PHOTOMETRIC NINHYDRIN METHOD FOR USE IN THE CHROMATOGRAPHY OF AMINO ACIDS

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For the investigations on the chromatographic separation of amino acids outlined in the preceding communication (1), it was necessary to have available a suitable quantitative method for the determination of the concentration of amino acids in the effluent from the column. For this purpose, the method should be sufficiently general to include the determination of most of the amino acids and peptides likely to be encountered in protein hydrolysates or other material of biological origin. The method should have as high a sensitivity as possible to permit the determination of low concentrations of amino acids in the effluent from the chromatogram. Also the laboratory procedure should be fairly simple to permit the method to be applied conveniently to large numbers of effluent samples.

It appeared probable that a photometric method would best fulfil these requirements. The two colorimetric methods of this type which had received the most study were the procedures based on the use of β-naphthoquinonesulfonic acid and ninhydrin (triketohydrindene hydrate) as reagents. For reasons which will be described, the ninhydrin reaction was selected for further investigation.

The color reaction between α-NH_2 acids and ninhydrin has been studied extensively in the past. It has been established that colored compounds are formed not only with amino acids, but also with peptides, proteins, and other classes of substances possessing free amino groups. The reaction is known to be extremely sensitive for qualitative work. In earlier attempts to render the color reaction quantitative (2–8), however, it has been found that the color yield per microgram of amino acid decreased markedly as the concentration of amino acid was reduced. In addition, the results have not been reproducible. In the present investigations, it has been observed that, when the color development is carried out in tubes exposed to the air, these difficulties appear to result primarily from the influence of dissolved oxygen. Improved results can be obtained when the reaction is performed in tubes evacuated to 20 mm. Under these conditions, the relationship between color yield and amino acid concentration is more nearly linear, although the deviations are still marked. By the addition of a strong reducing agent directly to the reaction medium, however, the oxidative side reaction has been eliminated. In preliminary experiments

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the reduced form of ninhydrin, as hydrindantin (9), was added. Subsequently, it proved simpler to add stannous chloride to the reaction mixture. The stannous chloride serves to reduce part of the ninhydrin, and the preparation of hydrindantin in crystalline form is not required. Although ascorbic acid used as an antioxidant increases the sensitivity of the reaction for qualitative use (10), it is unsuitable for quantitative work because it can also give colored condensation products with amino acids (*cf.* (11)).

When conditions had been established which would give thoroughly reproducible photometric readings with a given amino acid, a study was made with several amino acids to determine the effects upon the reaction of variations in pH, temperature, time of heating, and amounts of reagents. At the same time, the mechanics of the procedure were developed to permit the analysis of large numbers of samples in a routine manner.

The method still possesses one important disadvantage. Although reproducible results are obtained for a given amino acid, the different amino acids do not all yield the same amount of color per mole. For chromatographic analysis, this is not a serious disadvantage. In those instances in which the starch column can separate an amino acid completely from the other components of the mixture, the ninhydrin reaction can be made to give quantitative values by the use of a factor appropriate for the amino acid in question.

Some of the possible causes for variations in the color yield per mole have been studied. The absorption spectra indicate that all the α-NH_2 acids (except cysteine) give the same major colored end-product. This blue coloring matter was prepared by Ruhemann (9) by the reaction of ninhydrin with alanine and by the condensation of hydrindantin with ammonia. To this compound Ruhemann assigned the structure of diketohydrindylidene-diketohydrindamine. In the present work, the product has also been isolated from the mixtures obtained in the reactions of ninhydrin with glycine and glycylleucine. The substance crystallizes as the sodium salt from the

$$R-CH(NH_2)-COOH + 2\,C_6H_4(CO)_2C(OH)_2 \xrightarrow[\text{pH 5, 100° C.}]{\text{Na citrate buffer}} C_6H_4(CO)(C\text{-}ONa)C-N=C(CO)_2C_6H_4 + CO_2\uparrow + R-CH=O$$

α-NH_2 acid — Ninhydrin — Diketohydrindylidene-diketohydrindamine — Carbon dioxide — Aldehyde

citrate buffer solution used in the present experiments. The absorption spectrum of a standard solution of diketohydrindylidene-diketohydrindamine can be compared with the spectrum of the unfractionated reaction mixtures from ninhydrin and α-NH_2 acids. The comparison shows that in the leucine reaction, for example, the colored product is formed in only 93

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per cent of the theoretical yield. A modification of the method which would render the yields quantitative in all cases would be an improvement. For a given amino acid, the percentage yield of the colored product is independent of the initial amino acid concentration. This fact indicates that the low yield is characteristic of the mechanism of the reaction under the experimental conditions employed and is not due to destruction of part of the color by a trace of oxygen.

With proline and hydroxyproline, as shown by Grassmann and von Arnim (12), the reaction follows a different course than with the amino acids containing an α-NH_2 group. These two amino acids give products with a maximum absorption at 440 mμ. The present procedure can also be used to determine proline and hydroxyproline, although the sensitivity is less than in the case of the amino acids which form diketohydrindylidene-diketohydrindamine.

It has long been known that colorimetric ninhydrin methods are not specific for the NH_2 groups of amino acids. The NH_2 groups in peptides give good color development, many amines such as histamine and tyramine will react and the presence of hydrindantin, used in this procedure, causes NH_3 to give a nearly quantitative yield of the blue reaction product. For chromatographic experiments with amino acids and peptides, the generality of the reaction extends its usefulness. For work with unfractionated biological material, the lack of specificity would be a disadvantage, as was recognized by Harding and MacLean (2). The specificity of the photometric ninhydrin method is similar in a number of respects to that of the nitrous acid reaction for amino nitrogen. The method may be of value in instances when the nitrous acid reaction is useful. For the estimation of free amino acids in the presence of peptides, the photometric method, of course, lacks the specificity of the gasometric amino acid carboxyl determination of Van Slyke, Dillon, MacFadyen, and Hamilton (13).

The photometric ninhydrin method, with the present modifications which have rendered the results fully reproducible, appears to possess some advantages over β-naphthoquinonesulfonic acid procedures for those applications for which these methods are suitable. The reaction of amino acids with β-naphthoquinonesulfonic acid, as employed by Folin, recently modified by Frame, Russell, and Wilhelmi (14), and compared with the CO_2 method by Chinard and Van Slyke (15), involves the additional operation of bleaching of the excess reagent. The ninhydrin reagent solution possesses the advantage of being stable, and for routine use can be stored under nitrogen for a month or more. Fading of the color in the ninhydrin method proceeds at a much slower rate than that reported for the naphthoquinone procedure (15). The ninhydrin reaction yields the same end-product from all the α-NH_2 acids (cysteine excepted), whereas the chemistry of the β-naphtho-

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quinonesulfonic acid reaction is less well defined and the absorption maxima of the colored products obtained from different amino acids, though similar, are not identical (14).

Apparatus

Pipettes—For the pipetting of large numbers of small samples of amino acid solutions for analysis, modified self-adjusting transfer pipettes are used in 0.05, 0.1, 0.2, and 0.5 cc. sizes.[1] The accuracy of these pipettes is increased by operating them on a manifold connected to both compressed air and vacuum lines. For long series of analyses, this arrangement is also much more convenient for the operator. The arrangement of the pipetting stand is illustrated in Fig. 1. The manifold is made from three T-tubes. The third tube is mounted behind the rubber stopper (size 13). The connections are made with soft rubber hemocytometer pipette tubing. A slight vacuum (or pressure) is applied to the micro pipette by touching the top of the appropriate T-tube and greater vacuum (or pressure) by simultaneously pressing on the adjacent rubber tubing. The stop-cocks are closed only during the adjustment of the vacuum to about 60 mm. and the pressure to about 15 mm. Before use, the tip of the micro pipette should be bent, as in Fig. 1, and fire-polished to give a delivery time of 8 to 12 seconds at 15 mm. pressure. At this rate, and with wiping of the pipette tip before delivery, the reproducibility of delivery is 0.1 to 0.2 per cent. The hold up is about 2 per cent. A series of tubes from a chromatogram is run through without washing the pipette between samples. For each solvent employed the pipette must be calibrated gravimetrically. A table of calibration factors expressed in terms of the fraction of the rated delivery is prepared for each pipette. The metal holders for the 150 × 18 mm. sample tubes and photometer tubes are cut from brass tubing of 20 mm. inner diameter.

Photometer Tubes—For the chromatographic procedure, it has been necessary to accumulate a matched set of over 1000 tubes for use with the Coleman junior spectrophotometer, model 6-A. The tubes have been selected from strain-tested soft glass test-tubes, 150 × 18 mm., without lips.[2] A solution of methyl red in 0.03 N HCl is prepared of such a strength as to give a reading of 0.60 to 0.70 on the optical density scale when read at 525 mμ against a water zero. About 100 tubes are filled with 5 to 10 cc. of the methyl red solution. It is important that all tubes receive the methyl red solution from the same reservoir bottle. Pouring the solution

[1] The pipettes are made to the design of Dr. P. L. Kirk by the Microchemical Specialties Company, 1834 University Avenue, Berkeley 3, California (catalogue No. 283-B).

[2] Catalogue No. 9446, Arthur H. Thomas Company, Philadelphia, Pennsylvania.

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from tube to tube can introduce significant errors. The largest group of tubes giving readings within 0.005 unit of the same value is selected. The side of the tube facing the light source is marked temporarily at the time of the test, and subsequently with a glass-marking tool,[3] to indicate the correct position for the tube in the spectrophotometer. About a dozen of

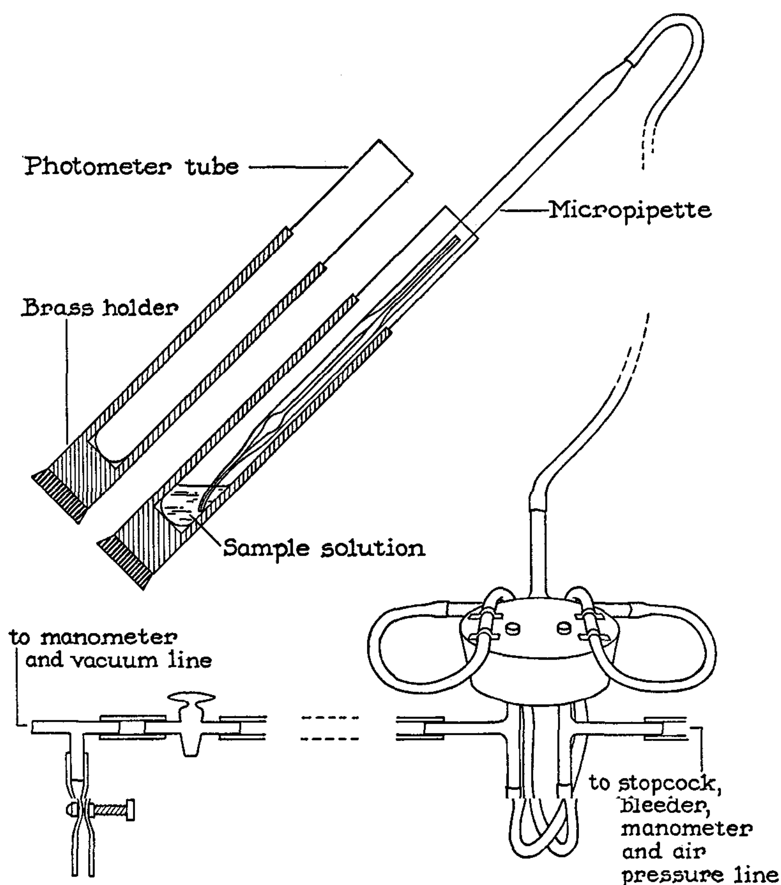

FIG. 1. Arrangement for the pipetting of samples for analysis

these tubes are marked as standards and reserved for use only in the checking of new sets.

In calibrating subsequent groups of 100 tubes against standard tubes filled with methyl red solution, each new tube is rotated in the instrument to determine whether it can be set to read within 0.005 unit of the standard

[3] Catalogue No. 3008, Chicago Wheel and Manufacturing Company, 1101 West Monroe Street, Chicago, Illinois, with additional grinding wheels No. 5-B-44.

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reading. The satisfactory tubes are appropriately marked for position. The selected tubes are permanently numbered in sets of 200 tubes each. Each set can be subjected to an additional test by observing the zero reading when the tubes are filled with distilled water. In general, tubes selected by the methyl red procedure have given uniform zero readings.

When measured by calipers below the rim, the tubes selected for this study had an inner diameter of 16.25 ± 0.15 mm. The outer diameter averaged 18.3 mm. Once a calibrated set has been selected with tubes of a given average diameter, such as 18.3 mm., it is necessary that subsequent lots of tubes obtained from the manufacturer have an average diameter in the same range. Under these conditions, twenty-five to 50 tubes per hundred may prove acceptable. If such specification is not made, some lots may run all high or all low.

When the sets have been handled in wooden or aluminum racks,[4] in order to protect the tubes from being scratched, and washed with boiling soap solution, the tubes have remained accurate during constant use for more than a year.

To cover the photometer tubes during the analysis, Aloe-Willett aluminum caps[5] are used.

Water Bath—A vigorously boiling water bath is required. An open bath can be used, but the amount of evaporation of the solvent (*cf.* Table III) from the tubes is less reproducible. The present experiments have been carried out in a specially designed, electrically heated, covered bath[6] with a constant level regulator. For these analyses, the bath is operated with the thermostat at the maximum setting, so that heat is constantly, not intermittently, applied. The tubes are immersed to a depth of about 2 inches. The rate of heat supply should be sufficient to bring the bath back up to 99–100° within 2 minutes after the insertion of a full rack of 50 tubes. Only one rack should be inserted at a time. The rear corners of the cover are bent downward slightly to allow the escape of steam without the collection of condensed water. A double hook handle for insertion and removal of the racks can be made from a $\frac{1}{4}$ inch metal rod.

Pipetting Machines for Reagents—For a small number of analyses, the reagents can be added by a burette or pipette. When large numbers of samples are being run, the ninhydrin solution can be stored under nitrogen in a 1 liter brown or red glass reservoir attached to a pipetting machine.[7]

[4] Suitable aluminum racks are described in the preceding communication (1).

[5] Catalogue No. JL-78300 (for 18 mm. tubes). A. S. Aloe Company, St. Louis, Missouri.

[6] Catalogue No. 405/3/R, Electric Heat Control Apparatus Company, 507 Fifth Avenue, New York 17.

[7] Model No. 40-SS-10, Brewer Automatic Pipetting Machine, Baltimore Biological Laboratory, Inc., 500 North Calvert Street, Baltimore 2, Maryland.

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The flexible connections are made with $\frac{1}{8}$ inch inner diameter Neoprene tubing. A 250 cc. dropping funnel, for use in filling the reservoir, is mounted on the bottle through a 3-hole rubber stopper. The reservoir is connected to the top of a second bottle of the same size by a U-tube, and the second bottle is connected through a 2-hole stopper to a third 1 liter bottle by a U-tube which reaches to the bottom of each. The second bottle is initially filled with water, and the air in the system is replaced by nitrogen introduced through the dropping funnel at the time the apparatus is set up. The reservoir can be refilled without replenishing the nitrogen, except when the apparatus is disassembled for cleaning. The pipetting machine is equipped with a 3 cc. syringe and set for 1 cc. delivery. The valves (ungrooved) may require regrinding by hand with a fine emery or rouge to insure smooth performance with organic solvents. Fire polishing of the glass delivery tip, when small volumes such as 1 cc. are being delivered, helps to eliminate a hanging drop or back flow.

A second pipetting machine, equipped with a 10 cc. syringe, is used to deliver 5 cc. of the diluent solution from a 2 liter storage bottle. The flexible connections can be made with 3/32 inch inner diameter Tygon tubing or $\frac{1}{8}$ inch Neoprene tubing. For convenience in the analysis of large numbers of samples, the reaction mixtures are diluted with a given volume of solvent from the pipetting machine to avoid the procedure of bringing the solutions to a prescribed volume in calibrated glassware.

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Reagents

Ninhydrin—To insure a low blank reading in the photometric procedure, the ninhydrin, prepared commercially according to the method of Teeters and Shriner (16), has usually been recrystallized within several months of the time of use. To 250 cc. of water, 100 gm. of ninhydrin are added. The hot solution is treated with about 5 gm. of decolorizing carbon. The filtrate is stored at 4° overnight. The ninhydrin is washed on the filter four or five times with 20 cc. portions of cold water. The air-dried crystals are stored in dark glass. The recovery is 85 to 90 per cent.

Citrate Buffer—The buffer, pH 5 (0.2 M), is prepared from 21.008 gm. of citric acid, $C_6H_8O_7 \cdot H_2O$ (reagent grade) and 200 cc. of N NaOH diluted to 500 cc. Several times this quantity can be prepared and stored in the cold with thymol. The pH of the buffer when diluted with an equal volume of water should be 5.0 ± 0.1.

Methyl Cellosolve—The samples of methyl cellosolve should give a clear solution when mixed with an equal volume of water[3] and should give a negative or very faint peroxide test with 10 per cent aqueous KI.

[3] Turbidity from several samples of methyl cellosolve was found to result from the fact that the solvent had been repackaged in lacquered cans. Purchase of the solvent packaged in glass or in the manufacturer's original containers is to be preferred.

Ninhydrin Solution—Dissolve 0.80 gm. of reagent $SnCl_2 \cdot 2H_2O$ in 500 cc. of the citrate buffer, pH 5. Add this solution to 20 gm. of recrystallized ninhydrin dissolved in 500 cc. of methyl cellosolve. Transfer the reagent solution to the reservoir bottle. If the system is not already filled with nitrogen, run a stream of nitrogen through the dropping funnel for about 30 minutes. When stored in this manner the solution can be kept for at least a month without deterioration. The adequacy of the preservation of hydrindantin in the reagent solution can be checked by determining the color yield on a standard amino acid solution of relatively low concentration (1 mM). If unrecrystallized ninhydrin is used, and the reagent solution is clarified with carbon, high blanks are likely to result from nitrogenous materials on the carbon.

If only a few analyses are being run at one time, and a pipetting machine is not required, the necessary small quantity of the reagent solution can be prepared for immediate use and the 1 or 2 cc. aliquots run into the photometer tubes from a burette.

Diluent Solution—Mix equal volumes of water and *n*-propanol (C.P.).

Standard Amino Acid Solutions—For the analysis of 0.1 cc. samples, 1.6 to 2.0 mM solutions of amino acids are convenient. The solutions are diluted 1:1 or 1:4 for analyses in which 0.2 or 0.5 cc. samples are required. When water-alcohol solvents are employed, the amino acids are dissolved in water and the solutions made to the appropriate volume with the dry organic solvent. A small amount of HCl is added to dissolve tyrosine and cystine. Blank solutions for each solvent mixture are prepared at the same time.

Procedure

In the case of standard solutions of amino acids, triplicate samples of the blank and the standards are pipetted into a series of photometer tubes. When the effluent from a chromatogram is being analyzed, a single sample is pipetted from each fraction. If an automatic fraction collector (1) has delivered the appropriate size of sample directly into the photometer tubes, the pipetting step is eliminated.

If the solutions for analysis are acidic, they should be neutralized (methyl red) to within ±0.1 cc. of 0.1 N NaOH. For a series of tubes from a chromatogram, a preliminary titration is made on a blank sample. Subsequently, 1.0 N NaOH is appropriately diluted so that 1 or 2 drops (0.05 to 0.1 cc.) from a burette will bring the sample within the desired limits. The prescribed number of drops of the alkali of adjusted concentration is added to each tube of the series prior to analysis. Acidic solutions will usually pick up a trace of NH_3 from the air. Since as little as 0.1 γ of NH_3 can be detected by the present procedure, it is important that the blanks and the analytical samples be handled under strictly parallel conditions.

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The pipetting machine for the ninhydrin reagent is checked for delivery by running five aliquots into a 5 cc. volumetric flask. This is done daily before use of the machine, and also serves to discard the solution which has been standing in the Neoprene tubing. The delivery should be within ±0.5 per cent of the set value. One or two aliquots of the ninhydrin solution (1 cc. for 0.1 and 0.2 cc. samples; 2 cc. for 0.5 cc. samples) are run into the photometer tubes. With samples in water or in solvents miscible with water, 1 cc. of ninhydrin solution can be used for 0.5 cc. samples if accuracy to the last few per cent is not important. Aluminum caps are placed on the tubes and the tubes are shaken to mix the reagent and sample. If the shaking is done by machine, the rack can be placed for 30 seconds on a reciprocal shaker[9] operating at about 240 excursions per minute.

The rack of tubes is heated for 20 minutes in a vigorously boiling water bath. The pipetting machine for the water-propanol diluent is checked for delivery with a 25 cc. volumetric flask. 5 cc. (±0.03 cc.) of the diluent are added to each tube. The stream is directed into the center of the solution to give good mixing. The tubes are wiped and transferred to a dry rack. The tubes are shaken by hand or by machine (1 minute). Readings are taken on the spectrophotometer starting at about 15 minutes after removal from the bath. Readings are essentially constant for 1 hour after completion of the reaction. The analyses should be run in groups of not more than 50 tubes to permit the readings to be completed within this time.

The tubes are read on the Coleman junior spectrophotometer, model 6-A, at 570 mμ. The blanks are read against a reference tube of 1:1 propanol-water. The average blank tube is chosen, and the rest of the series read with the instrument set on the blank determination as zero. The blank reading should be about 0.08 to 0.10 on the optical density scale for 1 cc. of reagent and 0.15 to 0.20 for 2 cc. of reagent. Proline and hydroxyproline are read at 440 mμ.

For tubes which read near to or above 1.00, the solutions and the blank are diluted with additional 5 cc. samples of the propanol-water solution. When the volume in the tube reaches 16 to 17 cc., shaking by machine is not satisfactory, and inversion of the Neoprene-stoppered tubes is required. If the readings are still off the scale (above 1.00), samples are pipetted into other photometer tubes for further dilution.

When an analysis is completed, the tubes are rinsed with water, a monel wire screen or a perforated aluminum cover being used to permit the draining of a full rack of 50 tubes in one operation. The tubes are half filled with 0.2 per cent aqueous solution of soap flakes. The rack of tubes is heated for 20 minutes in a boiling water bath. The tubes are rinsed three times with water and dried in an oven at 110°. The boiling soap solution is

[9] Catalogue No. 5855, Precision Scientific Company, Chicago, Illinois.

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required to remove the band of material that is deposited on the walls of the tubes when volatile solvents are used.

Calculations

A standard curve is plotted for 0.1 cc. aqueous samples of leucine at six concentrations varying from 0.5 to 2.0 mM. Before being plotted, the average values are divided by the pipette calibration factor for water to give corrected readings for 0.100 cc. samples. From the graph, a table is prepared giving the millimolar concentrations corresponding to optical density readings from 0.01 to 1.00, in steps of 0.01 unit. The concentrations are multiplied by 11.1/6.1 and 16.1/6.1 to give concentrations corresponding to the readings obtained after dilution of the 6.1 cc. with one or

TABLE I

Relationship of Optical Density to Leucine Concentration (Condensed Table of Leucine Equivalents)

Determined on 0.100 cc. aqueous samples in photometer tubes of 16.25 mm. inner diameter.

Spectrophotometer reading, optical density × 100	Leucine concentration, mM per liter			Spectrophotometer reading, optical density × 100	Leucine concentration, mM per liter		
	Volume of diluent added				Volume of diluent added		
	5 cc.	10 cc.	15 cc.		5 cc.	10 cc.	15 cc.
10	0.196	0.357	0.518	60	1.18	2.15	3.11
20	0.392	0.714	1.03	70	1.39	2.53	3.67
30	0.588	1.07	1.55	80	1.61	2.93	4.25
40	0.784	1.43	2.07	90	1.83	3.33	4.83
50	0.980	1.78	2.59	100	2.05	3.73	5.42

two additional 5 cc. aliquots of the diluent. A condensed format of the standard table obtained with tubes possessing an inner diameter of 16.25 mm. is given in Table I, which covers concentrations up to 5.4 mM. The procedure can be extended by manual dilutions to much higher concentrations. The curve follows Beer's law through readings up to an optical density of about 0.50. There is a deviation of 4 per cent from the straight line relationship at an optical density of 1.0.

For the other amino acids and related compounds, the color yields per mole can be expressed relative to the leucine value as 1.00. For 0.1 cc. aqueous samples, the millimolar concentrations read from Table I, corrected for pipette delivery, were divided by the millimolar concentrations of the standard solutions to give the yields summarized in Table II. Each amino acid and peptide listed in Table II was checked for correct elementary analysis and, whenever possible, for optical rotation (*cf.* (1)). The other sub-

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stances were obtained from commercial sources and were not purified before analysis.

For other than 0.1 cc. aqueous samples, the values given in Table I for millimolar concentration corresponding to a given spectrophotometer reading require correction for the changes in volume involved. Before

TABLE II

Color Yields from Amino Acids and Other Compounds on Molar Basis Relative to Leucine

Determined on 0.1 cc. aqueous samples of 2.0 mM solutions; heating time, 20 minutes; read at 570 mμ.

Compound	Color yield	Compound	Color yield
Alanine	1.01	Glutathione	0.76
Arginine	1.00	Glycine ethyl ester	1.00
Aspartic acid	0.88	Glycyltyrosine	0.88
Citrulline	1.03	Glycylphenylalanine	1.04
Glutamic acid	1.05	Glycylglycine	0.89
Glycine	1.01	Glycylleucine	1.05
Histidine	1.04	Leucylglycine	0.92
Isoleucine	1.00	Phenylalanylglycine	0.97
Leucine	1.00	Phenylalanine ethyl ester	0.98
Lysine	1.12	Histamine	0.65
Methionine	1.00	Taurine	0.97
Phenylalanine	0.88	Tyramine	0.64
Serine	0.94	Sarcosine	0.84 *Ca.*
Threonine	0.92	Glucosamine	1.00
Tyrosine	0.88	Creatine	0.03
Valine	1.02	Creatinine	0.03
Cysteine	0.15 *Ca.*	Dibenzylamine	0.04
Half-cystine	0.54	Glycine anhydride	0.01
Tryptophan	0.72 *Ca.*	Urea	0.03
Proline	0.05	Adenine	0.00
Hydroxyproline	0.03	*p*-Aminobenzoic acid	0.00
Ammonia	0.98 *Ca.*	Diethylbarbituric acid	0.00
Asparagine	0.94	Glucose	0.00
Glutamine	0.99	Uric acid	0.00

calculating the correction factors to be applied to the analysis of samples containing volatile organic solvents, it is necessary to determine gravimetrically the amount of evaporation during the analysis by weighing tubes before and after heating the reaction mixture for 20 minutes under the experimental conditions employed in an actual determination. With butanol-water and propanol-water samples, essentially the entire 0.1 to 0.5 cc. sample of solvent evaporates during the heating process. If acidic

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samples are neutralized before analysis, the volume of NaOH solution added must be included in the calculation. When each term is expressed in cc., the correction factors (F) are calculated as follows:

$$F = \frac{\text{(Sample volume + neutralizing solution + ninhydrin solution + diluent)} - \text{(loss by evaporation)}}{1.1 + \text{diluent}} \times \frac{0.100}{\text{sample volume}}$$

Representative factors for two organic solvents are given in Table III. As a first approximation, these factors, used in conjunction with the rela-

Table III

Factors for Different Sample Sizes and Solvent Systems

Factors by which the millimolar concentrations from Table I are to be multiplied to give corrected leucine equivalents.

Solvent	Sample size	Ninhydrin solution	Loss by evaporation		Factor†		
					Volume of diluent added		
					5 cc.	10 cc.	15 cc.
	cc.	*cc.*	*mg.*	*cc.**			
Water	0.1	1	(14)‡		1.000	1.000	1.000
	0.2	1	(19)		0.508	0.504	0.502
	0.5	2	(19)		0.246	0.225	0.217
Butanol-water§	0.1	1	94	0.10	0.984	0.991	0.993
	0.2	1	194	0.20	0.492	0.495	0.496
	0.5	2	395	0.45	0.231	0.217	0.212
Butanol-benzyl alcohol-water ‖	0.1	1	41	0.03	0.996	0.998	0.998
	0.2	1	62	0.05	0.504	0.503	0.502
	0.5	2	79	0.07	0.244	0.224	0.216

* Approximate.

† To be divided by the calibration factor of the pipette.

‡ The small loss from water samples is subtracted from the loss with organic solvent samples in the calculation of approximate volume change.

§ Butanol-water containing 150 cc. of water per liter ($d^{25} = 0.838$).

‖ Butanol-benzyl alcohol-water 1:1:0.288 by volume ($d^{25} = 0.936$).

tive yields of color listed in Table II, give satisfactory results for most of the amino acids in these solvents, if an accuracy greater than ±5 per cent is not required. The color yields given in Table II and the factors listed in Table III may also be used for approximate results with other solvents that may be tried with starch chromatograms. Under such circumstances, the factors given in Table III for water or the butanol-benzyl alcohol solvent may be used for relatively non-volatile solvents, and the butanol factors for volatile solvents.

However, for accurate quantitative work with those solvents which are

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selected as optimum for chromatographic analyses, the exact degree of evaporation must be determined experimentally, and the color yields obtained from a given amino acid must be checked by the user with standard solutions made up in the organic solvent. As may be seen from Table IV, the yields thus obtained may differ by a few per cent from the values obtained with aqueous samples given in Table II. The yields should be determined on the same size of sample being used in the chromatographic experiments, although no differences have been observed between 0.1 cc. and 0.5 cc. samples in the solvents studied thus far. For accurate work with solvents other than water, therefore, the concentration of amino acid given in Table I is multiplied by the appropriate factor from Table III and divided by the appropriate color yield from Table IV.

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TABLE IV

Color Yields from Amino Acids in Organic Solvent Solutions on Molar Basis Relative to Leucine in Water

Determined on 0.1 cc. to 0.5 cc. samples; heating time, 20 minutes.

Amino acid	Color yield	
	Butanol-water solvent	Butanol-benzyl alcohol-water solvent
Leucine	0.99	1.01
Isoleucine	1.00	1.03
Phenylalanine	0.85	0.86
Tyrosine	0.86	0.87
Methionine	1.00	1.01
Valine	1.01	1.03

The factors given in Table III and the color yields listed in Table IV are fully reproducible when once determined under given experimental conditions. Except with NH_3 and tryptophan, it is not necessary to run controls with each batch of analyses. If this were not the case, the application to chromatography would be rendered unduly complicated by a need for repeated controls. An occasional check on the recovery of leucine from a known solution serves to confirm the reproducibility of the procedure.

The calculation of the recovery of leucine from a synthetic mixture which has been subjected to chromatographic analysis is given in Table V. Suitable data sheets are mimeographed to facilitate the handling of the results. In this example, the automatic fraction collector has been used with a column 0.9 cm. in diameter to deliver approximately 0.5 cc. samples directly to photometer tubes.

When aliquots are pipetted for analysis from larger effluent fractions, the summations of the uncorrected millimolar concentrations are multi-

TABLE V

Data Sheet, Determination of Leucine

Solvent, 1:1:0.288 butanol-benzyl alcohol-water; ninhydrin solution, 2 cc.; fraction collector, 25 drops = 0.504 cc.; entire fraction analyzed; wave-length, 570 mμ; readings recorded as optical density × 100.

Fraction No.	Volume of diluent, 5 cc.		Volume of diluent, 10 cc.	Volume of diluent, 15 cc.	Uncorrected amino acid concentration†
	Read against 1:1 water-propanol	Read against blank fraction*			
					mM
11	22.0	0	0	0	
13	22.2	0			
15	21.1	−1			
35	22.0	0			
36	22.0	0			0.00
37	23.5	1.5			0.03
38	29.0	7.0			0.14
39		24			0.47
40		57			1.12
41		100	62		2.22
42		140	90		3.33
43		150	102	81	4.30
44		130	84		3.08
45		77			1.54
46		28			0.55
47	28.5	6.5			0.13
48	22.0	0			0.00
49	22.5	0.5			
50	22.0	0			

Integration: Fraction 43. $4.30 \times 0.216 \times 0.5$‡ $= 0.464$
Sum of Fractions 41, 42, and 44. $8.63 \times 0.224 \times 0.5 = 0.967$
" " " 37-42, and 46-47. $3.98 \times 0.244 \times 0.5 = 0.486$
Total = 1.917 micromoles.

Correction for color yield from leucine in this solvent, 1.917/1.01 = 1.898 micromoles = 0.249 mg. of leucine.

Leucine added, standard aqueous solution of a mixture of amino acids 40 mM with respect to leucine; 0.495 cc. (0.5 cc. pipette, delivery 99 per cent) diluted to 5.00 cc. and 0.485 cc. (0.5 cc. pipette, delivery 97 per cent) placed on the column in butanol-benzyl alcohol. Theoretical yield = 1.920 micromoles = 0.252 mg.; recovery = 98.8 per cent.

* Fraction 11.

† From the expanded form of Table I.

‡ If 0.5 cc. samples are pipetted, the factor becomes 0.216 × (volume of effluent fraction)/(pipette calibration factor).

plied by the appropriate factors from Table III and by the exact volume of an effluent fraction to give micromoles of amino acid. When the whole

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fraction is analyzed, as in this example, the sample volume is equal to the volume of an effluent fraction, and the two terms cancel out. At large effluent volumes, when the peaks cover twenty or more fractions, integrations can be obtained from the analysis of every second fraction.

In order to obtain a graphic picture of the fractionation, the uncorrected millimolar concentrations are used directly for preliminary plotting of effluent concentration curves. The graph gives information on the symmetry of the curves and the degree of fractionation. The curves are plotted before decision is made on the division of the peaks for integration. For publication, the curves in the preceding paper (1) have been replotted, each point being corrected to "leucine equivalents" by means of Tables I and III. When the peaks are completely separated, the curves could also be corrected for color yield, but in the case of incomplete separation of the components, this is not possible. In the preceding paper (1) the method is given for the calculation of the amounts of each component in a series of partially overlapping peaks.

Accuracy—When the calibrations have been carefully made both for the pipettes and the photometer tubes, the readings on 0.2 micromole of an α-NH_2 acid can invariably be reproduced to within 0.02 optical density unit, corresponding to an accuracy of about 2 per cent. In the chromatographic analyses, it is necessary to work, in part, below this optimum concentration range. In a large series of chromatograms, integration of the effluent curves has given recoveries of 100 ± 3 per cent, under favorable conditions, and 100 ± 5 per cent for peaks markedly below the optimum average concentration (1).

Reaction with Proline and Hydroxyproline—The preceding method can be used for the determination of proline and hydroxyproline by measuring the yellowish red products of the reaction at their absorption maximum of 440 mμ. However, the optical density readings are only one-fourth and one-seventh, respectively, of those obtained with equimolar solutions of the α-NH_2 acids. The color development is only 80 to 90 per cent complete in 20 minutes at 100°. Standard curves can be prepared for proline and hydroxyproline with a 30 or 40 minute heating period. In the chromatographic analysis, the tubes are usually heated for only 20 minutes along with the rest of the effluent samples. As a first approximation, millimolar concentrations can be calculated from Tables I and III, just as in the case of readings at 570 mμ, and the values can be converted to proline by multiplying by the factor 3.7 and to hydroxyproline by multiplying by the factor 7.2. The measurement of proline by the ninhydrin reaction is of course possible only when this amino acid is completely separated from the other amino acids, as it frequently is on the starch chromatograms. On the other hand, in view of their low absorption at 570 mμ, small amounts

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of proline and hydroxyproline can be present simultaneously with α-NH_2 acids without giving significant interference.

Reaction with NH_3—In the oxidative deamination of amino acids by ninhydrin, 1 equivalent of the reagent is reduced in the course of the formation of diketohydrindylidene-diketohydrindamine (9). If oxygen could be completely eliminated by evacuation of the system, the maximum color yields would be obtained from amino acids without the addition of any further source of reduced ninhydrin. The yield from NH_3 under these conditions would be low, since, of itself, NH_3 does not give rise to the reduced ninhydrin which is essential for the formation of the colored complex. This accounts for the fact that NH_3 does not react positively in a number of the colorimetric ninhydrin procedures that have been used (4, 5, 7). When hydrindantin exists preformed in the reaction mixture, however, as it does in the present procedure, the color yield from NH_3 is in the same range as that from the amino acids. In contrast to the amino acid reaction, which is independent of the concentration of hydrindantin above a certain minimum level, the color yield from NH_3 increases with the hydrindantin concentration. With the reagent solution used in the present procedure, the color yield from NH_3 reaches about 90 per cent of its maximum value. Since different batches of reagent solution may vary somewhat in hydrindantin content, a control determination on a known NH_4Cl solution must be run simultaneously if it is desired to obtain accurate values on NH_3 solutions by the photometric ninhydrin method.

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Experiments on Color Development

Isolation of Diketohydrindylidene-Diketohydrindamine—The product of the reaction of ninhydrin with glycine at pH 5 was prepared in order to compare its absorption spectrum with that of the unfractionated reaction mixture obtained in the ninhydrin analysis.

The amino acid (75 mg.), dissolved in 10 cc. of water, was heated for 20 minutes at 100° with 700 mg. of ninhydrin dissolved in 20 cc. of citrate buffer, pH 5 (0.2 M). The product (245 mg.) which crystallized from the cooled solution corresponded to 75 per cent of the theoretical yield of the sodium salt of diketohydrindylidene-diketohydrindamine. The same procedure carried out with the peptide leucylglycine (188 mg.) gave the same product in 40 per cent yield. For analysis the sodium salt (50 mg.) was recrystallized from about 15 cc. of hot 1:1 water-*n*-propanol.

$C_{18}H_8O_4NNa$ (325.2).	Calculated.	C 66.5, H 2.5, N 4.3, Na 7.08
Prepared from glycine.	Found.	" 66.2, " 2.6, " 4.4, " 7.07
" " leucylglycine.	"	" 66.6, " 2.6, " 4.3, " 6.94

Absorption Spectra—The absorption curves have been determined with 0.1 cc. samples of 2 mM aqueous amino acid solutions. The final vol-

ume of the reaction mixture was 6.10 cc. in photometer tubes of 16.25 mm. inner diameter. In Fig. 2 the curves for leucine, serine, and ammonia are compared with the absorption spectrum obtained from an equimolar solution of the crystalline sodium salt of diketohydrindylidene-diketohydrindamine. The solution was prepared by dissolving 1.065 mg. of the sodium salt in 100 cc. of a mixture of the ninhydrin solution and the propanol-water diluent in the proportions of 1:10 and was read against this solvent as the blank. The absorption spectra support the conclusion that the α-NH_2 acids and ammonia yield diketohydrindylidene-diketohydrindamine

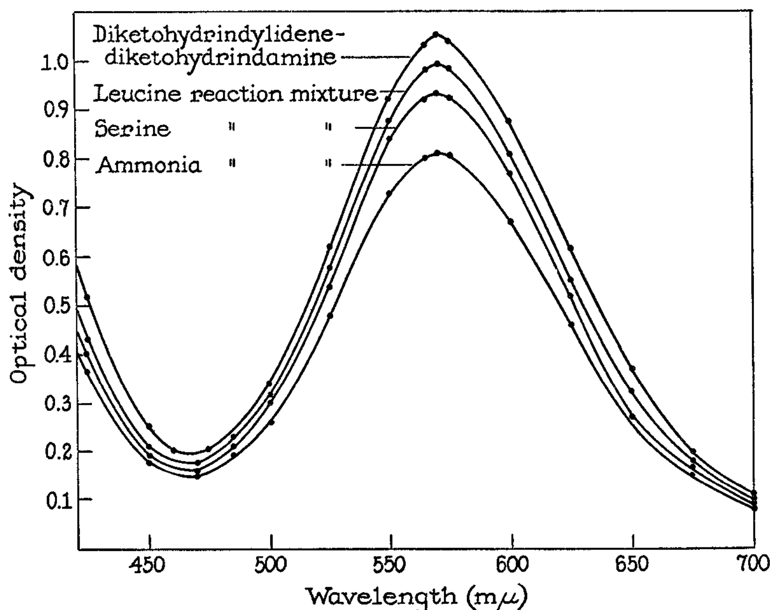

FIG. 2. Comparison of the absorption spectrum of diketohydrindylidene-diketohydrindamine with the spectra obtained after the reaction of ninhydrin with equimolar amounts of leucine, serine, and ammonia.

under the conditions of the determination. The curves obtained with the other α-NH_2 acids, except cysteine, and with peptides, are similar to those shown in Fig. 2. The major end-product is the same in all cases, exhibiting an absorption maximum at 570 mμ. The differences in the color intensities obtained with the individual amino acids arise from variations in the yield of this product. Relative to leucine, the reading of the pure sodium salt corresponds to a color yield of 1.07 (Table II). On this basis, leucine yields about 93 per cent of the theoretical amount of this product. The yields for phenylalanine and glutamic acid, for example, are 82 and 98 per cent.

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Color development with ninhydrin is not specific for amino acids, since a variety of primary amines and some secondary amines will give significant amounts of color. The structure of the end-products in these cases remains to be determined. The absorption curves obtained with histamine and with the N-methyl-substituted amino acid, sarcosine, are given in

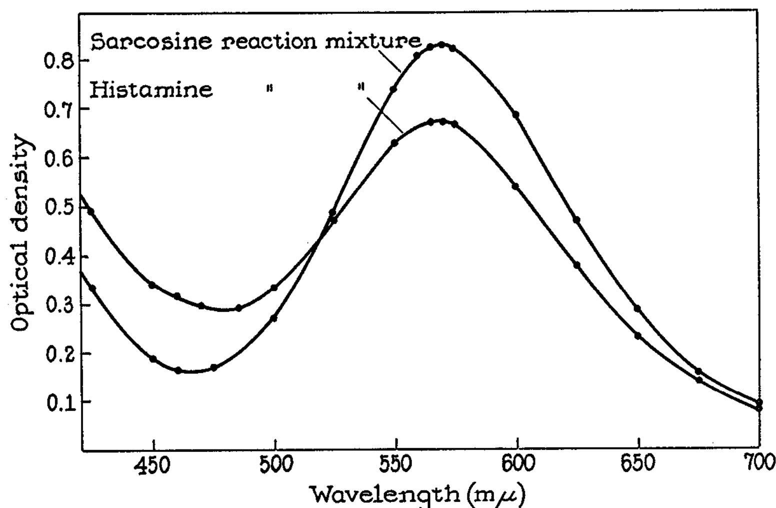

FIG. 3. Absorption spectra obtained after the reaction of ninhydrin with sarcosine and histamine.

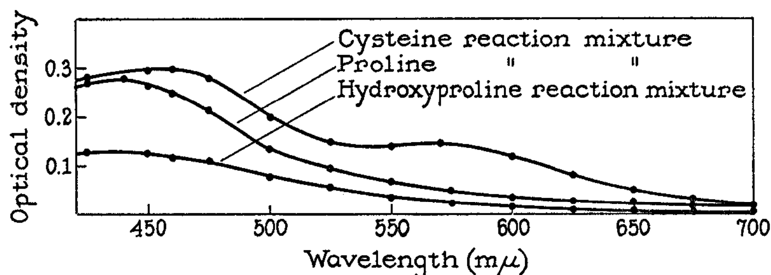

FIG. 4. Absorption spectra obtained after the reaction of ninhydrin with proline, hydroxyproline, and cysteine.

Fig. 3. Both compounds yield products with absorption maxima at 570 mμ.

Cysteine, which has been mentioned as an exception to the general reaction of the α-NH_2 acids, gives an absorption curve which is somewhat similar to that obtained with proline and hydroxyproline (Fig. 4). Neutral cysteine solutions, after standing for 24 to 48 hours, give the same color

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yield as cystine, with maximum absorption at 570 mμ. It is possible that the small amount of absorption in the range of 570 mμ observed with fresh solutions of cysteine may be attributed to the presence of some cystine in the reaction mixture. The compounds yielded by the prolines under these conditions presumably correspond to the structures assigned by Grassmann and von Arnim (12).

Variation of pH—The variation of color yield with the pH of the aqueous citrate buffer is shown in Fig. 5. The absorption maximum for the α-NH_2 acids is at 570 mμ over the pH range studied. The maximum color yield from leucine is obtained at pH 5, which has been chosen for the general procedure.

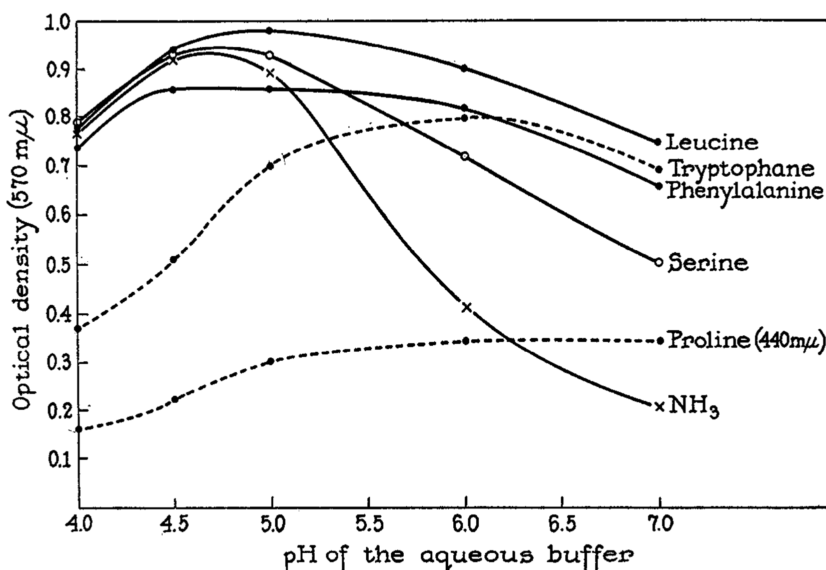

FIG. 5. Effect of pH on the intensity of color obtained after the reaction of ninhydrin with amino acids and ammonia.

The pH optima for the other α-NH_2 acids fall close to this value, with the exception of tryptophan which gives a maximum yield at pH 6. For most of the amino acids, a change in pH of 0.1 unit at pH 5.0 introduces less than a 1 per cent deviation in the optical density reading.

Rate of Reaction—The rates of color development have been determined for representative α-NH_2 acids over a range of pH from 4 to 7. In all cases, at 100° the reaction was complete in less than 20 minutes. The color yields were unaltered by an increase in the ninhydrin concentration. This result parallels the observations on the heating periods required for the complete liberation of CO_2 from amino acids at a ninhydrin concentration of 20 mg. per cc. in the gasometric method of Van Slyke, Dillon, Mac-

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Fadyen, and Hamilton (13). A more detailed study of the rate of color development has been made at pH 5. Constant readings are obtained with leucine in 5 minutes and with alanine in 10 minutes. The reaction with glycylphenylalanine and glycylleucine is complete in 20 minutes. On the other hand, phenylalanylglycine and leucylglycine reach only 90 per cent of completion in this time. Primary amines, such as ethylamine and ethanolamine, react still more slowly. The time of 20 minutes has been chosen as a heating period which gives constant readings with all of the α-NH_2 acids and can be expected to give reasonably high readings with most peptides possessing a free NH_2 group.

Temperature—The color yields are lower if the reaction is carried out at temperatures below 100°. For leucine, isoleucine, tyrosine, and phenylalanine, the optical densities were 4 per cent lower when the analysis was carried to completion in a water bath maintained at 95°.

Stability of Color—The rate of fading of the blue color is illustrated by the following average readings obtained on leucine samples at the specified times after removal of the photometer tubes from the heating bath: 15 minutes 0.835, 30 minutes 0.830, 45 minutes 0.835, 60 minutes 0.835, 1½ hours 0.815, 2½ hours 0.810, 4 hours 0.795, 5½ hours 0.785, and 22 hours 0.710. Thus, the color is stable for about 60 minutes, after which time there is a gradual fading, averaging approximately 1 per cent per hour. The end-product is not highly sensitive to oxidation by air, whereas, as noted below, an intermediate in the reaction appears to possess much greater sensitivity.

In early experiments, water was used as the diluent and marked fading was noted as a result of precipitation of the sodium salt of diketohydrindylidene-diketohydrindamine. The use of 1:1 water-*n*-propanol as the diluent serves to keep the relatively insoluble reaction product in solution.

Effect of Stannous Chloride—When 2 mM leucine samples are analyzed with a ninhydrin solution from which the stannous chloride has been omitted, the color yield is about half that obtained in its presence. At lower leucine concentrations the percentage decrease in yield is greater. By carrying out the reaction in vessels evacuated to 20 to 30 mm., as is done in the gasometric ninhydrin method (13), the color yield from 2.0 mM leucine solutions can be raised almost to the maximum value. At a leucine concentration of 0.05 mM, however, the results still run about 10 per cent low.

The first trials on the blocking of the oxidative side reaction by the addition of a reducing agent to the ninhydrin solution were made with hydrindantin. Consistent results were obtained at a hydrindantin concentration of 1 mg. per cc. The color yields were unaltered by a 4-fold increase in this concentration. Since hydrindantin can be prepared by the action

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of stannous chloride on ninhydrin, the addition of stannous chloride directly to the reagent solution was tried and found to give the same results. However, the presence of hydrindantin, which is highly insoluble in water, required the addition of an organic solvent which would keep this compound in solution during the course of the reaction and during storage of the reagent solution. Among the solvents tested, methyl cellosolve had the highest solvent power for hydrindantin. The solvent mixture chosen (1:1 water-methyl cellosolve) does not evaporate in the water bath at 100° and does not precipitate sodium citrate from the buffer.

SUMMARY

The reaction of ninhydrin with NH_2 groups to give diketohydrindylidene-diketohydrindamine has been utilized as the basis for a photometric determination of amino acids and related compounds in effluent samples from starch chromatograms. The color yields have been rendered fully reproducible by the incorporation of hydrindantin or stannous chloride in the reagent solution to eliminate oxidative side reactions. Although the color yield from a given amino acid is constant, the different amino acids do not all give the same percentage yield of the blue product. This fact does not prevent the accurate use of the method in chromatographic work in those cases in which the individual amino acids are separated from one another by the fractionation process.

Color development is obtained with a variety of compounds containing NH_2 groups, including amino acids, peptides, primary amines, and ammonia. For chromatographic work, the generality of the reaction extends its usefulness. For work with unfractionated biological material, the lack of specificity is a disadvantage.

The reaction is carried out at pH 5 and 100°. The absorption maximum of the blue product is at 570 mμ. On individual amino acids the accuracy is 2 per cent for samples in the range of 2.5 γ of α-NH_2 nitrogen. The mechanics of the procedure have been developed to permit the analysis of a large number of samples on a routine basis.

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PHOTOMETRIC NINHYDRIN METHOD FOR USE IN THE CHROMATOGRAPHY OF AMINO ACIDS

Stanford Moore and William H. Stein

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