate in buffers within the pH range 2·5–8·5. With both proteins the number of CH_3 groups introduced by repeated treatment with methyl sulphate was greater than could be accommodated on the free carboxyl groups of glutamic and aspartic acids, and it was suggested that certain 'activated' peptide linkages methylated as well as the free carboxyl groups of the salt-linkages. CH_3I and CH_3Br introduced fewer CH_3 groups into wool and silk fibroin than did methyl sulphate.

The present paper describes the extension of this work to the methylation of collagen and gelatin by similar methods, and the methylation with methyl sulphate and CH_3Br of wool which has been chemically modified by alkali, acid, nitrous acid and formaldehyde. In addition, the simultaneous acetylation and methylation of wool, silk fibroin and collagen by the combined action of methanol and acetic anhydride has been investigated.

METHODS

Methylation of collagen and gelatin with methyl sulphate and methyl halides

The collagen used in these investigations was a limed hide which had been delimed with acetic acid and then dehydrated in acetone. The gelatin was Coignet's Gold Label. The methylation procedure was similar to that described for wool and silk (Blackburn *et al.* 1941), with certain modifications to suit the properties of collagen and gelatin. Both proteins were cut into small pieces and acetate buffer was used for the methylations with methyl sulphate. At the end of each period of methylation about 1 g. of collagen was removed, washed in three changes of water and then shaken mechanically with water (25 ml.) for 1 hr. It was then dehydrated with acetone, air-dried and conditioned.* The moisture content of the collagen, the physical properties of which appeared to be unaltered, was determined by Barritt & King's method (1926). During methylation, the gelatin absorbed water and swelled greatly. The resulting gel and solution was dialyzed in cellophan against water for 3 days, the water being changed frequently. The resulting gelatinous mass was dried *in vacuo* below 60°.

Before methylation with either CH_3Br or CH_3I, the collagen was allowed to reach equilibrium with a borate buffer of pH 8, and then dehydrated with acetone and conditioned. The gelatin was treated dry as bought. After methylation, both proteins were rinsed in several changes of benzene and then conditioned. When proteins are methylated by either CH_3Br or CH_3I, one halogen anion is liberated for each CH_3 group introduced. To provide an additional measure of their degree of methylation, the halogen content of some of the methylated collagens and gelatins was therefore determined by the open Carius method (Peters & Van Slyke, 1932). The methylated gelatin

* All proteins 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. Although all the analyses were made on the conditioned proteins, the results are recorded as percentages of the anhydrous material.

was also analyzed for N by the Kjeldahl method. The CH_3 contents, determined by Baernstein's method (1932, 1936), were then calculated on the dry weight of gelatin, assuming the N content of gelatin to be 17·9%.

Methylation of chemically modified wools with methyl sulphate and methyl bromide

Unless otherwise stated, the wool used was similar to that described previously (Elsworth & Phillips, 1938). The chemically modified wools in which we are at present interested are those in which the chemical modification is restricted to certain groups in the keratin molecule. They were prepared as described below by methods unlikely to cause widespread decomposition of the wool.

Alkali-treated wool. Wool (100 g.) was boiled for 30 min. in 10 l. of 2% borax. It was then rinsed repeatedly in water, dried and conditioned. By this treatment the total-S (3·35%) and disulphide-S (2·88%) were reduced to 2·75 and 1·26% respectively, cystine being converted into lanthionine (Horn, Jones & Ringel, 1941, 1942 *a*, *b*; Mizell & Harris, 1943). The only other change likely to have been produced is the degradation of the serine and threonine residues, since those of silk have been shown by Nicolet, Shinn & Saidel (1942) to be susceptible to decomposition by alkalis. Analysis by the methods of Nicolet & Shinn (1941), Shinn & Nicolet (1941) showed, however, that a merino wool which originally contained 9·7% of serine still contained 9·3% after being boiled for 2 hr. in borate buffer at pH 9·6.

Acid-treated wool. The wool (100 g.) was boiled for ½ hr. with 1500 ml. of 0·5 N-HCl. The acid was replaced by 1500 ml. of fresh acid with which the wool was boiled for a further 15 min., then washed in several changes of distilled water, dried and conditioned. The amide-N of the wool had been reduced from 1·26 to 0·78%. These determinations were made by the method of Bailey (1937) and Lugg (1938) except that, in order to dissolve the wool completely, the period of hydrolysis was increased from 3 hr. to 4 or 5 hr. The acid-treated wool was brought to pH 5·4 in acetate buffer to remove HCl before methylation.

Deaminated wool. This was prepared by Bowes & Pleass's method (1939*a*), which deaminates only the terminal amino-groups of lysine.

Formaldehyde-treated wool. The action of formaldehyde on wool can be restricted to the terminal amino-groups of lysine and arginine or can include reaction with the disulphide-S of cystine (Middlebrook & Phillips, 1942). When the reaction was restricted to the amino-groups by treating the wool at ordinary temperature, it contained 1·14% formaldehyde, as determined by Bowes & Pleass's method (1939*b*), and acquired 1·7% CH_3 on methylation with methyl sulphate in phosphate buffer. Analysis then showed it was free from formaldehyde. A second sample of wool was treated with 50 times its weight of a 1% solution of formaldehyde at pH 6·9 and 70° for 18 hr. By this treatment its disulphide-S content was reduced from 2·80 to 2·49%.

The chemically treated wools, with the exception of the acid-treated wool, which was methylated in acetate buffer, were methylated with methyl sulphate in phosphate buffer, and also by exposure to CH_3Br after they had been buffered to pH 8·0 in borate buffer, 0·4 M with respect to H_3BO_3, and then air-dried (Blackburn *et al.* 1941).

Acetylation of wool with acetic anhydride

Air-dry wool was boiled under reflux with sufficient acetic anhydride to cover it completely. The reaction was allowed to proceed for either 30 min. or for 4 hr., the acetic anhydride was poured off the wool, which was soaked and rinsed in water until free from acetic anhydride, and then air-dried and conditioned. Wool acetylated for 30 min. in this manner was light yellow in colour. It gave a negative Millon reaction. When the wool was acetylated for 4 hr., it became deep brown in colour, but retained a considerable amount of its strength. The total acetyl content of wool acetylated for ½ hr. was 11·7%, and 13·8% after acetylation for 4 hr. Two further samples of wool acetylated for ½ and 4 hr. had total acetyl 12·6% (*O*-acetyl, 2·2%) and total acetyl 14·8% (*O*-acetyl 2·8%) respectively. A small amount of wool dissolved, giving the acetic anhydride a pale yellow colour.

Determination of acetyl groups

(*a*) *Total acetyl.* The wool (0·30 g.) was boiled under reflux for 4 hr. with 5 N-H_2SO_4 (10 ml.). The hydrolysate was distilled *in vacuo*, water being added from time to time, until 200 ml. of distillate had collected. This was titrated, whilst protected from atmospheric CO_2, with 0·02 N-NaOH. A sample of untreated wool was analyzed in a similar manner and the blank titration obtained was subtracted from the titrations of the distillates from the acetylated wools.

(*b*) *O-acetyl.* This approximate determination was based on the observations of Wolfrom, Konigsberg & Soltzberg (1936) and Synge (1939) that *O*-acetyl groups, in contrast to *N*-acetyl groups, are readily hydrolyzed by cold dilute NaOH. The acetylated and untreated wools (0·30 g. of each) were immersed in 20 ml. of 0·02 N-NaOH at room temperature. After 24 hr., 10 ml. of the alkali were removed and titrated with HCl against phenol red. The difference in the titrations of the alkali from the acetylated and untreated wools was taken as a measure of the *O*-acetyl groups in the acetylated wools.

Simultaneous acetylation and methylation of wool, silk fibroin and collagen with methanol and acetic anhydride

Ashley & Harington (1929) observed that thyroxine in ethanol at room temperature was converted into the ethyl ester of diacetyl thyroxine by the addition of acetic anhydride: phenylalanine similarly gave the ethyl ester of *N*-acetylphenylalanine. We have found that wool, silk fibroin and collagen can be simultaneously acetylated and methylated in a similar manner. The wool and silk fibroin (3 g.) were dried in a current of air at 115° for 10 min.; the collagen was dehydrated with methanol. They were then placed in methanol (100 ml.) and acetic anhydride (10 ml.) added. After 20 hr., the proteins were rinsed with water, allowed to stand for several hours in water which was changed frequently, dried at room temperature and finally conditioned.

Methylation of wool and silk fibroin with methyl p*-toluenesulphonate*

The wool or silk fibroin (3 g.) was dried in a stream of air at 115° and then heated under reflux with a solution of methyl *p*-toluenesulphonate (10 g.) in methanol (100 ml.)

Table 1. *Analyses of the products of methylation of collagen and gelatin with methyl sulphate and methyl halides*

Methylation with methyl sulphate		Methylation with CH_3I			Methylation with CH_3Br		
No. of methylations	CH_3 (%)	Total time of exposure (days)	CH_3 (%)	I (%)	Total time of exposure (days)	CH_3 (%)	Br (%)
			(*a*) Collagen				
2	0·56	5	0·34	—	10	0·85	5·42
6	1·17	11	0·42	—	76*	0·96	6·74
8	1·22	72	0·54	4·92	101	1·20	—
12	1·11	42*	0·67	5·76	—	—	—
14	1·27	69*	0·86	—	—	—	—
16	1·42	103	0·91	—	—	—	—
			(*b*) Gelatin				
10	1·74	15	0·44	5·66	15	1·09	8·15

* Collagen rebuffered and methyl halide renewed after determination of CH_3 had been made.

for 6 hr. The methylated proteins were squeezed, air-dried, extracted with methanol, dried, rinsed for 2·5 hr. in several changes of water, air-dried, and finally conditioned.

Methylation of wool and silk fibroin with methyl p-toluenesulphonate in the presence of alkalis

Wool or silk fibroin (6 g.) was dried in a stream of hot air at 115° and then heated under reflux for 6 hr. with methanol (200 ml.) to which methyl *p*-toluenesulphonate (20 g.) and anhydrous potassium carbonate (10 g.) had been added. The methylated proteins were squeezed, rinsed in water, acidified with acetic acid, air-dried, extracted with benzene, air-dried, rinsed in several changes of water, air-dried and finally conditioned.

RESULTS

Methylation of collagen, gelatin and chemically modified wools with methyl sulphate and methyl halides

As can be seen from Table 1, after 16 methylations with methyl sulphate, collagen contained 1·42 % CH_3, whilst gelatin, after 10 methylations, contained 1·74 % CH_3.

Gelatin contains 5·8 % glutamic acid, 3·4 % aspartic acid, 0·4 % NH_3-N and 0·97 % methionine (Dakin, 1920; Baernstein, 1932). Assuming that the NH_3-N isolated by Dakin (1920) is all present as amide-N, the methylation of the free carboxyl groups of glutamic and aspartic acids and the S-methyl group of methionine should together account for

$$(0{\cdot}59 + 0{\cdot}38 - 0{\cdot}35 + 0{\cdot}10) = 0{\cdot}72\ \%\ CH_3,$$

or 0·82 % CH_3 if the S-methyl group is converted into a sulphonium group. Since collagen takes up 1·42 % CH_3 and gelatin 1·74 % CH_3, when they are repeatedly methylated with methyl sulphate, some of the CH_3 groups become attached to centres other than the free carboxyl groups.

Table 2. *Analyses of the products of methylation of chemically modified wools with methyl sulphate and methyl bromide*

Methylation with methyl sulphate (in phosphate buffer)		Methylation with CH_3Br		
No. of methylations	CH_3 (%)	Total time of exposure (days)	CH_3 (%)	Br (%)
	(*a*) Alkali-treated wool			
2	0·82	28*	1·19	9·77
5	1·07	73†	0·94	—
9	1·55	114	1·51	—
14	1·69	—	—	—
16	1·45	—	—	—
18	1·68	—	—	—
	(*b*) Acid-treated wool			
1	0·39	22*	1·09	11·25
2	0·94	68†	1·00	—
3	1·41	109	1·50	—
6	2·08	—	—	—
9	2·39	—	—	—
11	2·74	—	—	—
12	2·90	—	—	—
	(*c*) Deaminated wool			
2	0·64	20*	1·16	7·18
5	1·25	58†	0·92	—
8	1·73	99	1·35	—
11	1·66	—	—	—
14	1·61	—	—	—
	(*d*) Formaldehyde-treated wool			
2	0·59	14†	0·94	8·20
5	1·17	28	1·16	—
9	1·47	—	—	—
13	1·64	—	—	—
15	1·51	—	—	—
17	1·58	—	—	—

* CH_3Br renewed after determination of CH_3 was made.

† Wool rebuffered and CH_3Br renewed after determination of CH_3 had been made.

The analyses of the chemically treated wools given in Table 2 show that mild alkali treatment, deamination and treatment with HCHO at 70° did not alter the degree to which the wool could be methylated with methyl sulphate. On the assumption that some methylation with methyl sulphate occurred at 'activated' peptide linkages, it would appear that these linkages were unaffected by the treatments given. Deamination and treatment with HCHO, under the experimental conditions employed, would only affect the terminal amino groups of the lysine side-chains. These are few in number (corresponding to about 0·25% CH_3) and this probably accounted for the small effect of both deamination and treatment with HCHO on the observed degree of methylation with either methyl sulphate or CH_3Br.

The acid treatment given, however, enabled the wool to take up 1·0% extra CH_3 when methylated with methyl sulphate. This may have been due partly to the esterification of carboxyl groups liberated from amide groups (the decrease in amide-N was equivalent to 0·51% CH_3) and partly to the esterification of carboxyl groups liberated by the hydrolysis of the main chains. Experiment showed that, when wool was boiled for 30 min. with 0·5N-HCl, at least 70% of the N passing into solution was NH_3-N: the amide-N of the wool showed a corresponding decrease. In contrast to methyl sulphate, CH_3Br did not methylate acid-treated wool to a higher degree than it does untreated wool.

Methylation of acetylated wool

When wool which had been acetylated for 30 min. was repeatedly methylated with methyl sulphate in phosphate buffer, the CH_3 introduced appeared to reach a limiting value of 0·8% (Table 3). The even lower limit of 0·6% CH_3 appeared to be reached when wool acetylated for 4 hr. was methylated, although 1·7% CH_3 can be introduced into untreated wool by 18 successive methylations (Blackburn *et al.* 1941). Methylation by the methyl halides was also greatly restricted.

The conversion of arginine into ornithine during the acetylation of wool

By Vickery's method (1940), 19·7% of the total N of the hydrolysate of the untreated wool was found to be arginine-N. On the other hand, only 9·5% of the total N of the hydrolysate of a wool which had been acetylated for 30 min. was arginine-N. It was evident therefore that arginine is destroyed when wool is acetylated.

On adding flavianic acid to the hydrolysate of a wool (50 g.) which had been acetylated for 4 hr., the precipitate obtained, which separated slowly during 2 weeks in a refrigerator, was found to be free from arginine diflavianate and to consist entirely of ornithine flavianate. This was recrystallized from hot water, dissolved in dilute HCl and the flavianic acid extracted with *n*-butanol (Pratt, 1926). The aqueous solution containing the base was concentrated under reduced pressure and decolorized with charcoal. The benzoyl derivative, prepared by the addition of benzoyl chloride and NaOH, after recrystallization from aqueous ethanol had m.p. 185–186° either alone or when mixed with *N*:*N'*-dibenzoyl ornithine (Boon & Robson, 1935; Karrer & Ehrenstein, 1926). (Found: N, 8·6. Calc. for $C_{19}H_{20}O_4N_2$: N, 8·25%.) It was optically inactive to light of λ 5893 in 0·1N-NaOH (*c*, 0·91; *l*, 50 mm.). Only 0·75 g. of dibenzoyl ornithine was isolated from the 50 g. of acetylated wool, but Vickery (1941) also found the isolation of ornithine from protein hydrolysates presents very special difficulties.

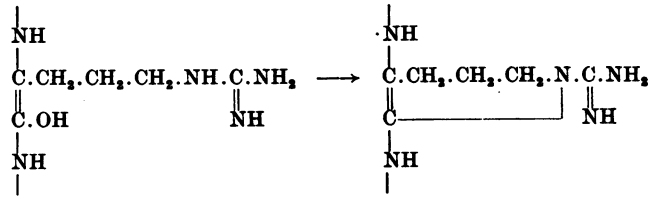
Since arginine is destroyed when wool is acetylated, it is reasonable to suppose that the ornithine isolated is derived from this source. Bergmann & Koster (1926) found that, on being heated with excess acetic anhydride, arginine was converted

Table 3. *Analyses of the products of methylation of acetylated wools with methyl sulphate and methyl halides*

Methylation with methyl sulphate		Methylation with CH_3I			Methylation with CH_3Br		
No. of methylations	CH_3 (%)	Total time of exposure (days)	CH_3 (%)	I (%)	Total time of exposure (days)	CH_3 (%)	Br (%)
		(*a*) Wool acetylated for ½ hr.					
2	0·35	14	0·27	—	14	0·34	3·34
5	0·60	35	0·38	—	35	0·44	4·29
9	0·70	—	—	—	—	—	—
14	0·78	—	—	—	—	—	—
18	0·86	—	—	—	—	—	—
		(*b*) Wool acetylated for 4 hr.					
3	0·42	14	0·16	—	14	0·19	2·64
7	0·57	35	0·27	—	35	0·35	3·76
14	0·64	—	—	—	—	—	—
16	0·57	—	—	—	—	—	—

into triacetyl anhydro-arginine, which with cold water gave immediately *dl*-acetamido-2-piperidone and diacetyl urea. Acid hydrolysis of the piperidone derivative gave *dl*-ornithine.

Dirr & Felix (1932) considered that the conversion of combined arginine into ornithine only took place during acetylation when the carboxyl group of the arginine was free (compare Bergmann & Koster, 1927; Felix & Dirr, 1928). If the assumption of Dirr & Felix (1932) is correct, then our experiments suggest that some of the carboxyl groups of the arginine in wool are also free. The available evidence is against this conclusion (Dauphinee & Hunter, 1930). In addition, the assumption of Dirr & Felix (1932) would seem to be unnecessary to account either for their own results with clupein or our results with wool, as ring closure not involving a free carboxyl group might occur if the peptide link adjacent to the arginine residues assumed the enolic form. Such enolization might cause loss of asymmetry of the α-carbon atom of the arginine and, as in our experiments, an optically inactive ornithine would be liberated by acid hydrolysis.



If ring closure occurs in this manner, the terminal amidine group would probably acetylate and on hydrolysis would be eliminated either as acetyl urea or its decomposition products.

The simultaneous acetylation and methylation of wool, silk fibroin and collagen with methanol and acetic anhydride

Table 4 shows that, when these proteins were treated with methanol-acetic anhydride, both CH_3 and acetyl groups were introduced.

Table 4. *Analyses of the products of the simultaneous acetylation and methylation of wool, silk fibroin and collagen with methanol and acetic anhydride*

Protein	CH_3 (less methionine CH_3) (%)	Acetyl (%) Total	Acetyl (%) *O*-acetyl	Molar ratio Total acetyl/CH_3	Molar ratio *O*-acetyl/CH_3
Wool	0·53	2·5	1·9	1·6	1·2
	0·73	—	—	—	—
	0·71	—	—	—	—
Silk fibroin	0·26	1·5	1·3	2·0	1·7
Collagen	0·64	—	—	—	—

The CH_3 content (0·79 %) of the wool methylated with methanol-acetic anhydride was unchanged by immersing the wool overnight in phosphate buffer of pH 6 (50 ml./g.) and decreased only to 0·67 % when exposed in a buffer solution to a current of steam. The CH_3 groups are therefore chemically combined with the wool. When a treated wool (0·59 % CH_3) was again treated with acetic anhydride in methanol, the CH_3 content rose to 0·75 %. Both *O*- and *N*-acetyl groups are introduced into proteins by methanol-acetic anhydride (Table 4). The acetylated and methylated proteins were turned yellow by nitrous acid at the same rate as were the untreated proteins, suggesting that the tyrosine hydroxyl groups had not been acetylated (Rutherford, Patterson & Harris, 1940). Amino-N determinations on the treated wool (Rutherford, Harris & Smith, 1937) showed that N_2 was evolved only very slowly by the action of nitrous acid, indicating that the terminal amino groups of lysine had been acetylated.

Table 5. *Analyses of the products of the further methylation with methyl sulphate and methyl halides of wool which had been acetylated and methylated with methanol and acetic anhydride*

Methylation with methyl sulphate (phosphate buffer) No. of methylations	Methylation with methyl sulphate (phosphate buffer) Total CH_3 (%)	Methylation with CH_3I Time of exposure (days)	Methylation with CH_3I Total CH_3 (%)	Methylation with CH_3I I (%)	Methylation with CH_3Br Time of exposure (days)	Methylation with CH_3Br Total CH_3 (%)	Methylation with CH_3Br Br (%)
0	0·77	0	0·77	—	0	0·77	—
2	1·31	11	1·04	4·53	11	1·01	4·72
4	1·35	0	0·74	—	0	0·74	—
6	1·48	32	1·02	5·02	32	1·31	5·11
8	1·50	—	—	—	—	—	—
10	1·43	—	—	—	—	—	—
12	1·53	—	—	—	—	—	—
14	1·48	—	—	—	—	—	—
16	1·66	—	—	—	—	—	—

It can be seen from Table 5 that when wool which has been treated with methanol-acetic anhydride is methylated with methyl sulphate it takes up further CH_3 groups slowly until the total CH_3 reaches the value normally attained on methylating wool with this reagent.

The methylation of wool and silk fibroin with methyl p-*toluenesulphonate*

Repeated methylation of wool with methyl *p*-toluenesulphonate in methanol caused the CH_3 content to increase gradually to 1·91 %, whilst when silk fibroin was methylated in a similar manner, 0·40 % CH_3 was introduced (Table 6). A wool which had been methylated three times with methyl *p*-toluenesulphonate (CH_3, 1·91 %) was remethylated with methyl sulphate (Table 7). The CH_3 content of the wool reached 3·22 %, which is greater by 1·2–1·5 % than the CH_3 content of a wool fully methylated with methyl sulphate.

The nitrous acid test of Rutherford *et al.* (1940) showed the tyrosine hydroxyl groups in the wool had not been methylated, but it was found that after methylation with the sulphonic ester, the wool (CH_3, 1·66 %) contained only 0·57 % amide-N as compared with 1·26 % for the untreated wool. Hence some of the observed additional methylation was due to methanolysis of glutamine and asparagine side-chains. The fall in amide-N (0·69 %) is equivalent to 0·74 % CH_3. Subtraction of this value from the total CH_3 of this wool gives 0·92 % CH_3 as the possible amount of CH_3 introduced at the other centres.

Table 6. *Analyses of the products of methylation of wool and silk fibroin with methyl* p-*toluenesulphonate*

Conditions of methylation		CH_3 (%) Wool	CH_3 (%) Silk fibroin
24 hr. at room temp.		0·34	0·10
6 hr. under reflux	(*a*)	1·11	0·40
	(*b*)	1·39	—
Successive treatments for 6 hr. under reflux	(first)	1·40	—
	(second)	1·78	—
	(third)	1·91	—

Table 7. *Analyses of the products of the further methylation with methyl sulphate and phosphate buffer of wool which had been methylated with methyl* p-*toluenesulphonate*

No. of methylations	CH_3 (%)
3	2·72
7	3·04
12	2·70
15	3·22

Table 8. *Analyses of the products of methylation of wool and silk fibroin with methyl* p-*toluenesulphonate in the presence of alkalis*

Wool: Conditions of methylation	CH_3 (%)	Silk fibroin: Conditions of methylation	CH_3 (%)
$MeOH/K_2CO_3$ 6 hr.	1·39	$MeOH/K_2CO_3$ 6 hr.	0·97
„ 6 hr.	1·04	Water/NaOH 2 hr.	0·87
„ 1·5 hr.	0·99	„ 4 hr.	1·00

The CH_3 contents of wool and silk fibroin after treatment with methyl *p*-toluenesulphonate and alkali are given in Table 8. The wool was markedly degraded by the 6 hr. treatment, but when the length of time of treatment was reduced to 1·5 hr., much less degradation took place, although the amount of CH_3 introduced was not much lower. Silk fibroin withstood the treatment without physical degradation and was similarly unaffected when the methanol and K_2CO_3 were replaced by 0·5 % aqueous NaOH. The nitrous acid test of Rutherford *et al.* (1940) showed that some methylation of the hydroxyl groups of the tyrosine in both the wool and the silk fibroin had taken place.

The results show that methylation with methyl *p*-toluenesulphonate introduces about the same amount of CH_3 as is introduced by methyl sulphate, but the reaction is less selective, and even in the absence of alkali protein hydrolysis is brought about by the *p*-toluenesulphonic acid which is liberated.

DISCUSSION

The CH_3 introduced into wool by treatment with methanol-acetic anhydride is limited in amount (about 0·7 %) and is removed slowly, as methanol, by regulated alkaline hydrolysis. Since amino-acids are esterified by this mixture (Ashley & Harington, 1929), it is probable that it also esterifies the carboxyl groups of the salt-linkages of wool, silk fibroin, and collagen.

By further methylation with methyl halides, the CH_3 content of wool, which has been methylated with methanol-acetic anhydride, can be increased to about 1·0 %, but when this value has been reached the methylation either stops or becomes very slow. The CH_3 content thus reaches the same value as that usually attained when untreated wool is methylated with methyl halides. It is probable therefore that during the initial relatively rapid methylation of wool with methyl halides the free carboxyl groups are esterified. In agreement with this conclusion, the CH_3 introduced by methyl halides is removed only slowly by hydrolysis.

Further evidence that both methanol-acetic anhydride and the methyl halides esterify the free carboxyl groups is provided by comparison (Table 9)

of the CH_3 introduced by these reagents with the CH_3 equivalent to the free carboxyl groups found by titration and as calculated from the aspartic and glutamic acid contents of wool, collagen and silk fibroin.

Table 9. *The CH_3 of proteins equivalent to free carboxyl groups as calculated (I) from free carboxyl groups determined from titration curves, (II) from free carboxyl groups of estimated aspartic and glutamic acids and amide-N contents, and as found by (III) methylation with methanol-acetic anhydride and (IV) methylation with methyl halides*

Protein	I	II	III	IV
Wool	1·17 (1)	0·96 (4)	0·75	1·0 (7)
Collagen	0·53 (2)	0·62 (5)	0·64	0·9
Silk	0·20–0·26 (3)	0·10 (6)	0·26	0·37 (7)

(1) Harris & Rutherford (1939); Steinhardt & Harris (1940). (2) Theis & Jacoby (1942). (3) Gleysteen & Harris (1941). (4) Speakman & Townend (1937). (5) Dakin (1920). (6) From diamino-acid content, Vickery & Block (1931). (7) Blackburn *et al.* (1941).

Methanol-acetic anhydride introduces rather less than the expected amount of CH_3 into wool, whilst the methyl halides introduce rather more than the expected amounts into all three proteins, but there is no wide disagreement.

When wool is methylated with methyl sulphate more CH_3 is introduced than by methylation with either methanol-acetic anhydride or methyl halides. Methylation with methyl sulphate appears to occur in two stages, the first being a relatively rapid initial methylation, after which the CH_3 content increases slowly to a maximum of about 1·7%. The CH_3 introduced in the earlier stage of the methylation is removed only very slowly by exposing the wool in pH 7 buffer to a current of steam, but by this means the CH_3 content of a wool containing 1·63% CH_3 can be reduced to 0·72% in 60 min., although a further 180 min. treatment is required to reduce it to 0·55% (Blackburn *et al.* 1941). It thus appears probable that methyl sulphate methylates two centres: the free carboxyl groups which give ester groups that hydrolyze slowly and a second centre which may be an activated peptide linkage that methylates slowly, giving methoxyl groups that are readily removed by hydrolysis.

When this second centre is methylated, sulphate becomes covalently linked to the protein. For example, after 1·66% CH_3 had been introduced into wool by 20 methylations with methyl sulphate in phosphate buffer, the wool contained 3·30% SO_4 by Mease's method (1934). When samples (2 g.) of this wool were immersed at room temperature in buffers (100 ml.) of pH 0·5–10·5 for 16 hr. only 0·24% SO_4 (calc. on the weight of the wool) was extracted by the buffer of pH 0·5: the remaining buffers contained only traces of sulphate. Barritt, Bowen, Goodall & Whitehead (1938) have shown that 0·5% aqueous solution of pyridine will extract all the sulphate from wool which has been steeped in dilute H_2SO_4. Experiment showed, however, that a fully methylated wool (SO_4, 4·69%) still contained 4·04% SO_4 after extraction with this solvent.

That sulphate is introduced into wool during the slower peptide methylation is in agreement with the fixation of sulphate during the methylation by methyl sulphate of acetylated wools and wools methylated with methanol-acetic anhydride. Acetylated wools react very slowly with methyl halides, but they undergo a restricted and slow methylation when treated with methyl sulphate. For example, wools acetylated for 30 min. and 4 hr. contained after 15 methylations in phosphate buffer CH_3 0·95%, SO_4 2·88%, and CH_3 0·67%, SO_4 2·30% respectively. Further, when a wool which had been simultaneously acetylated and methylated with methanol-acetic anhydride was methylated 15 times with methyl sulphate in phosphate buffer, its CH_3 content increased from 0·74 to 1·60% and 2·72% sulphate was introduced. The supposed peptide methylation involves the introduction into wool of about 0·7% CH_3 and if one assumes that one sulphate group were introduced for each CH_3 group, a fully methylated wool should contain 4·5% SO_4. Generally less than this amount was found, but this may be because, during the repeated methylations, the wool was in contact with buffer solutions for long periods.

Blackburn *et al.* (1941) suggested the peptide linkage methylated by methyl sulphate was a resonance hybrid of two electronic structures which approached a zwitterion in constitution. Since this suggestion did not explain why sulphate becomes covalently linked to wool during the methylation, it was later suggested (Blackburn, Middlebrook & Phillips, 1942) that oxazoline ring structures are formed by condensation between the hydroxy side-chains of serine and threonine and the activated peptide linkages. It was further suggested that during methylation with methyl sulphate cleavage of the oxazoline rings so produced took place, the enolized peptide linkage being *O*-methylated and the hydroxy groups of the liberated serine and threonine side-chains sulphated. The experimental evidence given above lends some support to these suggestions.

SUMMARY

1. When collagen and gelatin are methylated with methyl sulphate, peptide methylation as well as esterification of free carboxyl groups takes place. During peptide methylation, sulphuric acid becomes covalently linked to proteins. When exposed to

methyl iodide or methyl bromide, the free carboxyl groups are esterified, but peptide methylation only occurs slowly, if at all. Neither the peptide methylation nor the esterification of the free carboxyl groups of wool is prevented by treating it before methylation with dilute alkali, nitrous acid or formaldehyde. On the other hand, removal of amide groups by acid hydrolysis leads to increased esterification.

2. When wool is acetylated with acetic anhydride, the subsequent methylation of free carboxyl groups by either methyl sulphate or methyl halides is restricted. Treatment of wool and silk fibroin with acetic anhydride in methanol leads to *N*- and *O*-acetylation, simultaneously with the methylation of free carboxyl groups.

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Diplococcin, an Anti-bacterial Protein Elaborated by Certain Milk Streptococci

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Whitehead (1933) first showed that milk sometimes contains lactic acid-producing streptococci which inhibit the action of the very similar organisms used industrially as cheese starters. He further proved that the inhibition was due to a protein-like substance produced by the inhibiting organisms. It was not however made clear whether this substance was a constituent of the medium altered by bacterial