CXXX. THE DETERMINATION OF AMINO-NITROGEN USING A COPPER METHOD

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To determine the degree of hydrolysis of proteins by enzymes, as shown by the production of free α -amino-N, it is convenient to have a method which can be carried out rapidly. The methods of Van Slyke [1911; 1912], Sørensen [1907] or Willstätter & Waldschmidt-Leitz [1921] are generally used, but to each of these some objection has been raised. To obtain consistent results for enzymic digests with Van Slyke's method it is necessary to shake for 20–30 min. The other methods do not give very definite titration end-points. Richardson [1934, 1] has considered the principles underlying the formaldehyde, alcohol and acetone titration methods and has shown that the formaldehyde and alcohol methods measure the amino groups whereas the acetone method of Linderstrøm-Lang [1928] measures the carboxyl groups. In a second paper [1934, 2] he gives a full account of the limitations of the Van Slyke method, but concludes that it is to be preferred for dealing with coloured biological products. Of the various titration methods depending on the physico-chemical properties of complex mixtures none was considered wholly satisfactory.

Kober & Sugiura [1912; 1913] and Kober [1917] suggested a method for determining α -amino-N based on the formation of copper salts. Utkin [1933] further modified the method for determination of amino-N in protein decomposition products. Attempts to use the methods of Kober & Sugiura and Utkin in these Laboratories were not very successful. Pure amino-acids did not give theoretical results, and protein digests behaved variably. The copper salt method appeared to offer many advantages, and after further modification by one of us (C. G. P.) it was found to be possible to obtain correct results with certain amino-acids and repeatable results with tryptic digests of proteins. The procedure has certain limits as with other methods for determining amino-N; although, however, it is not entirely specific for amino-N, non-specific reacting substances are unlikely to be present in enzymic digests of proteins.

The method depends on the formation of soluble copper compounds through the reaction between the amino-acid or digest material and excess copper present in the form of copper phosphate. Kober & Sugiura used freshly prepared copper hydroxide and Utkin added copper chloride to the material under test, followed by sodium hydroxide to precipitate the excess copper. Our own observations showed that many of the difficulties encountered were due to the presence of copper in the form of hydroxide and it was not until we substituted copper phosphate that reliable results were obtained. The copper phosphate prepared as described in this paper gives no blank value due to traces of soluble copper. The amount of copper taken into solution by amino-acids or similar material is determined iodimetrically.

Some amino-acids form sparingly soluble copper salts under these conditions (e.g. cystine, methionine, tryptophan, leucine and phenylalanine) and for these

in pure state the method does not work. When mixed with other amino-acids most of these form mixed copper salts which are soluble, and can therefore be estimated by making mixtures with known amounts of a suitable acid (glycine or aspartic acid¹). The proportion of soluble to insoluble acid should be at least 4 to 1. With enzymic digests, the copper compounds appear to be completely soluble; this is also true for proteins hydrolysed with 20% HCl.

Both proline and hydroxyproline are estimated by the copper method, but not by that of Van Slyke. Ammonia, which interferes in the latter, does not affect the copper estimation. The accuracy of the method is easily determined for pure amino-acids, but can only be determined indirectly for more complex mixtures. Parallel determinations carried out by Van Slyke's method and the copper method have given good agreement on tryptic digests of fibrin provided that ammonia is absent and shaking with nitrous acid is continued for 20–30 min. The usual period of about 4 min. is insufficient with partly degraded proteins. In this paper we give the results of determinations on pure amino-acids showing the accuracy of the method and examples of its application to various forms of protein hydrolysates.

EXPERIMENTAL

Reagents required

Copper chloride. 27.3 g. dissolved in 1 l. of water (0.16 *M*).

Trisodium phosphate. 64.5 g. of disodium hydrogen phosphate dissolved in 500 ml. of CO₂-free distilled water; add 7.2 g. NaOH and when dissolved make to 1 l.

Borate buffer. 57.21 g. of sodium borate are dissolved in 1500 ml. water, 100 ml. of *N* HCl added, and the volume made to 2 l.

Copper phosphate suspension. The copper chloride solution (1 vol.) is added to the trisodium phosphate solution (2 vol.) and mixed well; 2 vol. of the borate buffer are added. This suspension appears to keep quite well and enough to last for several days may be prepared.

Thymolphthalein. 0.25 g. in 100 ml. of 50% ethyl alcohol.

Sodium thiosulphate. A stock solution approximately 0.1 *N* prepared by dissolving 49.6 g. of sodium thiosulphate in 200 ml. of CO₂-free distilled water and diluting to 2000 ml. The addition of 0.1% sodium borate keeps the solution stable and dilutions of 0.01 or 0.005 *N* are made from this stock using water containing borate.

Standard potassium iodate. 0.35675 g. of "Analar" potassium iodate dried at 110° for 1 hr. dissolved to 1000 ml. Used to standardize the thiosulphate solution.

Starch solution. Some difficulty was experienced in preparing a starch solution suitable for this method, but the following preparation works well. Soluble potato starch (20 g.) is mixed with about 50 ml. distilled water and 2000 ml. of boiling water are added. The solution is heated on a water bath and treated with 200 ml. *N* NaOH. When clear (after about 30 min.) the solution is cooled and 200 ml. *N* HCl are added. The reaction is adjusted to pH 7 and the solution shaken with 5 g. charcoal and filtered. The charcoal treatment should be repeated if necessary until the filtrate is water clear, after which solid NaCl is added to saturation.

¹ Under the conditions of these experiments aspartic acid forms a soluble copper complex different from the sparingly soluble copper salt commonly employed in the isolation of this amino-acid.

Method

The protein hydrolysate or amino-acid solution is measured into a 50 ml. volumetric flask; 4 drops of thymolphthalein are added, followed by *N* NaOH to a faint blue colour. Then 30 ml. of the copper phosphate suspension are added (measured quite roughly) and the volume made to 50 ml. with distilled water, well mixed and filtered through a no. 5 Whatman paper. The volume of the sample may be varied over wide limits but generally 5 ml. are used. The copper content of the filtrate is determined volumetrically as follows.

10 ml. of the filtrate are acidified with 0.5 ml. acetic acid and approximately 1.0 g. of KI is added.¹ The solution is titrated with standardized thiosulphate, 4 drops of starch solution being added towards the end of the titration. Each ml. of 0.01*N* thiosulphate is equivalent to 0.28 mg. amino-N. The amount of thiosulphate used is of course corrected for the dilutions involved; i.e. if 5 ml. of sample are used and 10 ml. of filtrate titrated, the volume of 0.01*N* thiosulphate $\times$ 0.28 gives mg. amino-N per ml. of original material. Where a shortage of material makes it desirable the volume may be reduced and the estimation carried out in 10 ml. volumetric flasks. In this case 6 ml. of the copper phosphate should be used and 2 ml. samples of filtrate titrated with *M*/500 thiosulphate.

RESULTS

Amino-acids

Solutions of each amino-acid were prepared from weighed amounts to contain 70–200 mg. of amino-N per 100 ml. Varying volumes from 2 to 10 ml. were used for the estimations. Table I shows the results obtained.

Table I

Amino-acid	Amino-N mg./10 ml.	
	Calculated	Found
Glycine	20.01	20.19
Alanine	20.10	20.19
Aspartic acid	20.02	20.25
Valine	A 9.98	10.32
	B 9.61	10.32
Tyrosine	12.00	12.04
Lysine	A 20.02	9.23*
	B 20.0	9.74
Arginine	9.98	7.72†
Tryptophan	2.12	7.16
+		
Glycine	5.04	12.50
Leucine	2.50	
+		12.90
Aspartic acid	10.00	
Methionine	2.55	12.55
+		
Aspartic acid	9.99	13.03
Phenylalanine	3.03	
+		13.09
Aspartic acid	10.00	

A and B samples from different sources.

* The ϵ group of lysine does not titrate by this method.

† The arginine was impure. The Van Slyke-N and copper values agreed.

¹ See Shaffer & Hartmann [1920–1] concerning the titration of copper iodimetrically.

The first seven acids all form soluble copper salts and present no difficulties. Tryptophan, phenylalanine, methionine and leucine were all estimated after mixture with a known excess of either glycine or aspartic acid. Lysine gives a value equal to half its nitrogen content, since the ϵ group is not determined by this method. Histidine was used as the monohydrochloride. Kober & Sugiura [1913] suggested that histidine formed a complex of the type $(\text{histidine})_3\text{Cu}_2$ and our results indicate this to be true. Four samples of histidine gave the results shown in Table II. The results found for histidine by the copper method multiplied by 0.75 give the α -amino-N value.

Table II. *Showing the results obtained with histidine monohydrochloride*

Histidine sample	Amino-N mg./10 ml. calculated	Theoretical for $(\text{histidine})_3\text{Cu}_2$	Found ml. thiosulphate $\times 2.8$
A	9.15	12.2	12.04
A	9.3	12.4	12.39
B	9.5	12.65	12.54
C	9.3	12.40	12.46
D	9.1	12.10	12.24

All the values given in Table I are the means of a large series of determinations. The estimations were made on varying volumes ranging from 2.0 to 10.0 ml., and at the lower end of the range the results were less accurate; exclusion of these results would have given a mean value nearer to the calculated value in most cases.

There appears to be a slight tendency to over-titrate with the thiosulphate, and for accurate work the end-point should be approached somewhat slowly.

Acid hydrolysis of proteins

The method has been used to study the rate of hydrolysis of protein by acids. Experiments on gelatin, zein, edestin and egg albumin were done, using

Fig. 1. Showing the hydrolysis of gelatin with 2, 5 and 20% HCl as measured by the copper method.

20% HCl at 100°. The materials were sealed in glass phials to avoid errors due to volume changes during heating. Results showing the course of hydrolysis are given in Figs. 1 and 2 where the amino-N determined by this method is

Fig. 2. Showing the hydrolysis of zein, edestin and egg albumin with 20% HCl as measured by the copper method. Zein •—•. Egg albumin ×—×. Edestin ○—○.

Table III. Showing the amino-acid content of gelatin, total N, and N estimated by the copper and Van Slyke methods

Figures for N represent g./100 g.

Amino-acid	g. present	Total N	Calculated N estimated by	
			Copper method	Van Slyke method
Glycine	25.5	4.75	4.75	4.75
Alanine	8.7	1.36	1.36	1.36
Leucine	7.1	0.76	0.76	0.76
Phenylalanine	1.2	0.10	0.10	0.10
Methionine	1.0	0.09	0.09	0.09
Aspartic acid	3.4	0.36	0.36	0.36
Glutamic acid	5.8	0.55	0.55	0.55
Histidine	0.9	0.24	0.11	0.08
Arginine	9.1	2.90	0.72	0.72
Lysine	5.9	1.18	0.59	0.59*
Proline	19.4	2.36	2.36	—
Hydroxyproline	14.4	1.54	1.54	—
Total	102.4	16.19	13.29	9.36

Theoretical copper value $13.29/16.19 \times 100 = 82\%$. Found 81%.

Theoretical Van Slyke value $9.36/16.19 \times 100 = 58\%$. Found 62.3%.

* Shaken for 4 min.; ϵ group of lysine not included.

Table IV. Showing N distribution figures for gelatin [Van Slyke, 1911-12] and the amounts estimated by the copper and Van Slyke methods

Fraction	% of total N	N estimated by	
		Copper method mg./10 ml.	Van Slyke method mg./10 ml.
Arginine	14.70	3.70	3.70
Histidine	4.48	1.98	1.49
Lysine	6.32	3.16	3.16*
Amino-N of filtrate	56.30	56.30	56.30
Non-amino-N of filtrate			
Proline	14.90	14.90	—
Hydroxyproline			
Total		80.04%	64.65%
		Found 81.0%	Found 62.30%

* Shaken for 4 min.; the ϵ group of lysine not included.

shown as a percentage of the total N. Hydrolysis of gelatin was also carried out with 5 and 2% HCl. Owing to its low solubility the zein was taken into solution in hot acid before it was filled into phials; some hydrolysis had already taken place at this stage. After these proteins had been hydrolysed completely with 20% HCl, the material left in the phials was bulked; estimations were made on the hydrolysate of the total N, and amino-N by the copper and Van Slyke methods. As a matter of interest we have given values deduced from the composition of the protein as given by Jordan-Lloyd & Shore [1938] and from the figures of Van Slyke [1911-12] for N distribution. Those for gelatin are given in full (Tables III and IV) and for the other proteins in summarized form in Table V. The difference between the values obtained by the two methods is large for gelatin owing to the high content of proline and hydroxyproline which are estimated by one method but not by the other.

Table V. *Showing summarized results for edestin and zein hydrolysates obtained by the Van Slyke and copper methods. Amino-N as % of total N*

Material	Method used	Calculated value from data	Found
Edestin hydrolysate	Van Slyke micro-method	57.4*	61.0
		61.58†	
	Copper method	60.0* 62.0†	62.3
Zein hydrolysate	Van Slyke micro-method	67.0*	74.6
	Copper method	75.0*	75.7

* Jordan-Lloyd & Shore [1938].

† Van Slyke [1911-12].

Tryptic hydrolysis

For this experiment fibrin was used as substrate and the digestion was carried out with (a) routine trypsin filtrate prepared from ox pancreas according to Cole & Onslow [1916] and (b) similar material treated with acid, based on the method of Northrop & Kunitz [1932], containing largely the proteinase. After 4 hr. digestion at 50° and pH 8, the materials were filtered and determinations made of the total N and amino-N by the Van Slyke and copper methods. Using the Van Slyke method, determinations were made after 4, 8, 16 and 32 min. shaking. Fig. 3 shows the results obtained; for this material at least 20 min. shaking was necessary before a constant result was obtained.

The two methods were also compared on material obtained from casein, deaminized with nitrous acid and afterwards digested with trypsin. The Van Slyke method gave, for 4 min. shaking, 8.95 mg. amino-N per 10 ml. and 10.56 mg. per 10 ml. after 30 min. shaking. The copper value was 10.59 mg. per 10 ml.

For each experiment the copper values quoted are the means of a large series of determinations, all of which agreed closely, in which varying volumes of material were used.

The value of the copper method for studying enzymic hydrolysis is also shown by an experiment on beef muscle. Cooked and well washed muscle was digested for 3 hr. with Merck's trypsin (treated according to Anson & Mirsky [1933]), and then for a further hour with crude untreated trypsin (Merck). Fig. 4 shows the results from copper estimations made during the digestion. After 3 hr. digestion with the purified enzyme (chiefly proteinase) the soluble

total N was 53.3 mg. per 10 ml. of which 9.15 mg. were amino-N (17.3%). 1 hr. after adding the crude trypsin the soluble total N increased to 98.8 mg. per 10 ml. of which 36.3 mg. were amino-N (36.7%).

Fig. 3.

Fig. 4.

Fig. 3. Showing comparative amino-N determinations by the Van Slyke (varying periods of shaking) and copper methods on two tryptic digests of fibrin. *A* = digested with crude trypsin. *B* = digested with proteinase trypsin. The dotted lines show the values obtained by the copper method.

Fig. 4. Showing the amino-N liberated during 3 hr. digestion with purified trypsin. At *A* crude trypsin was added. Determinations by the copper method. Substrate—cooked and washed beef muscle.

SUMMARY

A copper method for determining amino-N, which has been in routine use in these Laboratories since 1935, is described. This method has been found particularly suitable for determining the amino-N liberated by acid or enzyme hydrolysis of proteins. It gives quantitative results for pure amino-acids whose copper salts are soluble, and can be applied to some others which form insoluble copper salts. Some experimental results showing application of the method are recorded.

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