THE BASIC AMINO ACIDS OF SILK FIBROIN. THE DETERMINATION OF THE BASIC AMINO ACIDS YIELDED BY PROTEINS*

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Previous investigations of the basic amino acids yielded by human hair and by wool (1, 2) showed that the keratins of these closely allied tissues yield relatively high proportions of arginine but very low proportions of histidine; both tissues also yield high proportions of cystine. Because of this the determination of the basic amino acids presented special analytical difficulties that necessitated slight modifications in the technique employed. Available information regarding the composition of silk fibroin suggested that the unusually high proportion of monoamino acids, that results from the hydrolysis of this material, might also give rise to analytical difficulties, a study of which would be useful in the development of the methods of protein analysis. This was indeed found to be the case and the further interesting observation was made that the ratios of the yields of the three basic amino acids from silk fibroin are very closely similar to those from wool, although the total yield of bases from silk fibroin is only about one-tenth of that from wool.

Very little attention has been paid in the past to the basic amino acids derived from silk. Wetzel, in 1899 (3), obtained a trace of histidine, and Fischer and Skita, in 1902 (4), isolated somewhat under 1 per cent of arginine. Abderhalden, in 1922 (5),

* The data in this paper are taken from the dissertation submitted by R. J. Block in partial fulfillment of the requirement for the degree of Doctor of Philosophy, Yale University, 1931. A part of the expense of this investigation was borne by the Carnegie Institution of Washington, D. C.
found 1.5 per cent of arginine, 0.75 per cent of histidine, and 0.85 per cent of lysine by the Kossel and Kutscher (6) method; all three bases were secured as crystalline derivatives, but the proportions reported were probably calculated from the nitrogen content of their respective fractions, rather than from the weights of the derivatives, and the results may therefore be somewhat too high. Fürth and Deutschberger (7) obtained 1.52 per cent of arginine as the flavianate, but neglected to establish the purity of their product by analysis. It is clear, therefore, that the composition of silk fibroin, with respect to the basic amino acids, is still uncertain.

First Analysis—The silk fibroin employed contained 18.99 per cent of nitrogen, corrected for moisture and ash, and was free from sulfur. The sericin had been removed commercially; 1 439 gm. (corrected) were boiled with 3800 cc. of 8 N sulfuric acid for 16 hours; the hydrolysate was then diluted, neutralized to pH 4 with barium hydroxide, and the barium sulfate was removed and extensively washed. The hydrolysate was concentrated to 5 liters and silver oxide and dilute sulfuric acid were added alternately, according to Kiesel's (8) directions, until silver sulfate crystallized in considerable amounts from the warm solution. In spite of this the customary test for the presence of excess silver ion could not be obtained. Barium hydroxide was nevertheless added until a reaction of approximately pH 12 had been produced and the precipitate was removed. The filtrate was acidified and concentrated, and a second unsuccessful attempt to introduce an excess of silver ion was made. The precipitate, produced by making the solution alkaline with barium hydroxide, was added to the first precipitate and the material was worked up for histidine and arginine as described by Vickery and Block (2). The equivalents of 0.076 per cent of histidine and 0.22 per cent of arginine were isolated as flavianates.

The combined filtrates from the two silver precipitates were freed from barium and silver and treated with phosphotungstic acid in the usual way. In view of the low proportion of arginine found in the silver precipitate the phosphotungstate precipitate was investigated for arginine. No difficulty was experienced in

1 We are indebted to Dr. E. M. Shelton of Manchester, Connecticut, for this highly purified material.
introducing excess of silver ion into the acidified solution of the bases by the silver oxide technique, and the precipitate obtained at alkaline reaction yielded the equivalent of 0.52 per cent more arginine as the flavianate. No histidine could be detected. The filtrate from this silver precipitation was freed from reagents and treated again with phosphotungstic acid. Lysine was isolated from it, as picrate of decomposition point 265°, in an amount equivalent to 0.25 per cent of the fibroin.

It is evident therefore that, in order to precipitate arginine completely, it is essential to introduce a liberal excess of silver ion into the solution of the amino acids before this is made alkaline. Histidine, however, is completely precipitated even when silver ion is not present in excess as shown by the customary test.

Repeated experiences similar to that described have convinced us that silver sulfate, in spite of its manifest convenience, is not a suitable reagent to employ for the preliminary precipitation of arginine and histidine from protein hydrolysates.

A number of factors appear to affect the concentration of the silver ion that can be produced; among these are the acidity, the nature, and the concentration of the amino acids in the solution. There seems little doubt that silver enters into complex compounds with the amino acids but a satisfactory explanation of the phenomena observed has not yet been obtained.

Second Analysis—In view of the foregoing observations a hydrolysate of 440 gm. (corrected) of silk fibroin was prepared as before and was treated, at a volume of 12 liters, with a saturated solution of silver nitrate; 500 gm. of the salt were added before a strongly positive test for the presence of excess silver ion was secured. The solution was then made alkaline as usual; the precipitate produced yielded the equivalent of 0.7 per cent of arginine, as the monoflavianate of sulfur content 6.8 per cent (theory 6.56 per cent), and 0.06 per cent of histidine, as the diflavianate of sulfur content 8.13 per cent (theory 8.17 per cent). The filtrate from the silver precipitate was not investigated for lysine.

The results of these analyses are given in Table I together with previous analyses of silk collected from the literature; the proportions of bases isolated from wool are appended in order to draw attention to the close similarity in the ratios of the three bases to each other in these two proteins. The discrepancy between the
present analyses and those of others is probably largely due to a difference in method. The isolation method we have used leads to minimal values, while the calculation of results from nitrogen determinations leads to maximal values. It is probable that the true value is intermediate. It is also not improbable that silk of different origins may be differently constituted; this point should be further investigated.

*Estimation of the Basic Amino Acids Yielded by Proteins*

In the course of the studies by Vickery and Leavenworth of the silver precipitation method for the determination of the basic amino acids, a number of modifications and changes have been introduced from time to time that are described in detail in different papers (9–11, 1). It therefore seems desirable to give a brief description of what now appears to be the most generally applicable procedure. Unless otherwise noted it is to be understood that all precipitates are washed and all concentrations are carried out in vacuo. The volumes at which it is suggested that the different precipitations should be conducted are suitable in the case of proteins, such as edestin, that yield somewhat high proportions of bases; smaller volumes should be used where conditions warrant.

**Hydrolysis**—At least 50 gm. of protein are hydrolyzed by boiling for 24 or more hours with 8 times its weight of 8 N sulfuric acid. The hydrolysate is diluted and the greater part of the acid is removed as barium sulfate. As an alternative procedure the protein is boiled with 8 times its weight of 6 N hydrochloric acid,
the solution is repeatedly evaporated to a sirup, this is diluted, and the hydrochloric acid is removed as silver chloride by means of silver oxide in the presence of sufficient sulfuric acid to maintain the reaction somewhat more acid than pH 3. The silver chloride should be suspended in hot water, acidified with hydrochloric acid, and digested on the steam bath in order to recover any histidine that may have been precipitated. The extract is evaporated to dryness, the residue is taken up in water, and the hydrochloric acid is removed as before, the filtrate from the silver chloride being added to the main solution.

Silver Precipitation—The hydrolysate and washings of the inorganic precipitate are concentrated to a volume of 1 liter for each 50 gm. of protein. Silver nitrate is added, in concentrated solution, until the test for an excess of silver ion is strongly positive. Warm saturated barium hydroxide is then added until the solution is alkaline to alizarin yellow R (pH 11 to 12), the precipitate is centrifuged off and, without washing, is suspended in water and decomposed by hydrogen sulfide in the presence of a slight excess of sulfuric acid (pH 3 to 4). After removal of the silver sulfide and concentration to the same volume as before, silver oxide and sulfuric acid are added alternately until an excess of silver ion is present. The reaction is then brought to pH 7.2 by the cautious addition of cold saturated barium hydroxide solution (for details see (12), p. 119), the precipitate is centrifuged off and, without washing, is decomposed as before by hydrogen sulfide; in the presence of sulfuric acid. The solution is concentrated to about half the previous volume and the precipitation with silver at pH 7.2 is repeated (9). The precipitate is centrifuged and, if nitrate ion is found in the filtrate, must be washed until this can no longer be detected. It is then suspended in water, acidified to litmus with sulfuric acid, and decomposed with hydrogen sulfide; the solution is the crude histidine fraction. After concentration the reaction should be adjusted with barium hydroxide to approximately pH 4.

In order to remove the cystine that is precipitated along with the histidine (1), dry copper hydroxide (Kahlbaum), or dry copper carbonate, is added in excess to the boiling solution at a volume of approximately 250 cc. for each 50 gm. of protein. The solution is then allowed to digest on the steam bath for an hour, with
occasional stirring, and is finally chilled overnight. The filtrate
from the precipitate is freed from copper by hydrogen sulfide, is
concentrated, and the histidine is precipitated by mercuric sul-
flate in the presence of 5 per cent by weight of sulfuric acid (for
details see (10), p. 709), and finally isolated as the diflavianate.

Isolation of Arginine—The combined filtrates from the two
precipitates at pH 7.2 are acidified to pH 3 to 4 with sulfuric acid
and concentrated to a volume of 1 liter for each 50 gm. of protein;
if excess of silver ion is not present sufficient silver oxide is added
to provide this and the solution is then brought to pH 11 to 12
with warm saturated barium hydroxide. The precipitate is
washed free from nitrate ion, if this is present, by disintegrating
and centrifuging with very dilute barium hydroxide solution
(twice is usually sufficient), is suspended in water, acidified to
litmus with sulfuric acid, and is then decomposed with hydrogen
sulfide. The solution, after concentration, should be faintly acid
to Congo red (pH 4 to 5) and must be free from barium. It is
brought to a definite volume and the nitrogen content is deter-
mined. Arginine is isolated as the monoflavianate from a suitable
aliquot part (for details see (10), p. 709).

Isolation of Lysine—The filtrates from the two silver precipitates
at alkaline reaction are combined, acidified with sulfuric acid,
and the solution is saturated with hydrogen sulfide, or, as an
alternative procedure, is treated with an excess of barium sulfide.
After making certain that the barium has been completely pre-
cipitated the barium sulfate and silver sulfide are removed. The
solution is concentrated slightly to remove hydrogen sulfide and
neutralized to Congo red with sodium hydroxide; it is then further
concentrated to a volume of about 500 cc. for each 50 gm. of pro-
tein. The solution is made alkaline with sodium hydroxide, an
equal volume of alcohol is added, and the solution is again con-
centrated in vacuo at as low a temperature as possible to remove
ammonia. Great care must be exercised at this point; as little
excess sodium hydroxide as possible should be used. The con-
centrated solution is acidified with sulfuric acid, is filtered if
necessary, and brought to a volume of 500 cc. for each 50 gm. of
protein; sufficient sulfuric acid is added to make a concentration
of 5 per cent by weight and the lysine is precipitated by the addi-
tion of an excess of phosphotungstic acid. The precipitate is
removed, after standing overnight, and, without washing, is dissolved in 50 per cent acetone and decomposed by barium hydroxide. The barium phosphotungstate is thoroughly washed with warm and finally with hot water made alkaline with barium hydroxide, the solution is acidified with sulfuric acid, is concentrated to remove all acetone, and the lysine is precipitated a second time as phosphotungstate in the presence of 5 per cent of sulfuric acid. The precipitate is washed free from nitrate ion by disintegrating and centrifuging with a 2.5 per cent solution of phosphotungstic acid in 5 per cent sulfuric acid (three times are usually sufficient). After decomposition in acetone solution with recrystallized barium hydroxide the lysine is isolated as the picrate (for details see (10), p. 710). The double precipitation of lysine with phosphotungstic acid accomplishes a very considerable purification of the lysine fraction with respect to other amino acids as well as a complete elimination of inorganic ions.

SUMMARY

If silver sulfate is employed during the precipitation of arginine and histidine from protein hydrolysates that contain an unusually high proportion of monoamino acids, difficulty is experienced in introducing an adequate excess of silver ion; moreover, unless a considerable excess of silver ion is present a large part of the arginine may escape precipitation. These difficulties are avoided if silver nitrate is employed for the preliminary precipitation. Silver sulfate is then used for the reprecipitation and separation of the two bases and the laborious washing that is required to remove nitric acid from the precipitates is thereby largely avoided.

Silk fibroin has been found to yield 0.74 per cent of arginine, 0.07 per cent of histidine, and 0.25 per cent of lysine. A close analogy between the relative proportions of the bases yielded by silk fibroin and by wool has been noted.

A brief description of the most widely applicable method for the determination of the basic amino acids in proteins is given.

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