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SEPARATION OF PHOSPHOHYDROXYPYRUVATE, 3-PHOSPHOGLYCERIC ACID AND O-PHOSPHOSERINE BY PAPER CHROMATOGRAPHY AND CHEMICAL DERIVATIZATION

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SUMMARY

Novel chromatographic and derivatization techniques have been developed to investigate the levels and kinetics of 3-phosphoglyceric acid, phosphohydroxypyruvate and phosphoserine in plants. These techniques use derivatization reactions of the phosphorylated compounds with *o*-phenylenediamine and isonicotinic acid hydrazide, in combination with paper chromatography. Unexpected reactions of α-hydroxy acids have been encountered and circumvented.

INTRODUCTION*

The compounds phosphohydroxypyruvate, 3-phosphoglyceric acid and O-phosphoserine are under investigation in this laboratory as early intermediates in photosynthetic CO_2 fixation in plants[1]. These three substances constitute a chemical series of C-3 compounds that vary in composition only at the α-carbon (C-2). Carbons C-1 and C-3 consist of a carboxyl group and a phosphorylated ester of a primary alcohol, respectively. As a result of this close similarity in structure, and because of the relatively hindered condition of α-carbon, direct chromatographic resolution of these compounds is difficult to the degree that the separation of P-hydroxypyruvate from 3-PGA is cited[2] as "non-practical".

This paper describes the separation of these three compounds: P-hydroxypyruvate from 3-PGA and P-serine by chemical derivatizations and paper chromatography; 3-PGA from P-hydroxypyruvate and P-serine; and P-serine from P-hydroxypyruvate and 3-PGA by paper chromatography.

* Abbreviations: P-hydroxypyruvate = phosphohydroxypyruvate; RudP = ribulose diphosphate; 3-PGA = 3-phosphoglyceric acid; P-serine = O-phosphoserine; GAP = glyceraldehyde phosphate; F-1-P = fructose-1-phosphate, F-6-P = fructose-6-phosphate; F-1,6-dP = fructose-1,6-diphosphate; G-1-P = glucose-1-phosphate; G-6-P: glucose-6-phosphate; OPD = *o*-phenylenediamine; INH = isonicotinic acid hydrazide (isoniazid); BAW and EFW = developing solvents for paper chromatography described in methods.

EXPERIMENTAL

Materials

P-Hydroxypyruvate was prepared from the cyclohexylammonium salt of hydroxypyruvic acid phosphate dimethyl ketal according to the manufacturer's instructions (Sigma, St. Louis, Mo., U.S.A.). The hydrolysis procedure was carried out in an atmosphere containing toluene in order to inhibit the growth of micro-organisms. GAP was prepared from its barium salt by treatment of solutions (*ca.* 0.018 *M*) with an excess of Dowex 50W ion-exchange resin. 3-PGA (0.018 *M*) was prepared similarly from its trisodium salt. P-Serine (0.018 *M*) was dissolved in 1.5% formic acid. Spots of underivatized phosphate compounds were determined by use of phosphate reagent[3]. Derivatized products were also detected by their fluorescence when stimulated by long-wavelength UV light (Mineralite). Such fluorescence indicates the formation of a heterocyclic ring. The phosphate stain is considered to be sufficiently specific if the stained chromatographic spot is blue.

Preparation of OPD adducts

Some of these products were prepared according to Morrison[4] (Table I). Phosphorylated α-keto and α-hydroxy acids do not yield precipitable OPD adducts by the Morrison technique. These OPD adducts were prepared by dissolving OPD (50 mg, 0.47 mmoles) in 2 *N* HCl; 18 μmoles of P-hydroxypyruvate, GAP, 3-PGA or P-serine were then added as an additional volume of 1 ml and allowed to react in the dark for several days at room temperature.

TABLE I

PREPARATION OF OPD ADDUCTS OF NON-PHOSPHORYLATED ACIDS

The products were made by the Morrison technique[4], in 1.24 *M* acetic acid.

Reactant	*Amount (mmoles)*	*OPD (mmoles)*	*Acetic acid (ml)*	*Precipitate*	
				weight (g)	*color*
Ascorbic acid	22	74	150	none	—
Glyceric acid, hemicalcium salt	5	19	18	0.83	brown
Glyceric acid treated with 1 g of periodic acid before filtration	5	19	18	0.11	yellow
Glyoxylic acid sodium salt	11	56	43	1.97	grey
Hydroxypyruvic acid lithium salt	6.2	30	19	0.9	yellow-brown
α-Ketoglutaric acid monosodium salt	6	38	43	1.05	brown
Oxalic acid	39	38	35	4.0	green-gold
Oxalacetic acid	10	38	75	1.1	yellow
Sucrose	4.4	19	33	none	—
Sucrose treated with 1 g of periodic acid before filtration	4.4	19	33	0.42	black

Thin-layer chromatographic separation of OPD derivatives

A chloroform–methanol (98:2) system was used. The preparation (*ca.* 12.5 μg) was applied in methanol solution to a precoated silica gel plate [layer thickness, 0.25 mm; total dimensions 20 × 20 cm; Type 60F-254 (E. Merck, Darmstadt, G.F.R.)]. The plate was developed in one direction (16 cm). Compounds were detected using a long-wavelength UV light source (Mineralite).

Photodegradation of hydroxypyruvate-OPD complex

When hydroxypyruvate is treated with OPD[4] and irradiated with long-wavelength UV light it decomposes. If the hydroxypyruvate–OPD complex (hydroxypyruvate–quinoxalinol) is chromatographed on silica gel in the dark in one direction, then irradiated with long-wavelength UV light and run in the perpendicular direction, a change in migration occurs.

Freshly prepared hydroxypyruvate–quinoxalinol (0.5 mg) was applied to one corner of a precoated silica gel plate (see above). The plate was developed first in direction 1 with chloroform–methanol (98:2), dried in air and then irradiated at the closest distance possible with long-wavelength (360 nm maximum) UV light from two BLB 40 (Sylvania) lamps excited at twice the normal current (0.8 A) for 5 min. The plate was then developed in direction 2 (perpendicular to direction 1).

We found that the major component at R_F 0.63 breaks down to give a fluorescent product with an R_F of 0.36, the major product usually found when light is not excluded from the preparation. A hydroxypyruvate–quinoxalinol preparation which had not been protected from light gave three components (Table II) which have R_F values of 0.58, 0.36 and 0.14. Further photoconversion of the component at R_F 0.36 leads to components at R_F 0.16 and 0.14 which closely correspond in R_F to OPD derivatives of commercial glyoxylate– and oxalate–quinoxalinols (Table II). This suggests loss of C-3, and shows that quinoxalinols may suffer photochemical transformations and thus should be shielded from UV-containing light such as sunlight.

Separation of P-serine from P-hydroxypyruvate and 3-PGA by paper chromatography

This separation was achieved by use of a specially, developed paper chromatographic system which is less cumbersome, more rapid and more specific than the paper electrophoretic systems in present use[5–7]. The phosphorylated metabolites were spotted at a concentration of 60 μg per spot. The paper (Whatman No. 3) was developed to the top by ascending chromatography (255 mm). The developing solvent (EFW) was 95% ethanol–90% formic acid–water (652:21:168). All of the phosphorylated metabolites were in the acic form when applied. The detection of metabolites was accomplished by use of molybdate and ninhydrin staining reagents[3]. All of the R_F values are the means from at least five replicate measurements. The origins of spots were staggered on different chromatograms, although edge and middle effects on migration were negligible (Figs. 1 and 2).

Hydrolysis of P-serine

Aliquots of O-phospho-DL-serine (0.5 mg/ml), prepared in 2 *N* HCl, were treated for 16 h at (a) 93–95° or (b) room temperature. Samples (2.5 μg) were then developed in one direction on 0.1 mm thick cellulose TLC plates (Polygram Cel 300;

TABLE II

R_F DATA FOR OPD ADDUCTS OF NON-PHOSPHORYLATED ACIDS

Two different chromatographic systems were used for the identification: first, paper chromatography developed with solvent BAW on Whatman No. 3MM paper in the ascending direction; secondly, silica gel as the stationary phase developed with chloroform–methanol (98:2). Details in the Experimental section. Compounds were detected by a long-wavelength UV light source. The color of the observed fluorescence is indicated in parentheses.

Reactant	*Paper chromatography*		*Silica gel chromatography*	
	major	*minor*	*major*	*minor*
Glycerate	0.78 (yellow)	—		not done
Glycerate oxidized with periodate	0.78 (yellow)	—	0.08	0.15 0.44
Glyoxylate	0.78 (purple)	0.86 (lilac)	0.18	—
Hydroxypyruvate	0.87 (light blue)	0.84 (blue-green) 0.61 (blue)	0.36	0.58 0.14
α-Ketoglutarate	0.76 (yellow) 0.89 (blue)	— —	0.14 0.21	— —
Oxalic acid	not detected	(UV)	0.31	—
Pyruvate	not detected	(UV)	0.21	—
Sucrose oxidized with periodic acid	0.77 (yellow)	—	0.08	0.44

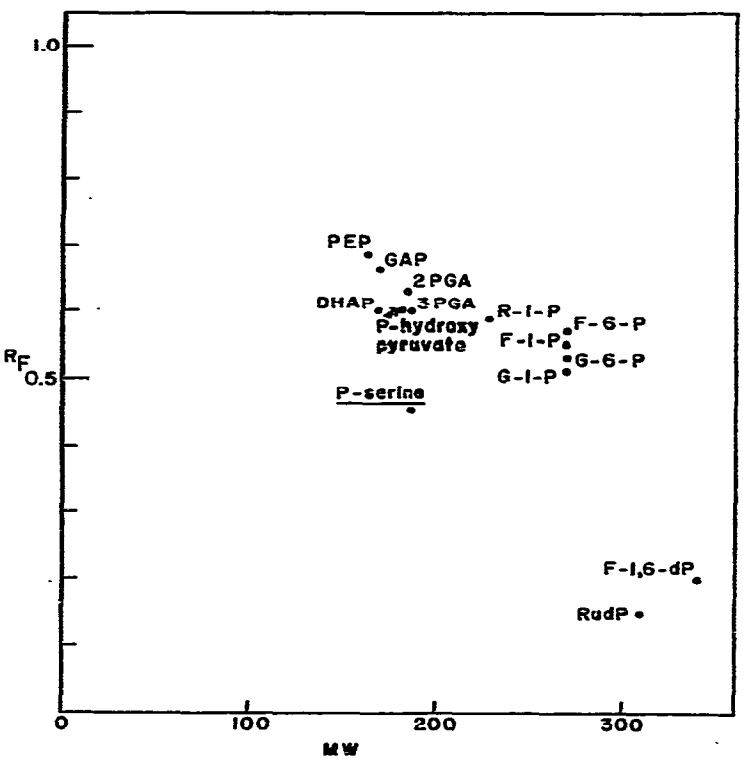

Fig. 1. Effect of an α-amino group on the chromatographic behaviour of phosphorylated metabolites. The paper (Whatman No. 3MM) was developed to the top by ascending chromatography (255 mm). The developing solvent was 95% ethanol–90% formic acid–water (625:21:168). Detection of amino acids was by ninhydrin stain[3]. R_F values are from the mean migration of two spots. Detection of phosphorylated derivatives was by phosphomolybdate stain[3]; R_F values are from the means of migration of at least five spots.

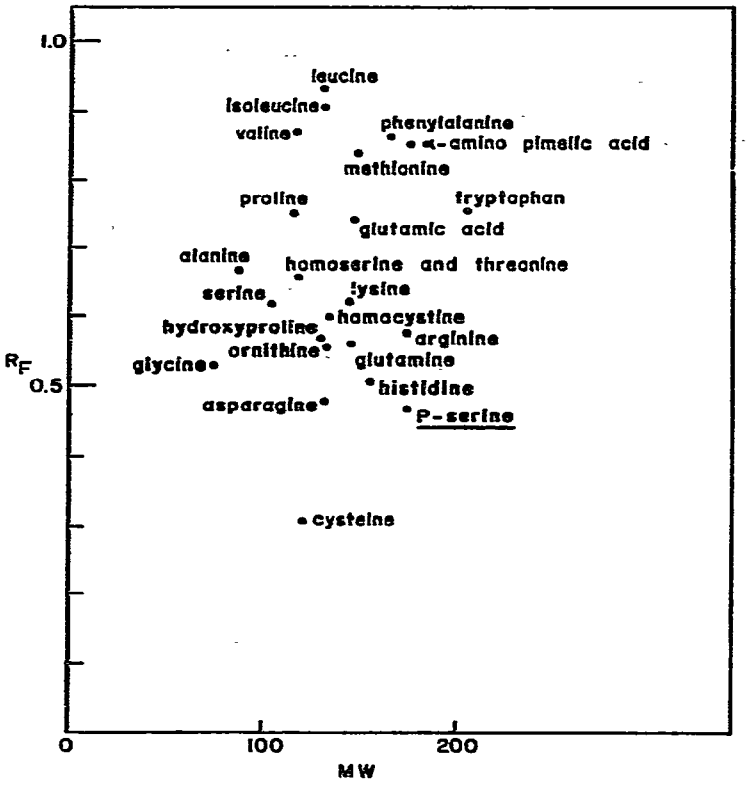

Fig. 2. Effect of an O-phospho ester on the chromatographic behaviour of amino acids. Experimental conditions as outlined for Fig. 1.

Macherey, Nagel & Co., Düren, G.F.R.) with 80% aqueous phenol (pH 5.4) and then stained by dipping in ninhydrin[3]. No serine was detected, but P-serine was quite prominent. Samples were then diluted with HCl to *ca.* 4 *N* and 0.33 mg/ml. Aliquots of the resulting solution were treated for 16 h at (c) room temperature or (d) 130° (autoclave). When samples (2.5 μg) were subjected to thin-layer chromatographic separation as above, sample c only contained P-serine with no serine being observed. Sample d contained only serine, and the hydrolysis was, within the limits of detection (ninhydrin dip), complete. The identities of the reactants and the products were confirmed by two-dimensional ascending paper chromatography in butanol–acetic acid–water (12:3:5) (BAW) and 80% aqueous phenol (pH 5.4). Detection of phosphate was accomplished by dipping in molybdate[3] as well as ninhydrin.

Separation of P-hydroxypyruvate from 3-PGA and P-serine by chemical derivatizations and paper chromatography

P-Hydroxypyruvate separation was accomplished by ascending paper chromatographic separation of the condensation products of P-hydroxypyruvate with OPD (Table III). P-Hydroxypyruvate, GAP, 3-PGA and P-serine and their corresponding OPD derivatives were applied to Whatman No. 3MM paper and the paper

TABLE III

CHROMATOGRAPHIC CHARACTERISTICS OF SOME PHOSPHORYLATED COMPOUNDS AND THEIR OPD CONDENSATION PRODUCTS

Separation was carried out on Whatman No. 3MM paper developed in the ascending direction with BAW, as described in the Experimental section.

Parent compound	*Untreated*		*OPD treated*	
	Migration (mm)	R_F	*Migration (mm)*	R_F
P-Hydroxypyruvate	60 ± 5 positive phosphate stain	0.24	173 ± 5 yellow fluorescence positive phosphate stain	0.68
GAP	81 ± 6 positive phosphate stain	0.32	172 ± 7 (not fluorescent) positive phosphate stain	0.68
3-PGA	54 ± 8 positive phosphate stain	0.21	150 ± 6 green phosphate stain	0.60
P-Serine	30 ± 2 positive ninhydrin and phosphate stains	0.12	34 ± 4 positive ninhydrin and phosphate stains	0.13
Standard reagent control (OPD)		—	141 ± 9 (brown in visible light)	0.56
Front			254	(1.0)

was developed by ascending chromatography with BAW. In each case, 0.8 μmoles of phosphorylated compound were applied as a spot, 12 μl at a time. The chromatograms were stained for phosphate with molybdate reagent, the stain was activated by IR irradiation followed by UV irradiation and the chromatogram was exposed to ammonia vapor. GAP–OPD and P-hydroxypyruvate gave definite blue stains after being treated with ammonia, but 3-PGA–OPD gave a green color because of the closeness in R_F value to that of the yellow-brown stain of OPD. All of the operations were carried out in dim light (Table III).

Separation of 3-PGA from P-hydroxypyruvate and P-serine

3-PGA, P-hydroxypyruvate and GAP, but not P-serine, react with isonicotinic acid hydrazide (INH). The INH adducts have different migration rates in BAW from their parent phosphorylated compounds. The adducts were run in direction 1 as their INH derivatives. 3-PGA was then regenerated from its IHN adduct by treatment with excess of benzaldehyde. P-Hydroxypyruvate and GAP were not regenerated by this treatment. Thus, on BAW rechromatography in direction 2,3-PGA is separated from P-hydroxypyruvate and GAP adducts and from P-serine (Table IV).

The INH complexes were prepared as follows. INH (50 mg, 0.36 mmoles) was dissolved in 1 ml of 4 *N* HCl (INH reagent); P-hydroxypyruvate, GAP, 3-PGA or P-serine (18 μmoles each) were added in an additional 1 ml of water. The reaction mixtures were warmed to 38° for 1 h. The complex-reversal treatment was carried out by dipping the chromatograms in benzaldehyde–light petroleum (b.p. 30–60°) (5:95) and permitting the chromatogram to dry in air for 2 days in the dark. Breakdown of the INH adducts was indicated by a change in R_F from that found in direction

TABLE IV

SEPARATION OF 3-PGA FROM P-HYDROXYPYRUVATE, GAP AND P-SERINE

P-Hydroxypyruvate, 3-PGA, GAP and P-serine were exposed to the INH reagent. Each chromatogram was spotted with two of the reaction mixtures: the 3-PGA reaction mixture and a reaction mixture containing one of the other compounds (P-hydroxypyruvate, GAP or P-serine). The chromatograms were developed first in direction 1, then washed with benzaldehyde–hexane solution and developed in direction 2. The migrations of each compound are expressed as R_F, R_{F1} for the first direction and R_{F2} for migration in the second direction. The solvent used was BAW, and the chromatographic support was Whatman No. 3MM paper. Experimental details are in the text.

Chromatogram	*3-PGA*		*P-hydroxypyruvate*		*GAP*		*P-serine*	
No.	R_{F1}	R_{F2}	R_{F1}	R_{F2}	R_{F1}	R_{F2}	R_{F1}	R_{F2}
1	0.77	0.30	0.58	0.56				
2	0.82	0.26	0.67*	0.52				
3	0.79	0.25	—	—	0.68	0.50		
4	0.80	0.27	—	—	0.68	0.50		
5	0.78	0.32	—	—	—	—	0.07	0.14
6	0.77	0.31	—	—	—	—	0.10	0.14

* Chromatogram front slightly skewed.

1 before benzaldehyde treatment to that found in direction 2 after this treatment (Table IV).

Chromatography was carried out in Whatman No. 3MM paper by the ascending technique, using BAW in both directions. Two spots were made on each chromatogram, in a staggered fashion at the lower right-hand corner of the chromatogram. One spot, the lowest and furthest to the right, contained 0.9 μmoles of the 3-PGA derivative; the other spot contained the same amount of one of the other INH complexes. Two replicate runs of each combination were made. Products were localized on the chromatogram by use of the molybdate staining reagent[3]. Since relatively small amounts of materials were applied (0.9 μmoles), the staining procedure was repeated three times. The chromatogram was exposed to ammonia vapor after each of the first two treatments; after the third application of the molybdate reagent, exposure to UV light was prolonged until all of the spots had been localized.

RESULTS AND DISCUSSION

Separation of compounds phosphorylated at C-3

In a mixture of phosphate esters, P-hydroxypyruvate can be separated from 3-PGA and P-serine by the reaction of the mixture with OPD, and subsequent paper chromatographic resolution of the derivatized and unchanged species using BAW as the developing solvent (Table III). Mixtures of this type from plant sources also contain GAP. The condensation products of GAP and P-hydroxypyruvate with OPD are co-chromatographed in this system. When the pulse-chase experimental system is used with $^{14}CO_2$ as the tracer, a relatively high proportion of the ^{14}C label in the phosphate ester fraction may be contained in GAP (ref. 1). For this reason, an aliquot of the mixture must be separated and the amount of label in GAP evaluated by the chromatographic system (BAW) in which unchanged GAP migrates at a different rate from unchanged P-hydroxypyruvate, 3-PGA and P-serine. 3-PGA forms

a complex with OPD, but this is not co-chromatographed with P-hydroxypyruvate (Table I). OPD complexes are readily detected by their fluorescence when excited by long-wavelength UV light. P-Serine does not react with OPD and its migration is not changed by the presence of OPD in the system.

The preparation of 3-GPA from P-hydroxypyruvate, P-serine and GAP is carried out using the BAW chromatographic system (Table IV). 3-PGA, P-hydroxypyruvate and GAP, but not P-serine, react with INH, and the R_F values of the resulting INH complexes differ from those of the unchanged compounds. Thus, by development in direction 1 with BAW, P-serine is separated from the INH complexes of P-hydroxypyruvate, GAP and 3-PGA. When treated with benzaldehyde in light petroleum the compounds do not move but the 3-PGA–INH complex is broken, while the P-hydroxypyruvate–INH and GAP–INH complexes remain stable. Thus, after this treatment, and development with BAW in direction 2, 3-PGA is separated from the INH complexes of P-hydroxypyruvate and GAP.

P-Serine can be separated from P-hydroxypyruvate, GAP and 3-PGA by a one-dimensional paper chromatographic system (EFW) (Fig. 1). Fig. 1 shows the separation of P-serine from other phosphorylated derivatives. Note that, when using ^{14}C-labeled leaf products, enhancement of separation of P-serine from hexose monophosphates is achieved by previous development with both BAW and 80% aqueous phenol (pH 5.4). Fig. 2 shows that P-serine is separated from other known previously labeled amino acids of photosynthetic pathways. This separation is also enhanced by predevelopment as described above. We also suggest that full extraction of P-serine may be accomplished by crushing and killing the plant material on the chromatogram[8], rather than the more common solvent extraction, concentration and spotting techniques. The relative insolubility of P-serine may contribute to its infrequent observation in plant extracts[1,9,10].

The methods described above permit the determination of P-hydroxypyruvate, 3-PGA and P-serine in the phosphate fraction of plant material. This separation of these three compounds has not been reported previously in plant materials. Previous paper chromatographic methods are subject to the problem of co-chromatography of P-hydroxypyruvate with 3-PGA and possibly of P-serine with hexose phosphates. Woods' precise and careful methods[11,12] are believed to be capable of separating P-hydroxypyruvate and 3-PGA, but the separation was not judged to be adequate for our uses. Paper electrophoresis will separate P-serine from other amino acids[5,7], but the separation from P-hydroxypyruvate and 3-PGA was not satisfactory. P-Serine may be separated from non-aminated compounds and then subjected to exchange resin chromatography. However, this procedure does not resolve P-hydroxypyruvate from 3-PGA.

At this point we have only used this technique in one plant, the bean (*Phaseolus vulgaris*), and for one purpose, $^{14}CO_2$ pulse-chase experiments. However, we believe that the results of this work, which show P-hydroxypyruvate and P-serine as important metabolites of early carbon fixation processes[1], demonstrate the usefulness of these procedures of separation.

CONCLUSIONS

3-PGA, P-serine and P-hydroxypyruvate were separated from each other and

from other photosynthetic intermediates by chemical derivatization and paper chromatography. These methods are designed, and have been used, for the separation of photosynthetic intermediates[1] under conditions where previous methods have been unsuccessful[2]. Additional observations include: evidence of previously unsuspected reactivities of phosphorylated α-hydroxy acids with OPD and INH; photodegradation of OPD derivatives; and conditions for acid hydrolysis of phosphoserine.

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