THE SEPARATION OF HEXOSEPHOSPHATES AND TRIOSEPHOSPHATES BY THIN-LAYER CHROMATOGRAPHY

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INTRODUCTION

Thin-layer chromatography has been used for the separation of a wide variety of substances[1–8]. Ease and simplicity of operation, rapidity and versatility are its outstanding characteristics. In the separation of sugars and sugar derivatives, as STAHL AND KALTENBACH[6] have noted, conversion from paper or column chromatography to thin-layer chromatography (TLC) cannot be accomplished without some modification of solvent systems, adsorbents and techniques. STAHL AND KALTENBACH[6] separated free sugars on Kieselgur G. DEFERRARI *et al.*[7] and GEE[8] used silica gel with a starch binder. After a preliminary investigation of silica gel in our laboratory, it was determined that cellulose powder without a binder was a suitable adsorbent for the TLC of sugar phosphates. A two-dimensional development, utilizing an acid with a water-immiscible solvent followed by a water-miscible system at right angles, gave small, concentrated spots with adequate resolution.

MATERIALS

Adsorbent

Cellulose powder MN 300 (particle size $< 10\,\mu$) – Machery, Nagel and Co., Düren, Germany. Obtained from Brinkmann Instruments, Inc., N.Y.

Solvents

Phase I[9]. Water-poor phase from mixture of: 60 ml *tert.*-amyl alcohol, redistilled 101.8°, 30 ml water + 2 g *p*-toluenesulfonic acid. In all solvent systems, the water employed was distilled-demineralized.

Phase II[10]. 66 ml isobutyric acid, redistilled 154.4°, 1 ml concentrated ammonium hydroxide, 33 ml water.

Solvents were obtained from Distillation Products Industries, Rochester, New York. Chemicals were reagent grade, used as received.

Apparatus

Rectangular chromatojars, 20 × 20 cm glass plates, 250 μ fixed-thickness spreader and aligning tray were purchased from Research Specialties Company, Richmond, Calif. Model No. V41 Mineralight (2537 Å) was obtained from Ultra Violet Products, Inc., South Pasadena, Calif.

Standards

Hexosephosphates and triosephosphates, Grade A purity, were purchased from Sigma Chemical Company, St. Louis, Mo., and decationized with the use of Dowex 50-X1, 50–100 mesh, hydrogen form. Standards were freshly prepared in concentrations from 20–300 mg/ml in aqueous solution for application.

Spray reagents

A. 0.2 *M* *m*-phenylenediamine dihydrochloride in 76 % ethanol[11].

B. 5 ml 60 % perchloric acid, 10 ml 1 *N* HCl, 25 ml 4 % ammonium molybdate, 60 ml H_2O[9].

C. 10 % $SnCl_2 \cdot H_2O$ in concentrated HCl freshly diluted 200 fold with 0.5 *M* H_2SO_4[12].

Other spray reagents were used[13–16] in the identification of individual phosphates, but were not incorporated into the routine detection procedure. Sprays were applied with the Universal Aerosol Spray Kit obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

METHODS

Preparation of plates

The plates were coated with a slurry prepared by mixing 15 g of cellulose powder and 90 ml of water with a mortar and pestle. After allowing 10 min at room temperature for the cellulose to set, the plates were activated by heating at 105° for 2 h. Until used, the prepared plates were stored over a desiccant in a closed chamber.

A suitable aliquot (1–10 λ) of each standard or mixture was applied to the lower left corner of a plate, approximately 2 cm from the cellulose edges, with a glass capillary. The spots were permitted to dry between applications to minimize origin size.

Development

Phase I solvent system was allowed a 30 min period to saturate the atmosphere in a chromatojar before chromatography of the plate was initiated. The plates were positioned in the jar for ascending development with the solvent flowing in the direction of slurry application. Six to 8 h were required for the solvent front to move 16–18 cm. The plates were then air dried at room temperature, usually overnight.

Phase II solvent system was given a 15 min saturation period in the chromatojar. The plates were placed at right angles to the first run for ascending development. Two to 4 h were required for the solvent front to move 14–18 cm. After air-drying with a hair drier, the plates were ready for the detection procedure.

Detection and identification

Detection of the hexosephosphates and triosephosphates was accomplished with a series of spray reagents which gave characteristic color responses (see Table I). R_F values and the position of individual standards relative to that of inorganic phosphate further aided in the identification of spots. The sprays were successively applied to each plate according to the following procedure and spots were marked as visualized.

Spray reagent A. After spraying, the plates were heated for 5 min at 110°. They were then viewed under ultraviolet light.

Spray reagent B. After spraying, the plates were dried with the hair drier before

heating for 5–8 min. at 110–120°. Sufficient heating was noted when the edges of the cellulose began to char.

Spray reagent C. This reagent was applied while the plates were still warm from the previous heating. While wet, the spots and colors were readily distinguished. As the plates dried, the background became pale violet. A subsequent spray with concentrated ammonium hydroxide restored the light background, but caused all spots to become dark blue.

RESULTS

The results are presented in Tables I and II and Fig. 1.

TABLE I

COLOR RESPONSES OF HEXOSEPHOSPHATES AND TRIOSEPHOSPHATES FOLLOWING APPLICATION OF INDICATED SPRAY REAGENTS

*Compound**	*Spray A** (under ultraviolet)*	*Spray B** (daylight)*	*Spray C** (daylight)*
PEP	U.V. absorbing spot	Blue	Blue
P_i	Not visible	Yellow-green	Yellow-green
2-PG; 3-PG	Not visible	Blue	Pink
F-6-P	Blue fluorescent	Blue-green	Blue-green
FDP	Not visible	Blue	Grey-green
G-1-P	Not visible	Yellow-green	Yellow-green
G-6-P	Blue fluorescent	Green	Green
Background	Violet	White	Light-blue, violet when dry***

* Abbreviations: PEP = phosphoenolpyruvic acid; P_i = inorganic phosphate; 2-PG = 2-phosphoglyceric acid; 3-PG = 3-phosphoglyceric acid; F-6-P = fructose-6-phosphate; FDP = fructose-1,6-diphosphate; G-1-P = glucose-1-phosphate; G-6-P = glucose-6-phosphate.

** See text for details.

*** After plate was completely dried, background was violet and spots were obscured to some extent. A light spray with concentrated NH_4OH restored the pale background and all phosphates were blue.

TABLE II

R_F AND R_{PO_4} VALUES IN PHASE I AND PHASE II SOLVENT SYSTEMS

*Compound**	*Phase I solvent system***		*Phase II solvent system****	
	R_F	R_{PO_4}	R_F	R_{PO_4}
PEP	0.87	1.18	0.23	0.90
P_i	0.74	1.00	0.27	1.00
2-PG; 3-PG	0.68	0.92	0.24	0.94
F-6-P	0.41	0.56	0.20	0.81
FDP	0.34	0.46	0.13	0.52
G-1-P	0.32	0.43	0.27	1.06
G-6-P	0.29	0.39	0.17	0.68

* See Table I for explanation of abbreviations.

** *tert.*-Amyl alcohol–water–*p*-toluenesulfonic acid (see text).

*** Isobutyric acid–ammonium hydroxide–water (see text).

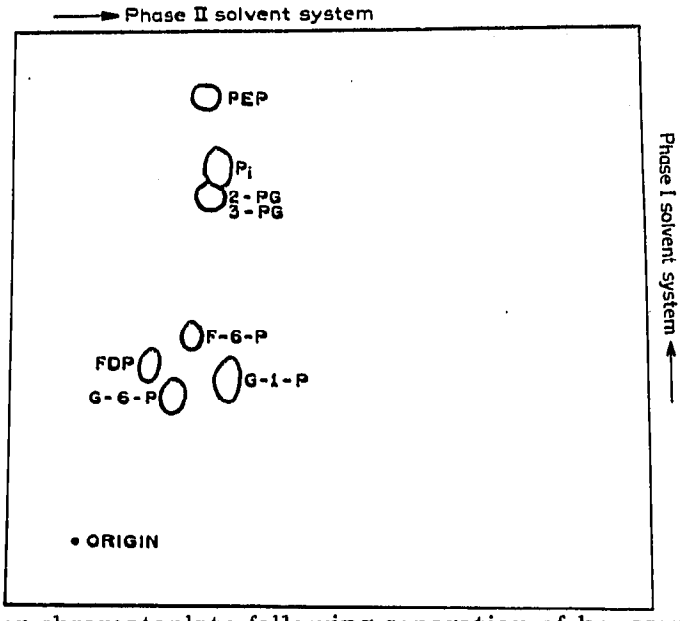

Fig. 1. Tracing of thin-layer chromatoplate following separation of hexosephosphates and triosephosphates. PEP = phosphoenolpyruvic acid; P_i = inorganic phosphate; 2-PG = 2-phosphoglyceric acid; 3-PG = 3-phosphoglyceric acid; F-6-P = fructose-6-phosphate; FDP = fructose-1, 6-diphosphate; G-1-P = glucose-1-phosphate; G-6-P = glucose-6-phosphate.

DISCUSSION

Experience in the laboratory has shown that methods for the separation of hexosephosphates and triosephosphates, which may be successfully applied in paper chromatography, may not be carried over to TLC with the same degree of success. The work reported is the culmination of many trials with different adsorbents, solvent systems and detection methods. It is pertinent to report these failures as well, since others may be spared the time spent in testing them.

It was found in these studies that the MN 300 cellulose powder gave the best resolution of the adsorbents tested, but was not free of impurities. Thus, in the development of each solvent system a yellow substance was observed which advanced with the front, but appeared not to interfere with the separation of the sugar phosphates. Attempts to remove the impurity by shaking or extracting the cellulose with diethyl ether were not successful. While cellulose for TLC obtained from Research Specialties Corporation, Richmond, Calif., was found to have fewer impurities, the resolutions were not as good as with the MN 300. Similarly, when Avicel (a microcrystalline cellulose sold by American Viscose Company) was tried as an adsorbent, the plates appeared thin, there were no impurities, and the resolution was not as good as was obtained with MN 300.

The solvent systems BANDURSKI AND AXELROD[17] and HANES AND ISHERWOOD[9] used in their paper chromatography methods failed to provide adequate separations on TLC, except for the *tert.*-amyl alcohol–*p*-toluenesulfonic acid–water system suggested by the latter authors. Furthermore, varying the proportions of *tert.*-amyl alcohol, water and *p*-toluenesulfonic acid in the Phase I system as well as the isobutyric acid, ammonia and water in the Phase II system did not improve the results. If anything, the results were not as good as those obtained with the solvent systems described as Phase I and Phase II.

Attempts were made to separate 3-phosphoglyceric and 2-phosphoglyceric acids, but these compounds migrated together while separating from the other sugar phosphates. Glyceraldehyde-3-phosphate appeared to separate, but four spots were obtained with the preparation used in these studies. Further attempts will be made to purify the preparation so that positive identification may be made. However, despite these difficulties, the good resolution of hexosephosphates and some triosephosphates offers a useful tool in the study of these compounds.

SUMMARY

The separation of hexosephosphates and triosephosphates may be obtained by use of TLC, using a cellulose adsorbent without binder. While the hexosephosphates resolved into well-defined spots, 3-phosphoglyceric acid and 2-phosphoglyceric acid did not separate.

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