CLXXIV. THE SULPHUR DISTRIBUTION OF PROTEINS

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WHEN current methods for the determination of cystine were applied to the hydrolysates of pasture proteins, certain anomalies appeared which demanded further investigation. In an initial attempt to assess the cystine content of these proteins, Pollard & Chibnall [1934] found that the values obtained by the Prunty [1933] modification of the Sullivan [1929] method were uniformly low and accounted for some 5–15% of the total S. They observed that the apparent cystine content, calculated from the mercaptide-S obtained in the Vickery & White [1933] method, gave values higher than those of the modified Sullivan method, and suggested the presence of a further amino-acid containing potential —SH groups. It is now known that such a difference might be explained on several grounds, e.g. inhibition of the Sullivan colour by other degradation products, particularly those of carbohydrate origin; defects in the Sullivan method itself; decarboxylation or deamination of a portion of the cystine. At that time no data were available for the amount of S contributed by methionine, but in the light of later analyses, as yet unpublished, it became apparent that the greater part of the S in these proteins was attributable neither to determined cystine, nor to methionine in spite of the greater values obtained by the Vickery & White method. Apart from any criticism directly applicable to the methods adopted for the determination of cystine, a more serious source of error with proteins in which the truly protein moiety is associated with comparatively large amounts of non-nitrogenous impurities, probably of carbohydrate origin, is the destruction of cystine during hydrolysis. These difficulties have led to a reinvestigation of the S distribution of pure and treated proteins and of proteins hydrolysed in presence of extraneous material, with a view to interpreting the results obtained with grass proteins. The preparations utilized were edestin, gliadin and various samples of wool and kemp. At the same time, newer methods for the determination of cystine have been investigated, and these were later applied to an examination of the S distribution of muscle proteins.

Of the known S-containing amino-acids, only cystine and methionine have been isolated from protein hydrolysates; the S distribution of a protein implies therefore, in the first instance, a determination of the partition of S between these two acids and its correlation with the S content of the protein and with that of its hydrolysate. Some account of previous work in this field is relevant. Following the observation of Barger & Coyne [1928] that the methylthiol group of methionine could be estimated by a measure of the volatile iodide produced on digestion with HI, Baernstein [1932, 1; 1934] elaborated a method for the general determination of methionine in proteins. Since methylimide and methoxyl groups do not occur in any known amino-acid derived from proteins on hydrolysis, it was assumed that the MeI produced by the action of HI arose entirely from the methylthiol group of methionine. Utilizing the method for

the determination of methionine in a series of 32 proteins and determining in addition their cystine contents, Baernstein [1932, 2] attempted to correlate the combined S content of these two acids with the total S content of the protein. In 10 cases the ratio 100 (cystine S + methionine S)/total S varied between 97 and 103 %, in 9 cases between 86 and 97 % and in 13 cases between 103 and 118 %. It is significant that Baernstein's cystine values, determined by his gasometric method [1930], are considerably higher than those obtained by other methods. Hess [1933] points out that the reaction upon which the method is based, viz. the interaction of iodine in strongly acid solution with reduced cystine, is not entirely specific. Even on the assumption that current colorimetric and gravimetric methods consistently under-estimate the cystine of proteins, it is difficult to accept the reliability of Baernstein's results when the ratio previously cited often exceeds 100, and as his methionine values are in fair agreement with later ones obtained by a method differing fundamentally in principle, it is safe to assume that the cystine values are largely responsible for the error.

In more recent papers [1936, 1, 2], Baernstein has adopted an entirely new and elegant procedure. When a protein is hydrolysed by HI, cystine is reduced to cysteine, and methionine is converted into the thiolactone of homocysteine. In acid solution, cysteine reacts with iodine, and the amount of iodine consumed gives a measure of the cystine originally present. At this juncture tetrathionate is added, and the system is made alkaline with ammonia. Under these conditions the thiolactone ring of homocysteine opens, and the liberated —SH group is oxidized by tetrathionate, which itself is reduced to thiosulphate. The amount of this latter, determined by iodine titration, gives a measure of the methionine originally present. Moreover, any H₂S and SO₂ arising during actual hydrolysis of the protein with HI is collected and estimated. In contrast to former results, the average S recovery is 97 %, but this figure includes S evolved as H₂S, amounting to 2.4–24.5 % of the total S. In our experience, proteins such as wool and kemp produce, on digestion with HI, considerable amounts of H₂S, which in these cases must arise from cystine, and whatever the source in other proteins, it is evident that the recovery of S in a form assignable to specific amino-acids is less than Baernstein's figures indicate.

Amongst other attempts to account for the whole of the S in proteins may be mentioned the work of Rimington [1929, 1, 2; 1930] and Barritt & Rimington [1931] who found that the cystine-S of animal hair and various wools, as determined by the Sullivan [1929], Folin & Looney [1922] and Folin & Marenzi [1929] methods, was identical with the total S. Subsequently, Barritt [1934] found that 2.4–4.8 % of the S of wool was also present as methionine, which Mueller [1923] had previously isolated in an impure form from wool hydrolysates. In the case of insulin, the whole of the S is present as cystine [Miller & du Vigneaud, 1937].

Claims for the presence of S-containing acids other than cystine have been made by Blumenthal & Clarke [1935], who observed that 2–7 % of the total S of 6 proteins examined was oxidized by bromine water to sulphate, arising possibly by oxidation of the thiolglyoxaline group of thiolhistidine. They distinguish yet another form of protein-S which is oxidized to sulphate by boiling HNO₃, but is stable to plumbite.

EXPERIMENTAL DETAILS AND CRITIQUE OF METHODS ADOPTED

Protein preparations. The edestin and gliadin were prepared according to accepted methods, the former being twice recrystallized; the N contents were 18.4 and 17.1 % respectively. Scoured samples of wool and kemp were obtained

from commercial sources and other samples from the raw fleece. The kemp fibre was hand-picked to prevent admixture with wool and only the middle portions of the wool fibre were utilized. All samples were defatted by washing in 5 changes of warm benzene, finally in light petroleum, and after drying were rendered ashless by steeping in several changes of distilled water containing 1 ml. of $N/10$ HCl per l.

Total S. In the earlier experiments, the protein was intimately mixed with 25 times its weight of a 1:1 mixture of Na_2CO_3 and Na_2O_2 and fused in a nickel tube. It was found exceedingly difficult to prevent violent explosions, and the method was modified to permit of slow fusion. 0.3 g. of protein (or its equivalent after hydrolysis) was digested in a small nickel crucible with 4 ml. of 10% NaOH and evaporated over a micro-flame to 1 ml. 4 g. of anhydrous Na_2CO_3 were then added and drying was continued overnight at 108° . The mass was now mixed with 4 g. Na_2O_2 , fused slowly over a micro-flame and finally brought to red heat. After cooling, the melt was dissolved in water, neutralized with HCl, filtered and the sulphate precipitated with BaCl_2 at a slightly acid reaction. Previous workers in this laboratory had found that oxidation of protein-S by the Benedict-Denis reagent was incomplete, giving results considerably lower than those obtained by alkali fusion. This method was therefore abandoned.

Methionine. The procedure of Baernstein [1932, 1] was slightly modified. The volatile iodide arising from the action of HI on the protein was washed in a suspension of red phosphorus containing H_2SO_4 and CdSO_4 and passed into 10 ml. of alcoholic AgNO_3 . In the initial stages of the reaction, small amounts of sulphide escaped the wash liquor and were retained by the silver solution. After 8 hr., the absorption tubes were detached, the AgNO_3 diluted with water, filtered through a washed gravimetric paper, and the precipitate and paper were finally washed free of silver ions. Filtrate and washings were diluted to 100 ml., and 20 ml. aliquots were titrated against $N/50$ KCNS. The precipitate of iodide and sulphide was boiled with dilute HNO_3 , and the amount of AgNO_3 regenerated from the sulphide was determined by titration, thus enabling the appropriate correction to be applied.

Although aware that a portion of the Ag precipitate is soluble in HNO_3 , Baernstein observes that only by omitting the treatment with acid is the Ag content of the precipitate equivalent to the methionine taken. Such omission is not in accordance with the recognized Zeisel procedure and its modifications, and seems dubious in view of the fact that different proteins evolve different amounts of H_2S which may or may not be efficiently trapped. After applying a correction for substances soluble in HNO_3 , it has been possible to obtain theoretical values for the MeO content of vanillin, and to obtain a 95% recovery of methionine. Baernstein's average methionine recovery was $97.6 \pm 1.4\%$.

Since most proteins are initially dried by alcohol and ether, there is some danger of obtaining volatile iodide from these sources if further drying is inadequate. In the case of a fibrous protein like myosin, it is difficult to remove the last trace of alcohol even at 108° . The values obtained by digesting the protein directly with HI may, however, be checked in the following way. The protein is hydrolysed for 7 hr. by boiling with 10 times its weight of $5N$ HCl and is then evaporated to dryness *in vacuo*, redissolved in water and re-evaporated to complete dryness *in vacuo* at 100° . During hydrolysis and evaporation, which are effected in the flask ultimately used for the HI digestion, all volatile alcohols are completely removed, and the degraded protein may be subjected to the Baernstein procedure.

Method of protein hydrolysis for cystine determination. The protein was gently refluxed with 10 parts of 5*N* HCl for 15 hr. The acid was neutralized in the cold to pH 2.5 with 20% NaOH, and the acid-insoluble humin was separated by centrifuging, washed 3 times with small amounts of water, and the combined liquors were diluted to a volume containing approx. 0.5 mg. of cystine per ml. and filtered clear. That the method of neutralization with alkali does not result in destruction of cystine was verified by subjecting cystine in 5*N* HCl to the same procedure and estimating the recovery by the methods to be outlined below.

Methods for cystine determination. Three methods were adopted, Sullivan's [1929], Lugg's modification [1933] of Sullivan's and Lugg's modification [1932] of the Folin-Marenzi [1929] method. These latter modifications have not generally been applied to the determination of cystine in protein hydrolysates. Sullivan's method itself, though employing a reagent specific for cystine, has been subject to criticism on account of the erratic colour variations to which it is prone. Some sources of error, recently summarized by Andrews & Andrews [1937], are due to inhibition of colour development by reducing agents such as sulphides, ascorbic acid, adrenaline, aldehydes and simple sugars. Rossouw & Wilken-Jorden [1934] emphasize the necessity of allowing a time interval of at least 20 sec. between the addition of naphthaquinone and sulphite. In a recent critique, Lee [1935] has shown that disregard of the pH of the medium in which colour development takes place invalidates the method, especially with proteins of low cystine content. Bushill *et al.* [1934], using the Zeiss photometer, found variations in the extinction coefficient of $\pm 25\%$ as compared with $\pm 8\%$ in the Lugg modification. In the latter method an attempt has been made to remedy some defects of the original Sullivan method, viz. differences of pH between standard and assay, and reduction of the colour intensity of the latter by amino-acids other than cystine. Both defects are overcome by use of a glycine buffer which "swamps" all other amino-acids. For equal amounts of cystine, Lugg's method gives a less intense colour than Sullivan's, and a relatively greater care must be exercised to ensure that the solutions, after pouring into the colorimeter cups, are completely reduced before comparison.

Lugg's modification of the Folin-Marenzi method, which in principle utilizes the reduction of molybdenum-free phospho-18-tungstic acid by cysteine, again ensures a rigid control of pH by use of a citrate buffer at pH 5.6, and so avoids all the disadvantages of colour production at an alkaline reaction. In the original Folin-Marenzi method, precipitation often occurs during the determination and there are no means of correcting for extraneous reducers. In Lugg's modification, precipitation is avoided, and an ingenious correction is applied for reducers other than —SH groups. In presence of HgCl_2 , these latter produce no colour with phosphotungstic acid, and the colour developed by other reducers is measured by artifice. Since, however, such extraneous coloration is affected to some extent by HgCl_2 , Lugg found it necessary to incorporate ZnCl_2 in the buffer, and this not only provides a heavy metal effect on weak reducers, so minimizing the influence of HgCl_2 , but prevents also the formation of precipitates with uric acid if this happens to be present. The presence of zinc inhibits to some extent the rate of colour development by cysteine, and to remedy this defect, NH_4Cl is also included in the citrate buffer. The method is rapid and capable of a high degree of reproducibility, whilst colour matching is more precise than with Sullivan's method or its modifications. The correction for extraneous reducers is of the order of 7–10% for most proteins of low cystine content, but for wool is almost negligible.

Mirsky & Anson [1934-35], in recent studies of the S—S and SH groups of proteins, have utilized a different modification of the Folin-Marenzi method, whereby colour development takes place in a phosphate buffer at pH 6.8 in presence of urea. The method does not include a correction for extraneous reducers, and in the absence of data as to its reliability, Lugg's method was adopted in preference. In the initial stages of the research, Prunty's modification of the Sullivan method was employed and was found to give results even lower than Sullivan's method itself. With coloured hydrolysates of low cystine content (e.g. grass protein and casein) Prunty's method may be applied with reasonable success, since the reduction of cystine by nascent hydrogen largely removes the colour contributed by humin.

Selecting a well-defined crystalline protein such as edestin, it is of interest to compare the cystine values reported by various workers with those now obtained.

Table I

Method	Observer	Cystine %
Mercaptide [Vickery & White, 1933]	V. and W.	1.25
Reduction of phosphotungstic acid in alkaline medium [Folin & Marenzi, 1929]	F. and M.	1.35
Reduction of phosphotungstic acid in alkaline medium [Folin & Looney, 1922]	F. and L.	0.75
Iodimetric [Okuda, 1925]	Sullivan and Hess	1.32
Iodimetric-gasometric [Baernstein, 1930]	B.	1.79
Sullivan [1929]	Sullivan and Hess	1.22
Sullivan [1929]	Author	1.14-1.17
Sullivan-Lugg [Lugg, 1933]	Author	1.26
Reduction of phosphotungstic acid in acid medium [Lugg, 1932]	Author	1.36

There is good agreement between the Folin-Marenzi, Folin-Marenzi-Lugg, and iodimetric methods on the one hand, and between the mercaptide, the Sullivan (as determined by Sullivan and Hess) and Sullivan-Lugg methods on the other. The first series includes (as cystine) any additional (S—S) groups which may occur in the protein molecule, but the Sullivan method is specific for cystine as such. With the exception of wool and kemp hydrolysates, the Folin-Marenzi-Lugg method gives values higher than the Sullivan-Lugg method, and this in turn higher than the Sullivan method. This disparity between the specific and unspecific methods may be due to several factors:

- (1) Presence of a S-containing amino-acid other than cystine and methionine.
- (2) Inhibition of the Sullivan colour by substances present in the protein hydrolysate.
- (3) Decarboxylation or deamination of a portion of the cystine.

An attempt was made to obtain evidence in support of this latter hypothesis. 0.13 g. of cystine was dissolved in 50 ml. of 5*N* HCl, and 5 ml. were added to 0.96 g. of edestin, which was then hydrolysed after further addition of 5 ml. of 5*N* HCl. Simultaneously, 0.96 g. of edestin was hydrolysed in absence of added

Table II

Wt. of edestin taken (g.)	Wt. of added cystine	Cystine estimated (mg.)					
		Folin-Marenzi-Lugg		Sullivan-Lugg		Sullivan	
		(a)	(b)	(a)	(b)	(a)	(b)
0.96	13 mg.	25.7	26.2	25.35	25.0	25.58	24.6
0.96	None	12.9	13.0	12.07	11.9	11.4	11.3
	Recovery	12.8	13.2	13.28	13.1	14.18	13.3

cystine. As standard, 10 ml. of the original cystine solution were partially neutralized and diluted to 50 ml., the final acid concentration being $N/10$. The hydrolysates were then neutralized and the cystine contents determined (Table II).

A theoretical recovery of cystine was possible by all three methods in spite of the fact that the total cystine estimated differed for each method. When cystine itself was subjected to the same conditions of hydrolysis the recovery was equally good ($99 \pm 2\%$), and all methods gave almost identical values, as is the case with hydrolysates of wool and kemp. If therefore the disparity between the Folin-Marenzi-Lugg and Sullivan-Lugg values in the case of many protein hydrolysates is due to the existence of deaminated or decarboxylated cystine, one must assume that part of the cystine has a reactivity, in the initial stages of hydrolysis (i.e. in the peptide stage), greater than that of free cystine, but that its reactivity in proteins of high cystine content is normal. It is known for example that the reactivity of cystine peptides is greater than that of cystine itself [Nicolet, 1935], and that in most globular proteins the appearance of $-SH$ groups readily occurs when the original specific configuration is modified during the degenerate stage of denaturation [Bailey, 1937; Astbury *et al.* 1935]. Wool fibre and insulin crystals on the other hand are stabilized systems, and it is possible that the reactivity of the molecule as a whole reflects the activity of the cystine.

Apart from the possible existence in protein hydrolysates of cystine in which one or more groups are modified, condensation reactions may occur in presence of certain extraneous materials, giving rise to products which are insensitive to the usual colour reactions. Roxas [1916] observed that cystine, boiled with glucose and HCl, gave rise to a humin precipitate containing 3% of the cystine-N, and that this figure could be increased by addition of proline to the system, despite the fact that proline, in absence of cystine, was not a source of humin. Dowell & Menaul [1919] found that cystine and tyrosine boiled separately with furfuraldehyde and 10% HCl for 2 hr. were deaminated to the extent of 85 and 90% respectively, whereas glycine was unaffected. The effect of a furfuraldehyde-yielding carbohydrate on cystine destruction was examined by hydrolysis of edestin in presence of 10% arabinose (Table III). This resulted in the production of a bulky humin precipitate (7-9% of the weight of protein taken) containing over 10% of the total S, and cystine was destroyed to the extent of 30%. The hydrolysate was found to contain substances which reduced phosphotungstic acid after addition of metabisulphite, thus involving a large correction for extraneous reducers. On this account the method is not very accurate with such hydrolysates, and gives a value equal to and not greater than that of the Sullivan-Lugg method. These results are in fact typical of those proteins containing a closely bound non-nitrogenous moiety, e.g. the grass proteins. The humin fraction of cocksfoot protein is extremely large and contains 29% of the total S, whilst only 57% of the S in the hydrolysate can be accounted for as cystine¹ and methionine. It appears therefore that condensation products of cystine are by no means concentrated in the humin fraction, but give rise to soluble substances which do not react to the Folin and Sullivan reagents. It must again be emphasized that cystine peptides may condense even more actively than cystine itself and condensation will be facilitated if the protein

¹ In confirmation of the destruction of the cystine of grass proteins by HCl hydrolysis, Lugg (private communication) records a 6-fold increase in the cystine content of cocksfoot protein using the Baernstein HI method [1936, 2], which eliminates humin formation. In spite of the higher value by this method Lugg believes that the cystine is still under-estimated.

contains free —SH groups in the form of cysteine. Native proteins such as haemoglobin and crystalline lens protein do contain SH groups according to Mirsky & Anson [1936], and these are subject not only to condensation with substances of an aldehydic nature [Schubert, 1936] but in the intact protein they may be oxidized partly to S—S groups and partly to sulphinic and sulphonic acids which do not reduce the phosphotungstic reagent [Lavine, 1936] or respond to the Sullivan reaction. The factors responsible for errors in the determination of cystine may now conveniently be summarized. (1) Errors in the methods adopted, e.g. inhibition of the colour by extraneous reducers in the case of the Sullivan reaction. (2) Losses occurring during hydrolysis. Under this heading must be included deamination, decarboxylation or condensation of cystine with substances of an aldehydic nature; oxidation and/or condensation of any pre-existing cysteine molecules. Such reactions may not necessarily occur with cystine or cysteine in the free form, but the increased activity of these amino-acids in peptide form probably enhances their susceptibility to reactions of this type.

COLLECTED RESULTS

The S distribution of edestin and other proteins is set out in Table III. The results are expressed in the following way: total disulphide groups by the Folin-Marenzi-Lugg method are expressed as $(S-S)$, cystine as determined by the Sullivan-Lugg method as C_s , methionine as M_s , total S of the protein as P_s , S of the hydrolysate as H_s . Values are now recorded for

$$[(S-S) + M_s]/P_s, [C_s + M_s]/P_s, [(S-S) + M_s]/H_s, [C_s + M_s]/H_s.$$

When a protein is hydrolysed, some of the S passes into the humin fraction, and traces of H_2S are evolved. In general, therefore, $P_s > H_s$ and since $(S-S) > C_s$ the value $[(S-S) + M_s]/H_s$ shows the maximum recovery of S, and $[C_s + M_s]/P_s$ the minimum, except in the cases of wool and kemp, where $(S-S)$ is approximately equal to C_s .

At least 6% of the S of edestin and 7% of the S of gliadin are unaccounted for as cystine and methionine. Apart from the possible presence of undiscovered S-containing amino-acids these figures include losses involved in the determination of cystine, already fully discussed, and approximately 3% error in the determination of methionine. If allowance is made for this latter error, it is evident that the amount of undetermined S-containing amino-acid remaining in the hydrolysate and expressed as a percentage of the protein is 0.17% for edestin and 0.28% for gliadin (assuming the S content of the amino-acid to be about 25%). The same edestin sample hydrolysed in presence of arabinose shows a S recovery in the hydrolysate of only 77% and almost 11% of the total S remains in the humin. The destruction of cystine amounts to 30% and of methionine to 20%. Similarly, the humin fraction of cocksfoot protein contains 29% of the total S, whilst only 56% of the S in the hydrolysate is attributable to cystine and methionine. In both cases, soluble unestimated condensation products of cystine appear to be present in the hydrolysate.

The analysis of samples of wool and kemp obtained from commercial sources points to a destruction of cystine occurring most probably during the scouring process. Only 90% of the total S of scoured wool was interpretable in terms of cystine and methionine, but a greater discrepancy was discovered in the sample of kemp, which gave a corresponding value of 36% and contained <2% of cystine. The source and history of a protein are especially important therefore if reliable estimates for its S distribution are to be made. The samples of wool and kemp obtained from the raw fleece gave analyses in agreement with those of

Table III. Sulphur distribution of various proteins

Weights in percentages of protein dry weight									
	S on hydrolysate after removal of humins at pH 2.5 (<i>H_s</i>)	S on protein (<i>P_s</i>)	Methionine on protein (<i>M_s</i>)	Methionine on HCl- hydrolysed protein (<i>M_s</i>)	Cystine				$\frac{C_s + M_s}{H_s}$ %
					Folin- Lugg (<i>S-S</i>)	Sullivan- Lugg (<i>C_s</i>)	Sullivan	$\frac{(S-S) + M_s}{P_s}$ %	$\frac{(S-S) + M_s}{H_s}$ %
Protein									
Edestin N = 18.4 %	0.91	0.93	2.3	2.3	1.36	1.26	1.14	94.4	91.5
Edestin + 10 % arabinose (a)	0.81	0.93	2.3	1.8*	0.9	0.9	0.9	77.6	77.6
(b)	0.83 (humins S = 0.10)	0.93	2.3	1.85*	0.9	0.9	0.85	77.0	77.0
Cocksfoot pro- tein (B.E.) N = 13.4 %	0.70 (humins S = 0.29)	1.01	—	1.66*	—	0.14	—	—	56.3
Gliadin, N = 17.1 %	1.12	1.19	1.6	1.6	2.6	2.44	2.4	92.8	89.0
Wool (Australian Merino 64 s) N = 16.3 %	3.53	3.63	0.6	0.6	12.5	12.5	12.5	98.2	98.2
Wool (scoured Merino) N = 16.2 %	3.60	3.73	0.65	—	11.7	11.6	11.6	90.8	90.0
Kemp (Welsh mountain sheep) N = 15.9 %	3.25	3.35	0.8	0.8	11.4	11.1	10.9	99.1	96.6
Kemp (scoured) N = 15.5 %	1.83	1.90	0.8	0.8	1.98	1.80	1.70	38.2	35.7

* These figures represent the methionine content of the hydrolysate after removal of humins at pH 2.5. For pure proteins, the methionine contents of the protein and its hydrolysate in presence or absence of humins are identical, i.e. no condensation products of methionine occur in the humin fraction.

Rimington and co-workers, i.e. 98–99 % of the total S was accounted for. A remarkable feature of the cystine values for wool and kemp is the agreement between the total disulphide and cystine, in spite of the fact that the colour correction for extraneous reducers in the former case is negligible. Rimington [1929] also obtained reasonable agreement between the Sullivan and Folin-Looney methods applied to various keratin hydrolysates. The methionine values of wool determined directly on the dry specimen and on the evaporated hydrolysate were in agreement with each other and with the value obtained by Barritt [1934] for a comparable sample of Western Australian wool. Thus in the well-defined keratins which have been investigated both by Rimington and the author, it appears that no S-containing acids other than cystine and methionine occur, except perhaps in very small amounts.

SUMMARY

1. Various methods for the determination of cystine in proteins are reviewed. For pure proteins, Lugg's [1932] modification of the Folin-Marenzi method is advocated as a measure of total disulphide, and for the estimation of cystine by a specific method, Lugg's [1933] modification of Sullivan's method is recommended, provided that the cystine content of the protein is greater than 0.5 %.

2. The S distributions of edestin, gliadin, wool and kemp have been investigated. Expressed as a percentage of the S in the hydrolysate, the S unaccounted for in terms of total disulphide (as cystine) and methionine amounts to 6–7 % in the cases of edestin and gliadin. In confirmation of the results of Rimington and co-workers, the S of wool and kemp is almost wholly attributable to cystine and methionine, the latter being present only in small amounts. In scoured samples of these keratins, the cystine content is low, owing probably to oxidation of the disulphide bond.

3. In the hydrolysates of wool and kemp, the total disulphide expressed as cystine agrees reasonably well with the cystine content (as determined by specific methods), but in the case of gliadin and edestin, the cystine figure is always lower. This discrepancy may arise either by presence of (*S*–*S*) groups other than cystine, by defects in the Sullivan method, or if part of the cystine is deaminated or decarboxylated during hydrolysis of the protein. When, however, cystine itself, or cystine added to edestin, is subjected to the conditions adopted for hydrolysis, 100 % recovery is obtained in both the Folin and the Sullivan modifications. It is suggested, therefore, that the reactivity of cystine in many proteins, like that of cystine peptides, is greater than that of free cystine, and deamination and decarboxylation may occur during the initial stages of hydrolysis when the cystine exists in peptide form.

4. Hydrolysis of proteins in presence of carbohydrate (as pentose) results in the production of large amounts of S-containing humin. There is a considerable destruction of cystine and methionine and condensation products of the former are apparent in the humin and the hydrolysate. The significance of these results in relation to the S distribution of the grass proteins is discussed.

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