THE ESTERIFICATION OF WOOL

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Summary

The reaction of wool with methanol, ethanol, n-propanol, and n-butanol in the presence of 0·1M HCl as catalyst has been found to be non-specific. In addition to the esterification of carboxyl groups, significant conversion of primary amide groups into esters occurs, especially with the higher alcohols. Experiments with [^{14}C]methanol show that the number of methyl groups bound during esterification considerably exceeds the methoxyl content, which in turn exceeds the ester content. Indirect evidence suggests that these extra methyl groups are introduced by *N*- and *O*-methylation of peptide bonds.

Esterification with methanol at 20°C is the best method for selectively modifying carboxyl groups in wool. Even under these optimal conditions a few amide side chains are esterified and the number of methyl groups bound still exceeds the ester content.

I. Introduction

Fraenkel-Conrat and Olcott (1945) first showed that it was possible to modify the carboxyl groups of proteins by treatment with methanol containing catalytic amounts of hydrogen chloride. The carboxyl groups of several proteins were completely esterified within 24 hr at room temperature, amino, phenolic, thiol, indole, peptide, and amide groups being unaffected. Under these conditions the carboxyl groups of wool react very slowly (Blackburn and Lindley 1948) but at 65°C all of the accessible carboxyl groups can be esterified in 6 hr (Alexander *et al.* 1951).

Recently, Kilpatrick and Maclaren (1969) found that amide groups, in addition to carboxyl groups, are modified during treatment of wool with boiling methanol containing hydrogen chloride. This casts doubts upon the specificity of the esterification procedure of Alexander *et al.* (1951). Esterification with boiling methanol has been used by numerous workers, who measured the changes in fibre properties (such as settability, water sorption, and dyeing behaviour) which resulted on esterification and then rationalized them in terms of changes in carboxyl content. It is therefore important to know the nature and extent of any side reactions accompanying the modification of carboxyl groups by esterification. We have examined in detail the esterification of wool with several alcohols and in this paper describe the side reactions which accompany the modification of carboxyl groups.

II. Experimental

(*a*) *Esterifications*

Wool fabric (MW 160, moisture content *c.* 10%) was esterified by treatment with methanol, ethanol, n-propanol, or n-butanol containing 0·1M hydrogen chloride (added as concentrated hydrochloric acid, unless otherwise stated) at 60°C for times in the range 0·5–50 hr. The liquor

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to wool ratio was 40 : 1. Acid concentrations of 0·2 and 0·5M were used in some experiments. Esterifications with methanol were also carried out at 20°C for 4 and 10 days. The treated fabrics were rinsed several times in distilled water, soaked overnight, allowed to dry at room temperature, and then conditioned at 20°C and 65% R.H. The moisture content at equilibrium decreased with increasing alkyl content. All analyses have therefore been corrected for moisture content and also for weight increase due to esterification. The chloride ion contents of esterified fabrics were $< 0{\cdot}5\%$, so corrections for weight increase due to chloride were not applied.

(*b*) *Analyses*

Carboxyl contents were determined by the method of Alexander *et al.* (1951), which entails titration of the wool from pH 7·0 to 1·7. Untreated fabric had a carboxyl content of 820 μmoles/g dry wool.

Amide contents were determined by estimation of the ammonia liberated afte hydrolysis of wool samples with 2M HCl for 3 hr at 100°C (Leach and Parkhill 1956). A value of 800 μmoles/g dry wool was obtained for untreated fabric. The loss in amide content which occurred during esterification was also estimated by analysis of the esterification mixture (after removal of the wool) for ammonia. Results obtained by the two methods were in good agreement.

Amino contents were determined by a colorimetric ninhydrin procedure (Caldwell, Leach, and Milligan 1969, and references therein), which gave a value of 215 μmoles/g for untreated fabric. Methoxyl contents were determined by the Australian Microanalytical Service by the Zeisel method. Thiol and disulphide contents were determined polarographically (Leach 1960). Amino acid analyses were determined using a Spinco amino acid analyser (model 120B). Acid hydrolyses were carried out with 6M HCl in sealed glass tubes for 24 hr at 110°C; alkali hydrolyses were carried out with 6M sodium hydroxide in sealed plastic tubes at 110°C for 24 hr.

(*c*) *Radioassays*

[^{14}C]Methanol was purchased from the Radiochemical Centre, Amersham, England, and n-[1-^{14}C]butanol from N. V. Phillips-Duphar, Holland. They were diluted appropriately with non-labelled alcohol before use. Specific activities were determined with a Packard Tri-Carb 314 EX liquid scintillation spectrometer after combusting the alcohols by the method previously described (Holt, Leach, and Milligan 1968). The [^{14}C]methanol was also assayed as its α-naphthylurethane and the n-[1-^{14}C]butanol as its phenylurethane. Both urethanes were radiochemically pure after one crystallization (from cyclohexane and light petroleum, respectively). The specific activity of the [^{14}C]methanol was 0·51 μCi/mmole (±2%) and of its α-naphthylurethane was 0·52 μCi/mmole (±2%). The n-[1-^{14}C]butanol had specific activity 2·12 μCi/mmole (±2%) and its phenylurethane 2·07 μCi/mmole (±2%). The close agreement of the specific activities of the alcohols with those of their corresponding derivatives establishes the radiochemical purity of the original alcohols.

Wool fabrics, after esterification with radioactive alcohols, were assayed for radioactivity by the combustion method (Holt, Leach, and Milligan 1968). The counting efficiency, estimated by internal standardization with added [^{14}C]benzoic acid, was 39·6–39·9% for all radioactive samples after combustion. Fabrics were also assayed "directly" (i.e. without combustion) by immersion in a modified scintillation cocktail (Holt, Leach, and Milligan 1968), the observed radioactivity being proportional to the [^{14}C]alkyl content estimated by the combustion method. The "direct" method was more convenient when counting a large number of fabric samples.

(*d*) O-*Methyltyrosine*

O-Methyl-L-tyrosine hydrobromide was prepared according to F.H.C. Stewart (personal communication) from *N*-benzyloxycarbonyl-L-tyrosine by treatment with dimethyl sulphate in alkaline solution. Removal of the benzyloxycarbonyl group with hydrogen bromide (2M) in acetic acid gave *O*-methyl-L-tyrosine hydrobromide as colourless plates, m.p. 220–221°C (with decomp.) after recrystallization from acetic acid. $[\alpha]_D^{21} -11{\cdot}3°$ ($c = 3$, H_2O). Found: C, 43·7; H, 5·2; CH_3O, 10·8%. $C_{10}H_{13}NO_3.HBr$ requires C, 43·5; H, 5·1; CH_3O, 11·2%.

(*e*) O-*Methylserine*

O-Methyl-DL-serine was prepared from ethyl acrylate by the method of Schiltz and Carter (1936) and purified via its 2-hydroxyl-1-naphthylidene derivative (Ando and Emoto 1961). It crystallized from aqueous ethanol as plates, m.p. 238°C (with decomp.). Found: C, 40·2; H, 8·0; N, 11·9; CH_3O, 25·0%. Calc. for $C_4H_9NO_3$: C, 40·3; H, 7·6; N, 11·8; CH_3O, 26·0%.

III. Results and Discussion

The rates at which carboxyl groups in wool are modified during esterification at 60°C are shown in Figure 1 for treatments with methanol, ethanol, n-propanol, and n-butanol. These results confirm earlier findings (Alexander *et al.* 1951) that the rate of esterification of carboxyl groups decreases markedly as the size of the alcohol increases. Figure 1 also shows that amides are progressively modified during esterification. The decrease in amide content corresponds well with the amount of ammonia

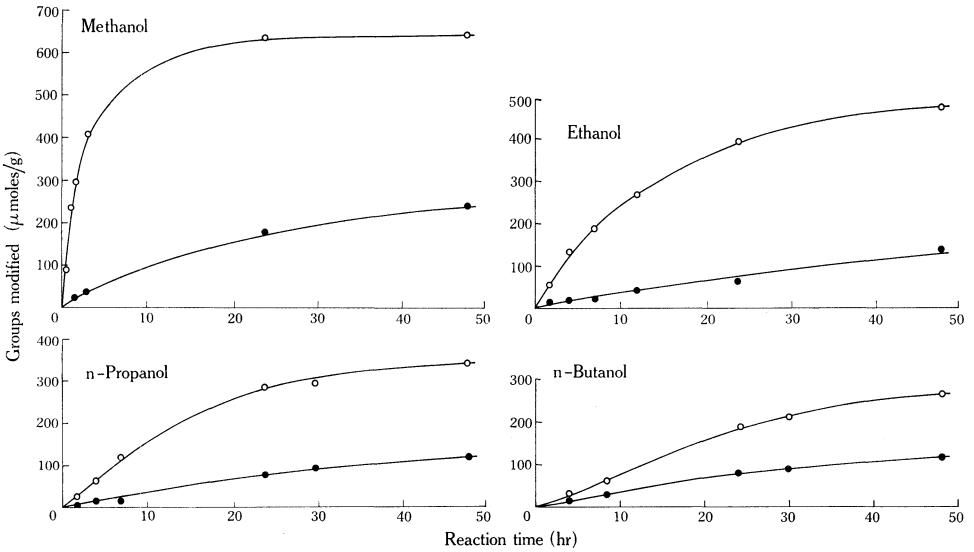

Fig. 1.—Rates of reaction of carboxyl (○) and amide (●) groups in wool with methanol, ethanol, n-propanol, and n-butanol at 60°C. 10M HCl was added to each alcohol to give a final acid concentration of 0·1M.

liberated into the esterification mixture, which is good evidence for the conversion of amides into esters; there is no evidence for the conversion of amides to *N*-methyl amides (see below). We are unable to say whether the amide groups are converted to esters directly or via carboxyl groups. Amide groups are modified during esterification with methanol at roughly twice the rate obtained with ethanol, n-propanol, or n-butanol (see Fig. 1). However, the rate of modification of carboxyl groups, relative to the rate of modification of amide groups, is much higher in the case of esterification with methanol than with the other alcohols. It follows that methanol is preferable to the higher alcohols for selectively modifying carboxyl groups with minimal modification of side-chain amides.

It has been shown previously that amide side chains in gluten are converted to esters by treatment with methanol, particularly using high concentrations (0·6–1·2M) of hydrogen chloride as catalyst (Beckwith, Wall, and Dimler 1963; Wall *et al.* 1967). Table 1 shows that the modification of amide groups in wool is also increased by increasing the concentration of acid used as catalyst. The number of carboxyl groups esterified increases too, but not proportionately. Many new amino groups are produced using hydrogen chloride in concentrations above 0·1M, indicating the occurrence of extensive peptide bond fission.

TABLE 1

CATALYSIS OF ESTERIFICATION BY HYDROGEN CHLORIDE: THE EFFECT OF ESTERIFICATION AT 60°C IN THE PRESENCE OF DIFFERENT ACID CONCENTRATIONS* ON CARBOXYL, AMIDE, AND AMINO CONTENTS

Alcohol	Time of Treatment (hr)	Acid Concn. (M)	Decrease in Carboxyl Content (μmoles/g)	Decrease in Amide Content (μmoles/g)	Increase in Amino Content (μmoles/g)
Methanol	3	0·1	408	38	0
		0·2	545	84	1
		0·5	602	162	7
Ethanol	24	0·1	392	64	3
		0·2	509	158	21
		0·5	499	318	52
n-Propanol	48	0·1	340	119	5
		0·2	419	233	41
		0·5	402	454	95
n-Butanol	48	0·1	267	118	17
		0·2	344	235	37
		0·5	310	542	141

* 10M HCl was added to the alcohol to give the appropriate concentration.

We have derived the total ester content of esterified wool by summing the loss in carboxyl and amide groups and the increase in amino groups (fission of peptide bonds produces equal numbers of amino and carboxyl groups). In the absence of other side reactions, the calculated ester content of wool esterified with [^{14}C]methanol should be the same as the methoxyl content and the [^{14}C]methyl content. Figure 2 shows that this is not so. After esterification at 60°C for 24 hr the methoxyl content* exceeded the calculated ester content by 110 μmoles/g.

This disparity between ester and methoxyl content has been observed previously for wools esterified with methanol (Blackburn, Carter, and Phillips 1941; Blackburn and Phillips 1944; Kilpatrick and Maclaren 1969). Kilpatrick and Maclaren suggested that the extra methoxyl groups could be present as methyl ethers of serine, threonine, or tyrosine residues. We would expect these ethers to be quite stable in

* The Zeisel method of analysis for methoxyl groups also estimates *S*-methyl groups. However, the thiol and disulphide contents of wool are unchanged by esterification and presumably the only *S*-methyl groups present are those of methionine, for which allowance (30 μmoles/g) has been made in all methoxyl contents quoted.

0·1M sodium hydroxide at room temperature (and have demonstrated this in the case of *O*-methyltyrosine and *O*-methylserine), whereas methyl esters would be

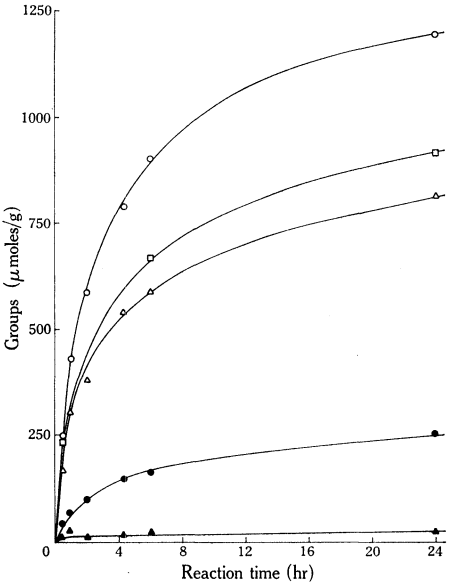

Fig. 2.—The [^{14}C]methyl (○), methoxyl (□), and ester (△) contents of wool fabric after esterification for different times at 60°C with [^{14}C]methanol containing 0·1M anhydrous HCl and the [^{14}C]methyl (●) and ester (▲) contents after subsequent treatment of the esterified fabric with 0·1M NaOH at 20°C for 18 hr.

rapidly hydrolysed. Figure 3 shows that virtually all of the ester groups in methanol-esterified wool were hydrolysed by 0·1M sodium hydroxide at 20°C within 18 hr.

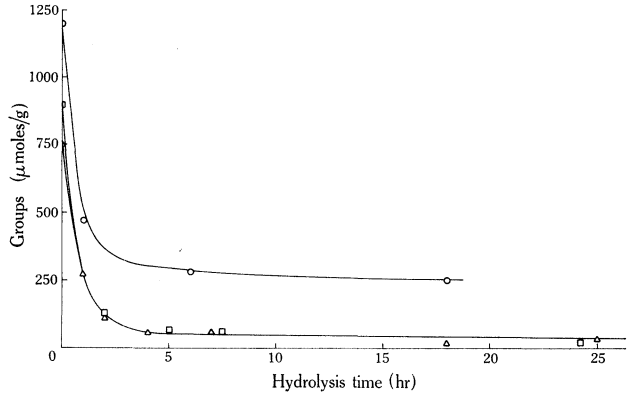

Fig. 3.—Effect of 0·1M NaOH at 20°C on the [^{14}C]-methyl (○), methoxyl (□), and ester (△) contents of wool which has been esterified for 24 hr at 60°C with [^{14}C]methanol containing 0·1M anhydrous HCl.

This treatment also caused the methoxyl content to fall to a very low level (*c.* 20 μmoles/g). It seems therefore that the extra methoxyl groups unaccounted for as methyl esters cannot be present as methyl ethers of serine, threonine, or tyrosine residues. Amino acid analysis of an alkali hydrolysate of esterified wool substantiates this conclusion. No *O*-methyltyrosine was detected despite the fact that 82% of this amino acid survives alkali hydrolysis in the absence of wool, 74% in its presence. Furthermore, no *O*-methylserine was detected in either acid or alkali hydrolysates

of esterified wool. Surprisingly, this amino acid is more stable in acid than in alkali (83% recovery after treatment with 6M HCl at 110°C for 18 hr, less than 5% recovery after treatment with 6M NaOH at 110°C for 18 hr).

The possibility that the extra methoxyl groups are due to adsorbed methanol, not removed by rinsing but displaced by alkali, was discounted by examining wool fabric which had been treated at 60°C for 24 hr with [^{14}C]methanol in the absence of hydrogen chloride. Rinsing with water removed virtually all radioactivity from the fabric within 1 hr.

A third explanation for the disparity between ester and methoxyl contents, advanced by Blackburn, Carter, and Phillips (1941) and Blackburn and Phillips (1944), invokes the occurrence of *O*-methylation of peptide bonds. Direct alkylation of amides normally gives *N*-alkyl derivatives but in some cases, e.g. ϵ-caprolactam, *O*-alkylation may occur preferentially (Benson and Cairns 1948). *O*-Methylamides, like the extra methoxyl groups in esterified wool, are readily hydrolysed and *O*-methylation of peptide bonds therefore remains a distinct possibility.

There is a further discrepancy in addition to that between ester and methoxyl contents. After esterification of wool with [^{14}C]methanol for 24 hr at 60°C, the total number of methyl groups bound, estimated by radioassay, exceeded the methoxyl content by 270 μmoles/g (see Fig. 2). These groups were resistant to mild alkaline hydrolysis, since virtually all remained after complete saponification of the ester groups present. Presumably these extra [^{14}C]methyl groups which resist alkali hydrolysis are *N*-methyl groups, since *C*-methylation seems very unlikely to occur under our conditions of esterification. *N*-Methyl groups in esterified wool could occur as the result of methylation of side-chain amides (asparagine and glutamine residues) or by methylation of basic amino acid residues, *N*-terminal residues, or peptide bonds.

N-Methylation of amide side chains would give methylamide groups, which on hydrolysis would yield methylamine. Wool which had been esterified with methanol at 60°C for 24 hr was subjected to acid hydrolysis and the hydrolysate was then made alkaline and steam-distilled. Ammonia and other volatile bases were collected by absorption in 0·1M HCl and then converted to 2,4-dinitrophenyl derivatives by treatment with 2,4-dinitrofluorobenzene in dimethyl sulphoxide containing triethylamine. Thin layer chromatography of the product on silica gel-coated plates showed the presence of 2,4-dinitroaniline and 2,4-dinitrophenol but no 2,4-dinitrophenyl derivative of methylamine was detected. The method was sufficiently sensitive to detect 20 μmoles of methylamide groups per gram wool. Hence it appears that no *N*-methylation of amide side chains has occurred during esterification.

Since there are few *N*-terminal amino acid residues (*c.* 20 μmoles/g) in wool (Thompson 1957), *N*-methylation of these could only account for a small fraction of the methyl groups not present as esters. Any *N*-methylation of the side chains of basic amino acids or *N*-methylation of peptide bonds should be detectable by analysis of an acid hydrolysate, since many *N*-methyl amino acids are moderately stable in hot acid (Ebata, Takahashi, and Otsuka 1966), whereas methyl esters are hydrolysed. Indeed, amino acid analysis of an acid hydrolysate of esterified wool ([^{14}C]methanol, 0·1M HCl, 60°C, 24 hr), followed by radioassay, showed the presence

of many [^{14}C]methyl amino acids in the effluents from both short and long columns. However, control experiments showed that these methyl amino acids did not necessarily arise during esterification. The amino acids obtained by acid hydrolysis of untreated wool with 6M HCl containing a little [^{14}C]methanol were found to contain considerable amounts of incorporated [^{14}C]methyl groups, most of which were stable to treatment with mild alkali. Therefore acid hydrolysis of esterified wool could cause [^{14}C]methyl groups to transfer from esters to other sites. This problem was overcome by removing ester groups by treatment with 0·1M NaOH before carrying out hydrolysis with 6M HCl. Evaporation of the hydrolysate gave a residue which contained *c.* 150 μmoles [^{14}C]methyl groups per gram of wool. Amino acid analysis and radioassay of the effluent indicated that at least 10 acidic, neutral, and basic [^{14}C]methyl amino acids were present in small amounts (*c.* 2–10 μmoles/g). No attempt was made to identify these due to the complexity of the chromatogram. The neutral and acidic methyl amino acids must have arisen at least in part by *N*-methylation of peptide bonds but the basic ones may have been formed by side-chain methylation of basic amino acid residues.

TABLE 2

EXTENT OF MODIFICATION OF CARBOXYL AND AMIDE GROUPS IN WOOL DURING ESTERIFICATION* WITH METHANOL AT DIFFERENT TEMPERATURES

Conditions of Esterification		Loss in Carboxyl Content (μmoles/g)	Loss in Amide Content (μmoles/g)
Temp. (°C)	Time (hr)		
20–25	96	465	17
20–25	240	580	53
60	5·0	465†	55†
60	12·5	580†	115†

* In presence of 0·1M HCl. † Interpolated from Figure 1.

We have also examined the esterification of wool with alcohols containing hydrogen chloride at lower temperatures. Appreciable modification of carboxyl groups occurred in 4 days using methanol, but there was little esterification with ethanol and virtually none with n-propanol or n-butanol. Table 2 shows the extents of modification of carboxyl and amide groups which occurred during esterification with methanol at room temperature. For the same level of carboxyl modification, fewer amides were modified during esterification at 20–25°C than at 60°C. However, esterification at 20°C using [^{14}C]methanol resulted in the binding of many more [^{14}C]methyl groups than can be accounted for as esters (see Table 3) and, in this respect, esterification at 20°C is no more selective for carboxyl groups than esterification at 60°C. In the absence of hydrogen chloride, methanol reacted with wool extremely slowly. After 8 days at room temperature, the total number of methyl

groups bound was only 44 μmoles/g. Not all of these were present as methyl esters, only 40% being cleaved by treatment with 0·1M sodium hydroxide.

TABLE 3

ESTER, METHOXYL, AND [^{14}C]METHYL CONTENTS OF WOOL AFTER ESTERIFICATION WITH [^{14}C]METHANOL* AT 20 AND 60°C

Conditions of Esterification		Calculated Ester Content†	Methoxyl Content‡	[^{14}C]Methyl Content
Temp. (°C)	Time (hr)	(μmoles/g)	(μmoles/g)	(μmoles/g)
20	96	435	549	623
20	240	555	618	751
60	2·4§	435§	472§	641§
60	5·0§	555§	624§	842§

* Containing 0·1M HCl. † Total loss of carboxyl and amide groups.

‡ Corrected for the contribution due to methionine. § Interpolated from Figure 2.

Esterification of wool with n-[^{14}C]butanol also resulted in the binding of more alkyl groups than can be accounted for by the ester groups introduced (see Fig. 4).

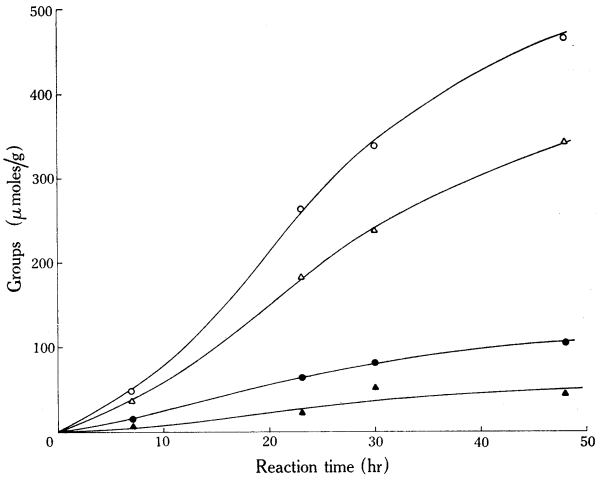

Fig. 4.—The [^{14}C]butyl (○) and ester (△) contents of wool after esterification for different times at 60°C with n-[1-^{14}C]butanol containing 0·1M anhydrous HCl and the [^{14}C]butyl (●) and ester (▲) contents after subsequent treatment of the esterified fabrics with 0·1M NaOH at 20°C for 18 hr.

Unlike methyl esters, n-butyl esters are not completely cleaved by treating the esterified wool with 0·1M sodium hydroxide for 18 hr at 20°C. Due to the difficulty of incorporating a large number of butyl groups into wool by esterification, no detailed analysis similar to that for methylated wool was carried out.

IV. ACKNOWLEDGMENTS

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