The Reactivity of the Combined Cystine of Proteins other than Wool

By H. LINDLEY, *Wool Industries Research Association, Torridon, Headingley, Leeds 6*

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Previous work has shown that the action of bisulphite solutions on wool leads to the subdivision of the combined cystine into two main fractions of differing chemical reactivity (Elsworth & Phillips, 1941; Middlebrook & Phillips, 1942*a*), each of which can be divided into two fractions showing smaller differences in reactivity. Further work on the reaction of wool with formaldehyde (Middlebrook & Phillips, 1942*b*, 1947) and alkalis (Cuthbertson & Phillips, 1945; Lindley & Phillips, 1945) has provided evidence that the combined cystine can be subdivided by reagents other than bisulphite. The effect of chemical modification of the wool on the magnitudes of the cystine fractions has been studied (Lindley & Phillips, 1947) and has helped to interpret the results in terms of protein structure.

In these circumstances it appeared important to determine whether the reactivity of the combined cystine varies in all cystine-containing proteins, or whether such variation is confined to the keratins or even to wool itself. The S distributions of a number of different types of keratin and of some other non-fibrous proteins have therefore been determined. The results obtained show that this variation in reactivity of the combined cystine is characteristic of the keratins and, with some slight reservations, of other cystine-containing proteins. The combined cystine of all the keratins examined has thus been found to be divisible into two main fractions: (*A* + *B*) defined as combined cystine which yields thiol plus *S*-cysteinyl sulphonate groups with NaHSO_3 and lanthionine with dilute alkalis, and (*C* + *D*) defined as being inert towards cold NaHSO_3 and giving combined α -aminoacrylic acid with alkalis. Fraction (*A* + *B*) of these proteins has been further divided into subfraction *A* which gives water-unstable thiol and *S*-cysteinyl sulphonate groups with NaHSO_3 , and subfraction *B*, which gives water-stable thiol and *S*-cysteinyl sulphonate groups. No attempt has been made to differentiate between subfractions *A* and *B* of the non-keratinous proteins.

THE (*A* + *B*) AND (*C* + *D*) FRACTIONS OF THE COMBINED CYSTINE OF VARIOUS KERATINS

EXPERIMENTAL

Materials. These call for no special comment beyond the fact that they were purified before analysis by successive extraction with ethanol and water, the sole exceptions to

this being the cow- and sheep-horn samples which are asterisked in Tables 1-4. After these latter had been partially analyzed, it was discovered that they had previously been boiled in water for some hours to remove the non-keratinous cores. New material was accordingly analyzed, but both sets of analyses are recorded for completeness. To facilitate penetration of the NaHSO_3 solution, all the horn samples were converted to fine shavings in a milling machine.

Analytical methods. These are identical with those previously described (Lindley & Phillips, 1945, 1947) with the addition of amide-N estimations, which were carried out as follows: 5 ml. samples of the hydrolysates (1 g. hydrolyzed for 4 hr. with 10 ml. 5*N*-HCl, filtered and made up to 50 ml.) were transferred to micro-Kjeldahl flasks and made just alkaline (bromocresol green) with 2*N*-NaOH; 5 ml. of 2% (w/v) borax were added to bring the pH to 8-9, and the NH_3 removed by distillation in a micro-Kjeldahl apparatus.

RESULTS

The S distribution of the keratins

The results of various S estimations on a variety of keratins are given in Table 1 together with the percentage of S unaccounted for either as cystine, cysteine, methionine, Br-oxidizable S or sulphate S. It will be seen that the keratins have a wide range of values for cysteine, cystine, total and unidentified S. Particularly noteworthy are the high cystine content of the dog hair and the high cysteine content of the two horn samples first analyzed. This high thiol content could not be duplicated on the other two samples by boiling them with water, and therefore this probably represents a real difference between the two specimens. The wide variation in the analytical figures of the various horn samples is of some interest.

Another point of importance is the wide range of values found for the unidentified S of the keratins. Thus the total S of dog hair, sheep horn and duck feathers can almost quantitatively be accounted for as cystine, methionine and Br-oxidizable + sulphate S, whereas calf, cow-tail and rabbit hair all contain approximately 0.8% S which remains unidentified. That this is not necessarily due to treatment of the keratin prior to analysis is established conclusively in the case of the rabbit hair. This particular specimen was obtained from the rabbit used to prepare the myosin for the later experiments and had no subsequent treatment other than extraction with ethanol and water. It also seems improbable that this unidentified S arises by oxidation of cystine

Table 1. *The S distribution of certain keratins*

(Results as % of protein dry wt.)

Keratin	Total S	Sulphate S + Br-oxidizable S	Thiol S	Disulphide S	Methionine S	Unidentified S (by diff.)
Dog hair	5.30	0.29	0.15	4.65	0.16	0.05
Rabbit hair	4.35	0.27	0.16	2.94	0.22	0.76
Calf hair	3.97	0.45	0.05	2.53	0.07	0.87
Cow-tail hair	3.93	0.28	0.05	2.71	0.10	0.79
Horse hair	3.71	0.19	0.00	2.83	0.19	0.50
Camel hair	3.28	0.09	0.00	2.74	—	—
Alpaca	3.93	0.14	0.00	3.34	0.08	0.38
Yak hair	3.85	—	0.00	2.78	—	—
Reindeer hair	—	—	0.00	2.40	—	—
Duck feathers	3.26	0.19	0.01	2.75	0.10	0.21
Cow horn*	4.17	0.37	0.54	2.37	0.08	0.81
Sheep horn*	—	0.19	0.34	2.68	—	—
Cow horn	3.77	0.07	0.22	2.94	0.14	0.40
Sheep horn	2.08	0.01	0.10	1.69	0.14	0.14
Foetal lamb's wool	3.64	0.08	0.13	2.95	—	—

* Pre-boiled in water.

Table 2. *The subdivision of the combined cystine of keratins into fractions (A + B) and (C + D)*

(Results in terms of S as % of protein dry wt.)

Keratin	Fraction (A + B)	Fraction (C + D) (by diff.)	Subfraction B	Subfraction A (by diff.)
Dog hair	2.52	2.28	0.64	1.88
Rabbit hair	1.63	1.47	0.40	1.23
Calf hair	1.13	1.45	0.35	1.10
Cow-tail hair	1.62	1.14	0.69	0.93
Horse hair	1.75	1.08	0.46	1.29
Camel hair	1.39	1.35	0.35	1.04
Alpaca	1.37	1.97	0.52	0.85
Yak hair	0.96	1.82	0.31	0.65
Reindeer hair	0.77	1.63	0.27	0.50
Duck feathers	2.04	0.72	0.86	1.18
Cow horn*	2.03	0.88	0.50	1.53
Sheep horn*	1.56	1.46	—	—
Cow horn	2.22	0.94	0.63	1.59
Sheep horn	1.20	0.59	0.34	0.86

* Pre-boiled in water.

during hydrolysis, since Baernstein estimations (in which the hydrolysis is carried out in a reducing medium throughout) gave values of 2.48, 2.72 and 3.16 % S respectively for the calf hair, cow-tail hair and rabbit wool compared with 2.58, 2.76 and 3.10 % S for the thiol + disulphide S by Shinohara estimation. Two-dimensional partition chromatography on filter paper of the hydrolysate of rabbit wool did not reveal the presence of lanthionine or any new amino-acid. A very faint spot corresponding to cysteic acid did occur, but was not of sufficient intensity to account for the unidentified S. Pending the result of further investigations, now in progress, the question of the chemical nature of this unidentified S must therefore be left open.

The subdivision of the combined cystine into subfractions A and B and fraction (C + D)

The various keratins were treated with 30 % (w/v) NaHSO₃ overnight, hydrolyzed and the thiol S content of the hydrolysates estimated to give fraction (A + B). Fraction (C + D) was calculated by subtracting this value from the disulphide S (Table 1). The results obtained are given in Table 2, together with the determinations of subfraction B and calculated values for subfraction A.

From Table 2 it will be seen that all the proteins contain both fractions (A + B) and (C + D) and that the magnitude of these fractions varies both absolutely and relatively. When the original

fractionation of the cystine S of wool was first reported (Middlebrook & Phillips, 1942*a*) some stress was laid on the approximate equality of fractions $(A+B)$ and $(C+D)$ of wool. The present analyses show that this is not a general property of keratins. By virtue of its very high cystine content, dog hair has the highest absolute value for both fractions $(A+B)$ and fraction $(C+D)$. The next highest absolute values for fraction $(A+B)$ are shown by duck feathers and cow horn, but if fraction $(A+B)$ is expressed as a percentage of either the total S or the total disulphide S, then duck feathers easily provide the highest value. This is of some interest since duck feathers are the only representative of the naturally occurring β -keratins here analyzed. The keratin with the lowest $(A+B)$ fraction both absolutely and relatively was reindeer hair, which was also undoubtedly the least impressive fibre of the varieties studied, being of very irregular cross-section, and with little tensile strength.

altered the cystine reactivity so that this subfraction behaved like subfraction *B*. A not unreasonable assumption, therefore, is that subfraction *B* is associated with amide groups of the wool. Accordingly the amide-N contents of the keratins were determined, Table 3, where it is seen that no obvious correlation exists between the magnitude of subfraction *B* of the various keratins and their amide-N contents.

THE $(A+B)$ AND $(C+D)$ FRACTIONS OF THE COMBINED CYSTINE OF SOME NON-KERATINOUS PROTEINS

EXPERIMENTAL

Materials. (1) Egg albumin: A commercial sample from British Drug Houses Ltd. and also a crystalline preparation made by the procedure of Kekwick & Cannan (1936). (2) Gliadin: This and the two succeeding proteins were gifts from Prof. A. C. Chibnall. (3) Edestin: Recrystallized.

Table 3. Ratio between fraction $(A+B)$ and total and disulphide S, and between subfractions *A* and *B*, together with amide-N values

Keratin	$(A+B)/\text{Total S}$	$(A+B)/\text{Disulphide S}$	A/B	Amide N (% of dry wt.)
Dog hair	0.48	0.53	2.9	1.11
Rabbit wool	0.37	0.53	3.1	1.07
Calf hair	0.28	0.44	3.1	1.20
Cow-tail hair	0.31	0.59	1.35	1.26
Horse hair	0.41	0.62	2.8	1.22
Camel hair	0.42	0.51	3.0	1.25
Alpaca	0.35	0.41	1.6	1.27
Yak hair	0.25	0.35	2.1	1.34
Reindeer hair	—	0.32	1.9	1.39
Duck feathers	0.62	0.74	1.4	1.31
Cow horn*	0.49	0.70	3.1	1.24
Sheep horn*	—	0.52	—	—
Cow horn	0.59	0.70	2.5	1.14
Sheep horn	0.58	0.67	2.5	1.20

* Pre-boiled in water.

Whilst fraction $(A+B)$ does not in general form any simple fraction of either the total disulphide S or total S a rather remarkable regularity comes to light when the relative magnitudes of subfractions *A* and *B* are compared (Table 3). In six of the keratins, dog hair, cow horn, rabbit wool, calf hair, horse hair and camel hair, subfraction *A* is approximately three times as great as subfraction *B*. In two of the keratins, yak hair and reindeer hair, the ratio is 2 to 1, whilst in cow-tail hair and duck feathers it is approximately 4 to 3; the alpaca gives an isolated figure of 1.6 (3) to 1, and the cow and sheep horn each give a ratio of 2.5 to 1. The ratio of subfraction *A* to subfraction *B* in the wool analyzed by Lindley & Phillips (1947) was 2 to 1.

Lindley & Phillips (1947) pointed out that subfraction *A* of the combined cystine of wool appeared to be intimately associated with the free carboxyl groups, since esterification of these carboxyl groups

(4) Ground nut globulin. (5) Rabbit myosin: Prepared from the leg muscle of the rabbit according to the method of Greenstein & Edsall (1940). (6) Insulin: British Drug Houses Ltd., crystalline. (7) Lactoglobulin: This was prepared according to Palmer (1934), but could not be induced to crystallize and was eventually precipitated from solution by the addition of ethanol. (8) Serum albumin: A crude commercial preparation (British Drug Houses Ltd.).

Treatment with NaHSO_3 . The protein (c. 0.5 g.) was treated with NaHSO_3 (1 g.) and water (3 ml.) overnight. Conc. HCl (5 ml.) was then added and the mixture boiled under reflux for 4 hr. Control experiments have shown that this method can be used to determine fraction $(A+B)$ satisfactorily for wool.

RESULTS

Tables 4 and 5 give the results of the analyses together with those previously reported by other investigators. The results were calculated on a dry weight basis calculated from the N content (Kjeldahl) of the hydrolysates. From these results it is apparent

Table 4. *The thiol and disulphide S content of protein hydrolysates*

Protein*	(Results in terms of S as % of protein dry wt.)						Reference
	Experimental results			Other investigators			
	SH	SS	SH + SS	SH	SS	SH + SS	
Egg albumin (commercial) (15.8% N)	0.07	0.45	0.52	—	—	—	—
Egg albumin (crystalline) (15.8% N)	—	—	—	0.37	0.28	0.65	Hess & Sullivan (1943)
Myosin (16.8% N)	0.17	0.12	0.29	—	—	0.48	Kassell & Brand (1938)
	0.0	0.19	0.19	0.29	0.04	0.33	Hess & Sullivan (1943)
	—	—	—	—	—	0.21	Bailey (1937 <i>a</i>)
Gliadin (17.4% N)	0.01	0.48	0.49	—	—	0.64, 0.69	Bailey (1937 <i>b</i>)
Edestin (18.6% N)	0.0	0.32	0.32	0.09	0.26	0.35	Hess & Sullivan (1943)
	—	—	—	—	—	0.38	Lugg (1938)
Lactoglobulin (15.6% N)	0.11	0.71	0.82	0.29	0.61	0.90	Brand & Kassell (1942)
Insulin (15.5% N)	0.0	2.60	2.60	0.0	3.32	3.32	Miller & du Vigneaud (1937)
Insulin HCl-HCOOH	0.0	2.96	2.96	—	—	—	—
Serum albumin (15.7% N)	0.0	1.25	1.25	0.08	1.57	1.65	Hess & Sullivan (1943)
Ground nut globulin (18.3% N)	0.02	0.20	0.22	—	—	—	—

* The N content used in the calculation of protein wt. is given in parenthesis.

Table 5. *Fraction (A + B) of the combined cystine of various proteins*

Protein	(Results in terms of S as % of protein dry wt.)		Maximum SH in protein	Reference
	SH of fraction (A + B)	SS + SH		
Egg albumin (commercial)	0.30	0.53	—	—
Egg albumin (crystalline)	0.15	0.36	0.34, 0.26	Greenstein (1938), Mirsky (1941)
Myosin	0.08	0.28	0.31	Greenstein & Edsall (1940)
Gliadin	0.26	0.56	—	—
Edestin	0.18	0.31	0.13	Greenstein (1939)
Lactoglobulin	0.38	0.81	—	—
Insulin	0.72	2.56	0.0	Greenstein (1944)
Serum albumin	0.70	1.08	0.09	Greenstein (1940)
Ground nut globulin	0.06	0.23	—	—

that the combined cystine of non-keratinous proteins can also be divided into fractions which are reactive and unreactive to NaHSO_3 solutions at pH 5 and room temperature. In most of these cases the (A + B) fraction is approximately half of the total disulphide, the principal exceptions being myosin and insulin, and with both these complications result from losses of S during hydrolysis. The confirmation of the finding of Miller & du Vigneaud (1937) that increased recovery of cystine from insulin can be obtained by hydrolyzing in the presence of formic acid is of interest since unpublished work by Mr W. R. Middlebrook in this laboratory has shown that this technique of hydrolysis does not affect the recovery of cystine from wool. Although the present value for the cystine content of insulin is lower than that of Miller & du Vigneaud it is in agreement with the work of Sahyun (1941) who prepared crystalline insulin with 2.96% total S. The present value has also been checked by Baernstein's procedure which gave 2.90% S as cystine + cysteine.

DISCUSSION

It has long been realized that the sulphur and cystine contents of keratins vary widely and that this variation occurs even within one type of keratin, e.g. wool. It seemed possible, however, that some regularities might be revealed by considering the differing cystine fractions separately, but the results obtained in the present work lend little support to this view. A possible exception to this is that subfraction A may be a simple multiple of subfraction B, but even this doubtful relationship is not constant throughout the keratins.

The observation that the combined cystine of the other proteins examined also shows differing reactivity towards NaHSO_3 solutions is important, since it suggests that the ultimate cause of this effect will be found in some relatively simple factor of protein structure. These results also make it extremely improbable that the differing reactivity

of the various fractions of the combined cystine of wool are directly due to the heterogeneous nature of wool. It was also considered possible that the ($A + B$) fraction of the non-keratinous proteins might be identical with the maximum titratable thiol groups found for these proteins in solution, but it cannot be claimed that the results lend much support to this suggestion. On this point the results are in conflict with those of Hess & Sullivan (1943) who found the cysteine content of the hydrolysates of a number of proteins to be equivalent to the titratable thiol groups of the intact protein. Unfortunately few independent estimations are available, but it would seem that in the case of myosin the present results are in agreement with the findings of Bailey (1937 *a, b*).

SUMMARY

1. It has been shown that the combined cystine of a number of keratins can be subdivided into fractions of differing reactivity towards NaHSO_3 solutions in a manner analogous to that previously found for the combined cystine of wool.

2. Evidence of a similar subdivision of the combined cystine S has been found for other non-fibrous proteins.

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Preparation and Antibacterial Action of some Compounds Structurally Related to Glutamic Acid. Their Application in Microbiological Determination of Small Quantities of Glutamine

By J. A. ROPER AND H. MCILWAIN, *Unit for Cell Metabolism (Medical Research Council), Department of Biochemistry, The University, Sheffield, 10*

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In studying the metabolism of glutamine (McIlwain, Roper & Hughes, 1948) use was made of compounds structurally related to glutamic acid which differentially inhibited certain of the reactions concerned. These inhibitors were selected from a larger group of analogues and derivatives of glutamic acid, whose preparation and properties are described in the present paper.

The substances concerned are listed in Table 1. Expectation that γ -glutamylhydrazine might be inhibitory was based on analogous findings with

respect to pantoylhydrazine (Madinaveitia, Martin, Rose & Swain, 1945; McIlwain & Hughes, 1945). Inhibition by α -amino- γ -methylsulphonylbutyric acid or 'methionine sulphoxide' was reported while the present investigations were in progress (Borek, Miller, Scheiness & Waelsch, 1946; Waelsch, Owades, Miller & Borek, 1946). Several examples are known of inhibition by homologues of biologically active compounds and this led to the examination of the α -amino dicarboxylic acids of Table 1.