# DIGESTION OF WOOL KERATIN BY PAPAIN-BISULPHITE-UREA AND RELATED SYSTEMS

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*Summary*

The digestion of wool in papain-bisulphite solutions is greatly enhanced by the addition of urea,† reaching completion within a few hours at 50° or 70°C. Both wool digestion and uptake of papain by wool from urea solutions proceed optimally at pH 7.

Urea-bisulphite solutions containing other proteinases such as ficin or bromelin, which are activated by reducing agents, partially digest wool at pH 7, and similar solutions containing pepsin partially digest wool at pH 3, but mould protease, trypsin, beef liver cathepsin, and pig pancreas extract display little activity in comparison with papain at all pH values between 5.7 and 9.6.

Replacement of sodium bisulphite in the digestion mixture with other sulphur-containing reducing agents reduces the amount of wool digestion at pH 7. Of a series of compounds related to urea, which were tested at equimolecular concentrations in the presence of papain and bisulphite, thiourea assisted digestion to a greater extent than urea, but the other compounds were less effective. Guanidine is only slightly inferior to urea, but partial or complete replacement of an amino group in the urea molecule, as in methyl urea and formamide respectively, lowers the activity greatly.

Horn and feather keratin and skin collagen are readily digested by papain-bisulphite-urea but silk fibroin and plasma fibrin are less affected.

## I. INTRODUCTION

Because of its pronounced resistance to attack by proteolytic enzymes, wool is much less readily damaged by living organisms, such as bacteria, moulds, and insects, than are most other protein materials. Under some conditions this resistance is overcome and it is important to identify such conditions and to determine the nature of the attack if means are to be sought for protecting the fibres from biological damage. Moreover, to prepare solutions and dispersions of wool of a type suitable for certain chemical studies, it is preferable to imitate the relatively mild enzymic digestion employed in nature rather than employ the more drastic and less specific chemical methods, for the former method is more likely to yield fragments of wool keratin in solution resembling intact keratin. For these reasons, particular interest attaches to the previously reported slow digestion of wool in solutions containing papain and bisulphite, which is the basis of the shrinkage resistance process developed by Middlebrook and

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† In this paper the term "digestion" signifies solution of wool through enzyme, physical, or chemical action without reference to the nature or fate of the substances removed from the fibres.

Phillips (1941). After 17 hr. incubation they found that the wool was reduced to a mass of cells, and H. Lindley (private communication) has observed complete digestion of the cortical cells from processed wool after prolonged incubation in papain-bisulphite solution at 65°C. In the present paper it will be shown that the addition of urea to the papain-bisulphite greatly assists attack on the wool to the stage of almost complete digestion within a few hours at 50° or 70°C. Wool is also extensively digested during incubation in other solutions containing similar combinations of sulphide-activated plant protease, or pepsin, reducing agent, and a compound structurally related to urea.

In some respects the present study is related to the work of Jones and Mechan (1943). These workers showed that 52 per cent. of wool substance was dissolved during 17 hr. incubation at 40°C. in 10M urea and 0.3M sodium bisulphite, but the remainder of the wool fibre was unattacked under these conditions.

## II. Materials and Methods

### (*a*) *Preparation of Wool*

The tips were removed from the staples of 50 lb. of 64's quality merino fleece and the remainder was extracted with ethanol for 7 hr. in a large brass Soxhlet apparatus, then immersed on four successive days in tap water, changing the water twice daily. On the fifth day distilled water was used and the wool was then squeezed well and dried in a current of warm air. Seeds and other impurities were removed from the scoured wool by hand-picking, and the product was stored in sealed metal containers.

### (*b*) *Proteinases and Reagents*

The papain used was a high quality commercial grade grown in Ceylon and supplied in the form of a slightly brown powder. Analysis of aqueous extracts showed that the powder contained 12 per cent. soluble ash. Rapid digestion was confirmed in some tests using samples from two other sources. The trypsin was from Allen and Hanbury, and the pepsin used was a "Difco" product. The mould protease powder also contained other enzymes from *Aspergillus oryzae* (Crewther and Lennox 1950), and was prepared from culture filtrate by low temperature precipitation with ethanol and freeze-drying. Crude bromelin was prepared by disintegrating pineapple tissues in the Waring Blendor, separating the juice by centrifuging and filtering, adding five volumes of ethanol at 2°C., centrifuging to recover the precipitate, drying in a vacuum over calcium chloride, and grinding the dry residue. Crude ficin powder was prepared by collecting in ethanol the latex from freshly picked green figs of the tree *Ficus glabrata* and treating the precipitate as described for bromelin. The ficin used in the experiments reported in Tables 4 and 5 was purchased from the Delta Chemical Works, U.S.A. Cathepsin was prepared from beef liver by the method of Anson (1936), the product obtained in the final dialysis being centrifuged to remove suspended matter and the solution freeze-dried. The pig pancreas extract used was freeze-dried. Enzyme extracts were prepared for the experiments by grinding the enzyme powders with water and centrifuging.

The chemicals used were all crystalline laboratory grade reagents except the sodium hydrosulphite, which was a technical grade. The hide powder was from Baird and Tatlock, and the silk fibroin and the fibrin were from Hoffmann La Roche.

### (*c*) *Measurement of Digestion*

Samples 1.00 g. of scoured dry wool were weighed and transferred to thick-walled 50 ml. centrifuge tubes containing 30 ml. of the test solution, previously adjusted to the desired pH with the aid of a glass electrode pH assembly, and stirred carefully to remove entrained air and distribute the wool uniformly through the solution. The tubes were incubated without agitation for 18 hr. at 50°C. If the wool was not broken into fragments during incubation, it was removed from the tube, washed, and squeezed repeatedly in running water. If disintegrated, the residue was filtered on a 7 cm. No. 541 Whatman filter paper on a Buchner funnel and washed well. If extensively degraded and partly gelatinized, the residue was recovered by centrifuging before suspending in water and filtering. The undigested material was dried for 5 hr. at 105°C. and allowed to equilibrate with the laboratory atmosphere overnight before weighing and calculating the percentage digestion.

TABLE 1

WOOL DIGESTION BY COMPONENTS OF PAPAIN-BISULPHITE-UREA MIXTURE ON WOOL AT 50°C., SINGLY AND IN COMBINATION

| Papain at 1% Concentration | Sodium Bisulphite at 0.1M Concentration | Urea at 4.0M Concentration | Wool Digestion (%) After 18 Hr. | Wool Digestion (%) After 41 Hr. |
|---|---|---|---|---|
| − | − | + | 8 | 3 |
| − | + | − | 3 | 2 |
| + | − | − | 2 | Nil |
| − | + | + | 5 | 5 |
| + | − | + | 4 | 3 |
| + | + | − | 18 | 45 |
| + | + | + | 92 | 100 |

Samples of the standard wool, heated for 5 hr. at 105°C. and then allowed to equilibrate as above, were found to be 2 per cent. lighter than unheated equilibrated samples, and estimation of the moisture content of air-dried wool over several months in this laboratory revealed a maximum variation of ± 1.5 per cent. The small error due to the effect of heating on moisture uptake by wool and variation in weight due to changes in the relative humidity would not affect the deductions drawn in this paper from comparison of the results of separate experiments, and moreover, most comparisons are made between results obtained in the same experiment. In a series of eight tests in one digestion experiment the mean percentage digestion and the standard deviation of the mean were 43.9 ± 0.4.

## III. Experimental

### (*a*) *Digestion of Wool by Papain in the Presence of Sodium Bisulphite and Urea*

A solution containing 1 per cent. papain, 0.1M sodium bisulphite, and 4.0M urea almost completely digested 1 g. wool after 18 hr. at 50°C. The superiority of this mixture to any of the components when tested singly or in various combinations is shown in Table 1. The small amount of undigested material was ribbon-like in appearance under the microscope and was identified by Dr. E. H. Mercer as cuticular sheaths (Mercer, Lindberg, and Philip 1949). Digestion in 4M urea and 0.1M sodium bisulphite at all pH values between 5.7 and

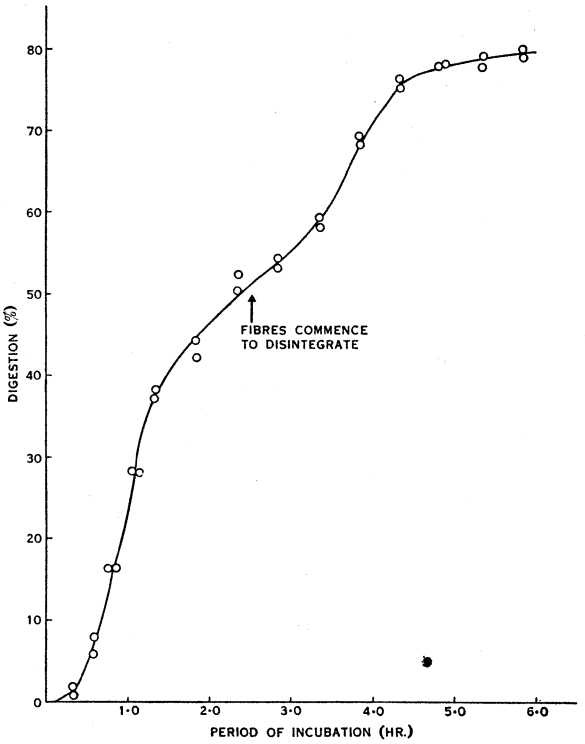


Fig. 1.—Progress of wool digestion in 1 per cent. papain, 0.1M sodium bisulphite, 3M urea during incubation at 50°C. and pH 7.0.

9.6 was inferior to that reported in the table for the papain solutions. All the amino acids of wool were detected on paper chromatography of the digest, but the separation of a white precipitate from the digest on dialysing or on adjusting the pH to 5.0-5.5, showed that portion of the wool protein was only partly degraded. This material is probably similar to that recovered by Blackburn (1950) from papain-bisulphite partial digests of wool.

The progress of digestion is shown in Figure 1. A second phase of rapid digestion followed disintegration of the fibres into fragments and cortical cells. From Figure 2, the digestion during 7 hr. incubation is seen to improve rapidly with increase in temperature above 22°C. Digestion was completed after 7 hr. at 70°C., but at 100°C. the papain was found to be inactive within 2 hr. Digestion at this temperature did not exceed 28 per cent.

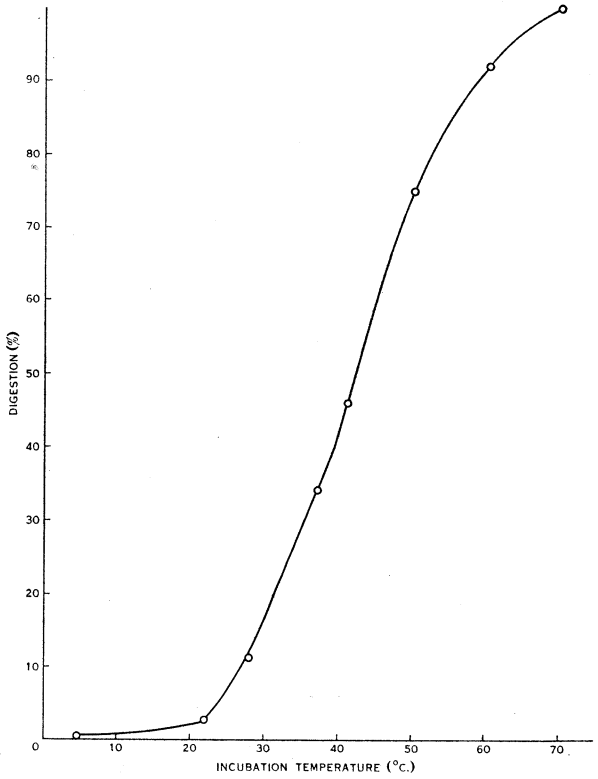


Fig. 2.—Digestion of wool in 1 per cent. papain, 0.1M sodium bisulphite, 3M urea at pH 7.0 after incubation for 7 hr. at various temperatures.

Reduction in the initial concentration of either the sodium bisulphite or urea reduced the percentage digestion during 18 hr. at 50°C., as shown in Figure 3, but it was necessary to reduce the initial papain concentration below 0.2 per cent. to produce a similar effect. Simultaneous dilution of all three components reduced the digestion much more sharply than dilution of any single component. When digested in papain-bisulphite in the absence of urea, the wool fibres were considerably weakened after 17 hr., and disintegrated into cortical cells after 41 hr.

By increasing the concentration of urea to 8M, 90 per cent. digestion occurred in 4 hr. at 50°C. (Fig. 4). With 5M urea 90 per cent. digestion

occurred in 4 hr. at 70°C., but the digestion was slightly less pronounced at higher urea concentrations at 70°C., suggesting that the enzyme itself was being inactivated at the higher urea concentrations.

By maintaining the urea concentration at 2M and increasing the sodium bisulphite concentration to 0.2M, complete digestion of the wool occurred in 2 hr. at 70°C., but at 50°C. only 40 per cent. digestion occurred during the same period and, even in 0.5M sodium bisulphite, digestion did not exceed 50 per cent. Solutions containing high bisulphite concentrations, like those containing high urea concentrations, probably inactivated the enzyme during incubation at these high temperatures.

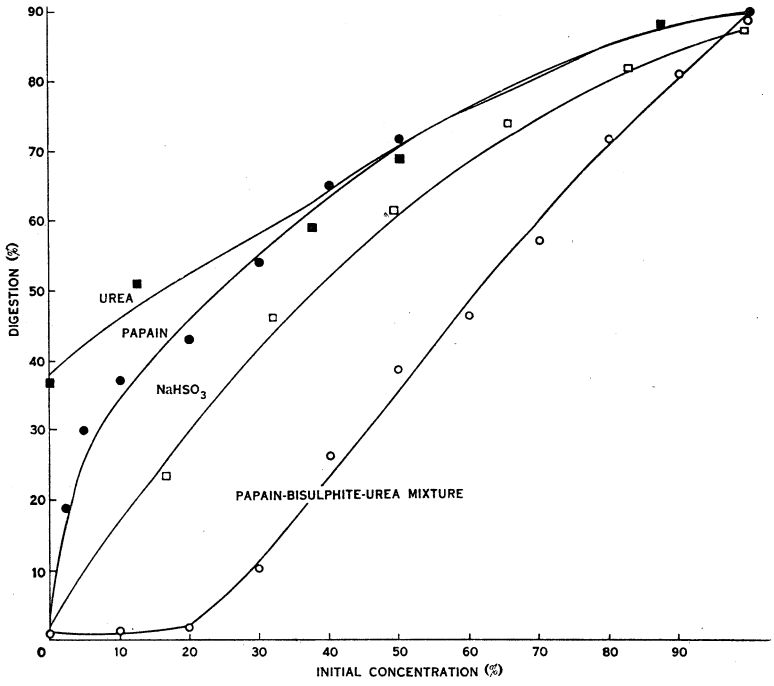


Fig. 3.—Effect of dilution of each constituent of the papain-bisulphite-urea solution on wool digestion after 18 hr. at 50°C. and at pH 7.0.

- O 2 per cent. papain-0.1M $NaHSO_3$-4M urea mixture diluted.
- ● 0.2 per cent. papain diluted in presence of 0.1M $NaHSO_3$ and 4M urea,
- □ 0.1M $NaHSO_3$ diluted in presence of 2 per cent. papain and 4M urea,
- ■ 4M urea diluted in presence of 2 per cent. papain and 0.1M $NaHSO_3$.

### (*b*) *Effect of pH on Digestion at Different Urea Concentrations*

The pH of a series of papain-bisulphite-urea solutions was adjusted to values between 4 and 10 with hydrochloric acid and sodium hydroxide before diluting them to 30 ml. and immersing the 1 g. wool samples. The final concentrations of the papain and the sodium bisulphite in all the solutions were

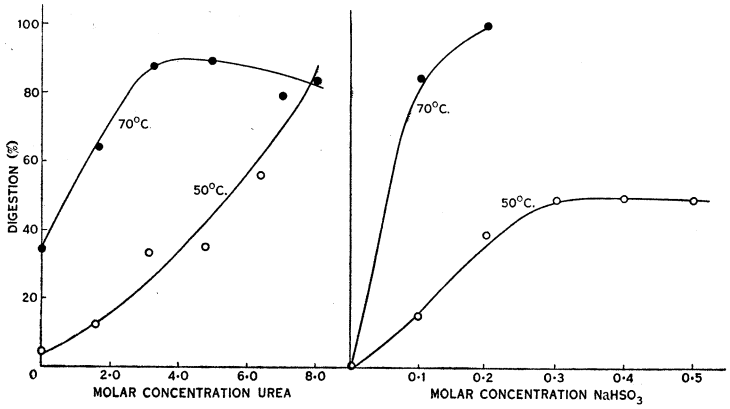


Fig. 4.—Effect of high concentrations of urea and bisulphite on digestion of wool by 1 per cent. papain after incubation for 4 hr. at 50°C. and 70°C. 0.1M $NaHSO_3$ was present in the urea series and 2M urea was present in the bisulphite series.

1 per cent. and 0.1M, respectively, but the urea was tested at different concentrations up to 4M. The pH values for maximum digestion decreased from 7.3

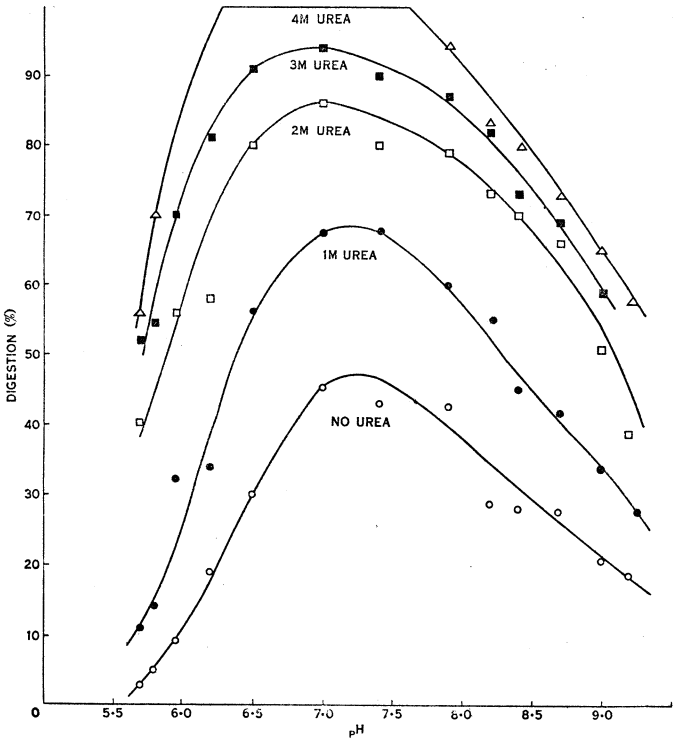


Fig. 5.—pH curves for solutions containing 1 per cent. papain, 0.1M $NaHSO_3$, and urea at the concentration indicated on each curve. The pH values reported were measured after 0.5 hr. and the digestions after 18 hr. incubation at 50°C.

to 7.0 with increase in the concentration of urea (Fig. 5). These pH values refer to readings obtained with the aid of the Beckman high temperature glass electrode 0.5 hr. after the commencement of incubation. As shown in Table 2, the pH values changed considerably immediately after the commencement of incubation, and to a lesser extent for some hours subsequently. When either papain or wool was omitted from the system, similar pH changes were observed and they are not therefore solely attributable to the uptake of ions on protein. Since wool digestion did not become properly established until after incubation for about 0.25 hr. (Fig. 1), the pH after 0.5 hr. probably approximates more closely to the mean pH for the digestion than any other.

TABLE 2

CHANGE IN pH OF 30 ML. 1 PER CENT. PAPAIN, 0.1M $NaHSO_3$, 3M UREA SOLUTION AFTER ADDITION OF 1 G. OF WOOL AND INCUBATION AT 50°C.

| pH Before Immersing Wool | pH After the Following Incubation Periods (hr.) | | | | |
|---|---|---|---|---|---|
| | 0.01 | 0.17 | 0.50 | 1.50 | 5.50 |
| 4.0 | 5.0 | 5.2 | 5.7 | 5.7 | 5.9 |
| 4.5 | 5.2 | 5.4 | 5.8 | 5.8 | 5.9 |
| 5.0 | 5.5 | 5.7 | 5.9 | 5.8 | 6.0 |
| 5.5 | 5.9 | 6.1 | 6.2 | 6.1 | 6.2 |
| 6.0 | 6.4 | 6.5 | 6.5 | 6.4 | 6.5 |
| 6.5 | 6.9 | 7.0 | 7.0 | 6.9 | 6.9 |
| 7.0 | 7.3 | 7.4 | 7.4 | 7.4 | 7.2 |
| 7.5 | 7.9 | 7.9 | 7.9 | 7.8 | 7.5 |
| 8.0 | 8.2 | 8.1 | 8.2 | 8.0 | 7.8 |
| 8.5 | 8.5 | 8.4 | 8.4 | 8.3 | 8.1 |
| 9.0 | 8.8 | 8.7 | 8.7 | 8.5 | 8.3 |
| 9.5 | 9.1 | 9.1 | 9.0 | 8.8 | 8.5 |
| 10.0 | 9.5 | 9.3 | 9.2 | 9.0 | 8.8 |
| 10.5 | 9.9 | 9.7 | 9.6 | 9.4 | 9.1 |

In the following experiment the reduction of wool digestion with departure of the pH from the optimum value was shown not to be due to inactivation of the papain. A series of solutions containing 1 per cent. papain, 0.1M sodium bisulphite, and 3M urea were adjusted to the same pH values as those reported in Figure 5, and then incubated for 18 hr. at 50°C. without wool. After incubation the pH values were all adjusted to pH 6.5 and the concentrations of the constituents were reduced by dilution to 0.9 of their initial values before adding 0.1 g. wool to 3 ml. of each and incubating for 18 hr. at 50°C. The digestion varied only between 80 and 85 per cent. between the initial pH values of 4.0 and 7.0 and decreased to 72 per cent. as the pH approached 10.5.

### (*c*) *Effect of pH on Uptake of Papain from Urea Solution*

Solutions of 0.2 per cent. papain and 3M urea were adjusted to the same pH values as in the digestion experiment. After incubating 1 g. wool in each for 1 hr. at 50°C., the wool was removed, squeezed between filter papers to

remove free liquid, transferred to solutions containing only 0.1M sodium bisulphite and 3M urea at pH 6.5, and incubated for 18 hr. at 50°C. Figure 6 shows that the digestion, and presumably also uptake of papain, were optimal at pH 7.0, there being practically no uptake of enzyme at pH 5.7.

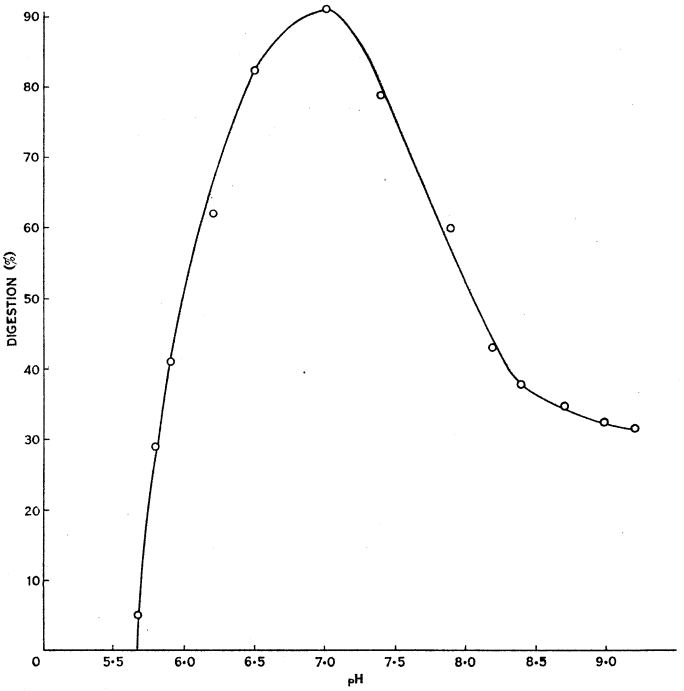


Fig. 6.—Influence of pH on uptake of papain by wool, as evidenced by digestion after 18 hr. at 50°C. in 0.1M $NaHSO_3$-3M urea, following uptake from 0.2 per cent. papain-3M urea solutions at various pH values. The pH values were measured after 0.5 hr. incubation.

In order to determine whether the uptake of papain on wool is reversed by lowering the pH, a series of papain solutions with concentrations ranging up to 1 per cent. were adjusted to pH 6.5 and 1 g. samples of wool were immersed in 30 ml. of each for 1 hr. at 50°C. The wool samples were removed from the solutions, free liquid was removed by squeezing between filter papers, they were washed by immersion in 30 ml. water for 1 hr. at 50°C., again partially dried, and transferred to 20 ml. water maintained at pH 4 by the addition of hydrochloric acid. Urea and bisulphite were added to 15 ml. of these extracts to give concentrations of 3M and 0.1M respectively after adjusting the pH to 6.5 and the volume to 30 ml.; 1 g. samples of wool were added to test the activity of these solutions by incubation for 18 hr. at 50°C. The original wool samples were partially dried and transferred to solutions containing 0.1M sodium bisulphite and 3M urea for detection of unextracted enzyme. Both sets of results are shown in Figure 7.

Although the two curves are not directly comparable, since only 20 ml. water was used for extraction and, of this, 15 ml. was taken for digestion, it is evident that extraction of the bound enzyme under these conditions was not complete.

*(d) Effect of Urea and Salts on Papain Uptake and Wool Digestion*

In the following experiment it is shown that urea influences the uptake of papain on wool. Incubation of 1 g. samples of wool for 1 hr. at 50°C. in 0.2 per cent. papain solutions at pH 6.5 containing nil, 1.0M, 2.0M, 3.0M, and 4.0M urea, followed by squeezing between filter papers and digestion for 18 hr. at 50°C. in 0.1M sodium bisulphite and 3M urea at pH 6.5, revealed an increase in wool digestion from 27 per cent. in the absence of urea to 53 per cent., corresponding to the increase in urea concentration. When urea was omitted from the bisulphite solutions, only 17-19 per cent. digestion occurred, showing that the improvement observed after transfer from papain-urea to 18 hr. bisulphite-urea digest solutions was due, not to urea taken up by the wool, but to papain uptake.

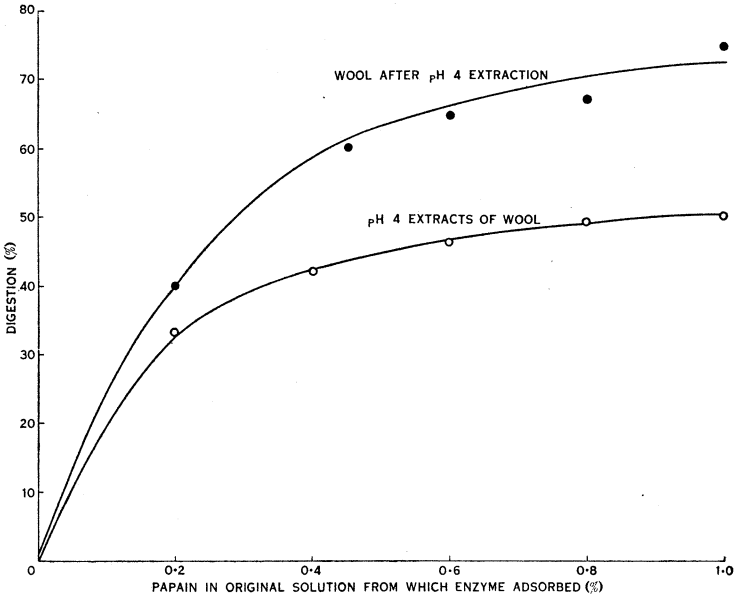


Fig. 7.—Partial removal of papain from wool at pH 4 after uptake at pH 7.0. Papain removed from wool estimated by adding 0.1M $NaHSO_3$ and 3M urea to the pH 4 extract and measuring digestion of wool after 18 hr. at 50°C. and pH 7.0. Papain not removed estimated by incubating extracted wool in 0.1M $NaHSO_3$ and 3M urea and measuring digestion after 18 hr. at 50°C. and pH 7.0.

In a similar experiment, to test the effect of sodium bisulphite on the uptake of papain on wool, the wool samples were immersed for 1 hr. in solutions at pH 6.5 containing 0.2 per cent. papain and sodium bisulphite ranging from 0.05M to 0.4M, before squeezing the samples and digesting them for

18 hr. in 0.1M sodium bisulphite and 3.0M urea at pH 6.5. The increase in bisulphite concentration was found to reduce the uptake of papain, as evidenced by a progressive reduction in wool digestion from 52 to 30 per cent. through the series. Omission of bisulphite from the 18 hr. urea digest solutions reduced the level of digestion throughout, but it revealed a progressive increase from 10 to 21 per cent. through the series. This increase was probably due to the uptake of bisulphite by the wool from the papain-bisulphite solutions.

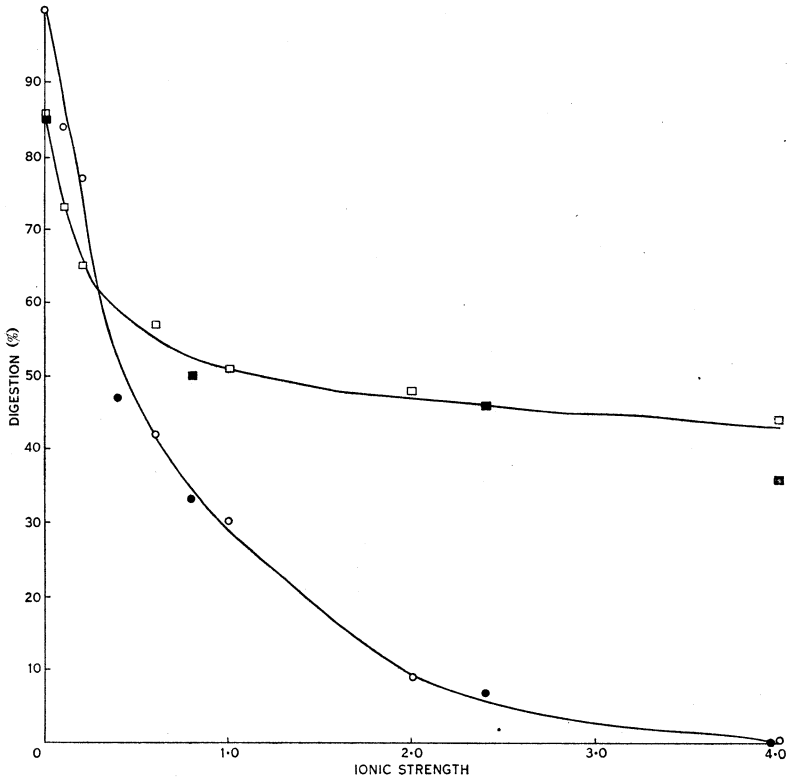


Fig. 8.—Inhibiting action of salts on wool digestion and papain uptake by wool at 50°C. and pH 7.0.

O Digestion of wool in 1 per cent. papain, 0.1M $NaHSO_3$, 3M urea containing NaCl,

● Digestion of wool in 1 per cent. papain, 0.1M $NaHSO_3$, 3M urea containing $MgSO_4$,

□ Digestion of wool in 0.1M $NaHSO_3$, 3M urea, after adsorption of papain in the presence of NaCl,

■ Digestion of wool in 0.1M $NaHSO_3$, 3M urea, after adsorption of papain in the presence of $MgSO_4$.

In all tests digestion was measured after 18 hr. at 50°C. and pH 7.0.

The amount of wool digestion was found to be lowered by dissolving in the solution containing 1 per cent. papain, 0.1M sodium bisulphite, and 3.0M urea, 0.1M, 0.2M, 0.6M, 1.0M, 2.0M, and 4.0M sodium chloride in one experi-

ment, and 0.1M, 0.2M, 0.6M, and 1.0M magnesium sulphate in another experiment, before immersing the 1 g. samples of wool in the solutions and incubating. As shown in Figure 8, increase in salt concentration reduced the wool digestion to zero. When the concentrations were expressed in terms of ionic strength, the effects of the two salts were indistinguishable.

To study the mechanism by which wool digestion was adversely affected by salts, 1 g. samples of wool were incubated for 1 hr. at 50°C. in 30 ml. 1 per cent. papain solutions at pH 6.5, containing the same concentrations of sodium chloride and magnesium sulphate as above, then squeezed to remove excess solution, and incubated for 17 hr. at 50°C. in 0.1M sodium bisulphite, 3M urea at pH 6.5 (Fig. 8). Since the wool digestion was not inhibited to the same extent as on direct addition of salts to the papain-bisulphite-urea solution, interference with papain uptake on wool is probably not the main mechanism of inhibition. However, the possibility should be recognized that urea or bisulphite may so affect papain or wool as to render their combination more susceptible to interference by salts than this experiment suggests. The inhibition of wool digestion observed in the latter experiment may have been due to the transfer of salts on the wool to the urea-bisulphite solution.

TABLE 3

PERCENTAGE WOOL DIGESTION BY VARIOUS PROTEINASES WITH AND WITHOUT BISULPHITE AND UREA AT pH 7.0 AFTER 20 HR. AT 50°C.

| Proteinase | 1% Proteinase | 1% Proteinase + 0.1M Sodium Bisulphite | 1% Proteinase + 4M Urea | 1% Proteinase + 0.1M Sodium Bisulphite + 4M Urea |
|---|---|---|---|---|
| Pepsin | Nil | Nil | Nil | 6 |
| Mould protease | Nil | 5 | Nil | 10 |
| Trypsin | Nil | Nil | Nil | 5 |
| Papain | Nil | 30 | Nil | 100 |
| Ficin | Nil | 10 | Nil | 65 |
| Bromelin | Nil | 5 | Nil | 25 |
| Beef liver cathepsin | Nil | Nil | Nil | 3 |
| Pig pancreas extract | Nil | Nil | Nil | 2 |

### (*e*) *Digestion Experiments with other Proteinases*

The plant proteinases ficin and bromelin partially digested wool during incubation at 50°C. in the presence of bisulphite and urea, but the other proteinases examined were ineffective at pH 7 (Table 3). The pH value-wool digestion relationships were determined for all the enzymes listed in Table 3 in the presence of 0.1M sodium bisulphite and 3M urea between pH 5.7 and pH 9.6, and for pepsin the range was extended down to pH 1. No greater digestion than that shown in Table 3 was revealed at other pH values for any proteinase except pepsin, which produced a maximum of 57 per cent.

digestion at pH 2.9 (Fig. 9).* In the absence of urea it produced maximum digestion of 48 per cent. at pH 1.8, showing that urea probably inactivated the pepsin rapidly in the previous experiment as the pH value was reduced below pH 2.9. Appreciable digestion at pH 1.8 in the absence of urea also suggests that hydrogen ions replaced urea as the denaturing agent at low pH values. In solutions containing sodium bisulphite and urea but no enzyme, maximum digestion of 15 per cent. was observed at pH 3.6. In solutions containing pepsin and urea but no bisulphite a maximum digestion of 7 per cent. was observed at the same pH value.

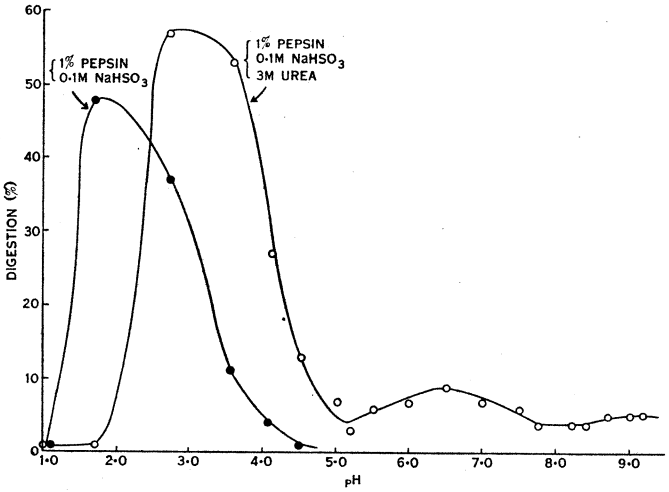


Fig. 9.—Curves relating wool digestion, after 18 hr. incubation at 50°C., with pH, after 0.5 hr. incubation at 50°C. in pepsin-bisulphite solutions with and without urea.

Incubation of wool for 1 hr. at 50°C. in 1 per cent. pepsin, 3M urea solutions, adjusted to cover a range of pH values, followed by squeezing to remove excess solution, transfer to solutions containing 0.1M sodium bisulphite and 3M urea at pH 3.0, and incubation for 18 hr. at 50°C., revealed maximum digestion of 7 per cent. corresponding to maximum uptake of pepsin at pH 3.0. This agrees fairly well with the pH of maximum digestion, but the uptake of pepsin was poor compared with the uptake of papain at the pH of maximum activity.

The effect of pH on digestion by ficin in the presence of 0.1M sodium bisulphite and 3M urea resembled its effect on papain except that the activity fell more sharply above pH 7.8 (Fig. 10), possibly owing to more rapid inactivation of the enzyme in alkaline solution.

Increasing the urea concentration up to 7.0M improved wool digestion by all the proteinases examined except ficin, which was apparently inactivated

* As in the experiment with papain, the pH values reported for the pepsin and ficin experiments refer to observations made 0.5 hr. after adding wool to the digestion mixture, that is, 0.5 hr. after the commencement of incubation.

by urea concentrations in excess of 5.0M (Fig. 11). Trypsin, beef liver cathepsin, and pig pancreas extract were the least effective, but the digestion curves for these proteinases and for pepsin rose more sharply at the high urea concentrations than at low concentrations.

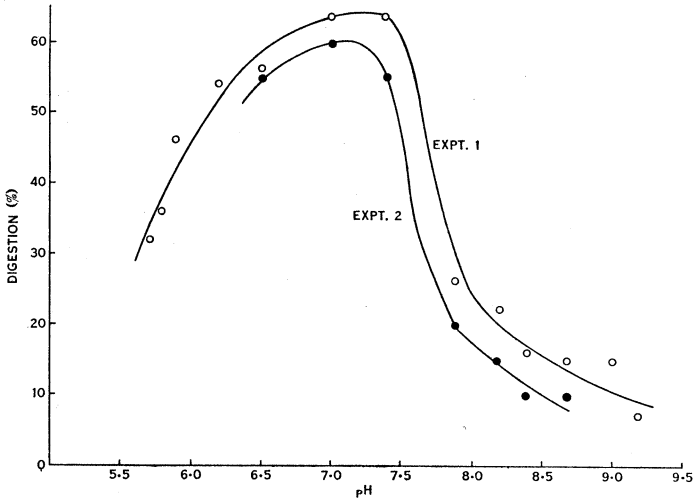


Fig. 10.—Curves relating wool digestion after incubation for 20 hr. at 50°C. in 1 per cent. ficin, 0.1M $NaHSO_3$, and 3M urea, with pH after 0.5 hr. digestion.

Although wool was completely digested in papain-bisulphite-urea, the digestion in pepsin-bisulphite-urea and ficin-bisulphite-urea did not exceed 57 per cent. and 64 per cent. respectively, even at optimal pH. Pepsin and ficin

TABLE 4

WOOL DIGESTION AFTER 18 HR. AT 50°C. IN PEPSIN AND FICIN SOLUTIONS CONTAINING HIGHER CONCENTRATIONS OF THE VARIOUS CONSTITUENTS THAN THOSE NORMALLY USED

| Enzyme Concentration (%) | $NaHSO_3$ Concentration (M) | Urea Concentration (M) | Digestion (%) | |
|---|---|---|---|---|
| | | | Pepsin at pH 2.7 | Ficin at pH 7.0 |
| 1 | 0.1 | 3 | 49 | 41 |
| 2 | 0.1 | 3 | 53 | 40 |
| 3 | 0.1 | 3 | 58 | 41 |
| 1 | 0.2 | 3 | 51 | 49 |
| 1 | 0.3 | 3 | 56 | 57 |
| 1 | 0.1 | 5 | 5 | 56 |
| 1 | 0.1 | 7 | 6 | 66 |
| 1 | 0.1 | 3 | 59* | 58* |

* The incubation period at 50°C. was increased from 18 to 41 hr. in these experiments.

were therefore re-tested at their optimal pH, but at the higher concentrations, also in the presence of higher concentrations of sodium bisulphite and urea and with a longer incubation period, in an unsuccessful attempt to digest the wool

completely (Table 4). The pepsin was inactivated at urea concentrations greater than 3M at pH 2.7 but the ficin was unaffected. The discrepancy between the results for ficin in Table 4 and those reported elsewhere in this paper for ficin at high urea concentrations is attributable to the fact that a purchased preparation of ficin, which displays greater resistance to urea inactivation, was used only in the experiments reported in Tables 4 and 5.

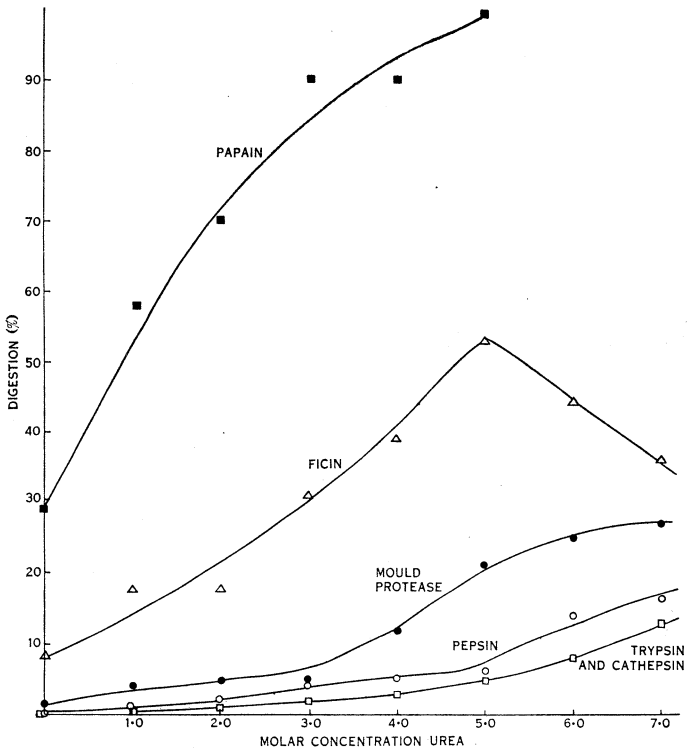


Fig. 11.—Effect of urea concentration on wool digestion during 10 hr. at 50°C. in 1 per cent. solutions of various proteinases at pH 7.0 containing 0.1M $NaHSO_3$.

The superiority of papain to the other proteinases when tested at 50°C. was also demonstrated at lower temperatures (Table 5). It cannot, therefore, be attributed to the greater thermal stability of papain.

*(f) Replacement of Sodium Bisulphite with Other Reducing Agents*

Owing to the difficulty of obtaining reducing agents in the pure state and of preventing oxidation, 1.0M solutions were prepared on the assumption that the compounds used were pure, and aliquots were taken and titrated with iodine. All solutions were then diluted to give the same titration as the sodium sulphide solution, immediately before adding 3 ml. of each to the papain-urea solution, adjusting to pH 6.5, and diluting to 30 ml. for measurement of wool digestion (Table 6).

Although it does not reduce iodine, potassium cyanide was included in the experiment because it is known to activate papain and it also reacts with the disulphide bonds in wool. Evidence that pretreatment with sodium thioglycollate, to produce partial reduction of wool, did not assist subsequent

TABLE 5

WOOL DIGESTION AFTER 18 HR. INCUBATION AT, AND BELOW, 50°C. IN SOLUTIONS CONTAINING 1.0 PER CENT. OF VARIOUS PROTEINASES, 0.1M SODIUM BISULPHITE, AND 3M UREA

| Enzyme | pH 0.5 hr. After Immersing Wool | Digestion (%) | | |
|---|---|---|---|---|
| | | 50°C. | 38°C. | 32°C. |
| Pepsin | 2.7 | 63 | 43 | 30 |
| Mould protease | 7.0 | 22 | 14 | 19 |
| Trypsin | 7.0 | 11 | 9 | 11 |
| Trypsin | 8.4 | 15 | 11 | 11 |
| Papain | 7.0 | 98 | 95 | 70 |
| Ficin | 7.0 | 62 | 23 | 23 |
| Beef liver cathepsin | 7.0 | 21 | 17 | 10 |

digestion by papain-urea appreciably was obtained by immersion of wool for 2 hr. at 50°C. in 0.2M sodium thioglycollate at pH 4.6, and at pH 6.5, then washing it in running tap water and finally transferring it to solutions containing 0.2 per cent. papain and 3M urea at pH 6.5; the wool digestions ob-

TABLE 6

WOOL DIGESTION BY SOLUTIONS CONTAINING REDUCING AGENTS AT CONCENTRATIONS OF EQUIVALENT IODINE TITRATION, IN PRESENCE OF 1 PER CENT. PAPAIN AND 3M UREA AT pH 7.0 AFTER 18 HR. AT 50°C.

| Compound | Each 1 ml. Reagent Solution Diluted to Following Volume to Equal Iodine Titration of $Na_2S$ Solution (ml.) | Digestion (%) |
|---|---|---|
| Sodium bisulphite | 3.73 | 38 |
| Sodium hydrosulphite | 3.07 | 32 |
| Sodium sulphide | 1.00 | 31 |
| Cysteine hydrochloride | 1.96 | 28 |
| Sodium thioglycollate | 1.77 | 24 |
| Potassium cyanide | —* | 7 |

* Not oxidized by iodine but tested without dilution.

served after 18 hr. at 50°C. were 6 and 26 per cent. respectively, which are no greater than occurred in the presence of papain-thioglycollate-urea at the same pH. In other instances the reduced wool was transferred to papain-urea solutions containing also 0.1M potassium cyanide, which is known to activate papain but does not confer wool-digesting action on papain-urea solutions at

pH 6.5. This modification caused the wool digestion to increase to 33 per cent. Thioglycollate reduction in solutions adjusted to pH 6.5 was shown to predispose the wool to digestion more than reduction at pH 4.6, but cyanide effected little further improvement. Thus reduction of the disulphide bonds in wool by thioglycollate in acid solution was less effective than sodium bisulphite reduction, judging from digestibility in papain at pH 7.0.

Although the data presented in this paper suggest that bisulphite is more effective than other reducing agents in assisting the digestive action of papain-urea solutions on wool, some reducing agents are more effective than bisulphite in alkaline solution. Experiments describing these observations will be reported in another paper.

### (*g*) *Replacement of Urea with Related Compounds*

Replacement of the urea in the papain-bisulphite-urea solution with closely related compounds showed that they too assisted digestion. Unrelated compounds, however, such as potassium thiocyanate and potassium trichloracetate, which were selected for test on the basis of their exceptional activity in lowering the shrinkage temperature of collagen (Lennox 1949), were only slightly effective. They were tested at concentrations ranging from 0.5M to 3.5M at pH 7.0 in the presence of 2 per cent. papain and 0.1M sodium bisulphite, but even after 3 days' incubation at 50°C. digestion at the most favourable concentrations, namely 1.0M potassium thiocyanate and 0.5M potassium trichloracetate, did not exceed 60 per cent. Urea at 3.5M concentration, on the other hand, caused complete digestion in 18 hr.

Substitution of various compounds for urea in the 1 per cent. papain, 0.1M sodium bisulphite, urea solution yielded the following wool digestions during 18 hr. at 50°C. at pH 7:

| | |
|---|---|
| 4M and 10M ethanol | 19 per cent. digestion |
| 4M semicarbazide hydrochloride | 4 per cent. digestion |
| 1.7M sodium salicylate | 1 per cent. digestion. |

The following compounds produced no digestion:

4M potassium thiocyanate, 4M ammonium chloride, 4M glycine, 4M hexamethylenetetramine, 4M alloxan, 10M lithium chloride, 0.4M cetyltrimethylammonium bromide, 0.4M sodium lauryl sulphate.

Of the compounds reported in Table 5 having molecular structures closely related to that of urea, thiourea, in which the O atom of urea is replaced by a S atom, was almost equally effective at only half the concentration, but low solubility prevented its use at higher concentrations. The superiority of thiourea was not apparent in the absence of bisulphite, for the percentage digestions by 1M urea and 1M thiourea were only 9 and 8 respectively, whereas in the presence of 0.1M $NaHSO_3$ in the same experiment, they were 63 and 81 respectively. Guanidine, having the urea O replaced by NH, was next in effectiveness, and then followed ethyl urea and methyl urea in which one of the H atoms of the $NH_2$ group in urea was substituted. Still less effective were urethane, formamide, and acetamide, in which an $NH_2$ group in urea has been entirely replaced, and finally acetamidine, which bears the same

structural relationship to guanidine as acetamide does to urea. The relative activities of thiourea, urea, and guanidine hydrochloride, shown in Table 7, were confirmed in another experiment in which the thiourea and urea had been recrystallized from water and the guanidine hydrochloride had been purified by precipitation from methanol by the addition of ether, as recommended by Greenstein and Jenrette (1942).

TABLE 7

WOOL DIGESTION AFTER 18 HR. AT 50°C. IN 1 PER CENT. PAPAIN, 0.1M SODIUM BISULPHITE SOLUTIONS CONTAINING COMPOUNDS STRUCTURALLY RELATED TO UREA, AT pH 6.5

| Compound | Digestion (%) | |
|---|---|---|
| | Expt. 1 | Expt. 2 |
| 2M Thiourea | 92 | 90 |
| 2M Urea | 69 | 66 |
| 4M Urea | 93 | 96 |
| 4M Guanidine | 73 | 74 |
| 4M Ethyl urea | 68 | 73 |
| 4M Methyl urea | 67 | 63 |
| 3M Urethane | 54 | 50 |
| 4M Formamide | 44 | 48 |
| 4M Acetamide | 41 | 44 |
| 4M Acetamidine | 19 | 12 |

### (*h*) *Action of Papain-Bisulphite-Urea on Other Fibrous Proteins*

The extent of digestion of keratinous materials other than wool and of other fibrous proteins is shown in Table 8. When tested in a coarsely ground state, the digestion of horn was much less than that reported in the table. The digestion of silk fibroin was optimal at pH 6.5, but the digestion of fibrin increased to 48 per cent. at pH 7.5. In the absence of urea only 5 per cent. of the fibrin was digested.

## IV. DISCUSSION

The favourable effect of urea on the digestion of wool keratin by papain-bisulphite is probably related to its well-known "denaturing" and solvent effects on globular proteins. Anson and Mirsky (1929) reported such action on haemoglobin, ovalbumin, and serum albumin, and Hopkins (1934) studied the action of urea on crystalline ovalbumin and observed that the effect was shared, though to a lesser extent, by many other compounds related to urea. He concluded that substitution of more than one of the two $NH_2$ groups in the urea molecule greatly reduced the activity. Burk and Greenberg (1930) observed that urea causes pronounced disaggregation of haemoglobin and edestin into units of lower molecular weight.

It seems likely that urea acts by competing with various groups of the protein for their place in hydrogen bonds, thereby reducing the cohesion of the structure. A reduction in the work of extension of wool and hair in the

presence of 5M urea was reported by Pasynskii and Blokhina (1950). Similarly, weakening of keratin by sodium bisulphite was shown by Carter, Middlebrook, and Phillips (1946) to occur under conditions which lead to the conversion of about half of the disulphide bonds contributed by the cystine residues to thiol and S-cysteine sulphonate groups. The bisulphite and urea, in conjunction with hydrolysis of peptide bonds by the papain, presumably disrupt the keratin sufficiently for the fragments to pass into solution.

TABLE 8

DIGESTION IN SOLUTION CONTAINING 1 PER CENT. PAPAIN, 0.1M SODIUM BISULPHITE, AND 3M UREA AT pH 7.0 OF KERATINOUS AND OTHER FIBROUS PROTEIN MATERIALS AFTER 18 HR. AT 50°C.

| Protein Material | Digestion (%) |
|---|---|
| Wool | 90 |
| Cattle horn filings | 96 |
| Duck feathers, finely ground | 87 |
| Hide powder | 100 |
| Sheepskin, shorn | 100 |
| Fibrin | 25 |
| Silk | 22 |
| Silk fibroin | 26 |
| Nylon | Nil |

The possibility that urea favoured papain digestion by increasing the dielectric constant of the medium is untenable since glycine, which greatly increases the dielectric constant, did not affect digestion. Urea may so affect the outer layers of wool as to facilitate their digestion and thereby allow ingress of enzyme and egress of soluble proteins, but apparently it also facilitates direct attack on the cortical cells, for when liberated after 2 days' incubation of wool in papain-bisulphite solution, they are digested less rapidly in the absence of urea than are the intact fibres.

Although bisulphite and urea both facilitate digestion of wool by papain, the observed differences in the rate of digestion of wool by various proteinases are partly attributable to differences in the degree of inactivation of the enzyme by urea or bisulphite. In general, those enzymes which are activated by reducing agents are stable to bisulphite; so also is pepsin. Trypsin, on the other hand, is inactivated by reducing agents (Grob 1946), and W. G. Crewther (unpublished data) has demonstrated partial inactivation of mould protease by sodium sulphite at concentrations corresponding to the bisulphite concentrations used in the present investigation. Many enzymes are known to resist high concentrations of urea. Steinhardt (1938), for example, showed that pepsin digests proteins in the presence of 4M urea, and in the Anson (1938) method of estimating trypsin and papain activity, urea is added to the solution to denature the haemoglobin substrate. Lineweaver and Schwimmer (1941) showed that papain retains its capacity for hydrolysing casein even

after incubation for 24 hr. at 30°C. in 9M urea. Of all the proteases examined, papain, which is activated by reducing agents and is also remarkably stable to denaturing influences such as heat and urea, produced the greatest digestion. The failure of ficin and pepsin to produce complete digestion of wool, even in the presence of three times the normal concentration of enzyme or bisulphite, more than twice the normal concentration of urea, or at temperatures below 50°C., or during prolonged incubation, showed that papain possessed some property, apart from pronounced resistance to high temperatures, bisulphite, and urea, that enabled it to digest rapidly all the components of wool. By virtue of their size or shape, papain molecules may be able to penetrate more readily than molecules of the other proteases to particular peptide bonds that are essential to the molecular structure of wool fibres. Alternatively, the more resistant components of wool may satisfy the particular substrate requirements of papain, but not of the other proteinases examined.

The action of urea on wool, in contrast with the effect of certain anions previously shown to be highly effective in lowering the thermal stability of collagen (Lennox 1949), suggests that the stability of the molecular network of keratin is less dependent on salt linkages than on hydrogen bonds and disulphide bonds, whereas collagen is stabilized mainly by salt linkages.

Elsworth and Phillips (1938) showed that the breakdown of disulphide bonds of wool in bisulphite solutions proceeded optimally at pH 5. The contribution of this reaction to the digestion of wool keratin is apparently less important than the uptake of papain which, like wool digestion, was most pronounced at pH 7. This optimal value is close to that reported by Middlebrook and Phillips (1941).

Titration curves for wool indicate that the isoelectric point of wool keratin is approximately pH 6.2 (Steinhardt and Harris 1940; Lemin and Vickerstaff 1946), whereas that for papain is approximately pH 9.0 (Balls and Lineweaver 1939). It is perhaps significant that the pH of optimal uptake of papain on wool and wool digestion lies between the isoelectric points of the enzyme and its substrate, for under these conditions they would be oppositely charged. Also, since the fibrous nature of the wool keratin would prevent folding and internal neutralization of charged groups of the type occurring in globular proteins near the isoelectric point, isoelectric keratin would be highly receptive for other ionized compounds such as papain. Northrop, Kunitz, and Herriott (1948) have similarly suggested that the pH optima for the digestion of proteins by pepsin and trypsin partly depend on combination of these enzymes with positively charged and negatively charged protein substrate ions respectively.

## V. Acknowledgments

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## VI. References


Anson, M. L., and Mirsky, A. E. (1929).—*J. Gen. Physiol.* **13**: 121.

Anson, M. L. (1936).—*J. Gen. Physiol.* **20**: 565.

Anson, M. L. (1938).—*J. Gen. Physiol.* **22**: 79.

Balls, A. K., and Lineweaver, H. (1939).—*J. Biol. Chem.* **130**: 669.

Blackburn, S. (1950).—*Biochem. J.* **47**: 443.

Burk, N. F., and Greenberg, D. M. (1930).—*J. Biol. Chem.* **87**: 197.

Carter, E. G. H., Middlebrook, W. R., and Phillips, H. (1946).—*J. Soc. Dy. Col., Bradford* **62**: 203.

Crewther, W. G., and Lennox, F. G. (1950).—*Nature* **165**: 680.

Elsworth, F. F., and Phillips, H. (1938).—*Biochem. J.* **32**: 837.

Greenstein, J. P., and Jenrette, W. V. (1942).—*J. Biol. Chem.* **142**: 175.

Grob, D. (1946).—*J. Gen. Physiol.* **29**: 219.

Hopkins, F. G. (1934).—*Nature* **126**: 328, 383.

Jones, C. B., and Mechan, D. K. (1943).—*Arch. Biochem.* **3**: 193.

Lemin, D. R., and Vickerstaff, T. (1946).—"Fibrous Proteins," p. 129. (The Society of Dyers and Colourists: Bradford.)

Lennox, F. G. (1949).—*Biochim. Biophys. Acta.* **3**: 170.

Lineweaver, J., and Schwimmer, S. (1941).—*Enzymologia* **10**: 81.

Mercer, E. H., Lindberg, J., and Philip, B. (1949).—*Text. Res. J.* **19**: 678.

Middlebrook, W. R., and Phillips, H. (1941).—*J. Soc. Dy. Col., Bradford* **57**: 137.

Northrop, J. H., Kunitz, M., and Herriot, R. M. (1948).—"Crystalline Enzymes." 2nd Ed. (Columbia Univ. Press: New York.)

Pasynskii, A. G., and Blokhina, V. (1950).—*Doklady Akad. Nauk.* S.S.S.R. **73**: 535. (*Chem. Abstr.* **45**: 684.)

Steinhardt, J. (1938).—*J. Biol. Chem.* **123**: 543.

Steinhardt, J., and Harris, M. (1940).—*Bur. Stand. J. Res., Wash.* 24: 335.