

THE CHROMATOGRAPHIC IDENTIFICATION OF SOME BIOLOGICALLY IMPORTANT PHOSPHATE ESTERS*

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The objective of the present work was to provide a means for separating and identifying phosphate esters involved in glycolysis in higher plants. Paper chromatography of phosphate esters has been employed by several workers, most notably Benson *et al.* (1) and Hanes and Isherwood (2). Benson's procedures were not primarily designed for the identification of phosphate esters and gave low R_F values for the phosphate compounds of particular interest to us. The unidimensional methods of Hanes and Isherwood do not result in adequate resolution of the complex mixtures such as are obtained from our plant materials.

The present procedure is based on two-dimensional chromatography with successive development in an acid and in a basic solvent. The solvents finally selected gave the best over-all resolution of the intermediates involved in plant glycolysis. Undoubtedly the resolution of certain pairs of compounds may be improved by suitable modifications. We have in addition made certain improvements in the procedure for locating the chromatographed materials.

EXPERIMENTAL

Reagents and Materials—

Acid solvent. Methanol 80 volumes, formic acid 15 volumes (analysis 88 per cent by weight), and H_2O , 5 volumes.

Basic solvent. Methanol 60 volumes, ammonium hydroxide 10 volumes (sp. gr. 0.9015), and H_2O 30 volumes.

Spray reagent. Hanes-Isherwood reagent (2). 5 ml. of 60 per cent

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(weight by weight) perchloric acid, 25 ml. of 4 per cent (weight by volume) ammonium molybdate, 10 ml. of *N* HCl, and 60 ml. of H₂O.

Paper. Schleicher and Schuell, No. 589, blue ribbon filter paper 28 cm. square was found satisfactory for our purpose.¹ Even analytical filter papers contain sufficient inorganic phosphate to give a visible band on development and spraying. The color is very light but there may be sufficient phosphate accumulated at the intersection of the bands produced in the acid and basic solvents to produce a spurious spot. If desired, this difficulty may be eliminated by washing the paper with the acid solvent before use.

Development of Chromatogram

Chromatography of phosphate esters is best accomplished with the free acids. Salts of the esters are readily converted to the corresponding acid and the metallic ions removed by shaking solutions or suspensions of the salts with a cation exchanger such as Dowex-50. A suitable quantity of solution containing the decationized compounds to be chromatographed is applied to one corner of the filter paper about 5 cm. from the bottom and side edge. It has been found desirable to limit the size of the spot to approximately 0.5 cm. in diameter. In practice this amounts to applications of 2 μ l. Since a minimum of 0.1 γ of P is generally needed for detection, it may be necessary to make multiple applications with dilute solutions, in which case the spot is air-dried each time. The paper is then formed into a cylinder and stapled together, avoiding contact of the adjacent edges. A cylindrical jar 15 cm. in diameter and 30 cm. tall, covered by a glass square and containing 100 ml. of the solvent, serves as a chromatographic chamber. A soft iron wire is inserted diametrically through the upper end of the paper cylinder, which is then suspended over the solvent by means of a magnet placed on top of the glass cover. We have found it advisable to use the acid solvent first and to conduct equilibration and development at 2°. After a 2 hour equilibration, the paper cylinder is dropped into the solvent by removing the magnet (3). The chromatogram is developed until the solvent front reaches the upper edge of the paper. *Usually 6 to 6.5 hours are required. The chromatogram is then thoroughly dried in front of a fan at room temperature. The paper cylinder is next reformed so that the second development will be at right angles to the first. The equilibration procedure is repeated with the alkaline solvent. Development in the alkaline solvent requires 12 to 15 hours.*

¹ The mention of special instruments or materials throughout this paper does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

Detection of Compounds

The paper is air-dried and then examined by appropriate methods. Carter (4) has shown that certain nucleotides may be detected on paper when viewed with short ultraviolet radiation (2536 Å). This suffices to locate adenosinetriphosphate (ATP), adenosinediphosphate (ADP), and adenosinemonophosphate (AMP).

The spray reagent used for the visualization of phosphorylated compounds is that of Hanes and Isherwood (2). The procedure for developing the color and hydrolyzing the esters has been modified, since, in our hands, their procedure does not serve to locate such difficultly hydrolyzable esters as phosphoglyceric acid. After the paper is sprayed at the rate of 1 to 2 ml. per 100 sq. cm., inorganic phosphate appears as a yellow spot. The paper is dried at 85° for 1 minute, at which time glucose-1-phosphate is revealed by a yellow to blue color. The paper is then exposed to ultraviolet radiation at a distance of about 10 cm. from a General Electric germicidal lamp (rated at 25 microwatts of 2537 Å radiation per sq. cm. at 1 meter) for 10 minutes. All of the organic phosphate compounds will now appear as blue spots, whereas inorganic phosphate produces a yellow-green color. An alternate method for developing the color consists of heating the paper for 5 minutes at 85° and autoclaving for 2 minutes at 8 to 10 pounds. The paper is now uniformly blue in color. When the chromatogram is exposed to ammonia vapors, the blue color of the background disappears, while the color due to phosphomolybdate blue remains.

Results

Table I gives the R_f values of some of the phosphate esters of glycolysis as well as ortho-, pyro-, and triphosphates in the acid and alkaline solvent. Fig. 1 illustrates the two-dimensional chromatographic separation of a mixture of inorganic phosphate, ATP, fructose-1,6-diphosphate, 3-phosphoglyceric acid (3-PGA), and 2-phosphoglyceric acid. These compounds are normally found in the "barium-insoluble fraction," prepared as described by Umbreit, Burris, and Stauffer (5). Fig. 2 shows the behavior of a mixture of some substances occurring in the "barium-soluble fraction" (5), namely glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, 3-AMP, and phosphoenolpyruvic acid. Inorganic phosphate was included as a marker. In our experience, the position of the spots relative to each other has proved more useful than R_f values. This is particularly true when complex impure mixtures are chromatographed (1). The R_f values, while fairly reproducible with a single batch of paper, are not the same for different grades of papers and, further, are affected by washing of papers.

TABLE I
R_F Values of Phosphate Esters

Compound	<i>R_F</i>	
	Acid solvent	Alkaline solvent
Adenosinetriphosphate.....	0.12	0.15
Adenosine-3-phosphate.....	0.17	0.32
Glucose-1-phosphate.....	0.27	0.60
Fructose-6-phosphate.....	0.34	0.44
Tripolyphosphate.....	0.37	0.06
Glucose-6-phosphate.....	0.38	0.48
Fructose-1,6-diphosphate.....	0.40	0.24
2-Phosphoglycerate.....	0.46	0.18
Pyrophosphate.....	0.46	0.05
3-Phosphoglycerate.....	0.50	0.35
Phosphoenolpyruvate.....	0.52	0.46
Orthophosphate.....	0.63	0.28

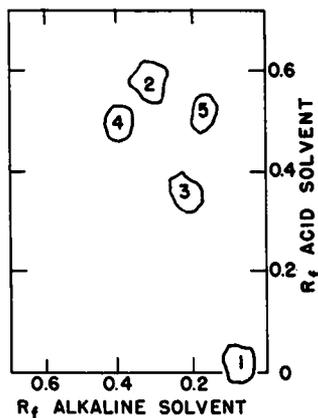


FIG. 1

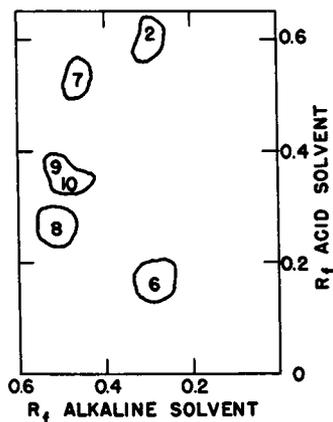


FIG. 2

FIG. 1. Two-dimensional chromatogram of glycolytic intermediates. "Barium-insoluble fraction:" 1, ATP; 2, orthophosphate; 3, fructose-1,6-diphosphate; 4, 3-phosphoglyceric acid; 5, 2-phosphoglyceric acid.

FIG. 2. Two-dimensional chromatogram of glycolytic intermediates. "Barium-soluble fraction:" 2, orthophosphate; 6, adenosine-3-phosphate; 7, phosphoenolpyruvic acid; 8, glucose-1-phosphate; 9, glucose-6-phosphate; 10, fructose-6-phosphate.

The identification of unknown constituents in mixtures is greatly facilitated when either the unknown compounds or known authentic samples are available with radioactive labels. After chromatographing a mixture of the unknown and known compounds, the exact coincidence of position

and shape of the spots on the chromatogram and the radiogram makes the identification of the unknown compounds highly probable.

The technique described above has found application in the study of the phosphorylated compounds formed by the incorporation of radioactive phosphate by pea seed meal, pea seed extract (6), and orange flavedo extract.²

DISCUSSION

Selection of Solvents

The selection of the solvents was based on the results of numerous tests carried out by the method of Rockland and Dunn (7) by employing small filter paper sectors developed in test-tubes. It was thus possible to survey quickly several hundred solvent combinations for their ability to resolve a mixture of glucose-1-phosphate, 3-PGA, and inorganic phosphate. The following solvents were tested singly or in certain combinations: water, methanol, ethanol, normal, and isopropanol, normal, secondary, tertiary, and isobutanol, isopentanol, furfuryl alcohol, glycol monomethyl ether, diethyl ether, isopropyl ether, dioxane, acetonitrile, acetone, diethyl carbonate, ethyl acetate, benzylamine, ethylenediamine, piperidine, pyridine, ammonium hydroxide, hydrochloric, formic, and glacial acetic acids. In summary these generalizations may be made. In the absence of water no movement occurred except with methanol, ethanol, glycol monomethyl ether, and acetonitrile. The R_F values for the esters in these solvents could be enhanced by the addition of water. However, a high concentration of water caused high R_F values and poor separation of the esters. Even with optimum amounts of water, poor resolution and broadly spread spots were obtained over the pH interval of 4 to 9. This difficulty was alleviated by the incorporation of acids or bases. Lugg and Overell (8) encountered a similar difficulty in the chromatography of organic acids, which they overcame by addition of "swamping" concentrations of a strong acid. The solvents which Hanes and Isherwood (2) found most suitable for the chromatography of phosphate esters were either strongly acidic or basic. In the present work the choice of acid or base, as of any solvent, was based first on the necessity for easy removal. Thus formic and hydrochloric acids were suitable from this point of view, but the use of hydrochloric acid resulted in excessive damage to the paper.

The order of the chromatographed compounds relative to one another was the same for all acid solvents used. Other orders are, however, obtained by the use of alkaline solvents, as was evident from the work of Hanes and Isherwood. Of all the bases listed, only NH_4OH and piperidine were found to be useful; the former was selected because of its lower cost

² Axelrod, B., and Bandurski, R. S., unpublished.

and greater volatility. Methanol, ethanol, *n*-propanol, acetonitrile, glycol monomethyl ether, and dioxane were all suitable as the neutral component of the solvent. Methanol was selected because it caused more rapid development than the other solvents named. The composition of the mixtures finally chosen was based upon experiments in which the proportions of the components were varied in small steps.

Confirmatory Identification of Phosphate Spots

The characteristic properties by which glucose-1-PO₄, inorganic phosphate, and the purine derivatives may be detected have already been discussed. These substances, if present, can serve as index compounds for the location of other esters. The various carbohydrate esters may be located by the use of specific carbohydrate sprays (9–11). It is sometimes useful to add one or more authentic phosphate esters in large amounts to obtain index spots. It is further possible to identify an unknown compound with a known by loading a second chromatogram with the known material and observing the augmentation of the spot.

SUMMARY

1. A two-dimensional chromatographic method for the identification of some phosphate esters of biological importance, including inorganic polyphosphates, is described.
2. An acid solvent consisting of methanol, formic acid, and water and a basic solvent containing methanol, ammonia, and water are used.
3. An improved method of color development involving ultraviolet light permits the hydrolysis of resistant esters and the minimization of background color.

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