STUDIES ON REDUCED WOOL

II. THE IDENTIFICATION OF TERMINAL *N*-ACETYLALANINE IN WOOL AND EXTRACTED WOOL PROTEINS

By I. J. O'Donnell* and E. O. P. Thompson*

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Summary

N-Acetylalanine has been isolated and identified in partial acid hydrolysates of wool and extracted wool proteins. Quantitatively it only accounts for a fraction of the acetyl groups present in these proteins. Isolation of *N*-acetylated peptides from enzymic digests of extracted wool proteins is complicated by the large amounts of pyroglutamic acid and pyroglutamyl peptides produced either during the hydrolysis or on subsequent passage through a column of Dowex 50 resin. No *N*-acetylated peptide could be isolated from either a pronase digest of reduced wool or a partial acid hydrolysate of wool.

I. Introduction

In a previous paper (O'Donnell, Thompson, and Inglis 1962) the presence of acetyl groups in wool and extracted wool proteins was demonstrated. The evidence suggested that they were located on α-amino groups, rather than on hydroxyl groups (serine, threonine, or tyrosine) or on ϵ-amino groups (lysine). The amount of acetic acid liberated on acid hydrolysis was such that *N*-acetyl amino acids appear to be the major end-groups of wool. As pointed out previously, positive evidence for the location of the acetyl groups can only be obtained by isolation and identification of the respective *N*-acetylated amino acids or peptides. The present paper reports attempts to isolate such acetyl compounds from enzymic and acid digests of wool and extracted wool proteins using the methods developed by Narita (1958).

II. Materials and Methods

The wool used was a Merino 64's top, MW129, which had been cleaned in the usual way (O'Donnell, Thompson, and Inglis 1962). The low-sulphur (SCMKA) and high-sulphur (SCMKB) protein fractions were prepared from this wool by reduction with mercaptoethanol followed by alkylation with iodoacetate and separation into high-sulphur and low-sulphur fractions by precipitation with citrate buffer (O'Donnell, Thompson, and Inglis 1962) at pH 4·4 in the presence of 0·5M potassium chloride (Gillespie, O'Donnell, and Thompson 1962).

Enzymic digestions of extracted wool proteins (1–2% solution) were carried out for 24 hr at 37°C in a pH-stat, or in 1% ammonium carbonate at pH 8·6–8·8. Enzymes used were chymotrypsin, pepsin, or pronase (1%, on the weight of the protein). The pronase was a product of Kaken Chemical Co., Japan. At the end of digestion the ammonium carbonate was removed by freeze-drying.

* Division of Protein Chemistry, CSIRO Wool Research Laboratories, Parkville, Vic.

Partial acid hydrolysis was carried out by standing 10 g wool in 160 ml of approximately 11N HCl for 72 hr at 37°C. This is the procedure of Gordon, Martin, and Synge (1941) (cf. also Sanger 1952) who showed that after this time the maximum number of dipeptides was present. The hydrochloric acid was removed by evaporation under reduced pressure.

The protein digests were dissolved in water and passed through a column (3 by 20 cm) of the acid form of Dowex 50–X2 resin, the unadsorbed fraction being eluted with water. This eluate was evaporated to dryness and, in the cases where large amounts of wool were used, passed again through the regenerated Dowex 50–X2 column. The unadsorbed fractions after freeze-drying to remove hydrochloric acid were loaded on a Dowex 1–X8 resin column in the chloride form. The column was washed with water and the eluate discarded. The bound materials were then removed from the Dowex 1–X8 by elution with 0·2N HCl. This eluate was dried by rotary evaporation and subsequent freeze-drying.

Ionophoresis was carried out under toluene by the method of Michl (1951) with Michl buffers as used by Sanger and co-workers (cf. Ryle *et al.* 1955). The dried chromatograms (or guide strips) were sprayed with 0·5% ninhydrin in 95% ethanol or treated with chlorine gas (Rydon and Smith 1952) and then sprayed with a solution of *o*-tolidine and potassium iodide in acetic acid (Reindel and Hoppe 1954). For Whatman No. 3MM paper, the most satisfactory results were obtained if the dried papers were first lightly sprayed with approximately 0·05M sodium tetraborate solution and then stood for 10 min in a jar full of chlorine (Zahn and Rexroth 1955). The paper was removed and waved around in the air for 1 min to remove excess chlorine. A corner was then tested with the *o*-tolidine–potassium iodide spray to find whether the background was reduced sufficiently for the rest of the paper to be sprayed. If the background was still too dark even after exposure to air for 2–3 min it could be reduced by Zahn and Rexroth's procedure of fleeting exposures to ammonia vapour. *N*-Acetylalanine and pyroglutamic acid were both readily located by the chlorine–potassium iodide procedure. It is of interest that Titani, Narita, and Okunuki (1962) found that *N*-acetylglycine was not readily revealed by their procedure.

The ionophoretic bands which were negative to ninhydrin but which reacted positively with the chlorine–*o*-tolidine–potassium iodide were located with guide strips and eluted. They were further purified either ionophoretically, or chromatographically in a solvent containing methyl cellosolve, 98% formic acid, butan-2-ol, and water in the volume ratio 2 : 1 : 8 : 3 (Eager and Savige 1963).

For quantitative estimation of the *N*-acetylalanine in a partial acid hydrolysate of wool (conc. HCl, 3 days at 37°C) the *N*-acetylalanine band was isolated ionophoretically and the amount of alanine estimated by a Spinco amino acid analyser after complete acid hydrolysis of the band.

The hydrazinolysis method (Akabori, Ohno, and Narita 1952; Niu and Fraenkel-Conrat 1955) was used for identification of the acetyl groups. A particular band, after elution from paper and evaporation to dryness, was heated in a sealed tube with anhydrous hydrazine at 100°C for 16 hr. After drying *in vacuo* over concentrated sulphuric acid the residue was analysed by one-dimensional chroma-

tography using either pyridine–aniline–water (9 : 1 : 4 v/v, Narita 1958) or collidine–α-picoline–water (5 : 3 : 2 v/v, Narita 1961). The acetyl hydrazide was detected with ammoniacal silver nitrate reagent (see Narita and Ishii 1962). Care had to be taken in evaporating the excess hydrazine as acetyl hydrazide is volatile under high vacuum, as has also been observed by Phillips (1963).

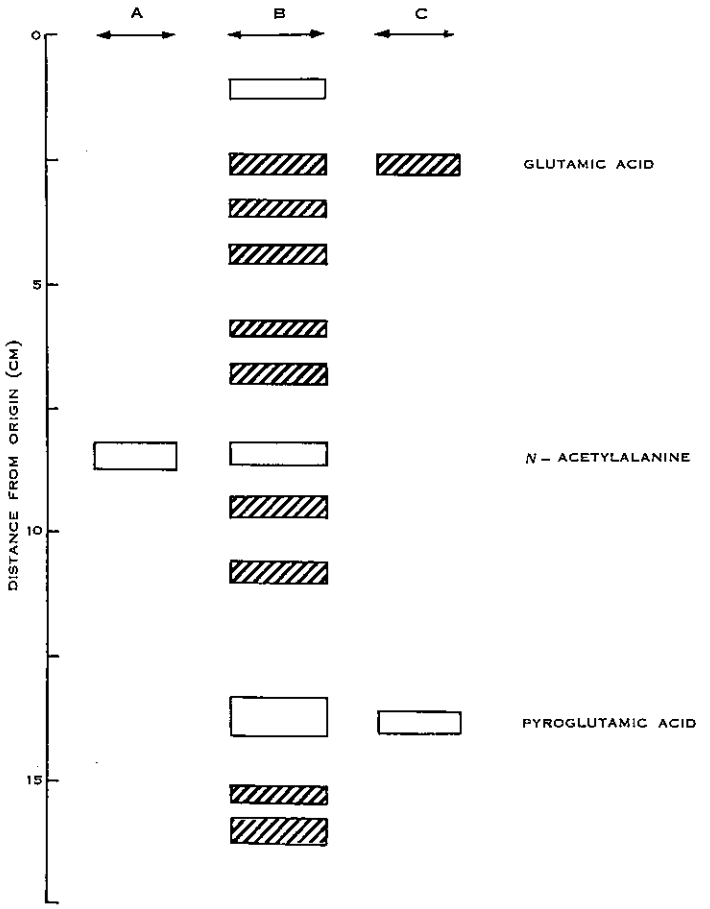

Fig. 1.—Ionophoretic pattern revealed by chlorine–potassium iodide of the acidic fraction of a partial acid hydrolysate of wool. Ionophoresis on Whatman No. 3MM paper at pH 3·6 for 1·5 hr at 50 V/cm. Migration towards the anode. The hatched areas also reacted positively with ninhydrin. A, *N*-acetylalanine; B, acidic fraction of a partial acid hydrolysate of wool; C, glutamic acid plus pyroglutamic acid.

III. Results and Discussion

After fractionation of a partial acid hydrolysate of wool on columns of Dowex 50 and Dowex 1 resins, ionophoresis at pH 3·6 of the acidic peptide fraction revealed only three bands which gave no ninhydrin reaction but gave a positive chlorine–potassium iodide reaction (Fig. 1). One of these was pyroglutamic acid and this and

also the slowest band gave only glutamic acid after complete acid hydrolysis. The second band is probably the diketopiperazine of glutamic acid. The third band on acid hydrolysis gave only alanine and ran in the same position as an authentic sample of *N*-acetylalanine, both ionophoretically at pH 3·6 and chromatographically. Hydrazinolysis of this spot produced a compound which chromatographed in the same relative position as acetyl hydrazide in the two solvents described above. This third spot is therefore *N*-acetylalanine.

Quantitative estimation of the amount of *N*-acetylalanine in a wool hydrolysate (conc. HCl, 3 days, 37°C) gave a maximum value of 5 μmoles per gram. This is only approximately 10% of the amount of acetic acid released by complete acid hydrolysis of wool (O'Donnell, Thompson, and Inglis 1962). Control experiments showed that *N*-acetylalanine was almost completely stable to the hydrolytic conditions of concentrated hydrochloric acid at 37°C, and in this respect shows a similarity to the stability of *N*-acetylglycine as reported by Titani, Narita, and Okunuki (1962).

In addition to the above three ninhydrin-negative bands, there were six or more bands which were ninhydrin-positive (Fig. 1). These were probably cysteic acid peptides derived from the small amount of cysteic acid present in raw wool.

It was of interest to see whether *N*-acetylalanine was present in both the high-sulphur and low-sulphur fractions of wool. Partial acid hydrolysis of the high-sulphur fraction, SCMKB, followed by fractionation with ion-exchange resins and paper ionophoresis revealed a relatively high content of *N*-acetylalanine and a low content of pyroglutamic acid; there were few other bands present. In contrast the low-sulphur fraction, SCMKA, gave a relatively weak *N*-acetylalanine band and a very heavy pyroglutamic acid band as well as numerous other ninhydrin-positive bands. The heavy pyroglutamic acid band is a reflection of the high content of glutamic acid in SCMKA but there is no obvious explanation for the much lower intensity of the corresponding band from SCMKB since their glutamic acid contents are at the most only different by a factor of two. It is possible that the proportion in each fraction of certain sequences, such as glutamylglutamic acid, which is the most abundant dipeptide found in wool (Consden, Gordon, and Martin 1949), gave rise to different behaviour between the fractions. No attempt was made to determine the amount of *N*-acetylalanine released by acid hydrolysis of the SCMKA and SCMKB.

It is possible that the *N*-acetylalanine detected represents only a fraction of the *N*-acetylalanine residues present in wool. If, for instance, the *N*-acetylalanine was linked to a basic amino acid residue, partial acid hydrolysis may liberate only a proportion of the *N*-acetylalanine; the remaining *N*-acetylalanyl-X (X = lysine, histidine, or arginine) would stay adsorbed to the Dowex 50 resin with the other neutral and basic peptides and would thus escape detection. Similarly if there were some *N*-acetyl-X this would not be detected.

No *N*-acetylated peptides that would extend knowledge of the *N*-acetylalanyl sequence were detected in the acid hydrolysate, but this may be a consequence of the amino acid adjacent to the alanine; for instance, if serine or threonine is the next amino acid along the chain, the –Ala–Ser– or –Ala–Thr– bond would break readily

in acid solution (cf. Sanger 1952) and no *N*-acetylalanylserine or *N*-acetylalanylthreonine would survive. Similarly *N*-acetylserine or *N*-acetylthreonine, if they were present as terminal residues, would not survive acid hydrolysis and therefore would not be detected. However, we found no evidence of these in enzymic digests of extracted wool protein SCMKA.

It is possible that *N*-acetyl amino acids other than *N*-acetylalanine are present in wool. From the results to date [reviewed by Narita and Ishii (1962), and O'Donnell, Thompson, and Inglis (1962)], it is apparent that a variety of *N*-terminal amino acids (glycine, serine, alanine, methionine, threonine) may be masked by acetylation. Incidentally pyroglutamic acid if present as an end-group in wool would not be detectable by the methods in current use.

Initially, attempts were made to isolate acetyl peptides from enzymic digests of reduced wool or reduced and alkylated wool or low-sulphur wool protein, using the general methods of Narita and co-workers. The enzymes used included chymotrypsin, pepsin, and pronase and, in general, the results were complicated by the presence of pyroglutamic acid and a number of pyroglutamyl peptides which were ninhydrin-negative and chlorine–potassium iodide-positive, and made fractionation and identification of *N*-acetyl amino acids or *N*-acetylated peptide compounds very time-consuming. Ready cyclization of *N*-terminal glutaminyl (Melville 1935; Sanger and Thompson 1953; Hirs, Stein, and Moore 1956; Smyth, Stein, and Moore 1962) or glutamyl peptides (Le Quesne and Young 1952) to pyroglutamyl peptides is well known, as is the effect of sulphonated polystyrene in promoting the conversion of glutamic acid to pyroglutamic acid (Moore and Stein 1951; Das and Roy 1962).

Although no *N*-acetylalanyl peptides were isolated and identified, there was strong evidence that one of the spots in the ionogram of a pronase digest of reduced wool was *N*-acetylalanine. None of the other spots contained alanine but they gave large amounts of glutamic acid on complete acid hydrolysis and presumably represented pyroglutamyl peptides.

IV. References

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