1106

[illegible] Colorimetric Procedures for Amino Acids

By [illegible]

1. Phenol Reagent Method

[illegible]

Reagents

Phenol reagent. One hundred grams of $Na_2WO_4 \cdot 2H_2O$, [illegible] g of $Na_2MoO_4 \cdot 2H_2O$, 700 ml of H_2O, [illegible] ml of [illegible] H_3PO_4, and [illegible] ml of concentrated HCl are boiled under reflux for [illegible] hours. Then [illegible] g of $Li_2SO_4 \cdot H_2O$, [illegible] ml of H_2O, and a few drops of Br_2 are added, and the solution is boiled for [illegible] minutes without the condenser. [illegible]

[illegible]

[illegible]

Methyl cellosolve. The methyl cellosolve should give a clear solution when mixed with an equal volume of water and should give a negative or very faint peroxide test with 10% aqueous KI [illegible]

Diluent solvent. Equal volumes of water and reagent grade n-propanol are mixed.

Solutions for analysis. Solutions of amino acids of [illegible] to 2.0 [illegible] or suitable concentrations of other compounds to give equivalent [illegible] are used for analysis of 0.1-ml. samples. [illegible] solutions should be neutralized to the end point of methyl red before analysis.

Procedure. Moore and Stein give details for application of the method to large numbers of samples with modifications as to volume and concentration of test solutions. They also give extensive details of special techniques and equipment such as automatic and self-adjusting pipets, selection of matched photometer tubes, water bath, mechanical shaker, and automatic pipetting machine. Users of the method interested in these details should consult the original article. The basic method described below is for 0.1-ml. samples of concentrations suitable for the described standard curve. Modifications can be made to accommodate the particular needs of the worker.

The sample (0.1 ml.) is placed in a 18 × 150-mm. photometer tube, and 1.0 ml. of ninhydrin solution is added. The tube is covered with an aluminum cap, and the contents mixed. A rack of tubes is heated for 20 minutes in a boiling water bath. Five milliliters of diluent is then added [illegible]

[illegible]

solutions must be determined by appropriate blank analyses. Color intensities of equimolar quantities of various amino acids relative to standard leucine vary. The relative color values of amino acids and some related compounds are given in Table I.[1] Moore and Stein[2] give correction factors for evaporation of solvent where water-organic solvent systems are analyzed. The accuracy of the method is 2% for individual amino acids with samples in the range of 2.5 γ of NH_3 nitrogen. No color is formed by equimolar quantities (relative to leucine) of adenine, p-aminobenzoic acid, diethylbarbituric acid, glucose, and uric acid. Creatine, creatinine, dibenzylamine, and glycine anhydride formed only 1 to 4% of the color intensity of leucine.

III. Copper Salt Method for Amino Acids and Peptides (620 mμ)

Principle. This spectrophotometric method is based on the blue color of the complex copper salts of amino acids and peptides. The copper salts of the amino acids are converted to the copper salt of alanine to eliminate the variation in color values of equimolar concentrations of copper salts of the amino acids. Peptide copper salts are also converted to the copper salt of alanine, the latter serving as a reference standard. The procedure described below is based on a method developed by Spies and Chambers[3] for studying rate and degree of protein hydrolysis.

Reagents

Copper chloride. 28 g. of reagent-grade $CuCl_2 \cdot 2H_2O$ per liter of H_2O solution.

Sodium phosphate. 68.5 g. of reagent-grade $Na_2PO_4 \cdot 12H_2O$ per liter of H_2O solution.

Sodium borate buffer, pH 9.1 to 9.2. 40.5 g. of reagent-grade anhydrous $Na_2B_4O_7$ is dissolved in 1 l. of H_2O, and the solution is filtered. Reagent-grade NaCl is dissolved in the buffer at the rate of 4 g. per 100 ml. This NaCl-containing solution is referred to as buffer solution.

Washed $Cu_3(PO_4)_2$.[4] To 10 ml. of sodium phosphate solution is added 20 ml. of $CuCl_2$ solution with swirling. The suspension is centrifuged for 5 minutes. The precipitate is washed twice by suspension in 60 ml. of buffer solution followed by centrifuging. The washed precipitate is suspended in 100 ml. of buffer solution. $Cu_3(PO_4)_2$ suspension in glass-stoppered bottles give maximum color after 4 days of storage, but slight decrease in color is obtained after 10 days of storage.

[1] R. Spies and D. C. Chambers, J. Biol. Chem. 161, 137 (1945).
[2] [illegible]

Alanine. Alanine is recrystallized with special care to prevent contamination with traces of insoluble foreign matter which might interfere with spectrophotometric analyses and to obtain analytically pure alanine for the standard curve.

Procedure. Be 5.0 ml. of test solution in a [illegible]-ml. glass-stoppered [illegible] is added 5.0 ml. of the $Li_2(PO_4)_3$ suspension. The suspension

TABLE II

[illegible] COLOR INTENSITIES PRODUCED BY [illegible] IN SOLUTIONS OF AMINO ACIDS ALONE AND WITH AMINO ALANINE AS COMPARED TO ALANINE AS 100%

Amino acid	Alanine equivalence, %	
	Without added alanine	With added alanine
[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
Aspartic acid	[illegible]	[illegible]
Glutamic acid	[illegible]	[illegible]
Glycine	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]
Hydroxyproline	[illegible]	[illegible]
Isoleucine	[illegible]	[illegible]
Leucine	[illegible]	[illegible]
Lysine monohydrochloride	[illegible]	[illegible]
Methionine	[illegible]	[illegible]
Phenylalanine	[illegible]	[illegible]
Proline	[illegible]	[illegible]
Serine	[illegible]	[illegible]
Threonine	[illegible]	[illegible]
Tryptophan	[illegible]	[illegible]
Tyrosine	[illegible]	[illegible]
Valine	[illegible]	[illegible]

[illegible]

[illegible] centrifuged in a capped [illegible] is carefully decanted [illegible] containing 200 mg. of alanine, [illegible] of the solution is deter- [illegible] of amino acids and peptides [illegible] and 5.0 ml. of water; then

200 mg. of alanine is dissolved in 9.5 ml. of this solution. Blank solutions for analysis of protein hydrolysates are made by mixing 5.0 ml. of buffer solution with 5.0 ml. of the diluted hydrolysate. After centrifugation, 200 mg. of alanine is dissolved in the decanted solution.

The standard curve is prepared with eight alanine solutions ranging from 5 to 40 mM, each solution differing by 5 mM. Conformity to Beer's

TABLE III

CHARACTERISTICS OF INTENSIVELY COLORED COMPOUNDS WITH AMINO ACIDS

Amino acid	Color of solution before adding copper solution	Maximum of test solution	Alanine equivalence of test solution, %	Ratio of mol. wt. to equivalent wt. of test compound	Formula of copper salt[a]
Alanine	Blue	0.0000	100.0	1.00	Cu_2A_2
Dichloroglycine[b] hydrochloride	Blue	0.0000	98.6 ± 0.0	2.00	Cu_2P
Alanylglycine	Blue	0.0000	100.0	2.00	CuP
Glycylalanine	Blue	0.0000	100.0 ± 0.4	2.00	CuP
Glycylglycylalanine hydrochloride	Blue	0.0000	100.0 ± 0.0	2.00	CuP
Glycylglycylglycine hydrochloride	Blue	0.0150	98.0 ± 0.0	2.00	CuP
Glycylleucylleucine	Green-blue	0.0150	99.0 ± 0.0	1.90	CuP
Glycylleucine	Blue	0.0150	98.0 ± 0.0	0.70	CuP_2
Glycylleucine	Blue	0.0150	100.0 ± 0.0	1.90	CuP
Leucylglycylglycine	Blue	0.0150	98.0 ± 0.0	1.90	CuP
Glycylglycylglycine	Purple	0.0150	100.0 ± 0.0	2.00	CuP
Glycylglycylleucine	Purple	0.0150	99.0 ± 0.0	1.90	CuP
Glycylleucylglycine	Purple	0.0150	98.0 ± 0.0	1.90	CuP
Glycylleucylleucine	Purple	0.0150	98.0	1.90	CuP
Leucylglycylleucine	Purple	0.0150	98.0 ± 0.0	1.90	CuP

[a] Calculated on the basis of chemical analysis and formula. Only P stands for ligand, the α-ketoimine.

[b] This solution would decolorize after adding 50 mg. of alanine, but over the entire range of concentrations is obtained. Acid solutions should be brought to between pH 7 to 8.1 before dialysis.

Discussion. The molar color equivalence of numerous amino acids differs from that of alanine from [illegible] as shown in Table I. Histidine gives an alanine color equivalence value of [illegible]. Tyrosine, phenylalanine, methionine, leucine, and tryptophan are insoluble in partially soluble copper salts. These amino acids can be analyzed by forming a mixed salt with glycine. Addition of glycine to analysis of protein hydrolysates is not necessary because of the small proportion of these refractory amino acids and the solubilizing effect of the other amino acids present. The

copper salt of cystine is too insoluble to be determined even with the mixed salt method. Dipeptides react with copper as though the component amino acids are free, whereas only two of the amino acids of tripeptides react with copper as shown in Table III. In general, only those amino acids of peptides or proteins react with copper that have a free carboxyl group adjacent to peptide-linked nitrogen or with a free amino group adjacent to a peptide-linked carboxyl group. Therefore, the copper-binding capacity of protein hydrolysates increases during hydrolysis until all the α-amino nitrogen is either free or in dipeptide form. Application of the method to the study of the rate of hydrolysis of proteins is described in the original paper.[11] Solutions (25 m*M*) of lactic acid, glucose, sucrose, mannitol, mannitol, and ammonium chloride give no color with copper in this test. Noninterference by ammonia is an advantage of the method. The average deviation of analyses is ±0.2%.

IV. Copper Salt Micromethod for Amino Acids and Peptides (Ultraviolet)

Principle. The method is based on the observation that copper salts of amino acids have an absorption maximum at 250 mμ. The procedure described below is based on a method developed by Spies[12] for studying protein hydrolysis.

Reagents

0.05 *M* copper chloride: 8.53 g. of reagent-grade $CuCl_2 \cdot 2H_2O$ per liter of H_2O solution.

Sodium borate buffer, pH 9.3 to 9.2: 46.5 g. of reagent-grade, anhydrous $Na_2B_4O_7$ is dissolved in 4 l. of H_2O solution and filtered. For pH 9.2 g. of reagent-grade NaCl is dissolved in 100 ml. of borate solution. This NaCl-containing solution will be referred to as buffer solution.

Alcohol: Analytically pure alcohol.

Procedure. To 3.0 ml. of buffer solution in a 15-ml. glass-stoppered centrifuge tube[13] is added 2.0 ml. of test solution. Then with pipettes of 0.05 *M* copper is added, and the tube is stoppered, shaken, and let stand for 10 minutes in a constant room temperature. For maximum accuracy the room temperature (25°) is maintained for all determinations. The mixture is centrifuged (2000 rpm) and stoppered while. The clear solution is poured into a quartz cuvette and transmittance is determined at 250 mμ. The ultraviolet-absorbing image of the copper salts is relatively insoluble

[13] Pyrex Z. Anal. Chem. 108, 241 (1937).
[14] Made from Kimble Glass Co., Toledo, Ohio.

in the presence of excess copper salt; therefore centrifugation at exactly 10 minutes after addition of the copper chloride is recommended. After centrifugation, the loss of absorbing capacity of the solutions is less than 3% in 60 minutes.

TABLE IV

ABSORBANCE SPECTRA OF COPPER SALTS OF 0.0002 M SOLUTIONS OF AMINO ACIDS AND MEASURED AT 230 mμ

Amino acid	Wavelength of maximum absorbency,* mμ	Alanine equivalence, % At 230 mμ	Alanine equivalence, % At wavelength of the maximum absorbency
Alanine		100.0	
Arginine	220–225	108.3	
Asparagine	220–225	86.9	
Cysteine[a]	222–225	80.7	83.7
Glutamic acid	220–225	100.8	
Glycine	220–227	100.3	102.9
Histidine	225–227[b]	98.4	97.1
Hydroxyproline	217–220	81.1	86.1
Isoleucine	220–222	102.7	
Leucine	220–222[c]	102.8	
Lysine	220–222	96.9	
Methionine	220–230	102.8	
Phenylalanine	220–225	97.1	
Proline	222–240	98.2	98.1
Threonine	220–222	100.7	
Tryptophan	[illegible]	94.1	
Tyrosine	220–225	94.7	95.7
Valine	220–225	99.4	

* Range of wavelength over which maximum absorbency occurred in not more than 10% of readings over 20 % transmittance.

[a] Average deviation of all results, 2.5%.

[b] A 0.0001 M solution was used.

[c] Maximum absorbency was found at lower wavelengths than given.

[d] Reading at 230 mμ was lower, however, with less accuracy.

Blank solutions are made by adding 2.0 ml. of the test solution to 2.0 ml. of buffer solution. A series of solutions of a freshly prepared mixture of alanine can be made with each assay to check for slight variations in absorbency due to distilled water or other causes because of the sensitivity of the method. This method employs blank necessary if maximum accuracy is not required or if experience shows it to be unnecessary.

The standard curve is prepared with alanine solutions in concentrations from 10 to 200 μM. Conformity to Beer's law over the entire range

of concentration to obtained long transmittance of absorbed given a known, [illegible] value of approximately 75 % due to absorption of copper salt in solution.

Discussion. The molar plotting equivalents of amino acids in [illegible] Table III, lines within 5%, and lines within 10% of the 100% value of standard, as shown in Table IV. Histidine gives an equivalent of [illegible] of 98 %, and tryptophan gives an equivalent of 107%. An [illegible]

TABLE IV

Analytical Recoveries of Certain Amino Acids Determined and Theoretical [illegible]

Amino acid	Weighed amount of amino acid	Amount found	Recovery	Theoretical amino acid	Recovery
	mg.	mg.	%	mg.	%
Alanine	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Hydroxyproline	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycine	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycylglycine	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycylleucine	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycylleucylleucyl acid	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycylleucylleucyl acid	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycylhydroxyvaline	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycylserine	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycylleucine	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycylglycylglycine	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycylglycylglycine	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycylglycylglycine	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycylglycylglycine	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycylglycylglycine	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycylglycylglycine	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Glycylglycylglycine	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

[illegible] based on the absorbance of [illegible] at [illegible] [illegible] a range of amounts determined.

advantage of this method is that the copper salt of amino acids [illegible] tyrosine, methionine, proline, and cysteine are all easily [illegible] the analysis without interference of the hydrolysis and physical [illegible] gray, [illegible] that 100-mm. cuvettes [illegible] [illegible] absorption differs from that of phenol, can be analyzed by applying an [illegible] correction for the spectrophotometer [illegible] amino acid being analyzed.

The absorbing capacity of the copper salt of the amino acids is [illegible] mg. is more than that of the copper salt of the corresponding peptide. [illegible] amino acids, as shown in Table [illegible]. This [illegible] absorption

provides the basis for adapting this method to study rate and degree of protein hydrolysis. Application of the method to the study of protein hydrolysis is described in the original paper, and use of the method in following proteolysis of cottonseed allergen has been described.[11]

Test solutions of [illegible] μM (twice the concentration of amino acid usually employed) in glucose, galactose, sucrose, mannitol, inositol, and ammonium chloride did not interfere with the test. Noninterference by ammonia is an advantage of the method. The necessity of avoiding traces of copper or amino acids in reagents, distilled water, or on glassware is obvious from consideration of the sensitivity of the method. Particular care must be taken to exclude contaminating copper from blank solutions.

The precision of the method determined from duplicate analyses of eight [illegible] μM alanine solutions is: standard deviation of the mean $\pm 1.1\%$, and standard deviation of a single pair of duplicates $\pm 3.2\%$.

[11] J. R. Spies, D. C. Chambers, E. J. Cookson, H. S. Burnton, and H. Stevens, J. Allergy [illegible] ([illegible]).

[illegible] Microbiological Determination of Amino Acids

By Esmond E. Snell

Introduction

The use of microorganisms for quantitative estimation of amino acids was a natural outgrowth of their earlier successful use in the determination of vitamins. The general principles on which such methods are based, an account of their development, and details of many individual assay methods, have been treated in [illegible].

The methods depend on employment of [illegible] individual amino acids are essential nutrients. In a basal medium lacking a single essential amino acid [illegible] the growth of such an organism is, within [illegible] amino acid added. The [illegible] can thus be determined by [illegible] measured in

[illegible] (1945).
[illegible]
[illegible] New York, [illegible]
[illegible]
[illegible] Amino Acids," [illegible]
[illegible] Physiol. Rev. [illegible]