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SERINE BIOSYNTHESIS FROM HYDROXYPYRUVATE IN PLANTS

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Abstract—Serine biosynthesis in plant extracts has been shown to occur via a transamination reaction between L-alanine and hydroxypyruvate. L-Alanine is the most active amino group donor of a number of amino acids that have been investigated. Green leaves are the richest source of the alanine : hydroxypyruvate transaminase.

INTRODUCTION

OTHER investigators have demonstrated that phosphoserine may be formed by a transamination reaction between phosphohydroxypyruvate and L-glutamate in both plant[1] and animal[2] systems. A second pathway in mammals for the biosynthesis of serine is by a transamination reaction between hydroxypyruvate and L-alanine.[3] This transaminase (L-alanine : hydroxypyruvate aminotransferase) has been shown to be specific for alanine as the amino group donor and has only slight activity with either glycine or glutamate. D-Glycerate dehydrogenase (D-glycerate : NAD oxidoreductase), the enzyme that catalyzes the oxidation of D-glycerate to hydroxypyruvate in the presence of nicotinamide adenine dinucleotide (NAD), is widely distributed in higher plants and is particularly active in green leaves.[4] In view of these results it was of interest to investigate the possible occurrence of the alanine : hydroxypyruvate transaminase in plants. Evidence is presented in this communication that green plants contain the transaminase with the same specificity for the amino group donor as the mammalian enzyme.

RESULTS

The distribution of plant alanine : hydroxypyruvate transaminase is given in Table 1. The highest activities were observed in green leaves. It is interesting to note that this distribution parallels that of D-glycerate dehydrogenase,[4] the enzyme that catalyzes the formation of one of the substrates for this transaminase.

Typical curves were obtained for the time course of the forward reaction (Fig. 1) and the enzyme concentration curve (Fig. 2). The pH optimum in borate buffer was found to be 8·9 (Fig. 3). With sodium barbital in place of borate buffer, the activity was essentially the same in the pH range 8·5–9·0.

Quantitative data for serine formation from hydroxypyruvate and various amino acids are presented in Table 2. L-Alanine was the most active of the amino group donors investigated. This is in agreement with the observations made on the mammalian enzyme[3] which, however, exhibited a much lower activity with glycine than found in the present studies. Individual control experiments were carried out with each of the amino acids in the absence of hydroxypyruvate. In no case was serine formation observed with the amino

[1] J. HANFORD and D. D. DAVIES, *Nature* **182**, 532 (1958).
[2] A. ICHIHARA and D. M. GREENBERG, *J. Biol. Chem.* **224**, 331 (1957).
[3] H. J. SALLACH, *J. Biol. Chem.* **223**, 1101 (1956).
[4] H. A. STAFFORD, A. MAGALDI and B. VENNESLAND, *J. Biol. Chem.* **207**, 621 (1954).

TABLE 1. DISTRIBUTION OF L-ALANINE : HYDROXYPYRUVATE TRANSAMINASE

Source	Total serine formed* (μmole)	Protein† (mg)
Beet Leaves (*Beta vulgaris*, L.)	7·3	3·2
Snap Bean Leaves (*Phaseolus vulgarus*, L.)	6·3	11·2
Parsley Leaves (*Petroselinum crispum*, Nym.)	5·1	2·7
Spinach Leaves (*Spinacia oleracea*, L.)	4·4	3·8
Broccoli (*Brassica oleracea*, L.)	2·5	3·1
Radish Leaves (*Raphanus sativus*, L.)	2·1	3·2
Lettuce Leaves (*Latuca sativa*, L.)	1·3	1·1
Endive (*Cichorium endivia*, L.)	1·3	1·8
Asparagus (*Asparagus officinalis*, L.)	0·5	3·0
Pea Pods (*Pisum sativum*, L.)	0·7	1·1
Peas (Green)	0·6	9·5
Snap Bean (Green pod and seed)	1·5	3·3
Radish Root	<0·2	1·0
Snap Bean Seeds	<0·2	12·2
Soybean Seeds (*Glycine max*, Merr.)	<0·2	13·0
Pea Seeds	<0·2	10·5

The incubation systems contained: L-alanine (50 μmole), hydroxypyruvate (50 μmole), pyridoxal phosphate (0·1 μmole) and enzyme in a total volume of 3·0 ml, 0·1 *M* phosphate buffer, pH 8·0.

* After one hour (see Experimental).
† Mg of protein in incubation system.

acids alone. Serine formation in the complete system was confirmed qualitatively by paper chromatography of the reaction mixtures.

As proof that the reaction observed was one of transamination, a balance study of reactants and products was made to establish the net stoichiometry of the forward reaction. The data (Table 3) clearly demonstrate that equimolar quantities of serine and pyruvate are formed and are accompanied by an equivalent decrease in the concentration of alanine and hydroxypyruvate.

The stoichiometry of the reverse reaction has not been determined. However, when serine and pyruvate are incubated with the transaminase in the presence of D-glycerate dehydrogenase and reduced nicotinamide adenine dinucleotide ($NADH_2$), the formation of hydroxypyruvate may be demonstrated (and followed) by the oxidation of the reduced coenzyme (Fig. 4). In order to demonstrate the formation of alanine, the other product

TABLE 2. SERINE FORMATION FROM HYDROXYPYRUVATE AND VARIOUS AMINO ACIDS

Amino acid added	Total serine (μmole)
None	0
L-Alanine	8·1
Glycine	3·8
β-Alanine	0
L-Glutamate	0·1
L-Aspartate	0
L-Leucine	0

The incubation systems contained amino acid (50 μmole) where indicated, hydroxypyruvate (50 μmole), pyridoxal phosphate (0·1 μmole) and enzyme (6·0 mg of protein) in a total volume of 3·0 ml, 0·1 *M* phosphate buffer, pH 8·0.

of the reverse reaction, this experiment was repeated with 1-C^{14}-labelled pyruvate and L-serine. After a 30-min incubation, the reaction was stopped by the addition of perchloric acid. An aliquot of the deproteinized reaction mixture was chromatographed on the amino acid analyzer and counted as described under Methods. A radioactive, ninhydrin positive

TABLE 3. STOICHIOMETRY OF THE FORWARD REACTION

Incubation time	Total concentration (μmole) in reaction system			
	Alanine	Hydroxypyruvate	Serine	Pyruvate
Zero time	19·1	18·6	0	0
1 hr	11·3	11·8*	7·2	7·1

The incubation system contained pyridoxal phosphate (0·1 μmole), enzyme (10 mg of protein), alanine and hydroxypyruvate (as indicated for zero time) in a total volume of 2·0 ml, 0·1 *M* phosphate buffer, pH 8·0

* Corrected for nonenzymatic decomposition as determined in control.

compound was detected in the alanine area. In an analogous experiment, in which alanine was added at the end of the incubation period, radioactivity accompanied the carrier alanine. On the basis of these experiments the products of the reverse reaction were identified as hydroxypyruvate and alanine.

DISCUSSION

Using soybean leaves (*Glycine max*, Merr., var. Hawkeye) Vernon and Aronoff[5] have reported that the only amino acids found to contain radioactivity from $^{14}CO_2$ during 15 and 50 sec photosynthetic periods are alanine, serine, and glycine, in order of decreasing specific and total activity. In addition serine has been found to incorporate radioactivity from $^{14}CO_2$ in similar experiments with *Scenedesmus obliquus*, leaves of barley (*Hordeum*

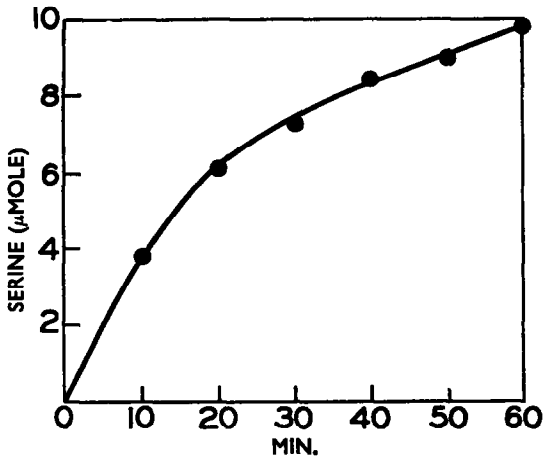

FIG. 1. TIME COURSE OF THE FORWARD REACTION
The reaction mixture contained the following in a total volume of 3·0 ml: 50 μmole of L-alanine; 50 μmole of hydroxypyruvate; 0·1 μmole of pyridoxal phosphate; 200 μmole of sodium barbital buffer, pH 9·0; and transaminase preparation (8·5 mg of protein).

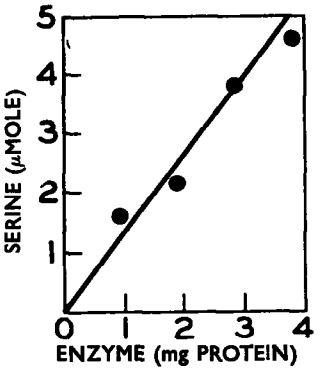

FIG. 2. ENZYME CONCENTRATION CURVE
The incubation conditions were the same as in Table 2, except varying amounts of transaminase were added.

[5] L. P. VERNON and S. ARONOFF, *Arch. Biochem.* 29, 179 (1950).

vulgare, L.) and geranium leaves (*Pelargonium sp.*).[6] Aronoff[7] has shown that glyceric acid is one of the primary organic acids produced during short term photosynthesis experiments (5–90 sec) in soybean leaves. The evidence presented in this communication for a plant alanine : hydroxypyruvate transaminase allows the formulation of the following series of

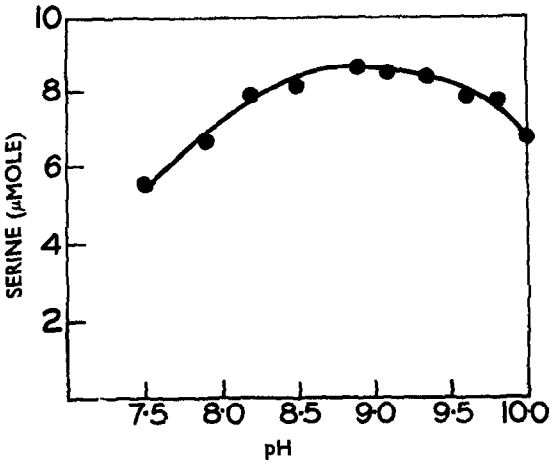

FIG. 3. THE pH OPTIMUM OF THE FORWARD REACTION
The incubation conditions were the same as in Fig. 1, except that barbital buffer was replaced by 100 μmole of borate buffer.

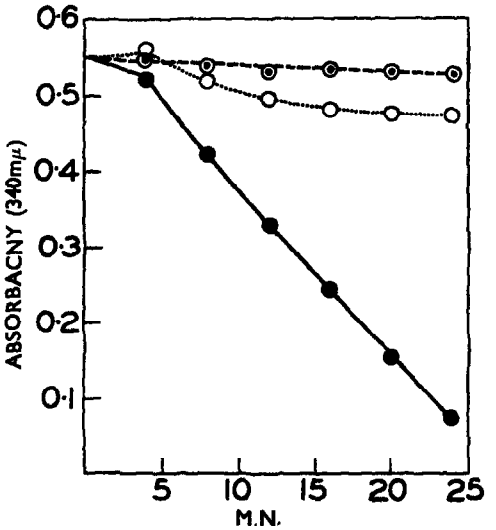

FIG. 4. TIME COURSE OF THE REVERSE REACTION
The complete reaction mixture consisted of 200 μmole of sodium barbital, pH 9·0; 0·1 μmole of pyridoxal phosphate; 0·27 μmole of $NADH_2$; 10 μmole of serine; 10 μmole of pyruvate, added last; plant D-glycerate dehydrogenase (0·7 mg of protein); transaminase preparation (3·8 mg of protein); in a final volume of 3·0 ml, contained in 1·0 cm cuvettes. The incubation was carried out at 25°. The above results have been corrected for the slight optical density change observed with a control containing all of the components except serine and pyruvate. ● .. ●, complete, serine plus pyruvate; O....O, pyruvate control; O....O, serine control.

[6] M. CALVIN, *J. Chem. Education* **26**, 639 (1949).
[7] S. ARONOFF, *Arch. Biochem.* **32**, 237 (1951).

reactions for the biosynthesis of serine: D-glycerate⇌hydroxypyruvate⇌L-serine. D-Glycerate could arise from either 2- or 3-phosphoglycerate by the action of a phosphatase. Joyce and Grisolia[8] have purified a non-specific phosphatase from wheat germ that dephosphorylates these two compounds. In addition, an acid phosphatase that acts on 3-phosphoglycerate has been partially purified from pea seed.[9]

Kretovich and Stepanovich[10] have reported that serine is synthesized from hydroxypyruvate in crude homogenates of wheat and pea seedlings. The process is associated with a decrease in the amount of a number of amino acids and amides in the enzyme preparations used. These authors suggest that transamination reactions may be involved. Preliminary studies in this laboratory have indicated that the alanine : hydroxypyruvate transaminase is present in wheat seedlings but at a much lower level than that found in green leaves.[11]

McConnell and Finlayson[12] concluded that serine is an excellent precursor of carbohydrate in wheat plants since more than one half of the total radioactivity derived from 3-^{14}C-L-serine in wheat kernels was found in starch. One way to explain their results would be by a reversal of the reaction sequence formulated above together with a D-glycerate kinase. Such an enzyme has been isolated from a Polish variety of rape seed (*Brassica campestris*, L.) by Ozaki and Wetter[13] and yields 3-phosphoglycerate.

In their discussion of the metabolic role of plant D-glycerate dehydrogenase Stafford *et al.*[4] suggested that it might function in an interconversion of serine and glyceric acid. This suggestion is supported by the demonstration of the plant L-alanine : hydroxypyruvate transaminase. An analogous transaminase is present in animal tissues.[3] With the recent demonstration of a mammalian D-glycerate dehydrogenase,[14,15] the enzymes necessary for the conversion of D-glycerate to serine have now been shown to occur in both plant and animal systems.

EXPERIMENTAL

Chemicals and materials

Crystalline lithium hydroxypyruvate was synthesized according to the method of Dickens and Williamson.[16] The following compounds were commercial preparations: L-alanine and L-serine (Sigma Chemical Company, St. Louis, Mo.); L-aspartic acid and β-alanine (Nutritional Biochemicals Corp., Cleveland, Ohio); L-leucine and glycine (Mann Research Laboratories, New York, N.Y.); L-glutamic acid and pyridoxal phosphate (Merck and Co., Inc., Rahway, N.J.); and NAD and $NADH_2$ (Pabst Laboratories, Milwaukee, Wisc.). Rabbit muscle lactate dehydrogenase was purchased from Sigma Chemical Company. D-Glycerate dehydrogenase was prepared from parsley leaves as described by Stafford *et al.*[4]

Preparation of plant material for enzymatic examination

Plants were grown in the garden or obtained from commercial sources. All plant parts were frozen before being ground. Seeds were soaked in distilled water overnight before freezing. The frozen plant materials were ground in a meat grinder, permitted to thaw and

[8] B. K. JOYCE and S. GRISOLIA, *J. Biol. Chem.* **235**, 2278 (1960).
[9] D. H. TURNER and J. F. TURNER, *Biochem. J.* **74**, 486 (1960).
[10] V. L. KRETOVICH and K. M. STEPANOVICH, *Doklady Akad. Nauk S.S.S.R.* **139**, 136 (1961).
[11] G. FLORES and H. J. SALLACH, unpublished results.
[12] W. B. MCCONNELL and A. J. FINLAYSON, *Can. J. Biochem. Physiol.* **39**, 1107 (1961).
[13] K. OZAKI and L. R. WETTER, *Can. J. Biochem. Physiol.* **38**, 125 (1960).
[14] J. E. WILLIS and H. J. SALLACH, *J. Biol. Chem.* **237**, 910 (1962).
[15] F. HEINZ and W. LAMPRECHT, *Z. physiol. Chem.* **325**, 280 (1961).
[16] F. DICKENS and D. H. WILLIAMSON, *Biochem. J.* **68**, 74 (1958).

the suspension strained through cheese-cloth. The plant juice was then cleared by centrifugation at 30,000 $\times g$ for 15 min. Aliquots of the supernatant solution were dialyzed against 0·01 *M* phosphate buffer, pH 7·4, containing 0·001 *M* 2-mercapteothanol, for several hours before they were assayed.

Transaminase preparation from parsley leaf

On the basis of enzymatic activity and its availability, parsley leaf was chosen as the source of the enzyme for a more detailed investigation. An eight- to ten-fold purification of the transaminase was achieved by the following procedure. All operations were carried out at 2°. Crude extracts of parsley leaf were prepared as described above. The extract was then dialyzed against 80 vol of 0·005 *M* sodium acetate buffer, pH 5·2, containing 0·001 *M* 2-mercaptoethanol, for 12 hr. The protein precipitate was removed by centrifugation at 30,000 $\times g$ for 20 min. The supernatant solution (100 ml) was brought to 30 per cent of saturation by the addition of solid ammonium sulfate. The suspension was stirred for 30 min to permit equilibration and then centrifuged at 30,000 $\times g$ for 15 min. The precipitate was dissolved in 10 ml of 0·01 *M* phosphate buffer, pH 7·4, containing 0·001 *M* 2-mercaptoethanol, and dialyzed overnight against 2 l. of the same buffer. This was the enzyme fraction used in all of the experiments reported except for the distribution studies. The enzyme preparation may be lyophilized at this point with little or no loss in activity.

Methods

Preincubation of the enzyme with pyridoxal phosphate and amino acid was conducted as described previously.[3] Reaction mixtures were incubated at 37° for 1 hr, unless otherwise indicated, and then stopped by the addition of 0·5 ml of 20% trichloracetic acid. Aliquots of the deproteinized solution were used for the determination of serine by the periodate method.[3] In the experiments in which the α-keto acids and alanine were to be measured, the reaction was stopped by the addition of 0·4 ml of 4*N* $HClO_4$. The protein was removed by centrifugation. Aliquots of the supernatant solution were neutralized with a known volume of 1*N* KOH, chilled and the precipitated $KClO_4$ removed by centrifugation.

Alanine, as well as serine, was assayed on the Beckman–Spinco amino acid analyzer.[17] Hydroxypyruvate and pyruvate plus hydroxypyruvate were determined by measuring the decrease in absorption of $NADH_2$ at 340 mμ in a Beckman DU spectrophotometer in the presence of plant D-glycerate dehydrogenase and muscle lactate dehydrogenase respectively, as outlined in detail elsewhere.[3] Protein was determined by the method of Lowry *et al.*,[18] using bovine serum albumin as the standard.

Radioactivity measurements of ^{14}C-labelled alanine were made using the Beckman–Spinco amino acid analyzer to which a Packard Tri-Carb Flow Monitor System was attached (Model 320E Flow Monitor and Model 317E Flow Detector, Packard Instrument Company, Inc., La Grange, Ill., U.S.A). This system has been described in detail by Rapkin and Gibbs.[19] Other individual details are presented with the individual experiments.

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[17] D. H. SPACKMAN, W. H. STEIN and S. MOORE, *Anal. Chem.* **30**, 1190 (1958).
[18] O. H. LOWRY, M. J. ROSENBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).
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