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Specific Identification of Hydroxyamino Acids on Paper Chromatograms of Protein Hydrolyzates

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► Individual methods are described for the specific identification of serine and threonine on paper chromatograms. The test for serine is based on the condensation of the formaldehyde, liberated by periodic acid oxidation, with acetylacetone, in the presence of ammonium salts to form the yellow, highly fluorescent, 3,5-diacetyl-1, 4-dihydrolutidine. Hydroxylysine, found only in a few proteins, also gives a positive response. Threonine is specifically detected in the presence of all amino acids by a test based on the reaction of acetaldehyde with sodium nitroprusside and piperidine, following oxidation of the amino acid with periodic acid.

SEVERAL METHODS have been described for detecting and confirming the identity of hydroxyamino acids on papergrams. The procedure suggested by Syngé (2) involves the detection, with Nessler's reagent, of ammonia liberated by periodate oxidation. This method will not differentiate between serine and threonine, and also requires that the paper be free from any ammonia that may have been employed in the solvent. The methods suggested by Buchanan, Dekker, and Long (1), and by Metzberg and Mitchell (6) are also utilizable, but these too will not distinguish between serine and threonine. In the procedure described,

serine is specifically detected in the presence of all commonly occurring amino acids including threonine.

IDENTIFICATION OF SERINE

The test is based on the fact that serine liberates formaldehyde when oxidized with periodic acid (8). The formaldehyde set free in the reaction is then condensed with acetylacetone in the presence of ammonium salts to form the yellow, highly fluorescent, 3,5-diacetyl-1,4-dihydrolutidine (?).

The finished chromatogram, dried at 50° C. or below, is sprayed carefully with a 0.035*M* solution of periodic acid in methanol containing 2 vol. % redistilled γ -collidine. When the sheet is visibly dry, it is sprayed with a solution containing 15 grams of ammonium acetate, 0.3 ml. of acetic acid, and 1 ml. of acetylacetone per 100 ml. of methanol. The latter solution is a modification of the reagent described by Nash (?) for the quantitative estimation of formaldehyde. The paper is allowed to develop at room temperature until the yellow spot indicating the position of serine is visible. This usually requires from 1 to 4 hours, depending on the concentration of serine. Under ultraviolet light, however, the spot is usually visible within 30 minutes after the application of the second spray. The approximate lower limit of detection for serine is 2 γ per

sq. cm. in daylight, and 1 γ per sq. cm. under ultraviolet light, after chromatography on Whatman No. 1 paper in the solvent system, methanol-water-pyridine (80 to 20 to 4) (9). The only other amino acid which gives a positive test under these conditions is δ -hydroxylysine. The limits of detection for this amino acid in white, and ultraviolet light, respectively, following chromatography under these conditions, are 1.5, and 1.0 γ per sq. cm.

Under similar conditions, L- or DL-threonine gives no spot in white light even in high (35 γ per sq. cm.) concentration. If the paper is examined under ultraviolet light after 24 hours, threonine is visible as a dark zone which cannot be confused with the yellow fluorescent serine spot. The dark threonine zone is due presumably to the formation of diacetyldihydrocollidine from the condensation of acetaldehyde with the reagent (?). However, approximately 15 to 20 γ of threonine are needed for detection under ultraviolet light, which renders its detection impractical with paper chromatography. A much more sensitive reaction for threonine is described.

The following compounds were examined, both in white and ultraviolet light, in concentrations ranging from 35 to 50 γ per sq. cm. All of the common amino acids, *o*-phosphoserine, *o*-phosphothreonine, *o*-phosphoethanolamine, methionine sulfone and sulfox-

ide, citrulline, cystathione, β -aminoisobutyric acid, β -alanine, taurine, γ -amino-*n*-butyric acid, methylhistidine, thiohistidine, sarcosine, cysteic acid, asparagine, glutamine, lanthionine, ornithine, and α -amino-*n*-butyric acid were negative under these conditions.

Compounds other than serine and hydroxylysine which responded to the test, and the approximate lower limit of detection (micrograms per square centimeter) in white, and ultraviolet light, respectively, were ethanolamine, 1.0 and 0.8; glycerol, 0.5 and 0.2; and L- α -phosphoglyceroethanolamine, 2.7 and 1.8.

SPECIFIC IDENTIFICATION OF THREONINE

The test depends on the fact that threonine liberates acetaldehyde when oxidized with periodate (5). The acetaldehyde is detected by applying a slight modification of the sensitive reagent described by Feigl and Stark (3).

The dry, finished chromatogram is sprayed with the periodic acid-collidine

reagent described for serine. When visibly dry, the sheet is sprayed carefully with a solution made by mixing, just before use, 1 volume of 5% methanolic solution of sodium nitroprusside with 1 volume of 20 vol. % piperidine in methanol. The threonine spot is indicated by a blue zone within 15 minutes. The sensitivity of the test under these conditions, which may not be optimal, is 1.5 γ of L- or DL-threonine per sq. cm., after chromatography under the conditions used for serine. All compounds except threonine which have been mentioned are negative in concentrations ranging from 35 to 50 γ per sq. cm. Higher concentrations were not tested.

The reactions described are not necessarily limited to the compounds reported which give a positive response. A review of the periodate reaction by Jackson (4) should reveal a number of compounds which would be expected to give a positive reaction on chromatograms containing biological materials other than protein hydrolyzates.

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