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151. Takeo Tsukamoto and Tetsuya Komori: Microanalysis of Amino Acids. III.[1] Modified Ninhydrin Reagent for Use in a Quantitative Determination by Paper Partition Chromatography (Supplement).

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In the preceding paper of this series,[2] an improved Ninhydrin reagent method, a method better than those hitherto used in paper partition chromatography,[3] was described.

Later detailed investigation on one- and two-dimensional paper partition chromatography disclosed that this method could be improved further in few respects. In the method reported earlier, ascorbic acid stabilized as a 2% solution in metaphosphoric acid is added with the same volume of Ninhydrin as a 2% solution in Methyl Cellosolve and heated for its reaction. This is occasionally unsatisfactory in result in that metaphosphoric acid, when mixed with Methyl Cellosolve, renders the Ninhydrin reagent cloudy, gives a high blank test value, and reduces the determinable proportion of amino acids under examination. Further examination disclosed that this turbidity is eliminated by the addition of a small quantity of water and shaking the mixture, and that emulsification is prevented and the blank test value minimized when metaphosphoric acid is used in 1% solution in water and Ninhydrin is lowered to 0.5% in concentration.

It was confirmed, further, that the filter paper used itself contains water-soluble ammonium compound, positive to Ninhydrin reaction, and that the previous removal of ammonia from the paper is necessary in paper partition chromatography. As a result, optical density of the blank test was kept constant at 0.04 (log I^0/I) or less when the reagent to be used in paper chromatographic procedure was kept in alkaline state by the addition of sodium hydroxide and left standing overnight in a reduced pressure over sulfuric acid in a desiccator before it was used. This method, worked out on the basis of these data has proved reliable as used in paper partition chromatography.

Experimental

Reagents

Preparation of Reagent I (Ninhydrin Reagent)—This reagent is a mixture of solutions A and B. Solution A: Ninhydrin (250 mg.) is dissolved in 25 cc. of Methyl Cellosolve, which has been distilled twice and added first with one-half its volume of 4% solution of KI to see that the mixture is a colorless or faintly yellowish solution, and then with an equal volume of water to ensure that a translucent solution is obtained. Into this Ninhydrin-Methyl Cellosolve solution, when these two conditions have been satisfied, N_2 gas is introduced continuously for 30 min.

Solution B: Ascorbic acid (99% or more in purity) (50 mg.), dried over H_2SO_4 in a vacuum desiccator, is dissolved in 25 cc. of 1% solution of metaphosphoric acid.

These two solutions, A and B, are brought together and added with a sufficient volume of 1% solution of metaphosphoric acid to bring the total volume to 50 cc. The diluted solution is kept in the dark (5°) for 1 day for its stabilization before it is used as reagent I.

Preparation of Reagent II (Citrate buffer solution of pH 5.3±0.02)—Citric acid monohydrate (2.10008 g.) is added with 20 cc. of *N* NaOH and diluted to 100 cc. with water. This solution is added with 25 cc. of 0.1*N* NaOH and the mixture, when found varying within ±0.02 in pH, is used as reagent II.

Procedure—Into a glass-stoppered test tube (1.5×15 cm.), 1 cc. of the Ninhydrin reagent (0.5% in

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1) Part II: This Bulletin, **5**, 590(1957).

2) T. Tsukamoto, T. Komori, N. Kinoshita: *Ibid.*, **5**, 363(1957).

3) L. Fowden: Biochem. J., **48**, 327(1951). cf. W. H. Stein, *et al.*: J. Biol. Chem., **176**, 337, 367 (1948); **192**, 663(1951); **211**, 893(1954); Anal. Chem., **30**, 1185(1958).

concentration), 1 cc. of distilled water, and 0.5 cc. of the citrate buffer were placed. The mixture was shaken thoroughly and heated at 100° for 20 min. in a water bath. This was cooled for 5 min. with tap water, 7.5 cc. of 50% EtOH added, shaken uniformly for 1 min., and this is used in the quantitative determination of amino acids, when found to be approximately 0.04 in its optical density at 570 mμ or when found ranging between 0.04 and 0.05 in its optical density at 570 mμ after the addition of some 5 mg. of ascorbic acid to it.

For actual use in the experiment, the Ninhydrin reagent and the buffer solution were added in the above described manner to 1 cc. of aqueous solution of amino acid (0.025~1.6 mμ*M*/cc.) and the mixture was shaken well before it was submitted to heat treatment.

The accidental deep color occurring occasionally during the measurement is probably due to the use of a contaminated apparatus in most cases. This phenomenon was identified as such from the extremely high value of the blank test. The test tube for use must be washed thoroughly first with tap water and then with distilled water before it is heat-dried and stored in an air-tight vessel.

Quantitative Analysis by Paper Partition Chromatography—The filter paper used in most cases was a Toyo Roshi No. 51 A (one-dimensional, 1.2×40 cm.; two-dimensional, 40×40 cm.). The substance under examination was spotted at a point 5 cm. from the bottom edge of the paper. The paper itself was saturated with solvent vapor at 23~25° during 2 hr.; the solvent was left to rise on the paper for a definite period, the paper was air-dried, washed with Et_2O, and treated with 0.06% EtOH solution of Ninhydrin for coloration. The position reached by each amino acid was determined by comparing it with the blank test paper. Development for a blank test was effected simultaneously and under the same conditions; each part of the paper where no amino acid was spotted was cut off under the same conditions, and, in two-dimensional determination, the mean optical density was obtained from 2 or 3 scraps of filter paper, 2 cm. square, which contained no amino acid. Into the tube containing each scrap of filter paper, 0.1 cc. of 0.1*M* NaOH was added and the tube was left under a reduced pressure overnight over H_2SO_4 in a desiccator. The residue was added with 0.45 cc. of 0.01*M* citric acid to bring its pH to ca. 5.3, 0.5 cc. of distilled water was added, and the mixture shaken thoroughly. To this solution, 1.0 cc. of Ninhydrin and 0.5 cc. of citrate buffer (pH 5.3) were added, the mixture was shaken thoroughly, and heated at 100° for 25 min. to effect Ninhydrin reaction. As in the case of amino acid, cooled and dissolved in water for its quantitative determination, the content of the tube so treated was diluted with 50% EtOH to 10 cc., shaken for 1 min., and optical density of this solution was measured by a Hitachi spectrophotometer Model EPU-2, provided with a cell of Corex glass of 10-mm. optical path. The test was made 2 hr. after the termination of heating.

Results and Discussions

The colored solution of proline and hydroxyproline had absorption maximum at 440 mμ, while that of other amino acids had its first absorption maximum at 570 mμ, and the second maximum at 406 mμ, with the Ruhemann purple developed invariably in it. When heated in the presence of 0.5% Ninhydrin, each amino acid showed its maximum optical density after a certain period of time before it began to fade and the time required for this coloring process to disappear varied with different amino acids. Accordingly, the rate in velocity between the coloring and fading reaction was dependent on the kind of amino acid reacting with Ninhydrin and each amino acid showed a characteristic optical density.

The optimal period of time required for the heating was 20 min., longer heating resulting in an increased blank test value. The temperature best adapted for the heating was 100°. It was noticed in some cases that an amino acid, 0.4 mμ*M*/cc. in concentration, lost 0.04~0.03 of its optical density when heated at 98° or so.

Amino acids spotted on a scrap of filter paper was not sufficiently colored in case the ingredients in the reaction mixture were not mixed thoroughly before heating.

The use of the Ninhydrin reagent, 0.5% or higher in concentration, was not likely to affect the coloring of amino acid (Fig. 3). The Ninhydrin used was 0.5% in concentration, because the blank test value tended to increase with higher concentration of Ninhydrin.

There was a definite difference in coloring between alanine and other amino acids in the same concentration (0.4 mμ*M*/cc.). In this determination, equation $Y=1.65\ fX$ was

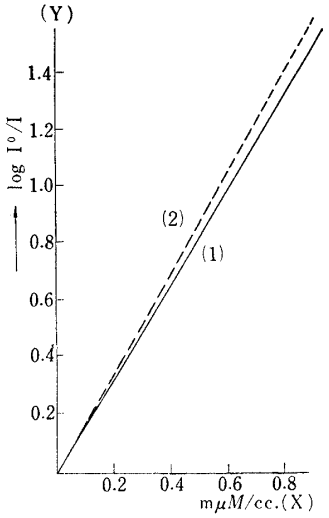

Fig. 1. Standard Curve of Amino Acids

Y = 1.65 fX
f = 1.00 Alanine ····(1)
f = 1.06 Serine·····(2)

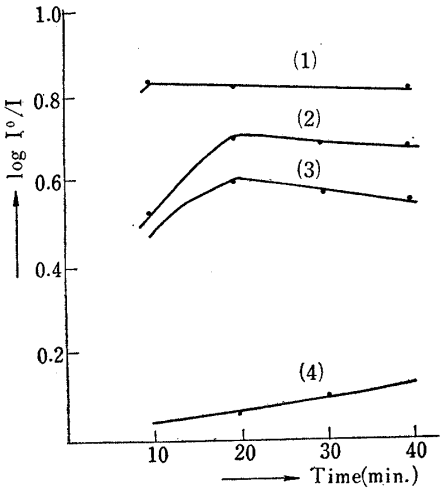

Fig. 2. Relationship between the Heating Time and Absorbance

Reaction mixture of glutamic Acid··(1)
Reaction mixture of serine·····(2)
Reaction mixture of alanine ·····(3)
Blank test ·····(4)

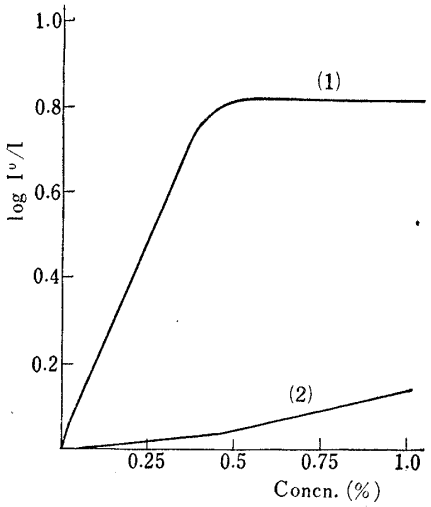

Fig. 3. Relationship between Ninhydrin Concentration and Absorbance

Reaction mixture of leucine··(1)
Blank test·····(2)

TABLE I. Relative Coloring (f) of Amino Acids and Other Compounds

Compound	f	Compound	f
Lysine	1.16	Serine	1.06
Histidine	1.10	Glycine	1.06
Arginine	1.08	Threonine	0.99
Tyrosine	1.17	Alanine	1.00
Phenylalanine	1.34	Valine	1.03
Tryptophan	1.22	Methionine	1.33
Proline	0.25	Isoleucine	0.97
Hydroxyproline	0.09	Leucine	1.13
Glutamic acid	1.16	Glutamine	1.14
Aspartic acid	1.15	Ammonia	0.77
Cystine (1/2)	0.64	Glucose	0.00

established, where f is the relative coloring of alanine, Y the optical density minus the blank test value for an amino acid under examination, and X the concentration of the amino acid. When the amino acid was alanine, the error arising from this formula was below 1.1%. The error recognized in the optical density determined from this formula for serine, 0.4 mμM/cc. in concentration, was invariably 0.1% or less.

In the case of quantitative analysis by paper partition chromatography, the optical density of blank test for a substance other than an amino acid developed on filter paper and treated in the manner described before was 0.13~0.16 (log I^0/I) in some cases and 0.20 (log I^0/I) or more in others when the reagent used was a routine one, and about 0.100 (log I^0/I) in all cases when the Ninhydrin reagent prepared as above was used. Detailed examination disclosed that the liquid substance extracted with hot water from the filter paper was positive to the Nessler reagent and that the ammonium ion present in the paper reacted with Ninhydrin. In a subsequent experiment, a piece of the filter paper used for paper partition chromatography, with 0.1N NaOH applied to it, was placed in a vacuum desiccator over H_2SO_4 overnight before it was measured and the optical density of blank test was the same 0.04~0.05 (log I^0/I) both when Ninhydrin was used after such pretreatment or when it was used with distilled water without such pretreatment. The fact proves that this Ninhydrin reagent is also available for quantitative analysis by paper partition chromatography when the spots for amino acids to be analyzed are completely separated from each other.

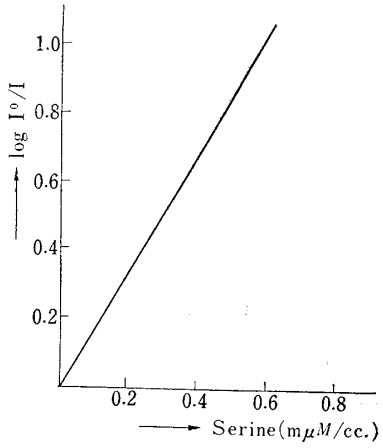

Fig. 4. Standard Curve of Serine (in pH 5.0) (One-dimensional paper partition chromatography : Toyo Roshi No. 51A)

Table II. Recovery Rate of Amino Acids separated by One-dimensional Paper Partition Chromatography[a)]

Amino acid	Recovery rate[b)]	Error (%)	Amino acid	Recovery rate[b)]	Error (%)
Cystine	0.967	±5.5	Methionine	0.830	±2.5
Aspartic acid	0.840	±4	Leucine	0.896	±5
Glutamic acid	0.907	±3	Phenylalanine	0.814	±4
Serine	0.915	±3	Lysine	0.902	±5
Glycine	1.151	±3	Histidine	0.798	±4.5
Threonine	0.992	±5	Arginine	1.009	±2.5
Alanine	0.955	±2	Proline	1.006	±5
Valine	0.907	±5	Tyrosine	0.809	±5

a) Ninhydrin reaction was carried out at pH 4.8 (ca. 0.4 mμM/cc.).

b) Recovery rate (R) $= \dfrac{\text{X} \quad (\text{m}\mu M/\text{cc.})}{\text{spot} \times \text{concn.} \ (\text{m}\mu M/\text{cc.})}$

$$\text{X} = \frac{\text{Y}}{1.65 \times \text{f}}$$

Recovery rates listed were obtained from average of 9 experiments.

In Table II is given the recovery rate of amino acids separated by one-dimensional paper partition chromatography. It was found that the amino acid was determined quantitatively by paper chromatographic procedure within the error of 5%.

The results of quantitative determination by two-dimensional paper partition chromatography will be described in a subsequent paper.

The authors express their thanks to Miss Y. Inoue for technical assistance in quantitative determination by paper chromatography.

Summary

A Ninhydrin reagent for use in the quantitative determination of amino acids separated by paper partition chromatography was worked out. It was confirmed that the best concentration of the reagent is 0.5%. The reagent had to be added with ascorbic acid as 1% solution in metaphosphoric acid if the optical density of the blank test was not to exceed 0.04~0.05 (log I^0/I). The blank test value was 0.04 (log I^0/I) or less when the reagent to be used in paper partition chromatography was kept in alkaline state by the addition of sodium hydroxide and left standing overnight in a reduced pressure over sulfuric acid in a desiccator before it was used. Amino acid spotted at a concentration of 0.08~0.4 mμM/cc. was recovered quantitatively within the error of 5%.

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