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The Determination of Amino-acids with Ninhydrin

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The colorimetric method for the determination of amino-acids based on the reaction with ninhydrin has been systematically examined. It has been found possible to obtain a stoichiometric reaction for most amino-acids with a quantitative yield of diketohydrindylidenediketohydrindamine, the probable end-product of the reaction, by a simple procedure suitable for routine analysis.

THE purple colour formed by the reaction of amino-acids in acid solution with ninhydrin (triketohydrindene hydrate) is attributed to the anion of diketohydrindylidenediketohydrindamine (DYDA).^{1,2} It seems that the first step in the reaction is the oxidative de-amination of the amino-acid with the formation of ammonia and the reduction of ninhydrin to hydrindantin. The ammonia then condenses with the hydrindantin to form DYDA.^{3,4,5} As recently pointed out by Troll and Cannan,⁶ this scheme is consistent with the fact that ammonia forms DYDA with ninhydrin only in the presence of a reducing agent capable of producing hydrindantin. It is, however, inconsistent with the fact that ammonia reacts more slowly and less completely than do amino-acids under the same conditions. It is therefore concluded that the de-amination and condensation are coupled in some way.

Moore and Stein⁷ developed the reaction as a convenient photometric method for the determination of amino-acids. They obtained consistent and reproducible results by the introduction of a reducing agent into the system to prevent the oxidation of the hydrindantin formed as a result of oxidative de-amination. The method was further simplified by Smith and Agiza⁸; but in both methods the reaction of different amino-acids with ninhydrin did not give a quantitative yield of DYDA, so that standardisation was required for individual amino-acids. Seeking an explanation for the apparent lack of stoichiometry of the reaction, Troll and Cannan⁶ sought conditions under which the rate of formation of DYDA was increased relative to its rate of destruction. Organic solvents were found to accelerate the development of colour to varying degrees, and ultimately an 80 per cent. solution of phenol in water, buffered at pH 5 with pyridine, was adopted as the most effective solvent. A solution of potassium cyanide was employed as the reducing agent rather than the less stable stannous chloride. The conclusion of Moore and Stein⁷ that pH 5 was optimal for reaction was confirmed. All amino-acids tested, except tryptophan and lysine, gave colours equivalent to 97 to 102 per cent. of that of pure DYDA in the same solvent. Troll and Cannan found, furthermore, that the absorption spectra of the colours obtained from amino-acids in their procedure were identical with that of DYDA.

For routine analysis this modified procedure has several disadvantages: it is difficult to control the pH accurately, high water blanks may be obtained and it involves the extensive use of a pyridine-phenol solvent. It has been found possible to retain the advantage of the quantitative reaction of Troll and Cannan⁶ by a simple procedure suitable for routine analysis.

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METHOD

MATERIALS—

Methyl Cellosolve, $\text{CH}_3\text{OCH}_2\text{CH}_2\text{OH}$ —Samples gave a clear solution when mixed with an equal volume of water and a faint or negative peroxide test with 10 per cent. potassium iodide.

Ninhydrin—To ensure a low blank reading in the photometric procedure the ninhydrin should be pale yellow with a greenish tint and completely odourless. It should dissolve in methyl Cellosolve to give a clear light yellow solution; if it does not meet these specifications, it may be recrystallised according to the method of Hamilton and Ortiz.⁹

Potassium cyanide—AnalaR reagent as supplied by The British Drug Houses Ltd.

Distilled water—Treat all distilled water with Permutit to remove traces of ammonia.

Amino-acids—For standardisation use synthetic products as far as possible; recrystallise all amino-acids at least once and dry over concentrated sulphuric acid *in vacuo* for 48 hours before use. Prepare stock solutions ($2 \times 10^{-2} M$) in 0.1 N hydrochloric acid and store in the cold with a little thymol to prevent fungal growth; prepare standard solutions ($2 \times 10^{-4} M$) by dilution with citrate buffer, pH 5 (0.2 M).

REAGENTS—

Citrate buffer, pH 5 (0.2 M)—Dissolve 21.008 g of citric acid, $\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$, in 200 ml of distilled water, add 200 ml of N sodium hydroxide and dilute to 500 ml; store in the cold with a little thymol.

Potassium cyanide, 0.01 M—Dissolve 0.1629 g of potassium cyanide in distilled water and dilute to 250 ml. This solution is stable for at least 3 months at room temperature.

Ethanol in water, 60 per cent. by volume.

Potassium cyanide - methyl Cellosolve solution—Dilute 5 ml of 0.01 M potassium cyanide to 250 ml with methyl Cellosolve. This solution is stable for at least 1 month at room temperature.

Methyl Cellosolve - ninhydrin solution—Prepare a 5 per cent. w/v solution of ninhydrin in methyl Cellosolve. This solution is stable for at least 6 months at room temperature.

Potassium cyanide - methyl Cellosolve - ninhydrin solution—Mix 50 ml of the methyl Cellosolve - ninhydrin solution with 250 ml of the potassium cyanide - methyl Cellosolve solution. The resulting solution is at first red, but soon becomes yellow. It should be stored overnight before use to ensure low blank readings and is stable for at least 1 week when kept in a stoppered flask at room temperature.

BLANK DETERMINATION—

Preliminary experiments indicated that to ensure low blank readings it was necessary to take precautions against contamination of all solutions by ammonia, especially the pH 5 buffer solution. The blank value at a volume of 5.7 ml, read against water, should not be greater than an optical density of 0.12, if reliable results are to be obtained.

PROCEDURE FOR DETERMINING AMINO-ACIDS—

Mix 0.5 ml of citrate buffer, pH 5 (0.2 M), with 1 ml of an amino-acid solution containing 0.05 to 5.6 μg of amino nitrogen. Add to this either 0.2 ml of the methyl Cellosolve - ninhydrin solution and 1 ml of the potassium cyanide - methyl Cellosolve solution or 1.2 ml of the potassium cyanide - methyl Cellosolve - ninhydrin solution. Heat the well mixed solution for 15 minutes at 100°C , and cool for 5 minutes in running tap water. Make up the solution to a convenient volume with ethanol, shake well, and determine the optical density, using a 1-cm glass cell, on a Unicam spectrophotometer SP500 at $570 m\mu$ for all amino-acids. Determine proline and hydroxyproline at $440 m\mu$. An E.E.L. portable colorimeter (Evans Electroelenium Ltd.) with No. L200 tubes and yellow filter No. 626 (maximum transmission at a wavelength of $570 m\mu$) is equally satisfactory and much more suitable for routine work involving measurements on a large number of tubes. Proline and hydroxyproline are determined with violet filter No. 621 (maximum transmission at $460 m\mu$). Read all tubes against blanks containing 1.5 ml of citrate buffer, pH 5 (0.2 M), and 1.2 ml of the potassium cyanide - methyl Cellosolve - ninhydrin solution subjected to the same procedure as above. Preliminary experiments indicated that the heating time

was not critical and that most amino-acids had reacted fully after heating at 100° C for 10 minutes. Furthermore, the boiling point of the water-methyl Cellosolve mixture is greater than 100° C; using tubes stoppered with a glass marble, evaporation gave losses during the heating period that were negligible. The colour was stable at room temperature for at least 3 hours before ethanol was added and for at least 1 hour after its addition.

ISOLATION OF PURE DYDA

DYDA was isolated, as its sodium salt, from aqueous solution by the method of Moore and Stein.⁷ For analysis 50 mg of the sodium salt was recrystallised from 15 ml of a water-n-propanol mixture (1 + 1). The crystals were dried over concentrated sulphuric acid *in vacuo* for 48 hours, and the millimolar extinction coefficient at 570 m μ was determined in the same solvent mixture as employed in the procedure described above. It was found to be 21.6 and is in agreement with that found by Troll and Cannan⁸ for the sodium salt of DYDA in a solution of pyridine, phenol and ethanol; and, as was also found by Troll and Cannan, the absorption spectrum of the sodium salt was identical with that of the unfractionated reaction mixture obtained from amino-acids in the ninhydrin analysis.

RESULTS

For each of the amino-acids various amounts containing from about 0.1 to 6.0 μ g of amino nitrogen were examined by the above procedure. The standard curves determined by using an E.E.L. colorimeter for amino-acids in the concentration range 0.05 to 2.8 μ g of amino nitrogen are shown in Fig. 1. The individual readings were reproducible to within 1 per cent. of the mean of four determinations for all amino-acids studied. From optical density measurements on the spectrophotometer the millimolar extinction coefficient of the reaction mixture, at 570 m μ , was calculated in each case, and since that of the sodium salt of DYDA was known the percentage yield of DYDA could be determined. The results are summarised in Table I.

TABLE I
EXTINCTION COEFFICIENTS AND ESTIMATED YIELDS OF DYDA

Amino-acid	Millimolar extinction coefficient at 570 m μ	Yield of DYDA, %
Alanine	21.6	100
Arginine	21.6	100
Aspartic acid	21.6	100
Asparagine	9.7	45
Cystic acid	21.4	99
Cystine	21.6	100
Glutamic acid	21.6	100
Glutamine	21.6	100
Glycine	21.6	100
Histidine	21.6	100
Leucine	21.6	100
isoLeucine	21.6	100
Lysine	23.4	108
Methionine	21.6	100
Serine	21.4	99
Threonine	21.6	100
Tryptophan	18.0	83
Valine	21.4	99
Phenylalanine	19.1	89
Tyrosine	19.1	89
Ammonia	7.1	33

It was found that all the amino-acids studied gave colours equivalent to 100 $\pm$ 1 per cent. of that of pure DYDA except tyrosine and phenylalanine (89 per cent.), tryptophan (83 per cent.) and lysine (108 per cent.). Ammonia reacted to yield a colour equivalent to only 33 per cent. of that of pure DYDA. This value for ammonia is, however, approximate and varies somewhat from one batch of reagent to another. Proline and hydroxyproline react with ninhydrin in an entirely different way; de-amination does not occur, no DYDA is produced, reducing agents are without influence and the imino-acid residue that results

from decarboxylation condenses directly with the ninhydrin to form a yellow end-product.¹⁰ Nevertheless, the same method can be used for their estimation, although the optical density readings are only about one-fourth of those obtained with equimolar solutions of amino-acids.

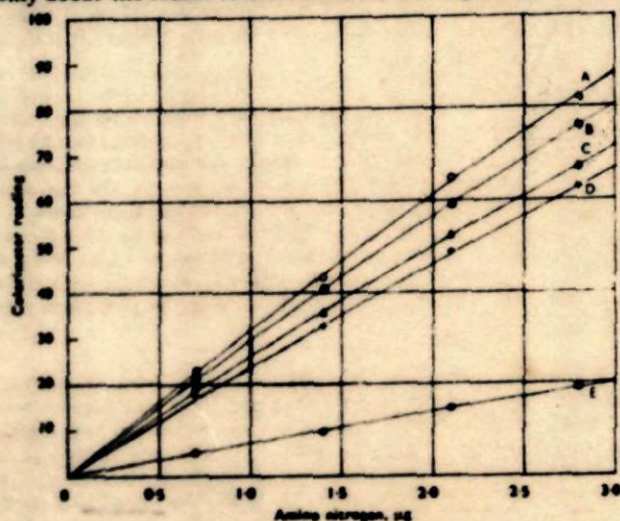


Fig. 1. Standard graphs determined on colorimeter with reaction mixture adjusted to 5.7 ml—

- A, lysine;
- B, alanine, arginine, aspartic acid, cysteic acid, cystine, glutamic acid, glycine, histidine, leucine, isoleucine, methionine, serine, threonine and valine;
- C, phenylalanine and tyrosine;
- D, tryptophan;
- E, proline.

DISCUSSION OF RESULTS

It is clear from the results shown that a stoichiometric reaction occurs for most of the amino-acids studied, with a quantitative yield of DYDA. The low yield with tryptophan is to be expected, since the optimum pH for reaction is in this case 6, and the high yield for lysine is probably brought about by the partial reaction of the terminal amino group. The yield of DYDA from tyrosine and phenylalanine is largely dependent on the relative proportion of water to methyl Cellosolve, for it is found that the reaction is almost complete when the relative proportion of water to methyl Cellosolve is increased from 1:0.8 to 1:0.4. The yields with the procedure described are however quite reproducible. The results for cystine indicate that only one-half of the amino groups react; it seems probable that one reacts and that the other is protected by cyclisation. Cysteine is known to be the only amino-acid that does not form DYDA; from it a yellow product similar to that given by proline is obtained.^{7,8} Cysteic acid on the other hand reacts normally.

The procedure described above has several advantages over that of Moore and Stein.⁷ The more complete reaction gives greater sensitivity and highly reproducible results, whilst the lower sensitivity to ammonia leads to smaller and more consistent blanks. Moreover a shorter and less critical heating period is required. There is also a considerable economy of ninhydrin, for only about one-half of the quantity is required compared with that used in the method of Moore and Stein, and furthermore the method avoids the complication of having to store reduced ninhydrin under nitrogen.

APPLICATION TO PROTEIN HYDROLYSATES

The procedure described was developed for the estimation of the amino-acids of protein hydrolysates separated chromatographically on ion-exchange resin columns by elution with buffers of progressively increasing pH, as in the method of Moore and Stein.¹¹ One-millilitre

fractions of effluent were obtained in optically matched tubes by using a capacity-change drop counter. Each fraction was treated with 0.5 ml of citrate buffer, pH 5 (0.2 M), to which *N* sodium hydroxide had been added so that the final pH of the treated fraction was 5. Then 1.2 ml of the potassium cyanide - methyl Cellosolve - ninhydrin solution were added as in the standard procedure. The tubes were well shaken and heated in racks of twenty-five at 100° C for 15 minutes. They were cooled in running tap water for 5 minutes, 3 ml of ethanol were added, the tubes were shaken and the colour intensity was read directly in an E.E.L. colorimeter, a No. 626 filter being used. In view of the low absorption of DYDA at 440 mμ it has been found possible to estimate proline when mixed with small quantities of other amino-acids. This is readily achieved on an E.E.L. colorimeter, No. 621 and 626 filters being used. No interference was noted from special chemicals such as benzyl alcohol, thiodiglycol and ethylenediaminetetra-acetic acid added to the columns to improve their performance. The complete reaction makes possible the more accurate calculation of the amount of an amino-acid present when one peak is incompletely separated from another; moreover, the low yield from ammonia ensures lower and more consistent blank readings, thus making possible the more exact determination of the amount of an amino-acid present under any particular peak. With the above procedure it has been found possible by using semi-automatic pipettes to analyse 100 samples in about 3 hours.

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Appendix

A CAPACITY-CHANGE DROP COUNTER

By R. E. RICKETTS

A SIMPLY constructed and reliable capacity-change drop counter is described. Fractions of 1 to 5 ml may be automatically collected, and measurements made over a period of several months indicate an accuracy of delivery better than ± 1 per cent.

It was found necessary to collect a large number of fractions of eluate from resin columns employed for the fractionation of protein hydrolysates. The equipment used was designed to operate in conjunction with a chromatographic table,¹ a set number of drops being counted into each test tube.

In view of the possibility of breakdown of unstable solutions owing to electrical contact, it was decided to use a capacity-operated switch. To guard against failure of valves or other components, two channels operating simultaneously were used, with "test channel" buttons to enable the operator to check, periodically, each channel separately.

The circuit diagram of the apparatus is shown in Fig. 2. Valves V_1 and V_2 are connected as a Hartley oscillator whose feedback depends on the ratio of C_1 and C_2 . Radio-frequency voltage from the oscillator is applied through C_3 to the thyratrons, V_3 and V_4 . The supply voltage to the oscillator is arranged to be 180° out of phase with that applied to the thyratrons, consequently rectification occurs at the grids of the thyratrons so charging C_3 negatively, thus biasing the