The Sulphur Balance in Wool

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During the past 30 years many samples of wool have been analysed for their total S, methionine, cystine and cysteine contents. All these analyses have produced a fair balance between the total S and the sum of the contributions of the three sulphur-containing amino acids, but, in general, a slight excess of sulphur was found (Marston, 1928; Rimington, 1929; Barritt, 1934*a*; Bailey, 1937; Schöberl & Rambacher, 1940; Cuthbertson & Phillips, 1945; Simmonds, 1954; Corfield & Robson, 1955; Leach, 1960). The small excess was usually attributed to experimental error, or to conversion of cystine S into hydrogen sulphide, sulphate, cysteic acid, lanthionine or free sulphur, but the possibility remained that some sulphur was present in a hitherto undetected form. With the advent of the chromatographic methods of amino acid analysis, it was shown that the amounts of *meso*-cystine plus cystine isolated from wool hydrolysates by chromatography were considerably less than the cystine contents determined colorimetrically (Simmonds, 1954; Corfield & Robson, 1955). This analytical discrepancy, too large for experimental error, cast doubts on the soundness of the colorimetric determination of cystine and refocused attention on the problem of the sulphur balance in wool.

To aid further investigation, and especially to ensure the unequivocal detection of all sulphur-containing substances present, a sample of ^{35}S -labelled wool was prepared (Lewis, Robson & Tiler, 1960). Chromatographic examination of its hydrolysate revealed the presence of several unidentified compounds that were subsequently shown to be derived from cystine during hydrolysis. The amounts of these compounds varied with hydrolytic conditions, but in acid hydrolyses of 24 hr. duration they formed together about 10% of the total ^{35}S activity. Since all of them gave positive reactions for either thiol or disulphide, their presence in wool hydrolysates accounted for the hitherto unexplained discrepancy between the amounts of cystine determined by chromatographic and colorimetric methods. The most abundant of these compounds has been identified as bis-(2-amino-2-carboxyethyl) trisulphide, and another of it is possibly bis-(2-amino-2-carboxyethyl) tetrasulphide (Fletcher & Robson, 1962*a*, 1963).

In this context the problem of the sulphur balance in wool has been re-examined by using a variety of methods for the determination of total S, methionine, cystine and cysteine.

MATERIAL AND METHODS

Australian Merino 64s quality wool. This was prepared according to the method of Corfield & Robson (1955). It was 'conditioned' over saturated NH_4NO_3 solution, and its moisture content determined by drying samples at 105°.

Colorimetric determination of cystine and cysteine. The method of Shinohara (1935), as modified by Fletcher & Robson (1962*b*), was used to determine both cystine and cysteine; the latter amino acid was also determined colorimetrically by the method of Liddell & Saville (1959).

Radiometric determinations of cystine plus cysteine. The method of Fletcher & Robson (1962*b*) was used.

Polarographic determinations of cystine and cysteine. The methods used were based on those described by Leach (1959) and by Allison & Cecil (1958).

Three solutions, A, B and C, were prepared for reaction with the wool. Solution A contained 0.04 mole of NH_3 , 0.16 mole of NH_4Cl , 0.40 mole of KCl, 0.20 mole of Na_2SO_3 , 0.5 m-mole of HgCl_2 and 50 mg. of gelatin/l. Solution B was the same as solution A except that 0.6 m-mole of phenylmercuric hydroxide was substituted for the HgCl_2 . Solution C was the same as solution B without the Na_2SO_3 .

For determinations of 'thiol plus disulphide', samples (100 mg.) of wool were shaken in stoppered flasks for periods between 1 and 14 days with either 100 ml. of solution A or 200 ml. of solution B. When thiol only was to be determined, samples (100 mg.) of wool were shaken with 100 ml. of solution C for periods between 1 and 14 days. After the required time the wool was removed by filtration through sintered glass, and a portion of filtrate was transferred to a polarographic cell (Human & Leach, 1956), maintained at 25°, for the recording of its current-voltage curve by using a modified Cambridge polarograph. Oxygen was removed from the solutions by a stream of oxygen-free nitrogen before measurement, and nitrogen flow maintained over the solutions during measurement. The characteristics of the dropping-mercury electrode were: t , 3.96 sec., and m , 1.51 mg./sec., at -0.583 v in 0.4 M-KCl. Current was plotted against concentration for HgCl_2 solutions in the range 0–0.4 mM in solution A, with a voltage of -0.40 v (against the saturated calomel electrode) on the dropping-mercury electrode. Similar curves were constructed for phenylmercuric hydroxide solutions with concentrations in the range 0–0.6 mM in both solutions B and C. The currents from the filtrates of the reaction mixtures were then read against the appropriate standard curves to give the excesses of mercury reagents present. Hence the 'thiol plus disulphide' and the thiol contents of the wool

Table 1. *Uptake of phenylmercuric hydroxide, and of mercuric chloride, by wool before and after scission of the disulphide bonds*

Samples (100 mg.) of wool were allowed to react with 100 ml. of phenylmercuric hydroxide (0.6 mm) at pH 8.9, or with 200 ml. of the same solution in the presence of sulphite, for the periods shown. The uptake of phenylmercuric hydroxide was followed by polarographic determination of the excess. The uptake of HgCl_2 by wool at pH 8.9 in the presence of sulphite was similarly determined. Each value is the mean of duplicate determinations. Details are given in the Materials and Methods section.

Period of reaction (days)	Uptake of $\text{Ph}\cdot\text{Hg}\cdot\text{OH}$ by wool at pH 8.9 ($\mu\text{moles/g. of wool}$)	Uptake of $\text{Ph}\cdot\text{Hg}\cdot\text{OH}$ and of HgCl_2 by wool at pH 8.9 in the presence of sulphite ($\mu\text{moles/g. of wool}$)	
		$\text{Ph}\cdot\text{Hg}\cdot\text{OH}$	HgCl_2
1	46	442	142
2	45	510	—
3	—	492	—
4	—	518	252
5	52	—	262
6	53	—	264
7	62	545	280
8	66	564	278
9	65	—	—
10	—	567	—
11	—	565	289
12	87	—	294
13	87	—	295
14	87	574	295
15	—	566	—

were deduced, the 'disulphide' content being obtained by subtracting the latter from the former.

Methionine. This was determined, on wool that had been hydrolysed under reflux for 24 hr. with 5.7N-HCl, by the automatic amino acid analyser described by Corfield & Robson (1962).

Total sulphur. The methods of Barritt (1934b), Niederl, Baum, McCoy & Kuck (1946) and Jones & Letham (1956) were used.

RESULTS

The amounts of phenylmercuric hydroxide taken up by virgin wool at pH 8.9 after increasing periods of time are given in Table 1, together with similar data for the uptake of phenylmercuric hydroxide and mercuric chloride by wool in the presence of sulphite. Phenylmercuric hydroxide will combine with thiol groups in a protein according to eqn. (1), and the uptake of phenylmercuric

$$\text{Prot}\cdot\text{S}^- + \text{Ph}\cdot\text{Hg}\cdot\text{OH} \rightarrow \text{Prot}\cdot\text{S}\cdot\text{Hg}\cdot\text{Ph} + \text{OH}^- \quad (1)$$

hydroxide by wool therefore should be a direct measure of the thiol present. The results in Table 1 show that for the first 2 days at least the uptake of phenylmercuric hydroxide is constant, but that thereafter the amount combined increases until after 12 days the uptake reaches a steady value

again. It is impossible to interpret these findings with certainty, but two explanations are suggested. The initial uptake of phenylmercuric hydroxide is by combination with the free thiol groups of wool, but in buffer at pH 8.9 some disulphide bonds are hydrolysed slowly and the additional thiol groups released combine with more phenylmercuric hydroxide. Leach (1960) had this in mind when he recommended that the determination of thiol in wool with methylmercuric iodide should be carried out at pH 7. An alternative explanation is provided by the observations of Allison & Cecil (1958), who found that human haemoglobin will take up more phenylmercuric hydroxide than is required for combination with thiol groups. Sulphite and thiosulphate, however, prevented the increased uptake by complexing with the mercury reagent. Thus it is possible that the increase in the uptake of phenylmercuric hydroxide shown by wool may be due to combination at sites other than thiol, but will not occur when sulphite is present. Whatever the true explanation may be the following assumptions have been made in calculating the amounts of thiol and disulphide in wool from the results shown in Table 1:

(a) The uptake of phenylmercuric hydroxide by virgin wool after 1 or 2 days' reaction at pH 8.9 represents the amount of free thiol present; this is shown as ' $\text{SH}(\text{Ph}\cdot\text{Hg}\cdot\text{OH})$ ' in Table 2.

(b) In the presence of sulphite the uptake of phenylmercuric hydroxide represents the sum of the free thiol plus the thiol produced by the scission of disulphide bonds in wool according to eqn. (2).

The thiol groups obtained from the reduction of these disulphide bonds react with phenylmercuric hydroxide according to eqn. (1).

(c) In the presence of sulphite the uptake of mercuric chloride also represents the sum of the free thiol and the thiol produced by the scission of the disulphide bonds in wool. The amount of free thiol is assumed to be the same as that determined with phenylmercuric hydroxide, and to combine with mercuric chloride according to either eqn. (3) or eqn. (4).

(d) All thiol groups derived from the scission of disulphide bonds are assumed to react with mercuric chloride according to eqn. (4).

On the basis of these assumptions the results shown in Table 2 have been derived from the data in Table 1. ' $\text{SS}(\text{Ph}\cdot\text{Hg}\cdot\text{OH}) \text{ A}$ ' is calculated from the average uptake of phenylmercuric hydroxide in

Table 2. Comparison of the amounts of methionine, cysteine, cystine and sulphur found in wool by a variety of analytical methods

Where applicable mean values $\pm$ S.D. are given.

Type and method of analysis	Wool hydrolysate	No. of analyses	S in wool (μ g.atoms/g.)		S in wool (%)	
			Cystine S	Cysteine S	Cystine S + cysteine S	Cystine S + cysteine S + methionine S†
Colorimetric (Shinohara, 1935; modified by Fletcher & Robson, 1962b)	4 hr. (HCl)	4	994 $\pm$ 6	32 $\pm$ 6	3.28	3.40
	16 hr. (HCl)	4	1007 $\pm$ 16	35 $\pm$ 3	3.33	3.45
	40 hr. (HCl)	4	904 $\pm$ 12	77 $\pm$ 15	3.13	3.25
Colorimetric (Liddell & Saville, 1959)	4 hr. (HCl)	3	—	37 $\pm$ 0.3	—	—
	16 hr. (HCl)	3	—	60 $\pm$ 8	—	—
	40 hr. (HCl)	3	—	21 $\pm$ 10	—	—
	12 hr. (H ₂ SO ₄)	3	—	31 $\pm$ 0.6	—	—
Chromatographic (Fletcher & Robson, 1963)	16 hr. (HCl)	1	906	16	2.95	3.07
	40 hr. (HCl)	1	890	19	2.91	3.03
Radiometric (Fletcher & Robson, 1962b)	16 hr. (HCl)	3	—	—	3.45 $\pm$ 0.07	3.57
	40 hr. (HCl)	5	—	—	3.42 $\pm$ 0.08	3.54
Polarographic (see Materials and Methods section)						
SH (Ph·Hg·OH)*		4	—	46 $\pm$ 2.5	—	—
SS (Ph·Hg·OH) A*		10	1040 $\pm$ 4	—	3.45	3.57
SS (Ph·Hg·OH) B*		10	982 $\pm$ 4	—	3.28	3.40
SS (HgCl ₂) A1*		8	988 $\pm$ 5	—	3.30	3.42
SS (HgCl ₂) A2*		8	1080 $\pm$ 5	—	3.60	3.72
					Total S in wool (%)	
Macro-gravimetric (Barritt, 1934b)					3.52 $\pm$ 0.04	
Micro-gravimetric (Niederl <i>et al.</i> 1946)					3.74 $\pm$ 0.06	
Micro-spectrophotometric (Jones & Letham, 1956)					3.71 $\pm$ 0.06	

* For the derivation of these results see the Results section.

† Methionine was determined in triplicate on a 24 hr. (HCl) hydrolysate of wool by automatic amino acid analysis (Corfield & Robson, 1962), and the methionine S (0.12% on the weight of the wool) added to the values in column 6 to give the results in column 7.

the presence of sulphite over 8–15 days; 'SS (HgCl₂) A1' is calculated from the average uptake of mercuric chloride over 11–14 days, by assuming that the free thiol reacts with mercuric chloride according to eqn. (3); and 'SS (HgCl₂) A2' is calculated from the same data, by assuming that the free thiol reacts according to eqn. (4).

The amount of disulphide S in the wool can also be calculated from the data in Table 1, on the assumption that phenylmercuric hydroxide, taken up in the absence of sulphite in excess of that assumed to combine with free thiol, is firmly bound to sites in wool other than thiol or disulphide, and that this binding cannot be prevented by sulphite. For example, after 14 days' reaction the uptake of phenylmercuric hydroxide by thiol groups derived from the scission of disulphide bonds by sulphite is given by $574 - 87 = 487 \mu\text{moles/g. of wool}$. (Amounts of phenylmercuric hydroxide combining with wool in the absence of sulphite after 10, 11 and 15 days' reaction have been interpolated as 72, 79

and 87 μmoles respectively for the purpose of these calculations.) The result is given in Table 2 as 'SS (Ph·Hg·OH) B'.

There is a further possibility that the increased uptake of phenylmercuric hydroxide with time may be due to the slow hydrolysis of disulphide bonds in the wool. If this is so the net result will be the same as for 'SS (Ph·Hg·OH) A'.

Table 1 of Fletcher & Robson (1963) includes the results of some chromatographic determinations of cystine and cysteine in wool hydrolysates. These have been incorporated in Table 2 of the present paper as they are pertinent to the subsequent discussion.

DISCUSSION

Accurate determinations of the total S, cystine and cysteine contents of proteins are still technically difficult in spite of the advances in methods of analysis that have been made during the past two decades (Cecil & McPhee, 1959). Difficulties were

encountered with the sulphur balances for insulin (Miller & Du Vigneaud, 1937; Du Vigneaud, Miller & Rodden, 1939) and for ribonuclease (Hirs, Stein & Moore, 1954; Hirs, Moore & Stein, 1956), but were resolved before the elucidation of the chemical structures of these proteins removed all doubts. Similar problems exist with the sulphur balances of papain (Finkle & Smith, 1958; Glazer & Smith, 1961) and of lysozyme (Jollès, Jollès-Thauraux & Fromageot, 1958; Jollès, Jollès & Jauregui-Adell, 1960), and these may only be solved when the chemical structures of these proteins are finally known.

Difficulties at least as great can be expected in arriving at a sulphur balance for wool: wool is not a homogeneous protein and, if pure wool is defined as the keratinous fibre synthesized in the skin follicles, it is doubtful if a sample of pure wool has ever been examined. When dirt, vegetable matter, suint and grease have been removed, and the weathered tips of the fibres discarded, the wool that remains can best be described as purified wool. Moreover, the sulphur content of wool is not invariable, and for purposes of comparison with other wool samples it is important to record the breed of sheep from which the sample under study is derived, the quality of the wool and the history of its treatment before analysis. In the present work a sample of Australian Merino 64s quality wool was used, and the word 'wool' is used in the discussion to refer to wool of this type.

Total sulphur. The total S content of wool, knowledge of which is a prime requisite for the establishment of a sulphur balance, was determined in the present work by three methods that have given good results in our hands when used on model compounds. However, the results in Table 2 show that the total S determined by the method of Barritt (1934*b*) is in poor agreement with the total S determined by the other two methods. Since the reproducibility of the results is high for all three methods, and the analyses were performed in duplicate by each of two analysts, it would appear that the differences are due to the methods rather than to personal errors. According to these analyses the total S content of the wool sample lies between 3.52% and 3.74%, but in discussion it is taken as the average value, 3.66%.

Cystine. By far the greater part of the total S in wool is present as cystine, the accurate determination of which is thus of paramount importance for the sulphur balance. The methods used to determine cystine in the present work may be divided into two classes, hydrolytic and non-hydrolytic; the hydrolytic methods and their results are discussed first.

During the acid hydrolysis of wool some cystine decomposes to give a complex mixture of sulphur

compounds; the most abundant of these is bis-(2-amino-2-carboxyethyl) trisulphide, another is probably bis-(2-amino-2-carboxyethyl) tetrasulphide, and several others remain unidentified (Lewis *et al.* 1960; Fletcher & Robson, 1963). All these compounds give positive reactions for either thiol or disulphide when tested by the appropriate Shinohara (1935) reaction, but some of them contain sulphur in excess of the amounts measured colorimetrically as disulphide S. For example, bis-(2-amino-2-carboxyethyl) trisulphide gives only about half as much colour in the Shinohara (1935) determination of disulphide as an equivalent amount of sulphur in the form of cystine. Some loss of cystine S as volatile compounds also occurs during acid hydrolysis; in experiments in which wool was hydrolysed in the presence of [³⁵S]cystine, activity losses of 2.4 and 6.1% were noted after 16 and 40 hr. of hydrolysis respectively (Fletcher & Robson, 1962*b*). Thus the chromatographic determination of cystine in a wool hydrolysate gives the true cystine content, whereas the colorimetric determination of cystine in the same hydrolysate gives only an apparent cystine content, comprising the disulphide contributions of cystine and cystine-decomposition products. The extent to which these two determinations differ depends on the hydrolytic conditions used, and can be gauged from the data in Table 2.

A radiometric method for the determination of cystine plus cysteine, that corrects intrinsically for hydrolytic losses of these amino acids, was described by Fletcher & Robson (1962*b*). This method gave good results with well-characterized cystine-containing proteins, e.g. insulin and lysozyme, and has given reproducible results with wool. According to the results in Table 2, when the methionine S is included the sulphur contributions of the three sulphur-containing amino acids total 3.56%. This value falls within the range of the determinations of total S.

Because of the difficulties presented by hydrolytic losses of cystine, alternative methods of analysis have been proposed. These include the determination of cystine as cysteic acid, as in the method of Schram, Moore & Bigwood (1954), the disulphide-interchange method of Glazer & Smith (1961), and the numerous methods based on quantitative reactions of thiol groups in proteins with a variety of reagents. The determination of cystine in wool as cysteic acid was not a success in our hands, giving erratic results owing to non-reproducibility of the oxidation stage of the method. Two non-hydrolytic methods, based on the titration of the thiol groups in wool with phenylmercuric hydroxide or with mercuric chloride, were more successful experimentally, but the amount of cystine in wool that they give

depends on the correct interpretation of the experimental data, which are given in Table 1. The amount of Hg^{2+} ion taken up by wool at pH 8.9 in the presence of sulphite after 5 or 6 days' reaction is in perfect agreement with the amount measured by Leach (1960) under the same conditions, and the amount of phenylmercuric hydroxide taken up by wool in the presence of sulphite after 2–4 days' reaction is in good agreement with the amount of methylmercuric iodide that Leach (1960) showed will combine under the same conditions. With both the mercury reagents, however, our results show an increased uptake up to a reaction-time of 12 days. From the results in Table 2 and the explanations given in the preceding section, it is obvious that the cystine content of wool, as measured by these methods, depends on an accurate determination of cysteine and on the stoichiometry of the reaction between the mercury reagents and the thiol groups. The determination of cysteine in wool is examined before the significance of the cystine determinations for the sulphur balance of wool is discussed.

Cysteine. There is considerable evidence for the presence of a small but significant amount of thiol in wool, but there are differences of opinion with regard to its origin. This thiol may have escaped oxidation to disulphide during the keratinization of the wool fibre; or the thiol may have been produced by the scission of some disulphide bonds during the time the wool fibres were on the sheep's back or during the cleaning processes used to remove dirt, suint and grease. It might appear unimportant which hypothesis is correct, because the amount of thiol is very small, but for a correct sulphur balance it would be essential to know whether the thiol groups occur in isolation, or whether each one represents half of a disulphide bond whose other sulphur atom suffers an unknown fate.

As with cystine, cysteine can be determined by hydrolytic and non-hydrolytic methods of analysis. That the cysteine content of a wool hydrolysate can be taken as only an approximation to the cysteine content of wool is well recognized (Schöberl, 1959). During hydrolysis, interaction between cysteine and tryptophan results in the formation of cysteine (Olcott & Fraenkel-Conrat, 1947), and it is possible that cysteine is slowly lost as hydrolysis continues, either by oxidation or, more probably, by interaction with hydrolytic artifacts. Although a detailed knowledge of these processes is lacking, it might be expected that results closest to the true value would be obtained with the shortest possible time of hydrolysis, and that the amount of cysteine present in hydrolysates of wool might attain a maximum and then decrease again. However, the results of the colorimetric cysteine determinations (Table 2) show a disturbing lack of reproducibility.

The two series of determinations by the Shinohara (1935) and Liddell & Saville (1959) methods should give comparable results as the hydrolytic conditions were the same for both series, but, apart from the 4 hr. hydrolysates, the agreement is poor. The amounts of cysteine in the wool hydrolysates originally examined by the Liddell & Saville (1959) method were confirmed by the Shinohara (1935) method, showing that the differences are due to variations in the cysteine contents of hydrolysates made under the same conditions and not to errors in the methods. Cuthbertson & Phillips (1945), using the method of Shinohara (1935) without modification, found 22–32 μmoles of cysteine/g. of wool after 4 hr. of hydrolysis. Unpublished work in this Laboratory has shown that at least one unidentified compound giving a positive thiol reaction is formed by the breakdown of cystine during the acid hydrolysis of wool, but this would not be expected to be present in significant amount after 4 hr. of hydrolysis. The conclusion may be drawn, therefore, that 4 hr. hydrolysates contain about 30 μmoles of cysteine/g. of wool, and that this is the best estimate of the cysteine content of the original wool that can be obtained by these methods, although, owing to the operation of opposing factors, a similar result might be obtained after longer times of hydrolysis.

Methods for the determination of cysteine by non-hydrolytic methods are in principle much more reliable. The reagent used must, however, combine quantitatively with the thiol groups and with no other groups in wool, and it is not easy to decide how completely these conditions have been fulfilled. Polarographic measurements of the uptake of phenylmercuric hydroxide by wool in the absence of sulphite give a value of 46 μmoles of thiol/g. of wool after a reaction time of 1 day at pH 8.9, and thereafter the amount increased with time to a maximum of 87 μmoles /g. of wool. According to Leach (1960) the amount of mercury reagent taken up by wool depends on the reagent and the pH: 35 μmoles of methylmercuric iodide were taken up by 1 g. of wool at pH 5.6, but 41 μmoles at pH 9.1. Leach (1960) believes that the increase uptake at the higher pH is due to hydrolytic scission of some disulphide bonds. If this is so there is no guarantee that scission of disulphide bonds does not occur at lower pH values. Leach (1960) considers that 35 μmoles of thiol/g. of wool is a reasonable estimate, but Human (1958), Burley (1956) and Burley & Horden (1957) found smaller amounts, within the range 18–30 μmoles of thiol/g., and Zahn, Weigmann & Nischwitz (1961) found 22 μmoles of *S*-dinitrophenylcysteine/g. after the reaction between wool and dinitrofluorobenzene. Thus the methods not involving hydrolysis do not agree very well among themselves, but indicate

a value of about 30 μ moles of cysteine/g. of wool, in agreement with the best estimate that can be arrived at from the hydrolytic methods.

Sulphur balance. The methionine S content of the wool sample was 0.12%, in complete agreement with previous analyses (Barritt, 1934*a*; Corfield & Robson, 1955; Cuthbertson & Phillips, 1945; Simmonds, 1954; Ward, Binkley & Snell, 1955). The sum of the methionine S and the contributions of cysteine and cystine, as determined colorimetrically in 4 hr. and 16 hr. hydrolysates of wool, accounts for only 93% of the total S content. The results in Table 2 show that methionine S, plus cysteine S and cystine S as determined polarographically, can account for between 93 and 102% of the total S according to the way in which the experimental data are interpreted. A similar calculation, by using the amounts of cysteine S and cystine S obtained by the radiometric method, accounts for 97% of the total S content of wool. Because the colorimetric methods give unreliable estimates of cysteine and cystine, owing to the changes that portions of these amino acids undergo during hydrolysis, and because there are uncertainties in the polarographic determinations, we regard the radiometric method as being the most sound fundamentally. The results are consistent with our view that all the sulphur in wool is in the form of the three sulphur-containing amino acids. However, additional sources of wool sulphur have been found by other investigators, and the probable origins of these demand attention.

Previous workers have shown that hydrolysates of virgin wool may contain cysteic acid, lanthionine, free sulphur, hydrogen sulphide, sulphate and bromine-oxidizable S (Cuthbertson & Phillips, 1945; Wibaux, Mazingue & Van Overbèke, 1960; Zahn, Kunitz & Hildebrand, 1960). The cysteic acid S and lanthionine S found were approximately equal, each representing 0.03% of the weight of the wool. We do not regard these amino acids as primary constituents of the wool fibre, but adhere to the general belief that they are produced from cystine under environmental conditions, and as such their amounts would be expected to vary according to the wool sample. No cysteic acid was found in our wool hydrolysates, and the sensitivity of our analytical methods places an upper limit of 0.02% on the cysteic acid S; lanthionine was not detected. However, on the basis of the findings of others the amounts of these amino acids, if present, could make a contribution of 0.06% to the total S of wool.

Sulphate, free sulphur and hydrogen sulphide are almost certainly hydrolytic artifacts from cystine, but there is some doubt as to whether all the sulphate in wool hydrolysates comes from cystine. Thus carrier sulphate added to a hydro-

lysate of ^{35}S -labelled wool removed 0.6% of the total ^{35}S activity on subsequent precipitation, which sets an upper limit of 0.02% to the weight of wool present as sulphate S, since there are dangers that other ^{35}S -labelled sulphur compounds may be co-precipitated. Other investigators have found much more sulphate than this in wool hydrolysates, so it is possible that sulphate of natural origin was present in our wool. When wool is hydrolysed for 16 hr. in the presence of [^{35}S]cystine about 2.4% of the ^{35}S activity is lost, and Bailey (1937) showed that wool lost 3% of its total S after 15 hr. of hydrolysis in acid. Presumably free sulphur and hydrogen sulphide form part of this.

Bromine-oxidizable S is that part of the total S in a wool hydrolysate that can be oxidized to sulphate by bromine water (Blumenthal & Clarke, 1935). Under the conditions used by these last authors, Thompson (1955) found only 93% conversion of cystine into cysteic acid, and obtained an indication that part of the cystine is oxidized to sulphate. Some sulphate, classified as bromine-oxidizable S, may come from this source. Bis-(2-amino-2-carboxyethyl) trisulphide is also present in wool hydrolysates, and yields one-third of its sulphur as sulphate on oxidation with bromine (Fletcher & Robson, 1962*a*), but the amounts of this trisulphide are insufficient to account for the 0.13% of bromine-oxidizable S present after 24 hr. of hydrolysis (Cuthbertson & Phillips, 1945).

The chromatographic examination of hydrolysates of ^{35}S -labelled wool (Lewis *et al.* 1960) and of wool hydrolysed in the presence of [^{35}S]cystine (Fletcher & Robson, 1962*b*) has revealed no ^{35}S -labelled substances that could not be accounted for as known constituents of the wool fibre, or as hydrolytic products from cystine, with the exception of two small radioactive peaks found on a starch chromatogram of an hydrolysate of ^{35}S -labelled wool. This work has not been extended, but the amounts of these substances represented less than 1% of the total ^{35}S activity.

From all these considerations we conclude that there are no sulphur compounds in wool other than cysteine, cystine and methionine, but traces of cysteic acid and lanthionine, which are formed from cystine during weathering, may be found in some samples of so-called virgin wool. The whole of the total S of wool has been accounted for in terms of these amino acids within the limits of the experimental errors.

Finally, we record some observations on the variation of total S content in wool from a particular breed, Merino wools of finenesses in the 60s to 70s range. In their critical review of the formation, composition and properties of keratins, Ward & Lundgren (1954) state that 'Sulphur or cystine contents discriminate species, individuals within a

species, nutritional status, follicles on an individual, and almost certainly, in some instances, the two sides of a follicle'. Evidence for variations in total S contents between wools from different species seems incontestable, and work by Ross (1961) supports the view that the total S content depends on nutritional status and on the individual animal. However, doubts have arisen with regard to the unequal distribution of S on two sides of a follicle (Leveau, 1959; Ryder, 1956; Schöberl, 1960), and we believe that variations in the total S content between wools of the same species may not be so great as they appear. If the estimated total S contents of Merino wools of 60s and 70s qualities obtained over a period of 36 years, by analysts of many countries using a variety of methods for S determinations and a variety of procedures for pretreatment of the wools, are averaged, the mean value is 3.61%. The recorded percentages, taken in chronological order, are: 3.62 (Barritt & King, 1926), 3.56 (Marston, 1928), 3.67 (Rimington, 1929), 3.72 (Barritt & King, 1929), 3.73 (Barritt, 1934*a*), 3.63 (Bailey, 1937), 3.45 (Cuthbertson & Phillips, 1945), 3.76 (Lindley, 1947), 3.68 (Simmonds, 1954), 3.70 (Ward & Lundgren, 1954), 3.38 (Corfield & Robson, 1955), 3.54 (Zahn *et al.* 1960), 3.46 (Leach, 1960), 3.66 (Wibaux *et al.* 1960) and 3.66 (the present work). Eleven out of the fifteen analyses are within 3% of the mean, and all but one within 5%. We consider that the recorded analyses are more likely to reflect inaccuracies in the S determinations than variations in the total S content, and that the total S content of Merino wools of 60s and 70s fineness has not varied over the past 36 years.

SUMMARY

1. The cystine, cysteine and total S contents of a sample of Australian 64s quality Merino wool have been determined by several different methods.

2. The colorimetric method for the determination of cystine in hydrolysates gives low results. This can be partially explained by the presence in the hydrolysate of decomposition products of cystine which give smaller colour yields than cystine, and by loss of volatile decomposition products.

3. A radiometric method for the determination of cystine plus cysteine allows correction to be made for loss of cystine during hydrolysis.

4. The polarographic determination of cystine and cysteine in intact wool can, with certain interpretations of the data, give results in agreement with the radiometric method. However, these interpretations are not unambiguous.

5. Three different and precise methods for total S gave significantly different results. The mean

value found, $3.66 \pm 0.12\%$, is close to the sum of the best values for cystine S, cysteine S and methionine S.

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Morphological Changes Associated with the Extrusion of Protein Induced in the Polymorphonuclear Leucocyte by Staphylococcal Leucocidin

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A survey of biochemical changes that occur when polymorphonuclear leucocytes are treated with leucocidin showed that although the normal impermeability of the cell membrane to many small molecules is retained there is a release into the medium of protein derived from the cytoplasmic granules (Woodin, 1961, 1962). When calcium is omitted from the medium the release of the granule proteins is prevented but the cell membrane simultaneously becomes more permeable to the soluble components of the cytoplasm (Woodin & Wieneke, 1963).

It has been suggested that the release of protein may have features in common with the extrusion phase of secretion in mammalian cells (Woodin, 1963). In the leucocyte the process is completed within 10 min. and is not accompanied by regeneration of the granules, so that the biochemical changes associated with protein extrusion are more easily followed. To aid the interpretation of the biochemical changes a study has been made of the morphology of the leucocidin-treated leucocyte and is reported in this paper.

METHODS

Materials. The preparation of leucocidin, polymorphonuclear leucocytes (referred to in this paper as leucocytes) and media for suspending the cells are described by Woodin (1961).

Effect of calcium on the appearance of the leucocidin-treated leucocyte observed with the light-microscope. Two suspensions of 3×10^7 leucocytes in 5 ml. of calcium-free Hanks medium were incubated with leucocidin at 37° for 10 min. and cooled to 0°. To one suspension calcium (75 μ l. of 0.11M-CaCl₂) was added and both suspensions were kept at 0°. Samples were placed on slides containing wells in Parafilm (Gladstone, Mudd, Hochstein & Lenhart, 1962) and were observed without a coverslip at $\times 200$ magnification under dark-ground illumination. After 30 min. at 0° samples were also observed under a coverslip at $\times 900$ magnification with dark phase-contrast illumination.

Preparation of material for examination in the electron microscope. Suspensions of 10^7 leucocytes were incubated alone or with leucocidin in 2 ml. of Hanks medium or calcium-free Hanks solution for various periods and cooled in ice. An equal volume of 2% (w/v) osmium tetroxide in sucrose-veronal buffer (Caulfield, 1957) or 0.6% KMnO₄ in 0.05M-veronal (sodium salt) buffer, pH 7.2, was added and the suspensions were maintained at 0° for 1 hr. The suspensions were centrifuged at 60g for 3 min. and the supernatant was rejected. The pellet was dehydrated and embedded in Araldite.

The granule fractions of normal and leucocidin-treated leucocytes were isolated by the method of Cohn & Hirsch (1960) from 5×10^8 cells that had been incubated in 10 ml. of Hanks medium for 10 min. A sample of the pellet obtained by centrifuging the granule fraction suspended in 0.32M-sucrose at 30 000g for 30 min. at 0° was fixed in 3 ml. of 1% osmium tetroxide in sucrose-veronal buffer at 0° for 1 hr. and then dehydrated and embedded in Araldite.

To demonstrate the penetration of ferritin or colloidal gold from the medium into the vesicles of the leucocidin-treated cell, suspensions of 5×10^8 leucocytes in 5 ml. of Hanks medium were incubated with leucocidin for 1 min. A portion (1 ml.) of a solution containing 100 mg. of

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