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Studies on Proteins, IV.

Action of Superheated Water on Proteins, III.

By

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In the previous article[1] of this subject the writers have pointed out that proteins when subjected to the action of superheated water, one part of which goes into solution, forming less complex compounds from the mother proteins, in which, from the study of the buffer values (dB/dP_H) of the solution, at least two compounds of complex nature were supposed to occur. To afford some evidences in favor of this present idea, the solution prepared from 3 gm. of edestin by heating 30 c.c. of water at 120° for 20 hours, and separated from the insoluble residue (A) by filtration, which showed $P_H=6\cdot9$, was acidified with $\frac{N}{100}$ HCl solution, and the precipitation of a substance (B) was completed at the isoelectric point $P_H=5$, which separated from the mother liquor (C), and both the substance (B) and the mother liquor (C) were determined of their nitrogen distribution as usual, and the results are shown in the Table I.

The substance (A), $C=52\cdot46$; $H=7\cdot20$; $N=15\cdot49$, which separated as an insoluble residue from the solution of $P_H=6\cdot9$, was supposed from the conception of the "isoelectric point" of an amphoteric electrolyte[2],

1. These Memoirs, A, **10**, 163 (1927).
2. J. Loew: Protein and the Theory of Colloidal Behavior, (1922), p. 6.

Table I.

	Edestin		Gliadin	
	Amide N.	Diamino N.	Amide N.	Diamino N.
Substance (B)	7·5 %	30·0 %	24·5 %	2·3 %
Mother liquor (C)	22·4 〃	28·1 〃	31·0 〃	4·1 〃
Insoluble part (D)	7·5 〃	7·5 〃	22·9 〃	7·0 〃
Soluble part (E)	7·7 〃	31·8 〃	22·2 〃	3·7 〃

to be a mixture of two or more substances of complex nature, since the solubility of genuine proteins or other amphoteric electrolytes in water is a minimum at the isoelectric point and increases as a rule when the hydrogen ion concentration of solution is shifted to either side of the point, to the more acid side or to the more alkali side, but for a different reason; namely, because protein salts or amphoteric electrolytes in general are more soluble in water than the non-ionized molecule, as this was already pointed out by J. Loew[1] and L. Michaelis[2] of proteins and amino acids.

As a matter of fact, the insoluble residue (A) was divided by treating with $\frac{N}{100}$ NaOH solution, into two parts, the insoluble part (D) and the soluble one (E), and the study of the nitrogen distribution of these two fractions, as we anticipated, verifies the above assumption, and also shows the complex substance (E) is the same as the one (B) which occurs in the solution. The protein of the amide nitrogen 10 % and of the diamino nitrogen 31·6 % was thus decomposed by the influence of superheated water into three parts, the substance (D) of the amide nitrogen 7·5 % and of the diamino nitrogen 7·5 %, the substance (B) of the amide nitrogen 7·6 % and of the diamino nitrogen 30 %, and a substance (C) of the amide nitrogen 22·4 % and of the diamino nitrogen 28 1 %, and they were separated from each other by means of their acidity. From the stand point of nitrogen distribution, the most insoluble substance (D) and the most soluble one (C) are quite different from the mother protein, from which they are derived, while the substances (E) and (B)

1. Loc. cit.
2. L. Michaelis and H. Davidsohn: Biochem. Z., **30**, 143 (1910).

similar to edestin in composition but not in solubility toward water and alcohol; the former being soluble in water and alcohol. By the action of superheated water, protein molecule was decomposed into fragments so as to distribute equally monoamino nitrogen to each fragment, but no any of other nitrogen atoms; the substance (D) is poor in diamino nitrogen content, but the substance (C) richest of amide nitrogens.

In the case of gliadin, the reaction products by the action of superheated water at 120° for 20 hours, can also be divided into three fractions; the most insoluble fraction, the most soluble fraction and an intermediate fraction, according to their solubility in a dilute acid solution.

The distribution of the nitrogen atoms of gliadin, the mother protein, in the three fractions being in every way equal, is quite different from the case of edestin as will be seen in Table I.

Casein, as was mentioned in the first report[1] of this subject, behaves toward superheated water in a quite different manner from other proteins, and the protein was divided into two parts, the insoluble residue which contains amide nitrogen 13·7 %, and diamino nitrogen 33·0 % and the soluble part of amide nitrogen 27·4 %; diamino nitrogen 12·9 % and the former dissolved completely in $\frac{N}{100}$ NaOH solution, but the latter solution gave no more precipitate on acidifying with the $\frac{N}{100}$ HCl solution, as the P_H value of the original solution has already exceeded the isoelectric point of the insoluble substance. The distribution of nitrogen in the insoluble part was reciprocated by that in the soluble one.

The nitrogen distribution of the soluble fraction resulting from the action of superheated water on proteins, which yields no precipitate in making the P_H value to 6·0, and also the comparative study of the fraction with the products of enzymatic hydrolysis of proteins, indicate that the solution should still contain various substances of complex nature, such as metaprotein, proteose, peptone, subpeptones, and aminoacids. The isolation of these fractions from the solution was regarded by the writers to secure some definite knowledge concerning the chemical changes occurring during the reaction by the superheated water, and also the constitution of proteins, and the following experiment were undertaken, following the method proposed by H. Wasteneys and H. Borsook.[2]

12 gm. of protein were heated with 120 c.c. of distilled water at

1. These Memoirs, A, **10**, 163 (1927).
2. J. Biol. Chem., **62**, 1 (1924).

120° for certain hours, and the solution separated from the insoluble residue by filtration, which yielded no precipitate on making the P_H value to 6·0 with dilute hydrochloric acid, made up to 100 c.c. and the total nitrogen content was determined. To 40 c.c. of the solution (A), 10 c.c. of trichloracetic acid were added and let stand for one hour, the precipitate (B) which was composed of metaprotein, was filtered and of the filtrate (C) the total nitrogen was estimated. 30 c.c. of the filtrate (C) were treated with a sufficient quantity of anhydrous sodium sulphate at 33° after the trichloracetic acid was removed by decomposing into carbon dioxide and chloroform, whereon proteose (D) was completely precipitated and filtered. 25 c.c. of the filtrate (E) was separated from the proteose (D) made up to 50 c.c., and the total nitrogen estimated. 25 c.c. of the filtrate (E) were mixed with 25 c.c. of 2·1 N sodium hydroxide solution, and 125 c.c. of a 20 % tannic acid solution dissolved in 0·1 N sulphuric acid solution, containing 20 % Na_2SO_4, and the mixture was thoroughly shaken and kept at 20° for 4—5 hours. Peptone (F) precipitated by the tannic acid was decanted off, and the total nitrogen of the solution was determined as usual.

From the total nitrogen content of each step of the fractions, the percentage of metaprotein, proteose, peptone and other fractions in nitrogen, was calculated, and the results are shown in the Table II.

Table II.

	Edestin		Gliadin		Casein	
Heating hours	6 hours	20 hours	6 hours	20 hours	6 hours	20 hours
Metaprotein fraction (C)	54·1%	35·3%	62·5%	11·0%	12·0%	24·3%
Proteose fraction (D)	21·9〃	31·2〃	35·8〃	25·2〃	25·4〃	31·6〃
Peptone fraction (F)	0·1〃	0·3〃	0 〃	18·7〃	0 〃	trace
Subpeptone, Polypeptides & Amino-acids.	23·9〃	32·7〃	1·7〃	45·0〃	52·5〃	47·0〃

The tannic acid filtrate was adjusted to P_H· 5·0 and nine times of its volume of 95 % alcohol added and the mixture allowed to stand for 24 hours, the precipitate centrifuged off and the remaining solution was treated with saturated alcoholic solution of zinc chloride to form precipitate subpeptone-like substances and filtered. Amino acids or polypeptides which remained in the final filtrate, were estimated by nitrogen determination.

Table III.

	Heating hours	6 hours		20 hours	
		Amide-N.	Diamino-N.	Amide-N.	Diamino-N.
Edestin	Metaprotein (C)	8·2 %	18·7 %	6·2 %	30·8 %
	Proteose (D)	3·5 〃	24·2 〃	6·3 〃	11·6 〃
	Peptone (F)	13·6 〃	11·7 〃	29·1 〃	9·8 〃
	Subpeptones	11·0 〃	0	A*17·2 〃 B 7·2 〃	8·0 〃 28·7 〃
	Polypeptides & Amino-acids	6·3 〃	20·9 〃	4·2 〃	45·0 〃
Gliadin	Metaprotein (C)	23·8 〃	0 〃	21·1 〃	2·1 〃
	Proteose (D)	24·2 〃	0	21·4 〃	1·8 〃
	Peptone (F)	—	—	13·1 〃	0
	Subpeptones	9·7 〃	15·4 〃	A*19·6 〃 B 17·0 〃	9·0 〃 0
	Polypeptides & Amino-acids	13·0 〃	50·0 〃	6·0 〃	69·0 〃
Casein	Metaprotein (C)	15·8 〃	25·4 〃	9·2 〃	17·1 〃
	Proteose (D)	8·9 〃	25·0 〃	6·6 〃	19·1 〃
	Peptone (F)	—	—	9·0 〃	0
	Subpeptones, Polypeptides & Amino-acids	19·2 〃	8·1 〃	21·9 〃	trace

* Subpeptone (A) is precipitate by means of alcohol and Subpeptone (B) is one by zinc chloride.

Of each precipitate, which fractionated from the solutions by means of the various reagents mentioned above, the various forms of nitrogen were estimated in order to get some idea of the chemical nature of these fractions, and the results are shown in Table III.

It was learned from the experimental results shown in the nitrogen distribution of the fractions resulted from proteins by the action of superheated water, that the substance of the amide nitrogen 7·5 and of the diamino nitrogen 30·0 and of its isoelectric point P_H 5, which was isolated from the insoluble residue and also from the soluble part yielded from edestin by superheated water, was supposed to composed of metaprotein,

amide nitrogen 6·2 and diamino nitrogen 30·8 and of proteose of the amide nitrogen 6·3 and of the diamino nitrogen 11·6.

The same relation with respect to the nitrogen distribution, is held among the derivatives of gliadin and also of casein. When the decomposition proceeded from proteose to peptone, there is a sudden change in the chemical composition of these substances as indicated in the nitrogen distribution; metaprotein, proteose and peptone from edestin show 6·2 and 30·8; 6·3 and 11·6; 29·1 and 9·8 for amide nitrogen and diamino nitrogen respectively, and accordingly in general, the fractions which correspond to subpeptones, polypeptides and amino acids, will naturally tend to predominate either diamino nitrogen or amide nitrogen due to the chemical nature of mother proteins.

Protein when subjected to the action of superheated water, will be transformed successively into metaprotein, peptone, subpeptone, proteose, polypeptides and amino acids, which are arranged in order of complexity of the molecule. The yield of the lower fractions, as shown in Table II, increased with time of heating, and therefore the chemical reaction on protein-complex proceeded with time and heating to yield substance of less complex nature on the one hand, and on the other to form an insoluble substance of more complex nature. The reactions of superheated water on proteins, in general, are like those of enzymatic hydrolysis, though the chemical nature of the cleavage products differ from the individual proteins.

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