N-ACETYL GROUPS IN WOOL AND EXTRACTED WOOL PROTEINS

By I. J. O'Donnell,* E. O. P. Thompson,* and A. S. Inglis*

[*Manuscript received March* 23, 1962]

Summary

N-acetyl groups have been shown to exist in wool and in proteins extracted from wool after reduction with mercaptoethanol followed by alkylation with iodoacetate or after oxidation with performic acid. The evidence suggests that these acetyl groups are located on *N*-terminal amino groups of peptide chains rather than on the ϵ-amino groups of lysine.

Acid hydrolysis with 12N sulphuric acid was found to be preferable to alkaline hydrolysis for the release of acetic acid from the *N*-acetyl groups, since alkaline hydrolysis produced volatile acids other than acetic acid.

I. Introduction

End-group determinations on wool and extracted wool proteins have revealed a variety of *C*-terminal and *N*-terminal amino acid residues but only in extremely small amounts. The extracted proteins show the same wide distribution of *N*-terminal residues as wool itself (for summary see Thompson 1957, 1959; Bradbury 1958). One possible explanation is that masked *N*-terminal and *C*-terminal groups are present. *N*-acetyl α-amino groups have recently been found in the following peptides or proteins: tobacco mosaic virus protein (Narita 1958), cucumber virus protein (Narita 1959*a*), melanophore-stimulating hormone (Harris 1959), bovine fibrinogen (Folk and Gladner 1960), turnip yellow virus protein (Harris and Hindley 1961), ovalbumin (Marshall and Neuberger 1961; Narita 1961), calf thymus histone (Phillips 1961), and cytochrome *c* (Margoliash and Smith 1961; Kreil and Tuppy 1961; Titani, Narita, and Okunuki 1962). This paper describes experimental evidence which shows that *N*-acetyl groups are also present in wool and extracted wool proteins.

The acetyl groups in wool were liberated by hydrolysis with both acid and alkaline reagents. Although Herriott (1935) found that alkaline hydrolysis was satisfactory for the determination of acetyl groups in acetylated proteins, our experiments show that acid hydrolysis followed by steam distillation is the preferable procedure, at least for proteins such as wool with a relatively high sulphur content and very low acetyl content.

II. Materials and Methods

(*a*) *Wool and Wool Proteins*

The wool used was a solvent-scoured Merino 64's top (MW129) which had been washed overnight with dilute sulphuric acid at pH 3, and then with water. This process should remove any acetate ions bound to the wool.

* Division of Protein Chemistry, C.S.I.R.O. Wool Research Laboratories, Parkville, Vic.

The *S*-carboxymethyl kerateines representing the low-sulphur (SCMKA) and high-sulphur (SCMKB) proteins were prepared from the above wool top after reduction and alkylation as described previously (Thompson and O'Donnell 1962*a*).

In preparations A and B (Table 1) the reduced wool was filtered and washed with 0·001N HCl before alkylation. Approximately 6% by weight of the wool (probably high-sulphur protein) was lost to the mother liquor and washings. In preparation C, where six separate 1-g samples of wool were reduced, four were subsequently treated as in preparations A and B while the remaining two samples were precipitated and washed with 0·4N trichloroacetic acid and then dialysed to remove residual reagents before alkylation. In preparation D the reduced wool was precipitated and washed with 0·4N trichloroacetic acid before dialysis in order to increase the percentage extraction of soluble alkylated proteins and also to recover a higher yield of high-sulphur protein (Thompson and O'Donnell 1962). For fractionation of extracted *S*-carboxymethyl proteins into high- (SCMKB) and low-sulphur (SCMKA) fractions two methods were used. In preparations A and B the pH was adjusted to 4·4 with 1N HCl in the presence of 0·5M KCl to precipitate the SCMKA fraction. Because of the rapid coagulation of the precipitate and the possibility of entrainment of high-sulphur protein from the supernatant, a 1·75M citrate buffer (pH 4·40, 20 ml/l) was used in preparations C and D, in the presence of 0·5M KCl. The physical characteristics of the precipitate were much improved, and following filtration the precipitate could be redissolved much more readily in 0·01M borate than those precipitated with hydrochloric acid. After reprecipitation of the SCMKA fractions they were dissolved in 0·01M borate.

The SCMKB fraction in the large volume of supernatant was recovered either by freeze-drying or trichloroacetic acid precipitation (final concentration 0·38N) followed by dialysis. Each fraction was well dialysed against 0·1M potassium sulphate–0·001M borate and finally against water before freeze-drying.

α- and γ-keratoses were prepared from oxidized wool as previously described (O'Donnell and Thompson 1959). The excess performic acid reagent was removed by dialysis against ice-cold water on a rotating dialyser before the soluble proteins were extracted at pH 8. Sulphuric acid was used to bring the pH to 4·0 to separate α- and γ-keratoses and these soluble proteins were finally dialysed against sulphate–borate as described above for the reduced proteins.

(*b*) *Procedures for Acetyl Determinations*

(i) *Total Acetyl Groups.*—For the determination of *N*-acetyl groups in proteins several methods of hydrolysis were combined with two different methods of distillation in order to establish optimum conditions for the analysis. Initially, the procedure recommended by Herriott (1935) for the determination of acetyl groups in acetylated proteins was used. This involved hydrolysis of the protein (0·5 g) for 5 days at 37°C in 0·35N alkali followed by repeated vacuum distillation at 50±5°C of the solution acidified with aqueous citric acid. The liberated acetic acid is titrated with alkali (phenolphthalein), and identified by its pK value determined from the magnitude of the back-titration with hydrochloric acid to the bromcresol green end-point (pH 4·7).

This distillation and titration procedure was also used after acid hydrolysis in which the protein (0·05–0·5 g) was either heated in a sealed tube at 105°C for 24 hr, or refluxed for 4 hr, with 2–5 ml 12N sulphuric acid. However, attempts to distil acetic acid under vacuum from 12N sulphuric acid instead of from citric acid were unsuccessful and gave such high and irregular blank values that experiments with this procedure were not pursued.

In subsidiary experiments the acetic acid liberated by acid and alkaline hydrolysis was recovered by steam distillation from sulphuric acid containing sodium sulphate in a modified Kjeldahl distillation unit and titrated alkalimetrically (Schöniger, Lieb, and Ibrahim 1954; Inglis 1958). This procedure was also used for additional experiments in which proteins were hydrolysed by refluxing with either 5N aqueous sodium hydroxide or 5N sodium hydroxide in 33% methanol.

In all cases appropriate blank values were determined.

(ii) O-*Acetyl Groups.*—*O*-acetyl determinations on wool were carried out as described by Herriott (1935) and the alkaline liquor distilled after acidification with citric acid.

(*c*) *Elimination of Formyl Interference*

Acetyl determinations on proteins isolated from wool oxidized with performic acid are complicated by the presence of *O*-formyl groups introduced by the performic acid reagent (cf. Narita 1959*b*; Smillie and Neurath 1959). In order to eliminate formic acid from the acetic acid solution obtained on acid hydrolysis of wool proteins, the steam distillate was neutralized with alkali, freeze-dried, and the residue oxidized with a mixture of 4 ml 5N chromic acid (168 g CrO_3 per litre) and 1 ml 36N sulphuric acid for 15 min at 100°C. The acetic acid alone remained after this treatment and was estimated after distillation from the chromic acid mixture.

This procedure of distillation from chromic acid was also used to show the absence of formic acid in distillates from hydrolysed wool.

(*d*) *Identification of Acetic Acid*

Acetic acid was identified in the distillates from an alkaline digest of 20 g wool. After three consecutive vacuum distillations of the digest from citric acid solution containing cadmium sulphate (Herriott and Northrop 1934) the volatile acids were neutralized with sodium hydroxide and freeze-dried. This distillation process was repeated on the residue to help remove some volatile compounds which are believed to be by-products of the alkaline decomposition of the cystine in the wool. From the alkali titration value the theoretical amount of phenyl phenacyl bromide to react with the volatile acid was calculated. This weight of reagent was added to the sodium salt dissolved in 1·25 ml water and 2·5 ml ethanol. The mixture was refluxed for 1 hr, rapidly cooled, and the residue recrystallized from 40% ethanol. The crystalline ester was compared with an authentic derivative of acetic acid prepared by the method of Drake and Bronitsky (1930).

TABLE 1

VOLATILE ACIDS DISTILLED FROM PROTEIN HYDROLYSATES

Results (columns 4 and 5) expressed as millilitres of 0·02N NaOH required to neutralize each distillate. Blank values have been subtracted from each titration value given

Sample	Dry Weight (mg)	Time of Hydrolysis	Titres (ml) after Alkaline Hydrolysis (0·35N NaOH, 37°C)*	Titres (ml) after Acid Hydrolysis (12N H_2SO_4, 105°C)	$10^{-3}\times$ Weight of Protein (g) containing 1 Equiv. of Acetyl
N-Acetyl methionine	14·5	24 hr		3·76¶	
Insulin†	135	24 hr		Nil	
N-Acetyl methionine +insulin†	14·5+240	24 hr		3·81¶	
Insulin†	225	5 days	(+$CdSO_4$) 0·6, 0·5, 0·15, total 1·25		
Wool	450	5 days	2·5, 1·58, 0·56, 0·48, 0·3, 0·1, total 5·52		
Wool	450	5 days	(+$CdSO_4$) 0·7, 0·3, 0·3, 0·4, 0·35, 0·15, total 2·20		
Wool	61	4 hr‖		0·16	19·1
	236	4 hr‖		0·56	21·1
	457	4 hr‖		1·04	22·0
	180	4 hr‖		0·49	18·4
	360	24 hr		0·94	19·1
SCMKA	450	5 days	1·2, 0·8, 0·59, 0·18, 0·12, 0·15, total 3·04		
SCMKA ex prep. A	465 (45%)§	24 hr		0·96	24·2
SCMKA ex prep. B	460 (48%)	24 hr		0·93	24·7
SCMKA ex prep. C	572 (45%)	24 hr		0·93	30·7
SCMKA ex prep. D	454 (58%)	24 hr		0·90	25·2
SCMKB	190	5 days	1·22, 0·7, 0·48, 0·32, 0·34, 0·46, 0·46, 0·04, total 4·02		
SCMKB ex prep. A	167 (4%)	24 hr		0·88	9·5
SCMKB ex prep. B	331 (6%)	24 hr		1·12	14·8
SCMKB ex prep. D	502 (12%)	24 hr		2·01	12·5
α-Keratose‡	450 (51%)	24 hr		0·68	33·1
γ-Keratose‡	450 (17%)	24 hr		1·74	12·9

* Acetic acid (4·3 ml, 0·02N), when vacuum-distilled at 50°C, gave titres 3·4, 0·65, 0·25, total 4·3.

† International sample No. 2189. Dialysis against potassium sulphate–sodium borate to remove contaminant anions had no effect on titres of subsequent distillates.

‡ Formyl groups removed as described in Section II(*c*).

§ Values in brackets are approximate percentages of wool that these fractions represent.

‖ Refluxed with 12N H_2SO_4.

¶ 99% theoretical value.

III. Results and Discussion

(a) *Alkaline Hydrolysis*

(i) *Total Acetyl Groups.*—The method recommended by Herriott (1935) for the determination of *N*-acetyl groups in acetylated proteins was found to be unreliable for the proteins studied here. This seems to be due at least in part to volatile acids produced by the alkaline degradation of sulphur-containing amino acids which are present in large amounts in comparison with the small quantity of acetyl groups. Although control experiments with added acetic acid showed that

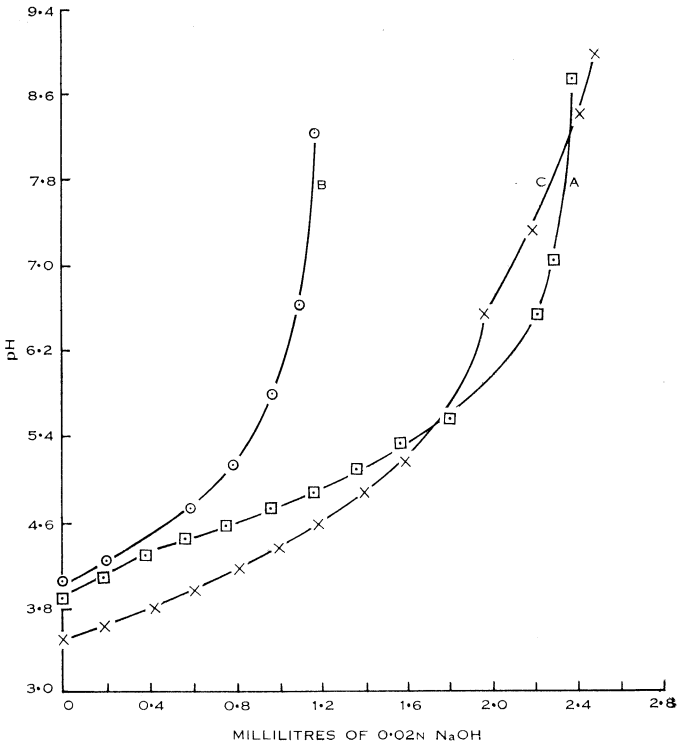

Fig. 1.—Titration curves of acetic acid (*A*), volatile acids steam-distilled from wool after acid hydrolysis (*B*), and volatile acids vacuum-distilled from wool after alkaline hydrolysis (*C*).

acetic acid could be recovered in three distillations by the vacuum-distillation technique (cf. Herriott 1935), Table 1 shows that the volatile acids obtained from alkaline hydrolyses of wool are recovered less efficiently. Herriott's criterion for identification of acetic acid was that the back-titration to pH 4·7 (bromcresol green) of the neutralized (phenolphthalein) volatile acid should be approximately half the forward titration. Although our results for wool met this criterion, titration with a glass-electrode revealed that products other than acetic acid were present, the shape of the titration curve (Fig. 1) being quite different from that of acetic acid. Purified insulin, which contains no acetyl groups and gives no volatile acids on acid

hydrolysis, also gave volatile acidic by-products after heating with alkali. In an effort to eliminate these artefacts cadmium sulphate was added before distillation. This addition, which had been used by Herriott and Northrop (1934), eliminated the pronounced buffering effect in the region between pH 5 and 8·5 that had previously appeared in the titration curve. However, the titration curve was still not exactly that of acetic acid. These results were obtained irrespective of the alkaline reagent or the distillation procedure. Alkaline hydrolysis was therefore abandoned for *N*-acetyl determinations. However, the vacuum-distillation procedure was more convenient for large-scale distillations and from the volatile products obtained from 20 g of wool a crystalline derivative of acetic acid (55 mg of the phenyl phenacyl ester, melting point and mixed melting point 110–112°C) was isolated. This product required only one recrystallization. The yield of product is only of the order of 20% but, in view of the fact that the bulk distillation of the wool digest is inefficient, this yield suggests that at least a large proportion of the acids present is acetic acid. The absence of small amounts of other small straight-chained carboxylic acids has not been proved.

(ii) O-*Acetyl Determinations.*—The determination of *O*-acetyl groups by alkaline hydrolysis at pH 10 (cf. Herriott 1935) and subsequent vacuum distillation from citric acid plus cadmium sulphate solution gave a titre which represented less than 10% of the total acetyl value obtained with acid hydrolysis. This indicates the presence of *N*-acetyl rather than *O*-acetyl groups or extraneous acetate.

(*b*) *Acid Hydrolysis*

Fraenkel-Conrat, Bean, and Lineweaver (1949) used 6N sulphuric acid hydrolysis at 100°C for release of acetic acid from acetyl groups in acetylated proteins. They recovered the acetic acid by vacuum distillation under conditions similar to those used by Herriott (1935) but reported that blank values for untreated proteins were in the range 1–3 equivalents per 10^4 g. As previously mentioned we were unable to satisfactorily distil sulphuric acid solutions under these conditions. However, the steam-distillation procedure gave low and reproducible blank values and *N*-acetyl methionine gave 99% recovery of acetyl groups after hydrolysis with acid. The presence of insulin during acid hydrolysis of *N*-acetyl methionine did not lower the recovery (Table 1). Furthermore titration curves of the distillates from acid hydrolysates of wool gave curves very similar to that of acetic acid (cf. Fig. 1). Since insulin gave no volatile acids by this method, acid hydrolysis is recommended as the more reliable procedure when determining small amounts of acetyl groups in proteins. Moreover, the reproducibility of acetyl determinations on wool, obtained under a number of different hydrolysis conditions, suggests that the hydrolysis procedures employed here gave quantitative fission of the acetyl groups. Table 1 lists the titres obtained for acid hydrolysates of wool and wool proteins.

(*c*) N-*Acetyl Content of Wool and Wool Proteins*

The values given in Table 1 show that 1 equivalent of acetic acid can be obtained from about 20,000 g of wool by acid hydrolysis. Corresponding values

for the extracted wool proteins are: SCMKA (1 in 26,000 g), SCMKB (1 in 9,500–15,000 g), α-keratose (1 in 33,000 g), and γ-keratose (1 in 13,000 g). The variation in the SCMKB values is probably linked with the different final yields of these proteins (see Table 1) and with the heterogeneity of high-sulphur wool proteins (O'Donnell and Thompson 1961; Gillespie 1962*a*). Because of the heterogeneity of these and also the low-sulphur wool proteins SCMKA (Gillespie 1962*b*) and α-keratose (O'Donnell and Thompson 1961; Thompson and O'Donnell 1962*a*), the absolute value of the number of grams of extracted wool proteins which contains one equivalent of acetic acid cannot be considered reliable. Furthermore, since the high-sulphur wool proteins contain more acetyl groups per gram than do the low-sulphur ones, contamination of the low-sulphur protein by high-sulphur protein could raise the apparent acetyl content of the low-sulphur protein. In the case of the low-sulphur protein α-keratose from oxidized wool, we know that there is some contamination by a protein resembling the high-sulphur protein (cf. Thompson and O'Donnell 1962*a*), and the high- and low-sulphur proteins from reduced and alkylated wool can also co-precipitate (Gillespie, O'Donnell, and Thompson 1962).

Since *N*-acetyl groups have been found in a variety of proteins as listed in Section I, mechanisms must exist for their biosynthesis and it is therefore not surprising that they are present in wool. Their detection in fibrinogen and wool, both members of the *k-m-e-f* group of proteins, suggests that they could also be present in myosin where the *N*-terminal residues are not equal to the *C*-terminal residues (Locker 1954).

The presence of acetyl groups on *N*-terminal α-amino acid residues in wool and extracted wool proteins can only be positively decided by isolation and identification of the corresponding *N*-terminal peptides (cf. Narita 1958). Such a study is at present complicated by heterogeneity in the case of wool proteins, but a study of *N*-acetyl sequences may be of value for following fractionation. There is no unequivocal method for separating *N*-acetyl terminal peptides since a basic amino acid residue adjacent or close to the *N*-acetyl terminal residue interferes with the application of the method of Narita (1958) which depends on the expected acidity of an *N*-acetyl peptide (cf. Harris and Hindley 1961). We do not think that the acetyl groups found here are located predominantly on the lysine residues since the ε-amino groups of wool (Middlebrook 1951) and extracted wool proteins are available to 1,2,4-fluorodinitrobenzene under mild conditions, e.g. in the low-sulphur proteins at least 90% of the ε-lysyl residues react. Also there is an excess of acetyl groups over lysyl groups in the high-sulphur protein SCMKB. The evidence strongly suggests that the acetyl groups present in wool and extracted wool proteins are *N*-terminal.

If the *N*-terminal end of the protein chains of wool and proteins extracted from it are masked by acetyl groups, the *C*-terminal ends of the chains may be masked also. Bradbury (1958) found that no large amounts of free amino acids were liberated from wool on hydrazinolysis, and his technique should have detected all amino acids not containing an amide group. We have found (Thompson and O'Donnell, unpublished data) that carboxypeptidase A does not liberate

sufficient asparagine or glutamine from SCMKA or α-keratose to indicate that they could be the predominant *C*-terminal residues. However, other amino acids are liberated in significant amounts and their origin is being investigated.

The molecular weight of the extracted wool proteins is still an unresolved problem (Gillespie *et al.* 1960; Woods 1961) and estimates range from 8000 determined by the surface-balance technique (Harrap 1956) to 50,000 in free solution in the presence of disaggregating agents. The *N*-acetyl contents which we have reported are consistent with molecular weights lower than 50,000.

IV. References

Bradbury, J. H. (1958).—*Biochem. J.* **68**: 482.

Drake, N. L., and Bronitsky, J. (1930).—*J. Amer. Chem. Soc.* **52**: 3718.

Folk, J. E., and Gladner, J. A. (1960).—*Biochim. Biophys. Acta* **44**: 383.

Fraenkel-Conrat, H., Bean, R. S., and Lineweaver, H. (1949).—*J. Biol. Chem.* **177**: 385.

Gillespie, J. M. (1962*a*).—*Aust. J. Biol. Sci.* **15**: 262.

Gillespie, J. M. (1962*b*).—*Aust. J. Biol. Sci.* **15**: 564.

Gillespie, J. M., O'Donnell, I. J., and Thompson, E. O. P. (1962).—*Aust. J. Biol. Sci.* **15**: 409.

Gillespie, J. M., O'Donnell, I. J., Thompson, E. O. P., and Woods, E. F. (1960).—*J. Text. Inst.* **51**: T703.

Harrap, B. S. (1956).—Proc. Int. Wool Text. Res. Conf. Aust. 1955. Vol. B. p. B–86.

Harris, J. I. (1959).—*Biochem. J.* **71**: 451.

Harris, J. I., and Hindley, J. (1961).—*J. Mol. Biol.* **3**: 117.

Herriott, R. M. (1935).—*J. Gen. Physiol.* **19**: 283.

Herriott, R. M., and Northrop, J. H. (1934).—*J. Gen. Physiol.* **18**: 35.

Inglis, A. S. (1958).—*Mikrochim. Acta, Wien* **1958**: 228.

Kreil, G., and Tuppy, H. (1961).—*Nature* **192**: 1123.

Locker, R. M. (1954).—*Biochim. Biophys. Acta* **14**: 533.

Margoliash, E., and Smith, E. L. (1961).—*Nature* **192**: 1121.

Marshall, R. D., and Neuberger, A. (1961).—*Biochem. J.* **78**: 31P.

Middlebrook, W. R. (1951).—*Biochim. Biophys. Acta* **7**: 547.

Narita, K. (1958).—*Biochim. Biophys. Acta* **28**: 184.

Narita, K. (1959*a*).—*Biochim. Biophys. Acta* **31**: 372.

Narita, K. (1959*b*).—*J. Amer. Chem. Soc.* **81**: 1751.

Narita, K. (1961).—*Biochim. Biophys. Res. Comm.* **5**: 160.

O'Donnell, I. J., and Thompson, E. O. P. (1959).—*Aust. J. Biol. Sci.* **12**: 294.

O'Donnell, I. J., and Thompson, E. O. P. (1961).—*Aust. J. Biol. Sci.* **14**: 461.

Phillips, D. M. P. (1961).—*Biochem. J.* **80**: 40P.

Schöniger, W., Lieb, W., and El Din Ibrahim, M. G. (1954).—*Mikrochim. Acta, Wien* **1954**: 96.

Smillie, L. B., and Neurath, H. (1959).—*J. Biol. Chem.* **234**: 355.

Thompson, E. O. P. (1957).—*Aust. J. Biol. Sci.* **10**: 225.

Thompson, E. O. P. (1959).—*Aust. J. Biol. Sci.* **12**: 303.

Thompson, E. O. P., and O'Donnell, I. J. (1962*a*).—*Aust. J. Biol. Sci.* **15**: 552.

Thompson, E. O. P., and O'Donnell, I. J. (1962*b*).—*Aust. J. Biol. Sci* **15**: 757.

Titani, K., Narita, K., and Okunuki, K. (1962).—*J. Biochem., Japan* **51**: 350.

Woods, E. F. (1961).—In "Structure de la Laine". Textes et discussions du colloque, July 1961. p. 143. (Institut Textile de France.)