A STUDY ON KERATIN

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Keratins are the proteins of epidermal and skeletal tissues which are insoluble in the usual protein solvents, not digested by trypsin or pepsin, and high in cystine content. Such a definition intentionally excludes fibroin, the major protein of silk. It will be shown in this paper that keratins can be converted into proteins soluble in alkali or acid, with a definite optimum pH of flocculation (which may be interpreted as an isoelectric point), and digestible by trypsin or pepsin. This is accomplished by breaking the disulfide bonds of the protein. Papers on the oxidation of keratins have been published by Lissizin (1), Stary (2), and Waldschmidt-Leitz (3), who used bromine, permanganate, and H₂O₂ as oxidants. Stary and Waldschmidt-Leitz have shown that the oxidized keratin is digested by trypsin. The oxidizing agents are not specific for the disulfide groups, but attack the protein molecule at other points, and they act very slowly. In contrast, the reductants will be shown to act very quickly and without bringing about any other appreciable chemical alteration than that concerned with the sulfur. These agents dissolve keratin only at alkaline reaction (pH 10 to 13), but the action is not due to alkali alone. Products prepared from the solutions behave as true proteins, and not as products of hydrolysis. Their solutions are precipitated by ordinary protein precipitants such as sulfosalicylic acid and lose this property when digested by trypsin or pepsin.

Reductants available for reduction of disulfide groups are thioglycolic acid, potassium cyanide, sodium sulfide, and sodium sulfite. The chemical process exhibited by these reagents on simple

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disulfide compounds such as cystine can be formulated as follows:

(1) \( R-S-S-R + 2HS-CH_2COOH \rightarrow 2R-SH + [S-CH_2COOH]_2 \) (see (4))
(2) \( R-S-S-R + HCN \rightarrow R-SH + R-S-CN \) (see (5))
(3) \( R-S-S-R + H_S + R-SH + H\_t \)
(4) \( R-S-S-R + H_2SO_3 \rightarrow R-SH + R-S-S-OH \) (see (6, 7))

Reactions 1 to 3 occur only in slightly alkaline solution; Reaction 4 is most rapid in neutral to mildly acid solutions.

When keratin, such as wool or feathers, is treated with thioglycolic acid at a pH of 10 or higher the reaction appears to be identical with that on simpler disulfides. The reaction is a simple reduction; no loss of sulfur occurs. The sulfhydryl protein can be reoxidized to the disulfide state, and this disulfide protein is still soluble in acid or alkali and digestible by trypsin or pepsin.

The action of cyanide on wool is not quite so simple. A higher alkalinity is required (pH 12 to 13) and the proteins lose sulfur; however, the substances obtained behave as true proteins, not as products of hydrolysis.

The dissolving action of sodium sulfide has been known for a long time and is used industrially. Küster and coworkers (8) and Merrill (9) have considered the dissolving of keratin as a hydrolysis. Pulewka (10) and Speakman (11) realized that the action is on the disulfide groups. However, substances obtained behave as true proteins. In contrast to the cyanide preparations they contain more sulfur than the native keratin, though the cystine determinations by the Folin and Marenzi (12) method give the same values as the wool. It is likely that the action of Na_2S on keratins is not identical with its action on simpler disulfides and seems to be analogous to the action of sulfite on cystine, and a polysulfide is formed which behaves as cysteine in the Folin and Marenzi procedure. When such a protein is redissolved in weak alkali and reprecipitated with acid, it undergoes a loss in sulfur,

* Dr. A. E. Mirsky called our attention to the use of thioglycolic acid as a protein reductant. Unpublished work from this laboratory has shown that cystine is reduced at mildly alkaline reaction by an excess of thioglycolic acid. The cysteine has been almost quantitatively recovered by converting it to benzylcysteine.

† Unpublished work from this laboratory has shown that cystine is reduced according to Reaction 3. The cysteine and disodium-disulfide have been recovered as benzyl derivatives.
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but always in such a way that the total sulfur exceeds the cystine sulfur calculated from the cystine determination on the same preparation.

Sodium sulfite does not dissolve wool at an appreciable rate at any pH; so Reaction 4 has no practical significance for keratin.

EXPERIMENTAL

Most of the experiments were performed with native sheep wool, freed from fat by ether extraction. Other experiments with chicken feathers gave similar results. The wool was analyzed for cystine by the Folin and Marenzi (12) procedure, for nitrogen by the Kjeldahl method, and for sulfur by bomb combustion (13). The results, corrected for moisture, but not for ash, are as follows: N 16.66, S 3.19, cystine 11.90, 11.86 (calculated according to sulfur content, 11.9).

Protein Obtained with Thioglycolic Acid

50 gm. of wool were dissolved in 2 liters of a 0.5 m thioglycolate solution. This solution was prepared by neutralizing 92 gm. of thioglycolic acid with twice the amount of concentrated NaOH necessary to neutralize it to the turning point of phenol red, and made up to 2 liters. (The pH of the solution is about 12.) After 3 hours at 30° practically all the wool had dissolved. The mixture was filtered and the filtrate precipitated with 2 moles of glacial acetic acid, the precipitate collected on a filter, immediately twice ground with acetone made acid with HCl, washed with ether, and dried in vacuo.

The yield was 30 gm. of dry white powder which was still contaminated with a small amount of thioglycolic acid. The protein was freed of the last traces of thioglycolic acid by suspending in water and dialyzing for 3 days against distilled water in a cellophane tube. The fact that the protein was free from thioglycolic acid was shown by the cobalt test described in the appendix. The protein obtained is insoluble in water or neutral salts, but is soluble in strongly acid or alkaline solutions. The dialyzed proteins gave directly a strong nitroprusside test. They are slowly oxidized by air in bicarbonate solutions. Then the nitroprusside test of the protein is positive only after addition of KCN. The
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oxidized protein can be reduced over again by thioglycolic acid in bicarbonate buffers.

Four preparations have been made from wool by treatment for 1, 3, 6, and 24 hours with thioglycolate solutions. No increased yield is obtained after 3 hours. On varying the pH of the thioglycolate the wool does not dissolve at pH 9.0; it dissolves slowly at 10.1, and rapidly at a pH of 11 or over. Control experiments with m sodium carbonate or ammonium hydroxide showed that no dissolving action occurred in the absence of thioglycolate.

Analyses of two of the preparations are given below. The cystine determinations were made after first oxidizing the hydrolyzed protein with 3 per cent H₂O₂ (as Dr. E. A. Mirsky suggested to us) and then following the Folin-Marenzi method. The nitrogen determination was by micro-Dumas. The figures are in percentage of the dry weight and are not corrected for ash.

<table>
<thead>
<tr>
<th></th>
<th>Treated with thioglycolate for 24 hrs</th>
<th>Treated with thioglycolate for 3 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfur</td>
<td>3.20</td>
<td>3.13</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>15.71</td>
<td>15.75</td>
</tr>
<tr>
<td>Cystine</td>
<td>11.8</td>
<td>11.9</td>
</tr>
<tr>
<td>&quot; calculated from sulfur</td>
<td>12.0</td>
<td>11.74</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>4.6</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Proteins Prepared by Potassium Cyanide

Wool does not appreciably dissolve in 1 M KCN solutions, even in days, and in such a solution it turns dark brown to black. In 0.1 N NaOH and m or 0.5 M KCN most of the wool dissolves in a few hours, and such solutions do not become black even after days. If the wool is first treated with NaOH, washed, and exposed to KCN, it does not dissolve. The preparation of the protein from the solution was analogous to that described above.

A protein was prepared from wool by treating 50 gm. of wool with 2 liters of m KCN in 0.1 N NaOH. The yield was 17.6 gm. of dry protein. The protein is similar to that obtained by thioglycolic acid. It is slightly more soluble, lower in sulfur and cystine content. It gives the nitroprusside test and does not lose this property even when its alkaline solution is exposed to the air for days. Preparations made by treatment with KCN for 1, 6, 24,
and 48 hours had sulfur contents of 1.3, 1.7, 1.8, and 1.4 respectively.

Analyses of two preparations are given below.

<table>
<thead>
<tr>
<th></th>
<th>Treated with KCN for 24 hrs.</th>
<th>Treated with KCN for 6 hrs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfur</td>
<td>1.8</td>
<td>1.73</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>15.99</td>
<td>14.79</td>
</tr>
<tr>
<td>Cystine</td>
<td>1.86, 1.87</td>
<td>4.7</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>4.7</td>
<td>4.7</td>
</tr>
</tbody>
</table>

*Preparation with Sodium Sulfide*

50 gm. of wool were dissolved in 2 liters of 0.5 M Na₂S. The protein was isolated as above. The S content was very high, due in part to free sulfur extractable by CS₂. However, the final preparations gave high constant values of S, and this could not be decreased by further grinding with CS₂. When the entire preparation of the protein is carried out under nitrogen, no free sulfur is formed, but the sulfur content of the protein is still high. The protein so isolated gave a negative nitroprusside test (even when prepared under nitrogen) unless first treated with KCN. The isoelectric zone is wide, but one preparation, as colloidal solution made by dialyzing the Na₂S-wool solution without previous precipitation, gave a sharp isoelectric point at pH 4.9.

The analyses of two preparations are given below.

<table>
<thead>
<tr>
<th></th>
<th>Sample A</th>
<th>Sample B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated with Na₂S for 48 hrs.</td>
<td>Treated with Na₂S for 6 hrs.</td>
</tr>
<tr>
<td>Sulfur</td>
<td>6.61, 6.55</td>
<td>5.36</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>12.99</td>
<td>13.62</td>
</tr>
<tr>
<td>Cystine</td>
<td>11.03</td>
<td>11.4</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>4.1-4.5</td>
<td>4.2-4.6</td>
</tr>
</tbody>
</table>

Sample A was redissolved in concentrated NH₄OH, filtered, and reprecipitated with acetic acid. On analysis the results obtained were: S, 3.93; cystine, 6.94 (cystine S, 1.85). Both the original and the reprecipitated protein, though differing in total S, con-
tain approximately twice the amount of sulfur as corresponds to the cystine sulfur.

_Solubility and Flocculation_

All the proteins described above are insoluble in water and resemble casein or denatured proteins. The dry proteins can best be dissolved in weak alkalis by grinding in a mortar with Na₂CO₃ or NaHCO₃. In dilute NaOH they dissolve readily, but lose sulfur. When the filtrate of wool dissolved in Na₂S is dialyzed directly for several days, the protein remains in solution, but it is precipitated by a trace of acetic acid. Among the dry proteins, the cyanide preparation dissolves most readily.

The isoelectric points were estimated by the method of Michaelis and Rona (14). The dry proteins were ground with NaCO₃ and water, and filtered, so that the solution was 0.1 m in Na₂CO₃ and 0.5 per cent in protein. To a series of test-tubes 1 cc. of protein solution and increasing amounts of acetic acid were added, so calculated that all the tubes had a total volume of 10 cc. and an arithmetical series of pH differing by 0.3 pH. The optimum of flocculation was taken as the isoelectric point, and its pH and that of the adjoining tubes was determined with the glass electrode. The results obtained are tabulated among the analyses above. The isoelectric points for these protein preparations are slightly lower than the isoelectric point of native wool. Speakman (15) and Elöd and Silva (16) by independent methods obtained the values of 4.8 and 4.9.

_Digestion by Trypsin and Pepsin_

Trypsin, purified by the method of Anson and Mirsky (17), was added to suspensions of the proteins in bicarbonate buffers at pH of 8.5 to 8.8, and maintained at 37°. Samples were withdrawn and precipitated with sulfoalicylic acid. The controls showed complete precipitation, and practically all of the nitrogen was in the precipitate. The digestion series showed progressively less precipitation, and after 2 hours 90 to 95 per cent of the nitrogen was in the filtrate. All of the proteins described above are digested by trypsin, and the thioglycolic acid preparation is digested when it is either in the sulphydryl or the disulfide state.

The dry proteins were suspended in dilute HCl, pepsin was
added, and the mixture had a pH of 1.30. The suspensions, maintained at 37°C, gradually became clear due to digestion by the pepsin. These solutions remained clear upon the addition of sulfosalicylic acid. Nitrogen determinations on the filtrates of control showed practically complete precipitation, and on the digestion series about 80 per cent digestion. All the proteins described above were similarly digested, and the state of the proteins as sulfhydryl or disulfide did not affect the result.

DISCUSSION

The keratins and fibroin are similar in their insolubility in the usual protein solvents, and in their indigestibility by pepsin and trypsin, but that they are essentially different in structure is shown by the agents which bring about their solution. Fibroin, though not soluble in water, dilute acids, or alkali, is easily dissolved in concentrated solutions of certain neutral salts such as calcium sulfocyanate or lithium iodide (18) and also in cold concentrated hydrochloric acid. These solutions can be easily reprecipitated, and the precipitate shows a fibrous structure. Wool cannot be dispersed in any of those solvents that act on silk, but it can be readily dissolved in a series of agents that split the disulfide bond, as alkaline solutions of sodium cyanide, sulfide, and thioglycolate. These solutions do not dissolve silk in a perceptible degree. The protein, reprecipitated from a solution of wool in one of these agents, is never fibrous but amorphous.

These properties are in good agreement with the known differences in chemical composition and structure of these two classes of proteins. The x-ray diffraction studies of Brill (19) and Meyer and Mark (20) show that fibroin consists of fully extended polypeptide chains, and these chains must be oriented parallel to each other chiefly by residual forces for fibroin contains no cystine and but very little diamino and dicarboxylic acids. The regularity of the fully extended chain of amino acids in silk seems to be sufficient

\[1\] Von Weimarn claims that wool can be dispersed in a concentrated boiling solution of calcium or lithium sulfocyanate at 180°C. It is true that the wool dissolves very slowly, but the solution becomes alkaline and the presence of sulfides can be demonstrated. So the action can be best interpreted as a dissolution by alkaline sulfides formed from the slow decomposition of sulfocyanate.
to bring about a tight fiber structure, without lateral chemical links, but this is not the case in the keratins. The x-ray studies of Astbury (21) on mammalian hairs have shown that these proteins are crystalline with partially contracted polypeptide chains. Speakman (22) has pointed out the importance of the lateral links between parallel polypeptide chains, the bond of the disulfide group of cystine, and the polar link of diamino and dicarboxylic acids. The stability of the keratin molecule depends on these bonds. Once these two types of bridges have been broken by chemical attack, the keratins behave in their solubility and digestibility by proteolytic enzymes as denatured proteins.

The sulfhydryl protein, produced by the action of thioglycolic acid on wool, can be reoxidized to the disulfide state by air, in moderately alkaline solution, or by hydrogen peroxide. The reoxidized protein, in spite of its disulfide bonds, is still soluble in alkali, is enzyme-digestible, and has about the same isoelectric point. In the reoxidation of the sulfhydryl protein, the original crystalline pattern is not reformed, the disulfide bonds are no longer rungs of a ladder connecting parallel polypeptide chains. This shows that though the disulfide bond is essential for the properties of native keratin, these properties cannot be ascribed to the purely chemical character of this group. It is the physical pattern of the keratin, with a definite spatial arrangement of peptide chains and disulfide bonds, which imparts to keratin its resistance to enzyme hydrolysis and dissolving agents.

Mention should be made of the fact that all of the agents used to dissolve wool require a higher alkalinity than to reduce a simple disulfide such as cystine. The pH used is never high enough to dissolve wool of its own accord in a comparable time, yet the necessity of a distinctly alkaline reaction is striking and demands an explanation.

The following hypothesis may be offered. The cross links between peptide chains in keratin are of two kinds, disulfide links and bridges formed by the electrostatic attraction of the NH$_3^+$ group of the diamino acids for the COO$^-$ group of the dicarboxylic acids. These salt-like bridges will be broken in alkaline solution by removal of a proton from the amino group. It appears that it is necessary to open these links before the disulfide groups may be reduced.
SUMMARY

Keratin dissolves in Na₂S, KCN, or thioglycolic acid at alkaline reaction. This effect is chiefly due to the splitting of the disulfide groups, which are essential for the maintenance of the fibrous structure of keratin. Chemically, the action of thioglycolic acid is the simplest; it simply reduces the disulfide to sulfhydryl groups with no other appreciable chemical change. The other reagents act in a more complicated way. The substances thus obtained are proteins. They are soluble in alkali or acid, with a definite isoelectric point, and they are digestible by pepsin and trypsin, even when secondarily the —SH group has been reoxidized to the —SS— stage, or when due to secondary reactions the sulfur content has been greatly changed.

Appendix

Test for Thioglycolic Acid—In order to test whether the protein prepared with thioglycolic acid is completely freed from this acid the following test was applied. A suspension of the protein in sodium pyrophosphate solution is mixed with a drop of a 1 per cent solution of cobalt sulfate. Any trace of free thioglycolic acid will develop, either immediately or after some time, on exposing the mixture to the air, a brown color. The sulfhydryl protein does not give this test, although it gives a positive nitroprusside test which is common for all sulfhydryl compounds. This test is based on the formation of strongly colored cobalti complexes of thioglycolic acid according to Michaelis and Schubert (23). The smallest amount of thioglycolic acid giving a distinct positive test in a volume of 1 cc. is approximately 0.05 mg. The oxidized form of thioglycolic acid, dithiodiglycolic acid, can be tested for with the same reagent, adding besides some Na₂SO₃ which reduces the disulfide to the sulfhydryl compound.

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