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Note

A thin-layer-chromatographic method for analysis of amino sugars in polysaccharide hydrolyzates

PAUL A. REBERS, G. E. WESSMAN,
National Animal Disease Center, ARS, U.S. Dept. of Agriculture, P.O. Box 70, Ames, IA 50010 (U.S.A.)

AND JOHN F. ROBYT
Iowa State University, Dept. of Biochemistry and Biophysics, Ames, IA 50011 (U.S.A.)

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Paper- and thin-layer chromatographic methods are well adapted for analysis of hydrolyzates containing neutral sugars because of their speed and simplicity. With amino sugars, streaking, diffuse spots, and poor separations are usually obtained[1]. The two amino sugars most frequently found in glycosaminoglycans and bacterial polysaccharides, D-glucosamine and D-galactosamine, are especially difficult to separate from each other by paper or thin-layer chromatography. However, the *N*-(2,4-dinitrophenyl) derivatives of the two have been separated in the presence of borate ions[2]. The free sugars have also been separated by two-dimensional t.l.c. on cellulose[3]. This note describes a one-dimensional t.l.c. procedure with K5 silica gel plates (Whatman Chemical Separations, Inc., Clifton, N.J.) that completely separates D-glucosamine, D-galactosamine, D-mannosamine, and D-muramic acid. It is an adaptation of the t.l.c.-silica gel procedure described by Gauch *et al.*[4] for the separation of mono-, di-, and tri-saccharides.

A brief description of the experiments leading up to the development of the procedure may be of value. The 17:3 acetonitrile–water system described by Gauch *et al.*[4] for use with silica gel completely separated L-*glycero*-D-*manno*-heptose, D-galactose, D-glucose, and L-rhamnose. It also separated glycine, alanine, and proline. However, the free amino sugars, D-glucosamine, D-galactosamine, and D-mannosamine remained at the origin with this system. The 2-acetamido-2-deoxy sugars, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-mannosamine migrated, but were not resolved. It was postulated that the free amino sugars bind to the silica gel through the amino groups, and that this binding might be overcome by introducing acid into the solvent system; addition of acetic acid to the solvent system caused the amino sugars to move. Three ascents on K-5 silica gel with 15:1:4 acetonitrile–acetic acid–water as solvent at 22° effectively separated D-glucosamine, D-mannosamine, and D-muramic acid, but D-glucosamine

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and D-galactosamine were incompletely separated. No improvement in the separation was observed by increasing the temperature to 40° during chromatography. Replacing the K-5 silica gel plates with Eastman Silica Gel Chromatography Sheet 6061 (Eastman Kodak, Rochester, NY) gave poorer separations: D-glucosamine, D-galactosamine, and D-mannosamine failed to separate from each other. Three ascents of solvent on the K5 silica gel plate with 15:4:1 acetonitrile–acetic acid–water increased the movement of the amino sugars, but D-glucosamine and D-galactosamine were incompletely separated. Several other mixtures of acetonitrile, acetic acid, and water were tried, but were not as good as those previously mentioned. Substitution of formic acid for acetic acid resulted in diffuse spots. Replacement of the acetic acid by propanoic acid resulted in slightly more-compact spots, but the separation was not improved.

D-Glucosamine and D-galactosamine were completely separated with 40:1:9 acetonitrile–concentrated ammonium hydroxide (15M)–water, but this mixture failed to separate D-glucosamine, D-mannosamine, and D-muramic acid from each other.

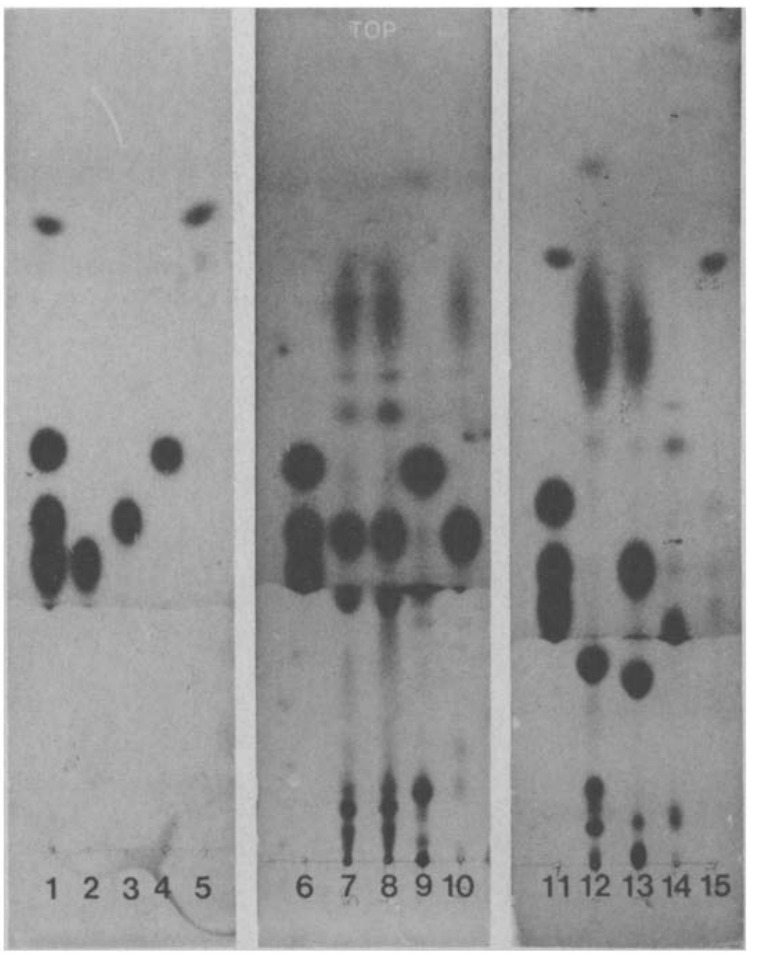

Fig. 1. Separation of amino sugar standards and sugars obtained from the hydrolysis of streptococcal polysaccharides by t.l.c. with ninhydrin as the detecting agent. 1 and 11. Separation of D-muramic acid, D-mannosamine, D-glucosamine, and D-galactosamine standards in descending order. 2. D-galactosamine standard. 3. D-glucosamine standard. 4. D-mannosamine standard. 5 and 15. D-muramic acid standard. 6, D-mannosamine, D-glucosamine, and D-galactosamine standards. 7 and 8. Hydrolyzates of type IV polysaccharide from strains EM-1 and 145A. 9. Hydrolyzate of type II polysaccharide from strain K131. 10. Hydrolyzate of type V polysaccharide from strain TG9. 12. Hydrolyzate of type VIII polysaccharide from strain 7121. 13. Hydrolyzate of type VII polysaccharide from strain 7585T. 14. Hydrolyzate of type VI polysaccharide from strain 0851T.

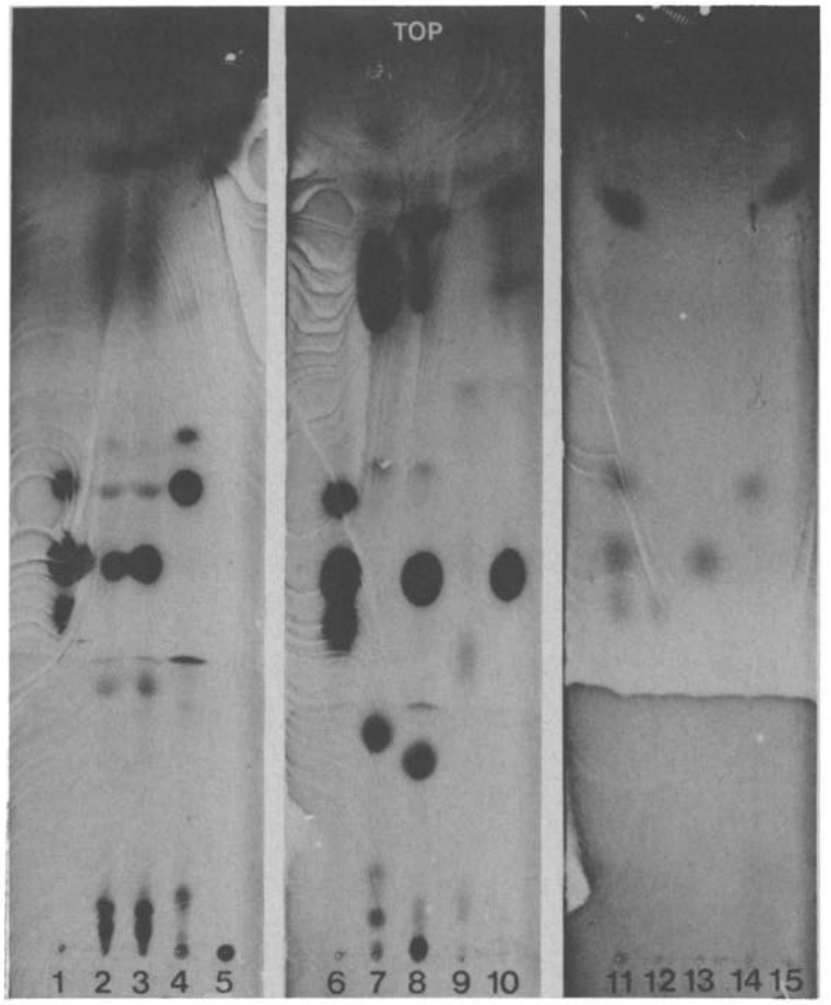

Fig. 2. T.l.c. separation of amino sugar standards and streptococcal polysaccharide hydrolyzate as in Fig. 1, but with substitution of silver nitrate reagent for ninhydrin as the detecting agent. 1 and 6. D-mannosamine, D-glucosamine, and D-galactosamine standards in descending order. 5. D-glucose and D-galactose standards in descending order. 2, 3, 4, 7, 8, 9, 10. Separation of streptococcal polysaccharide hydrolyzates from types IV, IV, II, VIII, VII, VI, and V, respectively. 11. D-muramic acid, D-mannosamine, D-glucosamine and D-galactosamine. 12. D-galactosamine. 13. D-glucosamine. 14. D-mannosamine. 15. D-muramic acid.

Addition of ethanol to the acetonitrile–acetic acid–water solvent mixture permitted the separation of a mixture of D-galactosamine, D-glucosamine, D-mannosamine, and D-muramic acid. Three ascents with 14:1:1:4 acetonitrile–acetic acid–ethanol–water gave a definite improvement, and 13:1:2:4 acetonitrile–acetic acid–ethanol–water gave complete separation.

Amino sugars were detected on separate plates with ninhydrin and with silver nitrate. Ninhydrin detects both amino acids and amino sugars, and silver nitrate detects reducing amino sugars and neutral sugars, but not amino acids. Of the compounds mentioned, only amino sugars would be detected by both reagents, and only those spots which were detected by both were considered to be amino sugars.

The separation of the standard amino sugars and of the polysaccharide hydrolyzates from 7 different isolates (6 types) of Group E *Streptococcus* are shown in Fig. 1 with the ninhydrin reagent and in Fig. 2 with the silver nitrate reagent. Preparation and purification of the Group E streptococcal polysaccharide (type specific) is described elsewhere[5]. D-Glucosamine was found in strains EM-1, 145A, TG-9, and 7585T, and D-mannosamine in strain K131. This is the first indication of the presence of D-mannosamine in a Group E polysaccharide.

EXPERIMENTAL

Separation. — Polysaccharides (1 mg) were hydrolyzed for 4 h at 100° with 1 mL M trifluoroacetic acid. These conditions were established by heating one of the polysaccharides with 1 or 2M trifluoroacetic acid for 2, 4, 6, and 8 h at 100°; qualitative t.l.c. showed the best results with the 4 h, M acid hydrolyzate. The hydrolyzates were taken to dryness in conical vials by placing them in a vacuum desiccator with "Drierite" (W. A. Hammond Drierite Co., Xenia, Ohio) overnight. The residues were dissolved in 50 μL of H_2O and 0.5-μL samples were applied to 5 × 20 cm Whatman K-5 silica gel plates on a line 2 cm from the bottom edge. After the first ascent (70–80 min), the plate was dried in a ventilated hood for 15 min, and then drying was completed by heating for 30 min at 37° in a convection oven. Three solvent ascents and three drying cycles were used. Oven drying was necessary to remove acetic acid. Better separations were obtained if the acetic acid was removed each time. Traces of acetic acid interfered with the reagents used to locate the sugars. Activating the plates before chromatography at 100° resulted in diffuse spots and poor separation.

Detection. — A dipping reagent of ninhydrin in acetone containing 1% pyridine was used to locate the amino sugars[6a]. The spots reached maximal intensity after 3–4 h at room temperature and then began to fade after 8 h. In order to stabilize the spots, the plates were stored under vacuum for 1 h after dipping. The spots were stable when the plates were stored in a vacuum desiccator, no change was observed even after 2 weeks. A dipping procedure with a solution of silver nitrate in acetone followed by a solution of sodium hydroxide in ethanol[6b] was used to locate reducing sugars and related compounds. Best results were obtained with slow, uniform dipping of the plate. It was difficult to avoid all traces of streaking with this reagent, but the sugars were nevertheless readily located.

The behavior of standard D-glucosamine and D-galactosamine dissolved in distilled water was compared with standards dissolved in M HCl. No differences could be seen, and it should be possible to examine hydrolyzates without the prior necessity of neutralization before chromatography.

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