CCXIV. INVESTIGATIONS ON THE SEPARATION OF THE DIGESTION PRODUCTS OF THE PROTEINS BY THE METHOD OF DIFFERENTIAL DIALYSIS.

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BEFORE Hofmeister [1902] enunciated what we now refer to as the peptide hypothesis, researches into the structure of the proteins had been confined almost entirely to attempts to characterise the proteoses and peptones which arise from them by the action of digestive enzymes or of acids and alkalis. This earlier work is now almost forgotten and will probably never be of more than historical interest, for the products obtained by tedious methods of fractionation were not what would be regarded to-day as definite chemical compounds. The peptide hypothesis caused the interest of chemists to swing from the more complex products of protein degradation to the final products, the amino-acids, and researches since that date have been concerned chiefly with the characterisation of these simple products and with the synthesis from them of more complex bodies of known chemical constitution.

Until quite recently it seemed that synthetic polypeptides furnished a type model of the proteins sufficiently accurate to account for most of their chemical characteristics, but with increasing knowledge of polypeptide structure has come increasing knowledge of the proteins themselves, especially of their physical character and of the way in which they are attacked by the proteolytic enzymes. The view that linkages other than the peptide linkage may play an important part in protein structure is growing, and it is probable that before long chemists will again have to turn their attention to the earlier products of degradation of proteins—products which are far more complex than any synthetic polypeptide, and which still retain some of the unique characters of the proteins themselves. The late Prof. Schryver, realising that the older salting-out methods of separation were too tedious and gave products too heavily contaminated with inorganic material to be of use for this purpose, suggested that an easier and more reproducible separation could be obtained by the differential dialysis of the digestion products through membranes of known permeability.

The present research on these lines is to be regarded as a preliminary investigation, for the quantities of material which have been dealt with were small. Sufficient evidence has been adduced however to show that a fractionation of the more complex products of protein digests is possible, and that the method may be of service in any future attempt to isolate and study the composition and properties of proteoses and peptones.

EXPERIMENTAL.

Membranes of graded permeability were made by the method of Pierce [1927], using ethylene glycol as a non-volatile water-soluble swelling agent. A 2 % solution of collodion instead of the 1 % recommended by Pierce was used, as this was found to give membranes which were strong enough to withstand unaided the pressure of the dialysing solution.

5 g. of dry nitrocellulose were placed in a 250 cc. measuring flask and 100 cc. of anhydrous alcohol-ether mixture (1 : 3) were added. After the nitrocellulose had passed into solution (2 days), varying quantities of specially dried ethylene glycol were added, followed by anhydrous alcohol-ether mixture to make 250 cc. The solution was well shaken and kept for use.

A plate of smooth glass of uniform thickness was first well cleaned and dried with a soft cloth. Ether-alcohol mixture was then poured over the surface and wiped off with dry filter paper so as to remove all traces of moisture and fine dirt. The cleaned plate was floated on mercury lying in a Petri dish which had been placed in a CaCl_2 desiccator. A ring or frame 9.6×6.0 cm. (previously dried in an oven at 100°) was placed on the floating plate, and 5 cc. of the collodion solution were poured over them from a dry pipette. The desiccator was quickly closed, and dry air was drawn through the apparatus at a uniform rate for 24 hours. A sulphuric acid trap attached to the exit tube effectively prevented the back entry of moisture. At the end of this time the membrane, still attached to the glass plate, was immersed in water. It then peeled off the plate quite easily and was washed in a stream of running distilled water for 48 hours. To obviate any differences in thickness the dialysing portion of the membrane was confined to the central 6.25×2.5 cm. only. The composition of the membranes is indicated in Table I; they became clogged if used more than once, and a fresh membrane was therefore employed for each experiment.

Table I. *Composition of the membranes for the dialysis of protein digests.*

No.	Nitro-cellulose g.	Ethylene glycol cc.	Alcohol-ether mixture cc.	Nitrocellulose/water ratio
4.0	2	2.0	98.0	1 : 0.996
4.25	2	2.5	97.5	1 : 1.247
4.50	2	3.0	97.0	1 : 1.502
4.75	2	3.5	96.5	1 : 1.763
5.0	2	4.0	96.0	1 : 2.043

The dialysing cells were specially prepared from hollowed out ebonite blocks and had internal dimensions of $6.5 \times 2.8 \times 1.2$ cm. The membranes

were placed between rubber washers and were then clamped one each side of the dialysing cell by means of ebonite rings which could be drawn towards each other by means of long bolts. The stirring which is essential for rapid dialysis was accomplished by passing in a steady stream of washed sterile air. In case of frothing liquids a few drops of capryl alcohol were put in the cells and the air was passed through bubblers containing capryl alcohol. The arrangement was found to be simple and effective.

These cells were completely immersed in distilled water in a specially constructed continuous dialysis apparatus, similar in design to that used by Schryver [1908], wherein the dialysate was continuously withdrawn and replaced by distilled water.

The first series of membranes prepared had nitrocellulose/water ratios ranging from 1 : 0.003 to 1 : 8.24. These were first tried on simple substances such as sodium chloride, ammonium molybdate, glucose, soluble starch, etc. and were found to have a satisfactory selective permeability. The first product of a protein nature to be examined was a "bactopeptone" prepared from meat, the fractional dialysis of which is given in Table II. The amino-N of each

Table II. *Fractional dialysis of bactopeptone.*

(Figures given are in percentages of total nitrogen.)

Membrane No.	Nitrocellulose/water ratio	Total N in the successive fractions obtained	Amino-N	Peptide-N	Non-amino-N
(1)	(2)	(3)	(4)	(5)	(6)
2	1 : 0.003	2.8	2.5	0.2	0.0
3	1 : 0.219	2.8	1.2	0.3	1.3
4	1 : 0.510	39.9	9.1	21.7	9.0
5	1 : 2.083	20.7	3.9	11.0	5.7
6	1 : 4.102	28.9	5.1	11.1	12.5
7	1 : 8.237	5.25	1.2	0.8	0.7

Table III. *Fractionation of bactopeptone by salt precipitation.*

Fraction		% of total N
(A) Primary proteoses	Precipitated by half saturation with Na_2SO_4 at 33°	13.8
(B) Secondary proteoses	Precipitated by full saturation of the filtrate from A	44.4
(C) Peptones	Precipitated by 14 % tannic acid at 20° at p_{H} 7 from filtrate of B	23.8
(D) Sub-peptones	Precipitated by 95 % alcohol from filtrate of C at p_{H} 5	12.4
(E) Simple peptides and amino-acids	Not precipitated by these reagents	5.5
Total		100.0

fraction was determined by the micro-method of Van Slyke, and the peptide-N in a similar way after hydrolysis for 20 hours with an equal volume of concentrated hydrochloric acid. Non-amino-N was obtained by difference, and represents the N which was not in amino- or peptide-form.

To compare the fractionation thus obtained with that given by the older precipitation methods, another sample of the bactopeptone was fractionated

by a modification of the method of Wasteneys and Borsook [1924], as shown in Table III. The two series of fractions thus obtained are correlated in Table IV. Agreement is fair, showing that differential dialysis, which introduces no

Table IV. *Comparison of the fractionation of bactopectone by dialysis and salt precipitation.*

(Figures given are percentages of total nitrogen of the bactopectone.)

Fraction	Dialysis	Precipitation
1	2.8	5.52 Amino-acids and simple peptides
2	2.8	
3	39.9	36.28 {12.4 Sub-peptones 23.8 Peptones
4	20.7	
5	28.9	44.4 Secondary proteoses
6	5.3	
7	0.7	13.80 Primary proteoses

foreign material such as Na_2SO_4 or tannic acid, can be used for the preparation of proteoses and peptones.

After the detailed examination of the fractions of the commercial peptone, the study of the differential dialysis of enzymic hydrolysates of pure proteins was undertaken. The proteins selected for preparing the digests, *i.e.* gelatin, egg-albumin, blood-fibrin, glutelin and caseinogen, were those which could easily be purified and which would be representative of typical groups.

The standard method of preparing a pepsin digest was adopted. Pepsin scales were added to a 2 % solution or suspension of protein in 0.4 % hydrochloric acid and the mixture was incubated at 37° with further additions of pepsin until digestion was complete. Usually a period of 3 months was required for maximum hydrolysis. The digest so produced was neutralised with sodium hydroxide, and the solution was concentrated *in vacuo* below 45°. The concentrated solution was made up so as to give a 10 % solution of the protein digest, and small amounts of chloroform and toluene were added to keep it aseptic. The p_{H} of the digest remained unchanged during the proteolysis.

On account of possible hydrolysis the distribution of nitrogen in the dialysate was not determined directly. It was computed by difference from the initial and final amounts of the forms of nitrogen present in the dialysis cell.

Peptic digest of gelatin.

In the preparation of the digests Coignet's Gold Label gelatin was used. In the present case it took 67 days to reach the point when further addition of pepsin produced no increase in free amino-nitrogen. The distribution of nitrogen in the digest was as follows: amino-N, 26.9 %; peptide-N, 38.4 %; non-amino-N, 34.7 %. It is to be noted that Frankel [1916] did not obtain, with pepsin, protein digests containing more than 20 % of the total amino-N as free amino-N, and consequently came to the conclusion that pepsin hydrolyses only about 20 % of the amide linkages of the proteins. The free amino-nitrogen of

the present digest of pure gelatin shows that (if the enzyme is allowed to act for a sufficiently long time) more than 40 % of the available amino-nitrogen is set free.

It is interesting to compare the non-amino-nitrogen content of the digest with that given by acid hydrolysis. The most recent determination of the distribution of nitrogen in gelatin by a modification of Van Slyke's method [Narayana and Sreenivasaya, 1928] shows 15.87 % arginine, 2.74 % histidine, and 18.8 % non-amino-non-basic nitrogen. Calculating from these figures, the total amount of non-amino-nitrogen is 32.53 %, in fairly close agreement with the 34.7 % obtained in the present digest.

Table V. *Fractional dialysis of a peptic digest of gelatin.*

(Figures given are in percentages of total nitrogen of the digest.)

	Fractions	Total N	Amino-N	Peptide-N	Non-amino-N
	Original digest	100	26.9	38.4	34.7
1	Dialysing out of membrane No. 4	9.8	3.6	1.9	4.3
2	Not dialysing out of No. 4 but dialysing out of No. 4.25	15.3	8.4	0.1	6.9
3	Not dialysing out of No. 4.25 but dialysing out of No. 4.5	42.8	9.1	21.7	11.6
4	Not dialysing out of No. 4.5 but dialysing out of No. 4.75	20.9	3.7	10.2	7.1
5	Not dialysing out of No. 4.75 but dialysing out of No. 5	0.8	0.4	0.0	0.4
6	Not dialysing out of No. 5	10.8	1.7	4.7	4.4

The nitrogen distribution in the various fractions (Table V) shows, as one would expect, a diminishing amount of amino-N and an increasing amount of peptide-N with increasing molecular weight. The non-amino-N is fairly evenly distributed, and does not suggest that proline or hydroxyproline is localised in any inner nuclei.

Tryptic digest of gelatin.

The digest was prepared in the following way. Coignet's Gold Label gelatin was dissolved in warm water and the solution was diluted to 2 %. Sufficient Na_2CO_3 was then added to make 0.4 %. Trypsin (1 g. per 100 g. of gelatin) was added and the solution was incubated at 37°. Further small amounts of trypsin were added from time to time during the next three months until the digest showed no appreciable increase in free amino-nitrogen.

The distribution of nitrogen in the digest shows amino-nitrogen 30.6 %, peptide-nitrogen 15.0 %, and non-amino-nitrogen 54.5 %. Neither gelatin nor its peptic digest when completely hydrolysed yields more than 34 % of the nitrogen in non-amino-form, so that the distribution of nitrogen is altered in favour of the non-amino-form by the prolonged action of trypsin. This curious fact might suggest the existence in the gelatin molecule of bonds which are hydrolysed in a different manner by pepsin or acid and by trypsin, an observation which is of interest in view of recent work on the structure of the gelatin molecule carried out in this laboratory [*cf.* Knaggs and Schryver, 1924].

Table VI. *Fractional dialysis of a tryptic digest of gelatin.*

(Figures are in percentages of the total nitrogen of the digest.)

	Digest	Total N	Amino-N	Peptide-N	Non-Amino-N
		100	30.6	15.2	54.2
1	Dialysing out of No. 4	42.4	11.0	10.5	20.9
2	Not dialysing out of No. 4 but dialysing out of No. 4.25	51.6	18.7	1.3	31.6
3	Not dialysing out of No. 4.25	6.0	0.9	3.4	1.7

Alternatively the large amount of non-amino-nitrogen may be due to diketopiperazines which are stable to hydrolysis with 20 % HCl. It is interesting here to recall that Blanchetière [1928] has observed extensive formation of diketopiperazine rings in a tryptic digest of egg-albumin. Whether these rings existed in the original gelatin molecule, or whether they are secondary products, cannot be profitably discussed here on the scanty evidence available.

A discussion of the individual fractions of the differential dialysis brings out certain interesting points.

Fraction 1. The distribution of nitrogen in this fraction shows that it contains most of the peptide-nitrogen of the digest. The peptide-nitrogen is therefore present almost entirely in the form of simpler polypeptides, which could, presumably, be hydrolysed almost completely by erepsin.

Fraction 2. This fraction is nearly half of the digest and consists mainly of amino- and non-amino-nitrogen which are present in the low ratio of 1 : 2.5. The peptide-nitrogen is almost negligible indicating that simple polypeptides must be absent. Linkages which may or may not involve nitrogen must be present in the compounds of this fraction, for although these contain amino-acids as components, it is clear that the amino-groups are all free, and that the acids must be linked in some way through their carboxyl groups. It is possible that the trypsin has attacked peptide bonds in the outer region of the protein molecule, leaving nuclei composed of amino-acids or other substances united by linkages that may not be of peptide nature. Such nuclei might be composed chiefly of amino-acids which do not contain reactive amino-groups—suggesting the “arginine nuclei” of Kossel [1901] or the kyrines of Siegfried [1904].

Peptic digests of egg-albumin, blood-fibrin, glutelin and caseinogen.

Full analyses of these digests have been made, and a condensed summary of the results is given in Table VII. In each case the fractions were obtained by using membranes similar to those referred to in Table I. The fractions show an increasing ratio of peptide-N to amino-N. The non-amino-N which, in the case of each digest, is approximately equal to the value which can be calculated from the known amino-acid composition of the respective protein, shows irregularities which seem worth recording.

One final observation which deserves mention is the high degree of proteolysis brought about by pepsin in all of these digests. Frankel [1916] found that practically all proteins he examined were acted upon by pepsin to the same

extent. In the present research it has been found that under the prolonged action of pepsin each protein is hydrolysed to a different degree and the maximum proteolysis obtained is much more than that obtained by Frankel (Table VIII).

Table VII. *Showing the fractional dialysis of peptic digests of certain proteins.*

(Figures given in percentages of total nitrogen of the digests.)

Fraction (Peptone)	Egg-albumin				Blood-fibrin			
	Total N	Amino-N	Peptide-N	Non-amino-N	Total N	Amino-N	Peptide-N	Non-amino-N
	100	24.0	62.2	13.8	100	24.2	46.0	29.8
1	8.7	1.7	3.1	3.9	9.7	4.6	2.1	3.0
2	26.5	6.2	17.6	2.7	5.9	0.6	3.0	2.3
3	40.4	12.4	27.0	1.0	22.3	10.5	11.3	0.5
4	24.4	2.7	15.5	6.2	12.8	0.3	4.0	8.5
5	—	—	—	—	22.8	5.5	10.5	6.8
6	—	—	—	—	26.5	2.7	15.1	8.7

Fraction (Peptone)	Glutelin				Caseinogen			
	Total N	Amino-N	Peptide-N	Non-amino-N	Total N	Amino-N	Peptide-N	Non-amino-N
	100	29.9	32.1	38.0	100	26.2	44.6	29.2
1	26.5	8.2	7.2	11.1	23.2	4.4	11.2	7.6
2	1.4	2.1	0.0	0.7	8.3	4.0	0.7	3.6
3	47.5	14.6	13.4	19.5	8.3	1.9	—	6.5
4	1.3	1.1	0.2	0.0	48.9	13.8	25.6	9.5
5	3.3	1.2	0.3	1.8	3.3	0.9	0.9	0.5
6	21.0	2.7	11.0	7.3	8.0	1.2	6.3	1.5

Table VIII. *Total amino-nitrogen of various proteins liberated by pepsin.*

Protein	Approximate time of proteolysis (months)	% of amino-N
Gelatin	3	41.2
Egg-albumin	3	28.0
Fibrin	4	34.5
Glutelin	4	48.0
Caseinogen	3	37.0

SUMMARY.

A method of differential dialysis suitable for the investigation of the digestion products of the proteins has been worked out.

The dialysing membranes of graded permeability are prepared from nitro-cellulose dissolved in an ether-alcohol mixture to which is added a suitable amount of ethylene glycol as a non-volatile water-soluble swelling agent [Pierce, 1927].

Peptic digests of gelatin, egg-albumin, blood-fibrin, glutelin and caseinogen as well as a tryptic digest of gelatin and "bactopeptone" have been fractionated by these membranes.

Fractions of increasing molecular complexity, as shown by estimations of free amino- and peptide-nitrogen, can be readily obtained, and the method

seems suitable for the preparation of these substances in amounts sufficient for more complete chemical analysis.

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