Reversed-phase paper chromatography of some substituted hydrazine derivatives of monosaccharides and related compounds

During the synthesis of some substituted hydrazine derivatives of monosaccharides and related compounds, it was desirable to chromatograph the isolated products and reaction mixtures. The compounds involved migrate in *n*-butanol–ethanol–water (10:1:2) (BEW) and in ethyl acetate–pyridine-water (8:2:1) (EPW), solvent mixtures used in this laboratory for chromatographing sugars and lactones, but either the resolution or the time required is not completely satisfactory. Hydrazones and osazones of monosaccharides are almost immobile in the *n*-heptane–phenoxyethanol reversed-phase paper chromatographic system used for the separation of 2,4-dinitrophenylhydrazones of aldehydes and ketones[1]. In the present study, a reversed-phase Zaffaroni-type solvent system used for the paper chromatography of steroids[2] gave rapid and satisfactory resolution of substituted hydrazine derivatives of monosaccharides and some organic acids and lactones. A number of systems of this general type were screened with typical sugar hydrazones and osazones. This paper reports the separations obtained with the best of these solvent systems—formamide–ethyl acetate–water (1:20:1) and formamide-impregnated paper (FEW).

Experimental

*Compounds and reagents**. Pure D-mannoheptulose was a gift from Dr. NELSON K. RICHTMYER, glucose was National Bureau of Standards sample No. 41, and the remaining commercially available sugars, organic acids, and lactones were used without further purification.

Eastman No. 1866 2,4-dinitrophenylhydrazine, No. 330 phenylhydrazine hydrochloride, and No. 1666 1-benzyl-1-phenylhydrazine hydrochloride were recrystallized before use.

The hydrazide, hydrazone, and osazone derivatives were prepared by the general methods referred to in Table I, column 2. Temperature of reaction, composition of solvent, and molecular ratio of reactants were modified as necessary.

Eastman No. 565 formamide and reagent grade acetone, ethyl acetate, and pyridine were used without further purification. Ethanol, 95 %, was distilled from magnesium turnings.

Preparation of chromatogram. Whatman No. 1 paper "for chromatography", cut 17 × 43 cm across the machine direction, was dipped twice into a mixture of 10 vol. formamide in 90 vol. acetone, drained momentarily, placed between four dry sheets of Whatman No. 1 paper on a smooth, flat surface, and slightly pressed to remove excess solvent. The impregnated paper was hung immediately in still air for 10 to 15 min. Not more than 2 μl of a freshly prepared solution of each compound was applied to the paper with a micropipet in spots 2 cm apart. The paper was suspended in a glass trough in the chromatographic jar, usually within one hour after impregnation, and migration was started immediately by the descending technique.

Benzylphenylhydrazine hydrochloride and phenylhydrazine hydrochloride were

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

TABLE I

R_G[a] VALUES OBTAINED BY REVERSED-PHASE CHROMATOGRAPHY

Solvent: Formamide–ethyl acetate–water (1:20:1, upper phase).
Paper: Whatman No. 1 "for chromatography" (descending).
Impregnation: Formamide–acetone (10% v/v).
Time of run: 2 h.
Temperature of run: 26°.
Detection: Acetone–silver nitrate — alcoholic potassium hydroxide.

Compound	*Method of preparation Ref. No.*	R_G
2,4-Dinitrophenylhydrazine		1.19
2,4-Dinitrophenylhydrazide of:		
Galactonic acid[b]	3	0.06
Gluconic acid[b]	4	0.09
Saccharic acid[b], bis,	4	0.52
Formic acid	5	1.04
Acetic acid	5	1.05
2,4-Dinitrophenylhydrazone of:		
Galacturonic acid	3	0.03[c]
Glucuronic acid	6	0.06[c]
Glucuronolactone	6	0.69
Mannoheptulose[b]	3	0.22
Galactose	3	0.27
Glucose	5	0.25
Mannose	3	0.28
Fructose	3	0.44
Arabinose	7	0.55
Lyxose	7	0.57
Ribose	7	0.63
Fucose	3	0.71
Rhamnose	3	0.85
2,4-Dinitrophenylosazone of:		
Glucose	5	1.02
Sorbose	3	1.05
Arabinose	5	1.16
Xylose	5	1.15
Fucose[b]	5	1.20
Rhamnose[b]	5	1.19
Phenylhydrazine[d]		(0.00, 0.96, 1.09)[e]
Phenylhydrazone of:		
Mannose	8a	0.21
Arabinose	8b	0.46
Phenylosazone of:		
Galactose	9	1.00
Glucose	8c	1.00
Sorbose	9	1.01
Arabinose	10	1.16
Lyxose	10	1.14
Rhamnose	9	1.19

(continued on p. 113)

TABLE I (*continued*)

Compound	*Method of preparation Ref. No.*	R_G
1-Benzyl-1-phenylhydrazine[d]		1.25[e]
1-Benzyl-1-phenylhydrazone of:		
Mannose	8d	0.76
Arabinose	8e	0.96
1-Benzyl-1-phenyl-phenylosazone of:		
Mannoheptulose	11	1.08
Glucose	11	1.16
Fructose	11	1.16
Sorbose	11	1.17
Arabinose	11	1.23
Rhamnose	11	1.23
Free sugar, acid or lactone:		
Uronic acids		0.00
Sugar acids (saccharic acid, and galactonic acid)		0.00–0.05
Lactones (galactonolactone, gluconolactone, glucuronolactone)		0.00–0.05
Heptulose		0.05–0.09
Hexoses		0.00
Pentoses		0.00–0.01
		0.01–0.05

[a] R_G = distance migrated by compound/distance migrated by glucose phenylosazone on same chromatogram.
[b] Derivative not reported previously.
[c] Spot not discrete, streaking or bearding.
[d] Prepared from recrystallized hydrochloride derivative, see text.
[e] Principal spots.

dissolved separately in 95 % ethanol containing an equivalent amount of potassium hydroxide and 50 to 100 μg of each free base was spotted on the paper. Twenty-five μg of 2,4-dinitrophenylhydrazine was spotted from pyridine.

The free sugars, acids, and lactones were dissolved in water, and their derivatives in pyridine. One to 5 μg (0.6 μl) of the sugar 2,4-dinitrophenylosazones, about 12.5 μg (2 μl) of the uronic acid 2,4-dinitrophenylhydrazones, and 25 μg (2 μl) of the other derivatives were spotted. Arabinose and mannose phenylhydrazones are somewhat labile in pyridine and were spotted as soon as they dissolved.

Chromatography. The solvent was a mixture of formamide–ethyl acetate–water (1:20:1 v/v/v), shaken and allowed to separate into two phases. Separation is rapid. The upper phase was used as the mobile phase, the lower phase was discarded.

The chromatographic chamber was a cylindrical glass jar 30.3 cm in diameter and 60.5 cm high, sealed with a weighted glass lid. The wall of the jar was lined with Whatman No. 1 paper thoroughly wet with mobile phase, and the bottom of the jar was covered with mobile phase. The chamber was equilibrated at least 16 h before use.

As soon as the papers were spotted, two papers were suspended from each of two glass troughs in the jar and the troughs were filled with solvent. To assure a well-equilibrated atmosphere, the paper liner was rewetted at the beginning of each

chromatographic run. The chromatograms were developed by the descending technique at room temperature (26°) until the solvent front was about 3 cm from the bottom of the paper, usually not more than two hours.

The presence of 2,4-dinitrophenylosazones on the chromatogram was established with certainty by their change from yellow to violet when the still damp chromatogram was exposed to ammonia vapor. After the chromatograms hung overnight in a well ventilated hood, visible spots were outlined, and colorless reducing spots were detected by successive dips in acetone–silver nitrate and alcoholic potassium hydroxide solutions.

Discussion and results

When synthesizing hydrazone and osazone derivatives of sugars and related compounds, the formamide–ethyl acetate–water system is helpful to establish quickly the extent of the reaction, the nature of the derivative formed, and the purity of the isolated compound. It is particularly helpful when preparing hydrazones of sugars because excess reagent and any osazone formed move well ahead of the corresponding hydrazone while unreacted sugar remains near the origin. Microgram quantities of hydrazones and osazones formed by the solvent diffusion technique for microscopic identification of sugars[6,7,10,12,13] may be transferred from the diffusion cell and chromatographed with this system to further confirm the identity of the sugar.

The R_G values of the substituted hydrazine derivatives studied by this reversed-phase system are given in Table I, column 3. The mobilities of the sugar hydrazones or osazones in the FEW system are in about the same order as those of the unreacted sugars obtained by descending chromatography in BEW, that is, in order of decreasing mobility: methyl pentose and glucuronolactone, aldopentose, ketohexose, aldohexose, heptulose, and uronic acid. The data in Table I show that the R_G values are similar for corresponding hydrazine derivatives of stereo-isomers. STOLL AND RÜEGGER[14] also observed this when chromatographing the *p*-nitrophenylhydrazones of sugars in water-saturated solvents.

Hydrazones resolve better than do osazones. Even so, when preparing the 1-benzyl-1-phenyl-phenylosazone derivatives listed in Table I, resolution was adequate to show if the corresponding phenylosazone derivative was also present in the reaction mixture. The 2,4-dinitrophenylhydrazones of mannose, fructose and arabinose resolved better in the FEW system than did the corresponding free sugars in BEW or in EPW; whereas the reverse was true for the 2,4-dinitrophenylhydrazones of mannose, glucose, and galactose.

In the FEW system spots are more discrete if the area of the spot is small, the paper is chromatographed as soon as possible after impregnation and the solvent flow is across the machine direction of the paper. Loading is important—25 μg (2 μl) gave a discrete spot for most of the derivatives but more than 5 μg (0.6 μl) of a 2,4-dinitrophenylosazone may streak excessively.

The R_F values from this system should not be considered absolute because it is difficult to impregnate the paper uniformly. For purposes of comparison or identification, an authentic derivative should be chromatographed adjacent to an unknown.

Acknowledgement

We thank Mr. GLEN F. BAILEY for confirming the identity of some of the derivatives by ultraviolet spectrophotometric analysis.

Western Regional Research Laboratory,
Western Utilization Research and Development Division,
Agricultural Research Service, U.S. Department of Agriculture,
Albany, Calif. (U.S.A.)

GERALDINE E. SECOR
LAWRENCE M. WHITE

1 W. S. LYNN, JR., L. A. STEELE AND E. STAPLE, *Anal. Chem.*, 28 (1956) 132.
2 V. R. MATTOX AND M. L. LEWBART, *Arch. Biochem. Biophys.*, 76 (1958) 362.
3 L. M. WHITE AND G. E. SECOR, *J. Am. Chem. Soc.*, 75 (1953) 6343.
4 R. T. WILLIAMS, *Biochem. J.*, 34 (1940) 272.
5 E. GLASER AND N. ZUCKERMANN, *Z. Physiol. Chem.*, 167 (1927) 37.
6 L. M. WHITE AND G. E. SECOR, *Anal. Chem.*, 31 (1959) 1273.
7 G. E. SECOR AND L. M. WHITE, *Anal. Chem.*, 27 (1955) 1998.
8 A. W. VAN DER HARR, *Anleitung zum Nachweis, zur Trennung und Bestimmung der Monosaccharide und Aldehydesäuren*, Gebrüder Borntraeger, Berlin, 1920; (a) pp. 149–150; (b) pp. 145–146; (c) p. 213; (d) p. 170; (e) pp. 167–168.
9 W. Z. HASSID AND R. M. MCCREADY, *Ind. Eng. Chem., Anal. Ed.*, 14 (1942) 683.
10 L. M. WHITE AND G. E. SECOR, *Anal. Chem.*, 28 (1956) 1052.
11 L. M. WHITE AND G. E. SECOR, *J. Org. Chem.*, 27 (1962) 2240.
12 L. M. WHITE AND G. E. SECOR, *Anal. Chem.*, 27 (1955) 1016.
13 L. M. WHITE AND G. E. SECOR, *Anal. Chem.*, 33 (1961) 1287.
14 A. STOLL AND A. RÜEGGER, *Helv. Physiol. Pharmacol. Acta*, 10 (1952) 385.

Received January 17th, 1964

J. Chromatog., 15 (1964) 111–115

Notes on semimicro preparative thin-layer chromatography

Details of preparative thin-layer chromatographic techniques used in this laboratory and mentioned in two recent papers[1,2] are described here since they are of general application. They have been used in the isolation of 2- to 5-mg quantities of material for subsequent identification by ultraviolet and infrared spectral studies and by chromatographic comparison.

Overdeveloping thin-layer plates

For the separation of contiguous spots and bands on thin-layer plates, a continuous, descending method was developed. It gave much better separation at higher loading than BRENNER AND NIEDERWIESER's method[3] and was easier in operation and gave better separation than repeated, ascending development. It resembles the continuous, descending methods by which STANLEY *et al.*[4] washed plates and which MISTRYUKOV recently described[5], as well as the descending method mentioned by BIRKOFER *et al.*[6]. It requires less special equipment than these published methods and makes use of the weight of the plate itself for providing contact with a soft, cloth wick. Although the apparatus is not as simple as that recently reported by BENNETT AND HEFTMANN[7], the method requires less handling of the delicate plates and is easier for routine use.