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Note

Ion-pair separation of 3-phosphoglyceric acid and 2-phosphoglycolate from ribulose-1,5-bisphosphate by high-performance liquid chromatography

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The compounds 3-phosphoglycerate (3-PGA) and 2-phosphoglycolate (2-PG) are products of ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase [EC 4.1.1.39][1,2]. This bifunctional enzyme catalyzes the primary carboxylation reaction in photosynthesis and the oxygenation reaction which leads to the production of glycolate, the substrate of photorespiration. In the carboxylation reaction, carbon dioxide combines with RuBP to form two moles 3-PGA (ref. 1). In the presence of oxygen, RuBP breaks down to one mole each of 3-PGA and 2-PG (ref. 2). The column chromatographic method which has been described previously for the separation of RuBP, 2-PG, and 3-PGA employs a low-pressure, gravity-flow, Dowex 2 (Cl^-) system[3]. Using the principle of ion-pairing, we have developed a high-performance liquid chromatographic (HPLC) method which permits the rapid isolation of 3-PGA from assay mixtures.

EXPERIMENTAL

Apparatus

Instrumentation for the development of this method included a Waters Assoc. (Milford, Mass., U.S.A.) Model ALC/GPC-244 liquid chromatograph which consisted of a Model 660 solvent programmer and two Model 6000A solvent delivery systems. Samples were initially monitored with either a Model 440 UV absorbance detector or a Model R401 differential refractometer interfaced with a Houston Instruments Omni-Scribe A5000 dual-pen recorder. Radioactive peak profiles were finally monitored as reported herein with a Beckman Model 100-C liquid scintillation counter following dropwise collection of eluate with a Model MFK Mini-Escargot Fractionator (Gilson Medical Electronics).

Reagents and sample preparation

The mobile phase was composed of an 0.05 *M* solution of tetrabutylammonium hydroxide (TBAH) adjusted to pH 7.3 with acetic acid. TBAH is available as an aqueous 40% methanol stock solution from Aldrich (Milwaukee, Wisc., U.S.A.). In

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initial studies, labeled 3-PGA was generated from RuBP and $^{14}CO_2$ in the RuBP carboxylase reaction: 0.6 m*M* RuBP, 10 m*M* [^{14}C]bicarbonate (0.5 μCi/μmole), 50 m*M* Tris (pH 8.2) and 0.025 mg enzyme (Sigma, St. Louis, Mo., U.S.A.) in a total reaction volume of 0.5 ml. Enzyme (5 mg/ml) was activated according to Lorimer *et al.*[4]. A 5-μl volume of the activated enzyme was then added to the reaction which was terminated after 5 to 10 min with perchloric acid. All procedures were performed at ambient temperature. [^{14}C]2-PG and [^{3}H]3-PGA were differentiated in the HPLC system using [1-^{14}C]RuBP or [5-^{3}H]RuBP as the substrate. Labeled RuBP was synthesized according to Wishnick and Lane[5] from [2-^{14}C]glucose and [6-^{3}H]glucose. With mixed oxygenase/carboxylase functions the ^{14}C-labeled substrate yields both labeled 3-PGA and 2-PG, while [5-^{3}H]RuBP yields only labeled 3-PGA (Fig. 1).

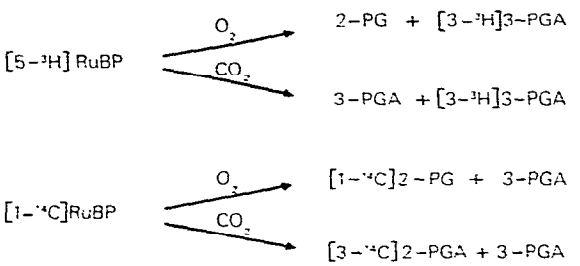

Fig. 1. Products of the bifunctional enzyme, RuBP carboxylase/oxygenase using [5-^{3}H] or [1-^{14}C]-RuBP with mixed co-substrates CO_2 and O_2. In the oxygenase reaction cleavage is generally represented as occurring between C-2 and C-3 of RuBP. In the carboxylase reaction O_2 condenses at C-2 with cleavage occurring at the same position.

Procedure

The 0.5-ml reaction volumes were quenched with 250 μl of 12% perchloric acid. The perchlorate-treated samples were neutralized to pH 7.3 $\pm$ 0.2 with 90 μl of 6 *M* KOH; the $KClO_4$ precipitate was compacted by centrifugation. Perchlorate treatment is essential to remove protein which would otherwise interfere with column performance and significantly reduce column life. Samples of 100 to 200 μl were injected directly into the chromatograph through the continuous-flow loop injector. Two 30 cm $\times$ 4 mm Bondapak C_{18} columns (Waters Assoc.) were used in series. The flow-rate was 1 ml/min; the pressure varied from 1200 to 1500 p.s.i. Under isocratic conditions with the 0.05 *M* tetrabutyl ammonium acetate (pH 7.3) as the mobile phase, the RuBP was retained on the column almost indefinitely for a series of runs. Tris and Mg^{2+} eluted with the solvent front. Five drops were collected per fraction with the Mini-Escargot fractionator which was interfaced with an event counter to accurately locate products. All procedures were performed at ambient temperatures.

RESULTS AND DISCUSSION

Labeled 3-PGA was resolved from contaminating compounds by ion-pair formation in which negatively charged carboxylate and phosphate groups "pair" with the positively charged tetrabutyl ammonium cation. As a result of its hydrophobic moieties, this cationic counterion exhibits an affinity for the hydrophobic Bondapak C_{18} stationary packing. The increased retention and differential migration of 3-PGA

in the presence of the pairing counterion is assumed to be either an ion-pair mechanism with formation of the complex in the mobile phase and subsequent retention[6] or adsorption of the hydrophobic pairing agent onto the reversed-phase packing material[7]. We were unable to develop a chromatographic system which would resolve all labeled compounds in either a single isocratic or non-isocratic run. Conditions which successfully eluted RuBP resulted in the co-elution of 3-PGA and 2-PG. The Dowex 2(Cl^-) method[3] reports that 3-PGA, 2-PG and RuBP elute in sequence using 0.05 *M* HCl–0.15 *M* NaCl as the buffer. We found that RuBP is retained on the column indefinitely and fails to elute even at higher ionic strengths and conditions of acidity. In the present HPLC system, the void volume was 6 min for a single isocratic run of 11 min. The retention times for 3-PGA and 2-PG were 8.25 and 9.1 min, respectively (Fig. 2). RuBP was completely retained on the column with no significant bleed-contamination in 10 consecutive runs. After a series of 5 runs, the RuBP was eluted in a 30-min flush with 100% methanol; no trace contamination remained on the column.

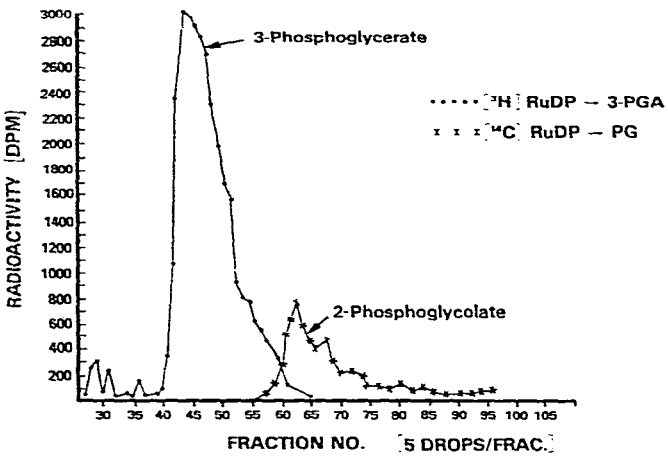

Fig. 2. Separation of 3-phosphoglycerate and 2-phosphoglycolate. [^{3}H]3-PGA was generated from [5-^{3}H]RuBP in the RuBP carboxylase reaction; the same product is produced by oxygenase activity. [^{14}C]2-PG was the product of the oxygenase reaction (high O_2 and an absence of CO_2) using [1-^{14}C]RuBP as the co-substrate. The chromatographic location of 3-PGA derived from labeled RuBP was confirmed by identifying the position of [^{14}C]3-PGA generated from unlabeled RuBP and $^{14}CO_2$, *i.e.*, [^{14}C]3-PGA is the only labeled product of such a reaction.

For continuous sample analysis we have established a regimen of 5 consecutive runs followed by the 30-min flush. For an 11-min run-period a 10-run cycle would require 3 h and 30 min per sample. A sample processor may be programmed to handle over 40 of these samples in a 24-h period.

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