Studies on Proteins, II.
Action of Superheated Water on Proteins, I.

By

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Taylor[1] 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 tha 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 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 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 protein molecule with the elimination of water according to the following equation:[2]

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

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.

The study of the action of superheated water on proteins was first

1 J. Biol. Chem., **1**, 345 (1906); On Fermentation, p. 223 (1907).

2 Robertson: The Protein; p. 141, 173 (1909).

tried by Wöhler[1], and his report was followed by contributions from Subain,[2] Lubavin[3] and others[4] on the isolation of cleavage products of proteins.

The next 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, tyrosin, aspartic acid, and atmid-bodies of complex nature in the reaction products of the proteins.

The extensive researches by R. Neumeister and R. H. Chittenden and F. S. Meara have given us much additional information 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 been content 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 adhearance to 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 unravelling 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.

No body paid any attention to the information of the insoluble substance from proteins by the action of superheated water, nor offered any examination of its relation to the atmid-bodies, all merely dwelling on the consitution of the latter substances. The present research was to heat pro-

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1 Lieb. Ann., **41**, 238 (1842).

2 Subain: Hoppe-Seyler: Medicin-chem. Untersuch. 480 (1871)

3 Lubavin: Ber. D. Chem. Ges., **10**, 2237 (1883).

4 Hammersten: Z. physiol. Chem., **7**, 227 (1883); Krukemberg: Sitzungsberichte der Jenaischen Gesellschaft für Medicin, etc., 1886; Aclemont: C. R., **105**, 222 (1887); Crismer: Host in Jahres Berichte f. Thierchemie für 1891, 19; Koukol-Yasnopolsky: Pflüg. Arch. Physiol., **12**, 85. R. Neumeister: Z. Biolog., **26**, 57 (1890); **36**, 420 (1896); E. Salkowski: Ibid., **34**, 190 (1896); **37**, 404 (1899); Blum u. Vaubel: J. prak. Chem., **56**, 396 (1897); **57**, 365 (1898); R. Bauer: Z. Physiol. Chem., **35**, 342 (1902); H. S. Steudel: Ibid., **35**, 540 (1902); R. H. Chittenden and F. S. Meara: J. physiol., **15**, 501 (1896).

teins with water in the isoelectric point, therefore, 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 co-workers.[1] The results of the analysis of the samples are shown in the following tables:

Table I.

	Edestin	Gliadin	Casein
C.....	51·30	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	1·1	3·5
Melanin-N.	0·1	0·1	0·2
PH[2]	6·3	5—6 (mean 5·7)	4·6

One gr. of protein was introduced with twenty c. c. of distilled water into a glass stoppered bottle of seventy c. c. capacity, which was previously washed with steam in order to remove completely any alkaline substance 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 120°. The bottle was opened after heating for a certain number of hours, and the fluid which had generally a 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 PH value of the solution was determined electrometrically of one part

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

2 PH 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 PH for edestin and for casein is 5·6 and 4·7 respectively, and Eto [J. Biochem., **3**, 373 (1921),] has given 6·6 for that of gliadin.

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 determined in the usual way, and the results shown in the following table.

Table II.

Quantities of Soluble and Insoluble Parts.

Edestin

Temperature		110°			120°			
Heating (hrs)		3	6	9	1	2	4	20
Sample (gr)		0.920	0.920	0.920	0.950	1.093	1.166	0.926
Soluble. part. (gr)		0.134	0.212	0.310	0.065	0.136	0.263	0.588
〃	(%)	14.3	22.6	33.0	6.9	12.5	22.6	63.4
Insol. part. (gr)		0.899	0.868	0.683	—	—	—	—
〃	(%)	97.7	94.3	94.2	—	—	—	—

Gliadin and Casein (120°)

Heating	(hrs)	2	6	17	2	6	15.5
Sample	(gr)	0.900	0.974	0.967	0.975	0.988	1.003
Soluble part.	(gr)	0.012	0.057	0.140	0.054	0.097	0.289
〃	(%)	1.3	5.9	14.0	5.5	9.9	28.8
Insol. part.	(gr)	0.923	0.933	0.837	—	0.941	0.727
〃	(%)	102.6	95.8	86.5	—	96.2	92.4

Table III.

Distribution of Nitrogen.

		Casein (120°)			Gliadin (120°)			Edestin (110°)		
Heating	(hrs).	2	6	17	2	6	15.5	3	6	9
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
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

Table IV.

Elementary Analysis of Insoluble Part and PH of the Solution

Heating (hrs).	Edestin (120°)			Gliadin (120°)			Casein (120°)		
	C	H	PH	C	H	PH	C	H	PH.
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

The constitution of the hydrolysate of protein by the action of superheated water can be divided according to their solubility in water, into two parts; the insoluble and soluble parts, 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 the table, 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 the table, exceeded in all cases the employed amount of the sample, indicating the hydrolysis of protein molecule occured in the digestion.

Although the insoluble part has the same appearance as the mother protein, they are different essentially in composition from each other, the insoluble part 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 varying with the external condition under which the protein was submitted to the chemical action; the substance derived from edestin shows gradual increase in carbon- and nitrogen-content with laps of the reaction time whilst that from casein and gliadin kept almost constant value.

These facts led the authors to the conviction that the chemical composition of the detached groups from protein should vary with each individual protein according to the amino-acids of which it was composed.[1]

1 G. Trier: Chemie der Pflanzenstoffe, 470 (1924).; O. Cohnheim: Chemie der Eiweisskörper III. Auf. 279 (1911).

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

When edestin was digested, the groups containing amide-nitrogen were removed mostly at the begining of the reaction from the protein molecule, whilst those containing 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 superheated 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.

It was a noteworthy as that the total amide nitrogen of the reaction products at the various stage 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 composed 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 take 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 toward 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 from the removal of the prominent parts from the protein complex, and secondarily, one part of other cleavage products soluble in water, forming simultaneously was subsequently converted another insoluble substance by condensation. The remainder of the soluble substances with amino-acids side by side in the solution with or without any chemical change in its structure.

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1 Bamberger and Berlé: Lieb. Ann., **273** 344(1893).

2 Hoppe-Seyler: Physiol. Pathol. Chem. Analyse, 9 Aufl. 315 (192·); A. Kossel u. S. Edlbacher: Z. physiol. Chem., **93**, 396 (1915); A. Windaus: Ber. D. Chem. Ges., **43**, 499 (1910).

The study of the PH 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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