VII. THE RELATION BETWEEN CYSTINE YIELD AND TOTAL SULPHUR IN WOOL.

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IN an investigation now in progress upon the nature of the sulphur linkage in wool, it became important to know whether the whole of the sulphur could be accounted for as cystine, or whether there existed other, hitherto undiscovered, amino-acids present in the protein molecule and containing the element sulphur in their constitution. Suggestions of such a nature have been made from time to time.

The kind of evidence usually brought forward as indicative of the presence of sulphur-containing substances other than cystine is divisible, broadly, into arguments of two kinds. First, differences have been noted and emphasised in the manner of reaction, towards alkaline solutions, of cystine and proteins. The latter lose all or a part of their sulphur with comparative ease, the sulphide ion being formed; cystine solutions under similar conditions suffer far less change. Moreover the fraction of the total sulphur which is eliminated as sulphide is not the same in all proteins but varies between wide limits [see Osborne, 1900; Fleitman, 1847, 1848; Krüger, 1888; Mörner, 1901].

The second class of arguments was based on the fact that in no case does the yield of cystine obtained from a protein after hydrolysis approach the value to be expected, were its sulphur present solely in this form. This aspect of the matter has been dealt with by Harris [1923] and by others. Cystine is not an easy amino-acid to isolate in quantitative yield, even from pure solutions, as Harris has shown, and its solubility, and therefore the difficulty of its separation, is enormously increased by the presence of other amino-acids [cf. Hopkins, 1921].

The methods available for its isolation have been reviewed by Barritt [1927], whose work represents the most recent attempt to obtain quantitative isolation from wool. Even under the most suitable conditions, in no case was he able to obtain more cystine than corresponded to 66 % of the total sulphur.

Since the work of Abel and his associates upon the sulphur of insulin, the question of the ratio of "labile" to "firmly bound" sulphur in proteins has taken on a new interest. In this connection Brand and Sandberg [1926] have

made the important observation that lability of the sulphur atom towards alkali differs enormously in the different compounds of cystine. Results of a similar kind have been obtained by Bergmann and Stather [1926]. On the other hand, du Vigneaud, Jensen and Wintersteiner [1928], working with crystallised insulin, found that after acid hydrolysis only about one half of the total sulphur could be accounted for as cystine, using the Sullivan [1926] colorimetric method for the determination of the latter, whilst Folin and Looney's [1922] colorimetric reagent gave a value consistently higher and approximating closely to that of the total sulphur. The Sullivan method is—so far as is known—specific for cystine, whilst the Folin and Looney reagent develops a colour with all substances in which the S—S linkage is present. Hence it seemed probable to du Vigneaud and his collaborators that there was present in insulin a substance which was not cystine but which contained sulphur in disulphide linkage.

Accordingly, for the purposes of the present investigation, it was decided to carry out cystine determinations upon acid hydrolysates of wool samples of different origin, using the two colorimetric methods mentioned above, and to compare the results so obtained with the cystine content reckoned by assuming all the sulphur of the wool to be present as cystine.

In every case examined a very close agreement has been found between these two values, all the sulphur of the wool being accountable as cystine measured by the Sullivan method (the most specific, short of direct isolation). The Folin and Looney values were found consistently to be slightly higher than the Sullivan values—and higher than the total sulphur—but this reagent is known to give a blue colour with tyrosine, hydroxytryptophan and other amino-acids.

The conclusion is drawn, therefore, that the whole of the sulphur of wool is liberated as cystine when the protein is hydrolysed by acid. The method is clearly applicable to all other proteins.

EXPERIMENTAL.

Raw wool samples were used in every case. These were degreased by washing twice in warm (50°) benzene, air-dried, and then washed thoroughly in cold 0.1 % saponin solution. Washing was effected by rinsing in many successive changes of distilled water, using a relatively large volume for each rinse. The wool was again air-dried by leaving it spread out very thinly in a warm dry atmosphere for several days. Samples were then taken for sulphur and moisture (regain) determination and at the same time for hydrolysis. Moisture content was determined by recording the loss in weight when a current of dry air was led over the sample contained in a specially designed drying bottle and held at a temperature of 105°. Total sulphur was determined by the Benedict-Denis method as used by Trotman and Bell [1926], or by the Carius method.

For hydrolysis 6 g. of air-dried wool were weighed out and added to 100 cc. of boiling 20 % hydrochloric acid. Boiling was maintained, using a sand plate and reflux condenser and samples of 5 cc. were withdrawn at intervals of $\frac{1}{4}$, $\frac{1}{2}$ hour, and thence at every hour from the time of commencement. Each sample was pipetted into a 50 cc. volumetric flask, diluted largely with water, and then rendered nearly neutral by the cautious addition of 5 cc. of 20 % sodium hydroxide, after which the total volume was made up to 50 cc. and the sample filtered. For cystine determination according to the Sullivan method 2 cc. of the sample were used, and a suitable amount of a standard 0.05 % cystine solution in 0.1 *N* hydrochloric acid, water being added to the standard if necessary to bring the volume to 2 cc. To each, 1 cc. of a 5 % sodium cyanide solution was added and after exactly 10 minutes 1 cc. of a 0.5 % solution of 1:2-naphthoquinone-4-sodium sulphonate and 5 cc. of 10 % sodium sulphite in 0.5 *N* sodium hydroxide. After 15 minutes each flask received 1 cc. of 2 % sodium hydrosulphite in 0.5 *N* sodium hydroxide. Colours were compared in a Bausch and Lomb colorimeter. It was found by experience that results were not dependable unless standard and unknown solutions contained very nearly the same quantity of cystine, and therefore a separate standard was made up for determination of each sample, allowing a difference in reading of not more than 15 %. The sulphite, hydrosulphite and quinone solutions were always freshly prepared, never being more than four hours old.

Using these precautions the method gave very satisfactory results. Since long boiling of cystine with strong acids converts it into an isomeric form differing from true cystine in crystalline form and in being optically inactive [Hoffman and Gortner, 1922], it was thought necessary to prepare some of this isomeric cystine and ascertain whether it was estimable by the Sullivan method. Accordingly 10 g. of cystine were boiled under reflux with 150 cc. of 20 % hydrochloric acid for 144 hours. The solution was filtered, decolorised with charcoal and the cystine precipitated by bringing to p_H 3.7 with sodium acetate. After recrystallisation it was analysed and compared with ordinary cystine by the Sullivan method. It gave 99.5 % of the colour of true cystine, an agreement which is well within the sensitivity of the method.

The Folin and Looney method was carried out employing 2 cc. of the test solutions and 2 cc. of the cystine standard. To each, 3 cc. of water were added, followed by 5 cc. of *N* sodium hydroxide and 10 cc. of fresh 20 % aqueous sodium sulphite. After 5 minutes, 3 cc. of the Folin and Looney reagent were added, and after waiting another 10 minutes the solutions were made up to a total volume of 25 cc. and compared.

The cystine used as standard was analytically pure ($S = 26.79$ %).

Total sulphur was determined upon 5 cc. of the hydrolysate by evaporating the hydrochloric acid in a silica dish on the water-bath adding 5 cc. of Benedict-Denis reagent and proceeding in the usual manner.

A typical result is shown in the curve, Fig. 1 (Crossbred 50's wool). The

liberation of free cystine proceeds rapidly and is complete within 5 to 7 hours. There is some indication of a fall after the maximum cystine value has been reached. This is in agreement with the findings of Merrill [1921], who also obtained the greatest yield of cystine from wool after 5 to 7 hours' hydrolysis. This destruction of cystine probably accounts for the fact that the maximum value is generally about 0.5 % short of the total sulphur figure reckoned as cystine. It hardly seems likely that any other amino-acid should be responsible for this very slight amount of extra sulphur. The values obtained by the Folin and Looney method correspond closely with the maximum Sullivan value with the exception of the first three or four points (1 to 2 hours). The fall from a value some 10 % higher than the final steady line is a constant feature observed in every experiment and can only be interpreted as indicating the formation of substances (possibly of phenolic nature) to which the reagent is

Fig. 1. Hydrolysis of wool (Crossbred 50's) by 20 % HCl.

sensitive and which are comparatively rapidly destroyed. Abderhalden and Fuchs [1913] have shown that tryptophan and hydroxytryptophan give a blue colour with the phosphomolybdate-tungstate reagent; they may equally well react in the present circumstance. It is well known that these substances are not stable under the conditions of an acid hydrolysis. The Folin and Looney values, it will be noticed, do not show a gradual increase in cystine as hydrolysis proceeds, but reach their maximum directly the wool is dissolved (within 10 minutes). As the reagent reacts with the disulphide linkage it would naturally react with cystine peptides and hence gives the form of curve to be expected.

Most of the preliminary work was done upon a Crossbred 50's wool, S = 3.46 %, but among other samples examined were a typical low sulphur wool—Devon lamb's wool, S = 3.34 %, and a high sulphur wool—Welsh mountain¹, S = 4.08 %, and also a fine and a coarse Turkey mohair. No difference in behaviour was found between these and other wools. The following table shows the results obtained.

¹ For these samples and the determination of their total sulphur contents, I am indebted to Mr Barritt, of this Association.

Table I.

Wool	Total S % dry wt.	Cystine in hydrolysate		Total S of hydrolysate (as cystine) g.	Ratio*
		By Sullivan's method (max. value) g.	By Folin and Looney's method g.		
Crossbred 50's I	3.46	0.3780	—	0.3602	+ 4.94
" " II	3.47	0.5700	0.5950	0.5815	- 1.99
" " III	3.54	0.5575	0.5673	0.5589	- 0.25
Devon lamb	3.34	0.5755	0.5825	0.6001	- 4.10
Welsh mountain	4.08	0.6300	0.6400	0.6277	+ 0.37
Turkey mohair	3.02	0.5978	0.6080	0.5948	+ 0.50
(coarse)					
Turkey mohair (fine)	3.18	0.5405	0.5564	0.5393	+ 0.22
Cape merino	3.67	0.5875	0.5963	0.5887	- 0.02

$$* \frac{\text{Total S (hydrolysate)} - \text{cystine (Sullivan)} \times 100}{\text{Total S}}$$

Precipitation of cystine by phosphotungstic acid.

Since du Vigneaud, Jensen and Wintersteiner [1928], found that their non-cystine, sulphur-containing substance could be approximately separated from cystine by precipitation of the latter with phosphotungstic acid, it was decided in the present case to adopt a similar procedure in order to check the results obtained.

40 cc. of an acid hydrolysate—containing in all 232 mg. of cystine—were diluted to 240 cc. and 15 g. of phosphotungstic acid added. The flask was stoppered and immersed in ice for 24 hours. The phosphotungstates were then filtered off, washed and dissolved in sodium hydroxide. Phosphotungstic acid was removed from this solution and from the filtrate by barium chloride in the usual way and excess of barium by sulphuric acid. Determinations were then carried out by the Sullivan and Folin and Looney methods and yielded the following results.

Phosphotungstate precipitate	Filtrate
Total cystine by Sullivan method. 117.5 mg. ...	55.0 mg.
Total cystine by Folin and Looney method. 120.6 mg.	65.0 mg.
Total tyrosine by Folin and Ciocalteu method [1927]	100.8 mg.
Equivalent colour as cystine ¹ ...	9.4 mg.

Wool contains about 3 % of tyrosine. The quantity present in this experiment gives an equivalent cystine value with the Folin and Looney reagent of 9.4 mg. in the filtrate. Tyrosine alone would therefore account almost entirely for the discrepancy observed between the figures in the last column. A second experiment gave similar results.

Determination of sulphur in wool from cystine yield.

From the quantity of cystine formed when a sample of wool is boiled with hydrochloric acid, the amount of sulphur originally present in the wool may

¹ 10 mg. tyrosine were found to give the same colour with the Folin and Looney reagent as 0.93 mg. of cystine.

be determined. The following example is taken from an actual experiment using a Crossbred 50's wool.

A known weight of wool was boiled for 7 hours in a Kjeldahl flask with 20 % hydrochloric acid. When cool, the hydrolysate was neutralised by sodium hydroxide, the solution filtered and, together with the washings, made up to 100 cc. Cystine was determined in 2 cc. of this liquid using the Sullivan method.

The moisture content of the wool was ascertained by weighing out a sample at the same time that the sample for hydrolysis was taken and proceeding as described earlier in this paper.

The following figures were obtained.

Weight of wool hydrolysed	...	...	0.5183 g.
Moisture content	...	...	13.27 % of wet wt.
Cystine in acid hydrolysate	...	...	57.75 mg.

whence

Sulphur content of dry wool	...	...	3.43 %.
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The sulphur content by the Carius method was found to be 3.46 %.

SUMMARY.

It may be concluded from these experiments that natural wools differing markedly in their sulphur content (3.34 % to 4.08 %—nearly the extremes of variation encountered) and also fine and coarse samples of Turkey mohair are all capable of yielding their entire sulphur as cystine when hydrolysed by acids. There is no evidence of any other sulphur compound entering into their constitution.

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