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97. Makoto Yokoo: Application of Azotometry. XIX.*1 Determination of Proline in Proteins.

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Proline is a peculiar natural amino acid having no primary amino group and it furnishes many interesting problems in its change *in vivo* and combination in proteins. For example, it is well known that proline changes into glutamic acid in animal tissues,[1] that ornithine is converted to proline,[2] and that the terminal amino acids of some kinds of salmine are all proline.[3] To solve these problems it is necessary to determine proline alone in the presence of other amino acids. Although a method for simultaneous determination of all amino acids, such as the ion-exchange chromatographic method of Stein-Moore, is necessary for the study of proteins, it is also necessary to determine each individual amino acid in the presence of other amino acids and there have already been reported many methods for this purpose, but no method which can determine proline precisely has been reported. Formerly the author announced that proline can be determined by azotometry for secondary amines[4] and the present report deals with the determination of proline present in proteins by the same method.

It is true that some attempts have so far been made to determine only proline present in proteins by utilizing the reaction of secondary amino group[5] in the pyrrolidine ring or the carboxyl group,[6] but they were always affected more or less by the coexisting other amino acids which behave like proline.

In the present method, a hydrolyzate of protein is allowed to react with nitrous acid in the presence of acetic acid to convert the proline therein into N-nitrosoproline, when all primary amino groups of the coexisting other amino acids are decomposed by nitrous acid and do not affect subsequent reactions. The N-nitrosoproline is then reduced with zinc dust and hydrochloric acid to N-aminoproline, which is determined by ferricyanide-azotometry. There are some natural amino acids besides proline, having the secondary amino group. For example, tryptophan, histidine, and prolidine have secondary amino group in their heterocyclic ring, and arginine also has it in the guanidine group, but the secondary amino group in an unsaturated ring such as that of histidine or tryptophan, and the secondary amino group in guanidine were found not to react with nitrous acid to produce the N-nitroso compounds. Although the -CONH- group in prolidine and

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*1 This constitutes a part of a series entitled "Application of Azotometry" by Masaharu Yamagishi.

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1) H. A. Krebs: Biochem. J., **29**, 2077(1935).
2) M. R. Stetten: J. Biol. Chem., **189**, 499(1951).
3) R. R. Porter, F. Sanger: Biochem. J., **42**, 287(1948).
4) M. Yokoo: This Bulletin, **6**, 64(1958).
5) M. Bergmann, C. Niemann: J. Biol. Chem., **122**, 577(1938).
6) W. R. Fearon, W. A. Boggarst: Analyst, **76**, 667(1951).

peptides sometimes reacts with nitrous acid to form the corresponding N-nitroso compounds, it does not interfere with the present method because the N-nitroso compounds are not reduced to the N-amino compounds.

Proteins to be subjected to the present method must be hydrolyzed beforehand and, though the hydrolysis can be effected by acid or alkali, alkali hydrolysis is more convenient for the following processing. Time required for complete hydrolysis of proteins is well known, but in the present method complete hydrolysis is not necessary and hydrolysis to such a degree as to liberate the secondary amino group is sufficient. In the present case, egg albumin, casein, and protamine sulfate were hydrolyzed with 5*N* NaOH at 100° and the liberated proline was determined at regular intervals to know the time required for complete liberation of proline. As a result, as shown in Fig. 1, the time was found to be ca. 6 hours for egg albumin, ca. 22 hours for casein, and

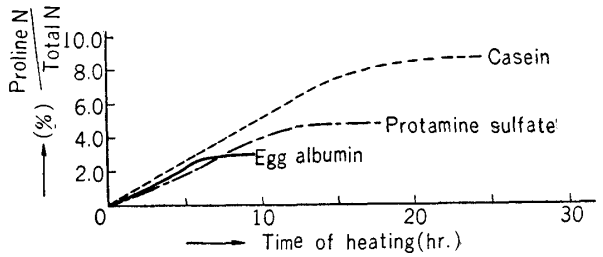

Fig. 1.

ca. 14 hours for protamine sulfate. Therefore, these proteins were subjected to the determination after being hydrolyzed for the respective period. It is well known that the hydrolysis of proteins requires a fairly long time and various amino acids in it are decomposed to some extent during this period. However it has never been shown that proline is decomposed during the hydrolysis and it is known that the pyrrolidine ring is very stable to acid and alkali. Protein was hydrolyzed as such and, after addition of a definite amount of proline under the same conditions and determination of the liberated proline, it was found that proline was not decomposed under the conditions of the present method.

Proline present in egg albumin, casein, and protamine sulfate was determined by the present method and the results were compared with the values reported in the literature. The content of amino acids in proteins is expressed in various ways, but as it is expressed most exactly by the ratio of nitrogen in amino acid to the total nitrogen, this method was adopted in the present work. The amount and proportion of amino acids in proteins are reported more or less differently according to workers and this may be due to difficulty in obtaining samples with the same quality. Especially the quality of proteins differs according to the method by which they are prepared. In the present case, proteins were prepared by the general method, i.e. egg albumin by precipitation with sodium sulfate,[7] casein from milk by isoelectric precipitation,[8] and protamine sulfate from the sperm of salmon by separatory centrifugation.[9] As will be shown in the experimental part, results were in good agreement with those reported in the literature, showing that the present method is reliable.

Method

I. Reagent—i) 5*N* NaOH ii) Glacial AcOH iii) 60% KNO_2 solution iv) Sulfaminic acid v) Zinc dust vi) Devarda alloy powder vii) conc. HCl.

II. Procedure—An amount of a protein is weighed accurately in a test tube with a stopper so

7) R. A. Kekwick, R. K. Cannan : Biochem. J., **30**, 227(1936).
8) O. Hammersten : Z. physiol. Chem., **7**, 227(1887).
9) A. Kossel, H. D. Dakin : *Ibid.*, **41**, 407(1904).

as to contain about 0.03~0.07 m.mole of proline and heated with 2 cc. of 5*N* NaOH at 100° for the fixed time. After cool the mixture is subjected to nitrosation with 2 cc. of glacial AcOH and 1 cc. of KNO_2 solution at 30° for 30 min., and the excess HNO_2 is decomposed with 0.5 g. of sulfamic acid. The mixture is reduced by shaking for 15 min. with 1 g. of Zn dust and 1.5 cc. of HCl, then for 15 min. more after addition of 15 cc. of HCl, and finally left standing for 30 min. with 0.5 g. of Devarda alloy powder with occasional shaking. The insoluble substances are collected, washed with water, and the combined filtrate and washing are diluted exactly to 25 cc. Two cc. of this solution is subjected to azotometry by oxidation with $K_3Fe(CN)_6$ and the volume of generated nitrogen gas is measured. When the value of nitrogen at normal state is taken as V, the quantity of proline is calculated by the following equation because the molecular weight of proline is 115 and 115 γ of proline generates 11.2 mm^3 of nitrogen gas.

$$\text{Quantity of proline} = \frac{V \times 115 \times 25}{11.2 \times 2 \times 1000} = 0.128 \times V \text{ (mg.)}$$

The ratio of nitrogen in proline to the total nitrogen is calculated after V is converted to weight value and total nitrogen of a sample is measured by Iwasaki's hypobromite-azotometry[10] after the sample is decomposed by the Kjeldahl method.

Experimental

Tables I to IV show the results of determination of proline in egg albumin, casein, and protamine sulfate. It was found from these results that proline is not changed under the conditions of hydrolysis used in the present method.

Table I. Determination of Proline in Egg Albumin

Sample (mg.)	Total N (mg.)	Total N (%)	Proline N measured (mg.)	Proline N / Total N (%)	Reported Data* Total (%)	Reported Data* Proline N / Total N (%)
101.4	16.02		0.471	2.94		
103.8	16.39	15.79	0.463	2.82	15.80	2.90
103.5	16.34		0.464	2.84		

* J.C. Rewis, N.S. Snell: J. Biol. Chem., **186**, 23(1950).

Table II. Determination of Proline in Casein

Sample (mg.)	Total N_t (mg.)	Total N_t (%)	Proline N measured (mg.)	Proline N / Total N (%)	Reported Data* Total (%)	Reported Data* Proline N / Total N (%)
42.5	6.63		0.537	8.10		
41.1	6.41	15.60	0.527	8.23	15.63	8.28
42.0	6.55		0.532	8.12		

* W.G. Gordon, W.F. Sennet, M. Bender: J. Am. Chem. Soc., **72**, 4282(1950).

Table III. Determination of Proline in Protamine Sulfate

Sample (mg.)	Total N (mg.)	Total N (%)	Proline N measured (mg.)	Proline N / Total N (%)	Reported Data[9] Total (%)	Reported Data[9] Proline N / Total N (%)
40.5	9.68		0.397	4.10		
40.9	9.78	23.9	0.432	4.42	24.1	4.30
41.2	9.85		0.458	4.58		

Table IV. Determination of Proline after Addition of Proline

Sample	m.g.	Proline added (mg.)	Proline N (mg.) Found	Proline N (mg.) Calcd.	Recovery (%)
Egg albumin	106.5	4.2	0.985	0.994	99.1
Casein	55.3	4.2	1.200	1.215	98.8
Protamine sulfate	61.8	4.2	1.143	1.156	98.9

10) K. Iwasaki: Juzenkai Zasshi, **42**, 2132(1937).

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Summary

Utilizing the reaction of its secondary amino group, proline in proteins was determined in the presence of other amino acids. The material proteins were first hydrolyzed and then allowed to react with nitrous acid to convert the proline therein into N-nitrosoproline, when all the other amino acids were changed into the corresponding hydroxy acids. N-Nitrosoproline was then reduced to N-aminoproline with zinc dust and hydrochloric acid, and finally the N-amino compound was determined by azotometry by oxidation with potassium ferricyanide. Incidentally, the secondary amino group in the ring of tryptophan, histidine, and prolidine, and the guanidine group of arginine do not interfere with the present method.

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98. Shoji Takemura: Chemical Studies on Antibiotics produced by Actinomycetes. IX. Racemomycin. (6). Structure of New Degradation Product of Recemomycin-O.*1

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In the previous reports of this series,[1,2] it was shown that four degradation products, β-lysine (I), roseonine (II), glucosamine (III), and an unknown reducing substance were obtained from the hydrolysate of the new streptothricin-like antibiotic, racemomycin-O. Structural studies of the fourth compound were carried out and the results are presented in this paper.

On the paper chromatogram, this compound is found at Rf 0.05 (BuOH–AcOH–H_2O= 4:1:5), negative to Ninhydrin reagent and giving a red color by treatment with alkali solution of triphenyltetrazolium reagent. Therefore, this compound is a new degradation product which has never been discovered in hydrolysate of streptothricin-group antibiotics.

This substance often cannot be detected on paper chromatogram when the paper strips were exposed to the air before developing or spraying of reagent. This observation shows that this compound is sensitive to oxidation. Furthermore, this compound is negative to various amine reagents. In order to isolate this compound, the hydrolysate was continually extracted with ether and the extract was concentrated to obtain an oily residue. The residue was distilled under a reduced pressure and the oily substance which gave Rf 0.05 on paper chromatogram was obtained. The freshly distilled oil is positive to the Tollens, aniline hydrogenphthalate, ammoniac-silver nitrate, and fuchsin-

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*1 This constitutes Part IX of a series entitled "Chemical Studies on Antibiotics produced by Actinomycetes" by H. Taniyama.

*2 Kowakae, Fuse, Osaka-fu (竹村庄司).

1) H. Taniyama, S. Takemura: This Bulletin, 8, 150(1960).

2) S. Takemura: *Ibid.*, 8, 154(1960).