reflux tube for half an hour at 100°; the solution turned brown and much gas was evolved. After it had stood for several hours, the solution became thick with crystals imbedded in a brown sirup. Ether was added to dissolve the sirup and the crystalline material was filtered off. The yield was about 4 g. It was purified by dissolving in alcohol and precipitating with ether. The shining flakes that formed proved to be only aniline hydrochloride, as the melting point (190°), a complete analysis and the deep violet coloration of the free base with bleaching powder showed.

Anal. Calcd.: C, 55.59; H, 6.2; N, 10.8; Cl, 27.36. Found: C, 55.69; H, 6.33; N, 10.84; Cl, 27.26.

When chloro-formanilide and methyl-urethan were heated at 50–60° for a half hour, an even larger yield of aniline hydrochloride resulted, and when they were left at room temperature for several hours, the same product slowly crystallized. At 150° the solution deposited only diphenyl-urea. This is undoubtedly due to the hydrolysis of phenyl isocyanate, into which chloro-formanilide decomposes at that temperature. Aniline hydrochloride may have resulted merely from the hydrolysis of chloro-formanilide alone, as it has been found that the latter substance, when exposed to the air for some time, slowly loses its odor, and a mass of the hydrochloride results.

Summary

1. The position of the phenyl group in ω-phenyl-biuret has been definitely proved.

2. Two new methyl-phenyl-biurets have been prepared. One is ω′-methyl-ω-phenyl-biuret. The other is probably *ms*-methyl-ω-phenyl-biuret.

3. Reasons are advanced for not assigning to Biltz' methyl-phenyl-biuret the structure $C_6H_5NHCONHCONHCH_3$ as he has recommended.

4. An unsuccessful attempt to synthesize ethyl N^{ms}-methyl-N^{ω}-phenyl-allophanate was made.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF YALE UNIVERSITY]

RESEARCHES ON PROTEINS. VII.[1] THE PREPARATION OF THE PROTEIN "SERICIN" FROM SILK[2]

BY ELBERT M. SHELTON[3] AND TREAT B. JOHNSON

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Silk occupies a very promient position among the organic resources of nature. Its commercial and economic importance is evidenced by the large amount of capital invested in manufacturing enterprises dependent upon it as a staple product of industry. From a scientific standpoint

[1] Previous publications in this series: Johnson and Kohman, THIS JOURNAL, **37**, 1863 (1915); **37**, 2164 (1915). Johnson, Hill and O'Hara, *ibid.*, **37**, 2170 (1915). Johnson, *ibid.*, **37**, 2598 (1915). Johnson and Hill, *ibid.*, **38**, 1392 (1916). Johnson and Daschavsky, *ibid.*, **41**, 1147 (1919).

[2] Constructed from part of a dissertation presented by Elbert M. Shelton to the Faculty of the Graduate School, Yale University, June, 1924, in candidacy for the degree of Doctor of Philosophy.

[3] Holder of the Cheney Organic Chemistry Research Fellowship, Yale, 1923–24.

it is a substance of biochemical interest on account of its formation by a living organism. It is recognized today by progressive silk manufacturers that a correct basis for permanent and economical results in the silk industry can be obtained only by encouraging and providing for thorough scientific research and study of the raw basic material of their industry.

Crude silk fiber as spun by the silk worm consists of a filament of the protein "fibroin" surrounded by a varnish-like coating known in the trade as silk gum. The relative proportions of these two components differ considerably in crude silk from different varieties of worms, but ordinarily the gum represents 15–30% of the raw fiber. This silk gum is described in the chemical literature as consisting for the greater part of the protein "sericin," accompanied by small amounts of coloring and mineral matter, fats, and a waxy substance of unknown structure. In the process of manufacture this gum is treated essentially as a waste product of the silk industry, and consequently very little attention has been paid to its chemistry as compared with the numerous investigations by various workers dealing with fibroin.

The recent advances as a result of scientific research in the field of protein chemistry have stimulated a renewed interest in the proteins found in silk. Hitherto it has been assumed from the available chemical and physical evidence that the fibroin of silk is to be considered as a distinct and characteristic protein substance. It is one of the few proteins which does not contain sulfur, and is characterized by its stability towards enzymes, being undigested by pepsin and trypsin. In other words, it is constructed of polypeptide or cyclic acid amide linkages which are extremely resistant to the action of proteolytic enzymes. Recent observations indicate, however, that very slight alterations in chemical structure produce a protein configuration which is digestible by enzymes.[4] Recently new physicochemical evidence has been produced which indicates that fibroin is not a chemical entity as previously considered, but is a mixture of two or more organic compounds. By application of Debye and Scherer's Röntgen-spectrographic method of crystal analysis Herzog and Jancke[5] have disclosed the interesting fact that crystalline substances are apparently held in solid solution in the fibroin filaments and their work has recently been confirmed by Brill.[6] The latter's interesting researches on X-ray analysis have led him to conclude that what we ordinarily consider to be pure fibroin is a mixture of at least two simple proteins, of which one is a crystalline substance. Brill has examined nine varieties of silk fibroin and concludes that the same crystalline structure functions in all.[7]

[4] F. Wessely, *Z. physiol. Chem.*, **135**, 117 (1924).

[5] Herzog and Jancke, *Ber.*, **53**, 2162 (1920); *C. A.*, **15**, 1311 (1921).

[6] Brill, *Ann.*, **434**, 204 (1923); *C. A.*, **18**, 381 (1924).

[7] A sample of specially purified Canton silk was sent by the authors to Professor

Support for the conclusions based on these spectrographic measurements has more recently been produced by Herzog and Kobel,[8] who have succeeded in reducing fibroin by intense grinding in a colloid mill to a condition whereby it has been possible to separate from it simpler polypeptide bodies of low molecular weight by simple extraction with water or alcohol.

In the light of these interesting observations it seemed probable that a more exact and critical study of the protein sericin would reveal here also the presence of a mixture of different substances of protein nature. It is indeed very questionable whether much dependence can be placed on the analytical results previously published and used as a basis of differentiation between fibroin and sericin. Most of the previous researches on sericin were carried out before the present methods of study of proteins had been developed. Thus far only about 40% of the sericin has actually been accounted for in the yields of amino acids isolated. In this paper we will report on our investigation of methods of preparation of sericin preliminary to extensive research on the chemical nature of this protein.

The Removal of Gum from Silk

The removal of gum from crude silk has been based entirely upon its property of solubility in hot water.

One of the earliest records of research on silk gum is that of Rigaut de Saint Quentin (L'abbé Collomb) who, as early as 1762, had investigated the removal of gum by dilute solutions of sodium carbonate instead of soap. In his book, "La Dissolution du Vernis de Soie," published in 1784, he announced that silk gum is soluble in boiling water. Another historic research was that of Roard,[9] who in 1808 investigated the action of light, water, alcohol, acids and alkalies on silk and recommended methods for removal of the gum. He obtained sericin by water extraction of the raw silk, followed by evaporation of the resulting protein solution.

The methods used by succeeding investigators have varied chiefly in the temperature of the water used. Mulder[10] boiled the silk in distilled water, renewing the water until the extract no longer gave a precipitate with gallic acid. He reported a loss of about 35% of the weight of the original silk. Cramer[11] obtained sericin by heating silk for three hours in water. Bondi[12] employed three successive one-hour extractions with boiling water. Most of the remaining investigators have operated at temperatures above 100° by heating in water under pressure. Wetzel[13] simply states that he heated the silk in water above 100°. Fischer and Skita[14] extracted silk for two successive three-hour periods with distilled water by heating in an autoclave at 118°. More

Brill for an X-ray analysis and he has reported by letter that this material exhibited the same crystalline properties that had been observed in the previous specimens examined.

[8] Herzog and Kobel, *Z. physiol. Chem.*, **134**, 296 (1924).

[9] Roard, *Ann. chim.*, **65**, 44 (1808).

[10] Mulder, *Pogg. Ann.*, **37**, 594 (1836); **39**, 498 (1837); **40**, 253 (1837).

[11] Cramer, *Jahresber.*, **1864**, 628; *J. prakt. Chem.*, **96**, 76 (1865).

[12] Bondi, *Z. physiol. Chem.*, **34**, 481 (1901).

[13] Wetzel, *ibid.*, **26**, 535 (1898).

[14] Fischer and Skita, *ibid.*, **35**, 221 (1902).

recently, Kondo[15] prepared sericin by autoclaving for three 3-hour periods under 2.5–3 atmospheres' pressure, while Türk[16] has subjected the silk to a three-hour heating in water at 145°. A contrast to these methods of operating is that of Anderlini[17] who, to avoid decomposition which might take place at higher temperatures, extracted silk for 25 days in water at 50–60°.

Since it was our purpose to compare the methods available for preparation of sericin, we have obtained it from the silk in a variety of ways. In all of the experiments to be described the raw silk has been in the form of frissons, the waste from the reeling of cocoons. The stock used was free from chrysalids and otherwise very clean and furnished a convenient and relatively inexpensive source of crude sericin. Because of the differences in the nature and composition of silks from different varieties of worms, we prepared all our protein for the present research from frissons of a European silk known as "Yellow Lombardy." Throughout our research all silk samples were treated under standard conditions in a conditioning oven before weighing.

The method of extracting frissons in water in an open kettle removed the greater part of the sericin, but the isolation of the protein from the resulting dilute solution is costly and not to be recommended as a laboratory procedure.

We next turned our attention to extraction of frissons in water at temperatures above 100°. In a series of experiments it was demonstrated that most of the sericin is removed by autoclaving for one-half hour under pressures of 600–700 cm. of mercury (14 lbs.). Increasing the time of treatment to two hours caused no greater loss in weight on the part of the frissons. Operating at as low pressure as 250 cm. of mercury (5 lbs.) produced nearly as good results except in the case where several lots of silk were to be treated successively in the same liquor. By autoclaving three or four lots of frissons in just enough distilled water to cover the silk we were able to obtain much more concentrated solutions of crude sericin than were possible by boiling in water in an open kettle. With such saturated solutions it is quite easy to apply successfully methods of protein analysis and separation and obtain large amounts of the protein at low cost. The advantage secured through autoclaving at a pressure of 250 cm. of mercury (5 lbs.) is not increased proportionately by operating at much higher pressures. A pressure of 500 cm. of mercury (10 lbs.) has proved a practical and efficient working condition.

The Isolation of Sericin from Aqueous Solutions of Silk Gum.—Two distinct methods have been employed for the recovery of sericin from its aqueous solution. The simpler consists of evaporation to dryness and was adopted by Mulder,[10] Bondi,[12] and Fischer and Skita.[14] The other

[15] Kondo, *J. Chem. Soc. Japan*, **42**, 1054 (1921); *C. A.*, **16**, 1508 (1922).

[16] Türk, *Z. physiol. Chem.*, **111**, 69 (1920).

[17] Anderlini, *Chem. Zentr.*, **1887**, II, 941; **1888**, I, 795.

method, the precipitation of the sericin from solution, has found greater favor, and has been carried out in a variety of ways. Cramer[11] and Wetzel[13] precipitated the sericin with lead acetate. Anderlini,[17] Türk,[16] and Kondo[15] took advantage of the insolubility of the sericin in alcohol. Türk precipitated the sericin at once by the addition of two volumes of absolute alcohol to the aqueous solution. Both Anderlini and Kondo added the alcohol by degrees to secure a fractional precipitation of the protein.

The recovery of sericin from solution is intimately associated with the subsequent processes for removal of impurities. Without going into detail concerning the procedure followed by each investigator, the refining processes may be summed up as having consisted of extractions in water, dil. acids or alkalies, alcohol and ether, together with precipitation of the sericin from solution by means of various reagents. Most of the previous investigators have based their methods upon the assumption that sericin is a single homogeneous compound. Anderlini isolated three fractions which he considered to be distinctly different, and which he designated as α, β and γ modifications. Kondo separated sericin into two fractions by successive precipitations with alcohol.

In our research many variations in the procedure for recovery and refining of the sericin have been tested. The method of recovery by evaporation to dryness has been least satisfactory of all. In addition to trouble from excessive foaming, there is abundant evidence of decomposition of the protein molecule on continued boiling of the aqueous solution, for an insoluble solid separates coating the walls of the kettle and forming a membrane over the surface of the solution, while the liquor gradually loses its capacity for forming a gel.

Mulder believed the material that separated during evaporation of an aqueous solution to be a coagulated albumin. Cramer considered the insoluble portion a decomposition product of the true sericin, while Bondi described sericin as existing in two modifications, soluble and insoluble, and enumerated the conditions favoring the transition of the protein from one to the other form. It will be one object of continued research on our part to determine the chemical relation of this insoluble fraction to the sericin as a whole.

Another method deserves a brief consideration here because of the evidence it also offers on the question of the homogeneity of sericin. We had observed that when a liquid immiscible with water is shaken with an aqueous solution of sericin, a very stable emulsion results. On standing, or by centrifuging the emulsion creams in a separate layer. The clear aqueous layer still contains a large amount of nitrogenous material which is precipitable in an excess of alcohol, while from the emulsion may be recovered a protein fraction showing a distinctly different physical behavior. This means of fractionation of sericin is convenient for application

to small lots of material, but without special centrifuge equipment it is not practicable for use on a larger scale.

Turning to the method of precipitation of sericin by alcohol, the following process was adopted after preliminary trials. The hot, concentrated sericin solution was poured directly from the autoclave into 7 or 8 volumes of 95% alcohol. After a few hours, the clear supernatant liquor was siphoned away from the white precipitate, carrying with it much of the wax, and coloring and mineral matter. Two or more subsequent washings of the precipitate with 95% alcohol render it sufficiently granular to permit suction filtration. The firm white cake obtained was then dried slowly over calcium chloride in a desiccator. A white, easily pulverized product resulted. Attempts to hasten drying by heating in a vacuum were unsatisfactory, yielding a product which was hard and glassy and colored deep brown.

When sufficient alcohol is used for precipitation, practically all of the protein present will be included in the sericin precipitate. On treating the latter with cold water we found that only a portion of it was soluble, and a considerable part dissolved slowly even in boiling water. In other words, this protein precipitates as a mixture apparently of the soluble and insoluble constituents of sericin already mentioned.

As an alternative method of fractionating the crude sericin, the process of "salting out" with various salts was tried. After preliminary trials the following process was applied to the recovery of sericin from its aqueous solutions. A sericin solution, obtained by autoclaving frissons for one-half hour at a pressure of 250 cm. of mercury (5 lbs.), was salted out by adding 15 g. of solid ammonium sulfate to each 100 cc. of solution. The gelatinous precipitate was found to be insoluble in cold water and could be washed until the washings gave negative tests for sulfates and proteins. In contrast to the behavior of crude sericin solutions, this gelatinous precipitate appears to be unable to support bacterial growth.

The clear filtrate obtained in the salting out of the first fraction still contained a considerable amount of water-soluble nitrogenous material, much of which was precipitated by complete saturation with ammonium sulfate. This was conveniently accomplished by evaporation of the solution under diminished pressure until it became saturated with the salt. The precipitate may be freed from ammonium sulfate by dialysis. The study of these protein fractions is now in progress and it is the purpose of subsequent research to determine for each fraction such characteristics as amino acid content and nitrogen distribution. We shall also report later on the action of pepsin on fibroin and sericin.

Summary

1. Improvements have been made in the method of removing water-soluble proteins from crude silk.

2. Further evidence has been presented showing that sericin is probably a mixture of at least two simpler proteins, one of which is soluble and the other insoluble in cold water.

3. The study of silk proteins will be continued along various lines with special emphasis on the action of proteolytic enzymes on the various protein fractions.

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THE ISO-ELECTRIC POINTS OF GLIADIN AND GLUTENIN

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The characterization of proteins is a difficult matter. The ease with which the protein complex can be changed and the lack of knowledge concerning the manner in which the different groups are linked together, render the study of this group of compounds one of the most difficult in chemistry. In the preparation and purification of these compounds the iso-electric point of each individual protein is of prime importance.

In some protein investigations undertaken in this Laboratory considerable difficulty was experienced in obtaining preparations of gliadin from wheat (*Triticum vulgare*) which would agree with one another in their chemical characteristics. The same was true of glutenin also.

Since the chemical properties of both of these compounds have not been fully investigated, it was thought advisable to undertake a more thorough examination of them. The iso-electric point was one of the constants investigated.

The iso-electric point of ampholytes is in general determined in two ways: (1) by calculation from the ionization constants of the ampholyte as (a) an acid and (b) a base, and (2) by electrocataphoresis in a series of solutions whose hydrogen-ion concentrations differ from one another.

In some preliminary work on gliadin it was found that the electrocataphoresis method was unsatisfactory for two reasons: (1) the iso-electric point was so near the neutral point (P_H 7.00) that solutions of acids of the requisite concentrations had not sufficient conductivity, and (2) gliadin was so slightly soluble in buffer solutions that the ordinary protein tests failed, and consequently the direction of migration of the solute could not be determined in this way.

Investigations with the hydrogen electrode showed, however, that gliadin was sufficiently soluble in buffer solutions to cause an appreciable change in the hydrogen-ion concentration of such solutions, provided that the molar concentration of the salt was sufficiently small, on account of the precipitating action of the salt on the dissolved gliadin, and at the same