STUDIES ON PROTEINS. II.[1]

ACTION OF SUPERHEATED WATER ON PROTEINS. I.

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Taylor[2] has observed that when pure sterile globulin from bovine serum was kept in distilled water at ordinary temperature for 18 months, it was hydrolysed to proteoses and partly polymerised into an insoluble form, and also that leucine may be recovered from a sterile suspension of casein in pure water after the lapse of a year or more. These facts indicate that the hydrolysis of proteins in neutral aqueous solution does occur at ordinary temperature, though the velocity of the autohydrolysis of proteins was very slow under these conditions. The influence of heat upon the autohydrolysis of proteins is of considerable interest, since it was a generally accepted view

(1) The first report has been published in *The Journal of Biochemistry*, 2 (1922), 365.
(2) *J. Biol. Chem.*, 1 (1906), 345; "On Fermentation," (1907), p. 223.

that the reaction of hydrolysis is accompanied by the evolution of heat and that the heat-coagulation of proteins was considered to consist in the polymerisation of the amphoteric proteins molecule with the elimination of water according to the following equation:

$$H\cdot X\cdot OH + H\cdot X\cdot OH = H\cdot X\cdot X\cdot OH + H_2O$$

and accordingly, the effect of applying heat to a protein solution must be to shift the equilibrium in the direction of polymerisation, as would follow from van't Hoff's law[1].

The study of the action of superheated water on proteins was first tried by Wöhler[2], and his report was followed by contributions from Subanin[3], Lubavin[4] and others[5] on the isolation of cleavage products of proteins.

The net result of these researches was (1) to establish that the protein body suffers fairly complete hydrolysis at the temperature of steam, and that the equilibrium between the protein-complex and the amino acids, which were regarded as the products of its hydrolysis, was shifted to the amino acids side; (2) to show the presence of leucine, tyrosine, aspartic acid and atmidbodies of complex nature in the reaction products of the proteins.

The extensive researches by R. Neumeister[6], and R.H. Chittenden and F.S. Meara[7] have given us much additional informations concerning the mode of cleavage of the protein molecule, though there are different opinions regarding the nature of the process which have revealed an important connection with the hydrolytic process brought about by the digestive enzymes and also by dilute acids. Many investigators mentioned above, have contented merely to examine the products, especially from the view point of physiology, to find some analogies between the action of superheated water and gastric or pancreatic digestion. Such a too close adherance to

(1) Robertson, "The Protein," (1909), pp. 141 and 173.
(2) *Lieb. Ann.*, **41** (1842), 238.
(3) Hoppe-Seyler, "Medicin-chem. Untersuch.", (1871), p. 480.
(4) *Ber.*, **10** (1877), 2237.
(5) Hammersten, *Z. physiol Chem.*, **7** (1883), 227.
Krukenberg, *Sitzungsber. der Jenaischen Gesell. für Medicin, etc.* (1886).
A. Clermont, *Compt. rend.*, **105** (1887), 222.
Crismer, *Jahres Berichte f. Thiechemie für 1891*, 19.
Koukol-Yasnopolsky, *Pflüg. Arch. Physiol.*, 12, 85.
R. Neumeister, *Z. Biol.*, **26** (1890), 57; **36** (1898), 420.
E. Salkowski, *Z. Biol.*, **34** (1896), 190; **37** (1899), 404.
R.H. Chittenden and F.S. Meara, *J. physiol.*, **15** (1896), 501.
Blum u. Vaubel, *J. prakt. Chem.*, **56** (1897), 396; **57** (1898), 365.
R. Bauer, *Z. physiol. Chem.*, **35** (1902), 342.
H.S. Steudel, *Z. physiol. Chem.*, **35** (1902), 540.
(6) Loc. cit.
(7) Loc. cit.

analogies might lead investigators into serious error as was already pointed out by Chittenden and Meara, and cause them to fail to pay attention to the unraveling of the constitution of proteins. Furthermore, it was doubtful whether the atmid-bodies would result in a definite chemical composition, in spite of the intensity and duration of heating by which they were produced.

Nobody paid any attention to the formation of the insoluble substance from proteins by the action of superheated water, nor offered any explanation of its relation to the atmid-bodies, all merely dwelling on the constitution of the latter substances. The present research, to heat proteins with water in the iso-electric point, therefore, was undertaken by the authors, to find some chemical relation between the insoluble substance and the atmid-bodies, to propose chemical constitution of protein, if possible, and also to explain the mechanism of the decomposition of protein by superheated water.

The materials employed in the present experiment were three different proteins; edestin, gliadin from wheat flour, and casein from cow's milk, which were prepared by the authors following the directions proposed by T. B. Osborne and his coworkers[1]. The results of the analysis of the samples are shown in the following table:

TABLE 1.

	Edestin	Gliadin	Casein
C.	51.3	53.78	53.39
H.	6.86	6.79	9.10
N.	18.63	17.65	15.55
Ash.	0.7	0.5	0.25
H_2O	6.43	4.1	3.67
Amide-N.	1.88	4.30	1.61
Monoamino-N	10.87	12.25	10.31
Diamino-N.	5.9	1.09	3.49
Melanin-N.	0.12	0.14	0.21
P_H[2]	6.3	5–6 (mean 5.7)	4.6

One gram of protein was introduced with 20 c.c. of distilled water into a glass stoppered bottle of 70 c.c. capacity which was previously washed with steam in order to remove completely any alkaline substance

(1) *J. Am. Chem. Soc.*, 25 (1903), 323.

(2) P_H of the pure protein was determined by the electrometric method of the solution which was prepared by shaking the protein with distilled water in a stoppered bottle for 24 hours. According to Michaelis, the P_H for edestin and for casein is 5.6 and 4.7 respectively, and Eto (*J. Biochem.*, 3 (1924), 373) have given 6.6 for that of gliadin.

which might be generated from the glass wall by the action of steam. The bottle was carefully stoppered, and heated in a thermostat which was kept at the constant temperature of 110° and 120°. The bottle was opened after heating for a certain number of hours, and the fluid which had generally an yellow colour, and gave a neutral reaction to litmus paper, was poured into a flask. The insoluble residue separated from the coloured solution, was washed with cold water, and then dried to constant weight at 105°. The solution and washings combined together, made up to 50 c.c., and the P_H value of the solution was determined electrometrically using one part of the solution. Another part of the solution was evaporated on a water bath to dryness and dried at 110° to constant weight and then analysed.

The elementary composition and the distribution of nitrogen of both the insoluble residue and the solution were determind in the usual way, and the results are shown in Table 2 and Table 3.

The constituents of the hydrolysate of protein by the action of superheated water can be divided, according to their solubilities in water, into two parts; the insoluble residue, and the soluble substances, and the latter composed of a substance of complex nature such as proteose, and of simple amino acids, and the quantity of the soluble substances as was indicated in Table 2, was increased in proportion to the reaction time. The rate of dissolution of protein in water, being different for individual protein, is greatest in edestin, and gliadin and casein were ranged in that order.

The total weight of the hydrolysate as will be seen in Table 2, exceeded in all cases the employed amount of the sample, indicating the hydrolysis of protein molecule occurred in the digestion.

Although the insoluble residue has the same appearance as the mother protein, they are different essentially in composition from each other, as indicated in Table 3, the insoluble residue resulting from edestin is rich in the content of carbon and hydrogen than the mother protein, while that from gliadin is poor in carbon but rich in hydrogen, and the insoluble substance derived from casein is rich in carbon but poor in hydrogen compared with the mother protein.

Generally, the insoluble substance has no definite chemical composition which varying with the external conditions under which the protein was submitted to the chemical action; the substance derived from edestin shows gradual increase in carbon and hydrogen-content with lapse of the reaction time, while that from casein and gliadin keep almost constant value.

These facts led the authors to the conviction that the chemical composi-

tion of the detached groups from protein should vary with each individual protein according to the amino acids of which it was composed[1].

Moreover, the study of the nitrogen distribution of the insoluble and soluble parts by the usual methods, supports the above idea with regard to the composition of the hydrolytic products, and also suggests the formulation of an hypothesis with respect to the nature of the process in which protein undergoes hydrolysis.

When edestin was digested, the groups containing amide nitrogen were removed from the protein molecule mostly at the beginning of the reaction, whilst those containning diamino nitrogen were detached gradually, and consequently the hydrolysis of the protein proceeded in a manner in which the distribution of nitrogen in the residue and solution will approach, in the progress of reaction, that in the original protein. Casein, however, shows quite different behavior toward super heated water, since the atomic groups containing diamino-nitrogen were removed mostly from the protein molecule at the first period of the reaction, and gliadin with respect to its behavior stands between these two proteins.

TABLE, 2.

			Casein (120°)			Gliadin (120°)			Edestin (110°)		
Heating (hrs.)			2	6	17	2	6	15.5	3	6	9
Sample (gr.)			0.8938	0.9740	0.9672	0.9749	0.9880	1.0028	0.9200	0.9200	0.9200
Solution (gr.)			0.012	0.057	0.14	0.054	0.097	0,289	0.134	0.212	0.310
„ (%)			1.34	5.85	14.00	5.5	9.9	28.8	14.3	22.6	33.0
Residue (gr.)			0.9228	0.9331	0.8365	—	0.9414	0.7265	0.8986	0.8678	0.6330
„ (%)			102.6	95.8	86.5	—	96.2	92.4	97.7	94.3	74.2
Distribution of N.	Solution.	Total N. (%)	—	17.0	15.0	16.3	14.4	16.2	16.2	18.7	17.8
		Amide N. (%)	—	4.5	2.6	—	4.6	5.0	3.0	3.4	2.7
		Diam. N. (%)	—	3.3	2.0	—	0.7	0.8	3.7	4.4	4.7
	Residue.	Total N. (%)	16.0	15.3	15.5	—	15.2	23.1	16.9	16.2	18.3
		Amide N. (%)	—	1.3	2.1	—	4.9	6.9	1.6	1.3	1.4
		Diam. N. (%)	—	3.0	5.1	—	0.8	1.5	5.4	4.9	5.3

(1) G. Trier, "Chemie der Pflanzenstoffe," (1924), p. 470.
O. Cohnheim, "Chemie der Eiweisskörper," 3 Aufl. (1911), p. 279.

TABLE, 3.

Elementary Analysis of Residue and P_H of the Solution.									
	Edestin (120°)			Gliadine (120°)			Casein (120°)		
Heating (hrs.)	Carbon	Hydrogen	P_H	Carbon	Hydrogen	P_H	Carbon	Hydrogen	P_H
0	51.36	6.86	6.3	53.87	6.79	5.7	53.39	9.13	4.6
1	51.80	7.55	7.3			—			—
6			—	52.27	7.11	6.8	54.29	7.34	4.7
20	52.46	7.20	6.9	52.19	6.85	6.4	54.27	7.17	5.0

It was a noteworthy fact that the total amide nitrogen of the reaction products at the various stages of the reaction, as will be seen in the case of edestin heated with water at 110°, exceeded that of the protein, and on the contrary there shows a corresponding diminution in the total diamino-nitrogen of the reaction products when compared with the original sample. These facts indicate the transformation of the diamino-nitrogen into amide-nitrogen, and also the phenomenon was noticed markedly in the case of casein, but in gliadin the reaction takes place in the least degree.

Such conversion of nitrogen compounds seems to be more probable when referred to the transformation of glyoxalin by the action of benzoyl chloride and alkali at 0° into bis-benzoyl amino ethylene[1], and also the behavior of tryptophane towards chemicals[2]. Consequently, the process involved in the appearance of the insoluble residue by the action of superheated water on protein was regarded as taking place in at least two ways.

First, there is a formation of an insoluble substance resulting by the removal of the prominent parts from the protein complex. Second, one part of other cleavage products soluble in water, formed simultaneously, was subsequently converted to another insoluble substance by the condensation. The remainder of the soluble substances exists with amino acids side by side in the solution, does or does not change its chemical structure.

(1) Bamberger and Berle, *Lieb. Ann.*, 273 (1893), 351.

(2) Hoppe-Seyler, "Physiol.-Pathol. Chem. Analyse," 9 Aufl. (1924), p. 315.
A. Kossel u. S. Edlbacher, *Z. physiol. Chem.*, 93 (1915), 396.
A. Windaus, *Ber.*, 43 (1910), 499.

The study of the P_H value of the individual reaction products and also their buffer action will be of some important service for the verification of the hypothesis with regard to the chemical reactions above mentioned, and the results will be described in detail in the next article.

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