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THE CAUSE OF AND REMEDY FOR CERTAIN INACCURACIES IN HAUSMANN'S NITROGEN DISTRIBUTION METHOD.

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The importance of proteins as life carriers in the plant and animal organism, as well as the significance of amino acids as simple substances out of which the protein molecule is built up, has long been recognized. The amino acids, along with the proteins assume added significance because of their importance in nutrition, as demonstrated by the more recent researches of Osborne[2] and Mendel, McCollum,[3] Van Slyke,[4] Hart,[5] Abderhalden[6] and their collaborators. It is for this reason that it is frequently desirable to analyze for the amino acids quantitatively. This can be accomplished by estimating the chemical groups characteristic of the different amino acids, according to the well-known method of Van Slyke,[7] or the diamino acids may be isolated and determined according to the method of Kossel[8] and Kutscher[9] and their collaborators, while the monoamino acids may be estimated according to Fischer's ester method.[10]

Whereas Van Slyke's method has the great advantage that it yields quantitative results with but a small amount of material (about 3 g. of protein) its disadvantage is that the time factor is not insignificant. On the other hand, analysis of proteins by the methods of Kossell and Kutscher and those of Fischer requires very much more material and time.

It is true that by Hausmann's method[11] the nitrogen distribution in the protein molecule can be determined in a very short time and with but one g. of protein material. But although the method yields fairly accurate results for acid amide nitrogen, the results for diamino and monoamino acid nitrogen are far from being accurate. Osborne and Harris[12] have essentially improved Hausmann's method by showing how

[1] We wish to express our thanks to Mr. K. S. Markley for some assistance in this work.

[2] *J. Biol. Chem.*, 12, 473 (1912); 13, 233 (1912); 17, 325 (1914); 18, 1 (1914); 20, 351 (1915); 26, 1, 293 (1916); 29, 69 (1917).

[3] *Ibid.*, 19, 323 (1914); 20, 415 (1915); 28, 211, 483 (1916).

[4] *Ber.*, 43, 3170 (1910); *J. Biol. Chem.*, 16, 231 (1913).

[5] *J. Biol. Chem.*, 21, 239 (1915); 26, 457 (1916); 29, 57 (1917); 31, 415, 445 (1917).

[6] *Z. physiol. Chem.*, 96, 1 (1915).

[7] *J. Biol. Chem.*, 10, 15 (1911–1912); 22, 281 (1915); 23, 411 (1915).

[8] *Z. physiol. Chem.*, 31, 165 (1900–01).

[9] *Ibid.*, 49, 318 (1906); 52, 108 (1907).

[10] *Ibid.*, 33, 151, 412 (1901).

[11] *Ibid.*, 27, 95 (1899); 29, 47, 136 (1900).

[12] THIS JOURNAL, 25, 323 (1903).

to determine humin nitrogen (which was not done by Hausmann) and by elaborating the exact conditions under which the diamino acids can be precipitated quantitatively. They also suggested calculating monoamino acids by difference rather than estimating them directly, since their direct determination by the Kjeldahl method, in the presence of a large quantity of phosphotungstic acid incidental to Hausmann's method, yields too low results.

In applying Hausmann's method on various occasions, the writer[1] found that while the figures for acid amide nitrogen were on the whole uniform and fairly accurate, those for diamino and monoamino nitrogen were not as uniform and sometimes could not be duplicated. Observations on Hausmann's method show that the more magnesium oxide is used in the distillation of the acid amide nitrogen, the more humin nitrogen is retained by the magnesium oxide. This is plainly visible, since the color of the residual liquid containing the basic and non-basic nitrogen is lighter when a larger amount of magnesium oxide is used. In this connection Hausmann[2] fails to specify the amount of magnesium oxide to be added to the evaporated hydrolyzed substance, while Osborne and Harris[3] recommend adding this reagent until it is in slight but distinct excess. However, because of the insignificant solubility of magnesium oxide in water (1:55368) and the slight alkalinity of its solution one is apt to use a considerable excess. This is especially true in the analysis of biological materials containing chiefly carbohydrates and but a small proportion of proteins and amino acids. Excessive use of magnesium oxide, however, involves significant errors, as will be shown in the experimental part of this paper.

Experimental.

Casein.—The casein used in these experiments was prepared from skim milk essentially according to the method of Hoppe-Seyler.[4] The purified, air-dried casein, contained 8.89% of moisture and 15.53% of nitrogen, calculated to the oven-dried substance. 1.2 g. quantities of air-dried casein were treated with 75 cc. of 20% hydrochloric acid and kept boiling under reflux for 10 hours. Each of the hydrolyzed portions was next evaporated on the steam bath, practically to dryness, transferred quantitatively to 800-cc. Kjeldahl flasks with 250 cc. of boiling hot water, cooled and distilled with various amounts of magnesium oxide previously mixed to a cream with 100 cc. of water. The distillate was titrated with 0.1 *N* sulfuric acid, in order to determine the acid amide nitrogen.

[1] S. L. Jodidi, THIS JOURNAL, 32, 396 (1910); 33, 1226 (1911); 34, 94 (1912); also Mich. Agr. Expt. Sta., *Tech. Bull.* 4 (1909); Jodidi and Wells, Iowa Agr. Expt. Sta., *Research Bull.* 3 (1911); Jodidi, Kellogg and True, *J. Agr. Research*, 15, 385 (1918).

[2] *Z. physiol. Chem.*, 27, 98 (1899).

[3] THIS JOURNAL, 25, 331 (1903).

[4] Hoppe-Seyler's *Handbuch d. Physiol. u. Pathol. Chem. Anal.*, Berlin, 1909, p. 488.

The magnesium oxide residue was now filtered off and thoroughly washed with boiling hot, ammonia-free water until free from hydrochloric acid. The filter and its contents were next put into a 500-cc. Kjeldahl flask, to which the residue in the 800 cc. flask was then quantitatively transferred by means of dil. sulfuric acid, and the whole analyzed by the Kjeldahl method, to obtain the proportion of "humin" nitrogen.

Filtrate and washings from the magnesium oxide residue were evaporated on the water bath to 100 cc., cooled to 20°, and treated with 5 g. of sulfuric acid and 30 cc. of a solution containing 5 g. of sulfuric acid and 20 g. of phosphotungstic acid per 100 cc. After about 24 hours the precipitate was filtered out and washed with a solution containing 5 g. of sulfuric acid and 2.5 g. of phosphotungstic acid per 100 cc. The washed precipitate and the filter were subjected to oxidation for 8 hours, according to the Kjeldahl method, which gave the nitrogen of diamino acids.

In all experiments a correction was made for the nitrogen present in the reagents.

The proportion of the monoamino acid nitrogen was found by subtracting from 100 the sum of the nitrogen per cents. obtained by the above operations.

The results representing the average of duplicate estimations are presented in Table I. Examination of the table shows that the percentage of the acid amide nitrogen is practically the same in all experiments, but that the percentage of nitrogen contained in the magnesium oxide precipitate is higher when a larger quantity of magnesium oxide is used for distillation, and the percentages of monoamino and diamino nitrogen are correspondingly lower. Since the hydrolysis of the various portions of casein took place under identical conditions, the amount of humin nitrogen formed should be the same in all cases. Hence, the fact that different quantities of nitrogen were retained by the magnesium oxide clearly indicates that diamino and monoamino acid nitrogen as well as humin, nitrogen, were adsorbed.

TABLE I.—NITROGEN DISTRIBUTION IN CASEIN.

Magnesium oxide used for distillation, G.	Nitrogen of acid amides.		Nitrogen of magnesium oxide ppt.		Nitrogen of diamino acids.		Nitrogen of monoamino acids.	
	Oven-dried casein, %.	Casein nitrogen, %.	Oven-dried casein, %.	Casein nitrogen, %.	Oven-dried casein, %.	Casein nitrogen, %.	Oven-dried casein, %.	Casein nitrogen, %.
4.0	1.60	10.28	0.74	4.78	2.79	17.97	10.38	66.84
2.0	1.59	10.21	0.41	2.60	2.96	19.03	10.59	68.17
1.0	1.58	10.15	0.27	1.72	..	...	...	...
0.5	1.59	10.22	0.20	1.30	3.27	21.05	10.47	67.43

If the humin nitrogen formed in the course of the casein hydrolysis were

insoluble it should be possible to separate and estimate its exact proportion; and the nitrogen present in the magnesium oxide would be simply adsorbed nitrogen of diamino and monoamino acids. To determine what actually happens, several more portions of 1.2 g. of air-dried casein[1] were hydrolyzed under exactly the same conditions as before. The hydrolyzed liquids, however, were evaporated on the water bath to dryness, taken up with hot water, filtered through a double filter and refiltered until the filtrate was perfectly clear. The black residue on the filter was then washed with boiling hot ammonia-free water until the filtrate was free from chlorine, whereupon the filter and residue were oxidized by the Kjeldahl method and the proportion of the insoluble humin nitrogen thus determined. The dark brown but perfectly clear filtrate and washings from the insoluble black residue were concentrated and used for distilling off, with the aid of magnesium oxide, the ammonia corresponding to the acid amides, etc., as already outlined. The data in question which represent the average of duplicate analyses are summarized in Table II.

TABLE II.—NITROGEN DISTRIBUTION IN CASEIN, WITH REMOVAL OF THE INSOLUBLE HUMIN NITROGEN.

Magnesium oxide used for distillation, G.	Insoluble humin nitrogen.		Nitrogen of acid amides.		Nitrogen in magnesium oxide ppt.		Nitrogen of diamino acids.		Nitrogen of monoamino acids.	
	Oven-dried casein. %	Casein nitrogen. %	Oven-dried casein. %	Casein nitrogen. %	Oven-dried casein. %	Casein nitrogen. %	Oven-dried casein. %	Casein nitrogen. %	Oven-dried casein. %	Casein nitrogen. %
4.0	0.03	0.18	1.58	10.26	0.77	4.96	2.95	19.16	10.07	65.46
2.0	0.02	0.11	1.57	10.22	0.32	2.06	3.07	19.93	10.42	67.68
1.0	0.01	0.08	1.61	10.46	0.23	1.48	3.29	21.38	10.25	66.60
0.5	0.02	0.16	1.59	10.32	0.11	0.72	..	...	...	...
Results of Osborne and Harris[2].....			1.61	...	0.21	..	3.49	...	10.31	...
Results of Hausmann[3].....			2.13	...	..	..	1.75	...	12.06	...
			2.07	...	..	..	1.61	...	11.81	...
			..	...	..	..	1.52	...	...	...
			..	...	..	..	2.33	...	...	...
			..	...	..	..	2.00	...	...	...
			2.10	(Ave.)	..	..	1.84	(Ave.)	11.93	(Ave.)

In glancing over this table it is readily seen that the proportion of insoluble humin nitrogen is insignificant, the data for acid amide, diamino and monoamino nitrogen fully corroborating the results already reported in Table I. While the successive treatment of the magnesium

[1] This casein had a moisture and nitrogen content slightly different from the casein used in the experiments already reported.

[2] THIS JOURNAL, 25, 349 (1903).

[3] *Z. physiol. Chem.*, 27, 103–4 (1899).

oxide precipitate with additional and larger quantities of water influences somewhat the relative amounts of nitrogen remaining in it and in the filtrate this influence is not considered great enough to affect the conclusions drawn.

When we compare those samples in Tables I and II, in which one g. of magnesium oxide was used for distillation, we find that these results agree very well with those of Osborne and Harris.

Hausmann's results differ considerably from those of Osborne and Harris, while ours are between the two. It is interesting that Hausmann obtained concordant duplicates in the estimation of acid amide nitrogen, while his figures for diamino nitrogen show considerable variations. In the light of the results in Tables I and II, it seems safe to assume that the considerable fluctuations in the diamino nitrogen obtained by Hausmann may have been due to different amounts of magnesium oxide used in the distillation, in addition to the fact that Hausmann did not quite succeed in rendering the conditions for the precipitation of diamino acids strictly quantitative.

Similar experiments carried out with egg albumin and gelatin[1] have led essentially to the same conclusions. The proportion of nitrogen found in the magnesium oxide precipitate was higher, and that of diamino and monoamino nitrogen was correspondingly lower, the more magnesium oxide was used for distillation, the amount of the latter being of no influence on the proportion of acid amide nitrogen.

Spinach.—In previous work[2] it was ascertained that the nitrogen of the spinach plant (*Spinacia oleracea*) is made up of proteins, and non-proteins (acids, amides, amino acids, etc.). For the following experiment we used air-dried, powdered leaves[3] from healthy spinach plants collected on the Childreth farm near Norfolk, Virginia. Several 5 g. portions were transferred to round bottom flasks to which 250 cc. of 20% hydrochloric acid was added and kept boiling under a reflux condenser for 10 hours. The contents of the flask were now filtered on a Büchner funnel provided with a cloth filter and washed with boiling hot, ammonia-free water until the filtrate was free from chlorine. The filtrate and washings were, on cooling, made up to a definite volume and shaken thoroughly. Several 500 cc. quantities of this solution, each containing 216.2 mg. of nitrogen, were now evaporated on the water bath to dryness, whereupon the separation of the acid amide, diamino nitrogen, etc., was effected as with casein. The results which are recorded in Table III

[1] The data in question may be omitted here for the reason that the egg albumin and gelatin used were not quite pure.

[2] Jodidi, Kellogg and True, *J. Agr. Research*, **15**, 393 (1918).

[3] The analysis showed them to contain 7.62% moisture and 5.00% nitrogen, calculated to the oven-dried substance.

fully corroborate the conclusions drawn from the experiments with casein, ovalbumin and gelatin.

TABLE III.—NITROGEN DISTRIBUTION IN SPINACH.

Magnesium oxide used for distillation. G.	Nitrogen of acid amides.		Nitrogen in magnesium oxide ppt.		Basic nitrogen.		Non-basic nitrogen.	
	Oven-dried Spinach. %	Spinach nitrogen. %	Oven-dried Spinach. %.	Spinach nitrogen. %.	Oven-dried Spinach. %.	Spinach nitrogen. %.	Oven-dried Spinach. %.	Spinach nitrogen. %.
4.0	0.88	17.61	0.26	5.28	0.79	15.81	3.06	61.30
2.0	0.89	17.77	0.19	3.77	0.81	16.22	3.11	62.24
1.0	0.88	17.68	0.11	2.13	0.85	16.98	3.16	63.21

An attempt to distill the evaporated hydrolyzed spinach with 0.5 g. of magnesia was unsuccessful, the amount of the latter being insufficient to render the substance alkaline.

Summary.

1. The proportion of acid amide nitrogen obtained by Hausmann's method, as modified by Osborne and Harris, is constant and does not depend upon the quantity of magnesium oxide applied to the distillation.

2. The percentage of nitrogen contained in the magnesium oxide precipitate is the higher, the greater the quantity of magnesium oxide employed in distillation.

3. Conversely, the proportion of monoamino and diamino nitrogen is the smaller, the larger the amount of magnesium oxide used in distillation.

4. In order to obtain uniform results and a minimum of "humin" nitrogen it is necessary to use the least possible amount of magnesia which is sufficient to render the substance to be distilled alkaline. In the case of plant and animal materials the uniform application of one g. of magnesium oxide seems to be satisfactory, while in the case of proteins 0.5 g. suffices.

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THE HYDROCHLORIC ACID COLOR METHOD FOR DETERMINING IRON.

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Iron occurs in determinable amounts in a surprisingly large number of materials. In the course of work on the raw materials for optical glass the determination of this element soon became the most pertinent criterion of quality, inasmuch as iron introduced into the glass produces color, with a corresponding absorption of light, and lowering of the usefulness of the glass.

In the preparation of solutions of these raw materials for the determina-