XLIV. INVESTIGATIONS ON THE HYDROLYSIS PRODUCTS OF FISH MUSCLE.

I. THE DIAMINO-NITROGEN OF COD MUSCLE.

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IN determining the diamino-nitrogen in the products of hydrolysis of proteins, Kernot and Knaggs [1928] showed that the results depend on several factors and, amongst them, on the temperature of precipitation and the temperature of the solution whilst the precipitate is settling.

Their results were confirmed by Daft [1929] who suggested that, in order to avoid discrepancies, a rigidly defined technique should be followed. Rosedale more recently [1929], in determining the diamino-content of fish muscle, came to the conclusion that results are not so much influenced by temperature as by the incompleteness of the hydrolysis, since in this case material which cannot be considered as consisting of diamino-acids is precipitated by phosphotungstic acid.

There is, however, no experimental evidence as to products of incomplete hydrolysis being precipitated with the diamino-fraction, but, as pointed out by Kernot and Knaggs [1928], proline, tryptophan, phenylalanine and hydroxy-aminobutyric acid have been found in this fraction.

The object of the present communication has been to find out how far the completeness of the hydrolysis could influence the results. If the hydrolysis is incomplete peptides may be found in the basic fraction but it is doubtful whether they could resist the prolonged action of a 20 % solution of sulphuric acid. This, however, would not be the case with some keto-piperazides which are so highly resistant to the action of acids and alkali that the question has arisen whether such products could be considered as primary constituents of the protein molecule. A preliminary attempt has been made to separate some of these products in order to find out whether they would be precipitated by phosphotungstic acid.

EXPERIMENTAL.

Cod muscle, from recently caught fish, was the material used for these experiments. The tissue was freed, as far as possible, from adjacent tissues and then dropped into boiling water, the boiling being continued for one hour. The coagulated muscular tissue was strained on a cheese cloth washed with

distilled water and suspended in chloroform water. After 24 hours the chloroform water was changed and the treatment repeated 5 or 6 times. It was then again strained, digested with 95 % alcohol and then twice extracted with absolute alcohol. After a further extraction with ether it was air-dried.

Estimation of the diamino-nitrogen.

Four portions of 25 g. each of the dried muscle were hydrolysed with 600 cc. of a 20 % solution of sulphuric acid for 12, 24, 48 and 96 hours respectively. The hydrolysate was diluted with water and barium hydroxide added until it was only very slightly acid to Congo red. The barium sulphate precipitate was removed by filtration and the precipitate digested four times with boiling water. These washings were added to the hydrolysate, and the combined filtrates, to which a small excess of barium carbonate had been added, were evaporated to dryness under reduced pressure. The residue was taken up with water, filtered, made up to a known volume and the nitrogen estimated by the Kjeldahl method.

Aliquot parts of these solutions were taken and made up to 100 cc. so that each contained 80 to 85 mg. of nitrogen.

The diamino-nitrogen was precipitated with phosphotungstic acid according to the method of Osborne and Harris [1903], the conditions of temperature being as follows.

(a) The hydrolysate and the phosphotungstic acid solution were separately heated to 100°, and after addition of the latter to the hydrolysate the solution was allowed to cool and stand in an incubator at 37° for 48 hours.

(b) The hydrolysate and the phosphotungstic acid were separately heated to 37°, and after precipitation kept in an incubator at 37° for 48 hours.

(c) The hydrolysate and the phosphotungstic acid were separately cooled to 0°, and after precipitation were allowed to stand at the same temperature for 48 hours.

The experiments were carried out in duplicate, and the average results are given in Table I.

Table I.

Time of hydrolysis i n hours	% of N precipitated by phosphotungstic acid		
	(a)	(b)	(c)
12	36.84	39.47	43.65
24	30.67	36.16	40.11
48	25.64	31.31	37.28
96	23.85	30.40	35.83

These results show that the percentage of diamino-nitrogen precipitated by phosphotungstic acid depends on the conditions of precipitation, and not only on the completeness of the hydrolysis.

Rosedale states that in the case of fresh fishes hydrolysis appears to be completed between 40 hours (carp) and 80 hours (tunny). We have found that the hydrolysis of the muscular tissue of fish may be considered as complete after 24 hours, as then the percentage of total amino-nitrogen

reaches its maximum. If the time of hydrolysis is prolonged this total diminishes while the percentage of nonamino-nitrogen increases, possibly owing to the formation of ring compounds.

In the following section we give a preliminary account of experiments carried out with the object of obtaining, if possible, some evidence on this subject.

Separation of some products of partial hydrolysis.

Abderhalden [1923] and his co-workers have, by means of solvents, isolated several products of the partial hydrolysis of proteins. The method consisted in evaporating the hydrolysate to dryness under reduced pressure, mixing the residue with sand and extracting it with solvents. By this method it is possible to use a great variety of solvents, but as all ketopiperazines are soluble in many solvents which are not miscible with water it was thought that a modification of the method suggested by Dakin [1912, 1920] could be used.

By extracting the hydrolysates from proteins with butyl alcohol, under the conditions described by Dakin, the proline fraction could never be obtained free from diketopiperazines, and in order to avoid this, Osborne, Leavenworth and Nolan [1924] suggested that the extraction should be carried out at room temperature. Even in these circumstances, however, ketopiperazines were found to be constantly present in the fraction.

In the following experiments, besides butyl alcohol, ethyl acetate and chloroform were used. The extractions were carried out by shaking the hydrolysates in a separating funnel with an excess of the solvent; they were repeated until a small portion of the solvent on evaporation left no residue. As in the previous section the experiments were carried out in duplicate and the figures given represent averages.

No attempt has been made, at this stage, to identify the different products obtained.

Ethyl acetate extract. The following percentages of the total nitrogen in the hydrolysate were extracted by this solvent.

Time of hydrolysis in hours	12	24	48	96
% N	2.02	0.25	0.16	0.10

This substance has all the characters of a diketopiperazine as it is practically insoluble in water, sparingly soluble in cold ethyl alcohol, but easily in hot ethyl alcohol, from which it crystallises on cooling in fine needles melting at 175° (not corrected). It is very soluble in chloroform and ethyl acetate, but little if at all soluble in hot dilute hydrochloric acid.

Butyl alcohol extract. This extract was evaporated to dryness *in vacuo* in small portions. The residue was taken up with alcohol, filtered through a Büchner funnel and the insoluble portion washed with small quantities of hot butyl alcohol. The two alcoholic filtrates were evaporated *in vacuo* to dryness and the residue taken up with absolute alcohol. This treatment was repeated again; the insoluble portion was separated by filtration and the

clear, red-brown, alcoholic solution was evaporated *in vacuo* until all the alcohol was removed. The residue was treated with warm water and a not inconsiderable amount of a brown product was removed by filtration on a Büchner filter. This residue was dissolved in absolute alcohol and the alcoholic solution evaporated *in vacuo* in tared dishes. The following percentages of the total nitrogen were thus obtained.

Time of hydrolysis in hours	12	24	48	96
% N	1.36	0.65	0.47	0.82

By treatment with ether part of this substance was dissolved. The ethereal solution evaporated in tared dishes gave the following results expressed as before in percentages of the total nitrogen.

Time of hydrolysis in hours	12	24	48	96
% N	0.70	0.36	0.21	0.56

This substance has all the properties of a ketopiperazine. It decomposes at 245° and then immediately volatilises.

The portion insoluble in ether was crystallised from absolute alcohol, from which it separated in fine hair-like needles; with the exception of the insolubility in ether it presented the same characters as the other substance. The ready solubility in ethyl acetate and chloroform, and its behaviour towards dilute solutions of acids and alkalis leave little doubt that it is a ketopiperazine.

Chloroform extract. A brown sticky mass was extracted by this solvent, which, owing to its semi-solid condition, was not weighed.

From these results it is evident that even after a prolonged hydrolysis ring compounds can be found amongst the products. But it is important to note that, in the case of the butyl alcohol extract, these compounds after reaching a minimum rapidly increase, thus showing that by prolonging the time of hydrolysis condensation takes place and this is confirmed by the increase of the nonamino-nitrogen.

SUMMARY.

1. The muscular tissue of the common cod was hydrolysed with sulphuric acid for varying periods. It was found that the percentages of basic nitrogen precipitated by phosphotungstic acid vary for a given period of hydrolysis with the conditions of precipitation, thus confirming the previous results obtained.

2. Evidence is given to show that even after a prolonged hydrolysis closed ring compounds or ketopiperazines are found in the hydrolysate, and that their percentage increases if the hydrolysis is prolonged.

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