LXVII. THE ESTIMATION OF THE DIAMINO-NITROGEN IN THE PRODUCTS OF HYDROLYSIS OF PROTEINS.

By JOSEPH CHARLES KERNOT and JOHN KNAGGS.

From the Fish By-products Research Laboratory, Imperial College of Science and Technology, London, S.W. 7.

(Report to the Food Investigation Board—Department of Scientific and Industrial Research.)

(Received March 6th, 1928.)

The discordant results obtained in protein analysis by following the method suggested by Van Slyke [1911, 1915] are attributed by every investigator to factors which arise in the precipitation and treatment of the phosphotungstates of the hexone bases.

Kutscher [1900] and Chittenden and Eustis [1900] state that the precipitation of diamino-acids by phosphotungstic acid is not complete. Osborne, Leavenworth and Brautlecht [1908] in their work on vegetable proteins also noted considerable discrepancies in the results obtained by direct estimation of histidine, arginine, and lysine by the methods suggested by Kossel and Kutscher [1901] and Kossel and Patten [1903], and the results obtained by estimating the same amino-acids by precipitation with phosphotungstic acid. Görtner and Hoffman [1925] found that the temperature at which the precipitation takes place has a considerable influence on the results. This was contradicted by Plimmer and Rosedale [1925] who state that no significant difference was produced by allowing the precipitate to stand at 0° instead of at room temperature. They also state that prolonging the time of standing of the precipitate has no particular advantage. In their opinion the origin of the discrepancies lies in the solubilities of the phosphotungstates.

The experience of the writers in the course of analytical work on a considerable number of proteins has confirmed the views mentioned at first, and the object of the present investigation has been to find out how far the results obtained could be influenced by the temperature at which the precipitation takes place, and by the concentration of the nitrogen in the hydrolysate.

In the course of these investigations ossein and the gelatin obtained from it were the materials used, in addition to dry cod-skins on which we were working at the time for other reasons. The ossein was carefully decalcified, treated for 60 days with 0·2 % NaOH, electrolysed, and treated finally with 0·5 % HCl for 4 days.

The different conditions under which the hydrolysis was carried out are given on the curves Figs. 1 and 2.

In every case, after hydrolysis, the hydrochloric acid, amide-N and humin-N were removed in the usual way, and the residue was taken up with water and filtered.

The solution was made up to a known volume and the nitrogen content determined by Kjeldahl's method.

Aliquot parts of this solution were taken, and the volume was adjusted to 100 cc. The diamino-acids were precipitated from this solution, which contained 5 % H_2SO_4, with an excess of phosphotungstic acid, following the method of Osborne and Harris [1903; see also Drummond, 1918], *i.e.* 30 cc. of phosphotungstic acid were added in each case so that the volume of solution used was constant throughout the experiments.

The basic fraction was precipitated under different conditions of temperature as follows:

(*a*) at 20°, filtered after 24 hours and washed;

(*b*) the solution was heated to 100°, the phosphotungstic acid was added, and the solution was allowed to cool and stand for 24 hours at laboratory temperature, then filtered and washed with a constant volume of liquid.

The mono-amino-N in the filtrate was estimated by the Van Slyke method for amino-N. Figs. 1 and 2 give the results obtained for the percentage of diamino-N.

It is evident from the curves that the following factors play a very important part in the precipitation of diamino-N by phosphotungstic acid.

(*a*) The concentration of the nitrogen in solution.

(*b*) The temperature of the solution during precipitation.

(*c*) The concentration of the acid used for the hydrolysis of the protein.

(*d*) The treatment with cold acid before hydrolysis.

The effect of the last-named factor has been discussed already in a paper by Knaggs [1923]. In the work there described the gelatin was hydrolysed with 20 % HCl but the concentration of nitrogen in solution was not varied, and the precipitation with phosphotungstic acid was carried out at laboratory temperature only.

(*a*) *Concentration of the nitrogen in solution.*

As the concentration of the nitrogen in solution is increased, the percentage of it precipitated by phosphotungstic acid increases also. At high concentrations, especially in the case of gelatin, there is a gradual decrease (Fig. 1, curve A). But this is not the case with ossein, which has been partially hydrolysed in the cold with 20 % HCl, as the amount precipitated increases regularly with the concentration. Ossein and cod-skins give a nearly constant value over a wide range of concentrations.

It is impossible to give a satisfactory explanation of the anomaly shown by gelatin as we have not an exact knowledge of the constituents of its hydrolysis products. The anomaly, however, may be due to influences of solubility.

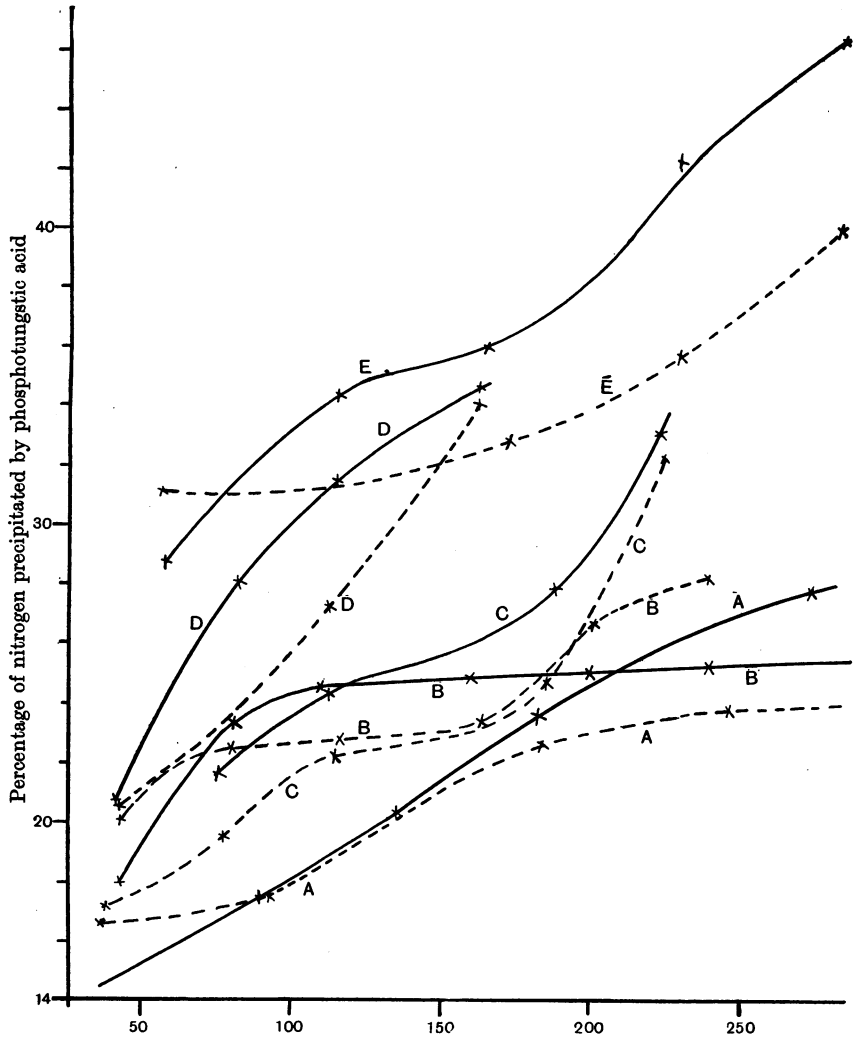

Fig. 1.

A. Gelatin from ossein hydrolysed by 20 % HCl.
B. Ossein hydrolysed by 20 % HCl.
C. Ossein treated with cold 20 % HCl for 10 days and hydrolysed.
D. Ossein hydrolysed by 5 % HCl.
E. Ossein treated with 20 % HCl at 25° for 10 days.

——— Basic nitrogen precipitated from cold solution.
– – – – – – „ „ hot „

(b) Temperature of the solution during precipitation.

The percentage of basic nitrogen precipitated from hot solution is generally lower than that precipitated at laboratory temperature. The experiments carried out with cod-skins which had been treated with a cold solution of 0·5 % HCl and then hydrolysed with the same concentration of acid (Fig. 2 D) do not seem to confirm this. The anomaly is probably due to incomplete hydrolysis.

(c) The concentration of the acid used for the hydrolysis.

The percentage of basic nitrogen obtained from the product of hydrolysis with weak acids is higher than that obtained from the precipitation of the diamino-fraction from the hydrolysis products for which more concentrated acids are used. This is shown to be the case for ossein in Fig. 2.

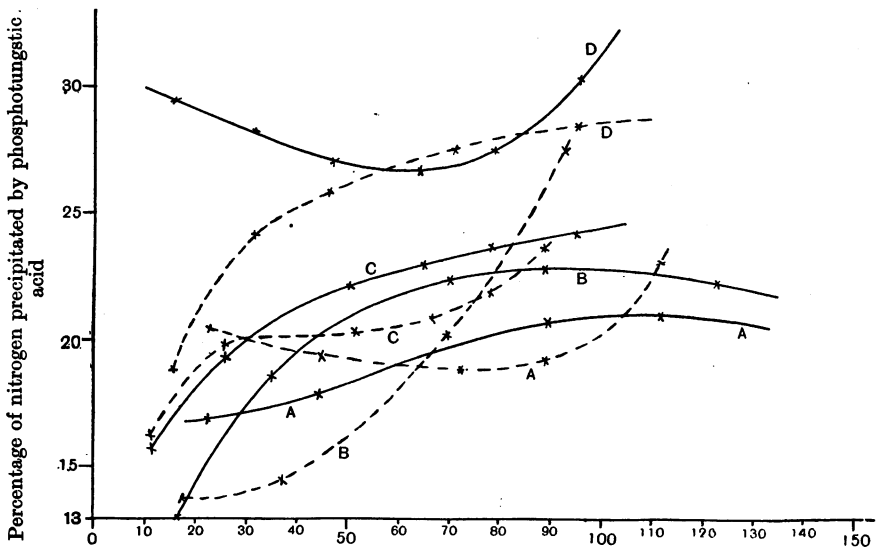

Fig. 2. Dry cod-skins.

A. Immediately hydrolysed with 20 % HCl.
B. Immediately hydrolysed with 5 % HCl.
C. Standing 10 days with 20 % HCl and then hydrolysed at 100° with 20 % HCl.
D. Standing 10 days with 0·5 % HCl and then hydrolysed at 100° with 0·5 % HCl.

------ Basic nitrogen precipitated from hot solution.
——— „ „ cold „

It is noticed that the basic values obtained from gelatin and ossein are very different. This seems to be evidence in favour of the view expressed by Emmett and Gies [1907] that collagen is not merely an anhydride of gelatin, as suggested by Hofmeister [1878], but an entirely different compound which produces gelatin by an intramolecular rearrangement.

(d) The treatment with cold acid before hydrolysis.

The increase shown in the percentage of basic nitrogen when the protein is allowed to stand with cold acid before hydrolysis is evidence of the fact that other products, probably ring-compounds, are precipitated by phosphotungstic acid. This has been observed in the case of ossein and cod-skins, as shown by Fig. 1 C and Fig. 2 C.

THE RESULTS FOR THE MUSCULAR TISSUE OF FISH.

In the course of investigations on fish muscular tissue, similar results to those given above were obtained. These are given in Table I.

Table I.

Muscular tissue of	N in solution mg.	% of basic N precipitated from cold solution	% of basic N precipitated from hot solution
Shark (red)	40·6	30·9	30·2
	81·2	35·6	31·9
	102·0	42·6	—
Shark (white)	40·8	35·3	32·2
	81·6	42·3	35·4
Cod	84·5	34·4	33·5
	120·0	45·2	—
Haddock	54·3	39·3	33·2
	108·6	44·3	43·1
	142·0	46·4	—

PRECIPITATION OF THE BASES AT 0°.

Experiments were carried out by precipitating the hydrolysate which had been cooled to 0° with phosphotungstic acid solution kept at the same temperature. For comparison aliquot parts of the same solution were precipitated at a temperature of 37°, and in each case the solutions were allowed to stand for 24 hours at the temperature of precipitation before filtration. This was carried out very quickly and the precipitates were washed with dilute phosphotungstic acid which had been kept at the same temperatures. The results are given in Table II.

Table II.

	Nitrogen in 100 cc. solution mg.	% diamino-N precipitated from solution cooled to 0°	% diamino-N precipitated from solution at 37°
Ossein. Allowed to stand 10 days with 5 % HCl and hydrolysed	182·8	37·4	21·7
Cod-skin dry. Hydrolysed with 20 % HCl	112·5	25·7	21·1
Hydrolysed with 5 % HCl	88·5	32·2	19·6
Allowed to stand 10 days with 20 % HCl and hydrolysed	64·9	38·4	23·5
Allowed to stand 10 days with 0·5 % HCl and hydrolysed	79·8	35·6	24·6
Skate-skins. Hydrolysed with 20 % HCl	50·4	20·8	13·9
Hydrolysed with 0·50 % HCl	52·0	24·4	15·5
Allowed to stand with 20 % HCl and hydrolysed	63·0	19·5	17·1

It will be seen from the above results that a considerably greater percentage of diamino-N is precipitated at 0° than at 37°, thus confirming the results of Görtner and Hoffman.

The problem is to establish whether the whole of the precipitate is due to products of a basic nature. This does not seem to be the case, however, according to the above-mentioned authors, who found, amongst the products precipitated, hydroxyaminobutyric acid.

Knaggs [1923] has previously shown how the variations obtained in the Hausmann numbers for gelatin depended on the preliminary treatment of the sample of gelatin or its precursors. In particular the diamino-N showed the greatest variations. It was suggested that the discrepancies were due to the formation of peptides which were resistant to hydrolysis and were precipitated by phosphotungstic acid.

Apart from this possible explanation the suggestion made by Plimmer and Rosedale [1925] that the differences observed may be due to the solubilities of the phosphotungstates in excess of the reagent is worth considering.

In the results shown in this paper it is noticeably the fact that a considerably higher percentage of diamino-N is obtained in all those cases in which the hydrolysis is carried out with dilute acids, thus showing that peptides which are more resistant to hydrolysis are precipitated by phosphotungstic acid. It is evident that there is a great difference in the solubility of these phosphotungstates at the different temperatures, apart from the fact that such phosphotungstates may be hydrolysed at high temperature and therefore will not precipitate on cooling.

Furthermore, it has been shown by different investigators that other hydrolysis products, apart from peptides, are precipitated by phosphotungstic acid. Schulze and Winterstein [1901] showed that certain amino-acids, especially phenylalanine, were precipitated by this reagent.

Görtner and Sandstrom [1925], using artificial mixtures of amino-acids, proved that the presence of proline and tryptophan led to errors in the basic fractions, these substances being partially precipitated.

Admitting that the errors may be due to the causes mentioned by the above investigators, we think that the presence of peptides precipitated by phosphotungstic acid offers a better explanation.

Investigations carried out in this laboratory have led to the isolation of at least two peptides in the diamino-fraction of gelatin from isinglass, one of them soluble in ethyl acetate and identified as alanylalanine (Speer, privately communicated).

The necessity of standardising the technique in the analytical study of the hydrolysis products of proteins by the Van Slyke method becomes more and more evident as the data available increase.

SUMMARY.

The percentages of nitrogen precipitated by phosphotungstic acid from the products of hydrolysis of a protein depend on several factors.

(*a*) The concentration of nitrogen in the solution.

(*b*) The conditions of precipitation, *e.g.* temperature of precipitation and temperature of the solution whilst the precipitate is settling.

(*c*) The concentration of the acid used for the hydrolysis of the protein.

(*d*) Period of contact of the protein with the cold acid before hydrolysis.

REFERENCES.

Chittenden and Eustis (1900). *Amer. J. Physiol.* **3**, Proc. xxxi.
Drummond (1918). *Biochem. J.* **12**, 3.
Emmett and Gies (1907). *J. Biol. Chem.* **3**, xxxiii.
Görtner and Hoffman (1925). *J. Amer. Chem. Soc.* **47**, 580.
Görtner and Sandstrom (1925). *J. Amer. Chem. Soc.* **47**, 1663.
Hofmeister (1878). *Z. physiol. Chem.* **2**, 299.
Knaggs (1923). *Biochem. J.* **17**, 473.
Kossel and Kutscher (1901). *Z. physiol. Chem.* **31**, 165.
Kossel and Patten (1903). *Z. physiol. Chem.* **38**, 39.
Kutscher (1900). *Z. physiol. Chem.* **31**, 215.
Osborne and Harris (1903). *J. Amer. Chem. Soc.* **25**, 323.
Osborne, Leavenworth and Brautlecht (1908). *Amer. J. Physiol.* **23**, 180.
Plimmer and Rosedale (1925). *Biochem. J.* **19**, 1005.
Schulze and Winterstein (1901). *Z. physiol. Chem.* **33**, 574.
Van Slyke (1911). *J. Biol. Chem.* **10**, 15.
— (1915). *J. Biol. Chem.* **22**, 281.