A New Micro-Estimation Method of Amino-Group.

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Introduction. Several method have already been published for the estimation of amino-group, the Van Slyke's method, however, is used most popularly.(1) That method, as is well known, needs a special apparatus for the measurement of nitrogen liberated by the reaction of amino-group with nitrous acid. The apparatus is somewhat complicated, and requires some skill in the treatment.

A new micro-estimation method of amino-group will be described in the following. The method consists of the same principle as that of the Van Slyke's but measures the residual nitrogen (non-amino nitrogen) instead of the liberated one, so no costly and complicated appratus is necessary. It is also possible to practise in a scale of micro-analysis.

At first the total nitrogen is determined by micro-Kjeldahl method in a definite quantity of the sample and then the residual nitrogen of the same sample, which is not reacted with nitrous acid.(2) The value of amino nitrogen is calculated as the difference of the total and the residual nitrogen thus obtained.

It was our chief problem how to deal with the excess of nitrous acid, its decomposition products and the secondary combined nitrogen (nitro, nitroso and ester) during the deamination reaction. The nitrous acid is decomposed and driven away with air stream(3) under the reduced pressure as quickly as possible after the deamination reaction.

The remaining nitrous acid is oxidized to nitric acid by hydrogen peroxide, which is then driven off by the vacuum distillation.(4)

At last a trace of the nitric acid is decomposed by ferrous sulphate. A proper amount of potassium permanganate solution is added before the vacuum distillation for the purpose of preventing the formation of ammonia. If there is any chloride compound, an unpleasant action will occur during the treatment with the permanganate. A method has been deviced to avoid this action.

Experimental. At first the total nitrogen contained in a definit

(1) Van Slyke, *J. Biol. Chem.*, **7** (1910) 34; **9** (1911) 185; **10** (1911) 15 **12** (1912) 275; **16** (1915) 281.
(2) Scott and West, *Ind. Eng. Chem., Analyt. Ed.*, **9** (1937) 50.
(3) Gomberg, Ber, (1903) 3281.
(4) Cambier et Leroux, *Compt. rend.*, **195** (1932) 1280.

quantity of the sample is estimated by the usual micro-Kjeldahl method. Then the residual nitrogen is determined by means of the deamination in the following manner.

The concentration of the sample solution is made so as it contains 0.2–0.6 mg. nitrogen per cubic centimeter. The sample solution (1–3 c.c.) is transfered into a Kjeldahl digestion flask, add 0.7 c.c. of 30% sodium nitrite solution and 0.35 c.c. of the potassium iodide solution. The latter is made by dissolving 4 grams potassium iodide in 2 c.c. of water and diluting to 100 c.c. by adding the glacial acetic acid. The reaction proceeds at room temperature, or at constant temperature if necessary, under continuous shaking. The relation between the temperature and the reaction time is shown in the following table.

Temperature (°C.)	15	20	25	30
Time (min.)	6-7	5–6	4-5	3.5–4

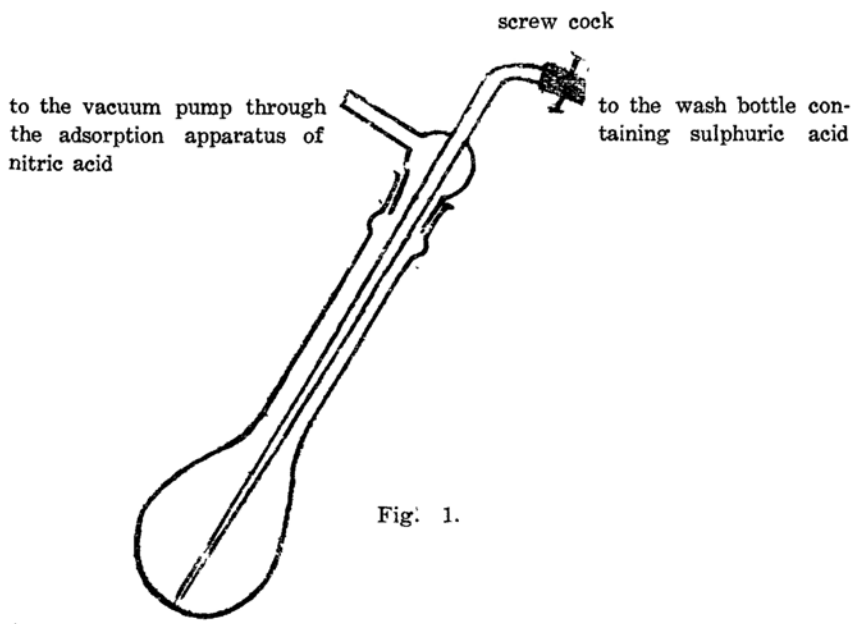

Fig. 1.

As soon as the deamination reaction is over, the flask is connected to the vacuum distillation apparatus as shown in Fig. 1. The bubbling, causing from the decomposition of the nitrous acid, ceases in about four minutes at room temperature. Then the air washed with the concentrated sulphuric acid is passed about one minute. Two drops of 35% hydrogen peroxide solution and one c.c. of 50% sulphuric acid are added, and the vacuum distilation is undertaken again. The air washed with sulphuric acid is passed cautiously during first one minute to change the remaining nitrous acid to nitric acid. The flask is, then, immersed in the warm water of 50°C. till its neck, and distilled in vacuum about 15 or 20

minutes. At last the air is passed slowly until the sodium sulphate crystallizes out, which takes about 2 minutes.

The residual hydrogen peroxide must be driven off completely by distillation. It is not necessary, however, to remove the hydrogen peroxide so completely if there is no substance which is easily converted into nitro- or nitroso-compounds. If the sample contains much substances which are easily converted into nitro- or nitroso compounds, such as tyrosin, tryptphan or phenylalanin etc., the treatment with potassium permanganate is necessary during the nitric acid distillation to prevent the reduction of the secondary combined nitrogen in these compounds into ammonia. Namely, after the above mentioned operations one c.c. of 50% sulphuric acid is added to resolute the sodium sulphate crystals once more, and then 0,5 normal potassium permanganate solution is added drop by drop with the aid of measuring pipette. At first, the dropped permanganate solution is instantly decoloured but after some time tne faint violet colour remains. After this state has been reached where the colour reamins unchanged during one minute, two or three times more permanganate solution is added.

Now the flask is again connected to the vacuum pump, and the temperature raises steadily from 50°C. to 100°C. A slow air stream is passed at the beginning, to prevent the bumping, and then distilled under high vacuum; the temperature reachs to 100°C. in 7 to 8 minutes. During the last one minute, a sufficient air is passed cautiously. Then one drop of hydrogen peroxide solution and one c.c. of distillation water are added, shaken well, and the vacuum distillation is resumed at 100°C. during some minutes. By these treatments the nitric acid is removed almost entirely. In the course of distillation the flask is shaken moderately and the air is passed adequately both at the beginning and the ending of the distillation.

It is necessary to add two c.c. of concentrated sulphuric acid and one or two drops of 25% ferrous sulphate solution in dilute sulphuric acid in order to detect and to decompose a trace of the remaning nitric acid. If the nitric acid remains still in considerable amount, the colour becomes brown by the addition of ferrous sulphate which soon decolours. In such a case one c.c. of water is added and the vacuum distillation must repeated at 100°C.

If the nitric acid is in a trace, which is proved by the reddish orange colouration, the flask is shaken moderately at 100°C. and under high reduced pressure. A drop of ferrous sulphate solution is added again for the assurance of the elimination of nitric acid. If the presence of nitric acid is not detected, a drop of hydrogen peroxide solution and a small quantity of copper sulphate are added to the mixture.

If there still remains nitric acid, the addition of ferrous sulphate

and the vacuum distillation must be repeated untill there remains no nitric acid. After these treatments the usual micro-Kjeldahl process is followed. The residual nitrogen, that is non-amino nitrogen and not reacted with nitrous acid, can thus be measured. The value must be corrected with the result of blanc test.

Notices.

I. *On the Use of Acetic Acid Solution containing 4% Potassium Iodide.* Kendrick and Hanke[5] improved the original Van Slyke's method of amino-nitrogen determination by use of the acetic acid solution containing 4% potassium iodide instead of the pure glacial acetic atid. We have undertaken the experiments on glycine, alanine, cystine and lactic acid. By use of the pure acetic acid we obtained the results that these substances give considerable amount of residual nitrogen, notwithstanding they contain no residual nitrogen. No such nitrogen, however, was detected when the acetic acid solution containing 4% potassium iodide was used. Only in the case of cystine, 10–20% residual nitrogen is still found. The secondarily generated nitrogen from cystine thus obtained is perhaps ammonia and is already generated in the course of the deamination reaction. The potassium permanganate treatment, therefore, shows no effect as shown in Table 2.

Table 2. Cystine.

Total nitrogen mg.	0.5 N. $KMnO_4$ c.c.	Residual nitrogen mg.	Ratio %
0.0826	0	0.0175	21.2
0.1652	0	0.0284	17.2
0.2913	0	0.0402	13.8
0.2913	0.2	0.0410	14.1
0.2913	0.6	0.0402	13.8

II. *The Removal of Nitrous and Nitric Acid.*

Obviously it is necessary to eliminate completely the surplus nitrous and nitric acid before the Kjeldahl digestion process. It is shown, from the results of experiments, that the reducing agents may not be used to decompose nitrous or nitric acid. For example, paraformaldehyde, hydrogen sulphide, sodium thiosulphate and sulphurous acid generate more or less ammonia. After all, the physical method is used and the ferrous sulphate is used only at the end of the treatment to detect and to decompose a trace of nitric acid.

III. *The Effect of Potassium Permanganate on the Analysis of Tyrosine and Tryptophane etc.*

(5) Kendrick and Hanke; *J. Biol. Chem.*, **117** (1937) 161.

Some amino-acids which are easily converted into nitro- or nitroso-compounds during the deamination reaction are apt to be reduced by the organic decomposition product and generate ammonia. Such a reaction occurs during the vacuum distillation of nitric acid at 50–100°C., and so the adequate amount of potassium permanganate solution is used to prevent the reduction. Table 3 shows the results of these experiments.

Table 3.

Sample Total nitrogen (mg.)	0.5 N. $KMnO_4$ c c,	Resid. nitrogen mg.	Ratio %
Tyrosine	0	0.1590	70.6
	0.6 faint red)	0.0565	25.1
0.2252	1.2	0.0136	6.0
	1.5	0.0108	4.8
Tyrosine	0	0.0708	62.8
	0.3 (faint red)	0.0348	30 9
0.1126	0.6	0.0152	13.5
	0.9	0.0008	0.00
Tyrosine	0	0.0236	42
0.0563	0.15 (faint red)	—	—
	0.3	0.0132	23.3
	0.45	0.0026	4.4
Tyrosine	0	0.0069	24
0.0282	0.075 (faint red)	—	—
	0.22	0.0072	25
Tryptophane	0	0.2661	102.0
0.2608	0.3 (faint red)	0.2268	87.0
	0.9	0.1736	66.5
Tryptophane	0	0.1309	100.0
0.1300	0.6	0.0866	66.4
Phenylpropionic	0	0.0550	
acid (2.405 mg.)	0.075 (faint red)	—	
	0.15	0.0327	
	0.22	0.0269	
	0.50	0.0126	

IV. *Effect of Potassium Permanganate to the Kjeldahl-digestion.*

The potassium permanganate was already used as an oxidizing agent of the Kjeldahl digestion. Usual amino-acids give theoretical values of nitrogen even under the presence of potassium permenganate. The substances which contain peptid bonds, guanidine groups and halides, however, give somewhat less nitrogen in the presence of potassium permenganate. The results are shown in Table 4.

Tabe 4.

Samples	Nitrogen (Absence of $KMnO_4$) mg.	Nitrogen (Presence of $KMnO_4$) mg.	0.5 N-$KMnO_4$ c.c.	Ratio %
Ammonia	0.4730	0.4728	2.5	100.0
Glycine	0.3928	0.3910	1.0	99.5
Glycyl-glycine	0.6930	0.5974	1.0	86.2
Tryptophane	0.4730	0.4728	2.5	100.5
Tyramine-HCl	0.2547	0.1537	2.0	60.3
Arginine-HCl	0.7624	0.3885	3.0	50.9
Guanidine-H_2CO_3	0.2690	0.1398	1.0	52.0
Glutokyrine	0.3668	0.3510	1.0	90.2

In our method, however, the potassium permanganate treatment is undertaken only during the vacuum distillation at 50–100°C., so the conditions are different. Table 5 show the effect of potassium permanganate treatment in our methods.

Table 5.

Sample	Nitrogen (without $KMnO_4$ treatment) mg.	Nitrogen (with $KMnO_4$ treatment) mg.	0.5 N-$KMnO_4$ c.c.	Ratio %
Arginin-HCl	0.4039	0.4061	1.0	100.5
Glycyl-glycine	0.6300	0.6244	1.0	99.1
Tyramine.HCl	0.2547	0.2294	1.0	90.1
Asparagine	0.6100	0.6105	1.0	100.0

We see that the influence of potassium permanganate is very weak excepting tyramine hydrochloride. On the influence of the chloride the results shown in Table 6 were obtained.

Table 6.

Glycine (N = 0.3942 mg.) added with 1 c.c. of 0.5 N-$KMnO_4$

Sodium chloride mg.	Nitrogen found (In presence of halide) mg.	Ratio %
12	0.3116	79.4
12 (Added few drops of 5% $AgNO_3$	0.3910	99.1
6	0 3683	92.3
6 (Added few drops of 5% $AgNO_3$)	0.3960	100.4
2	0.3872	97.5
0.5	0.3948	100.1

The influence of halide is small, providing its amount is not so great. The addition of a silver salt such as silver nitrate or silver sulphate is proved to be effective to exclude the halide errors. The reason of this

effect may be explained by the stability of silver chloride to potassium permanganate.

V *Results of Our Method.*

The results of the new modified micro amino nitrogen estimation method applied to the various amino-acids, proteins and their decomposition products are quite satisfactory as shown in Table 7. The amino nitrogen value of glycine is greater than the theoretical one by 7–8 % when the Van Slyke's method is used, while the present method gives the theoretical value. This method give a smaller error than Van Slyke's one in the case of cystine. When the sample contains much reducing organic substances such as carbohydrates etc., we had better to add the more amount of potassium permanganate than usual.

Table 7.

Samples	0.5 N-$KMnO_4$ c.c.	Total-N mg.	Resid N mg.	Amino-N mg.	Ratio %	Reference %
Glycine	0	0.2664	−0.0006	0.2670	100.2	100 (calc.)
						Van Slyke 107
Alanine	0	0.6253	0.0007	0.6246	99.9	
Cystine	0	0.2913	0.0402	0.2511	86.2	V.S. 108-14
(Table 2)	0.6	0.2913	0.0402	0.2511	86.2	
Tyrosine	0	0.1126	0.0703	0.0418	37.1	
(Table 3)	0.3	0.1126	0.0348	0.0778	69.1	
	0.9	0.1126	−0.0008	0.1134	100.7	
Asparagine	0	0.6100	0.2911	0.3118	51.3	50 (Calc.)
Tryptophane	0	0.4789	0.4557	0.0232	4.8	50 (Calc.)
	1.5	0.4789	0.3684	0.1105	33.7	
	2.0	0.4789	0.2640	0.2145	44.8	V.S. 40-60
Threonine (d. l-)	0	0.1701	0.0088	0.1613	94.9	
	0.32	0.1701	0.0011	0.1690	99.4	
Histidine-HCl	0	0.4164	0.2762	0.1402	33.9	33.3 (Calc.)
	0.3 (+Ag)	0.4164	0.2807	0.1357	32.6	
	0.5 (+Ag)	0.4164	0.2587	0.1577	37.9	
Arginine-HCl	0	0.7624	0.5665	0.1959	25.6	25 (Calc.)
	1.0	0.3946	0.2931	0.1015	25.7	
	1.0	0.1973	0.1395	0.0578	29.3	
Ammonia	0	0.3600	0.2783	0.0817	22.7	
Urea	0	0.4002	0.3395	0.0607	15.1	V.S. 7-8
	0.2	0.4002	0.3175	0.0823	20.5	
Guanidine 1/2 H_2CO_3	0	0.2706	0.2496	0.0210	7.8	
Tyramine-HCl	0	0.2547	0.1583	0.0964	37.9	
	0.45	0.2547	0.0938	0.1609	63.2	
	1.35	0.2547	0.0302	0.2245	88.2	
Tyrosine	1.0	0.0704	0.0005	0.0699	99.3	
Tyrosine + d-Glucose	1.0	0.0704	0.0055	0.0649	92 2	

Table 7.—(*Continued*)

Samples	0.5 N-$KMnO_4$ c.c.	Total-N mg.	Resid-N mg.	Amino-N mg.	Ratio %	Reference %
Tyrosine+ Arginine-HCl	0.9	0.1095 0.3840	0.2909	0.2026	41.1	41.7 (Calc.)
	0.45	0.0555 0.1946	0.1462	0.1039	41.5	
	0.15	0.0555 0.1946	0.1522	0.0979	40.1	
Tyrosine+ Arginine-HCl+ d-Glucose (1.5 mg)	1.0	0.0352 0.0324	0.0242	0.0434	64.2	64.0 (Calc.)
Tyrosine+ Arginine-HCl+ Glycine	0	0.0938 0.1748	0.0278	0.4855	94.5	95.2 (Calc.)
	0.55	0.2457	0.0215	0.4928	95.8	
Tyrosine+ Arginine-HCl+ Glycine	0.3	0.0563	0.0786	0.5376	87.2	88.0 (Calc.)
	0.7	0.0987	0.0755	0.5467	87.8	
	1.0	0.4612	0.6778	0.5384	87.4	
Tyrosine+ Histine-HCl	1.0	0.1205	0.1487	0.1815	54.7	
	2.0	0.2080	0.1362	0.1923	58.6	57.6
Phenyl-propionic acid	0	0.0000	0.0550			
	0.15	0.0000	0.0327			
	0.22	0.0000	0.0269			
Glycyl glycine	0	0.0139	0.2925	0.3214	52.3	(Calc. 50)
	0.28	0.6139	0.2925	0.4214	52.3	V.S. 60–65
	1.0	0.6139	0.2662	0.3477	56.6	
Glutokyrine[6]	0	0.3668	0.2624	0.1044	28.4	V.S. 29–30
	0.21	0.3668	0.2598	0.1070	29.2	
	0.60	0.3668	0.2616	0.1052	28.8	
Peptone	0	0.3577	0.2075	0.2302	38.5	V.S. 39.6
	0.21	0.4021	0.2416	0.1662	39.9	
	0.40	0.4021	0.2359	0.1662	41.3	
Casein (hydrolysed)	0	0.4160	0.1059	0.3101	74.5	
	0.3	0.4160	0.0807	0.3353	80.6	
	0.9	0.4160	0.0807	0.3353	80.6	
Casein	0	0.5568	0.5695	0.0127	-2.3	Lysine
	0.6	0.5368	0.5162	0.0206	3.7	amino N=3.2
	1.2	0.2734	0.2671	0.0113	4.1	
Gelatine	0	0.6289	0.6008	0.0281	4.5	Lysine amino N=3.2
	0.3	0.6289	0.5988	0.0301	4.8	
	0.6	0.6289	0.5810	0.0479	7.6	
Serum (horse)	0.4	0.2351	0.2187	0.0164	7.0	

$$\left[\text{Ratio} = \frac{\text{Amino N.}}{\text{Tot. N.}} \times 100\right]$$

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(6) Grassman, *Biochem. Z.*, **269** (1934) 211; **284** (1936) 177.