THE QUANTITATIVE ESTIMATION OF AMINO NITROGEN BY DETERMINATION OF BOUND COPPER WITH THE FLAME PHOTOMETER*

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(Received for publication, October 14, 1954)

The measurement of combined copper has been used in a number of procedures for the quantitative determination of amino acids and peptides. Pope and Stevens (1) as well as Schroeder, Kay, and Mills (2) determined the combined copper iodometrically. Martin and Mittelmann (3) analyzed for copper with the polarograph. Woiwod (4) used the spectrophotometer to measure the color produced by the reaction of copper with diethyl dithiocarbamate. Spies and Chambers (5) described a simple spectrophotometric technique in which the blue color obtained when an excess of alanine is added to the combined copper is utilized. In this laboratory, the iodometric determination was found troublesome, the Woiwod procedure lengthy, and the Spies-Chambers method not reproducible when used on plant tissue that yielded colored or turbid hydrolysates.

The present paper describes a simple and rapid method for estimating amino nitrogen in protein hydrolysates in which the combined copper is measured with the flame photometer. The results obtained by the flame photometric method are compared with those obtained by a modification of the Woiwod method (4).

Reagents and Solutions

Copper chloride solution. 14 gm. of reagent grade cupric chloride dihydrate per 500 ml. of water solution.

Sodium phosphate solution. 68.5 gm. of reagent grade trisodium phosphate dodecahydrate per liter of water solution.

Borate buffer, pH 9.1 to 9.2. 57.3 gm. of sodium borate decahydrate per 3 liters of water; filtered.

Sodium diethyl dithiocarbamate. 1 gm. dissolved in 50 ml. of water.

* Contribution No. 507, Department of Chemistry, Kansas Agricultural Experiment Station, Manhattan, Kansas. This work was supported in part by a grant from Eli Lilly and Company.

† From portions of a dissertation to be submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy.
The solution should be filtered; and refiltered before use if turbidity develops.

Washed copper phosphate. To 100 ml. of the sodium phosphate solution, 50 ml. of copper chloride solution were added with swirling, and the copper phosphate was removed by centrifuging. The residue was washed twice by resuspending in 150 ml. of sodium borate buffer and repeating the centrifugation. The copper phosphate was suspended finally in 250 ml. of sodium borate buffer, and 15 gm. of reagent grade sodium chloride were added to the suspension. This reagent was prepared according to the method of Schroeder et al. (2).

Copper standard solutions. A solution containing 2000 p.p.m. of copper was prepared by dissolving 2.682 gm. of reagent grade cupric chloride dihydrate in 500 ml. of distilled water. This solution was diluted to prepare standards containing from 1 to 40 p.p.m. of copper.

Isoamyl alcohol, Baker and Adamson, purified.

Amino acids were obtained from the following: DL-alanine and glycine, Eastman Kodak Company; L-arginine monohydrochloride, DL-aspartic acid, DL-histidine monohydrochloride, DL-isoleucine, DL-leucine, DL-methionine, and DL-threonine, Nutritional Biochemicals Corporation; L-glutamic acid, DL-phenylalanine, L-proline, DL-serine, DL-tryptophan, and DL-valine, General Biochemicals, Inc.; L-lysine, Merck and Company.

Phosphate buffer. 81.6 gm. of potassium dihydrogen phosphate and 23.0 gm. of sodium hydroxide were dissolved in 4 liters of water. The pH was adjusted to 8.4 with sodium hydroxide.

Protein Hydrolysates—Various plant tissues were hydrolyzed by a modification of the method of Melnick and Oser (6). A 1 gm. sample of the material to be hydrolyzed, 100 mg. of U. S. P. pancreatin (purified three times), and one or two crystals of thymol were placed in a 250 ml. beaker. 29 ml. of phosphate buffer and 40 ml. of water were then added to the beaker. The mixture was stirred 5 minutes as the pH was adjusted continuously to 8.4 with 5 per cent NaOH. The beaker was then covered with a watch-glass and set in a water bath maintained at 37°. The pH was readjusted to 8.4 at 12 hour intervals during hydrolysis. After the desired period of hydrolysis the hydrolysate was adjusted to pH 4.5 with glacial acetic acid and was boiled on a hot-plate for 3 minutes. The hydrolysate was then cooled, made to a volume of 100 ml., and filtered. After the filtrate was neutralized, the solution was ready for analysis.

Procedure

Flame Photometry—A Beckman model DU quartz spectrophotometer, equipped with a model 9200 flame photometry attachment and photomultiplier circuit, was used in the flame photometric work. The follow-
ing instrumental conditions were employed: wave-length 324.8 mµ, slit width 0.04 mm., oxygen pressure 16 pounds per sq. in., acetylene pressure 4 pounds per sq. in., maximal electrical sensitivity, sample feed rate, 1.56 ml. per minute.

The instrument was allowed to reach electrical stability before any analytical data were taken. The solutions to be analyzed were placed in 5 ml. beakers and atomized directly into the flame, and a series of three to five readings was taken on each sample. All transmittancy readings were corrected for a flame background with distilled water. Throughout this work, the background remained constant at 37 per cent transmittancy.

**Flame Photometric Copper Standard Curve**—This curve (Fig. 1) was prepared from data obtained by atomizing the copper standard solutions in the Beckman flame photometer. Per cent transmittancy, corrected for background, was plotted against copper concentration in parts per million to obtain the standard curve.

**Spectrophotometric Copper Standard Curve**—Portions of the same standards that were employed for flame photometry were used in preparing the spectrophotometric curve. The standards were diluted by pipetting 10 ml. of standard solution into 50 ml. volumetric flasks and making up to volume with water. To 10 ml. of this solution in a 50 ml. centrifuge tube equipped with a ground glass stopper was added 0.1 ml. of diethyl dithiocarbamate solution from a 1 ml. graduated pipette. 20 ml. of isoamyl

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**Fig. 1.** Flame photometric (△) and spectrophotometric (○) copper standard curves
alcohol were added to the solution in the centrifuge tube, and the mixture was shaken vigorously for 45 seconds and then centrifuged 5 minutes at 2000 r.p.m. A portion of the alcohol layer was removed with a pipette and its optical density determined at 435 μ. The calibration curve was obtained by plotting optical density, corrected for a water blank, versus the concentration of copper in the standard (Fig. 1).

**Development of Complex**—To 5 ml. of an amino acid solution or protein hydrolysate in a 15 ml. centrifuge tube were added 5 ml. of washed copper phosphate suspension. The tube was allowed to stand 5 minutes with occasional agitation and then centrifuged at 2000 r.p.m. for 5 minutes to remove the excess copper phosphate. A water blank also was carried through the reaction.

**Flame Photometric Analysis**—5 ml. of the supernatant liquid in the centrifuge tube were pipetted into a 50 ml. volumetric flask and made to volume with water. A portion of this solution was atomized into the flame photometer burner and its transmittance obtained.

**Spectrophotometric Analysis**—A 10 ml. aliquot of the solution used in the flame photometric analysis was diluted to a volume of 50 ml. 10 ml. of this solution were allowed to react with 0.1 ml. of diethyl dithiocarbamate solution. The reaction mixture was extracted with 20 ml. of isoamyl alcohol, and the optical density of the alcohol phase was measured as described above.

**RESULTS AND DISCUSSION**

**Alanine Amino Nitrogen Standard Curve**—Eleven alanine solutions containing from 10 to 400 mg. of amino nitrogen per liter were subjected to both the flame photometric and the spectrophotometric procedures. Alanine was chosen as the source of amino nitrogen, since Spies and Chambers (5) previously used it in expressing amino acid equivalence. The data obtained with these solutions were plotted versus the concentration of amino nitrogen to obtain the amino nitrogen standard curves shown in Fig. 2. The data also were referred to the proper copper standard curve (Fig. 1) to obtain the parts per million of copper bound, as shown in Table I.

From Table I it will be seen that alanine bound approximately theoretical amounts of copper over the entire range of amino nitrogen concentrations used. Theoretical binding is based on the observations of other workers (2–4, 7) that most amino acids, under conditions of this test, react to form compounds that can be represented by the formula CuA₂, where A equals an amino acid molecule.

**Amino Acids**—Sixteen solutions, each containing 300 mg. per liter of α-amino nitrogen of a given amino acid, were prepared. Also, a composite
Amino Nitrogen; mg/l.

Fig. 2. Flame photometric (Δ) and spectrophotometric (○) α-amino nitrogen standard curves.

Solution having the same amino nitrogen concentration was prepared in which the sixteen amino acids contributed equal amounts of α-amino nitrogen. The solutions were analyzed by both the flame photometric and spectrophotometric methods, and the readings obtained were referred to

Table I

Comparison of Copper Bound by Alanine Amino Nitrogen Determined by Flame Photometric and Spectrophotometric Methods

<table>
<thead>
<tr>
<th>Amino nitrogen mg. per l.</th>
<th>Copper bound</th>
<th></th>
<th>Spectrophotometry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Theoretical</td>
<td>Flame photometry</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p.p.m.</td>
<td>p.p.m.</td>
<td>p.p.m.</td>
</tr>
<tr>
<td>10</td>
<td>1.1</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>20</td>
<td>2.3</td>
<td>2.3</td>
<td>2.0</td>
</tr>
<tr>
<td>30</td>
<td>3.4</td>
<td>3.5</td>
<td>2.8</td>
</tr>
<tr>
<td>50</td>
<td>5.7</td>
<td>5.5</td>
<td>4.9</td>
</tr>
<tr>
<td>100</td>
<td>11.4</td>
<td>11.3</td>
<td>10.5</td>
</tr>
<tr>
<td>150</td>
<td>17.0</td>
<td>16.0</td>
<td>16.0</td>
</tr>
<tr>
<td>200</td>
<td>22.7</td>
<td>22.0</td>
<td>21.3</td>
</tr>
<tr>
<td>250</td>
<td>28.4</td>
<td>27.8</td>
<td>26.5</td>
</tr>
<tr>
<td>300</td>
<td>34.1</td>
<td>32.0</td>
<td>31.7</td>
</tr>
<tr>
<td>350</td>
<td>39.7</td>
<td>38.3</td>
<td>37.8</td>
</tr>
<tr>
<td>400</td>
<td>45.4</td>
<td>42.3</td>
<td>43.0</td>
</tr>
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</table>
the appropriate standard curve (Fig. 2). Good agreement between the two methods was obtained for all of the amino acids, as shown in Table II. The amount of \( \alpha \)-amino nitrogen in histidine was abnormally high. Histidine bound approximately 90 per cent of the amount of copper required for the formula \( \text{Cu}_2\text{A}_3 \). This agrees with Schroeder et al. (2) and Spies and Chambers (5).

### Table II

**Recovery of \( \alpha \)-Amino Nitrogen by Flame Photometric and Spectrophotometric Methods**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Flame photometry</th>
<th>Spectrophotometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Arginine monohydrochloride</td>
<td>100.7</td>
<td>95.7</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>97.7</td>
<td>102.3</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>97.7</td>
<td>100.7</td>
</tr>
<tr>
<td>Glycine</td>
<td>100.7</td>
<td>100.7</td>
</tr>
<tr>
<td>Histidine monohydrochloride</td>
<td>128.0</td>
<td>126.7</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>100.7</td>
<td>100.7</td>
</tr>
<tr>
<td>Leucine*</td>
<td>101.7</td>
<td>97.3</td>
</tr>
<tr>
<td>Lysine monohydrochloride</td>
<td>103.0</td>
<td>97.3</td>
</tr>
<tr>
<td>Methionine*</td>
<td>97.7</td>
<td>98.3</td>
</tr>
<tr>
<td>Phenylalanine*</td>
<td>97.7</td>
<td>101.3</td>
</tr>
<tr>
<td>Proline</td>
<td>97.7</td>
<td>94.3</td>
</tr>
<tr>
<td>Serine</td>
<td>100.7</td>
<td>102.3</td>
</tr>
<tr>
<td>Threonine</td>
<td>104.0</td>
<td>100.7</td>
</tr>
<tr>
<td>Tryptophan*</td>
<td>104.0</td>
<td>99.0</td>
</tr>
<tr>
<td>Valine</td>
<td>104.0</td>
<td>99.0</td>
</tr>
<tr>
<td>Composite</td>
<td>105.0</td>
<td>100.8</td>
</tr>
</tbody>
</table>

*The solution analyzed contained 2 parts of glycine nitrogen to 1 part of amino acid \( \alpha \)-amino nitrogen.*

Other workers (1, 2, 5) have found that phenylalanine, methionine, leucine, and tryptophan, when used alone, form insoluble copper complexes, but that they can be dissolved and separated from the excess copper phosphate by adding known amounts of glycine. In this work, 2 moles of glycine were added to each mole of the four amino acids listed above. No difficulty was encountered in the analysis of these amino acids under our conditions.

The results obtained with the composite solution of amino acids, without added glycine, indicate that insolubility of reaction products was not a problem in amino acid mixtures. This indicates the feasibility of determining mixed amino acids in protein hydrolysates by the flame photometric method. The recovery of 105 per cent \( \alpha \)-amino nitrogen in the
composite mixture can be explained in part by the presence of histidine. The average recovery of amino nitrogen by the flame method as calculated from the data presented in Table II is 0.5 per cent above the theoretical without histidine or 2.2 per cent with histidine. This compares with an average recovery by the spectrophotometric process of 1.0 per cent above the theoretical with histidine and a -0.7 per cent without histidine.

**Protein Hydrolysates**—Eight protein-containing plant products were hydrolyzed enzymatically as described above. The hydrolysates were analyzed by the flame photometric and the spectrophotometric methods and the data obtained were referred to the proper amino nitrogen standard curve. Other workers (1, 5) have shown that when protein hydrolysates form complexes with copper those components which are least soluble

<table>
<thead>
<tr>
<th>Time hydrolyzed</th>
<th>Sample</th>
<th>Flame photometry</th>
<th>Spectrophotometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrs.</td>
<td></td>
<td>mg. per l.</td>
<td>mg. per l.</td>
</tr>
<tr>
<td>43</td>
<td>Dehydrated bromegrass</td>
<td>200</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>Corn grain</td>
<td>60</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>Cottonseed meal</td>
<td>318</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>Hempseed meal</td>
<td>193</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>Sorghum grain</td>
<td>105</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>Soy bean meal</td>
<td>325</td>
<td>335</td>
</tr>
<tr>
<td>72</td>
<td>Dehydrated alfalfa</td>
<td>195</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td>Alfalfa silage</td>
<td>163</td>
<td>165</td>
</tr>
</tbody>
</table>

when by themselves are kept in solution by the others. Hence glycine was not added to any of the protein hydrolysates studied. Good agreement was obtained between the two methods (see Table III).

When hydrolysates of proteins contain a high concentration of the amino acids which tend to form insoluble copper complexes, it is theoretically possible that some amino acid is lost at the time of removal of excess phosphate. Even so, excellent agreement of results from these methods would still be obtained, just as in the case with the results reported in Table III, for the loss of amino acid would occur to an equal extent in the two procedures. However, others (1) have found that the Van Slyke method gives the same results that are obtained by a method which involves formation of copper complexes. It is assumed, therefore, on the basis of the good agreement reported in Table III that the results of the modification proposed here would be in good agreement with those by the Van Slyke procedure, at least with the hydrolysates tested.

**Concerning Flame Photometric Method**—Kingsley and Schaffert (8) have
shown that organic solvents, especially acetone, increase the sensitivity
of the flame photometer to calcium, potassium, and sodium. In this work,
acetone, dioxane, and ethyl alcohol were studied to determine their effect
on the sensitivity of the flame photometer to copper. When the organic
solvents were used in 50 per cent aqueous solutions, the corrected per cent
transmittancy was increased 3-fold for acetone, 2½-fold for dioxane, and
2-fold for ethyl alcohol. The instrumental conditions employed with the
organic solvents were the same as those when water alone was the solvent.
However, organic solvents were not used in this work, since the amount of
copper complex formed by the hydrolysates was sufficient to give adequate
readings with the instrumental conditions and the dilutions employed.

The flame photometric method was found to be more rapid than the
spectrophotometric method. The reaction with diethyl dithiocarbamate,
the extraction with alcohol, and the accompanying centrifugation are
eliminated. In analyzing for bound copper in the amino acids or protein
hydrolysates, it is necessary to adjust instrumental conditions to repro-
duce the original calibration curve. In some instances it is known that
organic material (9) or extraneous inorganic ions (10) may interfere in a
flame photometric analysis. However, no difficulty was encountered in
this procedure. This is substantiated by comparing the curve obtained
by analyzing solutions containing only the copper ion in distilled water
(Fig. 1) and that obtained by analyzing for copper bound with alanine
(Fig. 2). When a blank mixture containing all the components except
copper was analyzed under these conditions, a per cent transmittancy
equal to that of distilled water resulted. All the usual precautions as-
associated with good flame photometry techniques should be observed.
During the course of analysis, it is occasionally necessary to clean the
burner-atomizer assembly. In this study on amino nitrogen, it appears
that the flame photometric method and the spectrophotometric method
are equally reproducible.

SUMMARY

A simple, rapid method is described for the analysis of amino acids in
which they form complexes with copper and the copper is measured with
a flame photometer. Sixteen amino acids were studied. Results obtained
by the flame photometric method and by a spectrophotometric method
were compared. The application of the method to the determination of
amino acids in enzymatically hydrolyzed plant tissue is described.

BIBLIOGRAPHY
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