Notes

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Takeo Tsukamoto, Tetsuya Komori, and Nadao Kinoshita : Microanalysis of Amino Acids. I. A New Modified Ninhydrin Reagent.

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Of all the proposed methods for the quantitative determination of amino acids separated in a minute quantity by paper chromatography, the one[1~7] devised for colorimetric determination of the extract from the part of the paper on which the substance is developed excels the others in precision and accuracy.

Our investigation into the chemical structure of protein had to be started with determination of the free amino-acid content of protein hydrolysate and that of the free amino acid content of a given tissue.

Our comparative examinations having shown that none of the reagents known was satisfactory for the purpose, we undertook a reëxamination of ninhydrin as reagent and succeeded in finding a new, simple method by which the reagent can be used with precision and to the utmost of its sensitivity.

The photometric determination of the ninhydrin-produced colors of amino acid depends for its accuracy on the attainment of the highest possible color yield and on the stability of the colored end-product, Ruhemann purple. The principles set forth on the subject by Stein[8] and some others are now accepted rather extensively, but the reagents proposed for use are unsatisfactory in that, when used in paper chromatography, they produce a high blank test value, the coloring for amino acid of one identical mole is subject to increased variation, and that they are all over-sensitive to ammonia. It has recently been reported, though nothing more particular has been heard of since, that Cooking and others[9] succeeded in the quantitative determination of amino acid containing 0.05 to 2.8 μg./cc. of amino nitrogen by the use of 0.01*M* potassium cyanide as antioxidant against ninhydrin reaction.

The present examination has proved to some extent the reliability of this method, but it should be noted as its defect that the continuous variation in the purity of potassium cyanide results in a continual change in its potency as a reducing agent and affects the determination of the blank test value. The method employed by Yamagishi, *et al.*[10] for quantitative determination of several kinds of amino acid consists in insuring the formation of Ruhemann purple by adding ascorbic acid and pyridine to ninhydrin so as to inhibit decomposition of the intermediate product of ninhydrin reaction. However, none of these methods, with its merits and demerits, is adapted for use in paper chromatographic determination. Our reëxamination disclosed that ascorbic acid used by itself as a reducing agent was apt to give variation to the result of determination.

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1) L. Naftalin : Nature, **161**, 763(1948).
2) H.B. Bull, J.W. Hahn, V.C. Baptist : J. Am. Chem. Soc., **71**, 550(1949).
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Accordingly, metaphosphoric acid solution of ascorbic acid, in which ascorbic acid is stable, was added to 2% Methylcellosolve solution of ninhydrin, the mixture was kept at pH 5.0 by the addition of citrate buffer of pH 5.3, this being the optimum pH, as ascertained by Riffart[11] and the present authors. It was thus a reagent superior in precision and sensitivity to any conventional reagent and available in any quantitative analysis by paper chromatography. The minimum quantity of amino acid determinable by the use of this reagent is 0.025 mμM/cc., in the case of alanine, the determined value conformed to that determined by Lambert-Beer's rule, and the error did not exceed 5%. The reaction mixture in which the reagent has been used shows the presence of Ruhemann purple formed in it, as indicated by the absorption spectrum approximately at 570 mμ.

Experimental

Reagents

1) **2% Methylcellosolve Solution of Ninhydrin**—One g. of ninhydrin is dissolved in Methylcellosolve (50 cc.), previously redistilled and made negative to the peroxide test by the addition of 10% KI solution, the solution is left standing for 30 mins. while saturating nitrogen gas, and preserved in a brown-colored bottle. This reagent, kept in an ice box, remains effective for 1 week.

2) **Aqueous Solution of Metaphosphoric Acid** (2%)—Aqueous solution of metaphosphoric acid (20%), available for 1 month when kept in an ice-box. This is used in a 10-fold dilution.

3) **Ascorbic Acid (0.15%) dissolved in the Solution** (2)—Ascorbic acid is dried over H_2SO_4 and 75 mg. of the crystallized ascorbic acid, purity over 99%, is added to the 2% metaphosphoric acid solution to make a volume of 50 cc. This reagent is prepared anew every time it is needed.

4) **1% Ninhydrin Reagent**—A mixture of 50 cc. of reagent (1), and 50 cc. reagent (3). The total volume of the mixture is brought exactly to 100 cc. by the addition of a solution of metaphosphoric acid. Available for 2 days.

5) **Citrate Buffer Solution (pH 5.3)**—Citric acid monohydrate (10.0 g.) dissolved in distilled water (100 cc.) is added with a solution of NaOH (4.4 g.) in water (100 cc.), and brought to a total volume of 250 cc. with distilled water (pH 5.3±0.02).

Procedure—A standard aqueous solution of amino acid (0.025~0.8 mμM/cc.) (1 cc.) is placed in a graduated glass-stoppered test tube, citrate buffer (0.5 cc.) is added through a microburette, and 1% ninhydrin reagent (1 cc.) is added. The mixture is shaken gently and heated at 100° for 20 mins. in a water bath so as to bring the reaction to completion. The reaction mixture, now reddish purple in color, is cooled with tap water, the whole volume is brought to precisely 10 cc. with 50% EtOH, and shaken uniformly for a minute for complete aeration. The solution thus treated is deep purple instead of reddish purple. The optical density of the solution is determined at 570 mμ, within 1 hr. after the cooling process. The blank test value determined simultaneously by the above procedure is subtracted from the value of optical density, and the concentration of amino acid is determined by computation from this value, as shown in equation (1) (Fig. 2). Redistilled water is used as control solution. The Beckmann Type spectrophotometer eqipped with a cell of Corex glass of 10-mm. optical depth, used were Shimadzu, Type QB 50, 450 V, slit 0.01, or Hitachi, Type EPU 2, slit 0.04.

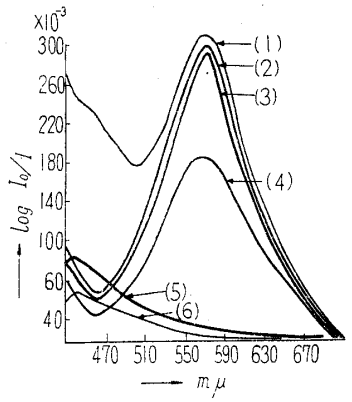

Fig. 1. Comparison of Absorption Spectra of Ruhemann Purple, after the Ninhydrin Reaction with 0.2 mμ*M* of Amino Acid

Cystine ·····(1)
Alanine·····(2)
Serine ·····(3)
Ammonia·····(4)
Proline ·····(5)
Hydroxyproline····(6)

11) H. Riffart : Biochem. Z., **131**, 78(1922).

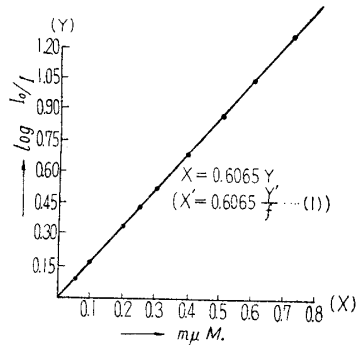

Fig. 2.
A Standard Curve of Alanine

Experimental Result and Conclusion

In alanine, relationship between the concentration, X (0.025~0.8 mμM/cc.), and the determined optical density, Y, is in a straight line, as shown in Fig. 2. The error was 5% or less. The relative coloring (f) for several different amino acids. as measured at 0.4 mμM are given in Table I. The photometric determination of proline and hydroxyproline was made at 440 mμ and the determined optical density, divided by that for alanine, at 570 mμ, gives the equation $X'=0.6065\frac{Y'}{f}$, making the quantitative determination of all amino acids possible. Freshly prepared 1% ninhydrin reagent can be used for 2 days, but as ninhydrin crystallizes out over a long period, the reagent loses its color-producing power by degrees with the crystallization of hydrindantine. This reagent cannot be used when it contains any crystals. The colors produced after 20 minutes of heating do not fade for 1 hour, but later their optical density begins to deviate at a rate of 0.005~0.010 per hour, and the consequent error ranges from 2~4% by concentration in the case of amino acid, so that the measurement should be finished within 1 hour.

Table I. Relative Coloring (f) for Amino Acids and Other Compound, determined on 1 cc. Aqueous Samples of 0.4 mμM Solutions

Compound	(f)	Compound	(f)
Alanine	1.00	Aspartic acid	0.95
Glycine	0.96	Tyrosine	0.96
Valine	0.99	Tryptophan	0.58
Leucine	0.99	Phenylalanine	0.94
Isoleucine	0.99	γ-Aminobutyric acid	0.50
Serine	0.99	Ammonia	0.67
Threonine	1.01	Glucosamine	0.78
Hydroxyproline	0.12	Glycobetaine	0.01
Proline	0.24	Choline	0.00
Cystine	1.12	Glucose	0.03
Methionine	1.01	Fructose	0.01
Lysine	1.03	Rhamnose	0.02
Arginine	0.94	Sucrose	0.01
Histidine	0.97	Glutamine	0.99
Glutamic acid	1.01	Asparagine	0.96

An example is as follorws : The top layer of a mixture of BuOH:AcOH:H_2O(volume ratio, 4:1:5) was used as a solvent; paper chromatographic procedure was carried out by the use of alanine; ethanol solution of ninhydrin (0.02%) was used for coloring. The colored spot on the filter paper was cut out and extracted with 1 cc. of water and the extract, when examined by the authors' procedure, showed that the recovery rate was 89.8%, the concentration ranging from 0.025 to 0.8 mμM and the mutual relation forming a complete straight line when recorded. The use of the procedure in chromatographical separation and quantitative analysis will be described on another occasion.

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Summary

The ninhydrin reagent for use in the quantitative determination of amino acids separated by paper chromatography was reëxamined and modified, and the conditions under which the reagent could be used most advantageously were determined. The potency of the ascorbic acid contained in the reagent was stabilized by the use of 2% aqueous solution of metaphosphoric acid, and the blank test value and the range of variation in the determined value were minimized. This modified method surpasses the original in that it is less expensive, less sensitive to ammonia, and easier to use, than the latter.

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Susumu Nonomura : Chemistry of Damnacanthus Genus. V.* Some Derivatives of Damnacanthal — Munjistin Dimethyl Ether.

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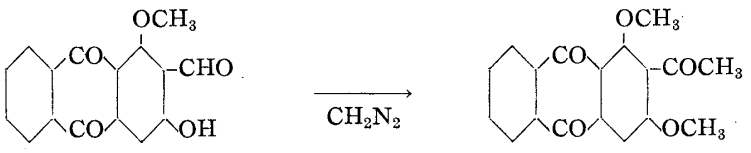
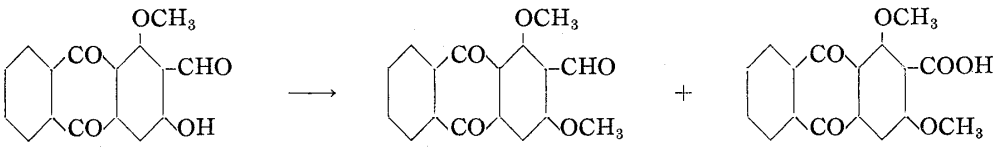
In a previous experiment[1] damnacanthal dissolved in acetone was methylated with diazomethane but the product (m.p. 175°) thereby obtained was not found to be a simple methyl ether derivative. This time, the product was prepared again and examined. It was proved that the aldehyde group of damnacanthal was changed into a methyl ketone group and the hydroxyl group was methylated. Its infrared absorption spectrum seemed quite reasonable for this structure.



In order to prepare the monomethyl ether of damnacanthal, it was heated with dimethyl sulfate and anhydrous potassium carbonate in acetone. The product was recrystallized from acetone and divided into two kinds of crystals. The one was a crude damnacanthal monomethyl ether (m.p. ca. 125°) and the other a damnacanthic acid monomethyl ether (m.p. 263°), i.e. munjistin dimethyl ether, which is a new substance.[2] Perhaps damnacanthal has been oxidized or it is a product of Cannizzaro reaction and the carboxyl group of munjistin is not esterified on account of the steric hindrance.



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2) E. Schunck, *et al.* : J. Chem. Soc., **33**, 422(1878).