Separation of phosphate esters by paper chromatography

Paper-chromatographic methods have been used extensively for the separation and identification of phosphate esters formed during photosynthesis[1] or from glycolysis of sugars and sugar derivatives by a variety of tissues[2,3]. HANES AND ISHERWOOD[4] made a thorough study of a variety of solvents, papers and conditions for the separation of phosphate esters. Their findings encouraged further studies and this resulted in reports of procedures by BANDURSKY AND AXELROD[5], BENSON *et al.*[6], and MORTIMER[7] which have found wide application.

Some of these procedures require extensive time intervals for resolution of esters, thus increasing diffusion of the spots on paper[1], while others require low temperatures[5]. This report presents a procedure for the separation of phosphate esters at room temperature using ethyl acetate–acetic acid–water[7] in one direction and methyl ethyl ketone–methyl cellosolve–ammonia[7] in the second direction.

Whatman 41 H or Schleicher and Schuell 589 blue ribbon paper was used without pretreatment throughout these studies. Standards were prepared from barium salts of phosphate esters purchased from Schwartz Laboratories, Inc. Barium was removed by treating a 2% solution of each ester salt with Dowex 50-H^+ (8% cross-linked 80–100 mesh). When phosphate esters were chromatographed from tissue incubations, it was first necessary to remove the cations in the deproteinized solution (containing trichloroacetic acid or $HClO_4$) by treatment with Dowex 50-H^+. Following this treatment, the centrifuged supernatant was used for paper chromatography. The methyl ethyl ketone solvent mixture, prepared from commercially available solvents, required pretreatment before use in order to remove unidentified impurities which caused rapid deterioration of the solvent mixture during chromatography. 7 Parts (by vol.) of methyl ethyl ketone were mixed with 2 parts of methyl cellosolve and this mixture was filtered through a bed of Dowex 50-H^+ previously drained free of excessive moisture by suction. Subsequently, 0.7 part of concentrated ammonia and 2.3 parts of water were added to 9 parts of the treated solvents, and this preparation was ready for chromatography.

Samples were applied to the paper in three or four 5-μl aliquots, the spots being dried between aliquots with a cold air blower. The chromatograms were first developed in an ascending direction with ethyl acetate–acetic acid–water (3:3:1, v/v/v) for 15 h, then dried, and developed in a descending direction at right angle with methyl ethyl ketone–methyl cellosolve–concentrated ammonia–water (7:2:0.7:2.3, v/v/v/v) for 22 h during which the solvent was permitted to drip from a serrated edge. The papers were dried and the phosphate esters located by one or a combination of the following spray reagents:

1. Acid molybdate spray was used to locate phosphate ions according to the procedure described by HANES AND ISHERWOOD[4]. Before spraying with this reagent it was necessary to spray the developed chromatogram with 10% trichloroacetic acid and allow the paper to hang for 1 or 2 h to facilitate hydrolysis, particularly of glycerol phosphate and diphosphoglyceric acid.

Abbreviations: FDP, fructose diphosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; DPG, 2,3-diphosphoglyceric acid; 2PG,2-phosphoglyceric acid; 3PG, 3-phosphoglyceric acid; GP, glycerol phosphate; R5P, ribose-5-phosphate; HP, hexose phosphates; P–P, polyphosphate esters; iP, inorganic orthophosphate.

2. For detection of carbohydrates, a 0.6 % ethanol solution of *p*-aminohippuric acid was used containing 0.3 % trichloroacetic acid[8]. The sprayed papers were then heated at 150° for 2–3 min, and the carbohydrates were located as fluorescent spots under an u.v. light. The sensitivity of this method is approximately 0.5 μg.

When this procedure was used for the separation of [^{32}P]phosphate esters from tissue incubations, autoradiograms were made by direct contact of dry paper chromatograms (before spraying with any reagents) with no-screen X-ray film for 48 h. The esters were then located by means of the developed X-ray, cut from the paper and pasted on metal or cardboard discs for radioactivity determinations with an end-window Geiger Müller tube.

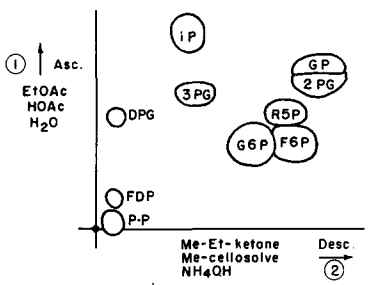

Fig. 1. Diagramatic representation of a two-dimensional separation of phosphate esters by paper chromatography.

Fig. 1 is a diagrammatic drawing of a two-dimensional chromatogram illustrating the location and separation of phosphate esters tested. This method of two-dimensional chromatography permits separation of FDP, DPG, 3PG, and polyphosphates (mainly coenzymes). Some esters, G6P and F6P, GP and 2PG, show some overlapping. This procedure gives good reproducible chromatograms under the stated conditions, and with ^{32}P-labeled esters allows quantitative evaluation of isotope distribution among the separated esters.

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