FURTHER STUDIES ON THE DIGESTION OF WOOL KERATIN BY PAPAIN-UREA: THE EFFECT OF ADDING COMPOUNDS THAT RUPTURE DISULPHIDE BONDS IN ALKALINE SOLUTION

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Summary

Whereas the optimum pH for digestion of wool by papain-bisulphite-urea is 7.0, a higher pH value is required for optimum digestion when bisulphite is replaced by sodium thioglycollate, cysteine hydrochloride, or potassium cyanide. Thioglycollate at pH 9.5 is superior to cysteine at the same pH or to bisulphite at pH 7.0 in promoting wool digestion by papain solutions containing urea. Cyanide is least effective. When wool is incubated in solutions of bisulphite, thioglycollate, cysteine, or cyanide in the absence of papain and urea, the extent of splitting of disulphide bonds runs parallel with the enzyme digestion observed in the presence of these reagents. The number of disulphide bonds split was measured by the iodosobenzoic acid method.

Wool digestion in a solution containing only fully active papain and urea is less than one-tenth of that observed when sodium bisulphite is also present. The cystine-free protein, collagen, is digested most rapidly in papain-thioglycollate-urea at acid pH values.

At all pH values the presence in the papain solution of both urea and a reagent rupturing disulphide bonds leads to greater wool digestion than either of these compounds used singly with papain. The combined action of sodium bisulphite and sodium thioglycollate in papain-urea solution increases wool digestion over a range of pH values.

I. INTRODUCTION

It was previously reported that the digestion of wool in papain-bisulphite solutions is increased by the addition of urea and reaches completion within a few hours at 50-70°C. (Lennox 1952). When the urea is replaced by structurally related compounds less marked effects on the digestion are observed except with thiourea. Replacement of the sodium bisulphite with sodium thioglycollate, cysteine hydrochloride, or potassium cyanide greatly reduces wool digestion at pH 7, but in alkaline solution, as will be shown in the present paper, these compounds increase digestion in papain solution both in the presence and absence of urea.

Marriott (1928), who was one of the earliest workers to study keratin digestion, reported that any substance capable of rupturing the disulphide linkage of cystine can function as an activator for the depilation of hides. Thus sodium sulphide and potassium cyanide in lime suspension caused "rapid rotting of hair," while sodium sulphite caused hair loosening. Partial solution of wool in

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0.5M sodium thioglycollate at approximately pH 12 and in 1M potassium cyanide containing 0.1N sodium hydroxide was similarly attributed by Goddard and Michaelis (1934) to the splitting of the disulphide bonds. No reference to the action of alkaline solutions of cysteine on wool has been found in the literature but this compound was shown by Mirsky and Anson (1935) to be capable of reducing the disulphide bonds in serum albumin although it was less effective for this purpose than thioglycollate. Cysteine has frequently been used to activate papain (Fruton and Bergmann 1940).

The simultaneous action of thioglycollate and proteolytic enzymes on wool has not been previously studied although Goddard and Michaelis (1935) showed that proteins prepared by extraction of wool with alkaline thioglycollate, cyanide, or sulphide and acidification of the extract were digested by trypsin and pepsin, and Routh and Lewis (1938) reported that the attack on the thioglycollate extractive "kerateine" was more rapid than that on finely powdered wool. Geiger *et al.* (1941) made similar observations using wool that had been reduced with alkaline thioglycollate or reduced and treated with methyl iodide to methylate the sulphydryl groups and prevent re-formation of the disulphide bonds. This product was almost completely digested by crystalline pepsin and crystalline chymotrypsin but was scarcely attacked by crystalline trypsin and was claimed to be completely resistant to crystalline papain.

II. Materials and Methods

Wool of 64's merino quality was used. The weathered tip was removed from the wool staples before Soxhlet extraction with ethanol and repeated washing with distilled water to remove wax and suint. Analytical grade chemicals were used throughout and the papain was a high quality product from Ceylon. All pH adjustments were made with hydrochloric acid or sodium hydroxide using a glass electrode pH assembly.

Digestion was measured by incubating 0.5 g. wool in 15 ml. of the test solutions in stoppered glass tubes at 50°C. and washing, drying, and weighing the undigested residue.*

The sulphydryl sulphur in partially reduced wool was measured by an adaptation (see Ellis, Gillespie, and Lindley 1950) of the iodosobenzoic acid method of Hellerman and Chinard (1941). In the present investigation immersion of 0.02 g. wool for 2 hr. in 20 ml. 0.002M iodosobenzoic acid was necessary for complete reaction with the sulphydryl groups. Unreduced reagent was estimated by the addition of potassium iodide and titration of the liberated iodine with 0.002N thiosulphate. Concentrations of sulphydryl sulphur estimated by the iodosobenzoic acid method were higher and less variable than the values determined by the Shinohara method (Shinohara 1935).

* In one experiment 56's Corriedale wool was incubated in 1 per cent. papain, 0.1M $NaHSO_3$, 3M urea. After 4 hr. at 50°C. and at pH 6.0, 6.5, 7.0, and 7.5 the digestion was only one-half to three-quarters of that observed with standard fine merino wool under the same conditions.

A sheep pelt was used as a source of collagen. The wool had been removed by application of a lime-sulphide depilatory to the flesh side and the pelt was delimed, bated, pickled in sulphuric acid and sodium chloride, freed of acid by repeated washing with a solution containing sodium acetate and sodium chloride, then with water, and finally dehydrated with acetone and air-dried.

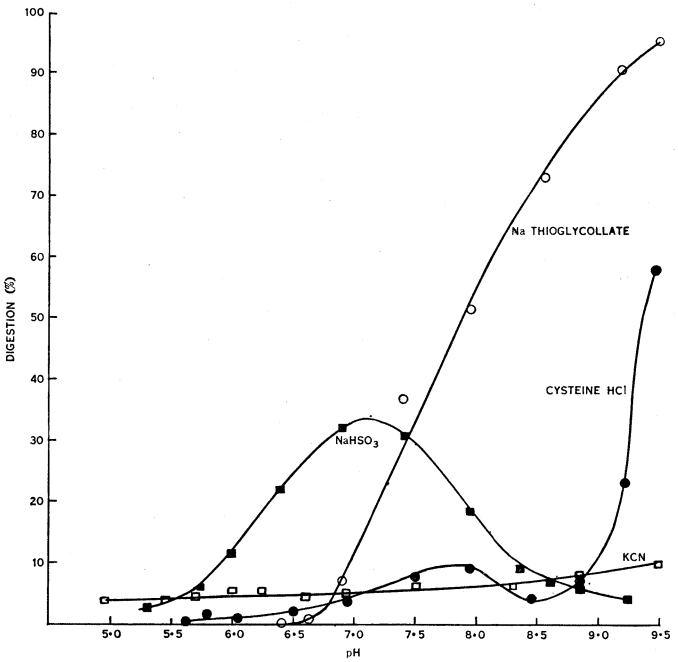

Fig. 1.—pH-wool keratin digestion in 1 per cent. papain, 3M urea solutions containing also various compounds that rupture disulphide bonds at 0.1M concentration. pH value measured after 0.5 hr. and digestion after 1.0 hr. incubation at 50°C.

III. Results

Figure 1 shows the effect of pH on the digestion of wool for 1 hr. at 50°C. in 1 per cent. papain, 3M urea solutions, containing in addition 0.1M sodium bisulphite, sodium thioglycollate, cysteine hydrochloride, or potassium cyanide. Sodium bisulphite produced maximum digestion at pH 7 while with the other reagents digestion increased as the solutions became more alkaline. Thioglycollate was the most effective agent under these conditions.

Figures 2, 3, 4, and 5 show the digestions obtained in similar solutions after 18 hr. incubation at 50°C. For comparison, curves showing the effect of omitting either urea or papain are also included. The pH values were measured 0.5 hr. after commencing the experiment. They were not separately determined for the papain-free and urea-free solutions but for purposes of graphing were assumed to change to the same values as those for the corresponding solutions containing all three components. The increase in digestion following

the incorporation of urea in the solution containing papain and compounds rupturing disulphide bonds was greatest with bisulphite and least with cyanide. The digests containing cyanide at pH values above 8.5 were reddish brown in colour. After 18 hr. at 50°C., the thioglycollate series revealed a second digestion maximum at pH 7 which was not detected after 1 hr. at 50°C.

No significant digestion was observed after incubation for 18 hr. at 50°C. in 1 per cent. papain, 3M urea solutions containing 0.1M ascorbic acid as the reducing agent. When 0.1M sodium sulphide was used as the reducing agent in the papain-urea solutions, two maxima of approximately 40 per cent. digestion were observed at pH 7 and pH 9, but the results were variable and are therefore not reported.

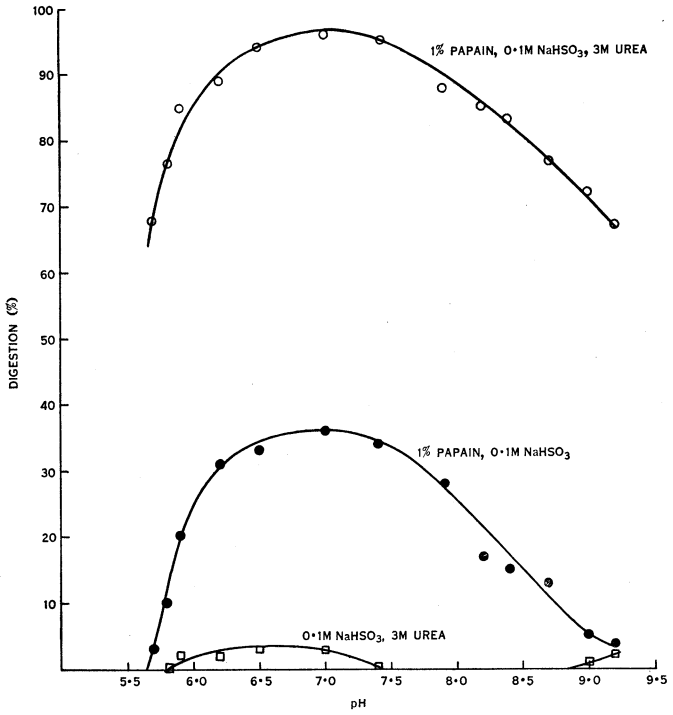

Fig. 2.—pH-wool keratin digestion in sodium bisulphite solutions containing also papain, urea, or both. Digested for 18 hr. at 50°C.

Sodium bisulphite and sodium thioglycollate together in the papain-urea solution increased digestion as shown in Figure 6. In this experiment the pH maxima were most clearly apparent after incubation for only 1 hr. at 50°C.

To enable the course of wool digestion in 1 per cent. papain, 0.1M sodium thioglycollate, 3M urea to be conveniently followed and compared with that previously reported for papain-bisulphite-urea (Lennox 1952) the initial pH was adjusted to 8.6. The amount of digestion after 6 hr. at 50°C. at this pH value was similar to that observed when bisulphite was used at pH 7.0, but the digestion proceeded more rapidly in the early stages of incubation (Fig. 7).

The change in the rate of digestion in the presence of thioglycollate is probably due to a fall in pH value to 7.0, which occurred within the first 2 hr.

The effect of pH on the formation of sulphydryl groups after incubation of 0.020 g. samples of wool for 1 hr. at 50°C. in 0.6 ml. portions of 0.1M solutions of the compounds previously tested in the papain-urea solutions was measured by the iodosobenzoic acid method and is shown in Figure 8. After incubation the wool was immersed for several hours in two changes each of 20 ml. distilled water (which had been stored under hydrogen) followed by one rinse in ethanol. In this way the reagents were removed with minimum risk of oxidizing the wool. Excess liquid was removed after each immersion by

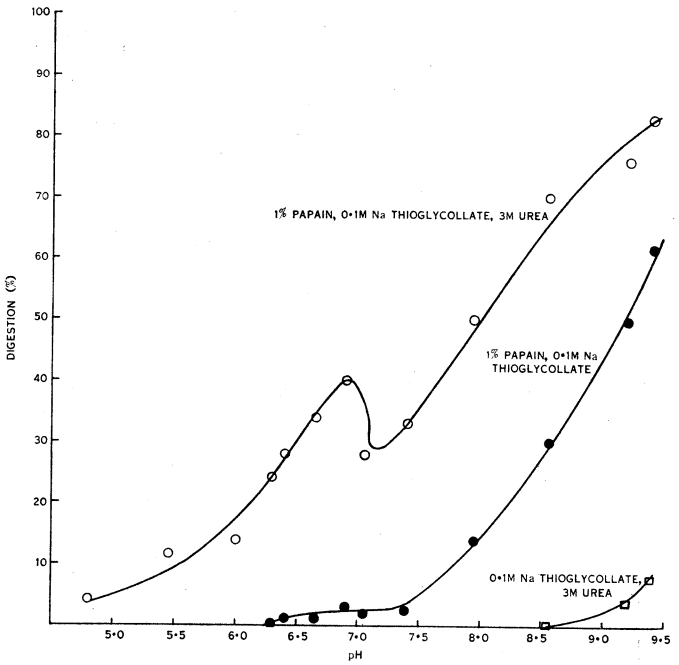

Fig. 3.—pH-wool keratin digestion in sodium thioglycollate solutions containing also papain, urea, or both. Digested for 18 hr. at 50°C.

repeatedly squeezing the samples between filter paper. Softening of the wool became evident as the pH value of the thioglycollate solutions increased above 9.0. The weight of wool was reduced in thioglycollate to 0.019 g. at pH 10.0 and to 0.017 g. at pH 10.5; at the latter pH most of the fibrous structure disappeared and the product became plastic. Hardening occurred on immersion in ethanol. In the cysteine solutions slight softening of the fibres was detected at pH 10.0 and 10.5. The form of the pH-thioglycollate reduction curve is similar to that reported by Patterson *et al.* (1941) although, because of the milder conditions employed, the extent of reduction is less. The formation of sulphydryl groups in the presence of potassium cyanide has been confirmed by increasing the concentration of this reagent to 1.0M, adjusting the pH value to

10.5, and incubating as before for 1 hr. at 50°C. Under these conditions the concentration of sulphydryl sulphur formed was doubled. Further confirmation

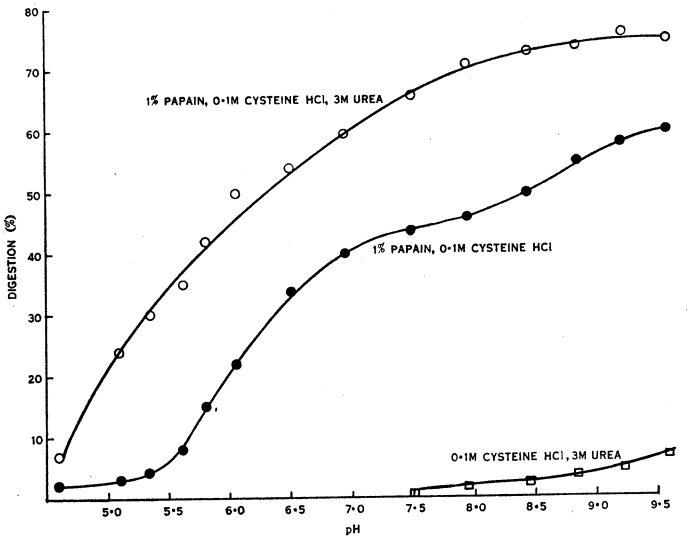

Fig. 4.—pH-wool keratin digestion in cysteine hydrochloride solutions containing also papain, urea, or both. Digested for 18 hr. at 50°C.

was obtained by incubating in potassium cyanide solutions as above, and staining with sulphydryl group reagents such as sodium nitroprusside. Such methods

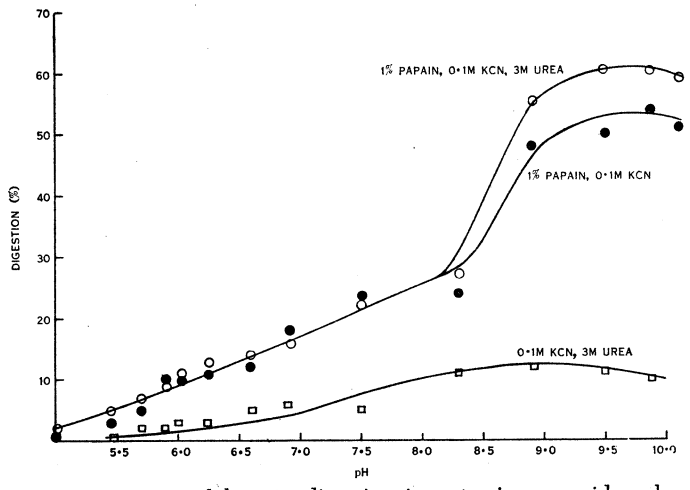

Fig. 5.—pH-wool keratin digestion in potassium cyanide solutions containing also papain, urea, or both. Digested for 18 hr. at 50°C.

also showed that material containing free sulphydryl groups was extracted from the wool by the potassium cyanide solution.

After reduction of wool at various pH values in 0.1M thioglycollate, as in the above experiment, incubation for 1 hr. at 50°C. in 1 per cent. papain, 3M urea at pH 7.0, produced the digestion shown in Figure 9. Prior reduction

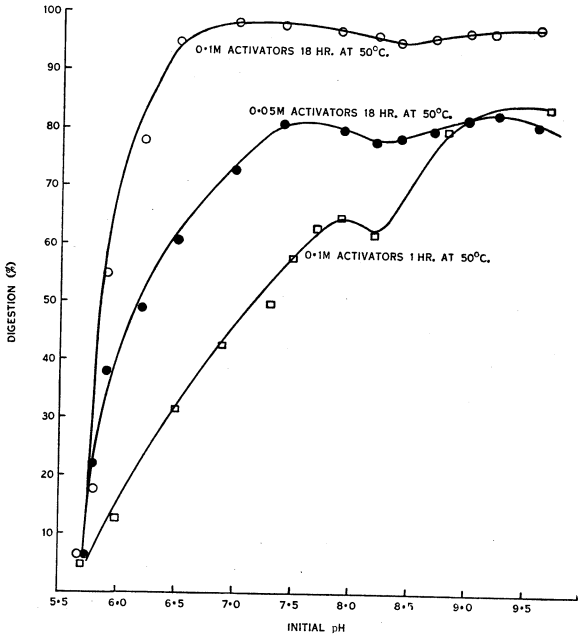

Fig. 6.—pH-wool keratin digestion in papain-urea solutions containing also the mixed activators sodium bisulphite and sodium thioglycollate.

TABLE 1

WOOL DIGESTION AND SULPHYDRYL SULPHUR IN WOOL AFTER INCUBATION FOR 1.5 HR. AT 50°C. IN THIOGLYCOLLATE SOLUTIONS WITH AND WITHOUT PAPAIN AND UREA

Wool Incubated in Solution Containing			Incubated at pH 7·0		Incubated at pH 8·0	
1 Per cent. Papain	0·1M Sodium Thioglycollate	3M Urea	Digestion (%)	Sulphydryl S (%)	Digestion (%)	Sulphydryl S (%)
−	+	−	Nil	0·83	6	1·38
−	+	+	Nil	1·03	4	1·47
+	+	−	21	1·16	66	1·66
+	+	+	50	1·41	82	1·06

apparently predisposed the wool to subsequent attack by papain-urea solution, but it will be noted that the digestion was less than that produced by the simultaneous action of papain, thioglycollate, and urea. Transfer from the 0.1M thioglycollate solution to the papain-urea probably allowed reoxidation of some

of the sulphydryl groups in wool to disulphide bonds. The enzyme and urea would then be unable to exert as great a disruptive action on the keratin structure as when they act in the presence of 0.1M thioglycollate. Similar treatment in sodium bisulphite solutions failed to predispose the wool to subsequent digestion in papain-urea.

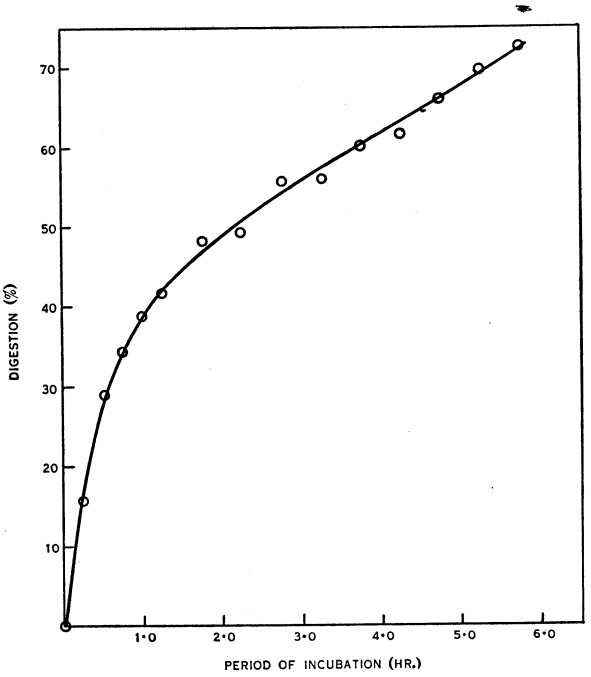

Fig. 7.—Progress of wool keratin digestion in 1 per cent. papain, 0.1M sodium thioglycollate, 3M urea during incubation at pH 7.5 (initial pH 8.6).

Greater reduction of disulphide bonds by thioglycollate in the presence of urea or papain than in their absence is apparent from the results in Table 1. The relatively low concentration of sulphydryl groups in the small residue remaining after digestion in papain-thioglycollate-urea at pH 8.0 indicates that keratin passing into solution was probably rich in these groups.

The pH-digestion curve for sheepskin collagen in 1 per cent. papain, 0.1M sodium bisulphite, 3M urea resembles that observed for wool and shows a pH optimum at approximately 7.0 (Fig. 10). The curve representing the digestion of collagen in 1 per cent. papain, 0.1M sodium thioglycollate, 3M urea differs markedly from the corresponding curve for wool keratin. Digestion was maximal in acid solutions and it diminished as the pH exceeded 7.5.

The initial pH values of the enzyme solutions in both series of tests were adjusted to values separated by 0.5 pH units between 4.0 and 10.5. The pH values reported in Figure 10 for collagen digestion, which were measured after the completion of incubation, reveal marked changes in some solutions and

entirely different changes for the two activators. The existence of four pH maxima in the thioglycollate series was confirmed in a second experiment.

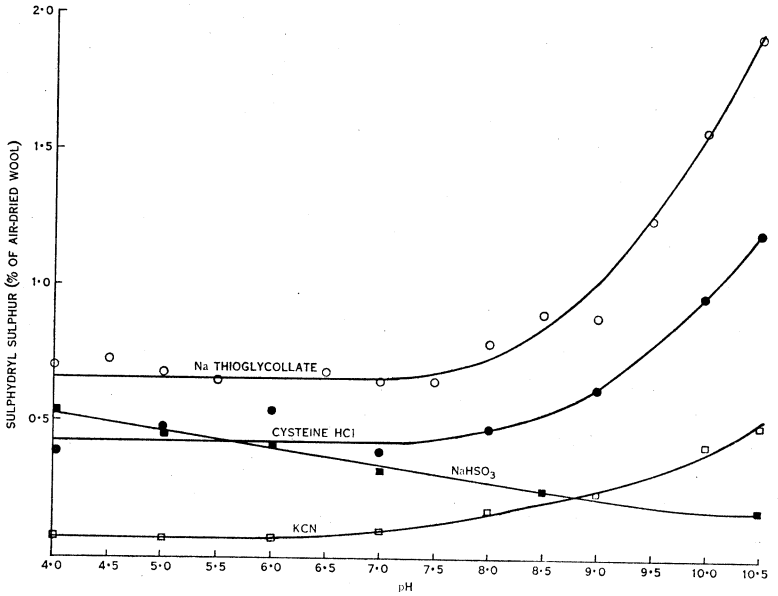

Fig. 8.—Effect of pH on reduction of disulphide bonds in wool by 0.1M solutions of compounds that rupture disulphide bonds. Incubated for 1 hr. at 50°C.

IV. Discussion

Schöberl and Hamm (1948) observed maximum digestion of wool in papain-cyanide solution at pH 9. Up to 40 per cent. was digested in 18 hr. at 60°C. and by repeated use of fresh papain solution the digestion was increased to 80 per cent. In the present study the pH optimum was shown to be 9.5-10.0.

Cuthbertson and Phillips (1945) showed that incubation of wool for 16.5 hr. in 1 per cent. potassium cyanide at 66°C. converted nearly all the combined cystine into combined lanthionine, the sulphur released during the process forming potassium thiocyanate. The development of a reddish brown colour during the digestion of wool at pH values above 8.5 in papain-cyanide, in the presence of urea, though to a lesser extent in its absence, may be due to the formation of a compound containing thiocyanate. Unlike ferric thiocyanate, however, it was not soluble in ether, even after precipitation of protein from the digest with trichloracetic acid. The pigment passed slowly through cellophane during dialysis and when subjected to paper electrophoresis it moved towards the anode more rapidly than the high molecular weight components in the digest, which stain with bromphenol blue.

It will be noted that the relative effectiveness of the various compounds in producing sulphydryl groups in wool resembles their contribution to wool digestion when incorporated in papain-urea solutions (Fig. 1) and incubated under identical conditions. In comparing the disulphide bond-rupturing agents

allowance must be made for the liberation by sodium bisulphite of only one sulphydryl group for each disulphide bond split, the other half of the cystine residue being converted to S-cysteine sulphonate (Elsworth and Phillips 1938). The same is probably true of the action of potassium cyanide. According to Cuthbertson and Phillips (1945), during the production of lanthionine cross-linkages in the presence of cyanide, one-half of each cystine residue yields a cysteine residue, which is presumably the source of the sulphydryl groups reported in Figure 8. In contrast to bisulphite and cyanide, thioglycollate and cysteine produce two sulphydryl groups from each cystine disulphide linkage, and if allowance is made for these differences in comparing the results in Figure

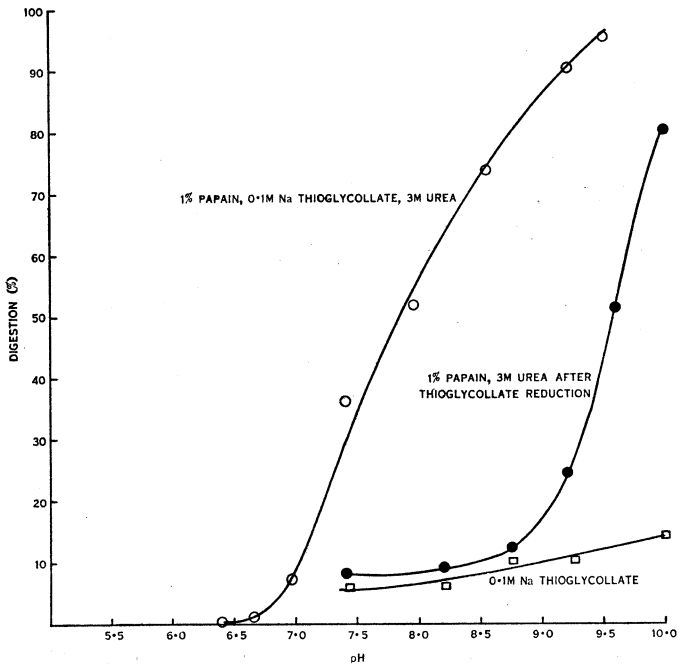

Fig. 9.—Effect of prior reduction of wool in thioglycollate at pH values shown on subsequent digestion by papain-urea at pH 7.0. Incubated for 1 hr. at 50°C.

8, bisulphite is found to split more disulphide bonds than the other three compounds in acid solution. Above pH 9 however, the relative order is: thioglycollate, cysteine, cyanide, and bisulphite, corresponding to their effects in increasing the papain-urea digestion of wool. Wool is comparatively resistant to papain-cyanide-urea solutions, even at alkaline pH values (Fig. 1), and this may be due to the formation of lanthionine cross-linkages. Comparison of the results in Figures 1 and 8 also suggests that the rupture of disulphide bonds should exceed a threshold value before wool digestion becomes appreciable. For thioglycollate and cysteine this corresponds to approximately 0.7 per cent. sulphydryl sulphur.

Further evidence that the action of the disulphide bond-rupturing agents on wool in the presence of papain and urea is not confined to the activation of papain, but also affects keratin directly, was shown by substituting a sample of fully active papain for the material normally used. When tested in solution containing 3M urea but no disulphide bond-rupturing agent, both samples of papain produced approximately 6 per cent. digestion during 6 hr. at 50°C., but when sodium bisulphite was added to a final concentration of 0.1M, digestion exceeded 70 per cent. Failure of ascorbic acid to increase the digestion of wool by papain-urea may be related to its comparatively high E'_0 at pH 7 which has been reported to be + 0.060 V. (Hellerman 1939); the corresponding values for cysteine and thioglycollic acid are approximately − 0.3 V. (Ryklan and Schmidt 1944).

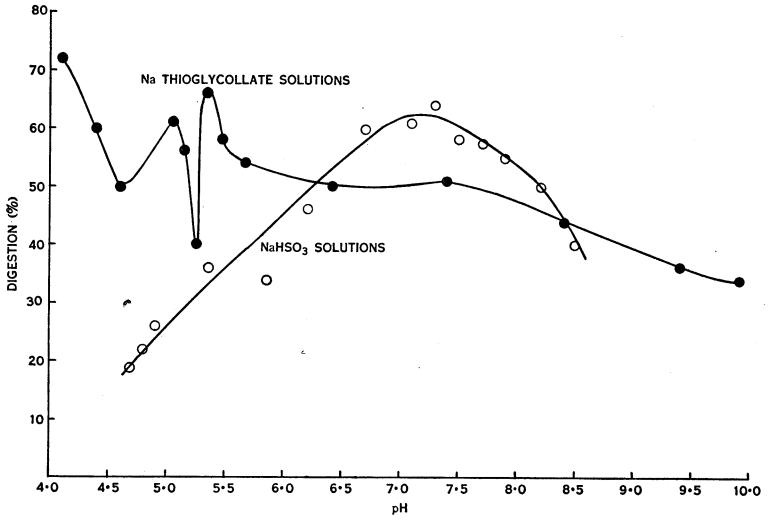

Fig. 10.—Digestion of skin collagen in papain-urea solutions activated with sodium bisulphite or sodium thioglycollate. O Incubated for 1.25 hr. at 35°C. in 1 per cent. papain, 0.1M $NaHSO_3$, 3M urea. ● Incubated for 0.75 hr. at 35°C. in 1 per cent. papain, 0.1M Na thioglycollate, 3M urea.

Since collagen contains no cystine the main role of sodium bisulphite and sodium thioglycollate during digestion of this protein in solutions containing also papain and urea would be that of papain activation.

V. Acknowledgment

The authors are indebted to Mr. T. A. Pressley for preparing the skin collagen used in this investigation.

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