LVIII. SOME NOTES ON THE DETERMINATION OF THE HAUSMANN NUMBERS OF PROTEINS.

By JOHN KNAGGS.

From the Biochemical Department, Imperial College of Science and Technology.

A Report to the Adhesives Committee of the Department of Scientific and Industrial Research.

(*Received June 6th, 1923.*)

THE modification of Hausmann's method by Osborne and Harris [1903] does not give consistent results for gelatin as the following determinations made with three different samples show.

Gelatin	% diamino N
Coignet's Gold label	31·7
	20·8
	20·1
	19·7
Coignet's Gold label which had been washed with frequent changes of water for 10 days	29·9
	29·9
	19·6
Gelatin extracted from ossein at 100°	33·7
	20·3

The percentage of diamino nitrogen varies considerably, and as these results were determined in duplicate under the same conditions and the duplicates were found to give the same result it was concluded that the variations were due to the difference in treatment to which the hydrolysed product was subjected before precipitation.

On referring to the literature it was found that variable results were obtained by different workers.

	% diamino N
Hausmann (1899)	35·83
Van Slyke (1911)	25·5
Bogue (1920)	22·75
	21·68
	22·1
	26·47

These variations are far larger than can be accounted for by differences in the samples analysed and must be due to some faults inherent in the method.

Attempts were therefore made to ascertain the cause of these discrepancies. It was thought that it was due to the formation of a polypeptide under certain conditions. [Compare Dakin, 1920.]

It was quite conceivable that gelatin, in the presence of cold acid, which produces only incomplete hydrolysis, might form certain polypeptides which are only hydrolysed with difficulty.

Therefore experiments were carried out in which the gelatin was heated immediately on adding the acid, and after allowing the gelatin to stand in contact with the cold acid for 24 hours at laboratory temperature.

It was found that the percentage of diamino nitrogen was always higher when the gelatin had been allowed to stand with cold acid for a few hours as the following results show:

	Coignet's Gold label	Gelatin from ossein
Immediately hydrolysed for 12 hours	20·6	25·03
Hydrolysed for 12 hours after standing 24 hours at lab. temp.	24·25	35·62
" " " " 37°	23·7	34·65

It was noticed that where the results were high a secondary precipitate was formed after filtration of the phosphotungstates. This is probably due to the formation of some product of hydrolysis of gelatin which gave a precipitate with phosphotungstic acid which was somewhat soluble in water so that variations in the results were obtained with very slight variations in the conditions.

Experiments were then carried out to determine the rates at which peptide scission and formation of amino groups takes place, with gelatin hydrolysed immediately after mixture with the acid, and after standing with cold acid solutions. It was found that the maximum amount was formed in one hour in the first case and not until after 3 hours in the second case, indicating the formation of resistant groups.

	% hydrolysis	
	A	B
	Gelatin hydrolysed immediately	Gelatin hydrolysed after standing with acid at lab. temp. 24 hours
0 mins.	4·5	8·9
15 "	60·9	—
30 "	62·6	46·6
45 "	68·3	—
60 "	75·8	65·8
1·5 hrs.	75·8	—
2 "	75·8	70·6
3 "	75·4	74·8
6 "	75·7	75·1
28·5 "	75·6	75·0

The rate of hydrolysis was followed by Van Slyke's method for amino nitrogen.

In the case of A the percentage of diamino nitrogen was also determined at various intervals and it was found that although the amino nitrogen reached its maximum after 1 hour the diamino nitrogen did not reach a constant until 12 hours.

Time of hydrolysis in hours	% amino N	% diamino N
3	0·93	36·1*
6	1·0	25·7*
9	1·06	36·6*
12	1·1	20·05
16	1·1	20·09
20	1·1	25·1*†
24	1·1	20·0
28·5	1·23	20·1

* The filtrate from the phosphotungstate precipitate on standing gave a further precipitate.

† This result was abnormal as conditions were not observed which were found out later to have an effect on the results, *i.e.* the solution was allowed to stand overnight with the acid before precipitating.

It was also found that if the solution containing the diamino acids were allowed to stand some time with 5 % H_2SO_4 abnormal results were obtained.

Gelatin was hydrolysed for 30 hours with 20 % HCl. The amide nitrogen and humin nitrogen were removed. The filtrate was concentrated to 100 cc. and 5 g. H_2SO_4 added. The following results were obtained:

	% diamino N	Type of precipitate
1. Diamino acids precipitated immediately solution was cold	20·4	Granular and easily filtered
2. Diamino acids precipitated after allowing solution to stand 24 hours with 5 % H_2SO_4	23·9	Flocculent ppt. and secondary ppt. which separated after standing
3. Diamino acids precipitated after allowing solution to stand 48 hours with 5 % H_2SO_4	25·1	,, ,,
4. Same as 3 only diamino acids precipitated in warm solution and allowed to stand 24 hours before filtering	20·1	Granular

The results indicate that some change takes place when the amino acids are allowed to stand with acid in the cold. Probably a polypeptide is formed which is precipitated by phosphotungstic acid.

Experiments were carried out to prove the above. The gelatin was hydrolysed for 3 hours with 20 % HCl. After the amide and humin nitrogen had been removed the solution was made up to 100 cc. in 5 % H_2SO_4 and heated for varying lengths of time at 120° in the autoclave. The diamino acids were precipitated with 20 % phosphotungstic acid on cooling.

Method of precipitating diamino acids	% diamino N	Type of precipitate
1. Add 3 cc. H_2SO_4 and 30 cc. of 20 % phosphotungstic acid after removing amide and humin nitrogen	36·04	Gelatinous
2. Add 3 cc. H_2SO_4 and heat under reflux for 1 hour before precipitating	24·00	Flocculent
3. Add 3 cc. H_2SO_4 and heat in autoclave for 1 hour before precipitating	24·1	,,
4. Heat for 2 hours at 120°	23·25	,,
5. ,, 3 ,, ,,	20·5	Granular
6. ,, 4 ,, ,,	20·2	,,

These results indicate that the correct values for diamino nitrogen may be obtained by hydrolysing immediately for 3 hours, heating the solution with 5 % H_2SO_4 in the autoclave for 3 hours at 120° and precipitating the diamino acids immediately on cooling.

If the solution is allowed to stand a few hours before adding the phosphotungstic acid solution the precipitate will be flocculent or gelatinous instead of granular as the following show:

Gold label gelatin hydrolysed 3 hours with 30 % HCl

Method of precipitating diamino acids	% diamino N	Type of precipitate
1. Evaporate to 100 cc. add 3 cc. H_2SO_4 and 30 cc. of 20 % phosphotungstic acid	25·8	Flocculent
2. Evaporate to 100 cc. add 3 cc. H_2SO_4 and heat for 3 hours in autoclave at 120°	20·6	Granular
3. As 2 above, but allow solutions to stand 24 hours before precipitation with phosphotungstic acid	30·5	Flocculent with secondary gelatinous ppt.
4. As 2 above; allow to stand 4 days before ppt.	24·1	Flocculent
5. As 4 but filter immediately on adding phosphotungstic acid	20·4	—
6. As 4 above but heat to boiling before ppt.	20·7	Granular

The experiment was repeated with Coignet's "Gold Label Gelatin" which was allowed to stand with the acid 48 hours at laboratory temperature before hydrolysing for 12 hours.

Method of precipitating diamino acids	% diamino N	Type of precipitate
1. Ordinary way by acidifying solution to 5 % H_2SO_4 and ppt. with 30 cc. of 20 % phosphotungstic acid	24·5	Flocculent
2. Add 3 cc. H_2SO_4 and evaporate to 100 cc. Allow solution to stand 4 days then heat to 100° and when cold add the phosphotungstic acid solution	24·0	,,
3. Evaporate to 100 cc. add 3 cc. H_2SO_4 and heat for 3 hours at 120° in autoclave and then ppt.	24·5	,,
4. Evaporate to 100 cc., add 3 cc. H_2SO_4 and heat for 3 hours at 120° in autoclave. Allow to stand at lab. temp. 2 days before ppt.	30·9	Gelatinous ppt.

The latter results show that when the cold acid is allowed to be in contact with the gelatin for some hours before heating some product is formed which resists hydrolysis even after 12 hours with 20 % HCl and a subsequent heating with 5 % H_2SO_4 at 120° in the autoclave before precipitation of the diamino acids.

In all the above cases where a high value for the percentage of diamino nitrogen was obtained the precipitate formed with phosphotungstic acid was flocculent or gelatinous, and took some time to settle, whereas those which were normal gave a granular precipitate which settled quickly and was easily filtered.

To obtain a correct value for the percentage of diamino nitrogen in the hydrolysis products of gelatin the following precautions should be observed:

1. On mixing the protein and the acid for hydrolysis, the mixture should be heated to boiling almost immediately for 12 hours.

2. The solution which contains the diamino acids should not be allowed to stand any length of time before the phosphotungstic acid solution is added. If the solution has been standing with the H_2SO_4 it should be heated to boiling for a few minutes and when cool the diamino acids precipitated with 20 % phosphotungstic acid solution.

A correct result for the percentage of diamino nitrogen may be obtained by hydrolysing the gelatin for 3 hours with 20 % HCl at boiling point and, after removing the amide and humin nitrogen, adjusting the solution containing the diamino acids to 200 cc. and adding 3 cc. H_2SO_4. This solution should then be heated for 3 hours in the autoclave at 120° and when cool the phosphotungstic acid solution should be added. This method cannot be applied when the complete analysis is required seeing that the amide group is not entirely broken down until the gelatin has been hydrolysed with 20 % HCl for 12 hours.

All the difficulties encountered hitherto, appear to be due to the fact that the hydrolysis products condense in the presence of cold acids and these condensation products cause the precipitation of gelatinous phosphotungstates.

Experiments were made to find another precipitant for the diamino acids other than phosphotungstic acid. The following alkaloidal reagents were tried: Bruckes' reagent, Mayer's reagent, cadmium iodide, tannic acid. None of these gave a precipitate with diamino acids in acid solution. Tannic acid only precipitates in neutral solution. This precipitate was examined and found to give the same result as the phosphotungstic acid precipitate.

The above work was carried out under the direction of Professor S. B. Schryver.

REFERENCES.

Bogue (1920). *Chem. Met. Eng.* **23**, 154.
Dakin (1920). *J. Biol. Chem.* **44**, 499.
Hausmann (1899). *Z. physiol. Chem.* **27**, 95.
Osborne and Harris (1903). *J. Amer. Chem. Soc.* **25**, 323.
Van Slyke (1911). *J. Biol. Chem.* **10**, 15.