CLI. A MICRO-METHOD FOR THE DETERMINATION OF THE HAUSMANN NUMBERS OF PROTEINS.

By KENNETH VIVIAN THIMANN.

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

(*Received October 11th, 1926.*)

The Hausmann numbers, or the determination of the partition of nitrogen, should be a rapid and convenient method for establishing definite criteria for a particular protein. This analysis, however, originally due to Hausmann [1899] and modified by Osborne and Harris [1903], has been subjected to criticism on several scores. In particular, several workers have found the estimation of the diamino-nitrogen, or basic fraction, to give unreliable results, which cannot always be duplicated. This fraction has been shown [Osborne, Leavenworth and Brautlecht, 1908] to correspond approximately with the amounts of the diamino-acids present in the protein, yet the results obtained by different workers vary so widely that it is evident that the composition of the precipitate is purely empirical and depends to some extent on the conditions.

Apart from the criticisms which were put forward in the early stages, and which have been answered by Osborne, Gumbel, and others, there are five main sources of error in the method as usually practised. Certain of these errors being exaggerated when the analysis is performed on the micro-scale, it became necessary to eliminate them as far as possible.

1. Jodidi and Moulton [1919] showed that the precipitate of humin and magnesia, filtered off after determination of the amide or ammonia nitrogen, contained an amount of nitrogen roughly proportional to the amount of magnesia used. Thus, in experiments on caseinogen,

Weight of MgO added	4 g.	Nitrogen in the precipitate	4·78 %
,, ,, ,,	2 g.	,, ,, ,, ,,	2·60 %
,, ,, ,,	1 g.	,, ,, ,, ,,	1·72 %

This absorption of nitrogen by the magnesia causes a corresponding decrease in the basic and monoamino-nitrogen found in the filtrate. On the other hand, the insoluble humin precipitate, if filtered off separately, before addition of MgO, contains only 0·1 % of the total nitrogen. It is clearly incorrect to regard this MgO precipitate as representing humin nitrogen, since it consists mainly of soluble nitrogen adsorbed on to the precipitate. Moreover, it has been found that thorough washing with hot water does not reduce the nitrogen in the

precipitate to an appreciable extent, so that the adsorption on to the magnesia is of a fairly close nature. The conclusion reached by Jodidi and Moulton is that if 1 g. of magnesia be used throughout, satisfactory results can be obtained, and they ascribe Osborne and Harris' concordant results to this fact.

2. In common with other workers, Jodidi and Moulton have overlooked the important question of the *amount of magnesium remaining in solution.* This is, of course, independent of the amount added, but depends upon:

(*a*) the amount of HCl remaining in the liquid after the evaporation, *i.e.* the completeness of the removal of the acid,

(*b*) the amount of amino-acid hydrochloride to be neutralised,

(*c*) the volume of water, in which magnesia has a very slight solubility (about 1 : 55,000).

It will, however, be shown that the presence of a variable amount of magnesium considerably influences the values obtained for the diamino-nitrogen. Other salts, or an excess of sulphuric acid, have a similar effect in increasing the amount of precipitate.

In a series of experiments gelatin was hydrolysed in 15 % H_2SO_4, diluted to three times the volume, and precipitated with phosphotungstic acid in presence of various salts.

	% nitrogen in the precipitate.		
	(*a*)	(*b*)	Mean
Salts absent	13·2	—	13·2
1 g. $MgSO_4.7H_2O$ added	15·0	15·2	15·1
1 g. NaCl added	18·8	18·6	18·7
1 cc. (extra) H_2SO_4 added	17·0	17·3	17·15

In the same way considerable variations in diamino-nitrogen were found to correspond with the quantities of alkali needed to make the solution alkaline for the amide determinations:

	Diamino-N of gelatin %	Diamino-N of edestin %
1·5 cc. 10 % NaOH	14·6	27·8
2·0 cc. 10 % NaOH	15·7	29·4

Thus the amount of nitrogen in the phosphotungstic precipitate varies with the amount of salt present. The errors involved are considerable, and may go far towards explaining the difficulty found in obtaining reliable values for the diamino-nitrogen.

The process, occasionally adopted, of redissolving the MgO in H_2SO_4 before filtering off the humin is therefore open to objection on the score that it involves the addition to the filtrate of a variable amount of salt and acid. In the method described below, a constant amount of NaOH is added to the solution, so that the quantities of salt and acid introduced are the same throughout, while the error due to the magnesia precipitate is avoided. The values for the amide nitrogen obtained by the use of NaOH do not differ considerably from those found with MgO, though on the whole slightly higher (see results for gliadin), while the humin nitrogen (the true insoluble matter) is so low as to be negligible

in some cases, as in gelatin. The minute amount of flocculent precipitate can, in the case of gelatin, be allowed to remain since it increases the diamino-N by less than 0·1 %.

3. The need for absolute standardisation of the conditions was shown by Knaggs [1923], according to whom the protein must be hydrolysed immediately on adding the acid—preferably thrown into the boiling acid. Further, the liquid must not be allowed to stand with the 5 % H_2SO_4 before precipitating with the phosphotungstic acid, or if it has stood, it should be heated to boiling or autoclaved and precipitated immediately on cooling.

The author has found the most reliable results to be obtained in the micro-method by heating the 5 % acid solution in the autoclave, cooling quickly and precipitating.

4. The concentration of the solution is an important factor. In the first place, if the precipitate is small, the effect of solubility is large, while if the precipitate is large and bulky, it is difficult to wash completely on the filter, and hence it may contain non-basic nitrogen from the filtrate. Experiments in redissolving and reprecipitating the phosphotungstates to avoid this were unsuccessful on account of the large amount of alkali needed to dissolve the precipitate and the consequent alteration of the equilibrium in the second precipitation. The whole mechanism of the precipitation appears to be a mass action phenomenon and is continuously variable, except within a limited range of concentration, which depends on the particular protein taken. Below this concentration precipitation is incomplete, even after allowing for the solubility of the phosphotungstates.

It is therefore essential to use the same amount of protein for the hydrolysis in each case, as nearly as possible.

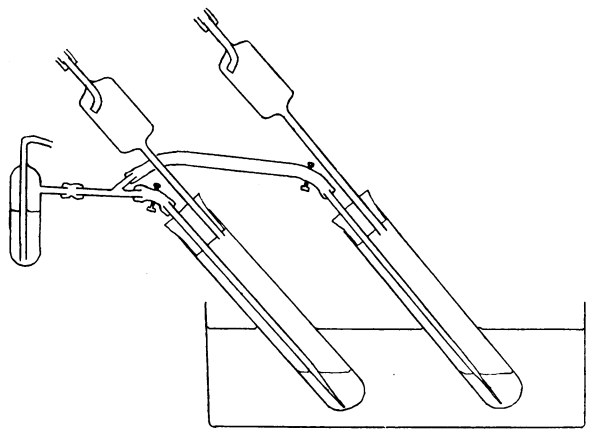
5. In addition, the temperature of the solution affects the precipitation, and while cooling to 20° is specified by Hausmann and by Osborne and Harris [1903], it has been found that 0° is a more convenient precipitating temperature for the purpose of this method.

Experimental.

The method is designed to deal with 3–6 mg. of total nitrogen in each determination, and hence for the hydrolysis some 15 mg. nitrogen should be used to allow two determinations. This corresponds to 90–100 mg. of dry protein, *i.e.* one-tenth the amount used in the ordinary method.

Hydrolysis. With dry material the protein powder is dropped into 30 cc. boiling 18–20 % HCl. Most of the experiments were carried out with 0·7 % gelatin solution (1 mg. N per cc.), in which case 15 cc. solution are poured into 15 cc. boiling 35 % HCl. The mixture is heated in a 100 cc. Kjeldahl flask under a reflux condenser for 18 hours. To avoid contamination by products dissolved from the rubber stoppers by the HCl, a hang-in glass condenser of simple design was used, large enough to fit closely into the neck of the flask, and through this water was circulated.

Evaporation of the acid. Two portions of 10 cc. each are pipetted out on cooling into hard glass boiling tubes (200 × 25 mm.), which are fitted with fine capillaries reaching almost to the bottom, provided with screw clips above to regulate the air passing through them (see diagram). Short exit-tubes are connected to small still-heads of oblong type (35 × 25 mm.), and thence to the pump. The two tubes are placed slantwise in a warm bath at 50°–60°, and the acid evaporated off under reduced pressure. The small amount of air which passes through the capillaries is brought through a bubbler of H_2SO_4 to remove ammonia. The liquid is brought as near dryness as possible—with a good pump in about 3 hours. Tubes and stoppers are then rinsed down with about 10 cc. of water, and a drop of phenolphthalein is added.



Apparatus for evaporation of the acid.

Amide nitrogen. 2 cc. of 10 % NaOH are then run in, followed by H_2SO_4 until just acid. 2 % NaOH is then added drop by drop until the indicator is just pink. The amide or ammonia nitrogen is then determined in an apparatus similar to that of Folin [1902] for determining ammonia in urine. In the first of three boiling tubes is placed H_2SO_4, in the second the alkaline liquid, while the third, which is fitted with a bubbler, contains 5 or 10 cc. $N/70$ H_2SO_4 to absorb the ammonia. The second tube is fitted with a small still-head to prevent loss, and is immersed in water at 40°–50°, and air is aspirated briskly through for 90 minutes. The receiving tube and bubbler are rinsed down, washed out into a small flask, and the solution is titrated at once with $N/70$ NaOH, methyl red being used as indicator.

Humin nitrogen. The solution is made acid with one drop of H_2SO_4, and the insoluble dark precipitate centrifuged off on a small centrifuge. 15 cc. urine tubes with pointed ends were found to be most satisfactory, using a water-driven centrifuge. In some cases the humin may be neglected if the liquid is clear and pale; otherwise the precipitate is washed three times with 1–2 cc. of water, centrifuging for 5–10 minutes between each washing. It is transferred with a few drops of water to a hard glass test-tube, and incinerated

with H_2SO_4 and potassium and copper sulphates, and the nitrogen estimated by Parnas and Wagner's [1921] modification of Pregl's method, which is used for all subsequent nitrogen estimations.

Diamino-nitrogen. 0·3 cc. concentrated H_2SO_4 is now added to the filtrate, the liquid evaporated down and made up to 10 cc. in a marked test-tube. If the solution has stood with the 5 % acid during the determination, the tube is plugged with cotton wool and autoclaved at 130° for 3 hours. If the process has been carried through without delay, however, the liquid is merely boiled. It is then cooled in ice, and 3 cc. of phosphotungstic acid solution (5 g. sulphuric acid to 20 g. phosphotungstic acid per 100 cc.) are added from a pipette drop by drop, with shaking, as soon as cold. After standing in ice overnight, the precipitate is centrifuged in a 15 cc. urine tube and washed four or five times with a total of 10 cc. phosphotungstic acid wash-liquid (5 g. sulphuric acid to 2·5 g. phosphotungstic acid per 100 cc.), the hard lump of precipitate being stirred up with a glass rod at each washing. After centrifuging for about 10 minutes each time, the liquid can be decanted accurately.

Finally, the precipitate is dissolved out of the cup with 2 cc. of 10 % NaOH, the precipitating tube being rinsed with a few drops of the same, and the nitrogen determined as described above.

Non-basic nitrogen. The combined solution and wash-liquid from above are made up to 50 cc., and the nitrogen estimated in two 10 cc. portions, since to incinerate the entire 4 mg. of nitrogen would be inadvisable. The two portions should agree within 0·1 cc. $N/70$ acid, as otherwise the error ($\times$ 5) is large.

Total nitrogen. This is determined on 2 cc. portions of the hydrolysate, the ratio of the 2 cc. (Ostwald) pipette to the 10 cc. pipette being carefully determined.

The accuracy of the incineration process was carefully checked, using quantities of pure tyrosine weighed out on the microbalance. It was found that up to 3 mg. nitrogen were incinerated and distilled without loss, while the reagent error from various blank determinations was of the order of 0·11 cc. of $N/70$ acid. This was duly allowed for. Parnas and Wagner's distillation apparatus is exceedingly convenient and accurate and was used throughout.

Some of the results obtained by this method are given below. As a general rule the amide nitrogen is higher, and the diamino-nitrogen lower, than given by the macro-method. The latter result is due to the presence of less salt at precipitation, and to more perfect washing on the centrifuge than is possible on a filter.

The gliadin was prepared from wheat flour, and the edestin from hemp seeds according to standard methods. The figures given for gelatin refer to ordinary gelatin in the case of Van Slyke and Hausmann, but to Coignet's Gold Label, purified by electrodialysis and flocculation, in the case of this method.

The entire determination can be carried through in about 3 days, which is roughly half the time occupied by the macro-method.

Protein	Author	Amide-N %	Humin-N %	Diamino-N %	Mono- & non-amino-N %	Total %
Gelatin	Hausmann [1899]	1·6	—	35·8	62·5	99·9
	Van Slyke [1911]	2·2	0·1*	25·5	71·2	99·0
	Knaggs [1923]	—	—	20·0	—	—
	Micro-method	2·6	0·0	17·6	79·6	99·8
Edestin	Van Slyke [1911]	10·0	2·0*	36·7	50·7	99·4
(hemp)	Osborne and Harris [1903]	10·1	0·7†	31·6	57·7	100·1
	Micro-method	12·1	0·3	28·8	58·7	100·0
Gliadin	Van Slyke [1911]	24·5	0·4*	6·2	68·9	100·0
(wheat)	Osborne and Harris [1903]	25·5	0·9†	11·7	61·7	99·8
	Micro-method	26·3	0·1	5·4	68·3	100·1

* Nitrogen in CaO precipitate. † Nitrogen in MgO precipitate.

Summary.

1. The various sources of error in the determination of the Hausmann numbers of the proteins are described.

2. The presence of an uncontrolled amount of salt is shown to be an important factor in determining the composition of the precipitate of the basic fraction.

3. A convenient micro-method for the determination of the Hausmann numbers is described, avoiding the various errors.

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

The author desires to express his thanks to the Department of Scientific and Industrial Research for a grant which enabled him to carry out this work.

References.

Folin (1902). *Z. physiol. Chem.* **37**, 161.
Hausmann (1899). *Z. physiol. Chem.* **27**, 95.
Jodidi and Moulton (1919). *J. Amer. Chem. Soc.* **41**, 1526.
Knaggs (1923). *Biochem. J.* **17**, 488.
Osborne and Harris (1903). *J. Amer. Chem. Soc.* **25**, 323.
Osborne, Leavenworth and Brautlecht (1908). *Amer. J. Physiol.* **23**, 180.
Parnas and Wagner (1921). *Biochem. Z.* **125**, 253.
Van Slyke (1911). *J. Biol. Chem.* **10**, 15.