A MODIFIED NINHYDRIN REAGENT FOR THE PHOTOMETRIC DETERMINATION OF AMINO ACIDS AND RELATED COMPOUNDS

By STANFORD MOORE and WILLIAM H. STEIN

(From the Laboratories of The Rockefeller Institute for Medical Research, New York 21, New York)

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The photometric ninhydrin method described previously (1) for use in the chromatography of amino acids has been the subject of further study during the development of the Dowex 50-X4 procedure (2). The adjustment of the pH of the effluent fractions prior to analysis required by the earlier method has been largely eliminated by increasing the strength of the buffer in the reagent 5-fold. A second modification involves the elimination of the stannous chloride which was previously added to form reduced ninhydrin (hydrindantin) in the reagent solution. Hydrindantin itself is now added directly, and this change avoids the precipitation of tin salts which occurs when the previous reagent is used to analyze samples of the phosphate buffers frequently employed in the chromatography of proteins (3). The effective concentration of hydrindantin has been raised to 0.3 per cent. As a result, the antioxidant effect of 1 ml. of the modified reagent is sufficient to prevent interference from the dissolved oxygen in a 2 ml. sample submitted to analysis. With 1 ml. fractions, 0.5 ml. of the ninhydrin reagent is adequate. The quantity of reagent required is thus reduced to one-half or one-quarter the amount formerly recommended (1).

Troll and Cannan (4) have recently described conditions under which the yield of blue color (diketohydrindylidenediketohydrindamine) in the reaction of ninhydrin with amino acids is raised to 100 per cent of theory in most cases. For this purpose, the reaction is performed in a predominantly organic solvent (phenol-pyridine-alcohol) having a maximal water content of about 20 per cent. The method, when applicable, has the fundamental advantage of giving maximal color yields. The present procedure, in which the reproducible color yield from leucine is 95 per cent of theory, appears to be more convenient for the analysis of the aqueous effluent fractions obtained in ion exchange chromatography.

Reagents

Ninhydrin—Several commercial sources provide ninhydrin sufficiently pure to be used without recrystallization (1). The reagent employed in this study was purchased from Dougherty Chemicals, 87–34 134th Street, Richmond Hill 18, New York.

Hydrindantin—The compound was prepared by the reduction of ninhydrin with ascorbic acid (*cf.* Abderhalden (5), West and Rinehart (6)). To 80 gm. of ninhydrin in 2 liters of water at 90°, add with stirring a solution of 80 gm. of ascorbic acid (Merck) in 400 ml. of water at 40°. Crystallization of hydrindantin starts immediately and is allowed to proceed for 30 minutes without further heating. During the next hour the solution is cooled to room temperature under running tap water. The hydrindantin is filtered off, washed well with water, and dried to constant weight over P_2O_5 in a vacuum desiccator protected from light. Yield, 75 gm. The compound should be stored in dark glass. The crystallization is carried out rapidly to give small crystals which will dissolve readily in methyl Cellosolve. The use of the anhydrous form rather than the air-dried dihydrate also increases the rate of solution in the organic solvent. (Hydrindantin thus prepared is available from Dougherty Chemicals.)

4N Sodium Acetate Buffer (pH 5.5)—To 2 liters of water, add 2720 gm. of $NaOAc \cdot 3H_2O$ (reagent grade) and stir on a steam or water bath until solution is complete. Cool to room temperature, add 500 ml. of glacial acetic acid, and make up to a volume of 5 liters. The solution (undiluted) should be at pH 5.51 ± 0.03. If final adjustment of the pH is necessary, 5 gm. of NaOH correspond to about 0.04 pH unit. The buffer can be stored at 4° without a preservative.

Reagent Solution—Dissolve 20 gm. of ninhydrin and 3 gm. of hydrindantin in 750 ml. of methyl Cellosolve.[1] The stirring should not incorporate air bubbles into the solution. Add 250 ml. of the buffer of pH 5.5 and immediately transfer the resulting reddish reagent solution to a 1 liter dark glass reservoir bottle arranged to permit the solution to be stored under nitrogen, as previously described (1).[2] The second and third bottles of the storage assembly are of 2 liter size. The 250 ml. dropping funnel used before (1) has been replaced by an inlet tube bearing a 6 mm. bore stop-cock and a small semiball joint, normally closed by a corresponding stopper. In refilling the reservoir, a bent glass tube reaching to the bottom of a beaker of the reagent is attached to the inlet tube through the ground joint connection. Slight air pressure is then applied to the third bottle to drive

[1] The solvent (monomethyl ether of ethylene glycol) is obtainable from the manufacturer (Carbide and Carbon Chemicals Corporation, 30 East 42nd Street, New York 17) or supplier (*e.g.*, Amend Drug and Chemical Company, 117 East 24th Street, New York 10) relatively free from peroxides. Each lot is tested for peroxide content by adding 2 ml. of the solvent to 1 ml. of freshly prepared 4 per cent aqueous KI. A colorless to light straw-yellow test is satisfactory.

[2] Evaporation from the concentrated salt solution may cause crystallization of sodium acetate on the syringe of the pipetting machine (1). The exposed section of the plunger should be wiped free of any appreciable deposit to prevent strain on the syringe assembly.

reagent remaining in the reservoir into the filling tube and to displace the air therein. The air pressure is replaced by suction to draw the reagent solution into the reservoir. Care should be taken not to introduce air into the assembly at the end of the filling operation.

The solution is not as stable on storage as the previous reagent. After 1 month, the blank readings have about doubled, and, unless the bottle has been stored in the dark, the color yields can run low by as much as 5 per cent. For this reason, not much more than a 1 week's supply of reagent should be prepared at a given time.

Procedure (*Cf.* (*1*))

For the analysis of 1 ml. or 2 ml. effluent fractions from ion exchange chromatograms (2), 1 ml. of the ninhydrin solution is used. The buffer is sufficiently strong so that preliminary adjustment of the pH of the samples is seldom necessary. If, however, more than 1 ml. of 0.1 N HCl or 0.1 N NaOH should be required to bring the effluent fractions to pH 4 to 6, addition of acid or alkali may be necessary to bring the pH of the samples to the optimum for color development. The capped tubes are shaken briefly (< 10 seconds) by hand or on a reciprocal shaker (1) and are heated for 15 minutes (accurately timed) in a covered boiling water bath (1). It is convenient to use 50:50 ethanol-water as a diluent rather than *n*-propanol-water (1). After dilution, the tubes (kept out of direct sunlight) are wiped dry and transferred to a dry rack, cooled to below 30° in front of an electric fan, and thoroughly shaken (about 30 seconds) before being read at 570 mμ (440 mμ for proline and hydroxyproline). Because the modified reagent contains a higher concentration of hydrindantin, more of the reddish color of this compound remains at the end of the heating period. In order to reduce the blank to the desired level, therefore, the tubes should be shaken sufficiently so that the major portion of the residual hydrindantin is oxidized by air. For this reason, the tubes are shaken uniformly in a rack, rather than individually. With the modified reagent, the blank readings for 2 ml. samples and 1 ml. of ninhydrin solution have been 0.05 to 0.10 on the optical density scale. If there is evidence that high blank readings are being caused by uptake of NH_3 during the period that the tubes are standing on the fraction collector, the cover of the machine should be lined with cloth impregnated with citric acid (7).[3] If effluent fractions are stored prior to analysis, they should be stoppered with corks treated with citric acid (7) and wrapped in aluminum foil.

The absence of tin salts in the reagent solution not only avoids the precipitation of tin phosphate when samples containing phosphate are ana-

[3] If ammonia interferes, it can be removed by rendering the samples alkaline and placing the tubes in a vacuum desiccator for a few hours before the ninhydrin analyses (2).

lyzed, but also eliminates the relatively insoluble deposit which was formed on the inner walls of the photometer tubes (1) with the previous reagent. As a result, the heating or brushing with detergent solution formerly required to clean the tubes is no longer necessary. After use, the tubes are rinsed once with tap water, three times with distilled water, and oven-dried. In an occasional chromatogram, a peak may be encountered that contains enough ninhydrin-positive material to cause precipitation of the colored reaction product. To clean tubes containing such a precipitate, brushing with a detergent solution is required.

Calculations

A standard curve is plotted in the manner previously described (1) by utilizing 1 ml. samples of leucine (0.05 to 0.2 mM) in 0.1 M citrate buffer at pH 5. For use with chromatograms, two direct reading tables are prepared in the format of Table I previously given (1), one for 1 ml. samples and 1 ml. of ninhydrin solution, and the second for 2 ml. samples and 1 ml. of the reagent. It is convenient to have twice the millimolar concentrations listed in the table for the 2 ml. samples. In this way both tables yield *micromoles* of amino acid directly, without the use of factors ((1) Table V), when the values corresponding to the points of a given peak on an effluent curve are added. Fractions that are off the scale of the table (above an optical density of 1.0 after dilution with 15 ml. of diluent) must be diluted further by hand before being read. The values taken from the final column of the table must then, of course, be multiplied by the appropriate dilution factor. When samples require neutralization or, in preparative chromatography, when aliquots from larger fractions are pipetted (1),[4] direct reading tables can be prepared for the special case, or appropriate volume corrections can be applied to the values read from a table for a different fraction size. In computing the volume corrections, no factor is required for the negligible loss by evaporation which occurs during the heating of the aqueous samples (1).

The color yields obtained with a number of ninhydrin-positive substances likely to be encountered in biological systems are listed in Table I. The values, as well as the color yields from peptides and proteins, are similar in most cases to those obtained with the previous reagent (1, 3). The yields were determined on 2 ml. samples of the compounds dissolved in the buffers in which they emerge from columns of Dowex 50-X4 in the current chromatographic procedure (2). Although the color yields are reproducible to ±2 per cent in a given laboratory, the exact values should be checked under the user's experimental conditions if maximal accuracy is desired.

[4] Transfer pipettes No. 283-R, Microchemical Specialties Company, 1834 University Avenue, Berkeley 3, California.

The molecular weight divided by the color yield provides the factor by which the chromatographic results (expressed in leucine equivalents in micromoles) should be multiplied to give micrograms of the substance in

TABLE I

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

Determined on 2 ml. samples of 0.1 mM solutions in the buffers (pH 2.2 to 5) in which the compounds emerge in the Dowex 50-X4 chromatographic procedure (2); heating time 15 minutes; read at 570 mμ. (The urea and creatinine were 3.0 mM.)

Compound	Color yield	Compound	Color yield
Aspartic acid	0.94	β-NH_2-isobutyric acid	0.44
Threonine	0.94	Carnosine	0.93
Serine	0.95	Citrulline	1.04
Proline (440 mμ)	0.225*	Creatinine	0.027
Glutamic acid	0.99	Cysteic acid	0.99
Glycine	0.95	Diaminopimelic acid (per 2 NH_2 groups)	1.24
Alanine	0.97		
Valine	0.97	Ethanolamine	0.91
Half cystine	0.55	Felinine	0.95
Methionine	1.02	Glutamine	0.99
Isoleucine	1.00	Glucosamine	1.03
Leucine	1.00	Glutathione (oxidized, half)	0.93
Tyrosine	1.00	Glycerophosphoethanolamine	0.50
Phenylalanine	1.00		
Ammonia	0.97	Hydroxylysine	1.12
Lysine	1.10	Hydroxyproline (440 mμ)	0.077*
Histidine	1.02	Methionine sulfone	1.02
Tryptophan	0.94	" sulfoxide	0.98
Arginine	1.01	1-Methylhistidine	0.88
α-NH_2-adipic acid	0.96	3-Methylhistidine	0.86
β-Alanine	0.50	Ornithine	1.12
Anserine	0.78	Phosphoethanolamine	0.43
Asparagine	0.95	Sarcosine	0.28
α-NH_2-*n*-butyric acid	1.02	Taurine	0.88
γ-NH_2-butyric acid	1.01	Urea	0.0314

* The readings taken at 440 mμ are first converted to "leucine equivalents" by using the same conversion table that is employed for the other amino acids measured at 570 mμ, and the concentrations of the imino acids are subsequently obtained by dividing by the above color yields.

question. Each of the first eighteen amino acids (Table I) was checked for purity by elementary analysis and by chromatography on Dowex 50. The other compounds were commercial or research samples possessing the correct elementary composition.

The color yields are relatively independent of the initial pH of the sample provided the substance analyzed is dissolved in 0.2 N buffers at pH 3 to 7. When, however, 2 ml. aliquots of a 0.2 N eluent of pH 2.2 are analyzed, the pH is lower than the optimum during the reaction with ninhydrin and the color yield is decreased. Taurine, for example, gives a color yield of 0.94 if dissolved in a solution at pH 3.1 and 0.88 if the sample is at pH 2.2. Cysteic acid has a color yield of 1.01 if the sample is at pH 5 and 0.99 for a sample at pH 2.2. In the cystine method of Schram *et al.* (8), cysteic acid gives a color yield of 0.99 when 1 ml. effluent fractions of 0.1 N monochloroacetic acid are analyzed without neutralization.

Maximal color yields with either 1 or 2 ml. samples are obtained for all of the common α-amino acids after heating for 15 minutes at 100°. Longer heating increases the color yield from the β-amino acids (Crokaert *et al.* (9)), proline, hydroxyproline, and sarcosine. With compounds such as these, for which the reaction is not complete in 15 minutes, the color yield is greater when 1 ml. fractions are analyzed, since the concentration of ninhydrin in the reaction medium is increased.

DISCUSSION

The proportion of methyl Cellosolve in the reagent solution has been raised from 50 per cent to 75 per cent in order to keep the increased quantity of hydrindantin in solution. The increase in the strength of the buffer has required the use of sodium acetate (*cf.* Boissonnas (10)), which is more soluble in methyl Cellosolve, in place of sodium citrate. With the stronger buffer, the pH at which maximal color yields are obtained in the ninhydrin reaction is very similar to that employed previously. Although the 4 N sodium acetate buffer is initially at pH 5.5, the pH drops to 5.1 when the buffer is diluted to 1 N, which is the actual normality employed in the reagent. With the previous reagent, maximal color yields were obtained at pH 5.0 with a 0.2 M citrate buffer.

Satisfactory recoveries of proline from chromatograms were obtained with use of the modified reagent (1) despite the low value of the color yield. With hydroxyproline, however, which has a much lower color yield, this would not be the case. Greater sensitivity for proline is exhibited by the procedure of Chinard (11), which utilizes the red color formed with ninhydrin in acid solution, and this method has been employed by Harfenist (12) to analyze the effluent from Dowex 50-X8 chromatograms. The special procedures of Neuman and Logan (13) and Troll and Cannan (4) (*cf.* Rogers *et al.* (14)) provide more sensitive tests for hydroxyproline.

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SUMMARY

An extension of earlier studies on the determination of amino acids and related compounds with ninhydrin has led to the development of a modified reagent composed of 2 per cent ninhydrin and 0.3 per cent hydrindantin in 3:1 methyl Cellosolve-4 N sodium acetate buffer (pH 5.5). The strength of the buffer eliminates or reduces the need for preliminary adjustment of the pH of the samples when the method is used for the analysis of effluent fractions obtained in ion exchange chromatography, and the higher hydrindantin concentration of the reagent solution permits economies in the use of ninhydrin.

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