ION EXCHANGE AND PAPER CHROMATOGRAPHY OF PHOSPHORYLATED HEXOSE ESTERS

By JASPER DULBERG, WILLIAM G. ROESSLER, TROY H. SANDERS, AND CARL R. BREWER

(From the Chemical Corps Biological Laboratories, Camp Detrick, Frederick, Maryland)

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The identification of phosphorylated compounds formed during metabolism by microorganisms has been aided significantly by paper chromatographic and ion exchange techniques. Cohen and Scott (1) used these techniques to separate and identify ribose-5-phosphate formed from 6-phosphogluconate by a yeast enzyme system. Horecker and Smyrniotis (2), investigating the same enzyme system, separated two pentose phosphate compounds, ribose-5-phosphate and presumably ribulose-5-phosphate, from the reaction mixture by the use of an ion exchange resin. Stadtman and Barker (3) used chromatography to identify the acyl phosphate esters formed by enzyme preparations of *Clostridium kluyveri*.

Experiments with radioactive carbon by Benson *et al.* (4) and by Aronoff and Vernon (5) have followed the sequence of formation of carbohydrate intermediates by plants. Preliminary separation of the metabolic intermediates was accomplished by ion exchange resins and the compounds were identified by paper chromatography combined with radioautography.

This report concerns the techniques of paper partition chromatography and ion exchange resins which were utilized for the identification of phosphorylated hexose esters occurring in fractions of bacterial fermentation products. Glucose-1-phosphate, glucose-6-phosphate, fructose-1,6-diphosphate, and fructose-6-phosphate were identified by these techniques. Experiments on the rates of hydrolysis of fructose-1,6-diphosphate and fructose-6-phosphate are also presented.

Methods

Ion Exchange Resins—Columns of ion exchange resins were employed to remove salts from fractions of fermentation products which contained salts, hexose phosphates, and free sugars. The deionization procedure was essential for preparation of material which could be subjected to chromatographic analysis satisfactorily. Partridge and Westall (6) used De-Acidite and Zeo-Karb[1] to remove ions from simple sugar solutions to obtain reliable paper chromatograms for the qualitative identification

[1] The Permutit Company, London, England.

of the sugars. Under different conditions, Amberlite IR-4 and Amberlite IR-100[2] were used by McCready and Hassid (7) to isolate glucose-1-phosphate from sugar mixtures. In the present work, Amberlite IR-4B and Amberlite IR-100 were used to deionize acid hydrolysates of the sugar esters.

The column of IR-4B, 1.5 cm. by 22 cm., was regenerated to its maximum capacity with 200 ml. of 1 N ammonium hydroxide at a flow rate of 10 ml. per minute, followed by a thorough rinsing with water. The fully regenerated column retained 16 m.eq. of 0.1 N hydrochloric acid at a flow rate of 10 ml. per minute before any detectable break-through of chloride ion occurred as tested with silver nitrate. IR-100 was used routinely to remove cations prior to chromatography in a column of the same size that was used with the anion exchange resin; it was fully regenerated with 100 ml. of 0.7 N hydrochloric acid at a flow rate of 10 ml. per minute followed by rinsing with 500 ml. of water.

A strongly basic anion exchange resin, IRA-400, was employed in some early attempts to separate glucose from glucose-6-phosphate. Both glucose and the ester were adsorbed by the resin and both compounds were eluted with 0.1 N hydrochloric acid, and hence no separation was accomplished with the IRA-400 resin. A less strongly ionized acid or lower concentrations of hydrochloric acid might have permitted a satisfactory separation. This problem was not studied further because the IR-4B resin was satisfactory.

Paper Chromatography—The method reported here was modified from that of Partridge and Westall (6). The adaptation and development of this method were essential for the identification of the sugar phosphates because no other chromatographic method reported was entirely applicable. The methods of Benson *et al.* (4) are applicable but require radioactive compounds. The Hanes and Isherwood (8) technique was investigated to a limited extent; the method, however, was difficult to employ and is as yet incomplete with respect to identification of sugar phosphates and other phosphate compounds which have similar R_F values. By the Hanes and Isherwood method one cannot distinguish between fructose-6-phosphate and fructose-1,6-diphosphate; this difficulty, however, was overcome in enzyme experiments by barium and alcohol fractionations.

Sheets of Whatman No. 1 paper, 15 cm. by 38 cm., were used in glass cylinders with 75 per cent aqueous phenol as the solvent. The ascending chromatograms were allowed to run 20 to 24 hours at room temperature. The paper was dried at 80–90°, then sprayed with the naphthoresorcinol reagent of Partridge and Westall (6) for spotting ketoses or with the aniline hydrogen phthalate reagent of Partridge (9) for spotting aldoses.

[2] Rohm and Haas Company, Philadelphia, Pennsylvania.

The method employed to hydrolyze fructose esters was based on the observations of Neuberg *et al.* (10) that fructose is slowly liberated when fructose esters are treated for several days at 37° in 1 N hydrobromic acid. The destruction of the liberated fructose was not appreciable under these conditions and the ketose was identified by paper chromatography after interfering anions and cations had been removed by ion exchange resins.

Phosphate was determined by the method of King (11).

EXPERIMENTAL

Changes in polarimetric readings and inorganic and total phosphate were observed in 1 N hydrobromic acid solutions of commercial preparations of fructose-1,6-diphosphate and fructose-6-phosphate held at 37° for 22 days in order to determine the rate of hydrolysis of these compounds.

Barium salts of the fructose esters were dried over phosphorus pentoxide under a high vacuum for 4 weeks at 55°. Our experience confirmed the observations of Neuberg *et al.* (10) that the fructose esters were difficult to prepare free of moisture and water of hydration. The following specific rotations are given by Neuberg *et al.* (10): $[\alpha]_D^{17} = +4.04°$ to $+4.15°$ for fructose-1,6-diphosphate (FDP), $[\alpha]_D^{19} = +3.58°$ for fructose-6-phosphate, and $[\alpha]_D^{20} = -92.4°$ for fructose (F). These constants were employed in a formula to determine the amount of fructose-1,6-diphosphate or fructose-6-phosphate hydrolyzed to fructose. Because the rotations of fructose-1,6-diphosphate and fructose-6-phosphate are approximately the same, the fructose-6-phosphate value was omitted in the calculation. The formula used to determine the amount of fructose-1,6-diphosphate hydrolyzed to fructose was as follows:

$$\alpha = \alpha_0 + 2\left[4.04\ (\text{gm. per ml. of FDP} - X) - 92.4\left(\frac{\text{gm. per ml. of F in FDP}}{\text{gm. per ml. of FDP}}\right)X\right]$$

where α is the observed reading with a 2 dm. tube, X is the gm. per ml. of FDP hydrolyzed to F, and α_0 is the zero correction determined from the observed zero time reading on the polarimeter and corrected to agree with the theoretical zero reading according to Neuberg *et al.* (10).

The data in Table I show that after 1 week approximately 5 per cent of the fructose-1,6-diphosphate or fructose-6-phosphate was hydrolyzed to fructose and that this rate continued for at least 3 weeks. The amount of phosphate converted to the inorganic form when fructose-6-phosphate was hydrolyzed to fructose agreed closely with that calculated from the rotation data. The phosphate in the 1 position of fructose-1,6-diphosphate is more rapidly hydrolyzed than the phosphate in the 6 position (10); this is substantiated by data on the rates and amounts of organic phosphorus converted to the inorganic form (Table I).

A preliminary experiment showed that the rate of hydrolysis of fructose-1,6-diphosphate at 42° was appreciably faster than at 37°; approximately 20 per cent was hydrolyzed to fructose in 1 week.

Barium salts of fructose-1,6-diphosphate, fructose-6-phosphate, glucose-1-phosphate, or glucose-6-phosphate, obtained either from commercial sources or from fermentations, were hydrolyzed and the free hexoses identified by paper chromatography.

TABLE I

Hydrolysis of Fructose-1,6-diphosphate and Fructose-6-phosphate at 37° with 1 N Hydrobromic Acid

Compound hydrolyzed	Days at 37°	$[\alpha]_D^{25}$	Phosphorus			Organic phosphorus converted to inorganic form	Calculated amount of ester hydrolyzed to fructose	
			Inorganic	Organic	Total			
			mg. per ml.	*mg. per ml.*	*mg. per ml.*	*per cent*	*per cent*	*mg. per ml.*
Fructose-1,6-diphosphate	0	+0.541	1.94	13.06	15.00	0	0	0
	4	+0.265	5.80	9.20	15.00	29.6	3.2	2.31
	7	+0.091	7.15	7.75	14.90	40.7	5.5	3.95
	11	−0.156	7.75	7.25	15.00	44.5	8.7	6.28
	14	−0.350	7.75	7.35	15.10	43.7	11.2	8.11
	18	−0.636	8.43	6.57	15.00	49.7	14.9	10.81
	22	−0.763	9.14	5.86	15.00	55.1	19.2	13.89
Fructose-6-phosphate	0	−0.007	0.16	7.34	7.50	0	0	0
	4	−0.338	0.41	7.09	7.50	3.4	3.3	2.10
	7	−0.461	0.51	6.99	7.50	4.8	4.8	3.00
	11	−0.686	0.62	6.83	7.45	6.9	7.4	4.67
	14	−0.915	0.72	6.78	7.50	7.6	10.0	6.29
	18	−1.064	0.91	6.59	7.50	10.2	11.9	7.47
	22	−1.301	1.15	6.35	7.50	13.5	14.7	9.23

The weight of dibarium fructose-1,6-diphosphate dissolved in 10 ml. of 1 N hydrobromic acid was 1.3010 gm., equivalent to 0.7242 gm. of free fructose-1,6-diphosphoric acid or 0.3837 gm. of fructose. The weight of barium fructose-6-phosphate dissolved in 10 ml. of 1 N hydrobromic acid was 0.9575 gm., equivalent to 0.6296 gm. of free fructose-6-phosphoric acid or 0.4362 gm. of fructose.

Identification of Fructose from Fructose-1,6-diphosphate—The washed and dried barium salt of a commercial preparation of fructose-1,6-diphosphate, or that found in the barium-insoluble fraction when fermentation products were fractionated with barium acetate and alcohol as recommended by Umbreit *et al.* (12), was dissolved in 1 N hydrochloric acid and held at 42° for 3 weeks to liberate fructose. 1 N sulfuric acid equivalent to the barium content was added to the hydrolysate and the barium sulfate precipitate was removed by centrifugation. Anions were removed from the supernatant solution by passage through a column of anion exchange resin (IR-4B) which had an acid-adsorbing capacity of approximately

twice the amount of mineral acid contained in the hydrolysate. The column was washed with 25 ml. of distilled water. The effluent and washings were concentrated to 1 ml. under a vacuum at 40–50°. When a poorly defined chromatogram was obtained because salts still remained in the solution, the concentrate was again deionized by passage through the cation (IR-100) exchange column and then through the anion (IR-4B) exchange column.

Paper chromatograms of this concentrated acid-free hydrolysate were prepared by placing repeated 0.005 ml. applications of the hydrolysate on a spot on Whatman No. 1 paper; each application was dried with a current of warm air. Parallel chromatograms of the concentrate were made on a single sheet of filter paper with the amounts at the separate spots ranging from 0.05 to 0.2 ml. Pure fructose and glucose also were placed on the same paper to provide control chromatograms.

Identification of Fructose from Fructose-6-phosphate—The barium salt of fructose-6-phosphate was analyzed by the same procedure used for the fructose-1,6-diphosphate.

Hydrolysis and Identification of Glucose-1-phosphate—The washed barium salts of glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate found in the barium-soluble, alcohol-insoluble fraction of fermentation products were dissolved in 1 N hydrochloric acid and made up to a measured volume. An aliquot was reserved for estimation of the 7 minute-hydrolyzable phosphate and 7 minute reducing value. The initial reducing value of the fraction was assayed as soon as possible. The remainder of the acid solution was placed in a boiling water bath for 9 minutes and then quickly cooled. The hydrolyzed glucose was separated from the unhydrolyzed glucose-6-phosphate (which was reserved for subsequent identification) by neutralizing the partial hydrolysate to pH 8.2 and precipitating the unhydrolyzed phosphate esters as the barium salts with 4 volumes of cold 95 per cent alcohol and chilling for 1 hour. The supernatant solution containing the hydrolyzed glucose was concentrated under a vacuum to remove the alcohol and a slight excess of 1 N sulfuric acid was added to precipitate the barium. The concentrated supernatant solution was deionized by passage through ion exchange columns of IR-4B and IR-100. The effluent solution was concentrated to about 1.5 ml. and spotted on filter paper by repeated applications of 0.005 ml. so that the total amounts ranged from 0.01 to 0.06 ml. Controls of pure glucose were applied to the same paper in parallel chromatograms.

Isolation and Hydrolysis of Glucose-6-phosphate—Glucose-6-phosphate was reprecipitated as the barium salt from the acid used to hydrolyze glucose-1-phosphate as described above. Glucose-6-phosphate is only 10 per cent hydrolyzed in 3 hours by 1 N hydrochloric acid at 100° and thus

is not affected appreciably by the conditions used to hydrolyze glucose-1-phosphate completely. The barium salt was washed twice with 90 per cent alcohol, dissolved in 10 ml. of 1 N hydrochloric acid, and partially hydrolyzed by heating in a boiling water bath for 3 hours. Darkening of the hydrolysate was observed, possibly because of the destruction of the fructose-6-phosphate. The barium was removed from the hydrolysate by the addition of a slight excess of 1 N sulfuric acid followed by centrifugation. To remove the mineral acids, the hydrolysate was passed through a column of IR-4B which had a capacity, as established by previous tests, sufficient to remove all of the mineral acids but to permit the leakage into the effluent of 25 to 50 per cent of the unhydrolyzed glucose-6-phosphate remaining in the acidic influent. The escape of glucose-6-phosphate with the free glucose from the IR-4B exchange column did not interfere with the subsequent analysis of glucose by chromatography; consequently, the resin columns were not modified to effect the complete separation of glucose from glucose-6-phosphate. The anion exchange resin was washed with 30 ml. of water and the combined washings and effluents were concentrated to 1.5 ml.

Paper chromatograms of the concentrated partial hydrolysate were prepared by spotting amounts ranging from 0.025 to 0.125 ml. Parallel control chromatograms of pure glucose were made on the same sheet of filter paper.

Results

Typical results obtained by paper chromatographic analysis are shown in Table II. Fructose-1,6-diphosphate or fructose-6-phosphate was hydrolyzed by 1 N acid at 42° for 3 weeks. The fructose was identified by the ketose reagent on the paper chromatogram at R_F 0.51. The pure fructose controls had R_F values of 0.52. The slight difference in the R_F of the liberated fructose in the hydrolysate was probably due to the salt effect of the small amounts of intact fructose esters present in the concentrate, as evidenced by the slightly pink spots at R_F 0.07.

Glucose-1-phosphate was characterized by the lability of the phosphate and the appearance of a glucose spot on the paper chromatogram. Well defined spots at R_F 0.38 were found on a chromatogram of the concentrate of the sugar liberated by hydrolysis in 1 N acid at 100° for 9 minutes; control spots of glucose gave the same R_F value. Commercially prepared glucose-6-phosphate or that found with glucose-1-phosphate and fructose-6-phosphate in the barium-soluble, alcohol-insoluble fraction of fermentation products was partially hydrolyzed by 1 N acid in 3 hours at 100°. Chromatographic analysis of the hydrolysate by means of the aldose reagent showed two well defined spots at R_F 0.38 and 0.08. The

brown spot at R_F 0.38 was the same in color and R_F value as that of the control of pure glucose, indicating that glucose-6-phosphate was partially hydrolyzed by the 3 hour treatment. The brown spot of the hydrolysate at R_F 0.08 was the unhydrolyzed glucose-6-phosphate which had leaked through the anion exchanger, as subsequent analysis of similar parallel spots proved.

Proof that the brown spot at R_F 0.08 was glucose-6-phosphate was obtained by comparing the R_F values of the unknown material and pure glucose-6-phosphate. Assays of material eluted from areas at R_F 0.08 in parallel chromatograms from one experiment showed 0.36 mg. of glucose-6-phosphate as estimated by reducing value and 0.25 mg. of glucose-6-phosphate as estimated by the organic phosphorus content.

TABLE II

Chromatographic Identification of Phosphorylated Sugar Esters

Compound	Hydrolysis in N HCl	Reagent	R_F	Control	
				R_F	Compound
Fructose-1,6-diphosphate	42°, 3 wks.	Ketose	0.51	0.52	Fructose
Fructose-6-phosphate	42°, 3 "	"	0.51	0.52	"
Glucose-1-phosphate	100°, 9 min.	Aldose	0.38	0.38	Glucose
Glucose-6-phosphate	100°, 3 hrs.	"	0.38	0.38	"
"	100°, 3 "	"	0.08	0.08	Glucose-6-phosphate

Chromatograms were run for 20 to 24 hours at room temperature, 24–27°, with 75 per cent aqueous phenol and Whatman No. 1 paper.

SUMMARY

Techniques for identifying fructose-1,6-diphosphate, fructose-6-phosphate, glucose-1-phosphate, and glucose-6-phosphate by paper chromatographic techniques were developed.

The use of ion exchange resins to remove interfering anions and cations from solutions of acid-hydrolyzed hexose phosphate esters was necessary to obtain satisfactory chromatograms.

Rates of hydrolysis of fructose-1,6-diphosphate and fructose-6-phosphate in hydrobromic acid at 37° were studied. Hydrolysis under these conditions was found to be practical for the subsequent identification of the liberated fructose by paper chromatography.

BIBLIOGRAPHY

1. Cohen, S. S., and Scott, D. D. M., *Science*, **111**, 534 (1950).
2. Horecker, B. L., and Smyrniotis, P. Z., *Arch. Biochem.*, **29**, 232 (1950).

3. Stadtman, E. R., and Barker, H. A., *J. Biol. Chem.*, **184,** 769 (1950).
4. Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A., and Stepka, W., *J. Am. Chem. Soc.*, **72,** 1710 (1950).
5. Aronoff, S., and Vernon, L., *Arch. Biochem.*, **28,** 424 (1950).
6. Partridge, S. M., and Westall, R. G., *Biochem. J.*, **42,** 238 (1948).
7. McCready, R. M., and Hassid, W. Z., *J. Am. Chem. Soc.*, **66,** 560 (1944).
8. Hanes, C. S., and Isherwood, F. A., *Nature*, **164,** 1107 (1949).
9. Partridge, S. M., *Nature*, **164,** 443 (1949).
10. Neuberg, C., Lustig, H., and Rothenberg, M. A., *Arch. Biochem.*, **3,** 33 (1943–44).
11. King, E. J., *Biochem. J.*, **26,** 292 (1932).
12. Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Manometric techniques and related methods for the study of tissue metabolism, Minneapolis, 185 (1945).

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