Carbohydrate Research, 43 (1975) 225–231

PAPER-CHROMATOGRAPHIC SEPARATION OF [^{3}H]BOROHYDRIDE-REDUCED HEXOSES FOR QUANTITATIVE DETERMINATION OF POLYSACCHARIDE COMPOSITION

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(Received February 10th, 1975; accepted for publication with revisions, April 1st, 1975)

ABSTRACT

An improved procedure for determination of composition of heteropolysaccharides by radiochromatography of sodium [^{3}H]borohydride-reduced hydrolyzates has been developed. The method is accurate and sensitive, is less cumbersome and more reliable than similar procedures currently in use, and permits, for the first time, compositional analysis of hydrolyzates containing both hexoses and uronic acids in a single radiochromatographic procedure.

INTRODUCTION

Because there is today such widespread interest in the study of heteropolysaccharides of plant, animal, or microbial origin, facile methods for their quantitative compositional analysis are continually being sought. At this Laboratory, extracellular microbial polysaccharides are being studied for their potential industrial usefulness. Most of the products studied thus far contain a uronic acid and two or three hexoses. The ideal analytical method for such substances is one in which a complete carbohydrate analysis could be achieved in a single procedure, and on a microscale.

Radiochromatography, because of its accuracy and sensitivity, is an attractive method for such analysis. Techniques for obtaining radioactive alditols from polysaccharide hydrolyzates by sodium [^{3}H]borohydride reduction have been described[1–3]. However, because paper-chromatographic separation of alditols derived from the common hexoses is poor, use of radiochromatography for analysis of carbohydrate mixtures has been limited. Conrad *et al.*[4] reported chromatographic techniques that utilize ion-exchange chromatography papers with various solvents to separate alditols of hexoses, pentoses, and amino sugars. However, their method for separating hexitols was found to be unsuitable for several reasons. First, the concentration of

*Agricultural Research Service, U. S. Department of Agriculture. The mention of firm names or trade products does not imply that they are endorsed or recommended by the U. S. Department of Agriculture over other firms or similar products not mentioned.

boric acid in the solvent for separation of hexitols needed to be controlled very precisely in order to avoid large variations in migration rates. In addition, the migration rates were found to vary with development time, which caused streaking and poor resolution. Finally, when uronic acid and hexoses were both present, as is the case in most anionic heteropolysaccharides, it was necessary to perform a preliminary separation in one chromatographic solvent and then elute the hexitol portion, and transfer to another chromatogram for resolution of the hexitols.

This paper describes an improved method for paper-chromatographic separation of D-glucitol, D-mannitol, D-galactitol, L-gulonic acid, and reduced derivatives of aldobiouronic acids. The method has been used successfully for radiochromatographic analysis of polysaccharide hydrolyzates and other carbohydrate mixtures. All of the foregoing materials can be separated by two-dimensional development; any two of the hexitols plus the acids can be resolved with single development. Problems encountered with Conrad's method are circumvented: no unusual care in solvent preparation is required and migration rates are constant and reproducible. Because transfer from one chromatogram to another is unnecessary, there is less chance of error through loss of sample. In addition, accurate analysis of polysaccharides containing uronic acids is achieved, without lengthy hydrolysis to break down aldobiouronic acids, by simply correcting uronic acid values for the amount of aldobiouronic acid measured. This procedure decreases the degradative loss of sugars upon extended hydrolysis. The method also has potential value for separation of other alditols that were not tested.

RESULTS AND DISCUSSION

Chromatography conditions and relative migration rates of D-glucitol, D-mannitol, D-galactitol, and L-gulonic acid are summarized in Table I. Separation of two hexitols can be achieved with a single development, either in Solvent I or Solvent II (see Experimental section). Separation of the three hexitols on a single

TABLE I

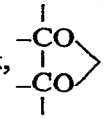
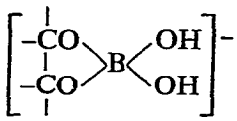
RELATIVE MIGRATION-RATES OF HEXITOLS AND GULONIC ACID

Compound	*Solvent I*[a]*, 20 h (vertical)*		*Solvent II*[a]*, 15 h (horizontal)*	
	Distance (cm)	$R_{D\text{-Glucitol}}$	*Distance (cm)*	$R_{D\text{-Glucitol}}$
D-Glucitol	22.2	1.00	27.4	1.00
D-Mannitol	18.8	0.85	27.9	1.02
D-Galactitol	18.6	0.83	22.0	0.80
L-Gulonic acid	5.1	0.23	2.5	0.09

[a]Solvent systems = I, 9:1:1 butanone–acetic acid–saturated boric acid; II, 3:2:1 ethyl acetate–pyridine–saturated boric acid. Paper was Whatman No. 1, dipped in saturated aqueous H_3BO_3 and dried before application of sample.

chromatogram requires two-dimensional development, with the first development always in Solvent I, in the machine, or vertical, direction of the paper. It should be noted, however, that Solvent II is effective only if the chromatogram has first been exposed to Solvent I. Otherwise, very little migration and no resolution is achieved. Therefore, when this solvent is used for single development, the chromatogram must first be dipped into Solvent I and allowed to dry before placing it in Solvent II. Solvent II gives more diffuse spots than does Solvent I. For this reason, Solvent I is always used for separations involving D-glucitol.

The most critical aspects of the procedure for separation of hexitols are impregnation of the paper with boric acid and use of saturated boric acid in the aqueous portions of both solvents. These steps provide a constant borate concentration, so that the composition of hexitol–borate complexes does not fluctuate. Isbell *et al.*[5] showed that complexing of diols with borate, as well as the type of complex formed, is affected by variation in concentration of hydrogen ion, of borate, and of the diol itself. Because chromatography paper itself is subject to borate-complex formation, excess borate must be available so that there is no competition for it throughout the development. With borate maintained at a high concentration while the diol concentration in the mobile phase is relatively low, the only other variable that can influence borate complexing is hydrogen-ion concentration. Thus, in any solvent system in which hydrogen-ion concentration is constant, migration rates will be constant.

Two types of borate complexes are favored under conditions of high borate and low diol concentrations—the weakly dissociated type A complex,  and the strongly ionized BD^- complex[5]  Because of the equilibrium $A + H_2O \leftrightarrows BD^- + H^+$, the type A complex will preponderate in the more acidic Solvent I, whereas in the more basic Solvent II, the type BD^- preponderates. The slow migration of neutral hexitols in Solvent II becomes rapid when the paper is pretreated with Solvent I and dried. Solvent II, which is basic, would be expected to promote BD^- formation, and this strongly anionic complex would not be expected to migrate far in a solvent of such low polarity. Pretreatment with Solvent I, however, promotes formation of the type A complex, which migrates more rapidly.

A significant modification in the borohydride-reduction procedure has also been made, wherein acetone instead of acid is used to decompose the excess of borohydride. This step prevents formation of the lactone of the reduced uronic acid. This is important for quantitative accuracy, as the lactone migrates similarly to D-glucitol in Solvent I. In addition, resolution is improved because the salt concentration in the sample is lower.

To ascertain the accuracy and reproducibility of the two-dimensional chro-

TABLE II

COMPOSITION OF HEXOSE MIXTURES

Mixture no.	*Glucose*			*Mannose*			*Galactose*		
	Composition (mg/ml)			*Composition (mg/ml)*			*Composition (mg/ml)*		
	Actual	*Calc.*	*% of actual*	*Actual*	*Calc.*	*% of actual*	*Actual*	*Calc.*	*% of actual*
1	6.94	7.35	105.9	7.16	6.92	96.6	6.85	6.72	98.1
2	6.19	6.52	105.3	6.40	6.34	99.0	6.45	6.16	95.5
3	6.88	6.74	98.0	6.95	6.90	99.2	6.58	6.78	103.0
Average percent of actual			103.1			98.3			98.9

matography, three mixtures of hexoses were reduced, chromatographed, and analyzed. The results are shown in Table II. Duplicate analyses were performed for each. Values are expressed for each duplicate in terms of mg/ml and in percent of actual values. Percentages of actual values were then averaged to determine the percent recovery and standard deviation that could be expected for each sugar.

The method was also applied to the compositional analysis of the polysaccharide produced by *Bacillus polymyxa*, NRRL B-1878, and values were compared with data obtained by other methods. Duplicate analyses were performed on 1-, 2-, 4-, 8-, 16-, and 24-h hydrolyzates. Radioactivity counting with these chromatograms revealed a spot near the origin arising from the alditol of the aldobiouronic acid. The amount of this component decreased with hydrolysis time, with proportional and corresponding increase in the amounts of gulonic acid and mannitol. Therefore, for the calculation of polysaccharide composition, the radiochromatographic data for mannitol and gulonic acid were each increased by an amount equal to the value for this aldobiouronic acid at the corresponding time. The full amount of correction was applied to both components because the aldobiouronic acid would be subject to twice as much ^3H labelling if it were completely hydrolyzed to its monomeric components.

Analytical results so calculated are given in Table III. All values are in excellent agreement with those obtained by gas chromatography of alditol acetate derivatives[7] or by carbazole analysis[8]. This result vividly demonstrates the value of the radiochromatographic method for analysis of polysaccharide composition, because a complete analysis can be made with a single procedure and with an accuracy equivalent to that from several existing procedures, none of which alone can provide analysis for all components present.

TABLE III

COMPOSITION OF POLYSACCHARIDE FROM *Bacillus polymyxa* NRRL B-1828

Method	D-*Glucose* (%)	D-*Mannose* (%)	D-*Galactose* (%)	D-*Glucuronic acid* (%)
Radiochromatography	37.3 $\sigma = 1.35$	36.4 $\sigma = 1.86$	13.8 $\sigma = 1.23$	12.5 $\sigma = 1.18$
Other procedures	37.9[a]	34.6[a]	12.3[a]	12.8[b]

[a]By gas chromatography[7]. [b]By carbazole analysis[8].

EXPERIMENTAL

Sample preparation. — Radioactive hexitols and glyconic acids were prepared essentially according to the procedure described by Conrad *et al.*[4] Fifty μl of 1% carbohydrate solution (in M HCl) was neutralized with an equal volume of M Na_2CO_3, and 10 μl of D-[^{14}C]glucose solution (0.1 mCi/ml, 3 mCi/mmole) was added as an internal standard. If the material to be analyzed contained uronic acid, the mixture was then heated for 30 min at 50° to convert any uronic acid lactone, formed during

acid hydrolysis, into the sodium salt. This mixture (5 μl) was then treated with 5 μl of 0.125M NaB^3H_4 (in 0.1M NaOH) (200 mCi/mmole) in a microreduction vial for 40 min at 50°. The excess of borohydride was decomposed with 5 μl of acetone. The entire sample, having ^{14}C activity of 30,000 c.p.m. and ^3H activity of 600,000 c.p.m., was then spotted directly on chromatograms. Samples tested were D-glucose, D-mannose, D-galactose, D-glucuronic acid, and the acid hydrolyzate (M HCl, 1–24 h, 100°) of the extracellular polysaccharide produced by *Bacillus polymyxa* NRRL B-1828.

Paper chromatography. — All chromatograms were made on Whatman No. 1 chromatography grade paper that had been dipped in saturated, aqueous boric acid solution and air-dried before application of the sample. Single-dimension chromatography was done on strips (57 × 2 cm) and two-dimensional separation on 46-cm squares, to which the sample had been applied at a point 35 cm from each leading edge. Solvents were 9:1:1 butanone–acetic acid–saturated aqueous boric acid (Solvent I)[6], and a modification of Conrad's solvent[4] (Solvent II), consisting of 3:2:1 ethyl acetate–pyridine–saturated aqueous boric acid. All development was at 20°. The time of development for Solvent I ranged from 20 to 30 h and for Solvent II from 14 to 16 h. When two-dimensional analysis was used, the first development was always made with Solvent I in the vertical direction. Descending development was used throughout.

Liquid-scintillation counting. — Quantitative measurements were made by direct liquid-scintillation counting on the paper, cut into segments and placed in 10 ml of liquid scintillation mixture (0.4% 2,5-diphenyloxazole in toluene). Samples were counted for 5 min or to a 2σ accuracy of 1% in a Beckman LS-250 liquid-scintillation counter.

For single-dimension work, the entire strip was cut into 1-cm segments and counted. The composition of mixtures and hydrolyzates was determined as described by Conrad *et al.*[4]. Total c.p.m. per peak was calculated, background was subtracted, and proper corrections were applied for the ^3H c.p.m. due to the presence of [^{14}C]-glucose as the internal standard. Comparison of ^{14}C c.p.m. to a known amount of [^{14}C]glucose gave percent recovery, and total μmoles of each sugar was calculated by comparison of total c.p.m. to that of a standard D-glucose sample in which specific activity in terms of c.p.m./μmole was known.

Two-dimensional chromatograms were cut into 5.1 × 35-cm strips so that the radioactive areas could be located with a Packard Model 7201 radiochromatogram scanner. These strips were then cut into three 1.7 × 35-cm strips and the radioactive areas cut into segments of 0.5 cm. The procedures used for calculation were the same as for single-dimension chromatograms, but the counting technique was modified because the hexitol spots were somewhat diffused. This diffusion caused detectable amounts of radioactivity to be deposited over a rather large area, thereby increasing the number of segments to be counted and introducing the possibility of error because of spots overlapping in the peripheral areas. However, most of the radioactivity was found in a comparatively small area. This fact, plus the fact that the size and shape of

each spot was consistent and characteristic, made it possible to determine total radioactivity from the proportion found in a given area of the spot.

Repetitive trials with each hexitol established the pattern of segments that best represented its migration behavior. To avoid errors caused by slight variations in migration distances, patterns were measured outward from the segment containing the greatest radioactivity. The best pattern was arbitrarily defined as the smallest number of segments containing at least 75% of the total radioactivity, reproducible within a standard deviation of less than 5%. The patterns established were: D-glucitol, +4 to −4 vertical, +1 to −2 horizontal (36 segments), standard deviation 4.0%; D-mannitol, +4 to −4 vertical, +1 to −1 horizontal (27 segments), standard deviation 1.3%; and D-galactitol +3 to −3 vertical, +1 to −2 horizontal (28 segments), standard deviation 2.9%. These patterns contained 83.3, 86.2, and 77.4% of total hexitol radioactivity, respectively. These factors were used in all subsequent experiments to determine total hexitol radioactivity. This method afforded considerable reduction in handling and in counting time, as the number of segments to be counted was at least halved in all cases, without loss of accuracy. No study of migration behavior was done for aldonic acids, which diffused little and their total areas could be measured conveniently.

ACKNOWLEDGMENTS

I thank Dr. H. E. Conrad of the Biochemistry Department, University of Illinois, for making some of his results available before publication; and my colleagues at this Laboratory, Drs. Allene Jeanes and P. A. Sandford, for encouragement and advice, and Mr. P. R. Watson for gas-chromatographic data.

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