PHOTOMETRIC ESTIMATION OF PROLINE AND ORNITHINE

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Some years ago Grassmann and von Arnim (1, 2) described in detail a red reaction product of proline and ninhydrin formed at pH 7; it was suggested that this reaction product might be of use as a measure of the concentration of proline in biological materials. Hydroxyproline gave a similar though not identical reaction product. More recently, Van Slyke et al. (3), in studies of the ninhydrin method for the gasometric estimation of free amino acids, reported that, at a pH of approximately 1.0, a red water-insoluble reaction product is formed by proline with ninhydrin; lysine forms a black product and ornithine a red one. No significant amounts of color are formed with most other amino acids at a pH near 1.0.

Under the conditions specified below, it has been found that the amount of colored product formed by the reaction of certain amino acids with ninhydrin at approximately pH 1.0 may be estimated spectrophotometrically and used as a measure of the amount of these amino acids present. The procedure is applicable to proline, ornithine, lysine, and hydroxylysine in pure solution. Most other amino acids and particularly hydroxyproline do not interfere significantly.

Procedure

Reagent Solution—Each ml. contains 0.4 ml. of 6 M H₃PO₄ and 0.6 ml. of glacial acetic acid; 25 mg. of ninhydrin¹ are added per ml. of this acid mixture. After the addition of the ninhydrin, the acid mixture is heated to about 70° to insure solution of the ninhydrin. The solution is stable for at least 24 hours; whether it is stable for longer periods has not been determined.

Color Development—To 1.0 ml. of the solution to be analyzed are added 1.0 ml. of glacial acetic acid and 1.0 ml. of the reagent solution. If the additions are made from burettes, a Silicone grease must be used to lubricate the stop-cocks. A sample blank is prepared by adding 1.0 ml. of glacial acetic acid and 1.0 ml. of acid mixture without ninhydrin to 1.0 ml. of the solution to be analyzed. A reagent blank is prepared by adding

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¹ Ninhydrin purchased from Dougherty Chemicals, 87-34 134th Street, Richmond Hill 18, New York, has been used without recrystallization.
1.0 ml. of the reagent solution and 1.0 ml. of glacial acetic acid to 1.0 ml. of the solvent of the amino acid. After the contents of the individual tubes have been mixed, the tubes are capped and heated in a constant temperature bath at 100° for 60 minutes. 1.0 ml. of glacial acetic acid is added to each tube; the tubes are then cooled to room temperature. The volume is adjusted to 5.0 ml. with glacial acetic acid. The optical densities are estimated at the appropriate wave-length. The readings are constant for at least 1 hour. The volumes stated above may be changed to meet the requirements of the available spectrophotometric equipment, but the proportions must be maintained. Standards are carried through the same procedure.

**Results**

**Absorption Curves of Solutions of Several Amino Acids after Reaction with Ninhydrin** Fig. 1 shows the molar absorption curves of solutions of proline, ornithine, lysine, natural and synthetic hydroxylysine, and cysteine after reaction with ninhydrin2 (Beckman DU spectrophotometer, 1 cm. path length, constant sensitivity setting at maximum, variable slit width setting). Several points may be emphasized. The curves for the natural and synthetic hydroxylysine are so nearly identical as to be indistinguishable on the scale of Fig. 1. As pointed out by Weisiger (4) and by Touster (5), this is substantiating evidence for the chemical identity of the natural and synthetic hydroxylysines. The proline and ornithine reaction solutions have absorption curves which are similar; if the molar absorption coefficients are plotted on a logarithmic scale with wave-length on a linear scale, it can be shown that the curves are essentially identical in shape but that the proline curve is displaced from the ornithine curve by a nearly constant proportion. (The molar absorption coefficient for proline is 89.8 per cent of the molar absorption coefficient for ornithine at λ = 515 mμ.) From this it is inferred that the reaction products of ornithine and of proline with ninhydrin may be identical under the conditions of this procedure but that the yield of reaction product is less with proline than with ornithine.

**Recovery of Reaction Products of Proline and of Ornithine with Ninhydrin**—Advantage was taken of the almost complete insolubility of these reaction products in water. 100 ml. of the reagent solution were added to

2 The proline, lysine, and ornithine were c.p. products purchased from the H. M. Chemical Company, Ltd., 144 North Hayworth Avenue, Los Angeles 36, California. High purified samples of ornithine and proline were supplied by Dr. Paul B. Hamilton; no significant differences in the absorption curves were found with these preparations. The natural and synthetic hydroxylysines were supplied by Dr. James R. Weisiger.
14.67 mg. of proline in 15 ml. of H₂O and to 15.41 mg. of ornithine monohydrochloride in 15 ml. of H₂O. The solutions were heated at 100° for 60 minutes. (A definite yellow color was noted in the vessel containing proline before the characteristic red color appeared; no yellow color was noted in the ornithine vessel.) To each solution were added 150 ml. of H₂O, and the solutions were stored at approximately 4° for 12 hours; the precipitates were then collected, dried, and weighed. Yields in mg. per mM of amino acid were 286.0 for ornithine and 255.2 for proline. The yield of proline reaction product was 89.2 per cent of that of the ornithine reaction product. Portions of the precipitates were accurately weighed and dissolved in hot glacial acetic acid. Absorption coefficients per gm. of reaction product per liter were 75.86 for proline and 75.41 for ornithine (Beckman DU spectrophotometer, 1 cm. cuvettes, λ = 515 mμ, 0.020 mm. slit width setting). The absorption coefficient of the proline reaction product was 100.6 per cent of that of the ornithine reaction product. No differences were detected in the two absorption curves in the range 320 to 620 mμ. These findings substantiate the inference that the major reaction products of ornithine and of proline with ninhydrin are identical but that the yield from proline is less than that from ornithine. The yellow product in the reaction with proline has not been identified.
**Interfering Substances**—After reaction with ninhydrin, the following substances did not yield significant increases in optical densities at \( \lambda = 515 \text{ m}\mu \) when present in molar concentrations 100 times as great as those of proline or ornithine: leucine, isoleucine, serine, valine, threonine, glutamic acid, glycine, alanine, \( \delta \)-aminovaleric acid, urea, creatine, creatinine, glycocyanine, glucosamine, ammonium chloride, pyrrolidonecarboxylic acid. The following substances did not yield significant increases in optical densities at \( \lambda = 515 \text{ m}\mu \) when present in molar concentrations 10 times as great as those of proline or ornithine: glutamine, histidine, cystine, methionine, tryptophan, arginine, tyrosine, hydroxyproline, aspartic acid, asparagine, phenylalanine, 1,4-diaminobutane, 1,5-diaminopentane. The following substances yield significant increases in optical densities at \( \lambda = 515 \text{ m}\mu \) when present in molar concentrations 10 times as great as those of proline or ornithine: citrulline, cysteine, lysine, and hydroxylysine. The interference by hydroxyproline depends on the purity of the sample: at equimolar concentrations the best sample of hydroxyproline gave an optical density reading 0.5 per cent that given by proline. The interference produced by arginine is probably not the result of production of ornithine during the reaction with ninhydrin: the absorption coefficient varies from sample to sample and there is no increase in the optical densities of the solutions when these are heated for longer than the specified 60 minutes. It appears that small and variable amounts of a contaminant, possibly preformed ornithine, may be present in the several samples of arginine examined. As is evident from Fig. 1, cysteine produces a characteristic color; this color is not produced with cystine. The cysteine reaction product has not been identified. The interference by citrulline may be due to hydrolysis of this amino acid to yield proline. Tryptophan gives a canary-yellow color, with a peak at \( \lambda < 320 \text{ m}\mu \).

**Agreement with Bouguer-Beer Law**—A linear relationship is obtained between concentration of amino acid and optical density in the range 0.02 to 0.1 \( \mu \text{M per ml.} \) for proline and ornithine and in the range 0.1 to 0.5 \( \mu \text{M per ml.} \) for lysine and hydroxylysine.

**DISCUSSION**

The applicability and limitations of the procedure are evident from the results. The procedure is best suited for the identification and estimation of the amino acids indicated above in those instances in which interfering substances have been removed, for example by chromatography, or in which interfering substances are known to be absent. The procedure has already been applied by several investigators (4–7). It must be emphasized that the possible interference of peptides and particularly of prolyl peptides has not been investigated.
It is tentatively suggested that the ornithine undergoes oxidative deamination and decarboxylation and that the resultant aldehyde group condenses with the terminal amino group with formation of a ring. This cyclic product then couples with 2 molecules of ninhydrin to form the colored product. The appearance of a yellow color in the reaction of proline, not apparent in the reaction of ornithine, suggests that a side reaction occurs. This could account for the fact that less color is produced by reaction of proline than by reaction of ornithine with ninhydrin.

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SUMMARY

A method is presented for the estimation of proline, ornithine, lysine, and hydroxylysine in pure solution and in the presence of certain other amino acids. Hydroxyproline does not interfere significantly in the estimation of proline. The applicability and limitations of the method are briefly discussed.

BIBLIOGRAPHY

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