Plant Physiol. (1977) 60, 109-114

Phosphoserine and Phosphohydroxypyruvic Acid

EVIDENCE FOR THEIR ROLE AS EARLY INTERMEDIATES IN PHOTOSYNTHESIS[1]

Received for publication December 14, 1976 and in revised form March 1, 1977

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ABSTRACT

Photosynthetic fixation of $^{14}CO_2$ in the bean *Phaseolus vulgaris*, cv. Pencil Pod Black Wax, resulted in the appearance of labeled compounds that were characterized as phosphoserine and phosphohydroxypyruvate by chromatographic separation and by the synthesis of chemical derivatives. In $^{14}CO_2/^{12}CO_2$ pulse-chase experiments these metabolites demonstrated the rapid pool saturation and depletion of ^{14}C characteristic of early intermediates in photosynthetic carbon fixation. They were present in sufficient amounts to account for about 35% of total carbon fixed in 1 minute.

The three compounds, P-hydroxypyruvate,[3] PGA, and P-serine, 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 do not differ in this series, and are part of a carboxyl group and the phosphorylated ester of a primary alcohol, respectively. As a result of this close similarity in structure, and because of the relatively hindered condition of the α carbon, direct chromatographic resolution of these compounds is difficult to the degree that the separation of P-hydroxypyruvate from PGA is cited as impractical (26). Partial separation has been achieved (30, 31) but the variable R_F values and small R_F differences were inadequate for our needs. Therefore, we developed other methods for separating P-hydroxypyruvate, PGA, and P-serine by chromatographic procedures and chemical derivatization. A more detailed analysis of the chemistry of these and related structures will be described elsewhere. This paper presents the results of $^{14}CO_2/^{12}CO_2$ pulse-chase experiments describing the kinetics of P-hydroxypyruvate, hydroxypyruvate, and P-serine together with the more conventional products of photosynthetic carboxylation and reduction in bean leaves.

MATERIALS AND METHODS

Plant Material. Beans (*Phaseolus vulgaris*, cv. Pencil Pod Black Wax) were planted in Perlite and grown in a greenhouse with supplementary nutrients. Primary leaves were used at the time the first trifoliate initials started to expand. The tip and some of the lateral lobes were removed from each leaf to make samples of identical size. Tests showed that this did not affect the rates of photosynthesis/unit area of leaf.

CO_2 Supply. Three leaves were placed with their petioles in a 10-ml beaker containing tap water and exposed to water-filtered incandescent light (1,000 μeinsteins m^{-2} sec^{-1}). The leaves were covered with a 260-ml bell jar containing air with about 400 μl/l CO_2, and 0.75 μCi $^{14}CO_2$ was introduced. The leaves were left for the required time at 25 C. The $^{12}CO_2$ chase was accomplished by removing the bell jar and allowing the $^{14}CO_2$ to disperse in a fume hood.

Preparation of Labeled Plant Material. When sampled, the plant material was dipped in dry ice-propanol to halt reactions. The petiole was snapped off and the rest of the leaf was crushed onto one corner of a sheet of Whatman 3MM chromatography paper (23 × 28 cm) using a stainless steel sampling die. The material was immediately killed by hot vapors from boiling 80% ethanol (2). The chromatogram was developed twice in the short direction with 0.8% (v/v) phenol in water (pH 5.4), and then twice in the same direction with BAW (butanol-acetic acid-water, 12:3:5, v/v/v). As monitored by radioautography and with phosphomolybdate and ninhydrin stains (20), this treatment divided the radioactive products of photosynthesis into three classes: (*a*) insoluble, remaining at the origin; (*b*) water-soluble, phosphate-positive, migrating slowly in phenol and BAW; and (*c*) water-soluble, ninhydrin-positive, phosphate-negative, fast migrating in phenol and BAW. These last two fractions were separated by a convenient endogenous fluorescent marker which was located by long wave UV irradiation (R_F 0.45). The UV irradiation of intermediates was minimized by rapidly scanning from the top of the chromatogram downward until reaching the marker, at which time the irradiation was discontinued. Each set of fractions *a*, *b*, and *c* (12 replicates for each of 12 time samples) was counted individually on paper in scintillation fluid. The amount of ^{14}C label in the parent phosphate fractions (fractions *b*) was statistically analyzed (Monroe 326 program 9216W). The data were found to fit best two parabolic regression curves, one for the pulse and one for the chase, which were statistically significant at levels of probability of 1% or less. The standard errors found were 0.14 and 0.095 × 10^6 dpm for pulse and chase, respectively. Fraction *b* pool size was 0.7 to 0.8 × 10^6 dpm/leaf. The recovery of ^{14}C in individual compounds assayed as described below was between 91% (fraction *c*) and 93% (fraction *b*) of ^{14}C applied to chromatograms.

All replicates of each fraction (*a*) or (*b*) were eluted with 0.8% (v/v) pH 5.4 phenol in water, pooled, and held frozen (−20 C). Dilute phenol was used to avoid bacterial contamination, whenever aqueous solutions of metabolites were prepared. In each of the methods described below, multiple aliquots were taken from each solution. The means of all analyses on each replicate are shown in Figures 1 and 2.

Relative Migration of Compounds Described. Relative migration (R_Fs) are described in Table I. Fraction *b* was found free from fractions *a* and *c* by two-dimensional chromatography

[1] Research supported by grants from the National Research Council of Canada to R. G. S. B.

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[3] Abbreviations: BAW: butanol-acetic acid-water (chromatographic solvent); EFW: ethanol-formic acid-water (chromatographic solvent); GAP: glyceraldehyde phosphate; HMP: hexose monophosphate; INH: isonicotinic acid hydrazide; OPD: *o*-phenylene diamine; PGA: 3-phosphoglyceric acid; P-hydroxypyruvate: phosphohydroxypyruvate; P-serine: *o*-phosphoserine; RuDP: ribulose diphosphate.

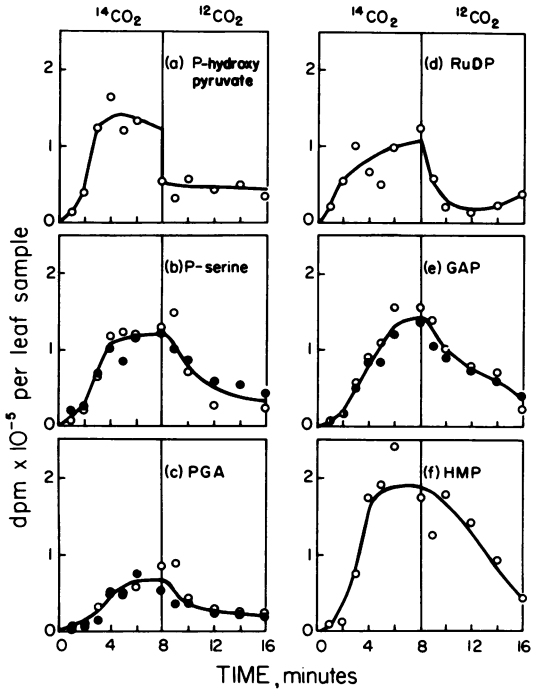

FIG. 1. Radioactivity in phosphorylated intermediates of fraction *b*. (a) P-hydroxypyruvate; (b) P-serine (○): by residue on OPD treatment and BAW development; (●): by EFW chromatography; (c) PGA (○): by OPD determination, (●): by reversible INH treatment; (d) RuDP; (e) GAP (○): by loss from R_F 0.35 to 0.45 on OPD treatment, (●): by EFW chromatography; (f) HMP.

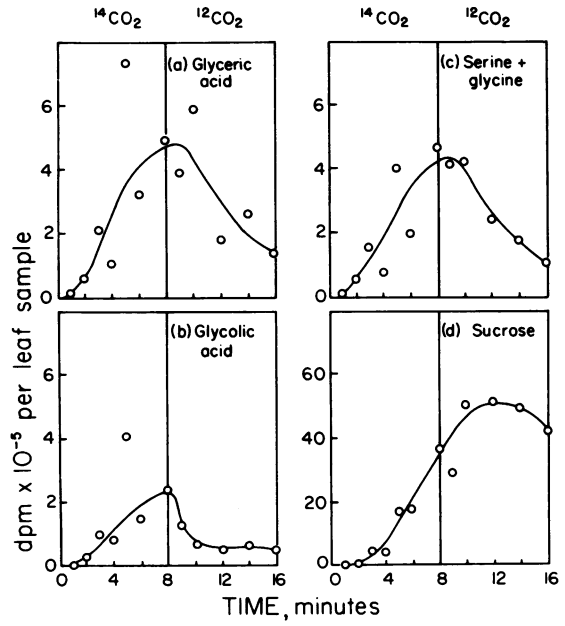

FIG. 2. Radioactivity in nonphosphorylated intermediates of fraction *c*. (a) glyceric acid; (b) glycolic acid; (c) serine + glycine; (d) sucrose.

(phenol *versus* BAW) as detected by radioautography. Fraction *c* was found free from fractions *a* and *b* by the same procedure.

Determination of Radioactivity. The radioactivity of samples was determined by liquid scintillation counting in PPO-POPOP-toluene (4 g: 0.4 g/l) or Omnifluor-toluene (4 g/l). Cpm were corrected to dpm by use of an external standard ratio calibrated quench curve. To avoid chemiluminescence, dark adaption was permitted before scintillation counting began.

OPD Condensation Products (Quinoxalinols). The OPD (4 mg/ml) reagent was prepared in 2 N HCl. An aliquot of the eluted phosphate fraction was treated with an equal volume of the reagent. The reaction was allowed to proceed for about 2 weeks at room temperature in darkness. Quinoxalinols are reported to be stable in the dark (17). Separation was carried out by ascending chromatography in the dark on Whatman No. 17 paper, developed once in BAW. The migration of standard P-hydroxypyruvate and hydroxypyruvate (P-hydroxypyruvate and hydroxypyruvate quinoxalinols) was detected by their fluorescence when excited by long wave UV light. The experimental sample sections were sliced from the chromatograms before UV irradiation of standards. Exposure of OPD-treated samples to UV or strong light was avoided, since we have observed photodecomposition of product quinoxalinols. Variation of migration between different sheets of paper was determined using 5,5',7,7'-indigotetrasulfonic acid (tetrapotassium salt) standards and by observing the yellow-brown color of untreated OPD and the pink, blue, and orange quinoxalinols formed. These spots were easily visible in dim light. Control reaction mixtures (without OPD and spotted in a final concentration of 1 N HCL) were run separate from OPD-treated samples since we observed that cross-contamination with OPD of samples chromatographed in the same tank was possible. The radioactivity in each section of the chromatogram was counted as described.

Determination of Sucrose, Serine-Glycine, Glycerate and Glycolate. These compounds, located in fraction *c*, were separated by standard two-dimensional chromatography (1, 13), located by autoradiography, and counted. The serine-glycine spot was eluted and rechromatographed in one direction twice on Whatman 3MM paper by the ascending technique using EFW-1 (95% ethanol-90% formic acid-water, 652:25:168, v/v/v). Serine and glycine were not separated, but a third component, also found in standards, was removed. The reference areas were cut away and stained with ninhydrin (20). The areas of the chromatograms corresponding to serine-glycine were decarboxylated to determine radioactivity in C-1 as described below.

Table I. Relative mobility of phosphorylated compounds, and some of their o-phenylene diamine (OPD) and isonicotinic hydrazide derivatives (INH) in different solvent systems and different papers. The term 'diphosphate' is the traditional description of the area of the chromatogram where RuDP is found, but which may contain other ^{14}C diphosphates when $^{14}CO_2$ pulse is long. The term HMP refers to unresolved mixes of G-1-P, G-6-P, F-1-P, and F-6-P. These terms are used to emphasize cochromatographic behavior of phosphorylated intermediates of photosynthesis. Please note positions of HMP and P-serine, and also Hydroxypyruvate-P and 3-PGA in solvent BAW.

	BAW		EFW-2
Paper (Whatman No.)	3MM	17	3MM
Compound		R_f	
'diphosphates'	0.07	0.09	--
RuDP	--	--	0.15
Fructose 1,6 diP	--	--	0.20
Serine-P	0.12	0.14	0.46
HMP	0.13	0.16	--
Glucose-1-P	--	--	0.51
Glucose-6-P	--	--	0.53
Fructose-1-P	--	--	0.55
Fructose-6-P	--	--	0.57
3-PGA	0.21	0.27	0.60
Hydroxypyruvate-P	0.24	0.28	0.60
Glyceraldehyde-3-P	0.31	0.38	0.66
OPD adducts of:			
3 PGA	0.60	0.68	--
P-hydroxypyruvate & GAP	0.68	0.77	--
INH adducts of:			
GAP	0.67	--	--
P-hydroxypyruvate	0.68	--	--
3-PGA (reversible)	0.80	--	--

Hydrolysis of P-serine to Serine. Ninhydrin decarboxylation of P-serine was found to be slow and not quantitative. Therefore aliquots of fraction *b* containing carried P-serine were treated under N_2 with 6 N HCl at 130 C (autoclave) for 16 hr. Serine was resolved from the hydrolysate by two-dimensional paper chromatography on Whatman 3MM paper using 80% (v/v) phenol (pH 5.4), and in the second direction 1-propanol-ethyl acetate-water, 7:2:4 (v/v/v) (13) run twice. Serine was located on reference chromatograms by ninhydrin, and radioactive serine from the sample hydrolysate was located by radioautography and by comparison with the reference chromatograms. Unhydrolyzed samples of fraction *b* had no detectable serine. The serine spots were cut out, their radioactivity determined, then they were decarboxylated.

Decarboxylation of Amino Acids. The sections of the paper chromatograms containing the amino acids in question were washed with toluene and dried on a pin board. To each, 0.1 ml of 0.23 M (pH 5.6) citrate (Na^+) buffer which also contained 50 μg/ml of carrier amino acid was applied and the papers were air-dried. Standard specifically labeled amino acids ([U-^{14}C]serine, [3-^{14}C]serine, [U-^{14}C]glycine, [2-^{14}C]glycine) were applied to paper and prepared in the same way. The total radioactivity of each sample was measured. The samples were then placed in test tubes (0.8 × 7.4 cm) and covered with 1 ml of a freshly prepared mixture of 65% (v/v) anhydrous DMSO containing 6% (w/v) ninhydrin and 35% (v/v) (pH 5.4) 0.23 M sodium citrate. This buffer was made 4 mg/ml in semicarbazide hydrochloride, which acts as a chemical trap for volatile aldehydes produced by ninhydrin action on amino acids. The final pH of this mixture was 7.7 due to the inclusion of DMSO in the mixture. Each test tube was placed inside a 12-ml conical centrifuge tube containing 1.5 ml of ethanolamine-methyl Cellosolve, 2:5 (v/v). The centrifuge tube was closed with a serum stopper and the apparatus was incubated at 37 C overnight. In the morning the mixture was a dark purple. After incubation and cooling, 50 μl of 10 mg/ml INH in 1 M H_2SO_4 was injected carefully with a syringe through the serum stopper into the small test tube. The reaction was allowed to proceed for about 30 min. Then 1 ml of 50% H_2SO_4 was added to the small test tube by injection through the stopper. The solution in the test tube bubbled, releasing CO_2 and turning from purple to yellow. This color change was attributed to the formation of an adduct of the residual carbon skeleton of the amino acid with semicarbazide and/or INH. The evolved CO_2 was absorbed by the ethanolamine solution and its radioactivity was determined. The background was measured from unlabeled samples quenched with ethanolamine-methyl Cellosolve. Standard amino acids of known isotopic distribution showed quantitative and complete decarboxylation and less than 0.5 contamination of the carboxyl fraction by other carbons from the molecules (Table II).

The data from 1 to 6 min were fitted to a second degree polynomial, with these resulting best fits for P-serine and serine, respectively: $y = 66.83 - 12.18179t + 1.45536t^2$, and $y = 10.32 + 11.15179t - 1.57679t^2$. In these equations y is per cent label in C-1 and t is time in min. Both linear and quadratic terms of these equations were significant at the 5% level. Examination of the linear and quadratic effects of both equations shows that the lines generated by these equations are simple mirror images of each other. The program used was the Statistical Analysis System, designed and implemented by A. J. Barr and J. H. Goodnight (North Carolina State Univ).

Determination of P-hydroxypyruvate. Aliquots of fraction *b* from each sample plus standard PHOP were reacted with OPD as described—a second series of aliquots was treated in the same way but OPD was omitted. The two series were divided into subaliquots and three subaliquots/time interval in each series were chromatographed on Whatman No. 17 paper with BAW. All operations were carried out under dim, indirect incandescent light. The area of the chromatogram corresponding to the condensation product of P-hydroxypyruvate and OPD (P-hydroxypyruvate quinoxalinol), R_F 0.72 to 0.81, was determined by comparison with authentic P-hydroxypyruvate quinoxalinol. The chromatograms were sliced to separate standards from sample P-hydroxypyruvate quinoxalinol. The location of the standard was determined by fluorescence under long wavelength UV light, and the radioactivity of this section from the sample chromatograms was measured. To determine the net radioactivity corresponding to P-hydroxypyruvate quinoxalinol, values for GAP-OPD condensation products and for the small amounts of label found without OPD treatment in this part of the chromatograms were subtracted. The background (minus-OPD) for this part of the chromatograms was about 200 dpm or less, and was never more than 16% of that found with OPD-treated samples. Untreated P-hydroxypyruvate co-chromatograms with PGA, but OPD-treated P-hydroxypyruvate runs ahead of PGA (Table I).

Table II. Decarboxylation of specifically labeled amino acids

	Total ^{14}C	Carboxyl ^{14}C	^{14}C in carboxyl
	dpm		%
(U-^{14}C)Serine	67,755	24,431	36.06
	44,170	15,588	35.29
	41,446	13,075	31.55
			Avs. 34.3
(3-^{14}C)Serine	29,883	161	0.54
	24,778	146	0.59
	57,951	209	0.36
			Avs. 0.5
(U-^{14}C)Glycine	4,048	1,968	48.62
	47,598	24,646	51.78
			Avs. 50.2
(2-^{14}C)Glycine	117,714	100	0.08
	153,846	201	0.13
	64,515	121	0.19
			Avs. 0.1

Determination of GAP. Aliquots of fractions *b* for each sample were treated with or without OPD as above, GAP cannot form a quinoxalinol, but we found that it does form a condensation product with OPD. Since the label corresponding to GAP on OPD treatment moves from a low R_F (0.35–0.45) to a high R_F (0.72–0.81), GAP can be determined by the loss of label from the lower R_F area. The background determined in the plus-OPD series (the radioactivity left at the low R_F) was subtracted from the amount of label originally found at the low R_F in the minus-OPD series. The difference is considered to be GAP. GAP was also determined by ascending paper chromatography in EFW-1 with good agreement.

Determination of PGA. Reversibility of hydrazide reaction products by excess ketones, usually benzaldehyde or formaldehyde, is a standard procedure of organic chemistry (18). This particular technique is based on the reversibility of the PGA-INH complex by benzaldehyde. P-hydroxypyruvate and GAP also form complexes with INH but these complexes are not reversible under our conditions. Aliquots (10 ml) of fraction *b* for each sample and also standards of P-hydroxypyruvate, GAP, PGA, P-serine, and phosphoglycolic acid (18 μg of each plus 1 ml of 0.8% phenol in water) were placed in separate beakers. Ethanol (95%, 53 ml) and concentrated HCl (25 μl) were added to each beaker. The solution was allowed to evaporate, with mild heat, reducing volume to about 1 ml, and evaporation was completed at room temperature. Complete desiccation at warm temperatures was avoided since the HCl-H_2O azeotrope is 5.6 N in HCl. Another aliquot (10 ml) of each fraction *b* was added, the beakers containing the standards received 10 ml of water. INH (0.185 mg/ml) was added in 50 ml of 95% ethanol-95%

formic acid, 19:1 (v/v), and the contents of the beakers were again allowed to evaporate. The contents of each beaker were transferred with ethanol and water washes to a Whatman 3MM paper chromatogram and developed twice in BAW in the long direction. The migration of standards was checked by rapid observation of the weak fluorescence of INH derivatives under long wave UV. The chromatograms were then washed twice in benzaldehyde-hexane, 1:19 (v/v), air-dried overnight, and developed once with BAW in the short direction. The chromatograms bearing fraction *b* were then air-dried again and radioautographed. The chromatograms of the standards were dried and sprayed with molybdate phosphate spray (20) producing a stain that was activated by short wave UV after IR treatment. The background was reduced by ammonium vapors. The process was repeated until staining was satisfactory. Radioactivity in the PGA spot was then determined.

Determination of HMP and RuDP. These were obtained from the corresponding areas of chromatograms of untreated samples developed with BAW or EFW-1. HMP was corrected for P-serine on BAW chromatograms, using data from EFW-1 chromatography.

Determination of P-serine. Aliquots of fraction *b* for each sample were chromatographed on Whatman 3MM paper with EFW-2 (95% ethanol-90% formic acid-water, 625:21:168, v/v/v). Standards of *o*-phospho-DL-serine and PGA (40 μg/spot) were run on each chromatogram. This solvent system was sufficient to resolve P-serine from the rest of the components found in fraction *b*. However, resolution of P-serine from unfractionated samples required predevelopment in the same direction with 80% phenol followed by BAW.

P-serine Formation as Affected by Oxygen. Fully expanded trifoliate leaves were allowed to photosynthesize as described earlier in a 260-ml bell jar with flowing N_2 containing 350 μl/l CO_2 and three levels of O_2: 1%, 21%, and 48%. After several min the flow of gas was stopped and 5 μCi of $^{14}CO_2$ was introduced into the bell jar. After 5 min the chamber was opened and leaf discs were cut and chilled at once in liquid N_2. The discs were crushed onto chromatograms and immediately treated with vapors of boiling 80% ethanol. Carrier P-serine (30 μg) was added to each chromatogram and a P-serine standard was placed beside the leaf disc on each chromatogram. The chromatograms were developed by the ascending technique, successively, in the same direction with 80% phenol, BAW, 80% phenol, and EFW-2, allowing 24-hr air-drying between development. The chromatograms were sliced into longitudinal strips and the position of P-serine standards was determined by ninhydrin dip (20). The strips of the chromatogram containing the samples were divided into three sections: insoluble material found at the origin, P-serine, and other soluble materials (balance). Three replicates were taken of all samples and the whole experiment was repeated.

RESULTS AND DISCUSSION

Details of the chemical characterization of P-hydroxypyruvate, hydroxypyruvate, and P-serine will be presented elsewhere. We feel that the procedures of the chromatographic separation of these compounds and their derivatives, as compared at every step with authentic compounds and their derivatives, provide sufficient evidence for their identification. Wherever two different methods were used to estimate any given compound (*e.g.* PGA, GAP, P-serine) they are identified on the figures by contrasting symbols; the two methods were almost always in close agreement.

Data for the pulse-chase kinetics of ^{14}C in the identified intermediates of photosynthesis are shown in Figures. 1 and 2. Fraction *b* contains phosphorylated compounds and includes the main intermediates of the photosynthetic cycle. P-hydroxypyruvate incorporated $^{14}CO_2$ in the pulse and lost it during the $^{12}CO_2$ chase in a manner consistent with an early role of this compound as a metabolite in photosynthetic carbon fixation (Fig. 1a). All other phosphorylated intermediates, RuDP, P-serine, GAP, PGA, and HMP (Figs. 1, b, c, d, e, f), saturate at about the same time, 4 min. We suggest that this is due to a complex PGA pool. Total cellular PGA does not saturate as fast as RuDP (19) and there is a rapid turnover of RuDP (16).

The principal components of fraction *c* are sucrose, serine-glycine, glycerate and glycolate. As expected, these compounds had pulse-chase patterns that showed later saturation in the pulse and slower disappearance of ^{14}C in the chase than did the metabolites of fraction *b* (Fig. 2). The glycerate pool (Fig. 2a) filled and flushed more slowly than PGA. This indicates that glycerate is not produced as an artifact of extraction by the dephosphorylation of PGA. Inspection of Figures 1 and 2 shows that P-hydroxypyruvate cannot be derived from glycerate. The kinetics of sucrose (Fig. 2d) is that of a metabolic end product.

The kinetics of fraction *a*, which contains the insoluble products of photosynthesis, is not presented here. Fraction *a* would contain mainly starch and a small amount of protein in an experiment of this duration, and it accumulated only a small proportion of the total label. It did not lose label during the chase, indicating that its main components were end products of metabolism.

The kinetics of $^{14}CO_2$ incorporation in these experiments is somewhat slower than that found by others (15). We attribute this to the relatively slow rate of $^{14}CO_2$ equilibration in the leaf cuvette, and the rather slow metabolism of the bean leaf (8, 27). Since the pulse was quite long (8 min) this does not affect the analysis of the pulse, and the chase was unaffected. Indeed, this slow incorporation amplifies the sequential differences between metabolite saturation curves, which accord with published data for the established metabolites (3, 27).

The data showing the results of the ninhydrin decarboxylation of P-serine and serine-glycine are presented in Figure 3. The serine-glycine fraction was not resolved, but was purified away from an unknown contaminant. Inspection of the chromatograms indicated that a relatively smaller proportion of glycine than serine was present in these spots.

The data presented in Figure 3 suggest three main points. First, serine and P-serine are not closely related metabolically. Second, the data suggest that serine-glycine was derived from the operation of the glycolate pathway (24) since the proportion of its total radioactivity in the carboxyls was initially low but rose to nearly 40% by 8 min. The third point is that P-serine behaves as if it were related to a product of C_3 carboxylation, whose normal labeling pattern is to be largely carboxyl-labeled, ini-

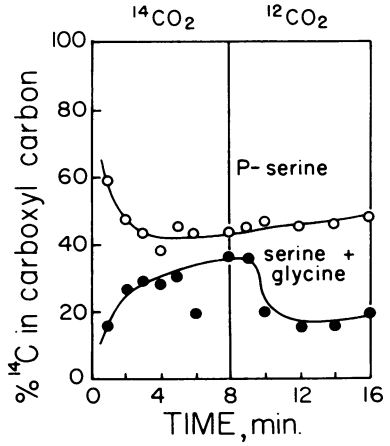

FIG. 3. Percentage of radioactivity in carboxyl groups of P-Serine and serine + glycine fractions.

Table III. The effect of oxygen on P-serine formation

O_2	Insoluble compounds at origin		P-serine		All other soluble compounds		Total	
%	dpmX10^{-3}±SD	% total ^{14}C	dpmX10^{-3}±SD	% total ^{14}C	dpmx10^{-3}±SD	% total ^{14}C	dpmx10^{-3}±SD	% total ^{14}C
0	156.4±41.1	(17.6)	67.7±29.7	(7.6)	666.9±235	(74.8)	891.0±385	(100)
0	160.8±23.3	(23.6)	66.7±9.6	(9.8)	455.1±106	(66.6)	682.2±431	(100)
21	72.0±32.4	(11.1)	50.4±7.1	(7.7)	528.6±113	(81.2)	651.0±149	(100)
21	125.5±56.0	(18.1)	42.4±6.8	(6.1)	527.2±175	(75.8)	695.1±122	(100)
48	92.3±13.5	(13.3)	61.5±18.4	(8.9)	541.1±72.0	(77.8)	694.9±102	(100)
48	82.1±11.6	(21.8)	38.1±10.0	(10.1)	256.4±57.6	(68.1)	376.6±70	(100)

tially, and to become equally labeled in all carbons by the time the pool is saturated with ^{14}C (approximately 8 min from Fig. 1b). It is likely that P-serine is derived from P-hydroxypyruvate, since P-hydroxypyruvate is the corresponding keto acid. In turn, this suggests that P-hydroxypyruvate is initially carboxyl-labeled, and is, itself, a product of, or derived from, C_3 carboxylation. The metabolic independence of serine and phosphoserine, and the importance of the latter as an early product of photosynthesis in maize, have recently been elegantly demonstrated by Chapman and Leech (4).

Experiments were conducted at different levels of O_2 in order to determine whether P-serine is associated with the metabolism of photorespiration. P-serine levels decreased slightly under increased O_2, but the percentage of ^{14}C entering P-serine was unchanged at all levels of O_2 (Table III). Thus, P-serine shows little indication of participation in photorespiration. P-serine might have a sparing action on C-1 loss from P-hydroxypyruvate or hydroxypyruvate (formed by phosphatase action (25) on P-hydroxypyruvate—which is not unlikely). This C-1 loss occurs spontaneously (10) and enzymically (6, 11, 12) with hydroxypyruvate, and has been found to occur spontaneously at a lower rate with P-hydroxypyruvate (unpublished observation). Whether this C-1 loss is large enough to contribute to photorespiration is not known. The C-2 of hydroxypyruvate may proceed down the metabolic paths of photosynthetic carbon fixation (21), and so may be a precursor carbon for photorespiratory CO_2. Thus, P-serine formation may conserve reduced carbon by decreasing the pool size of P-hydroxypyruvate. A role for serine as an endogenous chelating agent has also been proposed (23).

P-hydroxypyruvate and P-serine have so far escaped detection as important metabolites of photosynthetic carbon fixation, probably because P-hydroxypyruvate co-chromatographs almost exactly with PGA in all of the commonly used solvent systems (Table I). P-hydroxypyruvate is also very labile compared with PGA and the product of its dephosphorylation, hydroxypyruvate, is much more labile than glyceric acid (10). P-serine has been found before as a plant metabolite (29), but only recently has been considered an early product of photosynthesis (4). Several reasons for this are suggested. It is possible that P-serine has such a function only in beans and, perhaps, also in wheat (29) and maize (4), but this seems unlikely. It seems more probable that it has escaped detection because in the presence of metals, especially ferric ions, it forms chelates that are often insoluble (23). It is noted that in synthesis of P-serine acidic conditions and much larger volumes are used before cations are removed (9). When P-serine is prepared from phosphorylated proteins, by acid hydrolysis, cations are removed by precipitation at high pH (14). We found that commercially available P-serine is much less soluble than serine (2 mg/μl *versus* 300 mg/μl at 25 C). Also commercial preparations of P-serine yield acidic solutions with pH of 2 or less, which enhances solubility. In 80% ethanol, in which serine is much less soluble than water, commercial P-serine is even less soluble than serine. We suggest that this is due to the presence of metals (28). We also suggest that P-serine extracted from plants may be complexed to plant cations (7) which may make extraction of P-serine at the less acidic and neutral pH values difficult by the usual procedures. Furthermore, P-serine migrates to the general area of HMP in commonly used solvent systems, but not in EFW-2 and so might easily escape detection (Table I).

We have observed that its exact chromatographic location of P-serine tends to be variable (R_F 0.45 is the high limit, achieved with use of carrier 30 μg) and is believed to be determined by such factors as the amount loaded, the presence of cations (23), and the relative amounts of negative charges on different papers (Table I).

The possibility that the intermediates P-hydroxypyruvate and P-serine are artifacts of the extraction and characterization procedure seems remote. The pulse-chase kinetics of these compounds is distinct and quite different with respect to serine or glycerate which might serve as precursors. P-hydroxypyruvate and P-serine reach half-saturation before three min of $^{14}CO_2$ supply, while glycerate and serine saturate at later times. In general, there is little quantitative relationship between the curves for P-hydroxypyruvate and P-serine and those for compounds which might artificially give rise to them. The possibility of the condensation of serine with phosphate to form P-serine during extraction is negligible due to the known rigorous conditions of this reaction (14, 22).

The quantitative importance of P-serine and P-hydroxypyruvate as intermediates is suggested by their saturation levels of ^{14}C and turnover kinetics shown in Figure 1. The values in Table IV show that these two intermediates comprise 35% of the fixed ^{14}C in the photosynthetic intermediates at 1 min, and 18% at 6 min. Since they are intermediates in a cyclic process, the values in Table IV reflect pool size rather than the amount of carbon flowing through the pools. However, the data in Figure 1 and Table IV indicate that P-hydroxypyruvate and P-serine are major and early intermediates in photosynthesis.

Table IV. Radioactivity in intermediates (sucrose is not included) of bean leaf photosynthesis in $^{14}CO_2$

	one-minute sample		six-minute sample	
	^{14}C content dpm x 10^{-4}	% of ^{14}C in intermediates	^{14}C content dpm x 10^{-4}	% of ^{14}C in intermediates
P-hydroxypyruvate	1.2	19	13.3	9
P-serine	1.05	16	12.5	9
PGA	0.45	7	6.7	5
RuDP	1.8	28	10.0	7
GAP	0.6	9	13.3	9
HMP	0.6	9	24.2	17
Glycerate	0.4	6	31.7	22
Glycolate	0	0	14.2	10
Serine & glycine	0.3	5	19.2	13

Figure 1 indicates that P-hydroxypyruvate may be formed simultaneously with or independently from PGA, rather than being derived from it. The data in Figure 3 suggest that P-serine (and, presumably, its keto acid P-hydroxypyruvate) are formed as a result of a carboxylation reaction. This is in accord with the earlier proposal that P-hydroxypyruvate arises as the result of a carboxylation of RuDP (5).

A substantial amount of photorespiratory CO_2 might arise from the spontaneous or enzymic decarboxylation of P-hydroxypyruvate or hydroxypyruvate. While the data presented here are consistent with such a hypothesis, the proof must await additional evidence. However, these data strongly support an important role of P-hydroxypyruvate and P-serine in the early metabolism of carbon fixation in the primary leaf of the bean.

Acknowledgments—The authors are grateful to G. Couvillon and G. Ware for assistance with the statistical analyses, and to H. M. Vines for technical advice and support at the University of Georgia, Athens.

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